Neutrophil rolling and transition to arrest on inflamed endothelium are dynamically regulated by the affinity of the \( \beta_2 \) integrin CD11a/CD18 (leukocyte function associated antigen 1 (LFA-1)) for binding intercellular adhesion molecule (ICAM)-1. Conformational shifts are thought to regulate molecular affinity and adhesion stability. Also critical to adhesion efficiency is membrane redistribution of active LFA-1 into dense submicron clusters where multimeric interactions occur. We examined the influences of affinity and dimerization of LFA-1 on LFA-1/ICAM-1 binding by engineering a cell-free model in which two recombinant LFA-1 heterodimers are bound to respective Fab domains of an antibody attached to latex microspheres. Binding of monomeric and dimeric ICAM-1 to dimeric LFA-1 was measured in real time by fluorescence flow cytometry. ICAM-1 dissociation kinetics were measured while LFA-1 affinity was dynamically shifted by the addition of allosteric small molecules. High affinity LFA-1 dissociated 10-fold faster when bound to monomeric compared with dimeric ICAM-1, corresponding to bond lifetimes of 25 and 330 s, respectively. Downshifting LFA-1 into an intermediate affinity state with the small molecule \( \alpha_L \) subunit of LFA-1 domain allosteric inhibitor IC487475 decreased the difference in dissociation rates between monomeric and dimeric ICAM-1 to 4-fold. When LFA-1 was shifted into the low affinity state by lovastatin, both monomeric and dimeric ICAM-1 dissociated in less than 1 s, and the dissociation rates were within 50% of each other. These data reveal the respective importance of LFA-1 affinity and proximity in tuning bond lifetime with ICAM-1 and demonstrate a nonlinear increase in the bond lifetime of the dimer versus the monomer at higher affinity.

Neutrophils circulate in the bloodstream to sites of inflammation where they adhere and transmigrate through the endothelium as the initial step in combating infection and to facilitate wound healing. Recruitment from the circulation involves a multistep process of cell rolling, activation, and arrest. The heterodimeric integrin receptor LFA-1\(^1\) is composed of the \( \alpha_L \) (CD11a) and \( \beta_2 \) (CD18) subunits and is constitutively expressed in a low affinity conformation on the plasma membrane of leukocytes (1–3). Neutrophils encountering chemokines on inflamed endothelium are activated to shift LFA-1 from the low to high affinity conformation, which supports tight binding to endothelial ICAM-1. Increases in integrin affinity correlate in time with adhesion function as recently demonstrated in aggregation of cells expressing \( \alpha_4 \beta 1 \) and vascular cell adhesion molecule (4). ICAM-1 recognizes LFA-1 through an inserted (I) domain in the \( \alpha \) subunit. There is strong evidence correlating shifts in I domain conformation to affinity changes in binding ICAM-1. Mutations in I domain residues stabilized distinct structural conformations correlating to LFA-1 affinity. ICAM-1 equilibrium binding constants increase over 4 orders of magnitude ranging between low (i.e. 1600 \( \mu M \)), intermediate (i.e. 9 \( \mu M \)), and high affinity (i.e. 0.15 \( \mu M \)) (5). Further evidence linking allosteric shifts in I domain conformation to ICAM-1 binding is the activity of a class of allosteric small molecule antagonists engineered to inhibit LFA-1 function (6–9). Statin-derived small molecules such as lovastatin and LFA703 target the I domain allosteric site (IDAS) and abrogate LFA-1 recognition of ICAM-1 (8, 10). Another small molecule to the IDAS, BIRT377, was shown to inhibit rolling and adhesion of LFA-1 transfectants to ICAM-1 monolayers (11). BIRT377 and LFA703 appear to exert their actions through shifting LFA-1 into a bent conformation, rendering the I domain inaccessible and LFA-1 to a low affinity state (12). A second class of small molecules binds to the I-like domain in the \( \beta \) subunit of LFA-1 and indirectly regulates I domain affinity and ligand binding (10, 12). The small molecule XVA143 binds to the I-like domain and promotes cellular rolling by inducing an extended conformation that stabilizes an intermediate affinity associated with rolling of LFA-1 expressing transfectants in shear flow (11). We present here a new small molecule allosteric inhibitor that targets the IDAS and downshifts LFA-1 from a high to intermediate affinity. This small molecule is similar to the diaryl sulfide cinamamide antagonists (13). Allosteric small molecules provide a powerful tool for directing leukocyte adhesion; however, the interrelationships between bond kinetics, LFA-1 conformation, valence in binding ICAM-1, and adhesion stability remain ill-defined.

Concomitant with a shift in affinity is a rapid redistribution of LFA-1 into high density clusters on the plasma membrane.
We have reported recently that within seconds of activation, LFA-1 on neutrophils reorganizes from a uniform surface distribution to form both small punctate clusters (<1 μm²) and large caps (≈3 μm²) (1, 14). Clustering of LFA-1 on leukocytes tethered to inflamed endothelium in shear flow is a key step in adhesion strengthening and the transition from cell rolling to arrest (1, 14). To emulate affinity and molecular scale clustering of LFA-1 on activated leukocytes, we engineered a cell-free LFA-1 expression system by fusing the α-β subunits at the C terminus with an inserted leucine zipper motif. The C termini of two heterodimers were bound to each Fab arm of an anti-leucine zipper antibody covalently attached to the surface of a latex microsphere. We tested the hypothesis that two adjacent LFA-1 binding to an ICAM-1 homodimer could facilitate re-binding and exponentially prolong bond lifetime. Binding of fluorescent ICAM-1 to LFA-1 on beads was monitored in real time by flow cytometry while shifting LFA-1 conformation and affinity state with soluble agonists and antagonists.

Dissociation of monomeric ICAM-1 from high affinity LFA-1 was ~10-fold faster than dimeric ICAM-1. This difference was attributed to the ability of the dissociated leg of the ICAM-1 dimer to rebind to an adjacent LFA-1 as it is held in proximity by the remaining LFA-1 bond. Adhesion of neutrophils to beads presenting dimeric ICAM-1 in shear flow was sustained beyond 10 min, while monomeric ICAM-1 beads dissociated within 100 s. These data highlight the physiological significance of regulation of both LFA-1 affinity and LFA-1 spatial proximity in tuning bond lifetime and adhesion stability when binding to ICAM-1 homodimer.

**MATERIALS AND METHODS**

**Reagents—**The following antibodies were used: anti-leucine zipper (324C), anti-CD18 activating antibody (240Q) (1, 6, 15, 16), sterile blocking anti-LFA-1 (TS1/22) (17), anti-LFA-1 TS2/4 (18), recombinant LFA-1 heterodimer with an inserted leucine zipper, ICAM-1-flg produced as a chimeric human IgG containing two full-length ICAM-1 (molecular mass is 150 kDa as confirmed by native PAGE), anti-CD18 32C7 (1, 15), and LFA-1 small molecule allosteric inhibitor IC487475 of the p-arythio cin-amidines series that targets the I domain allosteric site of LFA-1. Inhibitory anti-CD54 (KIM-127) was purchased from Pierce. Blocking anti-Mac-1-2LPM19c was purchased from DakoCytomation, Glostrup, Denmark. Lovastatin sodium was purchased from Calbiochem. Anti-CD18 KIM12D (11, 19–21), which detects extended conformations of LFA-1, was a gift from Martin Robinson (Exploratory Research Cell Tech Therapeutics Ltd., Bath Road Slough, UK). Recombinant human ICAM-1 (monomeric full-length, molecular mass is 85 kDa) was purchased from Polysciences, Inc. (Warrington, PA). 500 μl of anti-Mac-1-2LPM19c was purchased from DakoCytomation, Glostrup, Denmark. Anti-Leu-10 was supplied by Calbiochem. 5 μl of anti-Leu-10 (100 μg/ml) was added prior to activation and incubated at 37 °C, 450 rpm for 10 min at room temperature in HEPES buffer, human serum albumin (0.1%), and CaCl₂ (1.5 mM). Kinetic experiments were performed as described for the LFA-1 microspheres.

**ICAM-1 Bead Assembly—**Carboxylate microspheres (diameter = 10 μm) were purchased from Polysciences, Inc. (Warrington, PA). 500 μl of beads were washed twice in 1.5 ml of MES buffer, pH 5.0 (Sigma), resuspended in 500 μl of MES, and sonicated for 15 min. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (Molecular Probes, Eugene, OR), was added at 1 mM, and beads were incubated for 5 min at room temperature and at 500 rpm. Monomeric ICAM-1 (70 μg/ml) or dimeric ICAM-1/Flg (32 μg/ml) was mixed with the beads for 1 h at room temperature and at 500 rpm. Glycine (Sigma) was added (10 mM), and beads were incubated for 10 min at 37 °C by flow cytometry. Blocking solution (Molecular Probes) was added to the beads for 10 min, and beads were washed and incubated for an additional 15 min. ICAM-1 beads were washed in PBS and resuspended in 1.5 ml of PBS. Site densities were obtained by Quantum Simply Cellular Beads (Bangs Laboratories, Fishers) to be ~6000 sites/μm² for monomeric and dimeric beads as identified by anti-CD54.

**Adhesion of Neutrophils to Beads Presenting Monomeric and Dimeric ICAM-1—**Neutrophils were mixed with 10-μm fluorescent latex beads (Fluoresbrite Carboxyl YG 10 Micron Microspheres) with ICAM-1 derivatized to their surface. Samples contained 1 × 10⁶ neutrophils/ml, 2 × 10⁶ beads/ml, and a small magnetic stir bar. Mac-1 blocking antibody 2LPM19c (test volume 10 μl) and/or 240Q (10 μg/ml) was preincubated for 10 min with the cell suspension without beads. For samples stimulated with IL-8 (5 nm), beads and stimuli were added immediately after the addition of ICAM-1 beads. Samples were maintained at 37 °C within a mixing chamber with a magnetic motor as described previously (22). The magnetic motor coupled with a magnetic stir bar created a shear field (shear stress ~1.0 dyn/cm²) within the test tube and initiated collisional interactions. Neutrophil capture of ICAM-1 beads was monitored by viewing the forward and right angle light scatter properties and gated in order to exclude unbound beads. Neutrophil-bead adhesion was quantitated on green fluorescence on fluorescence histograms. Quantal increases in fluorescence appeared as peaks in the fluorescence histogram corresponding to populations of neutrophils binding increasing numbers of beads (22). To distinguish relative levels of bead capture within the stimulated neutrophil population, neutrophil-bead interactions were...
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quantitated as the average number of beads per neutrophil according to Equation 1,

\[
\text{beads/neutrophil} = \frac{1}{N} \sum_{j=1}^{5} (NBj)
\]

(Eq. 1)

where \( N \) represents the number of nonadherent neutrophils, and \( NBj \) represents the number of neutrophil-bead aggregates bound to between 1 and 5 beads. Aggregates larger than 5 beads were not notably seen in this assay.

Dissociation of ICAM-1 beads from neutrophils was induced after 2 min by removing the cytometer sample tube for not more than 10 s during reading and adding inhibitor (1 \( \mu \)M IC487475, 100 \( \mu \)M lovastatin, 100 \( \mu \)g/ml TSI122, or 4 \( \mu \)l of 1:100 Me2SO/PBS). Cytometer reading resumed and dissociation was modeled as rate of bead/polymorphonuclear leukocyte disaggregation.

Data Analysis—Data were analyzed using Graphpad Prism version 4.0 for Windows (Graphpad Software Inc., San Diego). Constants \( k_{on} \) (association rate constant) and \( K_D \) (equilibrium affinity constant) was calculated as 4.0 for Windows (Graphpad Software Inc., San Diego). Constants were obtained by performing one-phase exponential decay (Y = specific binding \( \times e^{-kt} \) + nonspecific binding) and one-phase exponential association (Y = \( Y_{max} \) \( \times e^{-kt} \)) curve fits of the real time data, respectively. The \( k_{off} \) (association rate constant) was calculated as 1.0 for Windows (Graphpad Software Inc., San Diego). Constants were obtained by taking the average fluorescence over 5–25 s following the rate of change. \( K_{Dy} \), the equilibrium affinity constant, was calculated as \( k_{on}/k_{off} \) for kinetic experiments with ICAM-1 binding to the LFA-1 beads. \( K_{Dy} \) was calculated as the EC50 value in a dose-response curve for ICAM-1 binding to the neutrophils. Statistical significance was determined (\( p \leq 0.05 \)) by one-way analysis of variance with a Newman-Keuls multiple comparison post-test or by two-way analysis of variance.

RESULTS

LFA-1 expressed on the leukocyte membrane can shift its conformation in response to inside-out signaling via chemokine stimulation or extracellularly by addition of divalent cations (23), antibodies (16), and small molecules (12, 24). LFA-1 heterodimer captured at the C terminus by an antibody covalently attached to microspheres was predominantly expressed in a low affinity state but retained the flexibility to shift conformation and boost affinity for ICAM-1. Cell-free LFA-1 increased binding to dimeric ICAM-1/Ig by 150% over base line in response to the addition of divalent cations Mg2+ or Mn2+, but by only 20% in response to Ca2+. Addition of mAb 240Q, which is associated with allosterically stabilizing CD18 into a ligand binding conformation (1, 6, 15, 16), did not itself induce activation of LFA-1 on beads but in conjunction with Mg2+ augmented ICAM-1 binding by 50% above stimulation with Mg2+ alone.

ICAM-1/Ig binding was inhibited by pretreating Mg2+-activated LFA-1 with mAb TS1/22, which when bound to its epitope on the I domain sterically blocks ICAM-1 recognition (17, 20, 25). Addition of the allosteric small molecule IC487475, which binds with high affinity to the I domain (i.e. \(< 10 \text{ nM} \)), also abrogated ICAM-1 binding stimulated by Mg2+ (Fig. 1a). Pretreatment with mAb TS1/18, which binds to an allosterically sensitive domain on the \( \beta \) subunit (25), blocked 65% of ICAM-1 binding in the presence of Mg2+. A nonblocking control, anti-CDS1a TS2/4 (18), increased ICAM-1 binding in the presence of Mg2+ by 25% (data not shown). These data indicate that cell-free LFA-1 retains the capacity to increase affinity for the ICAM-1/Ig dimer through activation by divalent cation or by allosteric mAb 240Q. Moreover, ICAM-1 binding can be sterically blocked by TS1/22 or allosterically inhibited by binding of a small molecule to the I domain.

Antibodies that recognize specific epitopes on the \( \beta \) subunit can report on integrin conformation and activation. KIM127 binding has been correlated recently with the extended conformation of LFA-1 and is associated with intermediate or high affinity ligand binding (11, 19–21). This activation reporter was used to determine the mechanism by which IC487475 and 240Q allosterically alter the affinity for ICAM-1. In the absence of divalent cations, binding of KIM127 was 2-fold above an IgG isotype-matched control, indicating that a fraction of the LFA-1 adopted a conformation other than the bent low affinity state in the absence of stimulation (data not shown). Activation of LFA-1 with Mg2+ increased KIM127 binding by 40%, which was augmented to 60% upon addition of 240Q (Fig. 1c).

FIG. 1. Stimulation and inhibition of ICAM-1/Ig binding to LFA-1 on beads.a, ICAM-1/Ig-AlexaFluor488 binding to LFA-1 in the presence of Mg2+ (3 mM), Ca2+ (1.5 mM), Mn2+ (3 mM), and 240Q (10 \( \mu \)g/ml). Binding was detected by flow cytometry and expressed as fold increase of mean fluorescence intensity over untreated + S.E., \( n = 8 \) except Mn2+, where 3 cells indicated significant difference from Mg2+ alone. b, inhibition of ICAM-1/Ig binding to LFA-1 on beads by antibody and small molecule. Small molecule IC487475 was 1 \( \mu \)M; steric blocking anti-LFA-1 TS1/22 was 20 \( \mu \)g/ml, allosteric blocking anti-CDS1 TS1/18 was 20 \( \mu \)g/ml, and allosteric anti-LFA-1 TS2/4 was 20 \( \mu \)g/ml. Mg2+ (3 mM) and ICAM-1/Ig were added post-inhibition (-fold increase in MFI \( \pm \) S.E., \( n = 3, * \) indicates significant inhibition). c, detection of LFA-1 activation determined by binding of KIM127 in response to activation or inhibition with Mg2+, 240Q, or IC487475. (-Fold increase in MFI \( \pm \) S.E., \( n = 4, * \) indicates significant difference at \( p \leq 0.05 \)).
binding. This suggests that IC487475 can downshift LFA-1 affinity at the IDAS in the absence of inducing a bent conformation of the heterodimer.

Kinetic Analysis of ICAM-1 Binding by Fluorescence Flow Cytometry—Leukocytes can shift LFA-1 conformation and increase avidity within seconds of contact at vascular sites of inflammation (26). We applied fluorescence flow cytometry to detect with sub-second resolution the binding kinetics of ICAM-1-Alexa488 to suspensions of LFA-1 beads following addition of ICAM-1 into the cytometry tube (Fig. 2a). Binding kinetics for monomeric and dimeric ICAM-1 were modeled (see “Materials and Methods” for details), and the $k_{on}$ values were found to be statistically equivalent (Fig. 2b). The rate of association of both dimeric and monomeric ICAM-1 was increased by ~60% in the presence of the allosteric stabilizing mAb 240Q, denoted as stabilized. Binding was normalized to maximum mean fluorescence intensity. Association rate was modeled using a one-phase exponential association equation as defined under “Materials and Methods.” $b$, association rate constants, $k_{on}$, for ICAM-1/Ig binding to LFA-1 beads (mean ± S.E., n ≥ 3).

FIG. 2. Kinetics of monomeric ICAM-1 and ICAM-1/Ig binding to LFA-1 beads. a, representative curves for the kinetic association of monomeric ICAM-1 or dimeric ICAM-1/Ig. Binding occurred in the presence of Mg$^{2+}$, with and without activating antibody 240Q, denoted as stabilized. Binding was normalized to maximum mean fluorescence intensity. Association rate was modeled using a one-phase exponential association equation as defined under “Materials and Methods.” $b$, association rate constants, $k_{on}$, for ICAM-1/Ig binding to LFA-1 beads (mean ± S.E., n ≥ 3).

FIG. 3. Kinetics of dimeric ICAM-1/Ig dissociation from LFA-1 beads. Dissociation induced in the presence of the activators Mg$^{2+}$ or Mg$^{2+}$ with 240Q (stabilized). Curves were modeled to a one-phase exponential decay equation as described under “Materials and Methods.” Inhibitors added were as follows: IC487475, lovastatin, TS21/22, TS2/4, dilution 1:10 with excess unlabeled ICAM-1/Ig. $a$, representative curves of binding and dissociation kinetics of dimeric ICAM-1 binding to dimeric LFA-1 beads. LFA-1 was stimulated by Mg$^{2+}$ (3 mM), and dissociation was induced by injection of inhibitor through polyethylene tubing inserted into the cytometer tube at time point indicated by arrow. $b$, representative curves of ICAM-1/Ig dissociation by dilution, small molecule, or antibody in the presence of Mg$^{2+}$ or Mg$^{2+}$ and 240Q. $c$, dissociation rate constants, $k_{off}$, for ICAM-1/Ig from LFA-1 beads as computed from one-phase exponential decay equation (mean ± S.E., n ≥ 3, * denotes a significant increase in $k_{off}$, p ≤ 0.05).
nonblocking TS2/4, which binds to the β-propeller of the α subunit (18), did not induce dissociation of ICAM-1/Ig. Stabilizing CD18 by activation in the presence of Mg\(^{2+}\) and 240Q decreased \(k_{\text{off}}\) by 30% (Fig. 3c). These data demonstrate 1 domain-specific allosteric regulation of ICAM-1 bond lifetime from a high affinity of \(-300\) s down to \(-0.4\) s.

We next determined whether dimeric LFA-1 positioned on each Fab of the capture antibody bound more tightly to dimeric ICAM-1/Ig than to the monomeric ICAM-1. Dissociation kinetics in the presence of excess unlabeled ICAM-1/Ig or TS1/22 revealed that monomeric bonds are more transient than dimeric bonds (Fig. 4a). ICAM-1 dissociated at a rate of \(0.04\) s\(^{-1}\), a value 11-fold faster than dissociation of dimeric ICAM-1/Ig in the presence of unlabeled ICAM-1 (Fig. 4b). Stabilization of activated LFA-1 by 240Q slowed dissociation of monomer ICAM-1 by \(-30\%\), on par with that observed for stabilized dimer. Injection of IC487475 hastened dissociation by 4-fold, corresponding to a decrease in LFA-1 bond lifetime from \(-25\) to \(-7\) s (Table I). Injection of lovastatin hastened dissociation by 100-fold from the high affinity state of monomeric ICAM-1.

We next tested the dissociation of monomeric and dimeric ICAM-1 from monovalent LFA-1 that was covalently attached directly to the bead surface presumably as a single heterodimer. Kinetic analysis revealed dissociation of monomeric ICAM-1 at \(0.05\) s\(^{-1}\) and dimeric at \(0.05\) s\(^{-1}\) from high affinity LFA-1. Because dissociation from monovalent LFA-1 occurred at statistically equivalent rates, we concluded that divalent LFA-1 that was covalently attached to the plasma membrane, both of which bind ICAM-1 (1). To discriminate the binding kinetics of the LFA-1 ICAM-1 interaction, neutrophils were preincubated with mAb 2LPM19c that blocks Mac-1 binding to ICAM-1. In contrast to cell-free LFA-1, ICAM-1 binding to neutrophils was not activated by addition of Mg\(^{2+}\) alone. Therefore, both Mg\(^{2+}\) and 240Q were added to cell suspensions, and binding was measured in real time by flow cytometry. AlexaFluor488-conjugated ICAM-1 binds both specifically and nonspecifically to neutrophils, the latter defined as that nonblockable with anti-CD18. Therefore, only dimeric ICAM-1/Ig, which yielded a significant increase in specific binding, was examined (Fig. 5c). Addition of ICAM-1/Ig to neutrophils elicited binding kinetics qualitatively similar to cell-free LFA-1 on beads. Dissociation of ICAM-1/Ig was induced by injection of IC487475 or TS1/22, which decreased signal down to the background level of fluorescence (Fig. 5b), whereas addition of nonblocking anti-LFA-1 TS2/4 did not induce dissociation from neutrophils (data not shown). Dissociation of ICAM-1/Ig from high affinity LFA-1 on neutrophils was \(-0.02\) s\(^{-1}\), a value between those observed for monomer and dimer ICAM-1 on cell-free LFA-1 (Table I).

**Table I**

|         | \(k_{\text{on}}\) s\(^{-1}\) (×10\(^5\)) | \(k_{\text{off}}\) s\(^{-1}\) (×10\(^{-3}\)) |
|---------|-----------------------------------|----------------------------------|
| Monomer | 7 ± 3                             | 29 ± 3                           |
| Dimer   | 6 ± 1                             | 3 ± 0.2                          |
| Neutrophil | 11 ± 4                        | 17 ± 5                           |

**Fig. 4.** Kinetics of dissociation of monomeric ICAM-1 from LFA-1 beads. a, representative curves of monomeric ICAM-1 dissociation with and without activating 240Q (stabilized), by a 1:10 dilution with excess unlabeled ICAM-1/Ig, TS1/22, IC487475, or lovastatin in the presence of Mg\(^{2+}\) (3 mM). Curves were modeled to a one-phase exponential decay equation. b, dissociation rate constants, \(k_{\text{off}}\), for monomeric ICAM-1 from LFA-1 beads. Constants were calculated by Prism software from a one-phase exponential decay equation (mean ± S.E., \(n = 3\), * denotes a significant increase in \(k_{\text{off}}\) at \(p \leq 0.05\)).

IC487475 hastened the rate of dissociation by 4-fold that of steric inhibition with TS1/22 (Fig. 5c). ICAM-1/Ig dissociation induced by binding of allosteric or steric inhibitors was \(-2\)-fold faster on neutrophils as compared with beads. These data suggest that a fraction of the LFA-1 expressed on neutrophils either binds monovalently to ICAM-1/Ig or remains in a lower affinity state.

Equilibrium affinity constants were computed from binding kinetics of ICAM-1/Ig on beads yielding a \(K_D\) of 19 nM for stabilized (240Q) dimer, a value \(-10\)-fold lower than the \(K_D\) of 221 nM obtained for stabilized monomeric ICAM-1 (Fig. 6). In

**Fig. 5.**...
the absence of 240Q, the $K_D$ for dimeric ICAM-1 was $\sim 50$ nM. By comparison the $K_D$ for neutrophil binding to dimeric ICAM-1 was $\sim 150$ nM.

**Influence of LFA-1 Affinity and Valency on Neutrophil Adhesion**—We have reported previously that activation of neutrophils through chemokine signaling, or allosterically by mAb 240Q, induces high affinity and high density clusters of LFA-1 on the plasma membrane. These two components were critical for eliciting optimum adhesion efficiency of neutrophils on ICAM-1 expressing substrates in shear flow (27–29). To examine how ICAM-1 presentation as a monomer or dimer influences the efficiency of LFA-1-mediated adhesion of neutrophils stimulated with IL-8 or 240Q, we examined the kinetics of capture of beads coated with equivalent numbers of monomeric and dimeric ICAM-1 in sheared cell suspension. Cytometric based detection of neutrophil capture of fluorescent beads coated with ICAM-1 provides a continuous readout of the kinetics of LFA-1 avidity. In this case, the rate of ICAM-1 bead capture was quantitated over the 1st min following injection of agonist (Fig. 7a). In the absence of stimulus, monomeric beads did not adhere, whereas dimeric ICAM-1/Ig beads exhibited a low level of bead capture (Fig. 7b). Dimeric ICAM-1 beads bound with a 1-fold higher capture rate than monomeric ICAM-1 beads in response to stimulation with IL-8 or 240Q. A remarkable difference was in the stability of bead adhesion activated with IL-8. Beyond the 1st min of bead capture, monomeric ICAM-1 beads steadily dissociated from neutrophils, whereas dimeric beads were captured and remained stably bound throughout 10 min of observation (Fig. 7a). Activation of neutrophils with 240Q effectively prevented monomeric bead dissociation and boosted the rate of capture up to 3-fold for either type of ICAM-1 beads. These data indicated that both the affinity of LFA-1 and its valency in binding ICAM-1 directly influenced the efficiency and stability of neutrophil adhesion.

We next examined the nature of bond stability in dissociation of the dimeric ICAM-1/Ig beads by injecting soluble inhibitors directly into the sheared neutrophil bead suspensions (Fig. 7, c and d). We hypothesized that the stability of ICAM-1 bead adhesion is dependent on the continuous formation of LFA-1 bonds and that the rate of dissociation is indicative of these dynamics. Monomeric ICAM-1 beads dissociated at the same rate as those treated with TS1/22, suggesting that blocking rebinding of a single leg of ICAM-1/Ig facilitated bead dissociation at a rate expected for the decay of single LFA-1/ICAM-1 bonds. By comparison, IC487475 and lovastatin hastened the rate of dissociation by 40% and 2-fold, respectively, compared with TS1/22 dissociation. These data reveal how shifts into intermediate and low affinity influenced the stability of neutrophil adhesion. Furthermore, they revealed the importance of dimeric bond formation between LFA-1 and ICAM-1 in adhesion stability in shear flow.

**DISCUSSION**

Integrins may be thought of as gatekeepers in controlling the location and efficiency of leukocyte recruitment to vascular
Fig. 7. Time course of neutrophil capture and adhesion to monomeric and dimeric ICAM-1 beads. Mac-1 binding was blocked by prior incubation of neutrophils with antibody 2LPM19c. Neutrophils were left unstimulated, stimulated with 5 nM IL-8, or stimulated with 10 μg/ml activating anti-CD18 240Q. Dissociation was induced by addition of allosteric inhibitors IC487475, lovastatin, and TS1/22, or vehicle control of 0.1% Me2SO. a, number of monomeric or dimeric ICAM-1 beads captured per neutrophil over time. b, rate of monomeric or dimeric bead capture by neutrophils over the 1st min of adhesion. c, number of dimeric ICAM-1 beads captured per neutrophil over time with indicated inhibitor added after 2 min. Curves shown were activated with IL-8. d, dissociation rate constant after addition of inhibitors or Me2SO vehicle control to dimeric ICAM-1 beads (or monomeric ICAM-1 beads where indicated with no addition of inhibitor) from neutrophils stimulated with IL-8 (mean ± S.E., n ≥ 2, * denotes significant difference with p ≤ 0.05).

Sites of inflammation. To explore the relationships between integrin structure and function, we have engineered beads to express recombinant LFA-1 as a dimer at levels commensurate with that on leukocytes. This allowed a study of the influence of LFA-1 affinity and valence on bond lifetime and adhesion to ICAM-1 expressed as monomer and homodimer. Shifts in LFA-1 conformation from a low or intermediate to high affinity state were induced by divalent cations with the level of activation such that Mn2+ and Mg2+ exceeded Cu2+, in accordance to the hierarchy of activation observed previously (30, 31). ICAM-1 dissociation from high affinity LFA-1 I-domain was ~10-fold faster for monomeric than dimeric ICAM-1. A downshift in LFA-1 affinity was rapidly triggered by the binding of IC487475 orLovastatin. Despite binding to a similar domain in the IDAS, these small molecules stabilized very different states in LFA-1.

Addition of IC487475 decreased bond lifetime for both the monomer and dimer ~5-fold from that of high affinity dimer and monomer, respectively. Lovastatin decreased bond lifetime 1000-fold for the dimer and 100-fold for the monomer. These data reveal the capacity of neutrophils to regulate dynamically the affinity of LFA-1 over 4 orders of magnitude through shifts in conformation initiated at the I domain. This is greater than the 300-fold shift in dissociation rate observed for isolated I domain mutations (5). Our data indicate that a second mechanism for regulating adhesion kinetics is bond valency and that dimeric bond formation between LFA-1 and ICAM-1 homodimers can increase bond stability by an order of magnitude for high affinity binding. Supporting this is the ICAM-1 bead capture data. Although the rate of neutrophil adhesion to dimeric ICAM-1 beads was ~1-fold higher than for monomeric beads, adhesion stability exhibited greater sensitivity to bond valence. Conversion from transient (seconds) to stable adhesion (minutes) was only observed when neutrophils bound dimeric ICAM-1 beads.
ICAM-1 Valence Determines Lifetime of Adhesion to LFA-1

ICAM-1 Valence Determines Lifetime of Adhesion to LFA-1

Staunton, S. I. Simon, manuscript in preparation.

Published data (39) have demonstrated that ICAM-1 is expressed as a homodimer on inflamed endothelium. Spontaneous dimerization of ICAM-1 on the plasma membrane is believed to facilitate high avidity binding through LFA-1 (40, 41). This is supported by a 10-fold faster dissociation rate constant ($k_{off}$) for LFA-1 binding to monomeric versus dimeric ICAM-1 (33). In the present study, we show for the first time that the relative efficiency of neutrophil adhesion to ICAM-1 is ~60% greater in capture of dimer ICAM-1 than monomer at equivalent site density. This difference can be attributed to the capacity of the high affinity LFA-1 on neutrophils stimulated with IL-8, or allosterically induced by binding of mAb K1M127 (38). In separate studies, we have observed that IC487475 can support neutrophil rolling on inflamed endothelium.

LFA-1 Forms Bivalent Bonds with Dimeric ICAM-1 and Prolongs Adhesive Lifetime—Published data (39) have demonstrated that ICAM-1 is expressed as a homodimer on inflamed endothelium. Spontaneous dimerization of ICAM-1 on the plasma membrane is believed to facilitate high avidity binding through LFA-1 (40, 41). This is supported by a 10-fold faster dissociation rate constant ($k_{off}$) for LFA-1 binding to monomeric versus dimeric ICAM-1 (33). In the present study, we show for the first time that the relative efficiency of neutrophil adhesion to ICAM-1 is ~60% greater in capture of dimer ICAM-1 than monomer at equivalent site density. This difference can be attributed to the capacity of the high affinity LFA-1 on neutrophils stimulated with IL-8, or allosterically induced by binding of mAb K1M127 (38). In separate studies, we have observed that IC487475 can support neutrophil rolling on inflamed endothelium.

In the current study, we define the relationships between LFA-1 conformation, bond valency with ICAM-1, and bond/adhesion lifetime. A novel allosteric small molecule targeted to the IDAS revealed that LFA-1 dissociation can be dynamically regulated within this region into an intermediate affinity state. The data show for the first time that neutrophil adhesion to ICAM-1 can be controlled via the I domain and that formation of molecular scale clusters of LFA-1 is critical for increasing avidity. In this manner, neutrophil adhesion kinetics can be tuned from rolling to arrest and transmigration. Regulation over this process can also be exerted at the endothelial cell level, which can dynamically control site density and mobility of ICAM-1. ICAM-1 is present on microvillus projections that extend from the endothelial surface to form...
ICAM-1 Valence Determines Lifetime of Adhesion to LFA-1

a transmigratory cup during leukocyte recruitment (43, 44). Thus, gatekeepers may be present on the membrane of both the leukocyte and endothelium in governing the precise location and efficiency of recruitment.

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