Chemokine Stimulation of Lymphocyte $\alpha_4$ Integrin Avidity but Not of Leukocyte Function-associated Antigen-1 Avidity to Endothelial Ligands under Shear Flow Requires Cholesterol Membrane Rafts*

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VLA-4 and LFA-1 are the major vascular integrins expressed on circulating lymphocytes. Previous studies suggested that intact cholesterol rafts are required for integrin adhesiveness in different leukocytes. We found the $\alpha_4$ integrins VLA-4 and $\alpha_4\beta_2$ as well as the LFA-1 integrin to be excluded from rafts of human peripheral blood lymphocytes. Disruption of cholesterol rafts with the chelator methyl-$\beta$-cyclodextrin did not affect the ability of these lymphocyte integrins to generate high avidity to their respective endothelial ligands and to promote lymphocyte rolling and arrest on inflamed endothelium under shear flow. In contrast, cholesterol extraction abrogated rapid chemokine triggering of $\alpha_4$ integrin-dependent peripheral blood lymphocyte adhesion, a process tightly regulated by G-protein-coupled chemokine receptors (GPCR). Strikingly, stimulation of LFA-1 avidity to intercellular adhesion molecule 1 (ICAM-1) by the same chemokines, although $G_i$-dependent, was insensitive to raft disruption. Our results suggest that $\alpha_4$ but not LFA-1 integrin avidity stimulation by chemokines involves rapid chemokine-induced GPCR rearrangement that takes place at cholesterol raft platforms upstream to $G_i$ signaling. Our results provide the first evidence that a particular chemokine/GPCR pair can activate different integrins on the same cell using distinct $G_i$ protein-associated machineries segregated within defined membrane compartments.

To extravasate the bloodstream at specific sites of inflammation or antigen presentation, circulating lymphocytes must rapidly develop firm adhesion to specific endothelial ligands (1, 2). Lymphocyte arrest on vascular endothelia is nearly exclusively mediated by the integrins VLA-4 ($\alpha_4\beta_1$), its related integrin, $\alpha_4\beta_2$, and by LFA-1, counter-receptors for endothelial VCAM-1, MAdCAM-1, and ICAM-1, respectively. As these integrins occur in largely inactive states on circulating leukocytes, their in situ activation must take place within fractions of seconds at endothelial contact zones (3, 4). Apically expressed endothelial chemokines confer these integrins with high avidity and shear-resistant adhesion more efficiently than their soluble counterparts (4, 5). This suggests that chemokine-occupied GPCRs may transmit local signals to neighboring integrins at the lymphocyte plasma membrane. Segregation of the GPCR machinery, its proximal effectors, and their target integrins within preformed supramolecular structures may facilitate chemokine triggering of integrin avidity under shear flow (6).

Candidate membrane microdomains for such segregation of GPCR complexes are lipid rafts, cholesterol- and glycosphingolipid-enriched lipid microdomains, which serve as key signaling platforms in many cell types (7). Because lymphocyte rafts contain several key potential modulators of integrin adhesion stimulation by chemokines, including GPCR subsets and their associated G-proteins (8–10), PI3K and Src (11–13), we hypothesized that avidity stimulation of integrins by GPCRs should take place within or proximal to raft microdomains. Recently, thymocyte LFA-1 has also been shown to partition to rafts and undergo avidity modulation upon raft ligation (14). Lymphocyte rafts have been also implicated in $\beta_1$ integrin adhesion modulation by the T-cell receptor machinery (15) and by other agonists, as measured in prolonged static contacts (16). However, very little is known about the involvement of rafts in rapid stimulation of leukocyte integrin avidity by chemokines at endothelial contacts. We report here that although spontaneous integrin-mediated adhesion of resting PBL to inflamed endothelium under shear flow does not require raft integrity, chemokine triggering of VLA-4 or $\alpha_4\beta_2$ avidity to their respective endothelial ligands requires the integrity of cholesterol rafts. Interestingly, chemokine-triggered avidity of LFA-1 to endothelial ICAM-1 is insensitive to raft disruption by cholesterol depletion. Our results suggest that triggering of $\alpha_4$ integrin avidity by major GPCRs on resting T cells occurs through a cholesterol-dependent mechanism, not utilized by the LFA-1 integrin.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Recombinant soluble 7-domain human VCAM-1, sVCAM-1 (17), was kindly provided by Dr. R. Lobb (Biogen, blood lymphocytes; PTX, pertussis toxin; SDF-1$\alpha$, stromal derived factor-1$\alpha$; VCAM-1, vascular cell adhesion molecule-1; mAb, monoclonal antibody; PI3K, phosphatidylinositol 3-kinase; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid.

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‡ The abbreviations used are: VLA-4, very late antigen-4; MAdCAM-1, mucosal addressin cell adhesion molecule-1; Cyto D, cytochalasin D; ERK, extracellular signal-regulated kinase; GPCR, G-protein coupled receptor; HUVEC, human umbilical cord endothelial cells; ICAM-1, intracellular adhesion molecule-1; LFA-1, leukocyte function-associated antigen-1; MβCD, methyl-$\beta$-cyclodextrin; PBL, peripheral.
**RESULTS**

**Cholesterol Extraction from PBL Does Not Abrogate Intrinsinc Integrin Adhesiveness**—To test the involvement of cholesterol rafts in the regulation of selectin- and integrin-mediated adhesion of PBL to inflamed endothelium, freshly isolated PBL were perfused under physiological shear flow over a monolayer of TNF-α-activated HUVECs, a well established inflamed endothelial model. PBL establish persistent rolling on the endothelial cell monolayer, which is mediated primarily by E-selectin that is expressed on the luminal face of the monolayer. PBL capture and rolling on E-selectin are dependent on the expression of the CD18 integrin, while interactions with VCAM-1 induced on these endothelial cells (4). Interactions of PBL with VCAM-1 induced on these endothelial cells (4). Interactions of PBL with VCAM-1 induced on endothelial cells (4). Interactions of PBL with VCAM-1 induced on endothelial cells (4). Interactions of PBL with VCAM-1 induced on endothelial cells (4).
arrest on VCAM-1-bearing cell surfaces (Fig. 1C) or on purified VCAM-1 coated at different densities (Fig. 1D). Similar to VLA-4, α4β7 integrin-mediated tethering and rolling of PBL on MAdCAM-1 were also resistant to MβCD (Fig. 1E). Consistent with these results, α4 integrin expression was not affected by MβCD treatment (Fig. 1F). Thus, intact rafts are not required for the inherent ability of α4 integrins on resting T cells to interact with their respective ligands under shear flow and to spontaneously develop medium or high avidity to these ligands at rapid adhesive contacts.

**Cholesterol Extraction Abrogates Chemokine-stimulated α4 Integrin-mediated PBL Adhesion but Not Stimulation of LFA-1-mediated Adhesion**—Immovilized chemokines bind lymphocyte GPCRs and trigger robust VLA-4 integrin-mediated adhesion to VCAM-1 at static contacts through activation of pertussis toxin-sensitive Gα-protein machinery (4, 5, 23). We next asked how chemokine-stimulated adhesion of VLA-4 is affected by disruption of cholesterol rafts on PBL. The prototypic PBL chemokines, SDF-1α (CXCL12) and secondary lymphoid tissue chemokine (CCL21), co-immobilized with VCAM-1, trigger robust VLA-4-dependent adhesion of PBL at 1-min long static contacts (Fig. 2A). VLA-4-dependent adhesion triggered by both chemokines as well as by Mig (CXCL9) was entirely abrogated by MβCD pretreatment of PBL (Fig. 2A and data not shown), as well as by pertussis toxin pretreatment of lymphocytes (Ref. 4, and data not shown). The inhibitory effect of MβCD on chemokine-stimulated VLA-4 avidity was reversed in the presence of exogenous cholesterol (Fig. 2A), consistent with cholesterol depletion accounting for the inhibitory effect of MβCD. Similarly, MβCD pretreatment of murine B lymphocytes abrogated their ability to develop high avidity VLA-4-mediated adhesion to VCAM-1 in response to the B cell chemokine B cell attracting chemokine-1 (CXCL13, data not shown). Thus, although intact rafts are not required for spontaneous VLA-4 and α4β7 adhesiveness developed at rapid adhesive contacts, they are necessary for chemokine-stimulated VLA-4 adhesiveness. These results could suggest that GPCR signaling triggered by SDF-1α and secondary lymphoid tissue chemokine is severely impaired by MβCD treatment. Strikingly, however, both chemokines could normally trigger robust LFA-1-dependent PBL adhesion to purified ICAM-1, and such adhesion was insensitive to cholesterol extraction, yet sensitive to PTX pretreatment of lymphocytes (Fig. 2B, and data not shown).

In support of these results, both the expression level and intrinsic signaling activity of the SDF-1α GPCR (CXCR4) to ERK was fully retained in MβCD-treated PBL (Fig. 2, C and D). Spontaneous LFA-1-mediated PBL adhesiveness to ICAM-1 was also fully retained upon raft disruption (data not shown). Thus, Gαi protein-mediated enhancement of LFA-1-dependent adhesion by major chemokines is fully retained upon raft disruption in PBL, although Gαi stimulation of VLA-4 adhesiveness by identical chemokines is the same cells is totally abrogated.

**Cholesterol Rafts Are Necessary for Subsecond Gα-protein-dependent Enhancement of VLA-4 Avidity to Ligand under Shear Flow**—Chemokines also trigger VLA-4 avidity at subsecond contacts promoting lymphocyte capture to VCAM-1 under continuous shear flow (4). The involvement of rafts in these earliest signaling events of chemokines to target integrins on the lymphocyte surface was next investigated. In agreement with its suppressive effect on chemokine-stimulated VLA-4 adhesion at static adhesive contacts (Fig. 2A), cholesterol extraction from PBL impaired rapid SDF-1α stimulation of VLA-4 avidity to VCAM-1 at subsecond contacts and abrogated chemokine-triggered capture and immediate lymphocyte arrest.
on VCAM-1-bearing surfaces (cell-free or on the surface of a cell monolayer; Fig. 3, A and B, respectively). Cholesterol extraction also abrogated SDF-1α-stimulated Gα2-dependent (PTX-sensitive) transient tethers mediated by VLA-4 on low density VCAM-1 confirming that rafts are obligatory to chemokine-enhanced avidity regardless of the level of avidity stimulated or duration of the adhesive contact (Fig. 3C). Similar to VLA-4, rapid chemokine-triggered α4β7 avidity to MadCAM-1 was also abrogated in MβCD-treated lymphocytes (Fig. 3D), although spontaneous lymphocyte capture and rolling on MadCAM-1 were insensitive to cholesterol extraction (Fig. 3E). However, subsecond chemokine stimulation of LFA-1 avidity to isolated ICAM-1, required to trigger capture and immediate arrest of lymphocytes on ICAM-1 at low shear flow (Fig. 3F), was entirely insensitive to identical cholesterol extraction and raft disruption. Furthermore, robust SDF-1α-triggered integrin-dependent arrest of PBL captured on TNF-α-activated HUVEC was also insensitive to MβCD-mediated cholesterol depletion (Fig. 3F), in agreement with the major role of LFA-1 in rapid adhesion strengthening developed by lymphocytes on these HUVEC cells (24). Intact cholesterol-enriched rafts appear therefore obligatory for the ability of chemokine GPCRs to

**Fig. 2.** Cholesterol depletion abolishes α4 integrin but not LFA-1 stimulation by chemokines without interfering with GPCR expression and signaling. PBL were pretreated with MβCD as described in the legend to Fig. 1. A, adhesion of PBL to purified sVCAM (at 2 μg/ml) co-immobilized with heat-inactivated chemokines (–) or functional SDF-1α or secondary lymphoid tissue chemokine-1 (each at 2 μg/ml). Values depict the fraction of cells originally settled for 1 min that resisted detachment to a shear stress of 1 dyn/cm² for 5 s. Where indicated, PBL were pretreated with MβCD in the presence of cholesterol (2 μg/ml) for 10 min. B, adhesion of PBL to purified ICAM-1 (at 0.25 μg/ml) co-immobilized with heat-inactivated (–) or intact chemokine (SDF-1α at 2 μg/ml). Values depict the fraction of cells originally settled for 1 min on the substrates that resisted detachment by 1 dyn/cm² applied for 5 s. The results in A and B represent an average ± range of 2–3 fields of view. The experiments are each representative of three using multiple PBL donors. C, immunofluorescence flow cytometry of intact (black line) and MβCD (gray line) pretreated PBL stained with antibody to CXCR4 (rabbit polyclonal CXCR4 antibody, 10 μg/ml) followed by phycoerythrin-conjugated anti-rabbit (Jackson). D, effect of MβCD on chemokine signaling. SDF-1α (10 mM, 30 s) stimulation at 37 °C of ERK1/2 phosphorylation in PBL (intact or MβCD pretreated). Immunoblotting with anti-phosphospecific ERK1/2 (upper panel) and anti-ERK (lower panel) is shown. Intact PBL were also stimulated with SDF-1α in the presence of the CXCR4 blocker, AMD3100 (5 μM, 10 min) (44).

**Fig. 3.** Cholesterol depletion abolishes α4 integrin but not LFA-1 stimulation by chemokines at subsecond contacts. A, frequency of intact or MβCD-treated PBL interacting under shear stress of 1 dyn/cm² with sVCAM-1 (2 μg/ml) co-immobilized with heat-inactivated (–) or functional SDF-1α (2 μg/ml). The different tether categories were expressed as described under “Experimental Procedures.” The data represent mean values of determinations in two fields of view. One experiment representative of three is shown. B, frequency and category of tethers mediated by intact or MβCD-treated PBL interacting with a VCAM-1 expressing Chinese hamster ovary cell monolayer at 1.5 dyn/cm². SDF-1α (100 ng/ml) was overlaid on the monolayer and washed extensively before PBL perfusion. C, frequency of transient tethers initiated by PBL on low density sVCAM (1 μg/ml) co-immobilized with functional or heat-inactivated (–) chemokine (SDF-1α 2 μg/ml) under shear stress (1 dyn/cm²). Indicated PBL samples were pretreated with pertussis toxin (PTX) to block their Gαi signaling. D, frequency of intact or MβCD-treated PBL interacting under shear stress of 1 dyn/cm² with MadCAM-1-Ig (overlaid at 10 ng/ml) on protein A precoated with heat-inactivated chemokine (–) or functional SDF-1α as described under “Experimental Procedures.” E, frequency of intact or MβCD-treated PBL interacting, under low shear stress of 0.5 dyn/cm², with ICAM-1-α (overlaid at 0.5 μg/ml) and MβCD-Ig (overlaid at 0.5 μg/ml on protein A) co-immobilized with heat-inactivated chemokine (–) or functional SDF-1α (2 μg/ml). High avidity LFA-1 binding to ICAM-1 required for lymphocyte capture in the absence of selectins could be triggered only at this or lower shear stresses. The data shown in B–E each represent an average ± range of measurements in two fields of view. Each experiment shown is representative of two. F, frequency of intact and MβCD-treated PBL firmly arrested on TNF-α-stimulated HUVEC, overlaid with SDF-1α (100 ng/ml) at 1.5 dyn/cm². VLA-4 and LFA-1 integrins on intact PBL were pre-blocked with HP1/2 and TS 1/18 mAbs (20 μg/ml), respectively. Values are the mean ± range of two fields. A representative experiment of five is shown.
Raft Partitioning of Lymphocyte Integrins—This vastly different cholesterol dependence of α4 integrin avidity modulation could reflect preferential partition of these integrins together with their stimulatory GPCRs within cholesterol-containing raft domains. Strikingly, however, both α4 integrins and LFA-1 were largely excluded from detergent-resistant low-density lipid fractions recovered by sucrose gradient highly enriched with the raft marker p56Lck (Fig. 4A). Thus, raft partitioning of α4 integrin did not determine the cholesterol dependence of their GPCR-mediated avidity stimulation. Nevertheless, α4 integrins and LFA-1 appeared to be compartmentalized in distinct membranal domains of PBL as evident by their inability to co-localize (Fig. 4B). This dichotomy in MβCD sensitivity of chemokine-stimulated α4 integrin and LFA-1 avidity was not restricted to PBL. U937 monocytes and Jurkat T lymphoblasts express endogenous CXCR4 (4, 25). Similar to PBL, SDF-1−triggered VLA-4 avidity to VCAM-1 in both types of leukocytes was highly sensitive to cholesterol extraction (data not shown). SDF-1−triggered LFA-1 avidity in the same cells was, however, insensitive to raft disruption (Fig. 5A, and data not shown). Notably, a major fraction of LFA-1 in Jurkat cells (40%) was found to partition within Lck-enriched low-density lipid fractions (Fig. 5B). This further suggests that raft enrichment of LFA-1 does not render its chemokine/GPCR-mediated avidity stimulation more susceptible to raft disruption. Thus, the MβCD sensitivity of chemokine-stimulated α4 integrins, which is not shared by LFA-1, was not the result of a preferential partition of α4 integrins within rafts and did not occur even in cases where LFA-1 was present in rafts.

Raft Patching Is Insufficient to Trigger VLA-4 Avidity to VCAM-1 at Rapid Adhesive Contacts—Clustering of thymocyte rafts has been reported to stimulate integrin adhesiveness through activating PI3K (14). We therefore next asked whether raft clustering could play any stimulatory role in rapid VLA-4 adhesion stimulation. Because the levels of the raft sphingolipid marker, GM1, on resting PBL were low (data not shown), we used CD59, an abundant glycosylphosphatidylinositol-linked raft-enriched protein on resting PBL to induce raft patching (Fig. 6, top panel) and to test its effects on stimulation of VLA-4 avidity to VCAM-1 (Fig. 6, lower panel). Notably, under conditions where chemokines triggered robust VLA-4 avidity to VCAM-1, CD59 cross-linking had no proadhesive effect on VLA-4-dependent PBL adhesion to VCAM-1 (Fig. 6). Furthermore, murine pre-B 300.19 cells that express high levels of GM1 also failed to develop stronger VLA-4-mediated adhesion to VCAM-1 in response to GM1 patching by the GM1 receptor, cholera toxin B subunit, or to cross-linking of their glycosylphosphatidylinositol-linked protein, CD24 (data not shown). These results suggest that raft clustering on its own is insufficient to stimulate rapid α4 integrin avidity.
et al. anchorage of the antigenic determinant (4, 26, 27) was largely resistant to raft disruption by MβCD (Fig. 7B). Thus, CXCR4 surface levels and accessibility in PBL do not depend on integrity of cholesterol rafts. However, CXCR4-mediated PBL adhesion to high density anti-CXCR4 mAb under shear flow was substantially suppressed by cholesterol depletion by MβCD (Fig. 7B). A similar requirement for intact cholesterol rafts in PBL was observed when the lymphocytes were allowed to adhere to low density anti-CXCR4 mAb co-immobilized with the native CXCR4 ligand, SDF-1α (Fig. 7C). In this assay (Fig. 7D), the frequency and stability of cellular tethers formed on the surface-bound mAb serves as a readout of CXCR4 microclusters, spontaneous or chemokine-triggered (22). Consistent with this notion, neither soluble SDF-1α nor immobilized chemokine to an irrelevant GPCR, CCR7 (data not shown), could augment PBL adhesion to the low density anti-CXCR4 mAb (Fig. 7C). Both transient and firm PBL tethers on the mAb-coated substrates were stabilized over 0.1–0.5 s in a PTX-resistant manner (Fig. 7C, and data not shown). This indicated that CXCR4 reorganization by mAb and chemokine was completed within subsecond time frames of adhesive contact independent of Gβγ-protein activation. Taken together these results suggest that MβCD treatment of PBL abolishes SDF-1α-augmented CXCR4 clustering at subsecond contacts, a process that requires intact rafts, but not Gβγ-protein signaling.

Raft clustering and associated signaling depends on an intact actin cytoskeleton and involves enrichment of F-actin at sites of raft patches (28–30). Thus, SDF-1α-triggered PBL adhesion to anti-CXCR4 mAb may involve active clustering of rafts sensitive to disruption of the actin cytoskeleton. Indeed, short treatment of T lymphocytes with Cyto D abolished SDF-1α-triggered PBL adhesion to low density anti-CXCR4 mAb under shear flow (Fig. 8A). VLA-4 avidity stimulation by SDF-1α was also completely disrupted by the Cyto D treat-ment, even though the short treatment did not affect spontaneous VLA-4 adhesiveness to VCAM-1 in treated PBL (Fig. 8B). Nevertheless, identical Cyto D treatment had no effect on LFA-1 avidity stimulation induced by SDF-1α (Fig. 8C). These results therefore suggest that SDF-1α stimulates VLA-4 avidity through an MβCD-sensitive cytoskeletonally regulated CXCR4 clustering upstream to Gβγ signaling. This clustering of CXCR4 is not required, however, for SDF-1α to transduce Gβγ signals and optimally trigger LFA-1 avidity to ICAM-1.

DISCUSSION

Chemokines, in addition to orchestrating cell motility and chemotaxis, are potent stimulants of integrin avidity and adhesive processes (31). Although cholesterol- and sphingolipid-
endothelial VCAM-1 involves rapid induction of integrin clustering and affinity to endothelial ligands, this localized stimulation of integrins is designed to up-regulate signaling platforms in rapid chemokine signaling events has not been investigated to date. At endothelial contacts, leukocytes must respond within fractions of seconds to localized signals transmitted by endothelial displayed surface-bound chemokines to specific GPCRs on the leukocyte surface (3, 4). This localized stimulation of integrins is designed to up-regulate integrin clustering and affinity to endothelial ligands within subsecond contacts under the continuous presence of disruptive shear forces (4, 23, 25). Our studies on chemokine triggering of VLA-4 avidity in lymphocytes as well as in hematopoietic progenitors have also shown that VLA-4 avidity to endothelial VCAM-1 involves rapid induction of integrin clustering triggered preferentially by immobilized chemokines juxtaposed to the integrin ligand. Rapid chemokine signaling to VLA-4 therefore appears to depend on specialized GPCR rearrangement events proximal to the regulated integrin target. The present study provides the first indication that although both are transduced through G protein stimulation, VLA-4 and LFA-1 avidity stimulation by surface bound chemokines involves different GPCR rearrangement mechanisms. These rearrangements appear to operate upstream to the heterotrimeric G-protein activation step essential for the integrin avidity stimulation (Fig. 9) because they are insensitive to pertussis toxin blockage of G, signaling (Figs. 3C and 7C), but take place in the context of cholesterol rafts.

Subsets of CXCR4 have been reported to translocate into raft domains upon CXCR4 patching with human immunodeficiency virus envelope proteins (9, 33). Our results on CXCR4 suggest that the vast majority of this prototypic leukocyte chemokine receptor is excluded from rafts in PBL. These findings are in agreement with a recent study demonstrating CXCR4 exclusion from detergent-resistant raft fractions in resting and SDF-1α-treated T cells (34). Nevertheless, CXCR4, CCR7, and CXCR5, as well as other subsets of leukocyte GPCRs, are expected to operate in the vicinity of lipid rafts by way of association with their signaling units, the heterotrimeric G-proteins that partition into rafts through their G, subunits (10). Thus, our results are consistent with the notion that GPCRs, normally excluded from rafts, can utilize their raft-associated effectors to transmit subsecond signals from their cognate chemokines to target integrins on the cell surface. Notably, these target integrins, i.e. VLA-4 and ζ, can also be largely excluded from rafts and still respond to chemokine signals in the context of raft-associated effectors. Furthermore, in cells like the T cell line, Jurkat, where LFA-1 was found enriched within detergent-insoluble raft fractions, the integrin response to chemokine stimulation was still independent of cholesterol raft integrity, whereas VLA-4 response to chemokine was still dependent of intact cholesterol rafts, as in PBL (data not shown). Thus, the ζ-integrin response to a GPCR-mediated avidity stimulatory signal within a raft compartment

FIG. 9. Postulated mechanisms of chemokine-triggered avidity increase of VLA-4 and LFA-1 at rapid contact sites. Chemokine-triggered integrin avidity elevation involves two sequential steps: chemokine-induced rearrangement (clustering) of the GPCR (step 1) followed by activation of the associated G protein (G, signal, step 2). GPCR subsets clustered in the vicinity of raft microdomains (which are MβCD and Cyto D sensitive in the left part of the membrane) are required for successful transmission of the G, signal to ζ, integrins but are not required for triggering of LFA-1 avidity. GPCRs that do not undergo clustering within raft domains can still transmit their G, signals to LFA-1 although they are incapable of stimulating ζ, integrin avidity. For simplicity, integrins and ligands are not included in the scheme.
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does not require this integrin to partition into this compartment. The GPCR signaling machinery utilized by an integrin, rather than the partition of the integrin in rafts, appears to dictate whether chemokine-mediated stimulation of this integrin is dependent on raft integrity. Considering that cholesterol rafts are extremely small lipid entities with radii ranging from 10 to 30 nm (35), it is possible that GPCRs can transmit local signals to proximal integrin targets near such rafts within fractions of seconds, even if both the GPCR and the integrin do not co-partition in these rafts. We cannot exclude, however, the possibility that GPCR signals triggered preferentially by immobilized chemokines can mobilize $\alpha_4$ integrins but not LFA-1 into cholesterol rafts where their clustering is in situ stimulated by chemokine-occupied GPCRs. This process is unlikely to involve, however, GPCR signaling linked to Src activation, because this pathway is not involved in subsecond induction of either $\alpha_4$ or LFA-1 integrin avidity by chemokines (20).2

How then do cholesterol rafts serve as platforms of chemokine signaling to integrins and what is the basis for the raft segregation of chemokine signaling to $\alpha_4$ integrins as opposed to LFA-1? In PBL, VLA-4 and LFA-1 avidity regulation by chemokines at rapid contact sites appear to employ distinct $G$ protein-transduced machineries, even when stimulated by a common chemokine GPCR (4, 23). For instance, although both integrins do not require intact PI3K and classical protein kinase $C$ machinery to undergo avidity up-regulation by chemokines, LFA-1 but not VLA-4 avidity stimulation may depend on GPCR-triggered $Ca^{2+}$ mobilization (4, 23).2 Furthermore, LFA-1 and $\alpha_4$ integrins differ substantially with respect to both their surface distribution and cytoskeletal associations (36–38). It is therefore possible that a specialized GPCR effector of $\alpha_4$ avidity, which operates independently of PI3K, protein kinase $C$, or intracellular free $Ca^{2+}$, regulated by $G$ signaling within or proximal to raft platforms. Notably, disruption of the actin cytoskeleton abolished both CXCR4-dependent adhesion and $\alpha_4$ integrin avidity stimulation by SDF-1α without affecting LFA-1 avidity stimulation (Fig. 8). These results, and the finding that VLA-4 avidity stimulation is restricted to immobilized chemokines (4) suggest that a critical cytoskeletal-mediated raft-dependent GPCR clustering step must be coupled to $G$ signaling to optimally trigger $\alpha_4$ integrin avidity at second adhesive contacts (Fig. 9). In contrast to $\alpha_4$ integrin stimulation, enhancement of LFA-1 avidity by chemokines is triggered in the absence of CXCR4 clustering, because interference with this step by disruption of cholesterol rafts (Fig. 7, B and C) or by mild disruption of the actin cytoskeleton (Fig. 8A) did not affect the ability of CXCR4 to mediate optimal SDF-1α triggering of LFA-1 avidity (Fig. 8C). Because SDF-1α-triggered PBL adhesion to the anti-CXCR4 mAb as well as to the isolated integrin ligands takes place at $0.1–0.5$ long contacts, de novo actin polymerization events triggered by CXCR4 at such contact sites are unlikely to underlie $\alpha_4$ avidity stimulation. These subsecond contacts are much more rapid than earliest actin polymerization processes reported to date (39).

In contrast to the role of rafts in GPCR-mediated chemokine signaling to $\alpha_4$ integrins, we did not detect any raft requirement for $\alpha_4$ or other integrins to spontaneously capture lymphocytes from the flow and to generate high avidity to ligand at short-lived adhesive contacts independent of chemokine activation (Fig. 1). MβCD-treated lymphocytes could bind to VCAM-1, MadCAM-1, or ICAM-1 under shear flow at comparable efficiencies to intact lymphocytes (Fig. 1). $\alpha_4$ Integrin presentation and clustering on lymphocyte microvilli have been suggested to regulate lymphocyte capture on ligand under shear flow (26, 36). Microvilli structures on PBL appeared to remain largely intact in MβCD-treated cells, because both $\alpha_4$ integrin-mediated and L-selectin-mediated lymphocyte capture and rolling on respective ligands were not affected by cholesterol extraction (Fig. 1, $B$ and $D$).3 Our present data contrast recent reports suggesting that agonist-induced or constitutive LFA-1 adhesiveness in primary T cells, thymocytes, or in the Jurkat T cell line, is strongly suppressed by raft disruption (14, 16). In our hands, however, both spontaneous, phorbol ester-stimulated or mAb-activated LFA-1-dependent adhesion to ICAM-1 were entirely insensitive to cholesterol depletion (data not shown). Furthermore, raft clustering was shown to stimulate PI3K activity and thereby trigger LFA-1-mediated thymocyte adhesion (14). Raft patching also induces Src activation and downstream actin polymerization processes in adherent lymphocytes (29). However, spontaneous VLA-4 and LFA-1 adhesiveness developed by T cells at rapid adhesive contacts are insensitive to PI3K inhibition.4 Raft clustering alone is also insufficient to stimulate VLA-4 integrin adhesive- ness at these short-lived contacts (Fig. 6). Thus, the raft clustering events that have been reported to promote T cell adhesion do so via late post-ligand occupancy events rather than by directly enhancing integrin avidity to immobilized ligand. Furthermore, raft ligation alone was recently shown to translocate both free and ligand-occupied LFA-1 and VLA-4 integrins into rafts (16, 40) where these integrins and their cytoskeletal adaptors can be activated de novo or stabilized by raft-associated kinases (15, 41). In contrast, spontaneous integrin-mediated adhesiveness at rapid contacts do not appear to employ these slow adhesion strengthening processes. Rapid integrin-mediated adhesive contacts may rely on integrin molecules with preformed potential to generate high avidity to endothelial ligands independent of raft-associated machineries (42).

In conclusion, our results provide a first indication that chemokine signaling through a common GPCR to different integrins coexpressed on a given leukocyte can take place at different lipid microdomains. PBL rafts may stabilize an, as yet unidentified, GPCR effector that affects $\alpha_4$ avidity through a specialized rearrangement process not required for LFA-1 avidity stimulation. Spontaneous avidity generation of either $\alpha_4$ integrins or LFA-1 do not require, on the other hand, intact cholesterol rafts, ruling out preferential partition of $\alpha_4$ integrins or of their specific avidity regulatory effectors within these rafts. Thus, it is the integrin activating machinery, rather than the integrin itself, that requires intact rafts to convert chemokine signaling through GPCRs into productive integrin avidity stimulation at rapid contacts. The notion that GPCR effectors need to undergo specialized rearrangements to translate chemokine signals into avidity enhancement of particular integrins opens up new opportunities to selectively disrupt functional stimulation of distinct integrins by a given chemokine.

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