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Leukocyte Function Antigen-1, Kindlin-3, and Calcium Flux Orchestrate Neutrophil Recruitment during Inflammation

Neha Dixit,*† Min-Ho Kim,* Jan Rossaint,‡§ Itsukyo Yamayoshi,* Alexander Zarbock,‡§ and Scott I. Simon*†

Neutrophil arrest and migration on inflamed endothelium involves a conformational shift in CD11a/CD18 (leukocyte function antigen-1; LFA-1) to a high-affinity and clustered state that determines the strength and lifetime of bond formation with ICAM-1. Cytoskeletal adapter proteins Kindlin-3 and Talin-1 anchor clustered LFA-1 to the cytoskeleton and facilitate the transition from neutrophil rolling to arrest. We recently reported that tensile force acts on LFA-1 bonds inducing their colocalization with Orai1, the predominant membrane store operated Ca2+ channel that cooperates with the endoplasmic reticulum to elicit cytosolic flux. Because Kindlin-3 was recently reported to initiate LFA-1 clustering in lymphocytes, we hypothesized that it cooperates with Orai1 and LFA-1 in signaling local Ca2+ flux necessary for shear-resistant neutrophil arrest. Using microfluidic flow channels combined with total internal reflection fluorescence microscopy, we applied defined shear stress to low- or high-affinity LFA-1 and imaged the spatiotemporal regulation of bond formation with Kindlin-3 recruitment and Ca2+ influx. Orai1 and Kindlin-3 genes were silenced in neutrophil-like HL-60 cells to assess their respective roles in this process. Kindlin-3 was enriched within focal clusters of high-affinity LFA-1, which promoted physical linkage with Orai1. This macromolecular complex functioned to amplify inside-out Ca2+ signaling in response to IL-8 stimulation by catalyzing an increased density of Talin-1 and Kindlin-3 genes.

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that mediates stored operated calcium entry (SOCE) (23, 24). Orai1 is important for coordinating the transition from an arrested neutrophil to one projecting pseudopods and directionally migrating (10, 15, 25). This mechanism involves spatial regulation of calcium transients that occur proximal to integrin engagement and uptake of tensile forces imposed by shear stress (14, 22, 25). How Ca\textsuperscript{2+} flux is initiated by cooperation between high-affinity LFA-1 and Orai1 and how it elicits membrane clustering required for shear-strengthened neutrophil adhesion is the focus of this study.

LFA-1 affinity upshift and clustering requires the activity of Src family kinases and the cytoskeletal adapter proteins Talin-1 and Kindlin-3, which localize within membrane domains to alter LFA-1 function (4, 26). Talin-1 concentrates at focal adhesions along with paxillin to provide linkage of LFA-1 clusters to F-actin that in turn drives cell polarization (27). Whereas Talin-1 is necessary for LFA-1 extension from a low- to intermediate-affinity state, Kindlin-3 functions to stabilize LFA-1 at high affinity and induce submicrometer-size clusters. In T cells and neutrophils, it has been demonstrated that Kindlin-3 promotes cell spreading and adhesion strengthening (6, 28, 29). Because Talin-1 and Kindlin-3 bind to distinct sites on the cytodomain of the β2 subunit, they may physically interact to facilitate focal clustering of LFA-1 (30). Precisely how and in what sequence Kindlin-3 and Talin-1 interact with LFA-1 bonds as tension is taken up during cell deceleration to arrest and their role in initiating calcium flux via Orai1 remain key unanswered questions.

In this study, we applied total internal reflection fluorescence (TIRF) to image the spatiotemporal events associated with LFA-1 conversion from low to high affinity and its association with Kindlin-3 and Orai1 under hydrodynamic shear stress during neutrophil adhesion strengthening. We hypothesized that high-affinity LFA-1 bond formation and uptake of tensile force were necessary to catalyze downstream events required for neutrophil adhesion strengthening including Kindlin-3 recruitment and Orai1-mediated calcium flux. Tensile force facilitated physical association of Kindlin-3 with the cytodomain of LFA-1, and this step was necessary for local calcium influx and subsequent cytoskeletal assembly necessary for stable adhesion at stresses up to 40 dyne/cm\textsuperscript{2}. Knockdown of Kindlin-3 abrogated colocalization of Orai1 with high-affinity LFA-1 and diminished Ca\textsuperscript{2+} flux and the recruitment of Talin-1 to adhesive contacts. Knockdown of Orai1 or chelating intracellular Ca\textsuperscript{2+} flux eliminated the consolidation of LFA-1 into macroclusters, which correlated with diminished adhesion strengthening. These studies reveal how durable multivalent LFA-1/ICAM-1 bonds serve as mechanosensors that direct PMN cytoskeletal activity by transmission of tensile force to a supramolecular complex that triggers Ca\textsuperscript{2+} influx at sites of adhesive contact.

**Materials and Methods**

*Abs, small molecules, and other reagents*

Human and mouse ICAM-1-Fc and E-selectin-Fc were purchased from R&D Systems (Minneapolis, MN). Protein A/G was purchased from Pierce (Rockford, IL). 2-Aminoethoxydihydroxy borate (2-APB) was purchased from EMD Biosciences (San Diego, CA), resuspended in dry DMSO at concentration of 100 mM, and stored at –80°C under N\textsubscript{2}. Thapsigargin was purchased from Invitrogen (Carlsbad, CA) and resuspended to 1 mM in DMSO the same day as the experiment. Anti-CD18 327C, anti-LFA-1 TS2/4, and anti-CD18 (240Q) were obtained as a generous gift from Eli Lilly Corp (Indianapolis, IN). Anti-CD18 TS1/18, anti-CD45, and anti-Mac-1 ICRF44 were all purchased from BioLegend (San Diego, CA). Anti-CD18 IB4 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The small molecule lovastatin was purchased from Calbiochem. Polyclonal anti-mouse Orai1, anti-human and anti-mouse Kindlin-3, and anti-human and anti-mouse Talin Abs were purchased from Abcam (Cambridge, MA). Goat anti-rabbit–Alexa 488 polyclonal secondary Ab and normal goat serum were purchased from Invitrogen and from Thermofisher Scientific (Walhamp, MA), respectively. Abs were used at a saturating concentration of 5 μg/ml or per manufacturer’s instructions. Human promyelocytic leukemia cells (HL-60 cells) were purchased from American Type Culture Collection (Manassas, VA). Quantum simply cellular anti-mouse IgG beads were purchased from Bangs Laboratories (Fishers, IN). Control and Orai1 small interfering RNA (siRNA) were purchased from Qiagen (Valencia, CA).

**Neutrophil isolation**

Whole blood was obtained from consenting donors under an approved University of California, Davis, Institutional Review Board protocol and layered over PMN separation media purchased from Thermo-Fisher Scientific, as previously described (31). After centrifugation, PMNs were extracted from the appropriate density layer and washed with HEPES buffered salt solution. Mice heterozygous for expression of Orai1 were a generous gift from the laboratory of Dr. Anjana Rao (La Jolla for Allergy and Immunology, La Jolla, CA) (32). Mice were genotyped from tail clippings by PCR, and PMNs were isolated from the bone marrow of littermate Orai1\textsuperscript{+/−} and wild-type (WT) ICR strain mice as previously described (15, 33).

**siRNA transfection**

HL-60 cells were transfected with control scrambled siRNA or with Orai1-specific siRNA (Qiagen) by electroporation using the Amaxa Nucleofector 4D (Lonza) according to the manufacturer’s instructions. HL-60 cells were given fresh media 24 h before electroporation to maximize survival. Cells were pelleted from media, resuspended in high-resistance nucleofection buffer (Lonza) in the presence of 100 mM siRNA, and electroporated. Immediately after electroporation, cells were transferred to 37°C media containing 1.3% DMSO and differentiated for 3 d under influence of siRNA.

**Adhesion assays**

Human and mouse ICAM-1 and Abs 240Q, TS1/18, and CD45 were absorbed at 5 μg/ml to coverslips cleaned with piranha solution (34) and coated with aminosilane. PMNs were perfused through custom-made microfluidic chambers over substrates at 3 dyne/cm\textsuperscript{2} and imaged for calcium flux or fixed and labeled for Kindlin-3, Talin-1, and high-affinity CD18 (327C) as previously described (15). For adhesion-strengthening experiments, control, Talin, and Kindlin-3 short hairpin RNA (shRNA)–transfected HL-60 cells and control and Orai1 siRNA–transfected HL-60 cells were differentiated to a neutrophil phenotype over 3 d with 1.3% DMSO. Human isolated PMNs were allowed to settle over an ICAM-1 +240Q substrate derivatized on the substrate at 1:1 ratio (5 μg/ml each) to activate and stabilize high-affinity LFA-1 at adhesive contact sites. In adhesion-strengthening experiments, bone marrow–derived PMNs from Orai1\textsuperscript{−/−} and Orai1\textsuperscript{+/−} mice were isolated and allowed to settle on ICAM-1 substrate in the presence of Mn\textsuperscript{2+}. Cells were treated with Mac-1 excess blocking Ab in the media (ICRF44 for human and M1/70 for mouse) to ensure LFA-1–dependent adhesion. Shear was then ramped at 30–inter vals from 0, 4, 10, 20, to 40 dyne/cm\textsuperscript{2}, and the number of cells that remained adhered were measured over five separate fields of view at each shear level. To study the role of calcium flux in adhesion strengthening, PMNs were treated with 50 μM BAPTA or 100 μM 2-APB prior to each experiment.

**Real-time calcium imaging of PMNs in microchannels**

PMNs (human and mouse) were suspended at a concentration of 2 × 10\textsuperscript{6} /ml in HEPES buffered salt solution and labeled with fura 2-AM for 30 min at 37°C. Cells were then washed and resuspended in HEPES buffered salt solution. Labeled cells were perfused into a microfluidic channel and imaged as previously described (9, 35). Briefly, cells were drawn into the microfluidic channel at a calculated shear stress of 1 dyne/cm\textsuperscript{2} (i.e., venular magnitude of shear stress) and sequentially imaged during interaction with the substrate at alternating excitation by 340-nm and 380-nm light generated by a mercury arc lamp attached to a filter wheel with 0.1-s frequency excitation. Images were acquired with an Orca-ER camera (Hamamatsu) coupled to a Nikon 1200 microscope running Simple PCI 5.3 software. Image sequences were analyzed for the ratio between emission at 340 nm and 380 nm using custom macros written for Image Pro Plus 5.1. During analysis, the average intensity of each cell was identified in a confined area of interest around each cell for both the...
340-nm and 380-nm exposure. This method of cell identification and local overlap accounted for any motion of rolling PMNs during image acquisition.

**TIRF in-plane microscopy of LFA-1, Kindlin-3, and Talin-1 expression**

Human and mouse PMNs were treated with or without Mn²⁺ or lovastatin and perfused over ICAM-1, 240Q, TS1/18, or CD45 coated coverslips in a microfluidic flow chamber at a calculated shear stress of 4 dyne/cm², fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and labeled with primary and secondary Abs to specific proteins. Cells were imaged via TIRF microscopy, which excites fluorophores within a focal height of ∼100 nm above the glass coverslip–cell membrane surface. High-affinity CD18 receptor expression per cell was determined by comparing the mean fluorescence intensity (MFI) of bound 327C–Alexa 488 to a Quantum Simply Cellular calibration bead set containing defined numbers of goat anti-mouse binding sites on the surfaces of beads. From this analysis, a linear relation between MFI and receptor expression was determined to convert TIRF obtained by high-affinity CD18 fluorescence to receptor number (see Supplemental Fig. 2). An LFA-1 cluster was defined as an area of uniform fluorescence intensity registering two SDs above the MFI of the cell. Once this threshold was set for each cell, cluster area was measured in square micrometers. Average cellular MFI and cluster MFI for each condition was converted into average receptor numbers per cell or per cluster using the calibration bead set as described earlier.

**In vivo imaging of Orai¹⁺⁺ and Orai¹⁻⁻ PMN recruitment to wound sites**

EGFP–Orai¹⁺⁺ and EGFP–Orai¹⁻⁻ mice were generated by cross-breeding EGFP–lysozyme knockin mice with Orai¹⁺⁺ mice. Orai¹⁺⁺ and Orai¹⁻⁻ mice were anesthetized with 100 mg/kg ketamine/xylazine intraperitoneally, 10:1; Sigma), and the back skin hair was then removed with a mechanical shaver. After sterilization with 10% w/v povidone–iodine (intraperitoneally, 10:1; Sigma), and Orai1⁻⁻ mice were anesthetized with 100 mg/kg ketamine/xylazine (intrapерitoneally, 10:1; Sigma), and the skin biopsy punch (Robbins Instruments, Chatham, NJ). The in vivo imaging of EGFP neutrophils appearing on the site of the back skin wound was performed using the whole-body small animal fluorescence imaging system (Xenogen IVIS 100 system; Xenogen) as described recently (36). Mice were anesthetized with 2% isoflurane and then put into the imaging chamber of the system. The EGFP-expressing neutrophils within the wound area were visualized using a GFP filter (excitation at 445–490 nm and emission at 515–575 nm) at an exposure time of 1 s. Live Image Pro 2.3 software (Caliper Life Sciences) was used, and fluorescence intensities expressed as average radiance (photons per square centimeter per steradian) were measured by drawing a circular region of interest over the entire wound area.

**Immunoprecipitation of LFA-1 and CD45**

Human PMNs were isolated from whole blood or control and Kindlin-3 shRNA–transfected HL-60 cells that were differentiated for 3 d with 1.3% DMSO and allowed to settle on a 240Q, TS1/18, or CD45 Ab substrate in the microfluidic device in the presence or absence of shear stress. These cells bound to the microfluidic channel substrate were then sheared and lysed with IP lysis buffer containing protease inhibitor mixture in the channel. One milligram of protein lysates from cells bound to the substrate was collected from the microfluidic channel and incubated with anti–LFA-1 Ab TS2/4 or anti–CD45 Ab for 2 h on ice. Protein A/G agarose resin from Pierce classic IP kit was used to pull down LFA-1 or CD45, and the resin was washed four times with IP lysis buffer (Pierce). Samples were boiled in SDS-PAGE sample buffer, and equal concentrations of protein sample were subjected to electrophoresis. Polyvinylidene membranes were blocked with 5% milk for 1 h, probed with primary Abs and HRP-labeled secondary Abs, and developed using the ECL system.

**Statistical analysis**

Data analysis was performed using GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA). Differences between single pairs of conditions were analyzed for significance by two-tailed unpaired Student t test. For data sets derived from exclusively paired observations, p values were obtained by two-tailed paired t test. All error bars are mean ± SEM based on the number of independent experiments indicated in the figure legends.

**Results**

**Kindlin-3 is required for PMN adhesion strengthening through LFA-1**

Deceleration of neutrophils during the transition to a stable arrested state involves a rapid shift in LFA-1 from an extended intermediate affinity state that supports rolling to a fully activated high-affinity conformation (14, 37). Although it is known that Kindlin-3 and Talin-1 are involved in LFA-1 activation (6, 28, 29, 38), their precise role in adhesion strengthening of PMNs after arrest through high-affinity LFA-1/ICAM-1 bond formation has not been elucidated. Kindlin-3 and Talin-1 expression was knocked down by ~80% in promyelocytic HL-60 cells using lentiviral delivery of shRNA (Supplemental Fig 1). These HL-60 cells were then induced to differentiate to PMNs and assayed for their adhesive capacity. We endeavored to image LFA-1/ICAM-1 bond formation on PMNs activated by substrate-bound allosteric Ab 240Q, which initiates adhesion from the outside-in as CD18 is engaged and forms durable multivalent bonds (39). PMNs were perfused through the microfluidic flow channel and allowed to settle on substrates coated at a 1:1 protein ratio with ICAM-1–Fc and 240Q. This surface mimics an inflammatory substrate by shifting CD18 to high affinity upon contact, and in the presence of a function blocking Ab to Mac-1 receptor, activated high-affinity LFA-1 bond formation with ICAM-1 occurs at levels commensurate with IL-8 stimulation of CXCR (18). After attachment, shear was incrementally stepped up from 0 to 40 dyne/cm², and the fraction of PMNs that remained adherent to the substrate was measured (Fig. 1A). Kindlin-3 knockdown cells exhibited a 60% defect in adhesion strengthening compared with control scrambled shRNA–transfected HL-60. In comparison, Talin-1 knockdown cells bound robustly under high shear stress and exhibited only a ~20% defect in adhesion strengthening.

To determine whether the topography of LFA-1 bonds influenced adhesion strengthening, high-affinity CD18 was imaged by TIRF microscopy, which limits fluorescence detection to within ~0.1 μm of the plane of adhesive contact (Fig. 1B). Activated CD18 receptors were imaged by labeling adherent PMNs with 327C Ab, and the number of binding sites were quantified using a bead set calibrated at defined receptor density (Supplemental Fig. 2). HL-60–derived PMNs sedimented to the substrate under static conditions were bound by ~8000 high-affinity CD18 sites. These receptors spontaneously coalesced into numerous clusters containing an average of ~500 receptors (±100 receptors) of ~0.5 μm² in size (Fig. 1B, 1C). With the application of 4 dyne/cm² of shear stress for 2 min, PMNs maintained an equivalent expression level of high-affinity CD18. However, the smaller high-affinity clusters coalesced into macromolecular clusters of ~3000 receptors with an average area of ~3 μm². These often redistributed to the PMN uropod as cells adopted an elongated shape. Kindlin-3 knockdown in HL-60 expressed equivalent expression of high-affinity CD18 but exhibited impaired bond clustering in response to shear stress. In contrast, Talin-1 knockdown cells exhibited levels of bond clustering equivalent to control sheared cells, commensurate with the mild defect observed in adhesion strengthening. Thus, the consolidation of LFA-1 bond clusters within the plane of adhesion is dependent on application of shear stress and cytoplasmic association of Kindlin-3, but not Talin-1.

**Kindlin-3 associates with high-affinity LFA-1 under tension**

Kindlin-3 has previously been shown to associate with the β subunit of LFA-1 and to mediate clustering in response to acti-
vation through TCR signaling (29). Because tensile force acting on high-affinity LFA-1 promoted bond clustering, we next determined whether this required physical association with Kindlin-3. PMNs were allowed to sediment at low shear flow on a substrate coated with 240Q to activate high affinity CD18 or TS1/18 to stabilize LFA-1 in a low-affinity state (Fig. 2A). An equivalent number of LFA-1 were engaged within the plane of contact in the absence compared with the presence of shear stress, as confirmed by imaging with fluorescence Ab to LFA-1 and high-affinity CD18 (327C). The static condition correlated with a low albeit significant baseline level of Kindlin-3 expression. Application of shear stress to PMNs resulted in a 4-fold increase in high-affinity LFA-1 that corresponded to an ∼10-fold increase in Kindlin-3 expression. PMNs stabilized at low affinity via TS1/18 did not exhibit this increase in Kindlin-3 expression, cells were pretreated with excess anti-Mac-1 ICRF44 in all experiments. Data shown are mean ± SEM from three independent experiments.

Calcium flux via calcium release–activated channels is required for adhesion strengthening

Intracellular calcium flux initiated by membrane calcium release–activated channels (CRACs) cooperates with GPCR signaling in synchronizing PMN arrest and to shape polarization (15).
have previously reported that Orai1 is the predominant CRAC mediating Ca\(^{2+}\) influx in arrested PMNs (15). HL-60 cells were transfected with either a control scrambled siRNA or one specific to Orai1 and then differentiated into PMNs. Cells were settled on a substrate of ICAM-1–Fc and 240Q at low shear, and then stress was ramped in increments up to 40 dyne/cm\(^2\), and cell detachment was quantified. Orai1 knockdown HL-60 exhibited an \(
abla 50\%\) knockdown of Orai1 protein, which resulted in \(
abla 50\%\) defect in Ca\(^{2+}\) flux. These PMNs weakly adhered to the substrate with only \(
abla 40\%\) cells remaining firmly bound compared with \(
abla 90\%\) of the control siRNA–transfected cells (Fig. 3A, Supplemental Fig. 3A, 3B). Similarly, PMNs treated with BAPTA or 2-APB displayed a 2-fold decrease in Ca\(^{2+}\) flux compared with the control untreated PMNs and bound weakly to the substrate, with only \(
abla 20\%\) remaining adherent compared with 70% of control PMNs at 40 dyne/cm\(^2\) (Fig. 3A, Supplemental Fig. 3C). We next confirmed the defect in adhesion strengthening in PMNs isolated from Orai1\(^{+/+}\) versus Orai1\(^{-/-}\) mice. PMNs were activated with Mn\(^{2+}\) and allowed to sediment on an ICAM-1–coated substrate and assayed for adhesion strengthening. Orai1\(^{-/-}\) mice exhibited 30% less stably adherent PMNs compared with Orai1\(^{+/+}\) littermate controls at maximum shear stress (Supplemental Fig. 4A). This defect was LFA-1 dependent, as addition of a function blocking Ab to mouse B1 integrin abrogated the remaining 50% strongly adherent PMNs (data not shown). We conclude that Orai1 expression and its function in force-mediated Ca\(^{2+}\) flux is required for durable adhesion at high shear stress in both human and mouse PMNs.

We investigated whether the Orai1-mediated defect in adhesion strengthening translated into impaired PMN recruitment to a skin wound in a mouse model of acute inflammation. A full-thickness (6 mm diameter) sterile wound was created on the flank of lys-EGFP knockin mice whose mature PMNs upregulate lysozyme and express EGFP (EGFP-PMNs) (15). Calcium levels measured in PMNs from mice heterozygous or homozygous for Orai1 have previously been reported to express \(
abla 50\%\) that of WT littermates (10, 32). Lys-EGFP mice were crossbred with Orai1\(^{+/+}\) or Orai1\(^{-/-}\) mice to noninvasively quantify PMN recruitment using whole animal fluorescence imaging of EGFP-PMNs within the wound site (Supplemental Fig 4B, 4C). Whereas PMNs from both Orai1\(^{+/+}\) and WT control mice demonstrated peak infiltration into wound at 24 h, Orai1\(^{-/-}\) exhibited \(
abla 30\%\) less PMN recruitment to the wound compared with WT controls. PMN numbers in Orai1\(^{-/-}\) mice rapidly declined in the wound after 48 h, whereas the EGFP signal in Orai1\(^{+/+}\) was maintained significantly above Orai1\(^{-/-}\)-knockouts out to day 6 of injury. Spatial imaging of EGFP-PMN fluorescence within the wound revealed the highest concentrations in a circumferential zone just inside the margin of injury, and PMNs numbered significantly higher in the WT than Orai1\(^{-/-}\) mice. These data clearly reveal a defect in the capacity of PMNs to efficiently recruit into the wound margin and suggest that Orai1 participates in optimum PMN arrest and migration into a site of acute inflammatory injury.

To examine the mechanism by which Ca\(^{2+}\) flux played a role in mediating adhesion strengthening, we next examined the topography of high-affinity LFA-1 induced by membrane contact with 240Q and ICAM-1–Fc. Allosteric induction of high-affinity CD18 activated \(
abla 20,000\) sites per PMN, and this elevated level was attributed to the processes of siRNA transfection and subsequent differentiation into PMN (Fig. 3B, 3C). As in Kindlin-3- or talin-1 knockdown, this was not diminished by Ca\(^{2+}\) inhibition with BAPTA, 2-APB, or Orai1 knockdown. However, shear stress induced coalescence of LFA-1 bonds into macromolecular high-affinity clusters measuring \(
abla 3.5\) \(\mu\)m\(^2\) (\(\nabla 9000\) receptors) in isolated PMNs and \(
abla 1.5\) \(\mu\)m\(^2\) (\(\nabla 4000\) receptors) in HL-60 controls.

FIGURE 2. Kindlin-3 enrichment at sites of high-affinity LFA-1 requires presence of shear force. (A) Human PMNs in the presence of 327C mAb were perfused over a substrate coated with 240Q or TS1/18 at a shear stress of 4 dyne/cm\(^2\) or sedimented on the substrate under static conditions. Samples on a substrate coated with ICAM-1–Fc were also stabilized in a high-affinity state by treatment with Mn\(^{2+}\) or converted to low affinity by addition of lovastatin in the presence of Mn\(^{2+}\) and exposed to shear stress, or maintained under static conditions. Cells were fixed, permeabilized, and labeled for Kindlin-3. TIRF microscopy was used to detect expression of both Kindlin-3–labeled and 327C-labeled high-affinity CD18 sites limited to the plane of adhesive contact. (B) PMNs were isolated from whole blood and perfused over a substrate of 240Q, TS1/18, or ICAM-1 and labeled for Kindlin-3. TIRF microscopy was used to detect expression of both Kindlin-3–labeled and 327C-labeled high-affinity CD18 sites limited to the plane of adhesive contact. Western blot is representative of three separate experiments. Data shown are mean ± SEM from three separate experiments.
Coalescence of these clusters was abrogated by inhibition of Ca\(^{2+}\) flux and knockdown of Orai1, respectively. These data reveal that Ca\(^{2+}\) flux via Orai1 is necessary for subsequent consolidation of high-affinity LFA-1 into macromolecular clusters and concomitant adhesion strengthening of PMNs.

Kindlin-3, but not Talin-1, recruitment to adhesive contacts is independent of calcium flux

To define the intracellular mechanisms underlying impaired arrest strengthening, the relative capacity of Talin-1 and Kindlin-3 to recruit to adhesive sites and the dependence on Ca\(^{2+}\) influx through Orai1 was examined. PMNs were exposed to shear flow, fixed/permeabilized, and then labeled with fluorescent Abs to Talin-1 and Kindlin-3 before imaging by TIRF microscopy (Fig. 4). Immunofluorescence of human PMNs treated with BAPTA or 2-APB and of mouse PMNs isolated from the bone marrow of Orai1\(^{-/-}\) was compared with littermate WT controls. Activation was induced by 240Q on human PMNs, and Mn\(^{2+}\) was added to mouse PMNs to stabilize high-affinity bond formation between LFA-1 and ICAM-1 on the substrate. Pharmacological inhibition with 2-APB or BAPTA each significantly diminished the enrichment of Talin-1 at sites of adhesive contact (Fig. 4A). Likewise, Talin-1 enrichment was also decreased by \(\sim 1\)-fold in Orai1\(^{-/-}\) PMNs. In contrast, neither BAPTA, 2-APB, nor Orai1 knockout in mouse PMNs reduced Kindlin-3 enrichment within sites of adhesive contact on sheared PMNs (Fig. 4B). Thus, Talin-1 recruitment to LFA-1 bonds under tension is responsive to Ca\(^{2+}\) influx via Orai1, whereas Kindlin-3 is independent of intracellular Ca\(^{2+}\) levels.

Kindlin-3 is required for Talin/Orai1 recruitment and calcium influx at LFA-1 sites

Because Kindlin-3 redistribution to LFA-1 bonds is independent of intracellular Ca\(^{2+}\) flux, we hypothesized that physical association of Kindlin-3 with LFA-1 is required for Ca\(^{2+}\) influx during bond uptake of tensile force under shear flow. We first determined whether Ca\(^{2+}\) influx was defective in Kindlin-3 shRNA knockdown PMNs. Calcium stores were depleted by incubation with 1 mM thapsigargin in Ca\(^{2+}\)-free media, and HL-60–derived PMNs were then sheared on a substrate coated with TS1/18 or 240Q to stabilize LFA-1 binding sites at low or high affinity, respectively. To initiate the influx of Ca\(^{2+}\) through SOCE in arrested PMNs, a bolus of 1.5 mM Ca\(^{2+}\) buffer was perfused at 60 s (Fig. 5A). PMNs captured via high-affinity LFA-1 registered \(\sim 1000\) nM of Ca\(^{2+}\) flux compared with 500 nM in PMNs stabilized at low affinity. Ca\(^{2+}\) flux in Kindlin-3 knockdown cells bound via either low or high affinity LFA-1 remained at the baseline of 500 nM. In contrast, Talin-1 knockdown PMNs exhibited levels of Ca\(^{2+}\) flux not significantly different from high-affinity LFA-1 sites. These data suggest that Kindlin-3 is required for CRAC function induced by tensile force on high-affinity LFA-1, whereas Talin-1 functions downstream of Ca\(^{2+}\) influx. This prompted examination of whether Kindlin-3 physically recruits Talin-1 and...
Chemokine amplification of calcium flux is mediated by LFA-1, Kindlin-3, and shear stress

Neutrophils rolling on E-selectin and arresting on ICAM-1 under shear flow exhibit a 100-fold increase in sensitivity to IL-8 signaling compared with chemokine stimulation under static conditions as detected by the flux of intracellular Ca²⁺ on arrested cells (9). We investigated whether engagement of LFA-1/ICAM-1 bonds and Kindlin-3 function was necessary for this amplification in Ca²⁺ flux with increased dose of chemokine exposure of HL-60 arrested under shear stress. Differentiated control and Kindlin-3 shRNA-transfected HL-60 cells were loaded with fura 2-AM and perfused over an ICAM-1 substrate, and the maximum level of intracellular Ca²⁺ flux was recorded after IL-8 perfusion (Fig. 6A). Beginning at a baseline level of ~150 nM Ca²⁺ in adherent PMNs stimulated at 0.01 nM IL-8, a 10-fold increase up to ~1300 nM Ca²⁺ was detected at 1 nM IL-8, and this increase required the presence of shear stress acting on high-affinity LFA-1/ICAM-1 bonds. In contrast, Kindlin-3 knockdown cells barely registered an increase in Ca²⁺ flux under shear at maximum stimulation with 1 nM IL-8 (Fig. 6B). Talin-1 knockdown cells, however, exhibited equivalent levels of Ca²⁺ flux to the control cells at 1 nM IL-8 under shear stress. A direct correlation was observed between the level of Ca²⁺ influx and the relative size of high-affinity LFA-1 focal clusters supporting PMN arrest (Fig. 6B). These data suggest that IL-8–stimulated activation of LFA-1 involves not only an upshift to high affinity but also coalescence of focal clusters of LFA-1/ICAM-1 bonds in a process that is catalyzed by the level of chemokine-stimulated Ca²⁺ influx. Shear stress–imparted tensile force promotes Kindlin-3 association with LFA-1 and Orai1, which in turn mechano-transduced local Ca²⁺ influx that subsequently drives macromolecular clustering of LFA-1 bonds promoting durable adhesive contacts at sites of inflammation.

Discussion

Homodynamic shear stress provides an ever-present repulsive force on leukocytes adherent at sites of inflammation. Leukocytes exploit this tensile force that is transmitted across the membrane via durable LFA-1/ICAM-1 bonds to generate integrin-dependent outside-in signals that function to guide cytoskeletal assembly and cell migration (10, 11, 40, 41). Applying TIRF optics to image high-affinity LFA-1 bonds and their interaction with adapter proteins in the plasma membrane, we demonstrated that Kindlin-3 was central to conversion of mechanical force to intracellular signaling based on the following criteria: 1) Kindlin-3 association with LFA-1 was significantly increased at sites of high-affinity bond formation with ICAM-1 only in the presence of hydrodynamic shear stress. 2) Kindlin-3–mediated and Orai1-mediated calcium flux were not required for LFA-1 conversion to high affinity, but mediated the coalescence of bond clusters with ICAM-1 that supported shear-resistant adhesion. 3) Kindlin-3 association with LFA-1 was independent of cytosolic Ca²⁺, but necessary for shear-mediated Ca²⁺ influx via formation of a focal complex with Orai1. 4) Influx of Ca²⁺ through Orai1 was essential for Talin-1 recruitment to LFA-1 bond clusters, which in turn linked the nascent focal complex to the actin cytoskeleton. Together, these data highlight the essential role of Kindlin-3 in transducing mechanical force necessary for the spatial regulation of Ca²⁺ influx and cytoskeletal assembly at focal sites where LFA-1 forms multivalent long-lived bonds with ICAM-1 that mediate leukocyte firm adhesion on inflamed endothelium.

Tensile force and LFA-1 conformation regulates outside-in signaling

An upshift in LFA-1 affinity is signaled by ligation of chemokine to GPCRs that rapidly induce microclustering of high-affinity LFA-1, which is critical for PMN transition to stable arrest (2, 14, 20, 42). There is good evidence that stable bonds between high-affinity LFA-1 and ICAM-1 serve as focal sites of intracellular signaling that elicit PMN shape polarization (4, 43). We examined the nature of this outside-in signaling by capturing PMNs on a substrate presenting the allostERIC Ab 240Q that
binds to the IDAS domain of CD18 and promotes subsequent bond formation to colocalized ICAM-1–Fc heterodimers. Using microfluidic flow channels, we precisely incremented shear stress and examined the role of tensile force acting on either high-affinity bond clusters, those stabilized at low affinity with TS1/18, or tethered via non-integrin CD45 membrane receptors. We demonstrated that tensile force acting on high-affinity LFA-1 attachments was requisite for Ca2+-mediated receptor clustering, as equivalent numbers of high-affinity LFA-1 was detected in the contact region of PMNs adherent to the ICAM-1/240Q substrate under static conditions. Shear stress was required to elicit coalescence of 0.5-μm2 microclusters into ∼3-μm2 macroclusters in the absence of a significant increase in the density of LFA-1 measured at ∼3000–4000 sites/μm2. Moreover, LFA-1 clustering was impaired in Kindlin-3 or Ca2+-depleted PMNs, despite the presence of equivalent numbers of LFA-1/ICAM-1 bonds within the plane of adhesive contact. These data suggest that Kindlin-3 requires mechanical force to gain access to the LFA-1 cytodomain and subsequently provide linkage to Orai1.

LFA-1 mechanosignals via Orai1 and Ca2+ influx

Cytoplasmic Ca2+ functions as a versatile signaling molecule that functions to synchronize the transition from PMN rolling to arrest and shape polarization during its recruitment to sites of insult. GPCR engagement is followed by an intracellular Ca2+ burst mediated through phospholipase-C that releases intracellular stores via activation of inositol 1,4,5-trisphosphate associated with the ER (44, 45). Recently, we reported that cooperation between release of ER calcium stores and activation of Ca2+ influx in arrested PMNs occurs predominantly via Orai1 at sites of focal adhesion under tension (10, 15). In the current study, we demonstrated that chelating intracellular Ca2+ with BAPTA, impairing Ca2+ entry via SOCE with 2-APB, or in Orai1 knockdown PMNs, the size of LFA-1 clusters was diminished resulting in a defect in adhesion strengthening at shear stress above 4 dyne/cm2. We further demonstrated the importance of Orai1 function for efficient PMN recruitment during acute inflammation in a skin wound model. Comparing EGFP-PMN recruitment dynamics in Orai1-deficient mice with WT controls revealed that PMN emigration was significantly impaired and consistent with published findings that Orai1-defective PMNs are unable to induce LFA-1–mediated calcium flux and undergo F-actin–driven shape change and migration under shear flow (10). Together, this supports the concept that LFA-1 clustering and polarization is tightly regulated by release of intracellular Ca2+ and downstream linkage to cytoskeletal proteins including Talin-1, F-actin, and vinculin, all of which are necessary for optimal immune response of PMN (26, 46, 47).

LFA-1 bond clusters amplify chemokine signaling dependent on association with Kindlin-3 and Ca2+ flux

In the absence of shear stress, PMNs failed to elicit significant Ca2+ flux at maximal IL-8 stimulation, despite activation of ∼100,000 high-affinity CD18 per cell (41, 48, 49). The requirement of shear in activating calcium flux in HL-60–differentiated PMN is quali-
tatively similar to previous observations on freshly isolated human PMNs, wherein a 100-fold higher dose of IL-8 is necessary to elicit an equivalent level of Ca\textsuperscript{2+} flux under static versus shear flow conditions (9). The data support a mechanism by which engagement of high-affinity LFA-1 acts synergistically with chemokine receptor signaling to effectively amplify release of intracellular Ca\textsuperscript{2+}. Kindlin-3 association with high-affinity LFA-1 was necessary for this amplification in Ca\textsuperscript{2+} release in the presence of shear flow. We speculate that Kindlin-3 functions as a mechanosensor, as its association with LFA-1 did not itself require upshift to a high-affinity conformation or Ca\textsuperscript{2+} signaling, but rather was dependent upon tensile force applied to high-affinity bond clusters. Silencing expression of Kindlin-3 impaired LFA-1 clustering at adhesive contacts, which correlated with a significant decrease in adhesion strengthening. In contrast, silencing expression of Talin-1 did not alter high-affinity LFA-1 clustering and only slightly diminished stable adhesion at high shear stress. This suggests that Talin-1 may not play an eminent role in clustering of LFA-1, but may assist in reorganization of the actin cytoskeleton and reinforcement of LFA-1 attachment to the cortical cytoskeleton via adapter proteins such as paxillin and vinculin that associate with the integrin cytodomain (46, 50, 51). Our data demonstrate that Kindlin-3 recruitment to the \(\beta_2\)-cytodomain is necessary for optimum calcium flux by physically linking LFA-1 bond clusters and Orai1 at a point upstream of Talin-1 association. Recent evidence indicates that a \(\beta_2\)-specific NPXF motif is essential for triggering calcium signaling in Jurkat cells, and our data suggest that Kindlin-3 may function as the scaffold protein linking the \(\beta_2\)-integrin cytodomain to Orai1 during intracellular calcium mobilization (52). There are a number of cytoplasmic proteins that Kindlin-3 may interact with to activate calcium influx including STIM1, an ER luminal calcium sensor that facilitates clustering and spatial recruitment of Orai1 proximal to the ER (53, 54). Both calcium influx through Orai1 and Kindlin-3 are necessary for immune surveillance involving LFA-1-mediated signaling (38, 55, 56). Our data suggest that all three molecules contribute to mechanosignaling in PMNs. Future studies will focus on how Kindlin-3 facilitates this cooperation between phospholipase C–mediated calcium flux and Orai1 CRAC and which cytoskeletal proteins act as the conduit linking mechanical force to calcium flux.

In summary, we define a mechanism by which tensile force acting on LFA-1/ICAM-1 bonds mechanically catalyzes its association with Kindlin-3; a necessary step in linkage with Orai1 and initiation of Ca\textsuperscript{2+} influx at focal sites of adhesion. This latter process provides a feed-forward mechanism that is cooperative with chemokine signaling to promote coalescence of LFA-1 bond clusters and cytoskeletal reinforcement of adhesion via Talin-1 and F-actin. In this manner, durable multivalent bonds are anchored to the cytoskeleton and serve to strengthen adhesion and direct pseudopod projections that efficiently guide PMNs during transendothelial migration.
References


Supplementary figure 1: Talin-1 and Kindlin-3 knockdown by lentiviral transfection of shRNA in HL-60 cells. Control, Talin-1 and Kindlin-3 shRNA transfected cells were lysed before and after differentiation with 1.3% DMSO, 1mg protein lysate was run on SDS page and Talin-1 and Kindlin-3 protein was detected by western blot. Talin-1 and Kindlin-3 protein expression was normalized to GAPDH expression and revealed a 70% and 80% knockdown respectively. For blot images of Talin-1 knockdown in Talin-1 shRNA cells, the two blots are from the same lane.
Supplementary Figure 2: Correlating 327C MFI with antibody binding sites on beads at the contact site. 7.65 μm beads with increasing antibody binding sites were incubated with 327C mAb-Alexa-488 for 30 minutes and allowed to settle on a glass substrate. 327C fluorescence was imaged by TIRF at settings equivalent to other experiments measuring 327C expression and mean fluorescence intensity was quantified for each bead condition. 327C MFI at contact was plotted against average receptor numbers at contact site to obtain a linear relationship.
**Supplementary Figure 3**

**A.** HL-60 cells transfected with control or Orai1 siRNA and differentiated over 3 days with 1.3% DMSO were lysed and 1mg protein lysate was run on SDS page and Orai1 protein was detected by western blot. Orai1 protein expression was normalized to GAPDH expression and revealed a 50% knockdown. For blot images of Orai1 knockdown the two blots are from the same lane. **B and C.** PMN were isolated from whole blood or HL-60 cells were transfected with control and Orai1 siRNA and differentiated to form neutrophil like cells. Cells were labeled with Fura-2AM, PMN were treated with 50uM Bapta and 100uM 2-APB and all cells were perfused over an ICAM-1+240Q substrate and intracellular calcium flux was measured over 2 minutes. To ensure LFA-1 dependent adhesion, cells were pretreated with anti-Mac-1 mAb ICRF44. Maximum calcium levels were plotted and data shown is mean+/- SEM from 3 independent experiments.

**Supplementary Figure 3** *Orai1 knockdown, Bapta and 2-APB effectively inhibit intracellular calcium.* A. HL-60 cells transfected with control or Orai1 siRNA and differentiated over 3 days with 1.3% DMSO were lysed and 1mg protein lysate was run on SDS page and Orai1 protein was detected by western blot. Orai1 protein expression was normalized to GAPDH expression and revealed a 50% knockdown. For blot images of Orai1 knockdown the two blots are from the same lane. B and C. PMN were isolated from whole blood or HL-60 cells were transfected with control and Orai1 siRNA and differentiated to form neutrophil like cells. Cells were labeled with Fura-2AM, PMN were treated with 50uM Bapta and 100uM 2-APB and all cells were perfused over an ICAM-1+240Q substrate and intracellular calcium flux was measured over 2 minutes. To ensure LFA-1 dependent adhesion, cells were pretreated with anti-Mac-1 mAb ICRF44. Maximum calcium levels were plotted and data shown is mean+/- SEM from 3 independent experiments.
Supplementary Figure 4: Orai1 is required for PMN adhesion strengthening and migration to wound sites. A. Bone marrow neutrophils were isolated from Orai1^{++} and Orai1^{+-} mice, activated with Mn^{2+} and allowed to settle over an ICAM-1 coated substrate. Shear was ramped from 0, 4, 10, 20, 40, 80 dynes/cm² at 30 second intervals and number of cells remaining bound were counted and plotted. To ensure LFA-1 dependent adhesion, cells were pretreated with anti-Mac-1 M1/70 for all experiments. B and C. Skin wounds were created on the backs of EGFP-Orai1^{++} and EGFP-Orai1^{+-} mice and EGFP neutrophil fluorescence in the wound was tracked over 8 days. Representative images at Day 0 and 1 are shown. Data is plotted as mean+/− SEM from n=3 experiments.