Mac-1, but Not LFA-1, Uses Intercellular Adhesion Molecule-1 to Mediate Slow Leukocyte Rolling in TNF-α-Induced Inflammation[1]

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We have previously shown that Mac-1 and LFA-1 play a cooperative role in slow leukocyte rolling in inflamed vessels, and that, although both have a role in leukocyte adhesion, the contribution from LFA-1 exceeds that of Mac-1. In this study, we used mice deficient in ICAM-1 (ICAM-1^{null}) to study the function of ICAM-1 as an endothelial ligand for Mac-1 and LFA-1. The cremaster muscles of these mice were treated with TNF-α and prepared for intravital microscopy. We found that the average rolling velocity in venules was not different in ICAM-1^{null} mice (4.7 μm/s) compared with wild-type mice (5.1 μm/s). Similarly, leukocyte adhesion efficiency in ICAM-1^{null} mice (0.11 ± 0.01 mm) was similar to that in Mac-1$^{-/-}$ (0.12 ± 0.03 mm) mice but significantly increased compared with that in LFA-1$^{-/-}$ (0.08 ± 0.01 mm) mice and significantly reduced from that in wild type (0.26 ± 0.04 mm). When both LFA-1 and ICAM-1 were blocked, rolling velocity increased, and adhesion efficiency and arrest decreased. However, blocking both Mac-1 and ICAM-1 had no greater effect than either blockade alone. We conclude that endothelial ICAM-1 is the main ligand responsible for slow leukocyte rolling mediated by Mac-1, but not LFA-1. ***The Journal of Immunology,*** **2003, 171: 6105–6111.**

The localization of leukocytes to the site of inflammation requires several families of adhesion molecules. Leukocytes proceed through capture, rolling, slow rolling, and adhesion before they transmigrate through the vascular endothelium to access interstitial tissue (1–6). Although originally thought to mediate only adhesion and transmigration, β_2 (CD18) integrins have been shown to play an important role in the conversion from rolling to firm arrest, a step defined as slow rolling (2, 5, 7, 8). Recent work, both in vitro and in vivo, indicates that two of the β_2 integrins, LFA-1 (CD11a/CD18, $\alpha_L\beta_2$) and Mac-1 (CD11b/CD18, $\alpha_M\beta_2$), have distinct and cooperative roles in the inflammatory process. In mice lacking either LFA-1 or Mac-1, leukocyte rolling velocity is increased over wild type, but not to the levels of mice lacking all of the CD18 integrins (7). However, when both LFA-1 and Mac-1 are blocked, leukocyte rolling velocity approaches that seen in CD18$^{-/-}$ mice (7). In addition, although both LFA-1 and Mac-1 appear to be important in leukocyte adhesion, the contribution from LFA-1 is greater than that from Mac-1 (7, 9).

ICAM-1 on the vascular endothelium can serve as a ligand for both LFA-1 and Mac-1 (10, 11). However, ICAM-1 has only a small role in slow rolling (3, 12). Neutrophils treated with a mAb against LFA-1 show an 80% reduced adhesion to ICAM-1, but only a 40% reduced adhesion to endothelial cells (9), suggesting that endothelial cells express other functionally important LFA-1 ligands. Similarly, neutrophil adhesion under flow to ICAM-1-deficient endothelial cells is reduced, but not abolished (13). Other endothelial ligands for LFA-1 include ICAM-2 (14) and, recently, junctional adhesion molecule-1 (JAM-1)[3] (15). JAM-1 mediates LFA-1-dependent transmigration, but does not appear to play a role in adhesion under flow (15).

Dynamic regulation of integrin adhesive function through cellular activation is thought to be critical in order for leukocytes to arrest on the endothelium (1). It is thought that as a leukocyte rolls down the vasculature, exposure to chemoattractants presented on the surface of the endothelium progressively activates it (16). There is additional evidence suggesting that neutrophils also receive signals from binding to inflammatory adhesion molecules (8, 17). This progressive activation is essential in the conversion of rolling to firm adhesion (5).

Two previous studies investigated leukocyte recruitment in mice in which ICAM-1 was targeted by homologous recombination (18, 19). Although the common form of ICAM-1 is not detectable in these mutant strains, the production of splice variants that retain the binding domains for either LFA-1 or Mac-1 or both are found in both of these mutants (20). Recently, the entire coding region of ICAM-1 has been deleted, thus producing a true ICAM-1^{null} mouse (R. G. Collins and A. L. Beaudet, unpublished observations).

To begin to understand the molecular mechanisms involved in the conversion from rolling to firm adhesion, we designed experiments in mice lacking ICAM-1, LFA-1 (9), or Mac-1 (21) and use function-blocking mAbs to achieve blockade of combinations of these adhesion molecules. In addition to conventional intravital microscopy, we follow each individual leukocyte as it rolls along the venular tree until it adheres to or detaches from the endothelium.

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[3] Abbreviations used in this paper: JAM-1, junctional adhesion molecule-1; FtVII, fucosyltransferase VII.

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Materials and Methods

Mice

All mice appeared healthy in vivarium conditions. Experiments were conducted under a protocol approved by the University of Virginia institutional animal care and use committee.

Gene-targeted mice lacking LFA-1 (9) or Mac-1 (21) were obtained from Dr. C. Ballantyne (Department of Medicine, Baylor College of Medicine). Gene-targeted mice lacking the entire coding region of ICAM-1 (ICAM-1^{null}) were obtained from Dr. A. Beaudet. All mice were backcrossed into the C57BL/6 background for at least seven generations. Wild-type C57BL/6 mice were from Hilltop Labs (Scottsdale, PA) or The Jackson Laboratory (Bar Harbor, ME). We used male mice with a mean age of 20 wk and a mean weight of 31 g. The following eight groups of mice were studied: wild-type, ICAM-1^{null}, Mac-$1^{-/-}$, LFA-$1^{-/-}$, ICAM-1^{null} plus Mac-1 mAb, ICAM-1^{null} plus LFA-1 mAb, Mac-$1^{-/-}$ plus ICAM-1 mAb, and LFA-$1^{-/-}$ plus Mac-1 mAb (Tables I and II).

Reagents

Murine rTNF-α (0.5 μg/mouse) was obtained from R&D Systems (Minneapolis, MN). The blocking mAb M1/70 (22), specific for the α_M subunit of Mac-1 (rat IgG2b; 100 μg/mouse), and the blocking mAb 3C4 (23), specific for ICAM-2 (rat IgG2a,κ; 100 μg/mouse), were obtained from BD PharMingen (San Diego, CA). The LFA-1 mAb TIB-217 (24) (rat IgG2a,κ; 100 μg/mouse) and the ICAM-1 mAb YN1 (25) (rat IgG2b; 100 μg/mouse) were purified at the Lymphocyte Culture Center at the University of Virginia from hybridoma supernatant (American Type Culture Collection, Manassas, VA).

Systemic leukocyte counts were obtained from a 10-μl sample from the carotid cannula after discarding the first 20 μl. The capillary tubes were put in 90 μl of Kimura stain (11 ml of toluidine blue, 0.8 ml of 0.03% light green SF yellowish (Sigma-Aldrich, St. Louis, MO), 0.5 ml of saturated saponin (Sigma-Aldrich) in 50% ethanol, and 5 ml 1/15 M phosphate buffer (pH 6.4)) in a hemocytometer to obtain absolute numbers of leukocytes per microliter and the number of polymorphonuclear cells and mononuclear cells. Equivalent results were obtained using an automated blood cell counter (HemaVet 850FS; CDC Technologies, Oxford, CT).

Intravital microscopy

Two hours before exteriorization of the cremaster muscle, all mice were injected intrascrotally with 0.5 μg of TNF-α in 0.30 ml of isotonic saline. mAbs were injected i.p. immediately following TNF-α. Mice were anesthetized with ketamine hydrochloride (125 mg/kg; Sanofi Winthrop Pharmaceuticals, New York, NY), xylazine (12.5 mg/kg; TranquiVed; Phoenix Scientific, St. Joseph, MO), and atropine sulfate (0.025 mg/kg; Fujisawa, Deerfield, IL) i.p. Mice were kept at 37°C; trachea, jugular vein (for additional anesthetic), and one carotid artery (for blood sampling) were cannulated with polyethylene tubing (BD Biosciences, Sparks, MD). After surgery, the cremaster muscle was prepared for intravital microscopy, the epididymis and testis were pinned to the side, and the cremaster was superfused with a thermocontrolled (37°C) bicarbonate-buffered saline (131.9 mM NaCl, 18 mM $NaHCO_3$, 4.7 mM KCl, 2.0 mM $CaCl_2 \cdot 2H_2O$, and 1.2 mM $MgCl_2$) equilibrated with 5% CO_2 in N_2. All microscopic observations were made on a Zeiss intravital microscope (Axioskop; Carl Zeiss, Thornwood, NY), with a saline immersion objective (SW 40/0.75 numerical aperture). Venules between 15 and 70 μm were videotaped through a charge-coupled device camera system (model VE-1000CD; Dage-MTI, Michigan City, IN) for ~90 s/venule on a VHS recorder (AG-W1; Panasonic, Secaucus, NJ) for off-line analysis of leukocyte rolling velocity and adhesion data. The vessel centerline blood velocity was measured using a dual photodiode and a digital on-line cross-correlation program as previously described (26). Mean blood flow velocity (V_b) was approximated by multiplying the centerline blood velocity by a factor of 0.625 (27). Wall shear rate (γ_w) was estimated as follows: $\gamma_w = 2.12 \times 8 \times (V_b/d)$, where d is the diameter of the vessel, and 2.12 is a median empirical correction factor obtained from velocity profiles measured in microvessels in vivo (Table II) (28). There were no significant differences between any of the experimental groups for wall shear rate or venule diameter, thus ensuring that venules with similar hemodynamic parameters were compared.

Data obtained on videotapes during the experiments were analyzed off-line using a digital image processing system (26, 29). Vessel diameter and length were measured using electronic calipers. Adhesion was defined as a leukocyte that was stationary for >30 s. Adhesion numbers are expressed per unit surface area of the vessel, assuming cylindrical geometry. In vessels >40 μm, only one-half of the vessel is in sharp focus, and the sampled surface area was approximated as a half-cylinder. Rolling velocities were measured for 10 leukocytes per vessel picked at random by viewing the translation during 2 s.

For each of the groups studied, median leukocyte rolling velocity and the distribution of rolling velocities were determined. Because these data are nonnormal, statistical significance between groups was determined by a Mann-Whitney test at a significance level of $p < 0.05$. Overall adhesion efficiency was determined by dividing the number of adherent leukocytes per square millimeter by the number of peripheral leukocytes per microliter (unit, millimeter) as described (2). Statistical significance between groups was determined by a one-way ANOVA test followed by a t test at a significance level of $p < 0.05$. For the tracking analysis, tests on the proportions of adhered leukocytes were performed at a significance level of $p < 0.05$.

Leukocyte tracking

For the leukocyte tracking studies, after cremaster exteriorization, a venular tree of at least 1-mm length was located. An individual leukocyte, picked at random upon entering the tree from the capillary, was tracked using a motorized stage (MC2000; McAllister Technical Services, Coeur d'Alene, ID) and intravital microscopy. Each leukocyte made contact with the endothelium upon exiting the capillary and rolled along the endothelial wall. Each rolling leukocyte was followed until it adhered, detached from the endothelium, or was lost due to technical limitations of the tracking procedure. Between 5 and 320 measurements were made for each leukocyte, depending on the time rolled. A leukocyte was determined to be adherent as described above, that is, if it remained stationary for >30 s. A leukocyte was determined to have detached if it had not adhered to the endothelium, and it ceased to make visible contact with the endothelium, presumably returning to free flow. Considerable portions of leukocytes were lost in the tracking process. The majority of these occurred in crowded vessels when the leukocyte being tracked rolled above or below another leukocyte, and it was difficult to discern which was the tracked leukocyte. However, the proportion of leukocytes that were lost did not differ between groups, and thus, those leukocytes that were lost presumably did not affect the final analysis.

To compare the rolling behavior of adhered and detached leukocytes between groups, rolling distance and time were normalized by dividing the instantaneous value by the total time and distance for each leukocyte, respectively, as previously described (5). Each individual normalized curve was then averaged at 50 equidistant time points with the other leukocytes in its treatment group. This was performed separately for adhered and detached leukocytes within each treatment group. The averaged normalized adhered curves for each treatment group were plotted with the averaged normalized detached curves. These graphs allow for the comparison of the different characteristic behavior of leukocytes that adhere vs those that detach.

Table I. *Experimental groups and blood counts*[a]

Mouse Type	Number of Mice	Blood Leukocyte Concentration (cells/μl)		
		Total leukocytes	PMNs	MNCs
Wild type	7	2430 ± 500	1190 ± 380	1240 ± 160
ICAM-1^{null}	7	5980 ± 760[b]	3240 ± 640[b]	2750 ± 300[b]
Mac-$1^{-/-}$	11	7090 ± 1300[b]	4390 ± 920[b]	2710 ± 440[b]
LFA-$1^{-/-}$	12	7600 ± 1080[b]	4500 ± 790[b]	3110 ± 390[b]

[a] Data are presented as mean ± SEM. PMN, Polymorphonuclear cell; MNC, mononuclear cell.
[b] Significantly different from wild type.

Table II. *Microvascular parameters*[a]

Mouse Type	Treatment (mAb)	No. Venules	Average Venule Diameter (μm)	Wall Shear Rate (s^{-1})
Wild type		29	36.6 ± 2.4	620 ± 38
ICAM-1^{null}		23	30.6 ± 2.0	548 ± 36
Mac-1$^{-/-}$		23	39.0 ± 1.9	480 ± 35
LFA-1$^{-/-}$		39	40.9 ± 2.1	537 ± 33
ICAM-1^{null}	Mac-1	20	32.0 ± 1.8	495 ± 42
ICAM-1^{null}	LFA-1	10	34.2 ± 3.2	662 ± 49
Mac-1$^{-/-}$	ICAM-1	23	39.8 ± 2.0	652 ± 46
LFA-1$^{-/-}$	ICAM-1	28	37.2 ± 1.9	654 ± 45
Mac-1$^{-/-}$	ICAM-2	18	37.6 ± 2.0	567 ± 47

[a] Average venule diameter (micrometers) and wall shear rate (seconds^{-1}) are presented as mean ± SEM. Average venule diameter and wall shear rate measured in the same vessels as rolling velocities and adhesion.

Results

Rolling velocity distribution

To determine whether ICAM-1 modulated leukocyte rolling velocity either alone or in concert with its known ligands Mac-1 and LFA-1, rolling velocity was measured in mouse cremaster venules after 2 h of stimulation with TNF-α (Fig. 1). Leukocytes in wild-type mouse venules treated with TNF-α rolled at a median velocity of 5.1 μm/s. Leukocytes in ICAM-1^{null} venules rolled at a similar velocity to wild type, with a median rolling velocity of 4.7 μm/s.

To test whether ICAM-1 may be the rolling ligand for Mac-1 and/or LFA-1, ICAM-1^{null} mice were treated with mAbs to either Mac-1 or LFA-1, and Mac-1$^{-/-}$ mice and LFA-1$^{-/-}$ mice were treated with a mAb to ICAM-1. When ICAM-1^{null} mice were treated with a mAb to Mac-1, or Mac-1$^{-/-}$ mice were treated with a mAb to ICAM-1, rolling velocity did not increase significantly (Fig. 1). Because LFA-1 is still present in these mice, these data suggest that LFA-1 uses ligand(s) other than ICAM-1 as a significant ligand for slow leukocyte rolling. Conversely, when ICAM-1^{null} mice were treated with a mAb to LFA-1, or LFA-1$^{-/-}$ mice were treated with a mAb to ICAM-1, rolling velocity increased significantly from 4.7 to 8.4 μm/s and from 5.8 to 8.3 μm/s, respectively. These data suggest that Mac-1, which is still present, binds to ICAM-1 on the inflamed endothelium to mediate slow rolling. We also tested whether ICAM-2, another endothelial ligand for LFA-1, may be involved in slow rolling. To investigate the isolated contribution of LFA-1 to slow rolling, Mac-1$^{-/-}$ mice were treated with a mAb to ICAM-2. Rolling velocity in these mice did not change (5.8 vs 5.7 μm/s). These data suggest that ICAM-2 does not serve as a rolling ligand for LFA-1 in vivo.

Role of ICAM-1 in leukocyte adhesion

To establish the role of ICAM-1 in leukocyte adhesion, the number of adherent leukocytes was determined in ICAM-1^{null} mice. The number of adherent leukocytes was significantly higher (814 ± 99 adherent leukocytes/mm^2) in ICAM-1^{null} mice compared with wild-type and LFA-1$^{-/-}$ mice (684 ± 84 and 646 ± 74 adherent leukocytes/mm^2, respectively), but similar to Mac-1$^{-/-}$ mice (734 ± 98 adherent leukocytes/mm^2) (Fig. 2*A*).

However, these levels of leukocyte adhesion must be seen in the context of neutrophilia, which is present in ICAM-1^{null} (3-fold elevation), Mac-1$^{-/-}$ (4-fold elevation), and LFA-1$^{-/-}$ (4-fold elevation) mice (Table I). To account for the increased number of circulating neutrophils, adhesion data were normalized by the total number of circulating neutrophils as described previously (2). Although adhesion efficiency in ICAM-1^{null} mice (0.11 ± 0.01 mm) was significantly higher than that in LFA-1$^{-/-}$ mice (0.08 ± 0.01 mm), it was not different from that in Mac-1$^{-/-}$ mice (0.12 ± 0.03 mm) but was decreased from that in wild-type mice (0.26 ± 0.03 mm) (Fig. 2*B*).

Leukocyte tracking

To understand the cooperation between rolling and adhesion, individual leukocytes were tracked down a venular tree upon initial contact with the endothelium. Individual leukocytes were tracked (5–320 measurements per leukocyte) until they adhered to or detached from the endothelium. Those leukocytes that were lost due

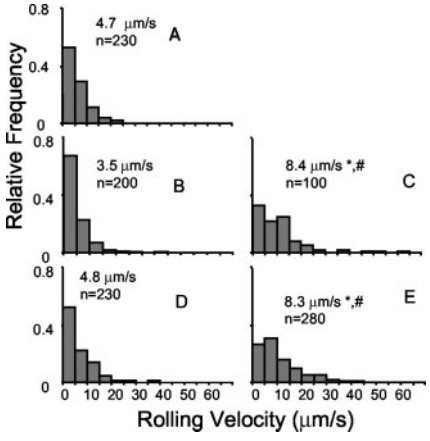

FIGURE 1. Rolling velocity histograms. ICAM-1^{null} (*A*), ICAM-1^{null} plus Mac-1 mAb (*B*), ICAM^{null} plus LFA-1 mAb (*C*), Mac-1$^{-/-}$ plus ICAM-1 mAb (*D*), and LFA$^{-/-}$ plus ICAM-1 mAb (*E*), *, Median rolling velocities that are significantly higher than wild type. #, Median rolling velocities that are significantly higher than their untreated counterparts.

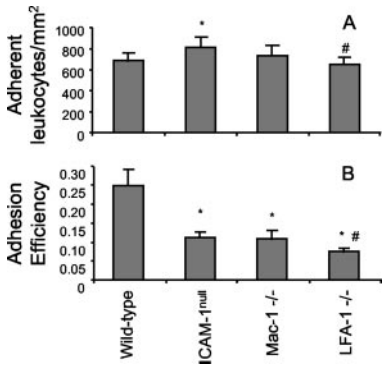

FIGURE 2. Adhesion. *A*, Number of adherent (>30 s) leukocytes per square millimeter in venules. *B*, Overall adhesion efficiency ((adherent leukocytes per square millimeter in venules)/(leukocytes per microliter in blood)). Data are presented as mean ± SEM. *, Values that are significantly different from wild type. #, Values that are significantly different from ICAM-1^{null}.

to technical difficulties were not included in the analysis. Twenty-one of 22 leukocytes (95%) tracked in the wild-type mice adhered to the endothelium (5). In LFA-$1^{-/-}$ mice, only 9 of 29 leukocytes (32%) adhered, and in Mac-$1^{-/-}$ mice, 11 of 21 leukocytes (52%) adhered. These results suggest that both LFA-1 and Mac-1 play a role in adhesion (Fig. 3) (7).

To further elucidate the role of ICAM-1 as a ligand for β_2 integrins, tracking analysis was performed on ICAM-1^{null} mice. Nine of 27 leukocytes (33%) adhered to the endothelium in ICAM-1^{null} mice. This is similar to levels seen in Mac-$1^{-/-}$ and LFA-$1^{-/-}$ mice. When ICAM-1^{null} mice were treated with a mAb to Mac-1, the percentage of leukocytes that adhered did not change significantly. The same result was obtained when Mac-$1^{-/-}$ mice were treated with a mAb to ICAM-1. These findings suggest that Mac-1 and ICAM-1 are binding partners in adhesion. However, when ICAM-1^{null} mice were treated with a mAb to LFA-1, the percentage of leukocytes that adhered decreased to 20 from 33%. A significant decrease of adhesion was observed when LFA-$1^{-/-}$ mice were treated with a mAb to ICAM-1 (5 vs 32%). These findings suggest that LFA-1 and ICAM-1 are in parallel adhesion pathways.

To examine the behavior of rolling leukocytes as they adhere to or detach from the endothelium, cumulative distance vs time plots were created for each group. To compare each group on the same graph, rolling distance and time were each normalized by dividing the instantaneous value by the total time and distance for each leukocyte, respectively (5). Next, distance-time tracings of all leukocytes that eventually adhered were averaged to form one curve, and the same was done for all leukocytes that eventually detached (Fig. 4). The convex shapes of the distance-time curves of those leukocytes that adhere show that these leukocytes slow down significantly before arrest and suggest that progressive activation may be occurring. The lack of the curved shape in those leukocytes that detach suggests that β_2 integrins and ICAM-1 are important in forming proper adhesions or in integrating inflammatory signals to form such adhesions.

To investigate whether duration of contact determined the eventual fate of an individual leukocyte, the total distance and time traveled by each leukocyte were recorded. Those leukocytes that ultimately detached from the endothelium remained in contact with the endothelium as long or longer in both distance and time as their counterparts that adhered, suggesting that it is not a lack of contact with the endothelium that causes leukocytes to detach (Fig. 5).

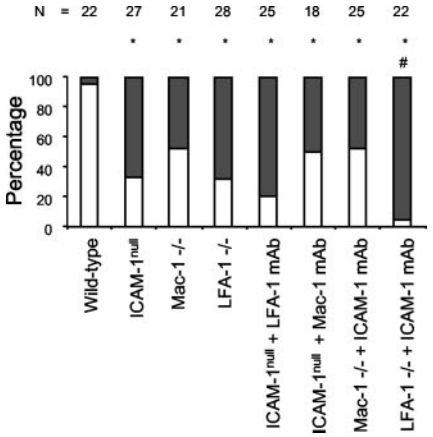

FIGURE 3. Percentage of leukocytes that adhered or detached. □, The percentage of leukocytes that adhered. ■, The percentage of leukocytes that detached. The number of leukocytes tracked per mouse treatment is indicated above the bars. ∗, Those percentages of adhered leukocytes that are significantly lower than wild type. #, Those percentages that are significantly lower than their untreated counterparts.

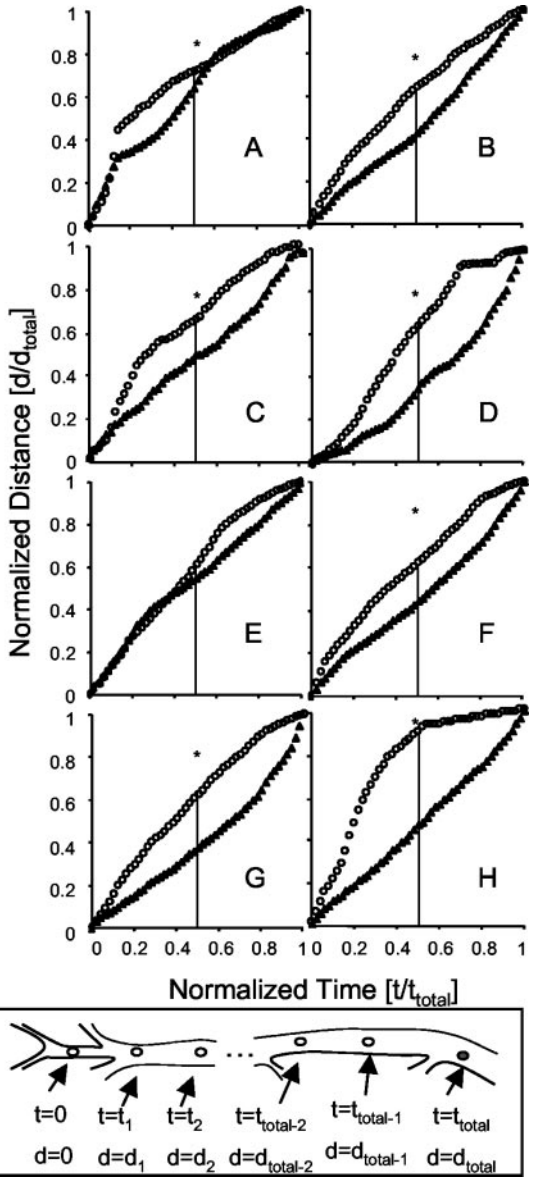

FIGURE 4. Normalized distance vs normalized time curves. Wild type (*A*), ICAM-1^{null} (*B*), Mac-$1^{-/-}$ (*C*), LFA-$1^{-/-}$ (*D*), ICAM-1^{null} plus Mac-1 mAb (*E*), ICAM-1^{null} plus LFA-1 mAb (*F*), Mac-$1^{-/-}$ plus ICAM-1 mAb (*G*), and LFA-$1^{-/-}$ plus ICAM-1 mAb (*H*). ○, The tracings of those leukocytes that eventually adhered to the endothelium. ▲, The tracings of those leukocytes that eventually detached from the endothelium. Schematic at the *bottom* shows the progression of a single leukocyte (○) rolling down a venular tree until it arrests or detaches (●). ∗, Significant difference between median values (vertical line).

Leukocyte arrest predicts adhesion efficiency

To test the hypothesis that the normalized parameter, adhesion efficiency, is a valid predictor of the actual ability of leukocytes to adhere, adhesion efficiency (from Fig. 2) was plotted against the percentage of leukocytes that adhere in a given population (from Fig. 3). As shown in Fig. 6, adhesion efficiency increases with an increase in the percentage of leukocytes that adhere. The linear regression of the data has a correlation coefficient of 0.5, indicating a positive correlation between adhesion efficiency and the percentage of leukocytes within a population that will adhere. In addition,

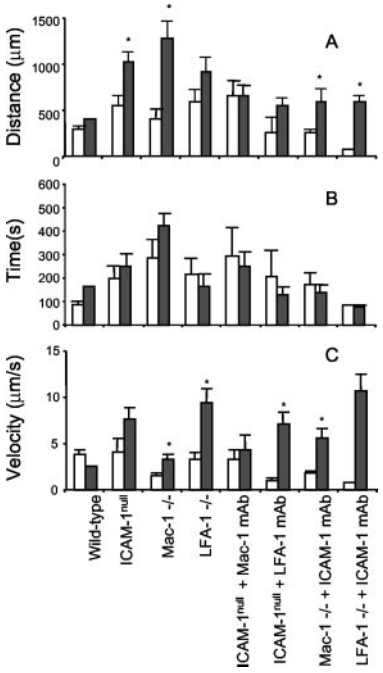

FIGURE 5. Average distance rolled, time rolled, and average rolling velocity. Distance rolled (*A*), time rolled (*B*), and rolling velocity (*C*). Rolling velocities are different from those in Fig. 1, because they are based on the total distance and time a leukocyte is rolling along the endothelium until it adheres or detaches. Because leukocytes often pause along the endothelium as they are rolling, these pauses are counted in the total time, whereas they are not in the analysis of Fig. 1. □, The averages of those leukocytes that eventually adhered. ■, The averages of those leukocytes that eventually detached. Data are presented as mean ± SEM. ∗, Those values in detached leukocytes that are significantly different from the values in adhered leukocytes.

a linear regression was performed on the data, and the slope of the line was found to be significantly different from zero, further indicating the positive correlation between adhesion efficiency and arrest. This suggests that adhesion efficiency is indeed an indicator of actual leukocyte arrest on the endothelium.

Discussion

This study shows that completely removing ICAM-1 from mice leads to no increase in average leukocyte rolling velocities in inflamed venules, unlike those seen after removal of LFA-1 or Mac-1 (7). Blocking Mac-1 in these mice leads to no further increase in leukocyte rolling velocity. In these mice, LFA-1 is functioning in the absence of ICAM-1, suggesting that ICAM-1 is not necessary for LFA-1-dependent slow leukocyte rolling. However, upon blockade of both LFA-1 and ICAM-1, leukocyte rolling velocity increases significantly, similar to levels seen in blockade of LFA-1 and Mac-1 (7), and arrest becomes very inefficient. This is consistent with ICAM-1 being an endothelial ligand for Mac-1 in the inflamed vasculature.

Unexpectedly, our data suggest that ICAM-1 does not appear to be a relevant LFA-1 ligand in leukocyte rolling and arrest. Similar to the defect seen in rolling velocity, blocking both ICAM-1 and Mac-1 does not result in a further decrease in leukocyte arrest, whereas blocking both ICAM-1 and LFA-1 does. Taken together with our finding that blocking ICAM-2 does not increase rolling velocity in Mac-$1^{-/-}$ mice, it appears that Mac-1 binds to ICAM-1, and LFA-1 binds to other, undefined ligands, to promote β_2-dependent leukocyte rolling and adhesion in the cremaster vasculature.

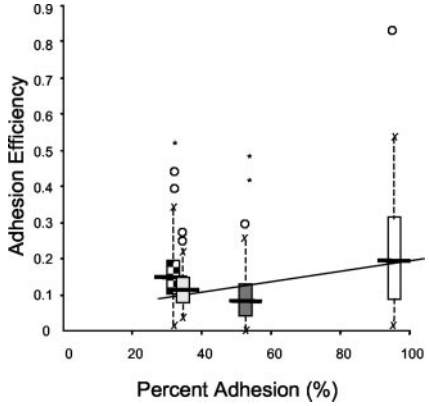

FIGURE 6. Correlation between adhesion efficiency and percent adhesion. Adhesion efficiency ((adherent leukocytes per square millimeter in venules)/(leukocytes per microliter in blood)) is plotted against the percentage of leukocytes that adhered as calculated from tracking individual leukocytes. Wild-type (open box), ICAM-1^{null} (light gray), Mac-$1^{-/-}$ (dark gray), and LFA-$1^{-/-}$ leukocytes (patterned). Horizontal thick lines represent medians, and the ends of the box mark first and third quartiles. Adjacent values are marked with an "x". ○, Mild outliers. ∗, Extreme outliers. The thin line represents least-square linear correlation.

Although the number of adherent leukocytes was not different from that in wild-type mice in ICAM-1^{null}, LFA-$1^{-/-}$, or Mac-$1^{-/-}$ mice, the leukocyte adhesion efficiency was. The adhesion efficiency parameter is calculated as the number of adherent leukocytes per square millimeter of venular endothelium divided by the number of peripheral blood leukocytes per microliter. This concept is based on the notion that the number of neutrophils that adhere to the inflamed endothelium depends on circulating neutrophil levels. Consistent with previous findings (2, 7), our data show that an increase in circulating neutrophils in adhesion molecule-deficient mice leads to a near-constant number of leukocytes adhering to the endothelium. Apparently, mice with defective neutrophil recruitment produce more neutrophils to compensate for reduced adhesion efficiency, a process regulated by IL-17 and G-CSF (30).

To further examine the role of ICAM-1, individual leukocytes were located as they exited a capillary into a postcapillary venule and followed down the venular tree until they either adhered to or detached from the endothelium. The percentage of leukocytes that adhered in the four groups correlated well with the adhesion efficiency parameter. This indicates that the observed slowing down of rolling leukocytes is indeed the process that promotes their eventual adhesion.

Previous work from our laboratory has shown that the β_2 integrins, LFA-1 and Mac-1, contribute to slow leukocyte rolling (5, 7). A number of in vitro experiments were aimed at delineating ICAM-1's role as a β_2 integrin ligand. Human vascular cell lines were transfected with fucosyltransferase VII (FtVII) and ICAM-1. FtVII catalyzes fucosylation of selectin ligands (31). When neutrophils rolled over this surface, rolling velocity decreased and adhesion increased as compared with neutrophils rolling on FtVII-transfected vascular cells alone, suggesting that ICAM-1 has a role in leukocyte rolling in adhesion. Adhesion on ICAM-1 was found to be entirely dependent on CD18, because a mAb to CD18 abrogated the adhesion effect (32).

In a plate-and-cone shearing device, neutrophils preincubated with mAbs to either LFA-1 or Mac-1 interacted with ICAM-1-expressing cells (33). Although both LFA-1 and Mac-1 bound to ICAM-1, LFA-1 accounted for the majority of cell adhesion under shear conditions. However, Mac-1 supported stable adhesion over

several minutes of chemotactic stimulation, suggesting that LFA-1 and Mac-1 may serve sequential rather than parallel functions (33). Mouse neutrophils isolated from bone marrow from CD18$^{-/-}$, Mac-1$^{-/-}$, or LFA-1$^{-/-}$ mice all showed decreased adhesion to an ICAM-1 monolayer (9). Interestingly, neutrophils treated with a mAb to LFA-1 showed an 80% reduction of adhesion to ICAM-1, but only a 40% reduction of adhesion to endothelial cells. By contrast, neutrophils treated with a mAb to Mac-1 showed a similar 30% reduction on either substrate (9). These findings are consistent with the idea that ICAM-1 is the main ligand for Mac-1 on endothelial cells, but LFA-1 has other endothelial ligands. We ruled out ICAM-2 as a potential ligand in mediating LFA-1-dependent slow rolling. In a recent in vitro study, blocking JAM-1 with a polyclonal Ab significantly inhibited neutrophil transmigration, but not arrest, under flow (15). Blocking both ICAM-1 and JAM-1 had no additional effect on neutrophil arrest beyond blocking ICAM-1 alone, suggesting that JAM-1 is not a relevant LFA-1 ligand for arrest under flow (15). Therefore, our findings suggest that one or more LFA-1 ligand(s) on endothelial cells remain to be identified.

The adhesion of β_2 integrins to the endothelium is thought to require integrin activation, exposing the ligand binding site in the inserted domain of the α-chain (34). The mechanism by which integrins are activated and whether ligand binding is a cause or consequence of activation are areas of active investigation. It has been suggested that, as neutrophils roll along the endothelial wall, they sample the endothelial surface, and, if sufficiently activated, adhere (16). In this study, we tracked individual leukocytes and studied their rolling behavior until they adhered to or detached from the endothelium. In those leukocytes that eventually adhered to the endothelium, a systematic slowing down occurred, suggesting, perhaps, an integration of signals and activation of leukocytes. Conversely, in those leukocytes that detached from the endothelium, no such slowing down occurred. However, these leukocytes traveled for similar or longer times and distances on the endothelium than their counterparts that adhered, suggesting that it is not a lack of contact with the endothelium causing these leukocytes to detach.

Whether clustering of integrins, which increases avidity, or conformational change, which increases affinity, is more important for regulating ligand binding has been debated (35). It is possible that avidity regulation may alter cellular adhesion independently of a change in affinity for ligand (36–39). Several groups have shown that leukocyte activation is accompanied by a conformational shift in integrins, and that this correlates with an increase in affinity for ligand (34, 40–48). Other groups have indicated that the avidity of integrins to their ligands is important, as evidenced by the relationship between pause times and adhesion (49). Understanding how these in vitro results correlate to the behavior of leukocyte integrins and their ligands on inflamed endothelium is an important next step.

In conclusion, ICAM-1 functions as a ligand for Mac-1, but not LFA-1, in leukocyte rolling and arrest on the inflamed endothelium. Leukocytes that adhere, regardless of available adhesion molecules, show a progressive slowing down before adhesion, whereas those leukocytes that detach do not.

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