Leukocyte Arrest During Cytokine-Dependent Inflammation In Vivo[1]

Eric J. Kunkel,[2] Jessica L. Dunne, and Klaus Ley[3]

Leukocyte rolling along the walls of inflamed venules precedes their adhesion during inflammation. Rolling leukocytes are thought to arrest by engaging β_2 integrins following cellular activation. In vitro studies suggest that chemoattractants may instantaneously activate and arrest rolling leukocytes. However, how leukocytes stop rolling and become adherent in inflamed venules in vivo has remained rather mysterious. In this paper we use a novel method of tracking individual leukocytes through the microcirculation to show that rolling neutrophils become progressively activated while rolling down the venular tree. On average, leukocytes in wild-type mice roll for 86 s (and cover 270 μm) before becoming adherent with an efficiency around 90%. These rolling leukocytes exhibit a gradual β_2 integrin-dependent decrease in rolling velocity that correlates with an increase in intracellular free calcium concentration before arrest. Similar tracking analyses in gene-targeted mice demonstrate that the arrest of rolling leukocytes is very rare when β_2 integrins are absent or blocked by a mAb. Arrest is ~50% less efficient in the absence of E-selectin. These data suggest a model of leukocyte recruitment in which β_2 integrins play a critical role in stabilizing leukocyte rolling during a protracted cellular activation period before arrest and firm adhesion. ***The Journal of Immunology*****, 2000, 164: 3301–3308.**

Leukocytes rolling on inflamed endothelium via the selectin family of adhesion molecules (1) are thought to require chemoattractant stimulation and cellular activation to arrest through engagement of their integrins (2–4). Activation-dependent arrest of rolling leukocytes has been part of the leukocyte recruitment paradigm for many years, and while many chemoattractants (e.g., fMLP, PAF, C5a, IL-8, eotaxin) are known to participate in the accumulation of leukocytes during inflammation, specific analysis of the rapidity, specificity, and efficiency of chemoattractant-induced arrest has only recently been reported (5–7).

The ability of surface-bound chemoattractants to mediate arrest of rolling leukocytes has been demonstrated thus far only in vitro. Rainger et al. (5) demonstrated that neutrophils rolling on cultured HUVEC treated with IL-8 or platelet activating factor stopped within less than 1 s and less than 15 μm after initiation of rolling. In a reconstituted system, Campbell et al. (6) showed rapid arrest ($<$1 s) of lymphocytes rolling on a substrate containing peripheral node addressin (a ligand for L-selectin) and ICAM-1 (a β_2 integrin ligand) when appropriate chemokines were co-immobilized. Similarly, monocyte chemotactic protein-1 and IL-8 were shown to mediate rapid arrest of monocytes rolling on endothelial cells in a flow chamber system (7). Rapid arrest of rolling leukocytes can be observed in vivo when IL-8 (8) or one of its murine homologues, macrophage-inflammatory protein-2 (MIP-2)[4] (9, 10), are injected adjacent to a venule using a micropipette. However, this mode of rapid activation may be typical of high local concentrations of chemoattractant, and rapid arrest of rolling leukocytes may not reflect the physiological process of leukocyte activation and arrest occurring during inflammation.

In previous work from our laboratory (11), we discovered that the number of adherent leukocytes during inflammation correlates with their venular transit time. This suggested that the amount of time rolling leukocytes remain in contact with the venular endothelium and are exposed to activating signals may determine arrest. To achieve wild-type levels of leukocyte adhesion, rolling leukocytes require an average rolling time of ~30 s to pass a 100-μm segment of venule; altering the rolling time using Abs against E-selectin or CD18 integrins reduced the number of adherent leukocytes. These data, gathered as population averaged data, suggest that prolonged rolling contact with the endothelium may be necessary to promote activation and trigger integrin-mediated arrest and firm adhesion.

Using the TNF-α-treated mouse cremaster muscle as a well-characterized model of acute cytokine-dependent inflammation (12–14), we have begun to examine the transition from rolling to firm adhesion under physiological conditions to understand this apparent requirement for long endothelial contact times. In this model, both P- and E-selectin are expressed on the vascular endothelium (14), and all three selectins (L-, P-, and E-selectin) contribute to leukocyte rolling (13, 15). About 97% of all rolling and adherent leukocytes in this inflammatory model are neutrophils (13). To examine the rolling-to-adhesion transition, we have developed a new method involving tracking of individual rolling leukocytes to test the hypothesis that rolling leukocytes may become activated during the rolling process, leading to progressively increasing engagement of β_2 integrins, and finally arrest and firm adhesion. In this paper we present evidence that under the conditions of in situ TNF-α stimulation, leukocyte adhesion occurs not

Department of Biomedical Engineering, University of Virginia Health Sciences Center, Charlottesville, VA 22908

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[2] Current address: Department of Pathology, Stanford University, 154-B VAMC, 3801 Miranda Avenue, Palo Alto, CA 94304. E-mail address: ejkunkel@cmgm.stanford.edu

[3] Address correspondence and reprint requests to Dr. Klaus Ley, Department of Biomedical Engineering, University of Virginia, Box 377 Health Sciences Center, Charlottesville, VA 22908. E-mail address: klausley@virginia.edu

[4] Abbreviations used in this paper: MIP-2, macrophage-inflammatory protein-2; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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as rapid arrest in response to chemoattractants, but a gradual deceleration process requiring increased β_2 integrin adhesiveness.

Materials and Methods

Animals

All mice used were between 8 and 16 wk old and healthy under barrier vivarium conditions, although spontaneous inflammatory skin lesions have been reported in older CD18$^{-/-}$ mice (16) when kept in conventional facilities. CD18$^{-/-}$ and E$^{-/-}$ mice were back-crossed into a C57BL/6 background and were gifts of Dr. A. L. Beaudet (Baylor College of Medicine, Houston, TX) and D. C. Bullard (University of Alabama, Birmingham). Control mice were age- and strain-matched C57BL/6 wild-type mice purchased from Hilltop Lab Animals (Scottdale, PA). All animal experiments were conducted under a protocol approved by the University of Virginia institutional animal care and use committee.

Reagents

Recombinant murine TNF-α was purchased from Genzyme (Cambridge, MA). The mAb GAME-46 against the common mouse β_2 integrin chain (30 μg per mouse i.v.) reported to block LFA-1 binding to ICAM-1, -2, and -3 and Mac-1 binding to ICAM-1 (17) was purchased from PharMingen (San Diego, CA). The mAb LAM1-101 (30 μg per mouse i.v.), which binds to murine L-selectin but does not inhibit rolling or lead to cell activation (18), was a kind gift of Dr. T. F. Tedder (Duke University, Durham, NC).

Intravital microscopy

Mice were pretreated 2.5 h before surgery with an intrascrotal injection of 0.5 μg murine recombinant TNF-α (Genzyme) in 0.30 ml isotonic saline, then injected with 30 mg/kg sodium pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL), 0.1 mg/kg atropine (Elkins-Sinn, Cherry Hill, NJ), and 100 mg/kg ketamine hydrochloride (Ketalar; Parke-Davis, Detroit, MI) i.p. for anesthesia, and prepared for intravital miscrosopy (13). The cremaster muscle was prepared as described and superfused with thermocontrolled (35°C) bicarbonate-buffered saline (12). Microscopic observations were made using an intravital microscope (Axioskop; Carl Zeiss, Thornwood, NY) with a saline immersion objective (either SW 20/0.55 or SW 40/0.75). Individual leukocytes were chosen randomly and without knowing their eventual outcomes as they exited 5-μm capillaries into postcapillary venules. Rolling leukocytes were tracked down a venular tree using a motorized stage (Märzhäuser, Wetzlar, Germany) while recording through a charge-coupled device (CCD) camera system (model VE-1000CD; Dage-MTI, Michigan City, IN) onto videotape for offline analysis (S-VHS recorder; Panasonic, Osaka, Japan). Distance-time tracings of tracked leukocytes were obtained at ~15 μm intervals and used to calculate instantaneous rolling velocity using a discrete center difference formula. The centerline erythrocyte velocity and venular diameter were measured after each point in the tree where two or more venules converged using a dual photodiode and digital on-line cross-correlation program. Mean blood flow velocities and wall shear rates were determined as described (13). In the venules studied, wall shear rate increased from small postcapillary venules (<10 μm diameter) toward larger draining venules (>60 μm).

Data analysis

For each tracked leukocyte, the dependence of leukocyte rolling velocity on time and wall shear rate was analyzed by a multiple linear regression with no transformation. Distance-time and velocity-time curves were fit to both linear (two-parameter) and exponential (two-parameter) models to determine the best fit. Average decelerations, rolling times, distances, and velocities between experimental groups were compared using an ANOVA followed by a Student-Newman-Keuls multiple comparison procedure. All statistical analyses were performed using NCSS Statistical Software (Kaysville, UT).

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements

Neutrophils were isolated from heparin-anticoagulated human venous blood over a Ficoll-Hypaque gradient and labeled with Fluo-3 (Molecular Probes, Eugene, OR) at a final concentration of 8 μM in PBS (with no calcium or magnesium) supplemented with 0.25% BSA and 0.1% glucose (Sigma, St. Louis, MO) for 30 min at room temperature. For flow cytometric measurement of calcium flux, labeled cells were resuspended at 1×10^6 cells/ml in calcium and magnesium-free PBS (with 0.25% BSA and 0.1% glucose). After measuring baseline fluorescence, chemoattractants were added to the tube of cells at the concentrations indicated and the change in cell fluorescence immediately recorded. Measurements were made on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software, version 3.1 (Becton Dickinson).

For in vivo calcium flux measurements, Fluo-3 labeled human neutrophils (1×10^7 cells/ml) were injected into small catheters (pulled PE10 tubing) placed in the iliac arteries of C57BL/6 wild-type mice. Stroboscopic (30 flashes/s) epifluorescence microscopy showed very dimly fluorescent cells entering the smallest postcapillary venules from capillaries. Stroboscopic illumination renders pixel intensity independent of rolling velocity, because the images of the cells are frozen in time due to the very short duration of each flash ($\ll$1 ms). Venules with fluorescent leukocytes were recorded on videotape using a SIT camera (SIT 66, Dage-MTI) without automtic gain control. Background tissue autofluorescence was negligible (and relatively constant throughout the tissue) at the low gains used here. To avoid major focal plane changes, venules were chosen in which leukocytes rolling along their top wall remained in focus while being tracked. Fluorescence intensity of individual leukocytes was measured repeatedly as the sum of all pixel intensities contained in a box measuring 120×120 pixels using NIH Image software on a Macintosh computer. In addition, distance-time data for each fluorescent cell were recorded.

Human LFA-1 can bind to murine ICAM-1 (19), and human neutrophils bind to murine E-selectin (20). Human neutrophils respond to murine MIP-2 and show chemotaxis (21). They also respond to two other ligands of murine CXCR2, murine KC (22), and murine granulocyte chemotactic protein-2 (GCP-2) (23), which could potentially be involved in activation of rolling leukocytes. Therefore, human neutrophils are likely to show many of the physiological responses relevant to rolling and attachment in the mouse system.

Results

We tracked leukocytes rolling in venules of the cremaster muscle of mice injected intrascrotally 2.5 h before surgery with recombinant murine TNF-α. A typical venular tree with diameter, length, velocity, and shear rate measurements is shown in Fig. 1. Venular trees were chosen randomly in each cremaster, with the single condition that they had adequate flow (flow velocities > 500 μm/s) in all segments from the smallest postcapillary venules (5–12 μm) to large draining venules (>150 μm). Leukocytes to be tracked were chosen without bias to final outcome (because their final outcome was unknown) by randomly picking a leukocyte exiting from a capillary into a postcapillary venule. We found that leukocytes made initial contact with the endothelium when they exited from small capillaries and then rolled along the walls of postcapillary and larger venules until they adhered, detached from the endothelium, rolled out of the cremaster vasculature, or were lost due to obstruction of microscopic view. We tracked a total of 127 rolling leukocytes (67 in wild-type mice under various conditions, 28 in CD18$^{-/-}$ mice, and 32 in E$^{-/-}$ mice) of which 34 were lost due to obstruction of the microscopic view. No tracked leukocyte rolled out of the cremaster vasculature. We based our analysis on all 93 leukocytes that had clear outcomes (adhered or detached).

To understand the molecular requirements of leukocyte arrest under physiological conditions, we tracked leukocytes in wild-type mice and mice with null mutations in the genes encoding the common β_2 chain of CD18 integrins (CD18$^{-/-}$) (16) or E-selectin (E$^{-/-}$) (24). We defined the efficiency of the rolling to adhesion conversion as the percentage of rolling leukocytes that became adherent. As in previous studies (11, 16), the number of circulating neutrophils in CD18$^{-/-}$ mice was highly elevated compared with other genotypes (Table I).

In wild-type mice, 21 of 23 leukocytes (91% efficiency) tracked in TNF-α-treated venules eventually became adherent on the inflamed endothelium (the efficiency could be as low as 74% in the unlikely case that all 7 leukocytes lost during tracking eventually detached). Arrest was dependent on TNF-α-induced inflammation, because none of the 10 leukocytes tracked in untreated control

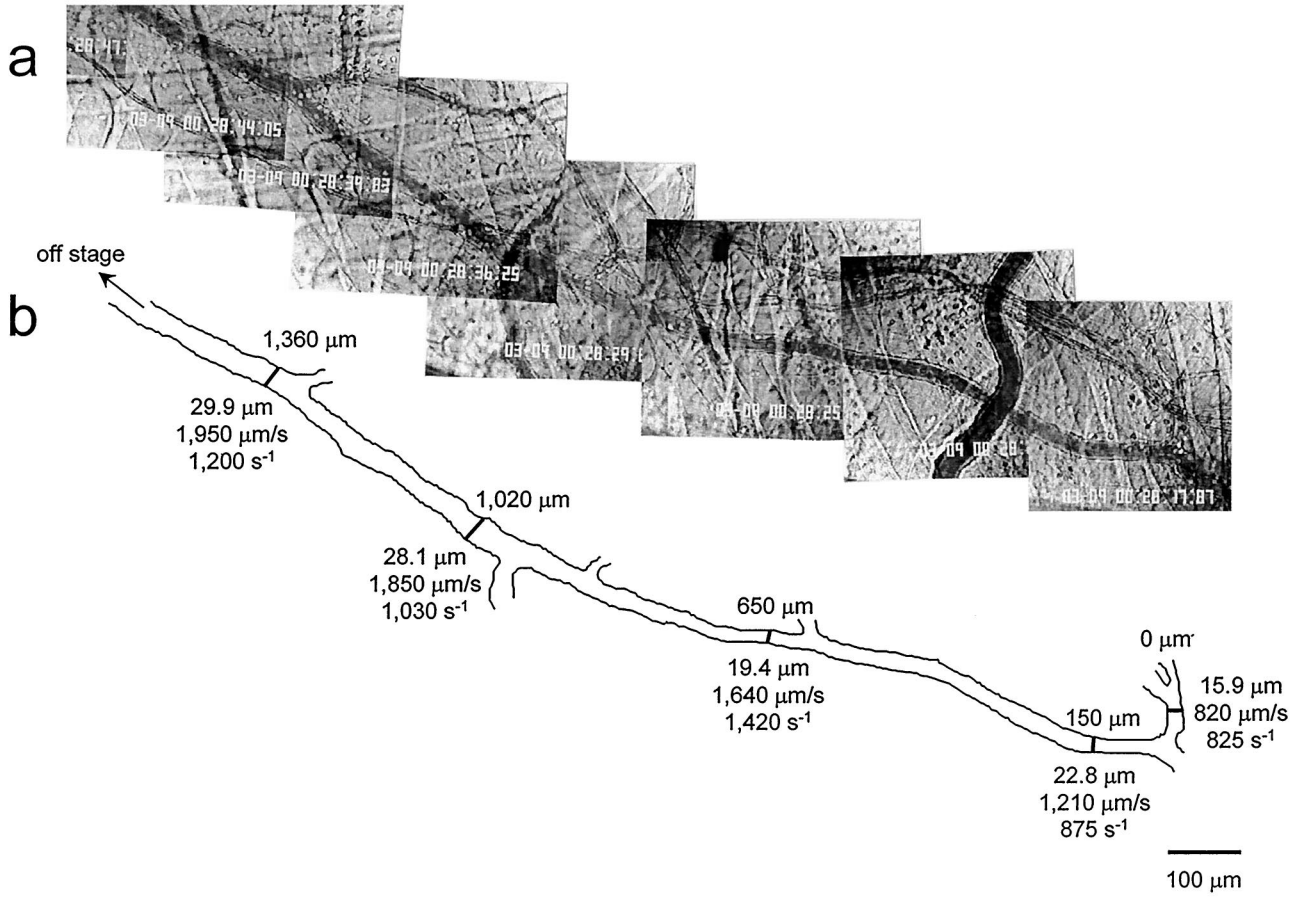

FIGURE 1. Typical postcapillary venular tree in the mouse cremaster muscle. Photomicrograph montage of partial postcapillary venular tree in mouse cremaster muscle (*A*) with the corresponding tracing (*B*) showing important hemodynamic parameters including distance along tree (*top*) and venular diameter, centerline velocity, and calculated wall shear rate (*bottom*) (×5 objective; bar = 100 μm).

mice became adherent. We analyzed the behavior of rolling leukocytes by generating cumulative distance vs time plots for each rolling leukocyte. A leukocyte steadily engaged in rolling would be expected to have a linear distance vs time curve (the slope is the average rolling velocity) and then a sudden decrease to zero velocity when activation and arrest occurred. However, almost all rolling leukocytes in TNF-α-treated wild-type mice had nonlinear distance vs time curves (where the slope of the curve decreases systematically along the curve), suggesting a decrease in average rolling velocity was occurring before arrest (Fig. 2). To assess whether rolling velocity was changing before arrest, we calculated the rolling velocity for every point on the distance vs time curve for all tracked leukocytes and conducted a multiple linear regression of velocity vs time and wall shear rate. This analysis was designed to detect a variation of rolling velocity with time and, as a control for hemodynamics, with wall shear rate. For instance, a negative correlation between rolling velocity and time reflects deceleration, a positive correlation reflects acceleration, and no correlation means that rolling velocity is not changing with time. A negative correlation between rolling velocity and wall shear rate demonstrates that leukocytes are decelerating even as wall shear rate increases, a positive correlation suggest that leukocyte are accelerating as wall shear rate increases, and no correlation means that rolling velocity is not affected by wall shear rate.

A majority (87%) of leukocytes rolling and becoming adherent in TNF-α-treated wild-type mice showed a significant negative correlation between rolling velocity and rolling time with an average deceleration of 0.28 ± 0.13 μm/s^2 (Table II). This means that an average rolling leukocyte slowed down by about 0.3 μm/s each second it was rolling. The starting velocity of these leukocytes was 9.0 ± 1.1 μm/s, and the average velocity over the full distance tracked was 3.9 ± 2.5 μm/s. The rolling velocities in wild-type mice did not correlate with wall shear rate. Leukocytes in wild-type mice rolled for 270 ± 58 μm (~30 cell diameters)

Table I. *Systemic leukocyte counts and differentials after TNF-α treatment*[a]

Genotype	Mice	Systemic count (per μl)	Leukocyte Differential	
			% Neutrophil	% Mononuclear
WT	4	5,330 ± 1,330	48 ± 8	52 ± 8
CD18$^{-/-}$	2	20,400 ± 4,300[b]	81 ± 6[b]	19 ± 6[b]
E$^{-/-}$	5	3,570 ± 500	61 ± 5	39 ± 5

[a] Data are expressed as mean ± SEM.
[b] Significantly different than values in other genotypes ($p < 0.05$).

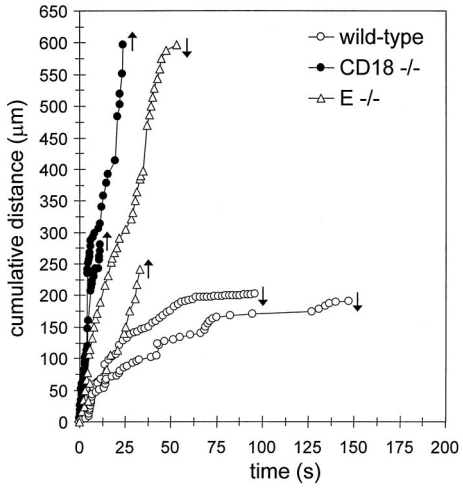

FIGURE 2. Distance-time curves for typical rolling leukocytes. Typical distance-time tracings for leukocytes from wild-type (○), $CD18^{-/-}$ (●), and $E^{-/-}$ (△) mice. Outcome indicated by arrows: ↓ adhere, ↑ detach. Tracings are from two representative leukocytes from each genotype.

before arrest and firm adhesion (Table III). In contrast, the two leukocytes in wild-type mice which detached from the endothelium correlated positively with time and shear rate. Although surprisingly few wild-type leukocytes detached during our observation period (too few for relevant analysis), the few that did detach from the endothelium behaved similarly to leukocytes from $CD18^{-/-}$ mice and wild-type mice treated with an anti-CD18 mAb (see below) in terms of rolling velocity correlation with time and shear rate.

To compare several leukocytes in the same graph, rolling distance, rolling time, and rolling velocity were each normalized to the range 0–1 by dividing the instantaneous value by the total time and distance for each leukocyte, respectively (Fig. 3). Interestingly, even though individual leukocytes rolled for varying distances and times (Fig. 2), normalization allowed the superposition of the various curves, highlighting the similarity of their behaviors. Leukocytes becoming adherent exhibited a conspicuous convex shape of the distance over time plot (Fig. 3*A*), reflecting the systematic decrease of rolling velocity (Fig. 3*B*). In contrast, two leukocytes that detached from the endothelium showed no such behavior (Fig. 3, *C* and *D*). Further statistical analysis of the full data set showed that the distance-time plot for most leukocytes eventually becoming adherent was best fit by an exponential model, and the data for detaching leukocytes was best fit by a linear model (data not shown). Deceleration before adhesion does not seem to be strictly necessary, because ~10% of leukocytes in wild-type mice arrested abruptly. The fact that most of the distance-time curves (and concomitantly, the velocity-time curves) were fit best by exponential curves suggests a rolling behavior characterized by continual deceleration of rolling leukocytes before arrest and firm adhesion.

The systematic decrease in rolling velocity of leukocytes that eventually became adherent suggested that progressive activation of rolling leukocytes may be causing the reduction of rolling velocity. To directly demonstrate activation of rolling neutrophils, we injected human neutrophils preincubated with Fluo-3, an indicator of $[Ca^{2+}]_i$ (25), through a local catheter placed in the femoral artery (26). We found that human neutrophils injected in such a manner rolled on the endothelium at a similar average velocity (~4 μm/s) as endogenous mouse leukocytes, suggesting the same or similar adhesion molecules mediating the interactions. Using flow cytometry, we found that human neutrophils labeled with Fluo-3 respond to human IL-8 and leukotriene B4 (LTB_4) with an increase in $[Ca^{2+}]_i$ as well as exhibiting homologous desensitization upon restimulation (data not shown). In addition, human neutrophils respond to even low doses (10 nM) of murine MIP-2 (Fig. 4), which is known to be present in TNF-α-stimulated inflammatory sites (27) and plays a major role in neutrophil recruitment in response to TNF-α (28). Based on these findings and previously published reports (19–23), the human neutrophils appear to be a good model for the behavior of mouse neutrophils in this system.

Fluo-3 is an intracellular fluorescent dye whose brightness is directly related to $[Ca^{2+}]_i$ (25). Using a high sensitivity SIT camera with the autogain turned off, we were able to see, for the first time, changes in intercellular calcium during leukocyte rolling. Fig. 5*a* shows a typical leukocyte that was very weakly fluorescent upon entering a postcapillary venule, became brighter as it rolled down the venule, and showed an additional burst of brightness when it finally became firmly adherent. Rolling leukocytes in vivo showed increases in maximal cell intensity of 4.1 ± 1.2-fold. Human neutrophils labeled with calcein acetoxymethyl ester (AM), a non calcium-sensitive dye, did not exhibit changes in intensity while rolling along the endothelium (data not shown). Fig. 5*b* shows an example of increasing $[Ca^{2+}]_i$ and decreasing rolling velocity for a single neutrophil. Although not all neutrophils showed such a clear and systematic increase of $[Ca^{2+}]_i$, the pooled data indicate a significant correlation between cell intensity and

Table II. *Multiple regression analysis of rolling leukocytes by genotype and outcome*[a]

Genotype	Outcome	Leukocytes		Major Dependence of Rolling Velocity on		Deceleration (μm/s^2)
		n	%	Time	Shear	
WT	Adhere	21	91	Neg	∅	−0.28 ± 0.13[b]
WT	Detach	2	9	Pos	∅	None
$CD18^{-/-}$	Adhere	0	0	—	—	—
$CD18^{-/-}$	Detach	24	100	∅	Pos	NS
$E^{-/-}$	Adhere	14	54	∅	∅	NS
$E^{-/-}$	Detach	12	46	∅	∅	NS

[a] Deceleration data shown as mean ± SEM of data pooled by outcome. Number of leukocytes in each group, *n*, and percent with each outcome, % are shown. Number of mice analyzed: wild type (WT) (5), $CD18^{-/-}$ (2), and $E^{-/-}$ (5). Neg, negative correlation; Pos, positive correlation; ∅, no correlation; NS, not significant. Best correlation for dependence of rolling velocity on time and wall shear rate based on data in Fig. 3.

[b] Significantly different from Detach in same genotype ($p < 0.05$).

Table III. *Average time rolled, distance rolled, and average rolling velocity*[a]

Genotype	Outcome	Time Rolled (s)	Distance Rolled (μm)	Average Velocity (μm/s)
WT	Adhere	86 ± 18	270 ± 58	3.8 ± 0.4
WT	Detach	140 ± 29	610 ± 200	5.0 ± 2.5
$CD18^{-/-}$	Adhere	—	—	—
$CD18^{-/-}$	Detach	12 ± 2[b]	270 ± 26	32 ± 7[c]
$E^{-/-}$	Adhere	40 ± 14	280 ± 49	14 ± 2[c]
$E^{-/-}$	Detach	41 ± 13	350 ± 110	11 ± 2[c]

[a] Data shown as mean ± SEM.
[b] Significantly lower than all other times rolled at $p < 0.01$.
[c] Significantly higher than wild type (WT):Adhere at $p < 0.05$.

rolling velocity (Fig. 5*c*). All neutrophils analyzed showed a negative correlation between their fluorescence intensity and their rolling velocity. The average slope was -0.3 ± 0.1 $(\mu m/s)^{-1}$, indicating that an average neutrophil would approximately double its fluorescence intensity from baseline for a decrease of rolling velocity by 3 μm/s. Plotting normalized distance as a function of time (Fig. 5*d*) reveals that the Fluo-3-loaded neutrophils behave very similarly to the unlabeled, endogenous neutrophils (Fig. 3), showing a systematic decrease of rolling velocity before neutrophil arrest.

We next explored the role of β_2 integrins in the arrest process by using $CD18^{-/-}$ mice (16). Of 24 rolling cells with clear outcomes, 24 detached, and none became firmly adherent (an efficiency of <5%). This finding shows that CD18 integrins are crucial in the rolling to firm adhesion process. Rolling neutrophils in $CD18^{-/-}$ mice showed a linear distance-time tracing (Fig. 2) with 70% of rolling leukocytes not decelerating and in fact increasing their rolling velocity with wall shear rate (Table II). The average rolling velocity was much higher in $CD18^{-/-}$ mice (32.0 ± 6.8 μm/s) than in wild-type mice (3.9 ± 2.5 μm/s) (Table III). This faster rolling led to a dramatically reduced transit time of only 12 ± 2 s in $CD18^{-/-}$ mice, compared with an average of 87 ± 22 s in wild-type mice (Table III). The total distance over which cells were tracked in $CD18^{-/-}$ mice (before detachment) was not different from wild-type mice (Table III). These findings provide a mechanistic explanation for data reported in vitro (29, 30) and in vivo (11, 31), showing that blockade of CD18 integrins can increase the rolling velocity of leukocytes. We confirmed this finding by blocking CD18 integrin function in wild-type mice with mAb GAME-46. Five of five tracked leukocytes came to arrest before GAME-46 injection, but only 3 of 20 arrested after GAME-46 injection (~15% efficiency). The velocity of rolling leukocytes also increased almost 4-fold after injection of GAME-46, consistent with the velocity increase seen in $CD18^{-/-}$ mice. Rolling leukocytes after GAME-46 treatment exhibited linear distance-time curves similar to those seen in $CD18^{-/-}$ mice, again demonstrating the role of CD18 integrins in rolling leukocyte deceleration and arrest. As a negative control, we injected a nonblocking

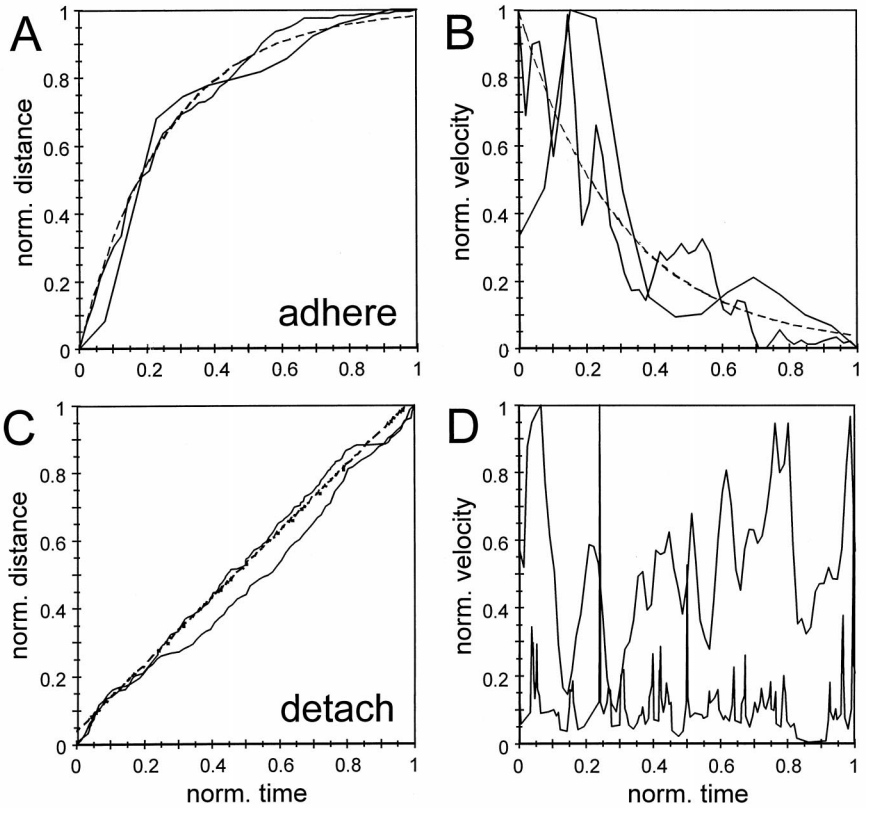

FIGURE 3. Wild-type leukocytes becoming adherent exhibit a gradual reduction in rolling velocity. Nondimensional distance-time tracings of two leukocytes, which eventually became adherent (*A*), showed nonlinear distance-time curves that were fit well with an exponential curve (dashed line). The corresponding velocity-time curves (*B*) demonstrated a definite decrease of velocity with time rolled, which was best fit with an exponential curve (dashed line). In contrast, leukocytes which broke off the endothelium or were lost (*C*) generally exhibited a linear distance-time curve (dashed line). For these leukocytes, the corresponding velocity-time curves (*D*) showed no decrease in velocity over time. Representative leukocytes are shown.

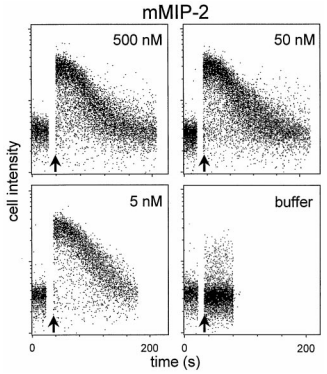

FIGURE 4. Human neutrophils respond to murine MIP-2. Human neutrophils loaded with Fluo-3 respond to several concentrations of murine MIP-2. Data are presented as FACS plots showing cell intensity changes with time. Arrows indicate time of chemoattractant or buffer addition.

L-selectin mAb (LAM1-101) (18) into wild-type mice. This Ab did not influence adhesion (5 of 5 tracked leukocytes with clear outcomes became adherent).

Finally, we investigated whether slow rolling mediated by engagement of E-selectin (10, 13, 32) was required for arrest of rolling neutrophils on inflamed endothelium. In $E^{-/-}$ mice, 14 of 26 rolling leukocytes (54% efficiency) eventually became adherent, whereas 12 cells detached. The distance traveled by rolling leukocytes in $E^{-/-}$ mice was similar to that in wild-type mice, but the average rolling velocity was significantly elevated to 14 ± 2 μm/s (Table III), consistent with previously published results (13). The endothelial contact time of rolling leukocytes was reduced to 40 ± 14 s, compared with 87 ± 22 s in wild-type mice (Table III). Interestingly, most leukocytes rolling in $E^{-/-}$ mice that eventually adhered did not exhibit a clear decrease in rolling velocity before arrest (Fig. 2), but a rather abrupt arrest after rolling a similar distance as wild-type leukocytes (Table II).

Discussion

In this paper we have identified the major adhesion molecule requirements necessary for the arrest of rolling leukocytes in vivo. We find that, on average, rolling leukocytes require 86 s of endothelial contact time before arrest and firm adhesion occurs. During this time, leukocyte rolling is stabilized by β_2 integrins and E-selectin. $[Ca^{2+}]_i$ levels increase systematically in rolling leukocytes and then show a further dramatic increase upon arrest.

The data presented in this paper provide direct evidence for and a mechanistic explanation of previous data demonstrating a correlation between rolling leukocyte transit time and the amount of firm adhesion (11). When leukocytes are rolling slowly on TNF-α-activated endothelium, CD18 integrins can participate in stabilizing leukocyte rolling, and thus prevent detachment of rolling leukocytes before they become activated enough to arrest. In fact, it is exactly because the activation process appears to be protracted that a relatively long endothelial contact time is necessary for efficient arrest and normal levels of firm adhesion.

We also present the first data demonstrating changes in intercellular calcium in rolling leukocytes. The gradual rise of $[Ca^{2+}]_i$ in rolling leukocytes may depend on chemoattractant stimulation, adhesion molecule cross-linking (for example, L-selectin), outside-in signaling through integrin binding, or any combination of these activation signals. Activation of rolling leukocytes appears to occur during the ~86 s that the leukocyte is rolling slowly along

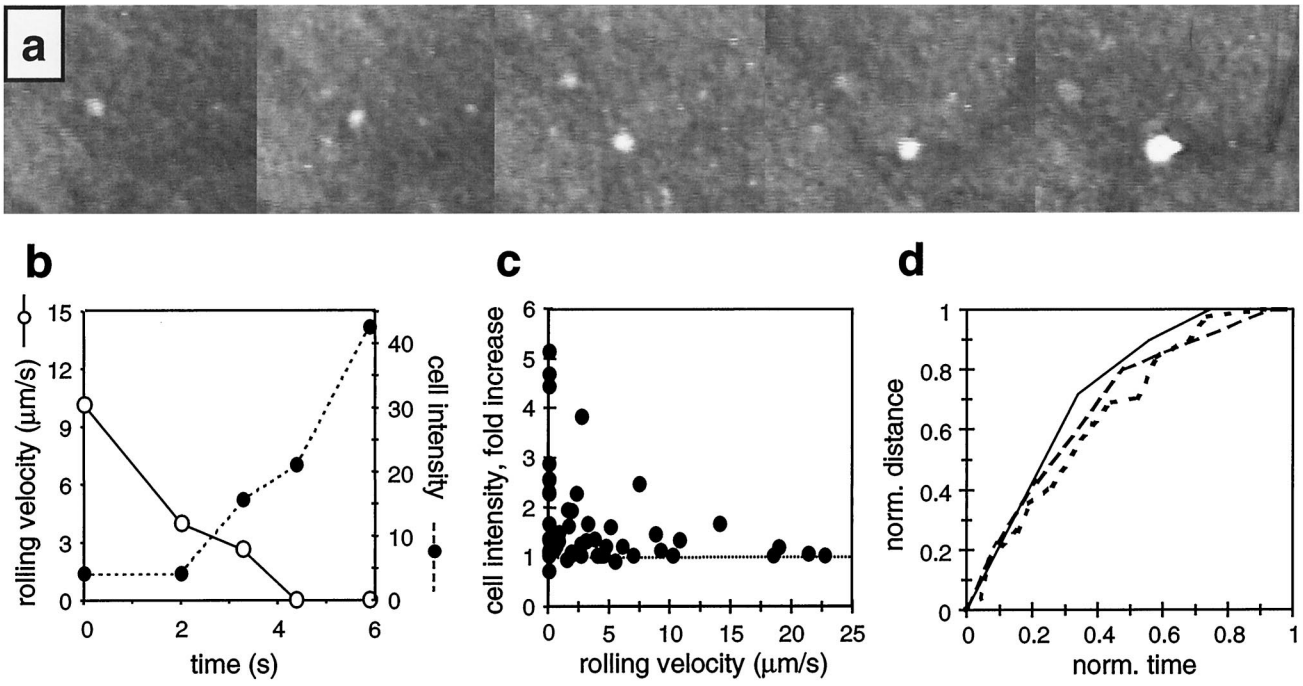

FIGURE 5. Activation of neutrophils while rolling. Fluo-3-labeled neutrophils injected into the femoral artery of mice and tracked through venules of the cremaster muscle were recorded by video microscopy to measure $[Ca^{2+}]_i$. *a*, An initially dimly fluorescent neutrophil showed increased fluorescence intensity as it rolled (first four panels), while maximum intensity was reached after firm adhesion (last panel). *b*, Rolling velocity (μm/s, ○) and cell intensity (arbitrary units, ●) for this neutrophil. *c*, Neutrophil intensity was expressed as fold increase over intensity recorded when first entering venule (=1) for all neutrophils measured. All cells showed an inverse relationship between rolling velocity and cell intensity, but to a varying degree. *d*, Nondimensional distance-time tracings for three representative Fluo-3 loaded neutrophils showed convex shape, indicating reduced rolling velocity before arrest (similar to unlabeled cells, Fig. 3*A*).

the endothelium, as opposed to during capillary transit. Any additional stimulation, for example by exogenous chemoattractant, would accelerate the cellular activation process and lead to rapid arrest and firm adhesion (10). Although more detailed studies of signaling events during leukocyte rolling will be necessary, our observation of a gradual increase of the $[Ca^{2+}]_i$ in rolling cells followed by a rapid rise upon arrest suggests that activation of rolling leukocytes is only partial. More complete activation under physiological conditions may require, or result from, firm adhesion, as suggested by the pronounced increase of $[Ca^{2+}]_i$ upon attachment.

Our observation of reduced efficiency of attachment and abrupt leukocyte arrest without a decrease in rolling velocity in $E^{-/-}$ mice is consistent with CD18 integrin involvement in leukocyte rolling and arrest. CD18 integrins have been shown to be unable to mediate leukocyte rolling independent of selectins (29), presumably because of the inability of CD18 integrins to bind rapidly to their endothelial ligands. However, it has been shown that the I domain of LFA-1, a β_2 integrin, can interact transiently with ICAM-1 and produce rolling interactions (33). This type of interaction may underlie the present observations. Leukocytes rolling on E-selectin appear to be moving slowly enough such that some CD18 integrins can bind to their ligands. In fact, the lack of cellular deceleration observed in $E^{-/-}$ mice suggests that the CD18 integrins are less efficient at decreasing rolling velocity in the absence of E-selectin, leading to a 3-fold elevation in rolling velocity in $E^{-/-}$ mice. When rolling leukocytes in $E^{-/-}$ become sufficiently activated (after rolling approximately the same distance as in wild-type mice), some of these leukocytes can arrest abruptly. This arresting mechanism is less efficient than the gradual deceleration process produced by CD18 integrin stabilization of the rolling process. These data are consistent with the moderate leukocyte recruitment defects seen in $E^{-/-}$ mice (10, 13, 32, 34).

Our data at first seems surprisingly at odds with previously published results showing rapid leukocyte arrest in response to chemoattractant stimulation (5, 6). One difference between these in vitro systems and the current in vivo observations may be the level of chemoattractant available to rolling leukocytes. In vivo, micropipette applications of IL-8 (8) or MIP-2 (9) can also cause rapid arrest of rolling leukocytes. However, this mode of leukocyte arrest seems to be rare in cytokine-induced inflammation in wild-type mice. TNF-α stimulation alone may not result in sufficiently high levels of chemoattractant expression to produce rapid leukocyte arrest. In a previous study, 10 ng of TNF-α injected into an air pocket of a mouse caused a 3-fold increases in MIP-2 or KC mRNA expression and produced 5–10 ng of secreted chemokine (27). In our model, chemokine concentration may vary along the venular tree, but it is currently not possible to measure the level of chemoattractant present on the surface of inflamed venules in vivo. Continuous superfusion of the tissue may also wash away some chemoattractant. However, we commonly find that leukocyte adhesion increases during the experiment, even with continual superfusion. If there were high levels of chemoattractant washout, adhesion would be expected to decrease, or at least remain constant as chemoattractant levels were reduced.

Our data demonstrate that activation of rolling leukocytes by chemoattractants (including chemokines) can be gradual, and not necessarily an all-or-none response as proposed using various in vitro models (5–7). The gradual activation response may be due to low local chemoattractant concentrations on the vascular endothelium in combination with adhesion molecule-mediated activation. One interesting implication of gradual activation is that a leukocyte, such as a neutrophil, with distinct receptors for multiple chemoattractants, could potentially integrate activation signals from several different chemoattractants (e.g., platelet-activating factor (PAF), MIP-2, KC) while rolling along the endothelium, thus reinforcing cellular activation through several different receptor signaling pathways.

In summary, the surprisingly long endothelial contact time needed for gradual activation of leukocytes rolling in inflamed venules in vivo requires a revision of the concept that neutrophil activation is always rapid and follows rolling (2, 3). Rather, for neutrophils, rolling and activation appear to be intimately intertwined to produce neutrophil recruitment into inflamed tissues. More detailed cell biological analysis of the interplay between chemoattractants, rolling receptors, and integrins in reconstituted systems will be needed to fully understand this unusual pattern of neutrophil activation observed during physiological inflammation.

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References

1. Ley, K. 1997. The selectins as rolling receptors. In *The Selectins: Initiators of Leukocyte Endothelial Adhesion*. D. Vestweber, ed. Harwood Academic Publishers, Amsterdam, p. 63.
2. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell 67:1033.*
3. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell 76:301.*
4. Hughes, P. E., and M. Pfaff. 1998. Integrin affinity modulation. *Trends Cell Biol. 8:359.*
5. Rainger, G. E., A. C. Fisher, and G. B. Nash. 1997. Endothelial-borne platelet-activating factor and interleukin-8 rapidly immobilize rolling neutrophils. *Am. J. Physiol. 272:H114.*
6. Campbell, J. J., J. Hedrick, A. Zlotnik, M. A. Siani, D. A. Thompson, and E. C. Butcher. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science 279:381.*
7. Gerszten, R. E., E. A. Garcia-Zepeda, Y. C. Lim, M. Yoshida, H. A. Ding, M. A. Gimbrone, A. D. Luster, F. W. Luscinskas, and A. Rosenzweig. 1999. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature 398:718.*
8. Ley, K., J. B. Baker, M. I. Cybulsky, M. A. Gimbrone, Jr., and F. W. Luscinskas. 1993. Intravenous interleukin-8 inhibits granulocyte emigration from rabbit mesenteric venules without altering L-selectin expression or leukocyte rolling. *J. Immunol. 151:6347.*
9. Morgan, S. J., M. W. Moore, G. Cacalano, and K. Ley. 1997. Reduced leukocyte adhesion response and absence of slow leukocyte rolling in interleukin-8 (IL-8) receptor deficient mice. *Microvasc. Res. 54:188.*
10. Ley, K., M. Allietta, D. C. Bullard, and S. J. Morgan. 1998. The importance of E-selectin for firm leukocyte adhesion in vivo. *Circ. Res. 83:287.*
11. Jung, U., K. E. Norman, C. L. Ramos, K. Scharffetter-Kochanek, A. L. Beaudet, and K. Ley. 1998. Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. *J. Clin. Invest. 102:1526.*
12. Ley, K., D. C. Bullard, M. L. Arbones, R. Bosse, D. Vestweber, T. F. Tedder, and A. L. Beaudet. 1995. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J. Exp. Med. 181:669.*
13. Kunkel, E. J., and K. Ley. 1996. Distinct phenotype of E-selectin deficient mice: E-selectin is required for slow leukocyte rolling in vivo. *Circ. Res. 79:1196.*
14. Jung, U., and K. Ley. 1997. Regulation of E-selectin, P-selectin and ICAM-1 expression in mouse cremaster muscle vasculature. *Microcirculation 4:311.*
15. Jung, U., and K. Ley. 1999. Mice lacking two or all three selectins demonstrate overlapping and distinct functions of each selectin. *J. Immunol. 162:6755.*
16. Scharffetter-Kochanek, K., H. Lu, K. E. Norman, N. van Nood, F. Munoz, S. Grabbe, M. McArthur, I. Lorenzo, S. Kaplan, K. Ley, et al. 1998. Spontaneous skin ulceration and defective T cell function in CD18 null mice. *J. Exp. Med. 188:119.*
17. Driessens, M. H. E., P. Vanhulten, A. Zuurbier, G. Lariviere, and E. Roos. 1996. Inhibition and stimulation of LFA-1 and Mac-1 functions by antibodies against murine CD18: evidence that the LFA-1 binding sites for ICAM-1, -2, and -3 are distinct. *J. Leukocyte Biol. 60:758.*
18. Steeber, D. A., P. Engel, A. S. Miller, M. P. Sheetz, and T. F. Tedder. 1997. Ligation of L-selectin through conserved regions within the lectin domain activates signal transduction pathways and integrin function in human, mouse, and rat leukocytes. *J. Immunol. 159:952.*
19. Johnston, S. C., M. L. Dustin, M. L. Hibbs, and T. A. Springer. 1990. On the species specificity of the interaction of LFA-1 with intercellular adhesion molecules. *J. Immunol. 145:1181.*

20. Norton, C. R., J. M. Rumberger, D. K. Burns, and B. A. Wolitzky. 1993. Characterization of murine E-selectin expression in vitro using novel anti-mouse E-selectin monoclonal antibodies. *Biochem. Biophys. Res. Commun. 195:250.*
21. Jerva, L. F., G. Sullivan, and E. Lolis. 1997. Functional and receptor binding characterization of recombinant murine macrophage inflammatory protein 2: sequence analysis and mutagenesis identify receptor binding epitopes. *Protein Sci. 6:1643.*
22. Bozic, C. R., L. F. Kolakowski, Jr., N. P. Gerard, C. Garcia-Rodriguez, C. von Uexkull-Guldenband, M. J. Conklyn, R. Breslow, H. J. Showell, and C. Gerard. 1995. Expression and biologic characterization of the murine chemokine KC. *J. Immunol. 154:6048.*
23. Wuyts, A., A. Haelens, P. Proost, J. P. Lenaerts, R. Conings, G. Opdenakker, and J. van Damme. 1996. Identification of mouse granulocyte chemotactic protein-2 from fibroblasts and epithelial cells: functional comparison with natural KC and macrophage inflammatory protein-2. *J. Immunol. 157:1736.*
24. Bullard, D. C., E. J. Kunkel, H. Kubo, M. J. Hicks, I. Lorenzo, N. A. Doyle, C. M. Doerschuk, K. Ley, and A. L. Beaudet. 1996. Infectious susceptibility and severe deficiency of leukocyte rolling and recruitment in E-selectin and P-selectin double mutant mice. *J. Exp. Med. 183:2329.*
25. Vandenberghe, P. A., and J. L. Ceuppens. 1990. Flow cytometric measurement of cytoplasmic free calcium in human peripheral blood T lymphocytes with fluo-3, a new fluorescent calcium indicator. *J. Immunol. Methods 127:197.*
26. Hafezi-Moghadam, A., and K. Ley. 1999. Relevance of L-selectin shedding for leukocyte rolling in vivo. *J. Exp. Med. 189:939.*
27. Tessier, P. A., P. H. Naccache, I. Clark-Lewis, R. P. Gladue, K. S. Neote, and S. R. McColl. 1997. Chemokine networks in vivo: involvement of CXC and CC chemokines in neutrophil extravasation in vivo in response to TNF-α. *J. Immunol. 159:3595.*
28. McColl, S. R., and I. Clark-Lewis. 1999. Inhibition of murine neutrophil recruitment in vivo by CXC chemokine receptor antagonists. *J. Immunol. 163:2829.*
29. Lawrence, M. B., C. W. Smith, S. G. Eskin, and L. V. McIntire. 1990. Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. *Blood 75:227.*
30. Abbassi, O., T. K. Kishimoto, L. V. McIntire, and C. W. Smith. 1993. Neutrophil adhesion to endothelial cells. *Blood Cells 19:245.*
31. Perry, M. A., and D. N. Granger. 1991. Role of CD11/CD18 in shear rate-dependent leukocyte-endothelial cell interactions in cat mesenteric venules. *J. Clin. Invest. 87:1798.*
32. Milstone, D. S., D. Fukumura, R. C. Padgett, P. E. O'Donnell, V. M. Davis, O. J. Benavidez, W. L. Monsky, R. J. Melder, R. K. Jain, and M. A. Gimbrone. 1998. Mice lacking E-selectin show normal numbers of rolling leukocytes but reduced leukocyte stable arrest on cytokine-activated microvascular endothelium. *Microcirculation 5:153.*
33. Knorr, R., and M. L. Dustin. 1997. The lymphocyte function-associated antigen 1 I domain is a transient binding module for intercellular adhesion molecule (ICAM)-1 and ICAM-3 in hydrodynamic flow. *J. Exp. Med. 186:719.*
34. Munoz, F. M., E. P. Hawkins, D. C. Bullard, A. L. Beaudet, and S. L. Kaplan. 1997. Host defense against systemic infection with streptococcus pneumoniae is impaired in E-, P-, and E-/P-selectin-deficient mice. *J. Clin. Invest. 100:2099.*