Shear and Time-Dependent Changes in Mac-1, LFA-1, and ICAM-3 Binding Regulate Neutrophil Homotypic Adhesion[1]

Sriram Neelamegham,* Andrew D. Taylor,† Harish Shankaran,* C. Wayne Smith,† and Scott I. Simon[2‡]

We examined the relative contributions of LFA-1, Mac-1, and ICAM-3 to homotypic neutrophil adhesion over the time course of formyl peptide stimulation at shear rates ranging from 100 to 800 s^{-1}. Isolated human neutrophils were sheared in a cone-plate viscometer and the kinetics of aggregate formation was measured by flow cytometry. The efficiency of cell adhesion was computed by fitting the aggregate formation rates with a model based on two-body collision theory. Neutrophil homotypic adhesion kinetics varied with shear rate and was most efficient at 800 s^{-1}, where ~40% of the collisions resulted in adhesion. A panel of blocking Abs to LFA-1, Mac-1, and ICAM-3 was added to assess the relative contributions of these molecules. We report that 1) LFA-1 binds ICAM-3 as its primary ligand supporting homotypic adhesion, although the possibility of other ligands was also detected. 2) Mac-1 binding to an unidentified ligand supports homotypic adhesion with an efficiency comparable to LFA-1 at low shear rates of ~100 s^{-1}. Above 300 s^{-1}, however, Mac-1 and not LFA-1 were the predominant molecules supporting cell adhesion. This is in contrast to neutrophil adhesion to ICAM-1-transfected cells, where LFA-1 binds with a higher avidity than Mac-1 to ICAM-1. 3) Following stimulation, the capacity of LFA-1 to support aggregate formation decreases with time at a rate ~3-fold faster than that of Mac-1. The results suggest that the relative contributions of β_2 integrins and ICAM-3 to neutrophil adhesion is regulated by the magnitude of fluid shear and time of stimulus over a range of blood flow conditions typical of the venular microcirculation. ***The Journal of Immunology*****, 2000, 164: 3798–3805.**

Neutrophil localization on the activated vascular endothelium is a critical step during the human body's immune response against bacterial infection and during inflammatory diseases. A two-step model for neutrophil-endothelial cell adhesion involving selectin-mediated tethering and rolling followed by integrin-mediated firm arrest is now well established (1, 2). Furthermore, recent studies have also proposed that neutrophils in the flow stream may be recruited by other neutrophils that are already firmly bound on the vessel wall (3–5). Such homotypic interactions have been demonstrated in vitro during neutrophil rolling on adherent neutrophils bound to IL-1-treated endothelial cells (3). Parallel plate flow chamber experiments using purified E-selectin and P-selectin as the substrate have demonstrated that rolling neutrophils can "nucleate" the recruitment of additional neutrophils from the flow stream through tethering interactions in part involving L-selectin binding to P-selectin glycoprotein ligand 1 (4, 5). Furthermore, in vivo studies in the mouse microcirculation have also suggested that leukocyte homotypic interactions may contribute at least in part to leukocyte localization (6).

Once recruited onto the vessel wall, rolling neutrophils are stimulated by ligation of inflammatory mediators including IL-8 and platelet-activating factor presented on the activated endothelium. This results in conversion of the neutrophil β_2-integrin molecules to an active state (7, 8) and their recognition of several members of the Ig family including ICAM-1 and ICAM-2 (9, 10). The interaction between the β_2 integrins and ICAMs is a critical step in the transition from cell rolling to firm arrest. Two members of the CD18 or β_2 integrin family, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), appear to account for ~50–70% of neutrophil firm adhesion (9, 11, 12). The observation that neutrophil-neutrophil interactions are prevalent in the vasculature suggest that integrin-mediated homotypic adhesion may also contribute to cell arrest in the vasculature. Analogous to neutrophil arrest on activated endothelium, we have reported that neutrophil homotypic adhesion is also dependent on L-selectin and β_2-integrins (13). Tethering through L-selectin is necessary for the formation of stable aggregates at shear rates above 400 s^{-1} (14). However, below this shear rate neutrophil adhesion can be supported entirely by activated β_2 integrins binding their ligands. This is supported by the observation that blocking L-selectin with Ab does not abolish cell adhesion at a shear rate <400 s^{-1} (14).

ICAM-3 is expressed on all populations of circulating leukocytes (15–18) and has been shown to bind LFA-1 on both resting and activated T cells (19, 20). It was recently demonstrated that ICAM-3 is a major LFA-1 ligand in the resting immune system (21), but not for Mac-1 or CD11c/CD18 (22). ICAM-3 on T cells appears to have the ability to signal intracellular functions, including the activation of β_1 and β_2 integrins following ligation (23). Although ICAM-3 is not expressed on resting or inflamed endothelium, it is inducible in some disease states, particularly in lymphomas (24, 25). The wide distribution of ICAM-3 on different leukocyte populations, its capacity to signal, and its distinct binding specificity suggests a crucial role in modulating immune response. However, the role of ICAM-3 in neutrophil adhesion function and its regulation with time after stimulation and applied shear

*Department of Chemical Engineering, Bioengineering Laboratory, State University of New York, Buffalo, NY 14260; †Department of Pediatrics, Section of Leukocyte Biology, Baylor College of Medicine, Houston, TX 77005; and ‡Division of Biomedical Engineering, University of California, Davis, CA 95616

Received for publication October 8, 1999. Accepted for publication January 14, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a grant from the United Engineering Foundation (to S.N.) and by Grants AI31652 (to S.I.S.) and AI43599 (to S.I.S.). S.I.S. is supported as an Established Investigator of the American Heart Association.

[2] Address correspondence and reprint requests to Dr. Scott I Simon, Biomedical Engineering, University of California, One Shields Avenue, Davis, CA 95616-5294. E-mail address: sisimon@ucdavis.edu

 0022-1767/00/$02.00

rate have not been examined. Furthermore, the relative contributions of LFA-1 and Mac-1 to neutrophil-neutrophil adhesion has also not been determined as a function of time and shear. In this manuscript, we examine these key features of LFA-1-, Mac-1-, and ICAM-3-mediated adhesion under defined hydrodynamic shear conditions.

Materials and Methods

Reagents

Anti-CD11b mAb, 60.1 $F(ab')_2$ (IgG1), was provided by Lora Whitehouse (Repligen, Cambridge, MA), anti-CD11a mAb R3.1 (IgG1) and chimeric ICAM-3-IgG (26) were gifts from Dr. Kei Kishimoto (Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT), anti-CD50 murine Abs ICR 1.1, ICR 4, and ICR 5.1 were provided by Dr. Donald Staunton (ICOS, Bothell, WA) (27), and anti-CD62L LAM1-3 was provided by Cell Genesis (Foster City, CA). Fluorescent mAbs to CD11b, 2LPM19c-FITC (Dako, Carpinteria, CA), to CD62L, SK11-FITC (Becton Dickinson Immunnocytometry, San, Jose, CA), and to CD45, 2D1-FITC (Becton Dickinson) were purchased commercially. Abs ICR1.1 and R3.1 were also conjugated with the fluorescent dye Cy3 using a FluoroLink-Ab Cy3 labeling kit from Amersham (Arlington Heights, IL). Fab fragments of some of the Abs (ICR1.1, LAM1-3, and R3.1) were also produced by digestion with papain and purification by passage over a protein A-Sepharose column using an ImmunoPure Fab preparation kit from Pierce (Rockford, IL). fMLP was purchased from Sigma (St. Louis, MO). The fluorescent nuclear dye LDS-751 was purchased from Molecular Probes (Eugene, OR).

Neutrophil homotypic adhesion studies

Neutrophils were isolated from human blood collected from healthy volunteers by venipuncture into a sterile syringe containing 10 U heparin/ml of blood. Neutrophils were isolated using a one-step Mono-Poly resolving density gradient (ICN Biomedicals, Costa Mesa, CA) as described previously (14) and kept at 4°C in a Ca^{2+}-free HEPES buffer before experimentation. The purity of isolated neutrophils was >90% and the viability measured by trypan blue exclusion was >99%. Before each experimental run, neutrophil suspensions (10^6 cells/ml) were incubated for 3 min at 37°C in buffer containing 1.5 mM Ca^{2+}.

Neutrophil aggregation experiments were performed using established cone-plate viscometry methodology followed by flow cytometry analysis of samples (28, 29). One of two viscometers from either Ferranti Electric (Commack, NY) or Haake (Paramus, NJ) were used. Both of these devices consist of a rotating 1°-cone placed over a stationary plate. The gap between the cone and plate is narrow and it ranges from <10 μm at the center to 610 μm at the outside edge. The entire experimental apparatus was maintained at 37°C. During the experiment, the neutrophil cell suspension was placed in the gap between the cone and the plate, stimulated with 1 μM fMLP, and shear was applied. The rotation of the cone at a fixed speed allowed the application of a uniform and linear shear field to the entire cell suspension. This shear rate is independent of position in the viscometer. At a defined shear rate (G), the shear stress (τ) applied on the cell suspension varies linearly with shear rate as: $\tau = \mu G$, where μ is the fluid viscosity (μ = .75 cp at 37°C). During the course of the experiment, 25-μl aliquots of the aggregating cell suspension were taken at fixed sampling points for up to 5 min after stimulation and fixed in 200 μl of cold 2% glutaraldehyde for flow cytometric analysis.

In blocking studies where mAbs were used, the cells were preincubated with the Ab for 15 min at room temperature before the experiment. Whole Abs (especially those to ICAM-3) were found to cause either higher than baseline levels of adhesion at low shear rates and/or sustained adherence of stimulated neutrophils. Therefore, Fab and $F(ab')_2$ fragments were used for the inhibition studies. Anti-L-selectin mAb LAM1-3 Fab (30 μg/ml), anti-CD11b/CD18 60.1 $F(ab')_2$ (30 μg/ml), anti-CD11a/CD18 R3.1 Fab (30 μg/ml), and anti-ICAM-3 ICR1.1 Fab (30 μg/ml) were used at saturation concentrations determined to inhibit neutrophil aggregation. Soluble ICAM-3 IgG was used at 40 μg/ml. None of the Abs or soluble molecule induced activation of neutrophils as judged by changes in the expression levels of L-selectin or Mac-1 on neutrophils (data not shown).

Flow cytometric detection of homotypic neutrophil adhesion

A FACScan flow cytometer (Becton Dickinson) was used to analyze the particle distribution of fixed cell suspensions. The neutrophil population was identified by gating on their characteristic forward scatter vs side scatter. Singlet neutrophils and aggregates were resolved using autofluorescence derived from glutaraldehyde fixation, and the particle distribution of neutrophil aggregates was determined using histograms of fluorescence intensity as described previously (30). The extent of homotypic adhesion (% aggregation) was determined based on the rate of depletion of singlet neutrophils according to the equation below:

$$\%\ \text{aggregation} = [1 - S/(S + 2D + 3T + 4Q + 5P + 6Sx)] \times 100$$

where the neutrophil aggregate sizes are given by S = singlets, D = doublets, T = triplets, Q = quartets, P = pentuplets, and Sx = sextuplets and larger unresolved aggregates. At low shear rates studied in this manuscript, particles larger than sextuplets typically comprised <10% of the total aggregates formed.

Estimating the adhesion efficiency of neutrophil homotypic aggregation

The details of the mathematical analysis used to estimate "adhesion efficiency" have been published elsewhere (29, 31). Briefly, the number of cell-cell collisions in the viscometer varies in proportion to the shear rate and as a square of the cell concentration. The extent of aggregation estimated above is therefore dependent not only on the activation of adhesion molecules on the neutrophils, but also on the physical parameters of the experimental system which control the number of cell-cell collisions. To delineate between the biological changes in adhesion molecule function and the physical effects of cell concentration and size, we quantified the adhesion efficiency for cell binding (29). Adhesion efficiency is defined as the fraction of total cell-cell collisions that result in aggregate formation and it is always ≤1: Adhesion eficiency= (number of collisions resulting in adhesion)/(total number of collisions)

Adhesion efficiency was estimated by fitting the kinetic data of homotypic neutrophil aggregation experiments over the first 30 s after the application of shear with a mathematical model. Briefly, the total number of collisions (denominator) is dependent on the cell concentration, applied shear rate, and cell radius, and it is estimated based on two-body linear collision theory. The number of effective collisions (numerator) is then measured based on the experimental aggregation kinetics and flow cytometric analysis. Adhesion efficiency estimated by this methodology is solely a function of the intrinsic biological properties of the cell that determine its adhesivity (14, 28, 29). Important among these properties are the number, affinity, and distribution of adhesive receptors expressed on the cell surface, their response to applied shear, and the time after stimulation.

Two-color neutrophil aggregation assay

In some experiments, cell adhesion was measured between two populations of neutrophils that were individually treated with different sets of blocking Abs. A modified protocol to the one described above for neutrophil homotypic adhesion was used for this assay. In this study, two aliquots of neutrophils at 3×10^6 cells/ml were simultaneously incubated with both the blocking Abs and spectrally distinct fluorescent markers for cytometric detection for 10 min at 25°C. One of these neutrophil aliquots was labeled with 5 μg/ml anti-CD45-FITC for detection in the green (FL1) fluorescence channel of the cytometer, the other was labeled with vital nucleic acid dye LDS-751 (4 μg/ml) for detection in the red (FL3) fluorescence channel. These labels do not alter cell function (28). Excess label was removed by a brief centrifugation (2–3 s at 2000 × g), and the two cell populations were mixed and incubated for 2 min in buffer containing 1.5 mM Ca^{2+} at 37°C. The combined sample was then stimulated with 1 μM FMLP and exposed to shear in the cone-plate viscometer. Aliquots of 25 μl were taken at desired time points and fixed in 100 μl of 0.5% cold paraformaldehyde for cytometric analysis of neutrophil aggregation kinetics.

Following addition of chemotactic stimulus, red, green, and red-green aggregates were observed. The fraction of particles in each population was quantified by drawing analysis gates on distinct aggregate species, as described previously (13). The rate of two-color aggregation was determined by dividing the number of cells in dual-fluorescent (heterotypic) aggregates by the total number of cells. The data were normalized by the extent of control aggregation performed in the absence of any Ab treatment. This parameter was denoted "two-color aggregation (fraction control)."

Quantifying receptor expression

To measure receptor expression, samples were labeled for 10 min at room temperature followed by 5 min at 37°C with either SK11-FITC to L-selectin or 2LPM19c-FITC to CD11b. The samples were then either stimulated with fMLP or incubated with anti-ICAM-3 mAbs at 37°C, and the changes in receptor expression were measured either by fixing the samples or using live flow cytometry. Isotype-matched labeled IgG Abs were used as controls for these experiments. Analysis of cellular events was performed using FACScan analysis software (Becton Dickinson).

In some experiments, where alterations in ICAM-3 expression level were monitored, neutrophils were incubated with 30 μg/ml Cy3-conjugated ICR1.1 Fab and the changes in expression level of this Ab following fMLP stimulation was monitored using live flow cytometry. Additional experiments were also performed with two other Abs to ICAM-3: mAbs ICR 4 and ICR 5.1. In these experiments, neutrophils were incubated with the anti-ICAM-3 Ab for 20 min at room temperature, washed, and then incubated with FITC-conjugated rabbit anti-mouse $F(ab')_2$ secondary Ab for 10 min. After a second wash step, the cells were stimulated with 1 μM fMLP and the changes in ICAM-3 expression level were monitored by measuring the level of secondary FITC-labeled Ab bound to the neutrophils.

The number of ICAM-3 receptors on the neutrophil surface was also quantified using a Quantum simply cellular microbead calibration kit (Flow Cytometry Standards, Research Triangle Park, NC) as described in detail earlier (28). Briefly, the uniform microbead standards have a calibrated number of goat anti-mouse IgG sites on their surface. When detecting ICAM-3 on the neutrophil, both the calibrated microbeads and isolated neutrophils were incubated with whole ICAM-3 Ab (ICR1.1) conjugated with Cy3 in the presence of 0.2% human serum albumin Ag. The number of binding sites on the neutrophil was determined by quantifying the fluorescence intensity of the cells, and this value was translated into the number of bound Ab using the microbead standards. Cy3 labeled isotype-matched mAb was used as control.

Statistics

Data were analyzed using ANOVA. The Student-Newman-Keuls test was performed to assess the significant differences in the data. A $p < 0.05$ was considered to be significant.

Results

ICAM-3 is a major ligand for LFA-1 during neutrophil-neutrophil adhesion at low shear rates

In a recent study in which we examined the contribution of L-selectin and β_2 integrins to neutrophil homotypic aggregation, we demonstrated that at low shear rates <400 s^{-1}, adhesion may be purely β_2 integrin dependent since blocking L-selectin alone does not abolish aggregation (14). At a shear rate of 100 s^{-1}, isolated neutrophils rapidly form aggregates when exposed to both shear and 1 μM fMLP, but do not aggregate in response to shear alone (Fig. 1*A*). Upon stimulation, ~25% of the neutrophils are incorporated into aggregates within 1 min of fMLP addition. Blocking L-selectin with LAM1-3 Fab does not alter aggregation kinetics at 100 s^{-1} (Fig. 1*A*). Homotypic neutrophil adhesion is dependent on both LFA-1 and Mac-1 (Fig. 1*B*). Addition of Abs to LFA-1 (R3.1 Fab) or Mac-1 (60.1 $F(ab')_2$) at saturating concentrations inhibit neutrophil aggregation by ~35%. Simultaneous addition of both Abs completely abolished homotypic aggregation over the entire range of shear rates applied from 50 to 3000 s^{-1}.

Recent studies have shown that ICAM-3 is a ligand for LFA-1 in lymphocytes (17). We examined whether LFA-1 binding to ICAM-3 plays a role in homotypic neutrophil adhesion. For these experiments, a blocking Ab to domain 1 of ICAM-3 (ICR1.1 Fab) was used. This Ab has been previously shown to inhibit the binding of JY cells to ICAM-3-coated substrates in a static adhesion assay (27). Addition of ICR1.1 Fab inhibited neutrophil aggregation to a similar extent as with blocking Ab to LFA-1 or Mac-1 (Fig. 1*C*). Simultaneous addition of Abs to LFA-1 and ICAM-3 did not further decrease cell adhesion. However, addition of blocking Abs to Mac-1 and ICAM-3 further inhibited cell adhesion, although not to background levels. The results indicate that although ICAM-3 is a major ligand for LFA-1 on neutrophils, another ligand(s) may also exist. Consistent with published data (22), Mac-1 does not appear to bind ICAM-3 on neutrophils or at least was not blocked by the anti-ICAM-3 Ab applied.

Aggregation studies were also performed with a soluble form of ICAM-3 expressed as an IgG molecule (26). We examined the ability of this chimeric ICAM-3 to inhibit β_2 integrin-mediated adhesion by competing for active LFA-1 sites. At a shear of 100 s^{-1}, 40 μg/ml ICAM-3 IgG blocked neutrophil homotypic aggregation by ~20 ± 5%. Simultaneous addition of ICAM-3 IgG and anti-Mac-1 inhibited aggregation by ~65 ± 10%. Taken together, the data indicate that ICAM-3 is the major ligand for LFA-1 during fMLP-stimulated neutrophil aggregation.

To compare the binding kinetics of LFA-1 and Mac-1 for their ligands on adjacent neutrophils, we computed the adhesion efficiency. This parameter is defined as the fraction of neutrophil-neutrophil collisions that result in stable aggregate formation, and it is derived from the kinetics shown in Fig. 1, *A–C*. Preincubating neutrophil suspensions with mAbs to LFA-1, Mac-1, or ICAM-3 reduced adhesion efficiency equally by about one-third in comparison to the control samples sheared and stimulated in the absence of Ab. An ~75% decrease in efficiency was observed when both Mac-1 and ICAM-3 were blocked with mAb. In comparison, addition of anti-LFA-1 along with anti-ICAM-3 did not diminish adhesion efficiency greater than either added separately. Complete inhibition was only observed on addition of both anti-LFA-1 and anti-Mac-1 Abs simultaneously. These results suggest that at the relatively low shear of 100 s^{-1} both LFA-1 and Mac-1 contribute equally to stable neutrophil adhesion.

Two-color analysis of neutrophil adhesion

To gauge the relative avidity of adhesion via LFA-1 binding ICAM-3 and Mac-1 binding to unknown ligand between individual populations of neutrophils, two-color adhesion experiments were performed. For these studies, one population of neutrophils was labeled green with anti-CD45 FITC and the other red with nucleic acid dye LDS-751. We have previously verified that the labels neither cause neutrophil activation nor interfere with cell adhesive function (28). Similar to the single-color experiments, the fraction of singlets recruited into aggregates between the two populations (percent two-color aggregation in Fig. 2) was computed at 30 s after stimulation. In response to shear alone the labeled neutrophils remain as discrete populations with no aggregate formation (data not shown). Following fMLP stimulation, both single-color aggregates as well as two-color red-green aggregates were formed as depicted previously (30). Fig. 2 is a plot of the extent of two-color aggregation for each Ab treatment normalized with respect to the control unblocked samples.

In Fig. 2*A*, LFA-1-dependent adhesion was assessed in the presence of excess anti-Mac-1. As observed in the single-color experiments and confirmed in two-color, blocking Mac-1 on both populations inhibited aggregation by one-third in comparison to the control samples (data not shown). Under these conditions, addition of anti-ICAM-3 or anti-LFA-1 to one population significantly decreased aggregation by an additional one-third (Fig. 2*A*). Under conditions in which LFA-1 is available on a single population, blocking ICAM-3 on both populations further reduced the level of aggregation by ~50%. However, this combination of blocking mAbs did not completely abrogate aggregation, implying that LFA-1 may bind an additional ligand(s) other than ICAM-3 between neutrophils.

Mac-1-dependent adhesion was examined in the presence of excess anti-LFA-1 to block both cell populations (Fig. 2*B*). Adhesion is inhibited by one-third from control on addition of anti-LFA-1 mAb as shown above in the single-color experiments, and it is unaffected by additional blocking of ICAM-3 on one or both populations of neutrophils. However, blocking Mac-1 on one population decreased cell adhesion by ~50%. The data indicate that the presence of either Mac-1, LFA-1, or ICAM-3 on one cell population results in equivalent levels of cell adhesion, which is

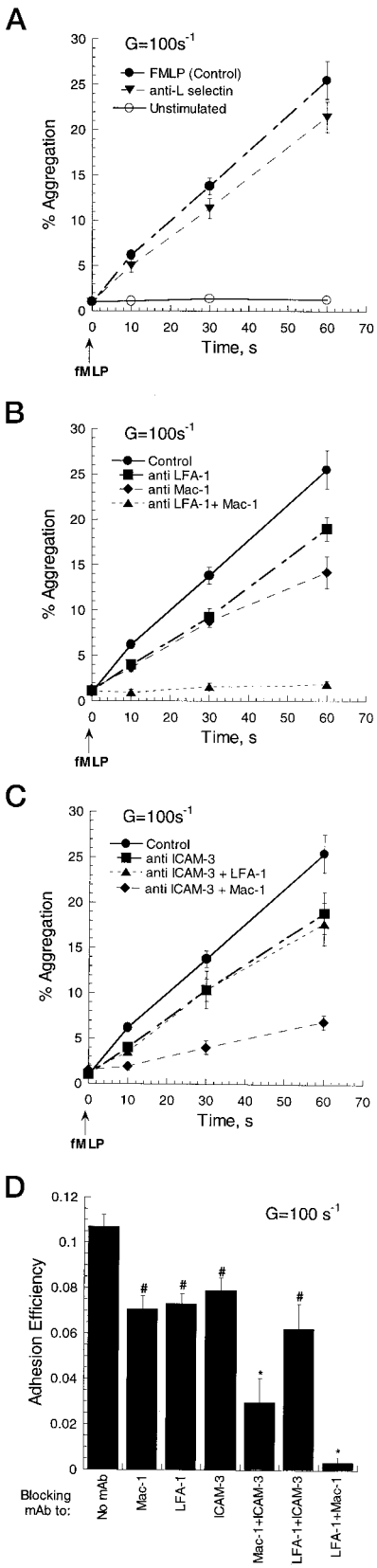

FIGURE 1. L-selectin- and β_2 integrin-dependent neutrophil aggregation. Neutrophil suspensions (10^6/ml) were stimulated with 1 μM fMLP and exposed to constant mixing in a cone-plate viscometer at a shear rate (G) of 100 s^{-1} upon addition of a panel of adhesion-blocking Abs to L-selectin (LAM1-3 Fab), Mac-1 (60.1 F(ab′)$_2$), LFA-1 (R3.1 Fab), and ICAM-3 (ICR1.1 Fab). Samples were removed at prescribed time points and fixed with 2% glutaraldehyde. Aggregate formation was quantified using flow cytometry as described in *Materials and Methods*. *A*, Selectin-independent adhesion at G = 100 s^{-1}. *B*, Contribution of β_2-integrin subunits (LFA-1 and Mac-1) to neutrophil aggregation. *C*, Contribution of ICAM-3 to neutrophil homotypic aggregation. *D*, A comparison of neutrophil adhesion efficiency for data presented in *A–C*. Statistics: *, $p < 0.05$ in comparison to all other treatments. #, $p < 0.05$ in comparison to no mAb control, Mac-1 + ICAM-3, and LFA-1 + Mac-1. Data are means ± SEM for n = 4–11 independent experiments.

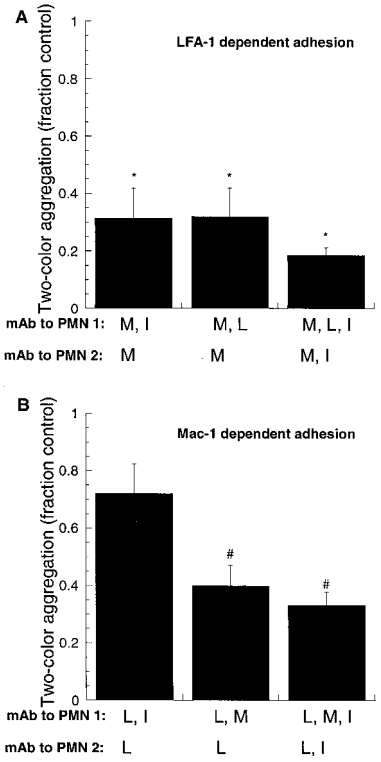

FIGURE 2. Two-color neutrophil aggregation kinetics. Two populations of neutrophils (PMN 1, PMN 2) at 3×10^6 cells/ml were labeled with spectrally distinct dyes, either CD45-FITC or LDS-751, for detection on the green and red channels of a flow cytometer. Both populations were preincubated with blocking Abs to Mac-1 (60.1 F(ab′)$_2$, denoted M), LFA-1 (R3.1 Fab, denoted L), or ICAM-3 (ICR1.1 Fab, denoted I), before mixing the two populations, stimulation with 1 μM fMLP and application of shear at G = 100 s^{-1}. Flow cytometry was applied to quantify cell aggregation kinetics using two-color analysis. Normalized two-color aggregation was quantified 30 s after application of shear as described in *Materials and Methods*. *A*, LFA-1-dependent adhesion was examined by incubating both PMN populations with excess anti-Mac-1 Ab. *, $p < 0.05$ in comparison to addition of excess anti-Mac-1 alone to both populations. *B*, Mac-1-dependent adhesion examined in the presence of excess anti-LFA-1 Ab in all of the runs. #, $p < 0.05$ in comparison to addition of excess anti-LFA-1 to both populations and ICAM-3 to one population (first bar in *B*). Data are means ± SEM for n = 2–4 independent experiments.

~30–40% of the control without Ab treatment. Therefore, it appears that LFA-1 binding to ICAM-3 at low shear contributes equally to cell avidity as Mac-1 binding to its ligand. Furthermore, Mac-1 does not appear to bind ICAM-3 on neutrophils.

Mac-1 is the predominant β_2 integrin receptor for neutrophil aggregation at high shear rates

Increasing shear rate during cell adhesion increases the tensile and shear stress applied on intercellular bonds, and it simultaneously

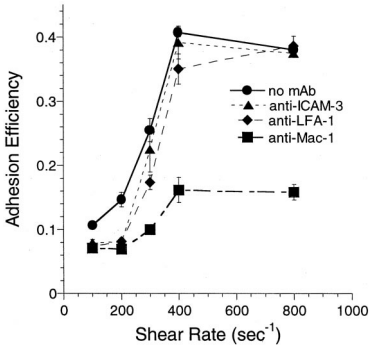

FIGURE 3. Dependence of LFA-1-, Mac-1-, and ICAM-3-mediated neutrophil aggregation on applied shear rate. Neutrophil suspensions (10^6/ml) were preincubated with Abs to either LFA-1 (R3.1 Fab), Mac-1 (60.1 F(ab′)$_2$), or ICAM-3 (ICR1.1 Fab), stimulated with 1 μM fMLP and sheared in a cone-plate viscometer over a range of shear rates. Homotypic neutrophil aggregation was measured using flow cytometry and adhesion efficiency was calculated. Data are means ± SEM.

reduces the average contact duration available for receptor-ligand bond formation during cell collision. These two features suggest that neutrophil adhesion efficiency should decrease with increasing shear. However, we have previously reported that the efficiency of homotypic neutrophil aggregation in the presence of L-selectin increases with shear up to a maximum over a narrow range between 200 and 400 s^{-1} and decreases beyond 800 s^{-1} (Fig. 3) (14).

Although our blocking studies demonstrate that the β_2 subunits LFA-1 and Mac-1 contribute equally to neutrophil adhesion at a shear of 100 s^{-1}, we examined how their relative contributions change at higher shear rates (Fig. 3). As shear rate was increased up to 300 s^{-1}, blocking LFA-1 or ICAM-3 was only half as effective at inhibiting aggregate formation as compared with anti-Mac-1. At 400 s^{-1} and beyond, adhesion was only slightly inhibited by anti-LFA-1 or anti-ICAM-3. In contrast, blocking Mac-1 resulted in greater inhibition as shear was increased reaching ~60% at 400 s^{-1}. Moreover, anti-Mac-1 was almost completely effective at inhibition (>90%) at a shear rate of 1600 s^{-1} (data not shown). Over the range of shear conditions between 400 and 1600 s^{-1} tested here, ~85–95% of the neutrophils were typically recruited into aggregates within 1 min of application of stimulus and shear. This large percent aggregation along with the almost complete abrogation of adhesion via anti-Mac-1 alone at the higher shears suggests that the differential contribution of LFA-1 and Mac-1 is uniformly exhibited on all neutrophils, and it is not limited to any single subpopulation of the cells.

We examined whether shear rate modulates the surface expression of LFA-1 or ICAM-3 and in this manner contributes to the decrease in their contribution with increasing shear. To determine this, we measured the expression level of LFA-1 and ICAM-3 in response to fMLP stimulation at a low (100 s^{-1}) and high (800 s^{-1}) shear rate. LFA-1 and ICAM-3 expression remained unaltered from the resting state upon stimulation with fMLP, consistent with previous reports (32, 33). The number of ICAM-3 molecules detected on neutrophils was 150,000 ± 20,000 (n = 2), and this expression level changed by <6 ± 3% upon 1 μM fMLP stimulation over a 5-min time course. Chemotactic stimulation at low or high shear also did not alter receptor expression of either ICAM-3 or LFA-1 by >5%. The data indicate that modulation in receptor surface expression does not contribute to the decrease in contribution of LFA-1 and ICAM-3 at high shear.

Table I. *Time-dependent changes in β_2 integrin avidity following fMLP stimulation*

Molecular Dependence	Efficiency at $t = 0$, E_o	Decay Constant, α
LFA-1 and Mac-1 dependent	0.15	0.006
LFA-1 dependent	0.08	0.012
Mac-1 dependent	0.10	0.004

Aggregate formation and stability via LFA-1 and ICAM-3 decreases rapidly with time of chemotactic stimulation

Distinct differences were observed between the pattern of adhesion over 5 min of fMLP stimulation at a shear rate of 100 s^{-1} in the presence of blocking Abs to either LFA-1, ICAM-3, or Mac-1 (Fig. 4*A*). Aggregation after 60 s of stimulation was not significantly different for untreated control compared with anti-LFA-1- or anti-ICAM-3-treated neutrophils. In contrast, blocking with anti-Mac-1 resulted in a diminished rate of aggregate formation by 60 s, no new aggregate formation over the succeeding 2 min, and complete disaggregation within 5 min of shear and stimulation.

To quantitatively measure changes in the contribution of LFA-1 and Mac-1 to neutrophil adhesion over the time course of fMLP stimulation, we added stimulus to the neutrophil suspension and delayed the application of shear mixing for a defined time period (Fig. 4, *B–D*). During this time interval between cell stimulation and application of shear, it is anticipated that neutrophils alter the affinity of LFA-1 and Mac-1 from their basal state. Furthermore, the absence of shear during this interval prevents neutrophils from aggregating. The kinetics of neutrophil aggregate formation for cells activated over a range of time intervals before application of shear is shown in Fig. 4*B*. Within 120 s after fMLP addition, a decrease in the rate and extent of aggregation is apparent. Blocking either LFA-1 or Mac-1 with mAb before stimulation (Fig. 4*C*) enabled assessment of their relative contribution. LFA-1-dependent adhesion was measured in the presence of anti-Mac-1 60.1 F(ab′)$_2$, whereas Mac-1-dependent adhesion was examined in the presence of anti-LFA-1 R3.1 Fab. Neutrophil adhesion following 120 s of stimulation appeared to be predominantly through binding of Mac-1 since the presence of anti-Mac-1 inhibited neutrophil aggregation to background levels (Fig. 4*C*). Anti-LFA-1 was only partially effective in blocking cell aggregation, indicating that although LFA-1 alone was not sufficient for enabling aggregation at 120 s of stimulation, it was necessary along with Mac-1 for optimum adhesion efficiency.

The contributions of LFA-1 and Mac-1 to the efficiency of aggregation at a shear of 100 s^{-1} over the time course of fMLP stimulation is plotted in Fig. 4*D*. Neutrophil adhesion via activation and binding of β_2 integrins clearly decreases with time after stimulation. Although either LFA-1 or Mac-1 exhibit an equivalent adhesion efficiency within seconds of fMLP stimulation, by 120 s Mac-1 accounts for all of the efficiency of aggregation. To quantitatively compare the decay in efficiency of LFA-1 and Mac-1, the data were fit to a first-order decay function, $E = E_0 e^{-\alpha t}$ (smooth curves in Fig. 4*D*). Here, E_0 is the baseline adhesion efficiency just after stimulation (i.e., at $t = 0$) and α is a coefficient that describes the rate of decay in efficiency. On comparing the value of α fit to the data (Table I), we observed that adhesion via LFA-1 decreases approximately three times faster than that via Mac-1. The results clearly show reversibility in the capacity of β_2 integrins to support aggregation with time of stimulation, and they suggest that the lifetime of adhesion through LFA-1 bonds are significantly shorter than that via Mac-1.

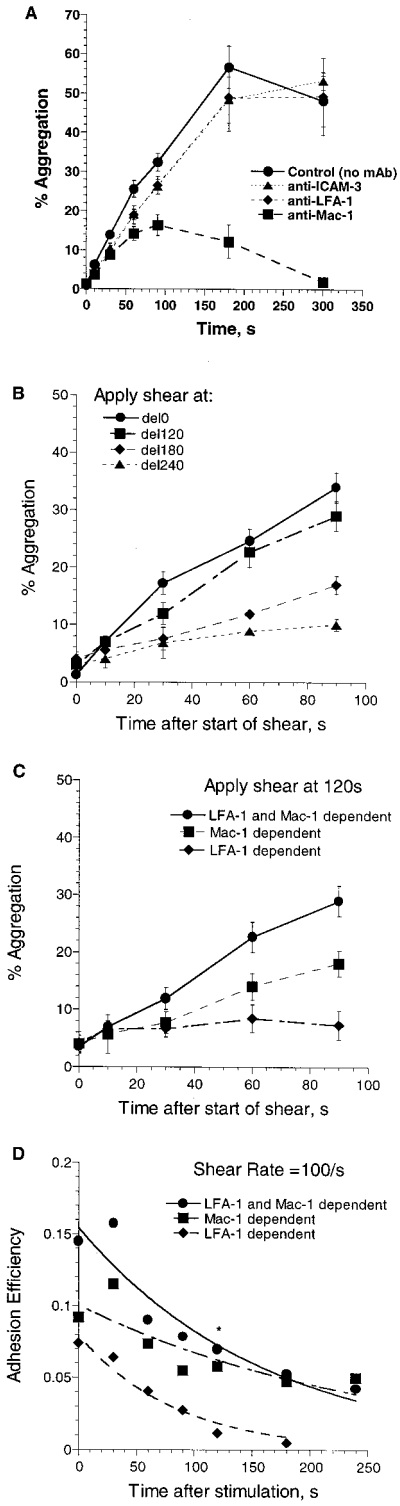

FIGURE 4. Time varying changes in contribution of LFA-1 and Mac-1 to neutrophil-neutrophil adhesion. Neutrophil concentration is 10^6 cells/ml for all experiments. *A*, Neutrophil aggregation kinetics at G = 100 s^{-1} over a 5-min time course after 1 μM fMLP stimulation in the presence of Abs to LFA-1 (R3.1 Fab), Mac-1 (60.1 F(ab′)$_2$), or ICAM-3 (ICR1.1 Fab). *B*, Neutrophils (10^6 cells/ml) were mixed and stimulated with 1 μM fMLP for fixed time periods (0, 120, 180, or 240 s) before application of shear at G = 100 s^{-1}. % Aggregation is plotted after start of shear. *C*, % Aggregation when shear was applied 120 s after fMLP stimulation either in the absence of any Ab (LFA-1 and Mac-1 dependent) or on addition of blocking Abs to Mac-1 (LFA-1-dependent adhesion) or LFA-1 (Mac-1-dependent adhesion). Data are means ± SEM. *D*, Adhesion efficiency for LFA-1- and Mac-1-dependent adhesion with time at G = 100 s^{-1}. Smooth lines represent curve fit to experimental data with a first-order exponential decay function. ∗, Adhesion efficiency for LFA-1-dependent adhesion is significantly different ($p < 0.05$) from that estimated in the absence of mAb or on addition of R3.1 Fab.

Discussion

Neutrophils adherent to purified selectin ligands, human vein endothelial cells, or platelet monolayers constituting the substrate of a parallel plate flow chamber exhibit the capacity to recruit other neutrophils traveling in the free stream (3–5, 34). Based on these observations, it is postulated that neutrophil-neutrophil adhesion under certain inflammatory conditions in the microcirculation may serve to amplify the number of neutrophils that tether and roll in venules. In this report, we focus on homotypic neutrophil adhesion via the β_2 integrin subunits LFA-1 and Mac-1, especially at low shear rates. Using cone-plate viscometry, we report that LFA-1, Mac-1, and ICAM-3 contribute approximately equally to neutrophil aggregate formation at shear rates on the order of 100 s^{-1}. Such purely β_2 integrin-dependent adhesion may be physiologically important at low shears that predominate in the pulmonary microcirculation and other organs, in particular under conditions of acute inflammation (35–37). Furthermore, these adhesion molecules may contribute to vascular occlusion via the formation of adhesive interactions between leukocytes and platelets (38). In support of this, neutrophils that are sheared together with platelets or perfused over a platelet monolayer have been shown to form aggregates that involve the β_2 integrins and ICAM-3 (34, 39).

ICAM-3 is a major adhesive ligand in neutrophil-neutrophil adhesion

Adhesion via Mac-1 and LFA-1 was found to exhibit very different dynamics with time of stimulation and applied shear rate. Using Fab fragments of Abs that block these receptors along with anti-ICAM-3 and a soluble ICAM-3-IgG chimera, we demonstrate that LFA-1 binds ICAM-3 as its major ligand in mediating homotypic neutrophil adhesion at low shear rates. The strongest evidence to support this was the two-color aggregation experiments in which blocking either LFA-1 or ICAM-3 on interacting populations resulted in equivalent inhibition. Moreover, blocking ICAM-3 had no affect on Mac-1-dependent adhesion in the presence of excess anti-LFA-1, leading to the conclusion that Mac-1 recognizes an as yet undefined ligand(s).

The role of ICAM-3 in signaling T lymphocyte activation and adhesion function is well established (21, 23). A few recent studies have also proposed that ligation of ICAM-3 can alter β_2 integrin function on neutrophils, although the mechanisms proposed are varied (40–42). In one study, it was proposed that anti-ICAM-3 Abs inhibit neutrophil adhesion induced by low levels of fMLP (<100 nM) by inhibiting Mac-1 up-regulation and L-selectin shedding (42). In another report, it is proposed that anti-ICAM-3 Abs increase neutrophil chemokine secretion via an Fc-dependent mechanism (41). A third study has reported that certain Abs to domain 1 of ICAM-3 spontaneously induce neutrophil homotypic aggregation via Fc-independent mechanisms (40). In our studies, we did not observe any significant change in neutrophil adhesivity due to the addition of the anti-ICAM-3 Ab ICR1.1 over a wide range of shear and incubation times. Incubation with ICR1.1 did not itself alter the expression level of L-selectin or Mac-1, nor did it either inhibit or augment the change in receptor expression following fMLP stimulation. Although some neutrophil aggregation (<5% of positive control with 1 μM fMLP) was observed in studies where neutrophils were preincubated with whole Ab (ICR1.1)

before application of shear, this was attributed to Fc-dependent binding.

Mac-1- and LFA-1-dependent adhesion exhibit distinct dynamics with shear

Previous studies on neutrophil adhesion via L-selectin indicate that the efficiency of tethering is dependent on the applied hydrodynamic shear (43, 44). We have also reported that at shear rates in excess of $\sim$100 s^{-1}, the formation of neutrophil aggregates in sheared suspension becomes increasingly dependent on tethering through L-selectin (14). At shear rates $>$400 s^{-1}, L-selectin is absolutely necessary for aggregation, whereas stable aggregate formation does not require tethering through L-selectin at shear rates $\leq$100 s^{-1}.

At the low shear rate, three adhesion receptors were found to support aggregation: Mac-1, LFA-1, and ICAM-3. Each contributed in equal proportion to avidity at 100 s^{-1}. However, their relative contributions changed with an increase in applied shear. At shear rates approaching 400 s^{-1}, aggregation through LFA-1 alone was barely detectable, yet its cooperation with Mac-1 was clearly evident since abrogation of aggregation required simultaneous addition of blocking Abs to LFA-1 and Mac-1. At higher shear rates ($>$1600 s^{-1}), blocking Mac-1 with Ab was sufficient to abolish aggregation. Based on these results, we conclude that Mac-1 and LFA-1 contribute equally to homotypic neutrophil adhesion at low shear rates. However, Mac-1 becomes the predominant molecular bond supporting aggregation at shear rates from $\sim$300 s^{-1} and higher.

Several mechanisms may account for the greater shear sensitivity in adhesion via LFA-1 as compared with Mac-1. Prominent among these mechanisms are the possibility that 1) The minimum duration of intercellular contact required for LFA-1 to mediate capture may be longer than that of Mac-1, i.e., Mac-1 binding kinetics may be faster than that via LFA-1. 2) The availability or number of active ligand-binding LFA-1 receptors may be less than Mac-1. Although LFA-1 and Mac-1 are expressed at equivalent levels on resting neutrophils, it is possible that fewer LFA-1 participate in neutrophil-neutrophil adhesion. 3) LFA-1-adhesive bonds may be weak as compared with Mac-1 bonds, and therefore more susceptible to rupture at shears above 100 s^{-1}.

Contrasting molecular events in neutrophil-neutrophil vs neutrophil-ICAM-1 adhesion

The relative contribution and dynamics of Mac-1 and LFA-1 in mediating homotypic neutrophil adhesion is very different from that observed in neutrophil capture of ICAM-1 expressing cells in shear mixed cell suspensions. In previous studies, we have demonstrated that LFA-1 accounts for most of the initial capture efficiency of an ICAM-1-transfected cell line under shear conditions (29, 31). The capacity of Mac-1 to support stable neutrophil adhesion to ICAM-1-expressing cells under shear was heavily dependent on the initial binding of LFA-1. Over a range of shear rates from 100 to 1000 s^{-1}, LFA-1 accounted for two-thirds of the adhesion efficiency whereas Mac-1 contributed less than one-third to efficiency (31).

ICAM-3 is expressed on neutrophils at a density of $\sim$850 sites/μm^2. This expression level is comparable to one of the ICAM-1 transfectant cell lines tested which expressed $\sim$750 ICAM-1 sites/μm^2 (28). In studies conducted with this cell line, LFA-1-mediated neutrophil capture of transfectants was detected up to a shear rate of 400 s^{-1}. In contrast, the current data indicate that homotypic neutrophil adhesion via LFA-1 and ICAM-3 decreased rapidly at shear rates $>$100 s^{-1} corresponding to collisional contact duration of $\sim$25 ms (14). This observation suggests that bonds between LFA-1 and ICAM-3 form with a lower affinity or are weaker under shear stress as compared with LFA-1 bonds to ICAM-1. These findings are corroborated by a recent report where it is shown that LFA-1 binding to soluble ICAM-3 is approximately nine times weaker than their binding to soluble ICAM-1 (26). Several explanations could account for the difference in binding kinetics of LFA-1 for the two ICAMs, including differences in their distribution on the cell surface, the nature of their interaction with the cytoskeleton, their response to stimulation, and their relative affinity for LFA-1. An intriguing mechanism is that ICAM-1 and ICAM-3 may bind to distinct sites within the I domain of LFA-1 (45). This proposition is supported by a recent observation that certain mAbs to LFA-1 preferentially inhibit ICAM-3 but not ICAM-1-mediated binding (20).

Time-dependent decay in adhesion via LFA-1 and Mac-1

Homotypic neutrophil adhesion in response to chemotactic stimulation has been reported to be reversible, decreasing with time after addition of stimuli (29, 46). It has previously been demonstrated that L-selectin shedding may play a role in this decay of neutrophil adhesivity (47). Furthermore, L-selectin shedding has also been implicated in regulation of neutrophil rolling velocity in vivo (48). In this manuscript, we demonstrate that besides the shedding of L-selectin, integrin deactivation may also contribute to the observed decrease in neutrophil avidity. Over the first minute of fMLP stimulation, Mac-1 and LFA-1 contributed equally to aggregate formation at low shear. After 120 s of stimulation LFA-1 did not contribute to adhesion, whereas Mac-1 retained the capacity to support $\sim$65% of the unblocked control aggregation. Within 5 min of stimulation, stable aggregates were completely supported by Mac-1 bonds and LFA-1 (or ICAM-3) appeared to play little or no role in cell adhesion. This decrease in LFA-1 avidity with time did not correlate with a change in its surface expression since the number of LFA-1 (or ICAM-3) receptors on neutrophils do not change significantly over this interval of stimulation. It is noteworthy that a similar decay in LFA-1-mediated adhesion to ICAM-1 transfectants was observed within $\sim$1–2 min after fMLP stimulation (28). Several potential mechanisms could contribute to the decay in β_2 integrin avidity following chemotactic stimulation, including changes in receptor distribution, mobility, affinity, and cytoskeletal association. We are currently exploring the mechanism of this decay.

This study has focused on the molecular dynamics supporting homotypic neutrophil adhesion via β_2 integrins under defined shear conditions. We demonstrate for the first time that LFA-1 binding to ICAM-3 contributes in equal measure with Mac-1 to avidity at shear rates on the order of 100 s^{-1}. At higher levels of shear, Mac-1 becomes the predominant molecule supporting homotypic adhesion. We have also demonstrated that Mac-1 supports adhesion over several minutes of chemotactic stimulation, whereas LFA-1-mediated avidity decays three times more rapidly. This quantitative information on the molecular dynamics and specificity in binding of the β_2 integrins and ICAM-3 may enable prediction of neutrophil function in the inflamed microcirculation and contribute to the development of rational clinical interventions.

References

1. Lawrence, M. B., and T. A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell 65:859.*
2. von Andrian, U. H., J. D. Chambers, L. M. McEvoy, R. F. Bargatze, K.-E. Arfors, and E. C. Butcher. 1991. Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte β2 integrins in vivo. *Proc. Natl. Acad. Sci. USA 88:7538.*

3. Bargatze, R. F., S. Kurk, E. C. Butcher, and M. A. Jutila. 1994. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. *J. Exp. Med. 180:1785.*
4. Walcheck, B., K. L. Moore, R. P. McEver, and T. K. Kishimoto. 1996. Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1: a mechanism that amplifies initial leukocyte accumulation on P-selectin in vitro. *J. Clin. Invest. 98:1081.*
5. Alon, R., R. C. Fuhlbrigge, E. B. Finger, and T. A. Springer. 1996. Interactions through L-selectin between leukocytes and adherent leukocytes nucleate rolling adhesions on selectins and VCAM-1 in shear flow. *J. Cell Biol. 135:849.*
6. Kunkel, E. J., J. E. Chomas, and K. Ley. 1998. Role of primary and secondary capture for leukocyte accumulation in vivo. *Circ. Res. 82:30.*
7. Zimmerman, G. A., T. M. McIntyre, M. Mehra, and S. M. Prescott. 1990. Endothelial cell-associated platelet-activating factor: a novel mechanism for signaling intercellular adhesion. *J. Cell Biol. 110:529.*
8. Detmers, P. A., S. K. Lo, E. Olsen-Egbert, A. Walz, M. Baggiolini, and Z. A. Cohn. 1990. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J. Exp. Med. 171:1155.*
9. Smith, C. W., S. D. Marlin, R. Rothlein, C. Toman, and D. C. Anderson. 1989. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J. Clin. Invest. 83:2008.*
10. de Fougerolles, A. R., S. A. Stacker, R. Schwarting, and T. A. Springer. 1991. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. *J. Exp. Med. 174:253.*
11. Smith, C. W., R. Rothlein, B. J. Hughes, M. M. Mariscalco, F. C. Schmalstieg, and D. C. Anderson. 1988. Recognition of an endothelial determinant for CD18-dependent human neutrophil adherence and transendothelial migration. *J. Clin. Invest. 82:1746.*
12. Doerschuk, C. M., R. K. Winn, H. O. Coxson, and J. M. Harlan. 1990. CD18-dependent and independent mechanisms of neutrophil emigration in the pulmonary and systemic microcirculation of rabbits. *J. Immunol. 144:2327.*
13. Simon, S. I., Y. P. Rochon, E. B. Lynam, C. W. Smith, D. C. Anderson, and L. A. Sklar. 1993. β_2-Integrin and L-selectin are obligatory receptors in neutrophil aggregation. *Blood 82:1097.*
14. Taylor, A. D., S. Neelamegham, J. D. Hellums, C. W. Smith, and S. I. Simon. 1996. Molecular dynamics of the transition from L-selectin- to β_2 integrin-dependent neutrophil adhesion under defined hydrodynamic shear. *Biophys. J. 71: 3488.*
15. Vazeux, R., P. A. Hoffman, J. K. Tomita, E. S. Dickinson, R. L. Jasman, T. John, S., and W. M. Gallatin. 1992. Cloning and characterization of a new intercellular adhesion molecule ICAM-R. *Nature 360:485.*
16. Fawcett, J., C. L. L. Holness, L. A. Needham, H. Turley, K. C. Gatter, D. Y. Mason, and D. L. Simmons. 1992. Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. *Nature 60:481.*
17. deFougerolles, A. R., and T. A. Springer. 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J. Exp. Med. 175:185.*
18. Cordell, J. L., K. Pulford, H. Turley, M. Jones, K. Micklem, I. A. Doussis, X. Tyler, K. Mayne, K. C. Gatter, and D. Y. Mason. 1994. Cellular distribution of human leucocyte adhesion molecule ICAM-3. *J. Clin. Pathol. 47:143.*
19. Hernandez-Caselles, T., G. Rubio, M. R. Campanero, M. A. del Pozo, M. Muro, F. Sanchez-Madrid, and P. Aparicio. 1993. ICAM-3 the third LFA-1 counterreceptor, is a co-stimulatory molecule for both resting and activated T lymphocytes. *Eur. J. Immunol. 23:2799.*
20. Landis, R. C., A. McDowall, C. L. L. Holness, A. J. Littler, D. L. Simmons, and N. Hogg. 1994. Involvement of the "I" domain of LFA-1 in selective binding to ligands ICAM-1 and ICAM-3. *J. Cell Biol. 126:529.*
21. Campanero, M. R., M. A. del Pozo, A. G. Arroyo, P. Sanchez-Mateos, T. Hernandez-Caselles, A. Craig, R. Pulido, and F. Sanchez-Madrid. 1993. ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathway. *J. Cell Biol. 123:1007.*
22. de Fougerolles, A. R., M. S. Diamond, and T. A. Springer. 1995. Heterogenous glycosylation of ICAM-3 and lack of interaction with Mac-1 and p150,95. *Eur. J. Immunol. 25:1008.*
23. Cid, M. C., J. Esparza, M. Juan, A. Miralles, J. Ordi, R. Vilella, A. Urbano-Marquez, A. Gaya, J. Vives, and J. Yague. 1994. Signaling through CD50 (ICAM-3) stimulates T lymphocyte binding to human umbilical vein endothelial cells and extracellular matrix proteins via an increase in β_1 and β_2 integrin function. *Eur. J. Immunol. 24:1377.*
24. Fox, S. B., G. D. Turner, K. C. Gatter, and A. L. Harris. 1995. The increased expression of adhesion molecules ICAM-3, E- and P-selectins on breast cancer endothelium. *J. Pathol. 177:369.*
25. Patey, N., R. Vazeux, D. Canioni, T. Potter, W. M. Gallatin, and N. Brousse. 1996. Intercellular adhesion molecule-3 on endothelial cells: expression in tumors but not in inflammatory responses. *Am. J. Pathol. 8:465.*
26. Woska, J. R., Jr., M. M. Morelock, D. D. Jeanfavre, G. O. Caviness, B. Bormann, and R. Rothlein. 1998. Molecular comparison of soluble intercellular adhesion molecule (sICAM)-1 and sICAM-3 binding to lymphocyte function associated antigen-1. *J. Biol. Chem. 273:4725.*
27. Sadhu, C., B. Lipsky, H. P. Erickson, J. Hayflick, K. O. Dick, M. Gallatin, and D. E. Staunton. 1994. LFA-1 binding sites in ICAM-3 contains a conserved motif and non-contiguous amino acids. *Cell Adhes. Commun. 2:429.*
28. Neelamegham, S., A. D. Taylor, A. B. Burns, C. W. Smith, and S. I. Simon. 1998. Hydrodynamics shear reveals distinct roles for LFA-1 and Mac-1 in neutrophil adhesion to ICAM-1. *Blood 92:1626.*
29. Neelamegham, S., A. D. Taylor, J. D. Hellums, M. Dembo, C. W. Smith, and S. I. Simon. 1997. Modeling the reversible kinetics of neutrophil aggregation under hydrodynamic shear. *Biophys. J. 72:1527.*
30. Simon, S. I., J. D. Chambers, and L. A. Sklar. 1990. Flow cytometric analysis and modeling of cell-cell adhesive interactions: the neutrophil as a model. *J. Cell Biol. 111:2747.*
31. Hentzen, E. R., S. Neelamegham, G. S. Kansas, J. A. Benanti, L. V. McIntire, C. W. Smith, and S. I. Simon. 2000. Sequential binding of CD11a/CD18 and CD11b/CD18 define neutrophil capture and stable adhesion to ICAM-1. *Blood. 95:911.*
32. Skubitz, K. M., K. Ahmed, K. D. Campbell, and A. P. N. Skubitz. 1995. CD50 (ICAM-3) is phosphorylated on tyrosine and is associated with tyrosine kinase activity in human neutrophils. *J. Immunol. 154:2888.*
33. Kishimoto, T. K., M. A. Jutila, E. L. Berg, and E. C. Butcher. 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science 245:1238.*
34. Rainger, G. E., C. Buckley, D. L. Simmons, and G. B. Nash. 1998. Neutrophil rolling on immobilized platelets migrate into homotypic neutrophil aggregates after activation. *Thromb. Haemostasis 79:1177.*
35. Martin, B. A., J. L. Wright, H. Thommasen, and J. C. Hogg. 1982. Effect of pulmonary blood flow on the exchange between the circulating and marginating pool of polymorphonuclear leukocytes in dog lungs. *J. Clin. Invest. 69:1277.*
36. Worthen, G. S., L. A. Smedly, M. G. Tonnesen, D. llis, N. F. Voelkel, J. T. Reeves, and P. M. Henson. 1987. Effects of shear stress on adhesive interaction between neutrophils and cultured endothelial cells. *J. Appl. Physiol. 65: 2031.*
37. Gaboury, J. P., and P. Kubes. 1994. Reductions in physiologic shear rates lead to CD11/CD18 dependent, selectin-independent leukocyte rolling in vivo. *Blood 83:345.*
38. Entman, M. L., and C. M. Ballantyne. 1996. Association of neutrophils with platelet aggregates in unstable angina: should we alter therapy? *Circulation 94: 1206.*
39. Konstantopolous, K., S. Neelamegham, A. R. Burns, G. S. Kansas, K. R. Snapp, E. L. Berg, J. D. Hellums, C. W. Smith, L. V. McIntire, and S. I. Simon. 1998. Venous levels of shear induces neutrophil-platelet and neutrophil aggregation in blood via P-selectin and β_2 integrin. *Circulation 98:873.*
40. Feldhaus, M. J., J. M. Kessel, G. A. Zimmerman, and T. M. McIntyre. 1998. Engagement of ICAM-3 activates polymorphonuclear leukocytes: aggregation without degranulation or β_2 integrin recruitment. *J. Immunol. 161:6280.*
41. Kessel, J. M., J. Hayflick, A. S. Weyrich, P. A. Hoffman, M. Gallatin, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 1998. Coengagement of ICAM-3 and Fc receptors induces chemokine secretion and spreading by myeloid leukocytes. *J. Immunol. 160:5579.*
42. Skubitz, K. M., K. D. Campbell, and A. P. N. Skubitz. 1997. CD50 monoclonal antibodies inhibit neutrophil activation. *J. Immunol. 159:820.*
43. Finger, E. B., K. Puri, R. Alon, M. B. Lawrence, U. H. von Andrian, and T. A. Springer. 1996. Adhesion through L-selectin requires a threshold hydrodynamic shear. *Nature 379:266.*
44. Lawrence, M. B., G. S. Kansas, E. J. Kunkel, and K. Ley. 1997. Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E). *J. Cell Biol. 136:717.*
45. van Kooyk, Y., M. E. Binnerts, C. P. Edwards, M. Champe, P. W. Berman, C. G. Figdor, and S. C. Bodary. 1996. Critical amino acids in the lymphocyte function-associated antigen-1 I domain mediate intercellular adhesion molecule 3 binding and immune function. *J. Exp. Med. 183:1247.*
46. Rochon, Y. P., and M. M. Frojmovic. 1993. Regulation of human neutrophil aggregation: comparable latent times, activator sensitivities, and exponential decay in aggregability for FMLP, platelet-activating factor, and leukotriene B4. *Blood 11:3460.*
47. Simon, S. I., J. D. Chambers, E. Butcher, and L. A. Sklar. 1992. Neutrophil aggregation is β_2 integrin- and L-selectin-dependent in blood and isolated cells. *J. Immunol. 149:2765.*
48. Hafezi-Moghadam, A., and K. Ley. 1999. Relevance of L-selectin shedding for leukocyte rolling in vivo. *J. Exp. Med. 189:939.*