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Detection of Bidirectional Signaling During Integrin Activation and Neutrophil Adhesion

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Abstract

Neutrophil arrest and migration on inflamed endothelium is dependent upon a conformational shift in CD11a/CD18 (LFA-1) from a low to high affinity and clustered state which determines the strength and lifetime of bond formation with intracellular adhesion molecule 1 (ICAM-1). Cytoskeletal adaptor proteins kindlin-3 and talin-1 anchor clustered LFA-1 to the cytoskeleton and support the transition from neutrophil rolling to arrest. We employ microfluidic flow channels and total internal reflection fluorescence microscopy to evaluate the spatiotemporal regulation of LFA-1 affinity and bond formation that facilitate the transition from neutrophil rolling to arrest. Methodology is presented to correlate the relationship between integrin conformation, bond formation with ICAM-1, and cytoskeletal engagement and adhesion strengthening necessary to achieve a migratory phenotype.

Keywords

Integrin; Selectin; ICAM-1; Intercellular adhesion molecule-1; Leukocyte function associated protein-1 (LFA-1); Macrophage protein-1 (Mac-1); Adhesion strengthening; G-protein coupled receptors; Cytoskeleton

1 Introduction

Leukocyte recruitment to sites of inflammatory insult is a multistep process governed by chemokines, selectins, and integrins that engage in a stepwise manner to initiate intracellular signals and adhesive bond formation [1–3]. Key stages in the neutrophil recruitment cascade include: rolling, capture, arrest, adhesion strengthening, polarization, and transmigration (Fig. 1). Integrins are transmembrane heterodimeric cell adhesion proteins with distinct combinations of α and β subunits. There are 18 α and 8 β subunits and thus together generate 24 heterodimeric forms. In neutrophils, β_2 integrins are most abundant and $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1) are of specific significance in transitioning neutrophils from rolling to arrest to a polarized and transmigrating phenotype. β_2 integrins are key adhesion receptors in this process as they perform both adhesion and signaling functions. In the circulation, β_2 -integrins are expressed on the membrane at low numbers (~100,000 copies) and in a low affinity state that rapidly shift to high affinity and increase in number, and surface density as they make contact with endothelium at sites of inflammation. This chapter will provide tools that facilitate quantitative measurement of the activation state of

neutrophils for clinical evaluation to delve the mechanism of recruitment in basic science studies of neutrophil acute response to inflammation.

1.1 Inside-Out and Outside-In Signaling of Integrin Activation

Affinity is regulated via allosteric changes in integrin structure that in turn modulate their potential to adhere to each other and inflamed endothelium. Activation of integrins can be achieved by engagement and rolling on selectins, which facilitates the initial capture of leukocytes on the endothelial surface [2, 3]. Specifically, E-selectin, P-selectin, and L-selectin are critical to leukocyte and lymphocyte capture and rolling through PSGL-1 and other glycosylated ligands. While E- and P-selectin are expressed on the endothelium, L-selectin is expressed only on leukocytes and is involved in secondary capture of neutrophils during recruitment [4, 5]. Selectins form adhesive bonds with their sialylated and fucosylated ligands with high on and off rates and require a threshold level of hydrodynamic shear stress to support rolling and subsequent signaling [6–8]. E-selectin binding to PSGL-1 activates tyrosine kinase Syk and MAPK, which together signal a shift in LFA-1 conformation to an extended and intermediate affinity state [9, 10]. This inside-out signaling of an intermediate affinity state in LFA-1 facilitates deceleration of neutrophil rolling on the endothelium and can trigger firm arrest in the presence of a sufficient density of ICAM-1 [8, 11]. Rolling on E-selectin is synergistic with the so-called inside-out signaling via GPCRs in activation of integrin-dependent arrest (Fig. 2). The mechanism is not completely elucidated but may involve calcium acting as a secondary messenger to amplify conversion of additional integrins to a high affinity state and facilitate their formation into focal clusters [1, 11–13].

Following selectin-dependent capture and rolling, an upshift occurs from a low affinity bent conformation to an extended conformation associated with intermediate affinity that can bind to endothelial ligands and effect deceleration of rolling leukocytes. Chemokines play a key role in inside-out signaling of an upshift in integrin conformation from intermediate to high affinity that is associated with adhesive stabilization, such that the leukocyte becomes resistant to tensile and shear repulsive forces of blood flow. In fact, it is control of the number and density of high affinity integrins and endothelial presentation of their cognate ligands that determines when and where leukocytes are recruited to emigrate during inflammation [11, 14–18]. Neutrophil receptors for chemokine binding such as CXCR1 and CXCR2 are linked to G-protein coupled receptor (GPCR) pathways that activate an upshift in affinity and focal clustering of Mac-1 and LFA-1 β_2 -integrins to initiate firm arrest and subsequent migration [19]. These integrins once activated to a high affinity state can bind ligand and themselves initiate outside-in signals to remodel the cytoskeleton facilitating the next step in the process of pseudopod extension and transendothelial migration [20].

1.2 Detection of Activation State of Integrins on Neutrophils

LFA-1 is the first integrin to be activated during a PMN's transition from rolling to arrest but is rapidly followed by upregulation of Mac-1 surface expression achieved by exocytosis of receptor from cytosolic granules. Both integrin subunits dynamically shift from low to intermediate to high affinity states, the latter of which is required to stabilize adhesion and activate intracellular signaling. These integrins link with the actin cytoskeleton on the inside

of the cell and can signal from the outside-in a change in cell shape and motility. Perhaps the most common means of gauging the activation state of a neutrophil is via detection of the number and conformational state of β_2 -integrins. Within seconds of stimulation with chemotactic factors at sub-nanomolar concentrations, corresponding to ligation of 10–100 GPCRs, upregulation of Mac-1 can be detected [21]. Thus, use of fluorescence imaging techniques can serve to elucidate the link between the numerical increase in activated integrins and adhesion and migratory function [11].

Shear stress is an omnipresent and necessary component of leukocyte recruitment and this chapter endeavors to describe techniques to image bidirectional signaling of integrins dynamically becoming activated upon ligand binding. The process by which they signal from the outside-in involves mechanotransduction of signals in a process that involves buildup of tensile force at focal sites of integrin adhesion in an arrested neutrophil [22, 23]. Shear forces exerted by flowing blood are known to play a significant role in neutrophil activation as it integrates signals derived from GPCR and selectin-ligand ligation and engagement of intermediate and high-affinity LFA-1/ICAM-1 bonds. Absence of shear causes impairment in neutrophil recruitment and the upshift in affinity of β_2 integrins that are catalyzed by the tensile forces transmitted from shear stresses under flow [3, 24]. Downstream effects of shear-induced adhesion has been demonstrated in leukocytes as evidenced by the absence of effective transendothelial migration of T cells under shear-free conditions even in the presence of appropriate chemokines [25]. However, shear forces have been shown to cooperate with chemokine-induced signals to enhance calcium signaling through bound selectins and integrins during neutrophil rolling and arrest [13]. Hence, there have been reports of shear force in mediating activation of integrins and promoting leukocyte TEM. Current data support the contention that bond force promotes the formation of a strong clustered adhesive complex between LFA-1, Mac-1, and their cognate ligands on inflamed endothelium. Transduction of directional shear forces is associated with normal mechanotaxis, a process that appears to assist in guidance of neutrophils as they migrate in a directionally biased manner perpendicular to the direction of shear or circumferentially along the blood vessel to nearest sites of transendothelial immigration. The methods that follow can be used to detect integrin number, topography, activation state, association with adaptor molecules that assist in linkage of cytoskeletal molecules, and correlation with neutrophil shape change and migratory behavior.

2 Materials

2.1 Substrate: Derivitization

1. Piranha solution: 95.0–98.0 % H_2SO_4 .
2. 30 % H_2O_2 solution.
3. Acetone.
4. 3-aminopropyltriethoxysilane.
5. Deionized water (diH_2O).
6. 35-mm diameter glass coverslips.

7. Plastic coverslip rack.
8. 60-mm diameter polystyrene disposable Petri dishes.
9. 70 % ethanol solution.
10. 35-mm aminosilanated coverslips.
11. Recombinant human ICAM-1 (rhICAM-1) (R&D Systems).
12. Recombinant mouse ICAM-1 (rmICAM-1) (R&D Systems).
13. E-selectin-IgG.
14. 240Q antibody.
15. TS1/18 antibody.
16. CD45 antibody.
17. Goat antihuman IgG F(ab')₂ fragments.
18. Phosphate-buffered saline without cations (PBS^{-/-}).
19. 1 % casein solution in PBS.

2.2 Flow Channel Assembly

1. 35-mm diameter glass coverslips with absorbed proteins.
2. Poly(dimethylsiloxane) (PDMS) microfluidic flow chambers and reservoirs.
3. Syringe pump with 1-mL syringe.
4. 20 cm of PE 20 tubing.

2.3 PMN Isolation

1. Whole blood obtained by venipuncture based upon an institutional review board approved human materials protocol.
2. Hemocytometer.
3. 12-mL syringe.
4. 16-G syringe needle.
5. PMN isolation media (Thermo Fisher Scientific).
6. Human serum albumin (HSA).
7. Hanks balanced salt solution (HBSS).
8. 1 M calcium chloride (CaCl₂) solution.

2.4 Analysis of the Integrin State

1. Primary antibody conjugated directly to fluorophore; free of sodium azide.
2. Fluorescent microscope with excitation and emission filter wheels, a high-sensitivity camera, and excitation/brightfield shutters.

3. Fluo-5F.
4. 4 % paraformaldehyde (PFA).
5. 0.1 % Triton X-100.
6. Secondary antibodies.
7. Human serum albumin (HSA).
8. L-Ascorbic acid.

2.5 Analysis of Cell Arrest and Adhesion Strengthening

1. HEPES-buffered saline: 10 mM KCl, 110 mM NaCl, 10 mM glucose, 1 mM MgCl_2 , and 30 mM HEPES, pH 7.4, 290 mOsm.
2. Fura-2 AM.

2.6 Analysis of Cytoskeletal Assembly

1. Talin-1 antibody (Abcam).
2. Kindlin-3 (Abcam).
3. Phalloidin (cell signaling).

3 Methods

3.1 Substrate: Derivatization

Clean coverslips with piranha solution and coat with aminosilane. Then derivatize either ICAM-1-IgG in presence or absence of E-selectin-IgG or antibodies that recognize a common or allosterically defined integrin conformation onto aminosilanated coverslips.

1. Fill two 600-mL glass beakers with approximately 450 mL of diH_2O each, and fill a 2-L glass beaker with approximately 1,500 mL of diH_2O .
2. Using tweezers, completely load plastic coverslip racks with clean 35-mm glass coverslips. Transfer assembled supplies to a fume hood.
3. Under a fume hood, add 300 mL of 95.0–98.0 % H_2SO_4 solution to a 600-mL beaker.
4. Add 150 mL of 30 % H_2O_2 solution to achieve a 2:1 ratio of H_2SO_4 to H_2O_2 , and let the solution stand for 5 min.
5. Carefully place coverslip racks into the piranha solution and let sit in solution for 15 min.
6. In two glass 100-mL beakers, add ~90 mL of acetone. Also add 100 mL of acetone to a 100-mL Nalgene beaker.
7. Aliquot 2 mL of 3-aminopropyltriethoxysilane into the Nalgene beaker containing acetone to achieve an aminosilane solution at a ratio of 1:50 aminosilane to acetone.

8. After the 15 min, transfer the coverslip racks from the piranha solution into one of the 600-mL beakers of water. Let them sit for at least 1 min.
9. Arrange your workspace: from left to right, place one of the 100-mL beakers with ~90 mL (sufficient volume to submerge) acetone, then the 250-mL Nalgene beaker with the aminosilane solution, and lastly the other 100-mL glass beaker containing acetone.
10. Carefully remove one coverslip rack from the water, submerge in acetone for 10 s, submerge in aminosilane solution for 5 min, submerge in the last acetone beaker for 10 s, and place into an unused 600-mL beaker of diH₂O.
11. Repeat **step 10** for the second rack.
12. Take the beaker of water with the racks in it over to the workstation. Remove racks. Blow-dry with air. Make sure to remove all water.
13. Once the coverslips are completely dry, place individual slips into 60 mm Petri dishes.
14. Absorb either recombinant ICAM-1, E-selectin, or antibodies 240Q (stabilizes CD18 at high affinity), TS1/18 (stabilizes LFA-1 at low affinity), or CD45 (control non-integrin), at 5 µg/mL to coverslips cleaned with piranha solution [26] and coated with aminosilane.
15. In a 1.5-mL Eppendorf tube, make a 250 µL solution of PBS^{-/-}, goat antihuman IgG F(ab')₂ fragments, and ICAM-1 or antibody. Adjust the volumes of goat antihuman IgG F(ab')₂ fragments and ICAM-1 or antibody added to achieve concentrations of 5 µg/mL, respectively.
16. Dispense 200 µL of the prepared solution onto an aminosilane-coated coverslip in a 60-mm Petri dish, and place coverslip at 4 °C for 1 h.
17. Use a pipette to wash the coverslip with 2 mL of PBS^{-/-}, aspirate, and repeat once.
18. Block nonspecific binding with casein by carefully suctioning proteins from the top of the coverslip (*see* Note 1) and then adding 200 µL of casein to the coverslip. Incubate at room temperature for 30 min.
19. Suction off casein, wash with 2 mL of PBS^{-/-}, suction off PBS, and repeat once. Add 2 mL of PBS to the coverslip and use for experiment.

3.2 Flow Channel Assembly

1. For a removable vacuum-sealed channel, soak all PDMS devices in separate Petri dishes containing sufficient deionized water to completely submerge chambers.

¹ For vacuuming excess solution from coverslips, an aspiration setup with primary containment will be sufficient. A sterile filter can be placed on the end of the plastic tubing that attaches to the laboratory vacuum spigot to prevent particulates from being aspirated. When vacuuming proteins off of the coverslips, be careful not to contact the surface of the coverslip with the tip of the aspirating pipette. It helps to tilt the Petri dish and displace the protein solution to one side of the slip and aspirate from there. Be careful not to fully dry the coverslip during aspiration.

2. Position a chamber with the channel-engraved surface facing down, and use needle nose tweezers to position the chamber against the wall of the dish. Keep the chamber under the level of the deionized water, but such that it is not in contact with the bottom of the dish.
3. Use a 200- μ L pipette to remove any air bubbles trapped within the channels. Position the pipette tip at the entrance to one of the channels on the top surface of the chamber and draw in with the pipette. Withdraw the pipette, expel the contents into the dish, and repeat for each channel.
4. Connect the PDMS device to its vacuum leads, and invert it such that several drops of deionized water can be dispensed onto the channel-engraved surface (*see* Note 2).
5. Use needle nose tweezers to place a protein-absorbed coverslip on top of the wetted PDMS and initialize the vacuum using a stopcock.
6. Use a stage heater to maintain experimental conditions at 37 °C. A heated and humidified enclosure provides optimal experimental conditions. For oil objectives, begin heating at least 1 h in advance of the experiment in order to allow optics to equilibrate at the experimental temperature.
7. To prevent desiccation of channels, place drops of deionized water on top of all channel access holes. Use blunt tweezers to hold down the PDMS and press fit fluid-filled reservoir and pump fluidic tubes into prepunched holes in the PDMS device.
8. Draw fluid through the microchannels into the pump tubing using the syringe pump. Set the volumetric flow rate of the syringe pump based on the desired fluid shear stress and Eq. 1.

$$\tau_w = 6\mu Q / wh^2. \quad (1)$$

where τ_w is shear stress, μ is fluid viscosity, Q is volumetric flow rate, w is channel width, and h is channel height (Fig. 3).

3.3 PMN Isolation

1. Dispense 4 mL of isolation media into a 15-mL conical poly-propylene tube using a serological pipette, taking care to minimize the amount of media that gets on the side of the tube.
2. Layer 4 mL of whole blood over separation media to achieve a 1:1 ratio of blood to isolation media.
3. Repeat **steps 1** and **2** to prepare a second tube for a 10 mL whole blood sample.

² It is not necessary to completely cover the bottom of the PDMS chamber with water. Simply dispense a few drops such that the channel entrances are covered and place the coverslip on top in the correct orientation. Dispensing too much water on the bottom of the chamber can result in improper coverslip alignment and difficulty attaining proper suction.

4. Centrifuge the tubes for 30 min at $760 \times g$.
5. In a 50 mL conical polypropylene tube, add 120 μ L of HSA to 30 mL of HBSS. In a 15 mL conical polypropylene tube, add 40 μ L of HSA to 10 mL of HBSS that will serve as the buffer to resuspend the PMN and use in the experiment later.
6. Use a 12-mL syringe with 16-G needle that has approximately 2 mL of HBSS/0.1% HSA drawn in from the 50-mL tube to extract the appropriate density layer from the centrifuged samples. Try to minimize collection of other blood constituents such as lymphocytes and platelets.
7. Wash the extracted cells by dispensing the syringe into the 50-mL HBSS/0.1 % HSA tube and centrifuging for 8 min at $190 \times g$ (*see* Note 3).
8. Dispense the supernatant and resuspend isolated PMN in 500 μ L of HBSS/0.1 % HSA from the 15-mL tube.
9. Use a hemocytometer to count cells, and suspend to a concentration of 2×10^6 cells/mL.
10. Add 1.5 mM CaCl_2 to the cell suspension just prior to the experiment. The cells remain viable and unactivated up to 4–6 h after isolation.

3.4 Analysis of the Integrin State

1. For real-time total internal reflection fluorescence (TIRF) calcium measurements, label cells with 1 μ M fluo-5 F for 30 min at 37 °C.
2. Fix PMN that were perfused over protein-absorbed glass coverslips in a microfluidic flow chamber with 4 % PFA.
3. Permeabilize the cells with 0.1 % Triton X-100 and label with primary (*see* Table 1) and secondary antibodies to specific proteins.
4. Excite arrested PMNs with a 488-nm laser using TIRF microscopy at 1 frame/s to observe changes in intracellular calcium in a focal section of the PMNs approximately 100 nm from the surface of the coverslip [27].
5. The topography of LFA-1, Mac-1, and high affinity CD18 can be imaged in real time during neutrophil rolling and arrest in shear flow with epifluorescence (within plane of focus) or total internal reflection fluorescence microscopy (within 100 nm of substrate).
6. Dilute neutrophils to a concentration of 1×10^6 cells/mL in HSA and 20 μ g/mL of the appropriate labeling control antibody. Preincubate neutrophils with nonblocking fluorescent monoclonal antibodies and allosteric inhibitors at 37 °C for 10 min.

³ Throughout the entire neutrophil isolation process and especially during this part of the procedure when transferring the isolated PMN to the 50 mL Falcon tube, be careful to minimize the chances of physically activating the cells. For example, dispense the PMN from the syringe at a slow but steady pace to prevent inadvertent activation as a result of imparted high shear stress.

7. Centrifuge the labeled neutrophils, and resuspend the pellet to a concentration of 1×10^6 cells/mL in HEPES-buffered saline containing 0.5 mM ascorbic acid, 1.5 mM Ca^{2+} , and 1 mg/mL of HSA.
8. Use these washed, labeled neutrophils as the sample for a microfluidic flow chamber by adding them to the inlet reservoir. Draw the sample into the flow chamber at the desired shear rate.
9. Optimize camera settings for the labeling conditions by taking single images of the first neutrophils that adhere to the substrate. When imaging moving cells, it is important to use the minimum exposure time necessary to obtain a clear image.
10. Once a number of neutrophils adhere to the underlying substrate, acquire images of immunofluorescence microscopy coupled with phase-contrast microscopy at one frame/s using a 60 $\times$ objective. Use an automated brightfield source shutter and an optical excitation filter wheel with filters appropriate for fluorescent labels (i.e., Alexa-488, Alexa-546, and PE labels) [11] (*see Note 4*).
11. The distribution of the labeled neutrophil surface epitopes may be processed and analyzed using Image-Pro (Media Cybernetics), MetaMorph (Molecular Devices), or National Institutes of Health (NIH) image analysis software. Defining clusters of epitopes as regions with signal intensity three standard deviations above the background intensity, one may quantify the number, size, and location of high-density adhesion molecules (i.e., β_2 integrins) on the neutrophil surface.

3.5 Analysis of Cell Arrest and Adhesion Strengthening

1. Suspend PMN at a concentration of 2×10^6 /mL in HEPES- buffered salt solution.
2. Label PMN with Fura-2 AM for 30 min at 37 °C.
3. Wash and resuspend cells in HEPES-buffered salt solution.
4. Perfuse labeled cells into microfluidic flow chamber at desired shear stress (4 dyn/cm² is the venular magnitude of shear stress).
5. Sequentially image cells over time with alternating excitation by 340 and 380 nm light generated by a mercury lamp attached to a filter wheel with 0.1 s switch time.
6. Images can be acquired with an Orca-ER camera (Hamamatsu Corp) coupled to a Nikon 1200 microscope running Simple PCI 5.3 software.
7. Analyze image sequences for the ratio between emission at 340 and 380 nm using custom macros written for Image-Pro Plus 5.1.
8. Identify the average intensity of each cell in a confined area of interest around each cell for both the 340 and 380 nm exposures. This method of cell identification and local overlap accounts for any motion of rolling PMN during image acquisition.

⁴ When a microscopic field becomes unsuitable for further imaging as a result of photobleaching, move the stage to another region of the flow chamber and resume imaging.

3.5.1 Adhesion Strengthening Experiments

1. Differentiate control, talin, and kindlin-3 shRNA transfected HL-60 cells to a neutrophil phenotype over 3 days with 1.3 % DMSO and human isolated PMN.
2. Treat cells with Mac-1 blocking antibody present in excess in the media (ICRF44 for human and M1/70 for mouse) to ensure LFA-1 dependent adhesion.
3. To study the role of Ca^{2+} flux in adhesion strengthening, treat PMN with Fura-2 to detect intracellular Ca^{2+} .
4. Block Ca^{2+} signal by chelation with 50 μM BAPTA or block CRAC channels with 100 μM 2-APB.
5. Perfuse cells and allow to settle over an ICAM-1 + 240Q substrate derivatized on the substrate at 1:1 ratio (5 $\mu\text{g/mL}$ each) to activate and stabilize high affinity LFA-1 at adhesive contact sites.
6. Ramp shear up at 30 s intervals from 0, 4, 10, 20, and 40 dyn/cm^2 .
7. Measure the number of cells that remain adhered over separate fields of view at each shear level.

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References

1. Campbell JJ, Hedrick J, Zlotnik A, et al. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science*. 1998; 279:381–384. [PubMed: 9430588]
2. Ley K. Integration of inflammatory signals by rolling neutrophils. *Immunol Rev*. 2002; 186:8–18. [PubMed: 12234358]
3. Simon SI, Green CE. Molecular mechanics and dynamics of leukocyte recruitment during inflammation. *Annu Rev Biomed Eng*. 2005; 7:151–185. [PubMed: 16004569]
4. Dwir O, Kansas GS, Alon R. Cytoplasmic anchorage of L-selectin controls leukocyte capture and rolling by increasing the mechanical stability of the selectin tether. *J Cell Biol*. 2001; 155:145–156. [PubMed: 11581291]
5. Taylor AD, Neelamegham S, Hellums JD, et al. Molecular dynamics of the transition from L-selectin- to beta 2-integrin-dependent neutrophil adhesion under defined hydrodynamic shear. *Biophys J*. 1996; 71:3488–3500. [PubMed: 8968618]
6. Thomas WE, Trintchina E, Forero M, et al. Bacterial adhesion to target cells enhanced by shear force. *Cell*. 2002; 109:913–923. [PubMed: 12110187]
7. Zhu C, McEver RP. Catch bonds: physical models and biological functions. *Mol Cell Biomech*. 2005; 2:91–104. [PubMed: 16708472]
8. McDonough DB, McIntosh FA, Spanos C, et al. Cooperativity between selectins and beta2-integrins define neutrophil capture and stable adhesion in shear flow. *Ann Biomed Eng*. 2004; 32:1179–1192. [PubMed: 15493506]
9. Zarbock A, Lowell CA, Ley K. Spleen tyrosine kinase Syk is necessary for E-selectin-induced alpha(L)beta(2) integrin-mediated rolling on intercellular adhesion molecule-1. *Immunity*. 2007; 26:773–783. [PubMed: 17543554]
10. Simon SI, Hu Y, Vestweber D, et al. Neutrophil tethering on E-selectin activates beta 2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. *J Immunol*. 2000; 164:4348–4358. [PubMed: 10754335]

11. Green CE, Schaff UY, Sarantos MR, et al. Dynamic shifts in LFA-1 affinity regulate neutrophil rolling, arrest, and transmigration on inflamed endothelium. *Blood*. 2006; 107:2101–2111. [PubMed: 16269618]
12. Alon R, Feigelson S. From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. *Semin Immunol*. 2002; 14:93–104. [PubMed: 11978081]
13. Schaff UY, Yamayoshi I, Tse T, et al. Calcium flux in neutrophils synchronizes beta2 integrin adhesive and signaling events that guide inflammatory recruitment. *Ann Biomed Eng*. 2008; 36:632–646. [PubMed: 18278555]
14. Beals CR, Edwards AC, Gottschalk RJ, et al. CD18 activation epitopes induced by leukocyte activation. *J Immunol*. 2001; 167:6113–6122. [PubMed: 11714770]
15. Constantin G, Majeed M, Giagulli C, et al. Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity*. 2000; 13:759–769. [PubMed: 11163192]
16. Kim M, Carman CV, Yang W, et al. The primacy of affinity over clustering in regulation of adhesiveness of the integrin $\alpha_1\beta_2$. *J Cell Biol*. 2004; 167:1241–1253. [PubMed: 15611342]
17. Sarantos MR, Raychaudhuri S, Lum AF, et al. Leukocyte function-associated antigen 1-mediated adhesion stability is dynamically regulated through affinity and valency during bond formation with intercellular adhesion molecule-1. *J Biol Chem*. 2005; 280:28290–28298. [PubMed: 15955822]
18. Bachmann MF, Kopf M, Marsland BJ. Chemokines: more than just road signs. *Nat Rev Immunol*. 2006; 6:159–164. [PubMed: 16491140]
19. Zarbock A, Deem TL, Burcin TL, et al. Galpha_i2 is required for chemokine-induced neutrophil arrest. *Blood*. 2007; 110:3773–3779. [PubMed: 17699741]
20. Alon R, Ley K. Cells on the run: shear- regulated integrin activation in leukocyte rolling and arrest on endothelial cells. *Curr Opin Cell Biol*. 2008; 20:525–532. [PubMed: 18499427]
21. Lum AF, Green CE, Lee GR, et al. Dynamic regulation of LFA-1 activation and neutrophil arrest on intercellular adhesion molecule 1 (ICAM-1) in shear flow. *J Biol Chem*. 2002; 277:20660–20670. [PubMed: 11929876]
22. Dixit N, Simon SI. Chemokines, selectins and intracellular calcium flux: temporal and spatial cues for leukocyte arrest. *Front Immunol*. 2012; 3:188. [PubMed: 22787461]
23. Dixit N, Yamayoshi I, Nazarian A, et al. Migrational guidance of neutrophils is mechanotransduced via high-affinity LFA-1 and calcium flux. *J Immunol*. 2011; 187:472–481. [PubMed: 21632714]
24. Alon R, Dustin ML. Force as a facilitator of integrin conformational changes during leukocyte arrest on blood vessels and antigen- presenting cells. *Immunity*. 2007; 26:17–27. [PubMed: 17241958]
25. Schreiber TH, Shinder V, Cain DW, et al. Shear flow-dependent integration of apical and subendothelial chemokines in T-cell transmigration: implications for locomotion and the multistep paradigm. *Blood*. 2007; 109:1381–1386. [PubMed: 17038526]
26. Mitchon LN, White JM. Growth and analysis of octadecylsiloxane monolayers on Al₂O₃ (0001). *Langmuir*. 2006; 22:6549–6554. [PubMed: 16830996]
27. Schaff UY, Dixit N, Procyk E, et al. Orai1 regulates intracellular calcium, arrest, and shape polarization during neutrophil recruitment in shear flow. *Blood*. 2009; 115:657–666. [PubMed: 19965684]

Fig. 1. Illustration of multistep process of neutrophil recruitment. Adhesion strengthening and migrating neutrophil shown with focal adhesions transmitting outside-in signals to adaptor and cytoskeletal proteins

Fig. 2.
Neutrophil engaging adhesion receptors and CXC chemokine. E-selectin and ICAM-1 engagement with respective neutrophil adhesion receptors are cooperative with chemokine signaling during rolling to arrest

Fig. 3.

Vascular mimetic microfluidic flow channels. A schematic of a 25 mm PDMS microfluidic disc assembled on a well of a 6-well plate. The pump line is connected to a syringe pump for variable control of the negative pressure flow rate at the exit of one of four flow channels used for each of four experiments. Two holes conduct a vacuum line to the spider webbed set of channels to anneal the flow channel to the substrate with negative static pressure. The hub of a syringe pump is affixed to a port connecting the inlet to the channel that serves as the reservoir for the neutrophil suspension. Dimension of an individual four-channel flow chamber provides a 100 μm height × 200 μm width rectangular flow area

Table 1

Summary of antibodies that can be used for the analysis of integrin activation state

Clone/subunit	Activation state	Activating/blocking/detection	Commercial source or reference
IB4/CD18	Common epitope	Detection	Santa Cruz Biotechnology
327C/CD18	Detects extended conformation epitope	Detection	Eli Lilly Corp
240Q/CD18	Induces extended conformation epitope	Activating	Eli Lilly Corp
TS2/4/CD11a	Common epitope	Detection	BioLegend
Mab24/CD18	Extended conformation epitope	Detection	Hycult Biotech
Kim127/CD18	Common epitope	Detection	ATCC
TS1/18/CD11a	Stabilizes bent or low affinity conformation	Blocking	BioLegend

Antibodies for detecting and inducing defined activation states of β_2 integrins