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Leukocyte Arrest During Cytokine-Dependent Inflammation In Vivo

Eric J. Kunkel, Jessica L. Dunne, and Klaus Ley

Leukocyte rolling along the walls of inflamed venules precedes their adhesion during inflammation. Rolling leukocytes are thought to arrest by engaging \( \beta_2 \) 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 \( \mu \)m) before becoming adherent with an efficiency around 90%. These rolling leukocytes exhibit a gradual \( \beta_2 \) 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 \( \beta_2 \) integrins are absent or blocked by a mAb. Arrest is \( \sim 50\% \) less efficient in the absence of E-selectin. These data suggest a model of leukocyte recruitment in which \( \beta_2 \) 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, etoxacin) 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 momentarily before arrest and firm adhesion. In a reconstituted system, Campbell et al. (6) showed rapid arrest of monocytes rolling on a substrate containing peripheral node addressin (a ligand for L-selectin) and ICAM-1 (a \( \beta_2 \) 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) (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 \( \sim 30 \) s to pass a 100-\( \mu \)m 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-\( \alpha \)-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 \( \beta_2 \) integrins, and finally arrest and firm adhesion. In this paper we present evidence that under the conditions of in situ TNF-\( \alpha \) stimulation, leukocyte adhesion occurs not
as rapid arrest in response to chemotactants, but a gradual de-
celeration process requiring increased β2 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 fa-
cilities. CD18−/− and E−/− mice were back-crossed into a C57BL/6 back-
ground and were gifts of Drs. 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 pur-
chased from Hilltop Lab Animals (Scottsdale, 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 β2 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 acti-
vation (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 intracranial 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 Lab-
oratories, Abbott Park, IL), 0.1 mg/kg atropine (Elkins-Sinn, Cherry Hill, 
NJ), and 100 mg/kg ketamine hydrochloride (Ketalar; Parke-Davis, De-
troit, MI) i.p. for anesthesia, and prepared for intravital microscopy (13).
The cremaster muscle was prepared as described and superfused with ther-
ocontrolled (35°C) bicarbonate-buffered saline (12). Microscopic obser-
vations were made using an intravital microscope (Axioskop; Carl Zei-
magger, 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 post-
capillary venules. Rolling leukocytes were tracked down a venular tree 
using after each point in the tree where two or more venules converged 
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 post-
capillary venules. Rolling leukocytes were tracked down a venular tree

Intracellular Ca2+ concentration ([Ca2+]i) measurements

Neutrophils were isolated from hepatic-anticoagulated human venous 
blood over a Ficoll-Hypaque gradient and labeled with fluo-3- 
(Binding Site, San Diego, CA) for 30 min at room temperature. For flow cyto-
meter and calcium flux measurements, labeled cells were resuspended in 
0.1% glucose). After measuring baseline fluorescence, chemotactants 
were added to the tube of cells at the concentrations indicated and the 

Results

We tracked leukocytes rolling in venules of the cremaster muscle of 
mice injected intrascrotally 2.5 h before surgery with recombin-
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 
exitating from a capillary into a postcapillary venule. We found that 
leukocytes made initial contact with the endothelium when they 
exitied 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 con-
ditions, 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 
β2 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 
E−/− 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 
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To understand the molecular requirements of leukocyte arrest under 
physiological conditions, we tracked leukocytes in wild-type 
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β2 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 in-
flamed 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
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/s² (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 mm (~30 cell diameters).

Table I. Systemic leukocyte counts and differentials after TNF-α treatment

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mice</th>
<th>Systemic count (per μl)</th>
<th>% Neutrophil</th>
<th>% Mononuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4</td>
<td>5,330 ± 1,330</td>
<td>48 ± 8</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>CD18⁻/⁻</td>
<td>2</td>
<td>20,400 ± 4,300</td>
<td>81 ± 6</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>E⁻/⁻</td>
<td>5</td>
<td>3,570 ± 500</td>
<td>61 ± 5</td>
<td>39 ± 5</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SEM.

*Significantly different than values in other genotypes (p < 0.05).
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. 3A), reflecting the systematic decrease of rolling velocity (Fig. 3B). 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 [Ca2+]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 (LTB4) with an increase in [Ca2+]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 [Ca2+]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. 5a 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 calcium acetoxymethyl ester (AM), a non calcium-sensitive dye, did not exhibit changes in intensity while rolling along the endothelium (data not shown). Fig. 5b shows an example of increasing [Ca2+]i, and decreasing rolling velocity for a single neutrophil. Although not all neutrophils showed such a clear and systematic increase of [Ca2+]i, the pooled data indicate a significant correlation between cell intensity and

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Outcome</th>
<th>Leukocytes</th>
<th>Major Dependence of Rolling Velocity on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Shear</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Deceleration (μm/s²)</td>
</tr>
<tr>
<td>WT</td>
<td>Adhere</td>
<td>21</td>
<td>91</td>
</tr>
<tr>
<td>WT</td>
<td>Detach</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>CD18−/−</td>
<td>Adhere</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD18−/−</td>
<td>Detach</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>E−/−</td>
<td>Adhere</td>
<td>14</td>
<td>54</td>
</tr>
<tr>
<td>E−/−</td>
<td>Detach</td>
<td>12</td>
<td>46</td>
</tr>
</tbody>
</table>

*a Deacceleration 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).
rolling velocity (Fig. 5c). All neutrophils analyzed showed a negative correlation between their fluorescence intensity and their rolling velocity. The average slope was $-0.36 \pm 0.1 (\text{mm/s})^2$, indicating that an average neutrophil would approximately double its fluorescence intensity from baseline for a decrease of rolling velocity by $3 \text{ mm/s}$. Plotting normalized distance as a function of time (Fig. 5d) 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 $\beta_2$ 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 $\approx 5\%$). 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 ($32.0 \pm 6.8 \text{ mm/s}$) than in wild-type mice ($3.9 \pm 2.5 \text{ mm/s}$) (Table III). This faster rolling led to a dramatically reduced transit time of only $12 \pm 2 \text{ s}$ in CD18$^{-/-}$ mice, compared with an average of $87 \pm 22 \text{ s}$ 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](http://www.jimmunol.org/)

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

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

$^a$ 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 $p < 0.05$.  

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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 E2/2 mice, 14 of 26 rolling leukocytes (54% efficiency) eventually became adherent, whereas 12 cells detached. The distance traveled by rolling leukocytes in E2/2 mice was similar to that in wild-type mice, but the average rolling velocity was significantly elevated to 14 ± 6 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 E2/2 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 β2 integrins and E-selectin. [Ca2+]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 [Ca2+]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...
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\textsuperscript{2+}] 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\textsuperscript{2+}], upon attachment.

Our observation of reduced efficiency of attachment and abrupt leukocyte arrest without a decrease in rolling velocity in E\textsuperscript{-/-} 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\textsubscript{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\textsuperscript{-/-} 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\textsuperscript{-/-} mice. When rolling leukocytes in E\textsuperscript{-/-} 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\textsuperscript{-/-} 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.

Acknowledgments

We thank Dr. A. L. Beaudet (Baylor College of Medicine, Houston, TX) and Dr. D. C. Bullard (University of Alabama, Birmingham) for the CD18\textsuperscript{-/-} and E\textsuperscript{-/-} mice, respectively; Dr. T. F. Tedder (Duke University) for the LAM1-101 Ab; and Nick Douris and Jennifer Bryant for animal husbandry.

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