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# Cells on the run: shear-regulated integrin activation in leukocyte rolling and arrest on endothelial cells

Ronen Alon[1] and Klaus Ley[2]

The arrest of rolling leukocytes on various target vascular beds is mediated by specialized leukocyte integrins and their endothelial immunoglobulin superfamily (IgSF) ligands. These integrins are kept in largely inactive states and undergo *in situ* activation upon leukocyte–endothelial contact by both biochemical and mechanical signals from flow-derived shear forces. *In vivo* and *in vitro* studies suggest that leukocyte integrin activation involves conformational alterations through inside-out signaling followed by ligand-induced rearrangements accelerated by external forces. This activation process takes place within fractions of seconds by *in situ* signals transduced to the rolling leukocyte as it encounters specialized endothelial-displayed chemoattractants, collectively termed arrest chemokines. In neutrophils, selectin rolling engagements trigger intermediate affinity integrins to support reversible adhesions before chemokine-triggered arrest. Different leukocyte subsets appear to use different modalities of integrin activation during rolling and arrest at distinct endothelial sites.

**Addresses**
[1] Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel
[2] Division of Inflammation Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037, United States

Corresponding author: Alon, Ronen (Ronen.alon@weizmann.ac.il)

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## Introduction

Leukocyte emigration at specific target sites is regulated by adhesive cascades mediated by three sequential and partially overlapping steps initiated by selectin-mediated capturing and rolling, followed by chemokine-triggered activation and integrin-dependent arrest on endothelial immunoglobulin superfamily (IgSF) ligands [1]. Different selectin and integrin family members, together with diverse endothelial-displayed chemokines, provide large combinatorial specificity to this process. Although adapted to operate under shear flow conditions [2,3] selectin and integrin bonds are differently regulated at leukocyte–endothelial contacts. Whereas the adhesiveness of the selectins and their cognate glycoprotein ligands is generally not modulated by *in situ* endothelial signals, the avidity of leukocyte integrins to their endothelial ligands is rapidly and reversibly regulated by chemokine-triggered signals [4] transduced via specialized G-protein-coupled receptors, GPCRs (for a detailed review on chemokines please refer to reference [5]). In many cases, endothelial chemokines trigger conformational integrin switches within a fraction of a second and at the actual site of leukocyte arrest by triggering inside-out signals and facilitating additional ligand-induced integrin activation events [6]. Recent studies suggest that shear forces exerted on the arrested leukocyte at the endothelial contacts facilitate chemokine-stimulated integrin activation [7•]. In addition to this apparently universal modality of leukocyte integrin activation, neutrophils and perhaps other myeloid leukocytes use their rolling interactions on specific endothelial selectins to ligate glycoproteins that stimulate specific tyrosine kinases to partially activate integrins during the rolling period [8•]. Whereas *in situ* integrin activation by arrest chemokines is abrupt and highly localized within the direct endothelial site of arrest [9], these weaker selectin-triggered kinase signals appear to stimulate integrins on the entire surface area of the rolling leukocyte in a gradual manner. Selectin-mediated rolling also increases the probability of chemokine and integrin ligand encounter by leukocytes and thereby facilitates integrin activation even without triggering signaling effectors. In this review, we focus on recent works that shed light on the molecular and mechanical basis of leukocyte integrin activation in blood vessels and discuss their physiological outcome during lymphocyte and neutrophil rolling and arrest on various endothelial targets.

## Initial selectin-mediated leukocyte capturing to blood vessels—a force regulated process

Selectins are the main receptors that mediate the initial capture of circulating leukocytes to vascular endothelial surfaces [10]. Leukocyte capture is followed by rolling adhesions, which proceed from seconds (for most leukocytes) to a few minutes (neutrophils) [11]. The selectins comprise a three-member family with highly conserved N-terminal C-type lectin and epidermal growth factor (EGF)-like tandem domains that bind sialyl-Lewis$^{x}$-like carbohydrate ligands [10]. L-Selectin is expressed on most circulating leukocytes and is the key receptor that initiates leukocyte capture events in high endothelial venules in secondary lymphoid tissues and at peripheral sites of injury and inflammation. L-Selectin can also bind leukocyte ligands, particularly PSGL-1, and this

interaction can enhance the capture of leukocytes by intravascular adherent leukocytes. P- and *E*-selectins are inducibly expressed in both acutely and chronically inflamed endothelial beds, and in many of these settings their contribution is redundant.

Selectin-mediated adhesions are characterized by fast on-rates and off-rates and exceptional resistance to disruptive shear forces exerted on the leukocyte at the vessel wall [12]. Selectins undergo conformational changes upon ligand binding that decrease their off-rate under tensile forces, making them 'catch bonds' [13]. Recent evidence suggests that the lectin-EGF interdomain hinge in L-selectin and *P*-selectin may critically regulate this stabilization via force-stabilized extension [2,3]. While *E*-selectin may also need to utilize shear forces to stabilize extension, this selectin often generates instantaneous multivalent bonds with ligands presented on closely spaced multivalent glycans [10]. Increasing evidence suggests that in order to load forces, L-selectin, *P*-selectin, and their glycoprotein ligands need to be properly anchored to the cytoskeleton either directly or indirectly through engagements with submembranal assemblies [14,15]. Selectins and their ligands are localized to microvillus-like projections, favorable sites of leukocyte–endothelial collisions [16] and possibly major sites of integrin activation (Figure 1). The topographic distribution of selectins and ligands on both leukocyte and endothelial cell microvilli is thought to be important not only for increasing their effective association rates [16], but also as a means to dissipate a portion of the dissociation energy exerted on individual adhesive bonds along the microvillus axis.

## Leukocyte integrin activation at endothelial contacts

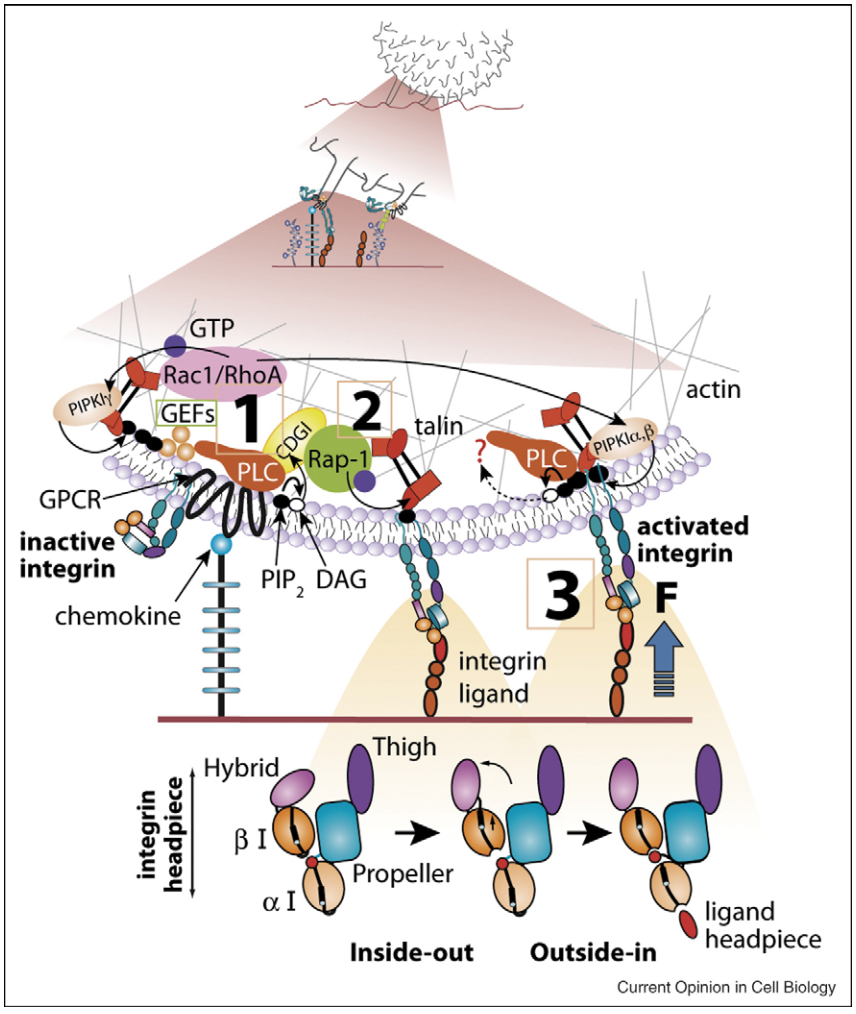
### Chemokine signals to leukocyte integrins—conformational changes in the ectodomain triggered by and coupled to cytoskeletal associations

Under normal flow, selectin bonds cannot arrest, on their own, rolling leukocytes. Leukocyte arrest is nearly exclusively mediated by members of the integrin superfamily [17]. Both *in vivo* and *in vitro* studies in numerous leukocyte–endothelial model systems have established that leukocyte arrest results from an abrupt and local activation of integrin adhesiveness to cognate endothelial ligands [18–20]. This rapid triggering of integrin adhesiveness is preferentially transduced by leukocyte GPCRs through local triggering of heterotrimeric G proteins, primarily of the Gi/o subtype, and in neutrophils specifically $G\alpha_{i2}$ [21], as they encounter immobilized, endothelial-presented chemoattractants, primarily chemokines and some lipid attractants (For reviews please refer to [20,22]). As in other cell types, integrin conformation regulation by GPCRs involves bidirectional activation initiated by inside-out conformational activation and reinforced by ligand binding (outside-in activation, Figure 1) [17].

*In vivo* analysis suggests that integrin-activating endothelial-displayed chemokines and chemoattractants transduce their signals to integrins on rolling lymphocytes and monocytes in an abrupt rather than in a stepwise manner [19,23]. Integrin heterodimers on the majority of circulating leukocytes are kept in an inactive conformation via a cytoplasmic clasp that can be disrupted by the binding of the talin head domain to the integrin β subunit tail [24]. The most detailed information about integrin conformational activation comes out from structural and kinetic analysis of $\alpha_v\beta_3$ and LFA-1-mediated interactions with cognate ligands [17] (Figure 1). The LFA-1 α and β subunits were shown to move apart in response to overexpression of the talin head or to a chemokine signal [25], a change that results in release of multiple constraints on the integrin headpiece [17]. LFA-1 activation by chemokine signals as well as by ICAM-1 under shear flow was also shown to depend on intact talin [19]. Introduction of mAb probes for integrin ectodomain extension and headpiece activation also allowed to probe chemokine-induced conformational LFA-1 switches in real time [19]. These and other studies suggest that stimulatory chemokines capable of triggering integrin-mediated leukocyte arrest ideally function when encountered by the stimulated leukocyte in juxtaposition to the integrin ligand, since upstream exposure of rolling leukocytes to immobilized chemokine signals is insufficient to trigger integrin adhesiveness at a downstream field [26]. These results collectively suggest that GPCR engagement by an arrest chemokine can trigger a signaling cascade that activates neighbor integrin(s) *in situ* in an instantaneous manner via a talin-dependent mechanism and that prior encounters of chemokine signals during the rolling phase are not necessary for integrin activation [9,19]. In T lymphocytes interacting with chemokines and ICAM-1 under shear flow, full stimulation of high LFA-1 affinity by prototypic arrest chemokines occurs within $<0.4$ s of cell contact with surface bound ICAM-1 [19] and appears to involve only small subsets of the entire surface expressed integrin. The chemokine signal is thought to first trigger a reversible extension of the LFA-1 integrin that dramatically increases the association rate of its headpiece for a nearby surface-immobilized ICAM-1 molecule [17]. This, and subtle conformational changes in the integrin β subunit hybrid domain [17] locally prime the integrin headpiece for a subsequent and instantaneous ligand-induced rearrangement, resulting in full activation (opening) of the headpiece I-domains [19] (Figure 1).

Recent studies on T lymphocytes indicate that certain chemokines can trigger VLA-4 and LFA-1 adhesiveness even in the absence of integrin extension and induction of headpiece conformational epitopes associated with high affinity recognition [27,28], suggesting that chemokine

**Figure 1**




Rapid GPCR-mediated integrin activation by endothelial-immobilized chemokine; involvement of Rap-1 and Rho GTPases. A proposed intracellular signaling cascade from a chemokine (blue ball) bound to a GPCR to the LFA-1 integrin (blue heterodimer) within a single microvillus of a lymphocyte rolling on L-selectin ligands. The front (right) microvillus is occupied by L-selectin ligand, whereas integrin activation takes place during occupancy and stretching of the rear microvillus, which may be co-occupied via L-selectin (not drawn). Canonical bidirectional activation of LFA-1 from inactive bent state to a semi-active extended state (unbent ectodomain with the two headpiece I-domains in closed conformation with low affinity to ligand) and to high affinity extended state (unbent integrin with both of the I-domains stabilized in an open state, see the ribbon diagram of the various LFA-1 headpiece states in the lower panels) is shown. Upon initial encounter of the proper endothelial-bound chemokine, the leukocyte GPCR is activated within milliseconds and transmits signals converting a nearby inactive (folded) integrin to its extended conformation, probably through Gβγ-triggered phospholipase C (PLC)-mediated hydrolysis of PIP2, activation of CalDAG-GEFI (CDGI), its target Rap-1 and the downstream effector talin (steps 1,2). GPCR-triggered GEFs of RhoA and possibly of Rac1 co-activate downstream PIP2-generating kinases (PIPKIα, β, γ). Local increase of PIP2 directly activates talin binding to and unclasping of the inactive integrin, rendering it extended (step 2) and facilitating its full outside-in activation by ICAM-1 (step 3). Force exerted on the ligand-occupied integrin facilitates this outside-in activation by opening the I-domains on both the α and β subunits (lower panels) and both CDGI activated Rap-1 and PIP2 activated talin may further stabilize the unclasped integrin. Integrin homodimers may interact with pre-existent ligand dimers (not shown) and increase binding avidity. Similar events can take place in lymphocytes and other leukocytes rolling on P and *E*-selectins (not shown). The degree of integrin extension, headpiece activation by ligand, force loading and headpiece rearrangements, and ligand-induced dimerization is likely to differ among distinct integrins, GPCRs, cell types, and species. The lower panels were modified from reference [17].

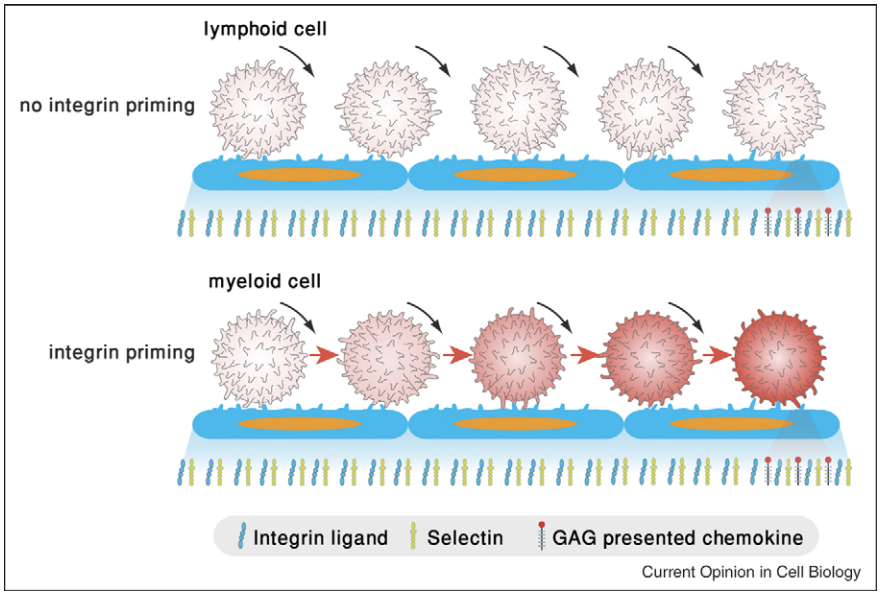
signals may facilitate integrin activation also via rapid post-ligand-binding stabilization rather than by an *a priori* conformational activation of the integrin ectodomain. With the increasing evidence that integrins undergo direct activation by forces [29•,30], it becomes apparent that the ability to rapidly respond to and undergo activation by shear forces relies also on proper anchorage of the integrin to the cell cytoskeleton. Anchorage of the $\alpha_4\beta_1$ heterodimer to the actin cytoskeleton facilitates mechanical stabilization of VLA-4-VCAM-1 bonds under

external forces without increasing VLA-4 affinity to soluble ligand [31]. A recent study indicates that immobilized chemokines locally stiffen membrane compartments nearby VCAM-occupied VLA-4, and thereby strengthen VLA-4-VCAM-1 bonds (Schmitz, submitted for publication). Extended LFA-1 is thought to be pre-anchored to the lymphocyte cytoskeleton and can undergo further anchorage upon occupancy by ICAM-1 [32•], highlighting the possibility that chemokine signals can stimulate integrin adhesiveness under shear flow by anchoring integrins right after they bound their cognate extracellular ligands. Following arrest, chemokine signals may within seconds increase integrin clustering with additional endothelial ligand molecules probably by acting on mobile integrin subsets [26]. The consequence of these versatile activation modalities is that subsets of VLA-4-VCAM-1 and LFA-1-ICAM-1 as well as Mac-1-ICAM-1 bonds can support both rolling and firm adhesions depending on their basal affinity states [26,27,33] as well as on the degree by which the integrin ligand can rearrange and activate the headpiece of its cognate integrin [9]. This capacity is likely to also depend on the exposure time and local availability of the activating chemokine [26].

## Selectin signaling to integrins—a global activation modality before leukocyte arrest

Recently, engagements of neutrophil PSGL-1 during active rolling on *E*-selectin and to a lesser extent on *P*-selectin were demonstrated to activate the tyrosine kinase Syk that rapidly transmits weak reversible activation signals to subsets of LFA-1 on rolling neutrophils [8•]. The PSGL-1-triggered signals stabilize these LFA-1 subsets at intermediate affinity states (i.e. unbent extended integrins with their headpiece in a closed conformation with low affinity to ICAM-1) that mediate rolling adhesions rather than firm arrests. Unlike the abrupt integrin activation events transduced by local GPCR signals, *E*-selectin-triggered signals require seconds of rolling during which LFA-1 conformations are globally triggered over the entire surface of the rolling leukocyte (Figure 2). These selectin engagements lower the threshold for subsequent arrest induction by low concentrations of immobilized chemokine [8•]. Under these conditions, neutrophils can roll slowly for minutes without becoming adherent. Although LFA-1 clearly engages its ligand ICAM-1, PSGL-1-mediated LFA-1 activation does not result in full ligand-induced LFA-1 activation. Neutrophils rolling for minutes on inflamed

**Figure 2**




Accumulating vs. abrupt switches in integrin avidity states in rolling leukocytes. *Upper panel*: A lymphocyte rolls via PSGL-1 or other endothelial selectin ligands (not shown) but fails to undergo integrin activation by successive rolling engagements owing to insufficient triggering of kinases, PLCs, and secondary messengers. When lymphocytes encounter high density of arrest chemokines juxtaposed to integrin ligands, their integrins are locally and instantaneously activated by $G\alpha_i\beta\gamma$ signals as delineated in Figure 1. *Lower panel*: A rolling neutrophil and possibly other myeloid leukocytes can integrate weak integrin activation (priming) signals through engagements of *E*-selectin ligands including PSGL-1, which trigger phosphorylation and activation of spleen tyrosine kinase Syk [8•,58]. Within seconds, LFA-1 and possibly other integrins are stabilized in an extended intermediate affinity state on the entire plasma membrane. These integrins can form reversible adhesive bonds with endothelial ICAM-1 sufficient to slow down selectin-mediated rolling. Like the lymphocyte, when the rolling neutrophil encounters high density of arrest chemokines, its integrins can undergo robust bidirectional activation via $G_i$ signals. Post arrest, ligand-driven integrin microclustering can immediately follow to further stabilize the integrin-mediated contact. Co-ligation of multiple selectin ligands and integrins trigger both Src and Syk kinases to further activate integrin avidity and adhesion strengthening [59].

endothelial cells also accumulate cytosolic $Ca^{2+}$, followed by a large rise in $Ca^{2+}$, upon arrest and integrin engagement [34]. It is unclear, however, whether Syk and $Ca^{2+}$-dependent signals triggered by rolling engagements of neutrophils cooperate in this integrin activation modality. Another unresolved question is why selectins do not contribute to integrin activation in lymphocytes, including memory lymphocyte subsets that express *E*-selectin binding PSGL-1 glycoforms but fail to activate their LFA-1 without chemokine signals (Alon, unpublished). One possibility is that lymphocyte ZAP-70, Syk, and Src kinases cannot substitute for neutrophil Syk, and Src kinases (Zarbock and Ley, unpublished). Another open question is to what extent lymphocytes as well as neutrophils and monocytes utilize L-selectin to signal and activate leukocyte integrins before leukocyte arrest on target endothelium. L-selectin signaling to integrins, including integrin translocation to the plasma membrane, has been argued to control various adhesion strengthening events in neutrophils [35]. There is no doubt, however, that in both myeloid and lymphoid cells, selectin-mediated rolling facilitates integrin-mediated leukocyte arrest by topographical means, that is, by microvilli flattening that enhances chemokine encounter by the rolling leukocyte.

### GTPases implicated in spontaneous and in chemokine-stimulated integrin adhesiveness under shear flow

Both inside-out and outside-in leukocyte integrin activation stimulated by arrest chemokines are triggered by specialized chemokine-occupied G-protein-coupled receptors (GPCRs) [20,36,37]. Increasing evidence suggests that full integrin activation requires the GPCR-transduced signals to activate integrin affinity and clustering as well as to stabilize integrin–ligand complexes in properly anchored states to allow these complexes to undergo optimal activation by shear forces and develop high detachment [9]. The relative contribution of each of these modalities to the activation of specific integrins by specific GPCRs is likely to vary between different types of integrins (LFA-1 versus VLA-4), GPCRs, leukocytes and species [20,36,37]. Conserved players in GPCR signaling to integrins at leukocyte endothelial contacts are Rap and Rho small GTP-binding proteins, which undergo direct activation by chemokine signals [36] and talin, a key integrin cytoskeleton linker that controls both integrin affinity and anchorage states [19,24,27,38•] (Figure 1). The most upstream element of chemokine signaling to integrins is PLC [37]. Although knockouts of the major Gi activated PLCs in neutrophils, PLC$\beta_2$ and PLC$\beta_3$ do not exhibit major trafficking defects possibly due to redundancy, pharmacological blockage of total PLC activity in platelets, monocytes and neutrophils impair integrin activation by GPCR agonists [21,39,40,41•]. A key target for PLC activity underlying GPCR-mediated integrin activation in these cells as well as in lymphocytes is CDGI [39,41•,42•,43], a $Ca^{2+}$ and DAG-dependent GEF for the ras-like small GTPase Rap1. This GTPase has emerged over the past few years as a key regulator of rapid integrin activation by a variety of inside-out signals [44]. The central role of its activator, CDGI, in integrin activation in platelets, neutrophils, and lymphocytes is highlighted by patients suffering from a rare adhesion deficiency syndrome termed LAD-III [41•]. LAD-III patient cells exhibit reduced levels of this GEF and suffer from global defects in integrin activation by multiple GPCR agonists. These defects include abrogated GPCR-transduced affinity modulation of the platelet integrin $\alpha_{IIb}\beta_3$ and the lymphocyte integrin LFA-1, the loss of $\beta_2$ integrin-mediated neutrophil arrest on endothelial ligands under shear flow [41•], and deficiencies in both LFA-1 and VLA-4-mediated lymphocyte arrest. Loss of CDGI in mouse platelets and neutrophils mimic the LAD-III syndrome [42•], but murine lymphocytes lack this GEF [39,41•], highlighting species-dependent differences in GPCR/Rap-1-mediated inside-out integrin activation.

GPCR-stimulated Rap-1 appears to trigger high integrin affinity by activating talin [45] (Figure 1). Talin1, the major leukocyte talin isoform, is recruited by activated Rap-1 and its effector RIAM to the vicinity of $\beta_3$ and $\beta_2$ integrins and switch these integrins into high affinity and high avidity states [45]. Although this Rap-1 regulation of talin was so far established only in prolonged inside-out integrin activation processes, it is plausible that CalDAG-GEFI-activated Rap-1 can also locally modulate talin–integrin associations during the earliest integrin activation events underlying GPCR-triggered platelet activation [46] and GPCR-stimulated leukocyte arrest on vascular endothelium. In addition to being recruited by RIAM, talin can be directly activated to bind and stimulate integrins by local changes in the plasma membrane lipid PtdIns(4,5)$P_2$ (PIP2). This phosphoinositide not only activates talin [47] but is a key substrate for PLC-mediated CDGI activation and Rap-1 stimulation [39]. In addition, Rap1 can upregulate LFA-1 avidity through recruitment of a direct effector, RAPL, to the cytoplasmic interface of the LFA-1 $\alpha$ chain [48]. Consistent with this possibility, initial LFA-1-mediated arrest of RAPL null T cells on HEVs is normal, whereas subsequent adhesion strengthening is reduced (Kinashi, personal communication). Interestingly, PIP2 production can be driven by combined activities of a talin-associated PtdIns(4)P 5 kinase, PIPKI$\gamma$ [49], and two other PIP2 generating enzymes, PIPKI$\alpha$ and PIPKI$\beta$, which may be activated by GPCR-stimulated Rho proteins. Indeed, rapid lymphocyte and neutrophil integrin activation by endothelial chemokine signals require intact RhoA [50]. *In situ* chemokine-activated RhoA may locally elevate PIP2 near integrins and thereby activate talin both directly and indirectly, that is, via a PLC-CalDAG-GEFI-Rap-1-RIAM axis [45]. Rho GTPases can also remodel actin

filaments and elevate their bundling within integrin–GPCR signalosomes, thereby stiffening the cortical cytoskeleton near ligand-occupied integrins, which, in turn, may facilitate mechanical activation of these integrins (Figure 1). Lastly, Rap-1 and RhoA may activate target integrins by sequestering integrin partners that impose constraints on integrin unclasping and activation (Figure 1). It is therefore possible that full integrin activation only occurs when both Rap-1 and RhoA are co-activated by chemokine signals. Recent *in vitro* results further suggest that the relative contribution of Rap-1 and RhoA signaling to integrin activation can substantially vary with the type of integrin and with the stimulatory GPCR [28,43] within distinct integrin–GPCR signalosomes.

### Rapid integrin activation machineries: preformed GPCR assemblies on leukocytes

As integrin activation by surface-bound chemokines under physiological conditions is a highly localized event which takes place over a fraction of a second, chemokines must be able to organize a localized network of interacting proteins, possibly within supramolecular structures localized on leukocyte-surface microvilli, preferential sites of leukocyte–endothelial contacts under shear flow (Fig. 1) [16]. Indeed some integrin-activating GPCRs are clustered on lymphocyte microvilli [51]. Each supramolecular assembly of a given GPCR may consist of distinct combinations of regulatory GTPases, their GEFs, and GAPs, as well as of GPCR scaffolds like filamins, myosins, and β-arrestins together with integrin effectors like talin and talin-binding cytoskeletal adaptors like vinculin and α-actinin. Notably, VLA-4 and LFA-1 are topographically segregated on the leukocyte surface [52]. It is therefore possible that a given GPCR scaffold and an integrin target communicate in distinct membranal compartments. In addition, GPCRs may exist as distinct homodimers or heterodimers. Different GPCR assemblies are known to associate with different heterotrimeric G proteins and their associated effectors and may therefore signal differently to their integrin targets. For example, a single chemokine, CCL5, transduces rapid integrin avidity stimulatory signals to monocytes and effector T cells through its GPCR CCR1 but fails to activate integrins through a different receptor, CCR5 on the same cells [53].

## Conclusions and perspectives

Chemokine signals are the most efficient endothelial-displayed triggers of integrin activation on leukocytes. Myeloid cells and subsets of activated lymphocytes can also integrate signals from endothelial selectins [35], immunoreceptors [54] neighbor integrin ligands [55], integrin-associated adhesion receptors, [56] and regulatory integrin partners [57] to further strengthen integrin adhesiveness at endothelial sites of arrest. In spite of increasing information on numerous potential effectors involved in rapid integrin activation processes on different leukocyte subsets, we still do not know how these effectors cooperate in facilitating post arrest adhesion strengthening events. Reversible changes both in integrin conformation, cytoskeletal associations, and outside-in signaling to different actomyosin effectors must be co-regulated during the first few seconds of these critical post arrest processes. Future studies must dissect these complex activation programs and their variations among different types of leukocytes and endothelial beds in order to generate a road map of molecular networks that control initial integrin-mediated arrest, subsequent adhesion strengthening, as well as motility from the arrest site to the final site of transendothelial migration.

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