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RhoA Is Involved in LFA-1 Extension Triggered by CXCL12 but Not in a Novel Outside-In LFA-1 Activation Facilitated by CXCL9

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Chemokines presented on endothelial tissues instantaneously trigger LFA-1-mediated arrest on ICAM-1 via rapid inside-out and outside-in (ligand-driven) LFA-1 activation. The GTPase RhoA was previously implicated in CCL21-triggered LFA-1 affinity triggering in murine T lymphocytes and in LFA-1-dependent adhesion strengthening to ICAM-1 on Peyer’s patch high endothelial venules stabilized over periods of at least 10 s. In this study, we show that a specific RhoA 23/40 effector region is vital for the initial LFA-1-dependent adhesions of lymphocytes on high endothelial venules lasting 1–3 s. Blocking the RhoA 23/40 region in human T lymphocytes in vitro also impaired the subsecond CXCL12-triggered LFA-1-mediated T cell arrest on ICAM-1 by eliminating the rapid induction of an extended LFA-1 conformational state. However, the inflammatory chemokine CXCL9 triggered robust LFA-1-mediated T lymphocyte adhesion to ICAM-1 at subsecond contacts independently of the RhoA 23/40 region. CXCL9 did not induce conformational changes in the LFA-1 ectodomain, suggesting that particular chemokines can activate LFA-1 through outside-in post ligand binding stabilization changes. Like CXCL9, the potent diacylglycerol-dependent protein kinase C agonist PMA was found to trigger LFA-1 adhesiveness to ICAM-1 also without inducing integrin extension or an a priori clustering and independently of the RhoA 23/40 region. Our results collectively suggest that the 23/40 region of RhoA regulates chemokine-induced inside-out LFA-1 extension before ligand binding, but is not required for a variety of chemokine and non-chemokine signals that rapidly strengthen LFA-1-ICAM-1 bonds without an a priori induction of high-affinity extended LFA-1 conformations. The Journal of Immunology, 2008, 180: 2815–2823.

The arrest of tethered or rolling leukocytes on target endothelium is nearly exclusively mediated by members of the integrin superfamily and their endothelial Ig superfamily ligands (1). Activating cytokines are key players in this arrest, because integrins do not mediate firm shear-resistant adhesions unless activated in situ at the leukocyte-endothelial interface. Key checkpoints in integrin activation on many types of leukocytes include the rapid modulation of integrin affinity and valency to endothelial ligands (2–4). In situ activation of the αβ integrin heterodimer is rapidly triggered by the binding of specialized endothelial chemokines to G protein-coupled receptors (GPCRs) (5–9). In vitro studies suggest that lymphocyte GPCRs such as CXCR4, CCR7 and CXCR3, when occupied by surface-presented rather than soluble ligands, can transmit a series of cytoplasmic signals that result in LFA-1 activation (8, 10). This signaling occurs within subseconds through Gi heterotrimeric G protein activation. A critical step in integrin activation is the almost instantaneous stabilization of an extended conformational state that must be rapidly coupled to the ligand-induced activation of the integrin headpiece (10, 11).

Many of the downstream Gi targets suggested to mediate this initial integrin activation step are distinct from those participating in rapid integrin activation at lymphocyte-endothelial contacts subjected to shear forces as they are involved in more prolonged integrin mobility, clustering, and cell-spreadings events (12). Only few Gi targets triggered by endothelial-displayed chemokines have so far been implicated in the initial, rapid integrin activation events of lymphocytes. Among them, the small GTPase RhoA was shown to be involved in rapid LFA-1 activation by the CCL21 and CXCL12 chemokines in lymphocytes (13). RhoA-mediated LFA-1 activation was argued to involve two modalities, the first, activation of a high-affinity LFA-1 state capable of binding soluble ICAM-1 (14), and the second, triggering of LFA-1 lateral mobility. Using Trojan peptides with sequences corresponding to distinct effector regions of RhoA, each able to block RhoA-dependent signaling in a domain-selective manner, it was shown that RhoA-triggered LFA-1 affinity and lymphocyte adhesion strengthening on ICAM-1 involves the 23/40 effector domain of this GTPase (13). A distinct RhoA effector region, encompassing residues 92/119, was shown to regulate LFA-1 mobility and was argued to augment firm lymphocyte adhesions to low-density ICAM-1 (13). Nevertheless, the contribution of each RhoA modality to the very
initial LFA-1-mediated arrest step on ICAM-1 and the mechanism by which specific RhoA regions regulate LFA-1 affinity, i.e., by instantaneous stabilization of an extended conformational state taking place under shear stress conditions and/or by ligand-induced integrin activation have not been elucidated. An alternative effector system that regulates LFA-1 valency (avidity) and adhesiveness to ICAM-1 are diacylglycerol (DAG)-dependent protein kinase C (PKCs) (10, 15–17). Although neither classical (DAG and Ca²⁺-dependent) nor novel (DAG-dependent but Ca²⁺-independent) PKC isoforms play a role in rapid chemokine-triggered LFA-1 activation (10, 13, 14), recent studies implicated classical PKCs in LFA-1 activation by selectin ligands on subsets of T cells, leading to their arrest on ICAM-1 (17). Activation of PKCs with the DAG analog PMA drives LFA-1 clustering in T lymphocytes without a noticeable increase in LFA-1 affinity to soluble ICAM-1 (14). PKCs were argued to release LFA-1 from cytoskeletal constraints (18), increase the lateral mobility of LFA-1, and thereby enhance the ability of the integrin to generate high-avidity contacts with both low- and high-density ICAM-1 (14). In contrast, recent studies suggested that PMA switches T cell LFA-1 from an inactive bent state to an extended state with high affinity to ICAM-1 (19). Thus, the role of DAG-dependent PKCs in LFA-1 conformational activation and in the up-regulation of active LFA-1 clustering potentially involved in the earliest lymphocyte contacts with ICAM-1 subjected to shear stress conditions has not been elucidated.

In the present study, we used cell-permeable Trojan peptides containing the two major RhoA effector domains 23/40 and 92/119 to specifically interfere with the function of these two RhoA regions in primary human T lymphocytes undergoing in situ activation by various stimuli. Lymphtocytes pretreated with these RhoA-interfering peptides were allowed to interact under continuous shear flow with surface bound ICAM-1 or a mAb probe for extended LFA-1. Our results show that the Rhoa 23/40 region is critical for the ability of LFA-1 to undergo rapid extension and acquisition of high affinity to ICAM-1 in response to in situ CXCL12 signals. We also found that LFA-1 undergoes robust activation by the inflammatory chemokine CXCL9 through a novel G·β-dependent, RhoA 23/40-independent mechanism that does not involve integrin extension or acquisition of high-affinity conformation. Another mode of rapid LFA-1 activation, which occurs via DAG-dependent PKCs, also does not involve conformational extension of LFA-1, nor is it associated with enhanced active LFA-1 clustering, and can take place in the absence of intact RhoA signaling. Thus, our results collectively suggest that particular chemokine signals activate, within subseconds, the RhoA 23/40 region to induce an LFA-1 extension and thereby trigger LFA-1-mediated lymphocyte arrest on ICAM-1. Other agonists drive an alternative modality for facilitating rapid LFA-1 adhesiveness to ICAM-1, which does not involve an a priori inside-out LFA-1 extension and is independent of the 23/40 RhoA region.

Materials and Methods
Reagents and mAbs
RhoA-blocking peptides corresponding to the 23/40 and 92/119 regions of the protein have been previously characterized (20) and were synthesized as previously described. The rhodamine-labeled phosphatidylserine 4.5-bisphosphate (PIP2)-binding peptide, PBP10 (rhodamine B-QRLFQQVKGR) corresponding to gelsolin residues 160/169, and the control peptide (rhodamine B-QRL) (20) were a gift from Dr. P. A. Janney (University of Pennsylvania, Philadelphia, PA). Recombinant ICAM-1-Fc fusion protein, human CXCL12, and human CXCL9 were purchased from R&D Systems. PMA, BSA (fraction V), Ca²⁺⁻ and Mg²⁺⁻free HBSS, EDTA, HEPES, and Ficol-Hypaque 1077 were obtained from Sigma-Aldrich. Human serum albumin (fraction V), bisindolomaleimide I (Bis), and pertussis toxin were purchased from Calbiochem. CMFDA, CMTMR, and an Alexa 488 labeling kit were obtained from Molecular Probes. The anti-α₄ integrin subunit mAb TS2.4 and the anti-β₇ integrin high-affinity neoepeptide, 327C mAb, were gifts from T. Springer (Harvard University, Cambridge, MA). The anti-β₂ integrin extension epitope KIM127 was a gift from M. K. Robinson (UCB-Celltech, Slough, U.K.). The LFA-1-I domain allosteric IDAS-specific inhibitor A308926 and its control compound A270412 were gifts from D. Staunton (21). TS2.4-Alexa568 was a gift from F. W. Luscinakas (Harvard Medical School, Boston, MA).

Cells
Human PBL (obtained from healthy donors) were isolated from citrate-anticoagulated whole blood as previously described (8) and consisted of ~90% CD3⁺ T lymphocytes. This study was approved by the Institutional Review Board of The Weizmann Institute of Science. Memory subsets of T lymphocytes were isolated by depletion of CD45RA⁺ naive T cells by staining cells with CD45RA-FITC (IQ Products) and using a negative cell isolation kit (MACS; Miltenyi Biotec) according to the manufacturer’s instructions 2 h before functional experiments.

Flow cytometry
Cells treated with 25–50 µM of the RhoA-inhibiting peptides were stimulated for either 1 or 10 min with 10 nM CXCL12 at 37°C in the presence of Abs against the LFA-1 extension and high-affinity neoepitopes KIM127 and 327C, respectively. Cells were then washed at 4°C, stained with PE-conjugated secondary Ab (Jackson ImmunoResearch Laboratories), and analyzed by FACScan (BD Biosciences). For analysis of induction of KIM127 and 327C after PMA or CXCL9 stimulation, washed T lymphocytes were either left intact or stimulated with 100 ng/ml PMA, or 10 nM of either CXCL12 or CXCL9 at 37°C for the time intervals described in the legends in the presence of the appropriate reporter mAb. Cells were washed at 4°C, stained with PE-conjugated secondary Ab (Jackson ImmunoResearch Laboratories), and analyzed with FACScan.

Measurement of high-affinity LFA-1 states
Induction of LFA-1 high-affinity state by CXCL12 was evaluated by measuring binding of soluble 125I-ICAM-1 as previously described (14). In brief, recombinant ICAM-1-Fc fusion protein (R&D Systems) was iodinated with 125I-Na and the binding assay was performed at 37°C. Lymphocytes were stimulated for 30 s with chemokines (1 µM) and 125I-ICAM-1 (40 µg/ml). The binding reaction was stopped by rapid centrifugation of the cell suspension layered on oil cushion as previously described (14).

Imaging of fixed lymphocytes
T lymphocytes were either left intact or stimulated for 1 min with 100 ng/ml PMA at 37°C before fixation with 4% paraformaldehyde/2% sucrose and subjected to staining with the anti-α₄, mAb (TS2.4-Alexa568). Serial Z-stacked (0.4 µm/section) confocal imaging was performed with Delta Vision (Applied Precision) with a ×60 oil-dipping objective, aperture 1.42. Deconvolution and image analysis were performed using Softworx (Applied Precision). An LFA-1 cluster was defined according to size (~0.5 µm²) and fluorescence intensity (at least 2-fold greater than the cell mean fluorescence intensity (MFI)).

In vitro shear flow assays
The preparation of all chemokine-containing adhesive substrates in a parallel plate flow chamber (260-µm gap) was performed as previously described (22–24). Briefly, polystyrene dishes were coated with 1 µg/ml protein A followed by coimmobilization of either heat-inactivated or intact CXCL12 or CXCL9, each at a concentration of 2 µg/ml for 3 h at 4°C. Substrates were subsequently overlaid with various concentrations of ICAM-1-Fc. Ligand densities were assessed as previously described (25). Isolated T cells were washed in 5 mM EDTA containing medium and were suspended in H/H medium (HBSS containing 2 mg/ml BSA and 10 mM HEPES, pH 7.4) in the presence of the indicated peptides or reagents. For RhoA inhibition, PBL were pretreated with 25 µM of the inhibiting peptides for 30 min at 37°C. For DAG-dependent PKC inhibition, PBL were pretreated for 30 min at 37°C with 5 µM Bis. The LFA-1 domain was inhibited by pretreatment of cells for 5 min with the allosteric IDAS-specific inhibitor A308926 (21), or its control compound, A270412, or carrier alone (DMSO). All flow experiments were performed at 37°C. The entire period of cell perfusion was recorded as described elsewhere (22). All cellular interactions with the adhesive substrates were determined by computer-assisted tracking of individual cell motion in at least two fields of view (each 0.17 mm² in area). Transient tethers were defined as cells that attached briefly (<3 s) to the substrate; arrest (firm) tethers were defined as tethered cells immediately stopping for at least 3 s (22). More than 90% of
Transient tethers lasted <1 s. Frequencies of adhesive categories in differently pretreated cells were determined as a percentage of cells flowing along 0.9-mm paths in the focal plane of the substrates. For assessment of in situ conformational changes of lymphocyte surface LFA-1 at contacts lasting for less than a second, PBL were perfused over surface-bound KIM127 (coated at a concentration of 0.2 μg/ml, coimmobilized with 2 μg/ml inactivated or active chemokine) as previously described (22). Data from representative experiments were expressed as the mean range of two fields of view.

**Intravital microscopy analysis of lymphocytes in Peyer’s patches**

Naive lymphocytes (~70% T, 30% B cells) were isolated from peripheral lymph nodes and Peyer’s patches from young BALB/c mice (Harlan Breeders). Isolated lymphocytes were resuspended at 5 × 10⁶/ml in bicarbonate-free DMEM supplemented with 20 nM HEPESS/5% FCS (pH 7.1), treated with the peptides of interest (50 μM, 60 min at 37°C), and subsequently labeled with either CMFDA or CMTMR for 30 min at 37°C as described elsewhere (14). After washing, 3 × 10⁵ labeled cells, resuspended in PBS and 10% FCS (pH 7.2) were injected i.v. through the tail vein. In situ video microscopic analysis was conducted in HEV in the secondary lymphoid organ Peyer’s patch shortly after injection as previously described (14). Cell behavior was analyzed over a period of 20–30 min starting at 2 min after i.v. injection. Film strips of digitalized frame sequences were generated with a time delay between frames of 12.5 ms. Image analysis was performed with an automated module in NIH Image. Adhesive interactions lasting 1, 3, and 10 s were scored. The murine studies have been approved by the Comitato etico Università degli Studi di Veron.

**Results**

RhoA regulates earliest LFA-1-mediated lymphocytes arrest on HEVs in vivo

RhoA has been implicated in CCL21-induced LFA-1-mediated arrest of murine lymphocytes on HEVs within Peyer’s patches (PP-HEV) (13). In that study, the ability of rolling lymphocytes to arrest and strengthen adhesion under continuous shear stress conditions for at least 10 s was probed. The 23/40 region of RhoA was found to be essential for both arrest and subsequent adhesion strengthening of murine T lymphocytes on HEVs. Because the role of this critical RhoA region in the very initial LFA-1-mediated events, i.e., transient interactions lasting 1 s or stable arrests lasting 3 s was never investigated, we used a frame-by-frame analysis of videotaped segments of T lymphocytes rolling and arresting on HEVs to test the effect of a RhoA 23/40-interfering peptide on these earliest LFA-1-mediated events. RhoA inhibition was found to disrupt both transient interactions lasting 1 s and the subsequent stable arrests lasting 3 s (Fig. 1), since inhibition of the 23/40 region of RhoA in T lymphocytes interacting with the HEVs caused a dramatic decrease in both fractions (58 and 60% inhibition, respectively) compared with the lymphocytes treated with a control P1 peptide. Notably, all LFA-1-mediated interactions, including the shortest ones, were eliminated by pertussis toxin pretreatment of the T lymphocytes, consistent with the notion that all types of LFA-1-mediated adhesions in this model are mediated by Gt signaling triggered by HEV-displayed chemokines. Importantly, RhoA inhibition did not interfere with lymphocyte rolling, a process mainly dependent on lymphocyte L-selectin but not on LFA-1 (26). These data demonstrate that the 23/40 region of RhoA plays a key role in both transient and stable LFA-1 adhesiveness triggered by endothelial chemokines in lymphocytes rolling on PP-HEVs. These events precede the subsequent adhesion strengthening events established over 10 s or more.

RhoA regulates CXCL12-induced LFA-1 extension and subsecond induction of LFA-1 adhesiveness to ICAM-1 under shear stress

To test whether and how RhoA regulates LFA-1 activation in human lymphocytes, we treated human CD3⁺ PBL with the two distinct P1-linked RhoA-inhibiting peptides and measured their adhesiveness under flow conditions to ICAM-1 coimmobilized with the prototypic chemokine CXCL12. As expected (10), CXCL12 coimmobilized with either low- or medium-density ICAM-1 increased the percentage of transient and firm LFA-1 adhesions when encountered by flowing T cells even without a prior selectin-mediated rolling step (Fig. 2A). On low ICAM-1, the RhoA 23/40-interfering peptide (P1–23/40) eliminated all CXCL12-triggered LFA-1 interactions (Fig. 2A, left), while on medium as well as on high ICAM-1 densities, P1–23/40 reduced CXCL12-triggered interactions by ~50% (Fig. 2A, right, and data not shown). Notably, the RhoA 92/119-interfering peptide (P1–92/119) did not affect rapid CXCL12-triggered LFA-1 adhesion to ICAM-1 at any density tested (Fig. 2A and data not shown) in contrast to its ability to suppress chemokine-triggered LFA-1-mediated adhesion to low-density ICAM-1 measured under prolonged static conditions (13). This interfering peptide did, however, markedly reduce lymphocyte motility over CXCL12-ICAM-1 substrates under shear flow, a process taking place 2–3 min after arrest (data not shown). Importantly, neither RhoA 23/40 or 92/119 had any effect on adhesion to ICAM-1 in the absence of the chemokine (Fig. 2A, inset, and data not shown), demonstrating that the intrinsic ability of LFA-1 to bind, rearrange, and generate high affinity and avidity to ICAM-1 (outside-in signaling) was normal. Thus, the RhoA 23/40 but not the 92/119 region regulates the rapid inside-out LFA-1 activation transduced within subseconds by surface bound CXCL12.

Since this rapid inside-out activation was shown to require the transition of LFA-1 from a bent to an extended state, we next tested whether RhoA regulates in situ LFA-1 extension. Integrin extension was probed using the extension reporter mAb KIM127, coimmobilized with CXCL12 (10). Consistent with its interference with CXCL12-triggered LFA-1 adhesions to ICAM-1, P1–23/40 abolished the rapid CXCL12-induced formation of extended LFA-1, recognized by surface bound KIM127 under shear flow, while P1–92/119 did not (Fig. 2B). The P1–23/40 peptide also reduced CXCL12 and CCL21 triggering of the KIM127 epitope as well as triggering of a second high-affinity β₂ activation reporter, 327C, under shear free conditions (Fig. 2C and data not shown). As reported earlier, CXCL12 does not trigger T cell interactions to surface-bound 327C (10) and, therefore, in situ triggering of this

**FIGURE 1.** Immediate arrest on PP-HEV requires RhoA signaling. Intravital microscopy was performed in PP-HEV. Lymphocytes were treated with buffer (−), or with 50 μM control peptide P1, or the RhoA inhibitory peptide P1–23/40. Treated lymphocytes were labeled with either CMFDA or CMTMR and injected into mice, and their subsequent interactions with the endothelium were categorized according to duration of arrest events lasting either 1 s (○), 1–3 s (□), or at least 10 s (■). Each arrested lymphocyte was assigned to one of the three categories. Percentages depicted above bars indicate the decrease in interactions of P1–23/40-treated cells compared with lymphocytes treated with control P1. Values are the mean percentage of total interacting cells (rolling plus rolling and arrested cells) in three experiments with five to seven vessels counted per experiment. Error bars are S.D. * p < 0.01.
The 23/40 RhoA region, but not the 92/119 region, regulates chemokine-triggered LFA-1 conformational change. A, Frequency and strength of tethers mediated by PBL interacting with low (85 sites/μm²; left) or medium (190 sites/μm²; right) ICAM-1-Fc densities coimmobilized (Imm.) with heat-inactivated (−) or functional (+) CXCL12 (2 μg/ml) at a shear stress of 0.5 dyn/cm². PBL were left untreated (−) or pretreated with 25 μM control peptide P1 or the RhoA inhibitory peptides P1–23/40 or P1–92/119. The mean ± range of two fields is depicted. Results are representative of 11 experiments. A two-tailed paired Student's t test for the arrested fraction of P1-treated vs arrested fraction of P1–23/40-treated T cells yielded a p value of 0.0039 and 0.0052 (on ICAM-1 coated at 85 and 190 sites/μm², respectively). A similar comparison of an arrested fraction of P1-treated vs P1–92/119-treated T cells gave p values larger than 0.5. B, Effect of the RhoA inhibitory peptides on the frequency and strength of tethers mediated by PBL interacting with the high ICAM-1-Fc density coated at 1900 sites/μm². More than 90% of transient tethers lasted <1 s. Results are representative of six experiments. C, CXCL12-triggered induction of the LFA-1 extension epitope detected by the reporter mAb KIM127 (top panel) or the high-affinity LFA-1 epitope detected by the mAb 327C (bottom panel), in PBL pretreated with 50 μM of either P1 or P1–23/40, assessed by FACS staining. Data are presented as MFI. D, RhoA controls high-affinity LFA-1 binding to soluble ICAM-1 triggered by CXCL12. Human lymphocytes were pretreated with the indicated peptides, each at 25 μM, and were then stimulated for 30 s with buffer (no agonist, n.a.) or with 1 μM CXCL12. 125I-ICAM-1 binding to cells was determined in three experiments and mean values are depicted. At 50 μM, P1–23/40 (but not of P1–92/119) inhibited ICAM-1 binding by 70%. Error bars are SD. *, p < 0.01. E, Frequency and strength of tethers mediated by PBL interacting with ICAM-1-Fc coated at 190 sites/μm² coimmobilized (Imm.) with heat-inactivated (−) or functional (+) CXCL12 at a shear stress of 0.5 dyn/cm². Inset, frequency and strength of tethers mediated by PBL interacting with a high ICAM-1-Fc density (1900 sites/μm²) at a shear stress of 0.5 dyn/cm². PBL were pretreated with 40 μM of a PIP2-sequestering peptide (PBP10) or a control peptide (QRL). Data are expressed as the mean ± range of two fields of view and are representative of three experiments.
The inflammatory chemokine CXCL9 triggers LFA-1 adhesiveness without triggering high-affinity conformation in a RhoA 23/40-independent manner. A. Lack of CXCL9-triggered induction of LFA-1 extension epitope detected by the reporter mAb KIM127 (top panel) or high-affinity LFA-1 epitope detected by the mAb 327C (bottom panel) in PBL, assessed by FACS staining. Data are represented as MFI. Results are representative of three experiments. Frequency and strength of tethers mediated by PBL interacting with low (85 sites/μm²) or medium (190 sites/μm²) ICAM-1-Fc densities coimmobilized (Imm.) with heat-inactivated (−) or functional (+) CXCL9 (2 μg/ml) at a shear stress of 0.5 dyn/cm². PBL were left untreated (−) or pretreated with 25 μM of the control peptide P1 or the RhoA inhibitory peptide P1–23/40. B and C (top insets), Effects of P1–92/119 on CXCL9-induced PBL adhesion to low- and medium-density ICAM-1, respectively. C (bottom inset), Effect of Bis and pertussis toxin (PTX) on CXCL9-induced PBL adhesion to medium-density ICAM-1. The mean ± range of two fields is depicted. Results are representative of five experiments.


due to this end, we used a gelsolin-derived peptide that sequesters PIP₂ (20). PIP₂ sequestering significantly reduced CXCL12-triggered adhesiveness of LFA-1 to ICAM-1 at a degree comparable to RhoA 23/40 inhibition (Fig. 2, A and E). Therefore, intact PIP₂ is required for optimal RhoA 23/40 effector-mediated LFA-1 activation by CXCL12.

The inflammatory chemokine CXCL9 triggers LFA-1 adhesiveness in a RhoA-independent manner and without triggering integrin extension

Previously, we observed that in contrast to the homeostatic chemokines CXCL12 and CCL21, the inflammatory chemokine CXCL9 only weakly stimulates conformational changes of LFA-1 detected by the reporter mAb 327C (24). Reassessing the ability of CXCL9 to rapidly stimulate LFA-1 extension and acquisition of high affinity within short-term (1 min) exposure to soluble CXCL12, CCL21, and CXCL9, we found that CXCL9 failed to trigger any noticeable conformational activation of the integrin measured either with the 327C or KIM127 reporter mAbs (Fig. 3A). Although it did not induce LFA-1 conformational changes, CXCL9 could trigger robust Gi-mediated activation of LFA-1 adhesiveness to both low and medium densities of ICAM-1 under shear flow conditions (Fig. 3B). Notably, the RhoA 23/40 region was not involved in this CXCL9-triggered LFA-1 activation modality, consistent with a specialized role of this RhoA region in integrin extension (Fig. 3B). Similarly, the 92/119 region of RhoA was not involved in CXCL9-induced LFA-1 activation (Fig. 3B, inset, and C, top inset). CXCL9-triggered LFA-1 activation did not involve activity of DAG-dependent PKCs, potential integrin regulators triggered by GPCRs signals (12), as evident from the insensitivity of CXCL9-stimulated LFA-1 to the potent DAG-PKC inhibitor Bis (Fig. 3C, bottom inset). Notably, the ability of CXCL9 to stimulate LFA-1 adhesion was enhanced by shear force, since in the absence of continuously applied shear stress, a much smaller fraction of T cells developed LFA-1-mediated contacts with identically immobilized ICAM-1 and CXCL9 (data not shown). Thus, CXCL9 signals may use an alternative modality of LFA-1 activation, which possibly involves ICAM-1 outside-in activation of LFA-1 under external shear forces.

Because CXCL9 affects mainly the T memory subset within the human PBL populations characterized in our assays (28), it was possible that the insensitivity of the CXCL9-responsive T cells to RhoA 23/40 inhibition reflected a general RhoA independence of LFA-1 activation in this T cell subset. We therefore next tested whether LFA-1 expressed on a CD45RA⁻ memory subset still requires the RhoA 23/40 region for activation by the CXCL12 chemokine. Notably, the RhoA 23/40 peptide blocked LFA-1 activation by CXCL12 in this memory subset to the same extent as in total resting T lymphocytes (data not shown). Thus, the inability of 23/40 peptide to block CXCL9-triggered LFA-1 adhesiveness to ICAM-1 reflects a property of CXCL9 (and its receptor, CXCR3)-mediated activation rather than an exclusion of RhoA signaling in memory T cells.

PMA facilitates LFA-1 activation via DAG-dependent PKCs, without inside-out conformational changes and independent of the RhoA 23/40 region

LFA-1 activation can occur via exposure of lymphocytes to non-GPCR agonists. A prototypic class of such LFA-1 activators are members of the DAG-dependent PKC family (14, 29). Although rapid chemokine-induced integrin activation does not involve DAG-dependent PKCs (Ref. 10 and Fig. 3C, bottom inset), this integrin activation pathway may occur in lymphocytes exposed to multiple costimulatory signals (16, 17, 30). In T lymphocytes, DAG-dependent PKC activation by the DAG analog PMA can stimulate firm adhesiveness of LFA-1 (10) under shear stress conditions, and RhoA was previously implicated in this PKC-mediated integrin activation (13). Therefore, we next tested whether the 

FIGURE 3. The inflammatory chemokine CXCL9 triggers LFA-1 adhesiveness without triggering high-affinity conformation in a RhoA 23/40-independent manner. A, Lack of CXCL9-triggered induction of LFA-1 extension epitope detected by the reporter mAb KIM127 (top panel) or high-affinity LFA-1 epitope detected by the mAb 327C (bottom panel) in PBL, assessed by FACS staining. Data are represented as MFI. Results are representative of three experiments. Frequent and strength of tethers mediated by PBL interacting with low (85 sites/μm²), B or medium (190 sites/μm²), C ICAM-1-Fc densities coimmobilized (Imm.) with heat-inactivated (−) or functional (+) CXCL9 (2 μg/ml) at a shear stress of 0.5 dyn/cm². PBL were left untreated (−) or pretreated with 25 μM of the control peptide P1 or the RhoA inhibitory peptide P1–23/40.
RhoA 23/40 region is involved in PMA-induced activation of T cell LFA-1 under shear stress conditions. Inhibition of the RhoA 23/40 region did not alter PMA-induced adhesiveness of LFA-1 and neither did inhibition of the 92/119 region (Fig. 4A and data not shown). In contrast, and as previously shown (10), PMA-induced LFA-1 activation was totally suppressed by Bis, a blocker of both classical and novel DAG-dependent PKC isoforms, even at the highest ICAM-1 density tested (Fig. 4A, inset). Thus, the RhoA 23/40 effector region is not involved in PMA-induced PKC-mediated activation of LFA-1 under shear stress.

PMA has been demonstrated to induce LFA-1 extension measured by the LFA-1 extension epitope KIM127 (19, 31–33). Nevertheless, the short (1 min) PMA treatment, which was sufficient to potently activate LFA-1 adhesiveness (Fig. 4A), failed to induce any in situ extension of LFA-1 on the surface of T lymphocytes (Fig. 4B and C). Thus, PMA induces LFA-1 activation via a mechanism that is distinct from CXCL12-triggered RhoA-mediated LFA-1 extension. The short PMA treatment also failed to trigger the β-specific activation epitope detected by the 327C reporter mAb (data not shown), ruling out activation of this regulatory domain by

FIGURE 4. PMA induces a RhoA 23/40-independent LFA-1 activation that is not associated with conformational changes or clustering. A, Frequency and strength of tethers mediated by PBL interacting with ICAM-1-Fc coated at 60 sites/µm² at a shear stress of 0.5 dyn/cm². PBL were left intact (−) or prestimulated with 100 ng/ml PMA for 1 min as indicated. Inset, Effect of Bis on PMA-induced PBL adhesion to ICAM-1-Fc coated at 300 sites/µm² at a shear stress of 0.5 dyn/cm². Results are representative of five experiments. B, Frequency and strength of tethers mediated by PBL interacting with the KIM127 reporter mAb (1 µg/ml) at a shear stress of 0.5 dyn/cm². PBL were left intact (−) or prestimulated with PMA as described in A. N.S., not significant. Results are representative of three experiments. C, CXCL12 (10 nM)- and PMA (100 ng/ml)-triggered induction of LFA-1 extension epitope detected by the mAb KIM127, measured at 1 min and assessed by FACS staining. Cii, PMA (100 ng/ml)-triggered induction of LFA-1 extension epitope detected by the mAb KIM127, assessed by FACS staining, and measured at the indicated time points. Data in C and Cii are representative of MFI. Cii inset, Effect of 1- and 10-min PMA stimulation on intact or on Bis-treated PBL adhesion to ICAM-1-Fc (170 sites/µm²) at a shear stress of 0.5 dyn/cm². Results in C and Cii are representative of three to five experiments. D, Fluorescence images of representative PBL stained with the LFA-1 mAb TS2.4. PBL were either left untreated (basal) or treated with PMA (100 ng/ml) for 1 min. Bar, 5 µm. Di, Average number of LFA-1 clusters found in T cells either left untreated (basal) or treated with PMA (100 ng/ml) for 1 min and fixed. Cluster density was determined as indicated in Materials and Methods. N = 13. E, Frequency and strength of tethers mediated by PBL interacting with ICAM-1-Fc coated at 60 sites/µm² at a shear stress of 0.5 dyn/cm². PBL were pretreated with 2.4 nM of the allosteric I domain IDAS inhibitor A308926 (I) or its control compound A270412 (C). Where indicated, PBL were prestimulated with 100 ng/ml PMA for 1 min in the presence of the IDAS inhibitor or the control compound. Data in A, B, and E are expressed as the mean ± range of two fields of view and are representative of three experiments each.
rapid PMA signals. Importantly, induction of the KIM127 epitope by PMA was observed only after prolonged stimulation, ranging from 10 to 30 min ((Ref. 19) and Fig. 4Cii) and resulted in higher induction of LFA-1-mediated adhesions (Fig. 4Cii, inset, and data not shown). Thus, thus PMA stimulation that is sufficient to induce immediate LFA-1-mediated lymphocyte capture and arrest on ICAM-1 can take place without prior LFA-1 extension. Importantly, the long-term stimulation with PMA (10 min), which induced LFA-1 extension, activated LFA-1 adhesiveness in a manner independent of the integrity of the 23/40 RhoA region, but entirely dependent on DAG-dependent PKC activity (Fig. 4).

Activation of LFA-1 is modulated not only by affinity (i.e., conformational changes), but also by changes in integrin valency (i.e., due to enhanced lateral integrin mobility and increased density per area of the plasma membrane). Indeed, PMA was suggested to elevate LFA-1 mobility (14, 18) and thereby to facilitate LFA-1 clustering with ICAM-1. Nevertheless, the short PMA treatment that induced LFA-1 to immediately arrest T cells on ICAM-1 did not increase the degree of LFA-1 clustering on intact T cells before their immediate arrest (Fig. 4, Di and Dii).

In order for LFA-1 to become fully activated in response to immobilized chemokines and to be able to support lymphocyte arrest, allosteric activation of the I domain within the integrin’s headpiece must occur (10). We therefore hypothesized that in the absence of ICAM-1-independent, inside-out conformational changes or enhanced clustering, PMA must elevate LFA-1 adhesiveness via an outside-in (ICAM-1-induced) activation (opening) of the LFA-1-I domain. We therefore tested whether this allosteric activation of the I domain is crucial also for PMA-induced LFA-1 adhesiveness. As seen in Fig. 4E, locking the I domain in the closed inactive state using an allosteric inhibitor abolished PMA-induced LFA-1 adhesiveness to ICAM-1. Taken together, PMA induces LFA-1 activation without inducing LFA-1 extension or clustering before the contact. However, LFA-1 activated by PMA is primed to undergo rapid opening of its I domain upon binding ICAM-1. Collectively, our results suggest that both CXCL9 and PMA, although targeting distinct machineries, share the ability to stimulate LFA-1 adhesions by facilitating post ligand binding events, rather than a conformational change in the LFA-1 ectodomain before ligand binding.

Discussion

Immovilized chemokines displayed on blood vessel endothelia activate, through specific leukocyte-expressed Gi-coupled receptors, cytoplasmic effectors which switch leukocyte-expressed integrins from inactive to active states (10). The most important integrins that mediate adhesion of lymphocytes to the endothelium are LFA-1 and VLA-4, which bind to their ligands ICAM-1 and VCAM-1, respectively. The small GTPase RhoA has recently been implicated in cell motility, polarization, spontaneous integrin adhesiveness, and integrin activation by cytokines (34–36); however, its contribution to rapid integrin activation by chemokines critical for the arrest of rolling leukocytes on target endothelial beds (37) has only recently been addressed. The cardinal contribution of RhoA to in vivo integrin activation was demonstrated by the inability of murine lymphocytes to develop firm LFA-1-mediated adhesion to PP-HEVs, a process known to involve CCL21-triggered Gi-mediated integrin activation, under conditions where RhoA signaling was disrupted (13). Selective inhibition of RhoA domains was achieved with three novel cell-permeable peptides that bind distinct effector regions within the RhoA protein and block their interactions with downstream RhoA effectors; 23/40, 75/92, and 92/119 (38). Since the inhibiting peptide derived from the 75/92 region of RhoA is not involved in integrin activation, and after ruling out a role for the RhoA 92/119 region in rapid LFA-1 activation, we decided to focus our study on the RhoA 23/40 region, previously implicated in the regulation of murine LFA-1 affinity and mobility by CCL21 and CXCL12. In the present study, we demonstrate that the RhoA 23/40 domain is critical for the earliest CCL21-triggered LFA-1 activation events measured in vivo as well as for a CXCL12-induced LFA-1 extension step critical for integrin adhesiveness to ICAM-1 under shear flow, but is not involved in spontaneous or CXCL9-induced LFA-1 adhesiveness to ICAM-1 in human T lymphocytes. We also find that CCL21 and CXCL12, on one hand, and CXCL9 on the other hand, use different modalities to activate LFA-1 adhesiveness, whereas CCL21 and CXCL12 trigger LFA-1 conformational changes before ligand binding and thereby increases the pool of extended LFA-1 readily available for interacting with surface-bound ICAM-1 (10). CXCL9 triggers a unique LFA-1 activation process that takes place without conformational changes before ligand binding and therefore may rely on spontaneously extended subsets of LFA-1 that preexist on T cells and preferentially engage with ICAM-1 (4, 39). We also show that an alternative chemokine-independent, PKC-induced pathway of LFA-1 activation also does not trigger integrin conformational extension or integrin clustering before ICAM-1 binding and takes place independently of RhoA.

One of the most intriguing results of this study is the inability of CXCL9 to deliver LFA-1 extension signals via its GPCR, CXCR3. Notably, the ability of CXCL9 to trigger outside-in activation of these LFA-1 subsets is facilitated by application of force on the LFA-1-ICAM-1 complex, reminiscent of recent findings that highlight the importance of external forces in general integrin activation by chemokines (40). In this context, CXCL10, which binds CXCR3 at higher affinity than CXCL9 (41), was able to increase both the KIM127 and 327C activation epitopes on a subset of T cells and this conformational regulation was entirely RhoA-dependent (data not shown), further demonstrating the tight linkage between inside-out conformational LFA-1 activation and RhoA 23/40 activity. This is a first suggestion that a given GPCR can facilitate outside-in LFA-1 activation when occupied by a weak chemokine agonist but can trigger both inside-out conformational integrin activation and outside-in activation by ligand when occupied by a strong chemokine agonist. Whether these results apply to other pairs of chemokines and GPCRs remain to be seen, but it is likely that when a chemokine signal is intrinsically weak (e.g., low-affinity binding of CXCL9 to CXCR3), it is insufficient to induce integrin conformational switch via RhoA, before ligand binding, but can still facilitate outside-in integrin activation.

The ras-like small GTPase Rap1 has recently emerged as an important regulator of early integrin activation by chemokine signals (42–44). Its crucial role in LFA-1 triggering is also evident from the latest description of a human genetic defect, leukocyte adhesion deficiency (LAD-III) (44), in which the expression of the Rap1 GEF, CalDAG-GEF1, is impaired (45). RhoA may be implicated in local generation of PIP2 (27, 46, 47), a substrate for CalDAG-GEF1 activation (48). Interestingly, CXCL9-triggered LFA-1 activation, as well as PMA activation of the integrin, although RhoA 23/40 independent, still required intact CalDAG-GEFI because it was largely defective in LAD-III T lymphocytes (R. Pasvolsky, S. W. Feigelson, A. Sagiv, V. Grabovsky, S. S. Kilic, A. Etzioni, and R. Alon, manuscript in preparation). Thus, both RhoA-dependent and RhoA-independent LFA-1 activation by chemokines are tightly regulated by the Rap1 activator CalDAG-GEFI. Furthermore, since inhibition of RhoA did not reduce LFA-1 adhesiveness by >50%, it appears that even with arrest chemokines that trigger LFA-1 affinity via RhoA, this GTPase may...
generally need to cooperate with Rap1 to optimally trigger earliest LFA-1 activation.

An open unresolved question is the identity of the RhoA effector implicated in LFA-1 affinity regulation. Binding of GTP by Rho proteins is accompanied by a conformational change in two regions, switch I and II (49). Effectors known to bind at or nearby the RhoA 23/40 region, which overlaps with switch region I, include mDia (50), PKN, ROCK Citron (38, 51, 52), and phospholipase D (53). We have ruled out a role for ROCK in rapid LFA-1 activation by chemokines (10, 13). Another potential target of RhoA is PIP2 that catalyzes the synthesis of PIP2, a key regulator of the integrin-activating adaptor talin (54) implicated in chemokine-mediated LFA-1 activation (10, 27, 55–57). Using a compound that sequesters PIP2 from membranes (20), we found that PIP2 is involved in CXCL12-induced adhesiveness of LFA-1, implying this key regulatory lipid as a target of the RhoA 23/40 effector domain. Importantly, we cannot rule out the possibility that the effect seen with the gelsolin-derived PIP2-sequestering peptide resulted from its association with talin and α-actinin 4 (58) and potential blockage of their interactions with LFA-1.

In addition to the crucial roles played by arrest chemokines in triggering specialized integrin-activating Gi signaling machineries at the plasma membrane, members of the PKC family (59–61), particularly DAG-dependent PKCs, have been implicated in early LFA-1 activation events (10, 15, 17). DAG-dependent PKC activation may be triggered by prolonged rolling on endothelial selectins either by occupancy of selectin ligands alone (17) or in combination with cytokine receptors (16). The mechanism by which PKC triggers integrin activation is not fully understood; however, the potent PKC agonist PMA induces phosphorylation of numerous cytoskeletal proteins, which enhance cytoskeletal anchorage of LFA-1 (18). In addition, PKC induces phosphorylation of Thr758 in the β2 chain of LFA-1, which can modulate LFA-1 interactions with the actin cytoskeleton (62, 63). Previous studies tested the effect of PMA on LFA-1 activity after prolonged exposure periods (10–30 min) and demonstrated that this stimulation induces LFA-1 conformational changes detected by various reporter mAbs (10, 19, 21, 64). Importantly, although 1 min stimulation of T cells with PMA is sufficient to trigger rapid LFA-1 adhesiveness to ICAM-1, it does not induce conformational changes of LFA-1. Accordingly, PMA stimulation of T cells does not increase LFA-1 affinity to soluble ICAM-1 (14). Notably, neither short nor long PMA pretreatment of T cells induced any noticeable LFA-1 clustering before lymphocyte contact with ICAM-1. This is in agreement with previous findings, suggesting that PMA-facilitated LFA-1 clustering is favored by cell-cell interactions and is driven by LFA-1 occupancy by ligands (65). Although formation of preformed rather than ligand-induced clusters was suggested to increase LFA-1 avidity to ICAM-1 (66), PMA is unlikely to promote LFA-1-mediated T cell arrest on ICAM-1 through such a mechanism. Taken together, our data suggest that short term PMA-induced LFA-1 activation is a PKC-driven process that does not involve conformational activation of LFA-1 before ICAM-1 binding and is not the cause of an enhanced preformed clustering of LFA-1. Because ICAM-1 can rearrange LFA-1 via an outside-in activation mechanism (32), we suggest that PMA-triggered PKC activation may facilitate this ICAM-1 outside-in activation of LFA-1 and thereby also drive rapid postligand occupancy clustering. Indeed, PKC-facilitated LFA-1 adhesion to ICAM-1 was absolutely dependent on allosteric activation of the I domain, most probably by ICAM-1.

In conclusion, we have demonstrated both in vitro and in vivo a key role for RhoA in a subsecond induction of LFA-1 extension triggered by two homeostatic chemokines (CXCL12 and CCL21) during rapid lymphocyte arrest on ICAM-1 under shear flow. We also unraveled an alternative LFA-1 activation modality by an inflammatory chemokine (CXCL9) that does not involve such induction of LFA-1 extension and is not mediated by RhoA. The RhoA-LFA-1 signalosome is also dispensable for a chemokine-independent modality of LFA-1 activation by DAG-dependent PKCs, a modality that also takes place independent of LFA-1 extension and acquisition of high-affinity conformation. Importantly, our findings extend the notion that LFA-1 activation can be achieved not only by an a priori inside-out conformational activation of the integrin on the plasma membrane. Rather, outside-in activation of the integrin by its own ligand can take place at subsecond contacts and this activation can be facilitated by either Gi chemokine signals or by Gi-independent DAG-dependent PKC signals. Future studies should identify in other cell types and other chemokine-integrin pairs the relative contribution of the RhoA axis to rapid LFA-1 extension in particular and to chemokine triggering of LFA-1 adhesiveness in general.

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Disclosures

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References


