Role of Primary and Secondary Capture for Leukocyte Accumulation In Vivo

Eric J. Kunkel, James E. Chomas, Klaus Ley

Abstract—Leukocyte accumulation during inflammation depends on the concerted action of selectin and integrin adhesion molecules, which promote capture, rolling, and arrest of these cells on activated endothelium. In addition to interacting with endothelial cells, leukocytes can also adhere to already adherent leukocytes through an L-selectin–dependent mechanism. Initiation of adhesion through this mechanism has been called nucleation and leads to characteristic geometric patterns (ie, clusters and strings) of adherent leukocytes in flow chambers. We have used intravital microscopy of tumor necrosis factor-α (TNF-α)–treated mouse cremaster muscles to quantitatively investigate the potential role of leukocyte-leukocyte adhesion in initiating and maintaining the leukocyte clusters that are commonly observed in inflamed venules. Our data show that in TNF-α-treated venules with diameters between 23 and 108 μm, leukocyte adhesion occurs in clusters that are 19 to 50 μm long and 8 to 44 μm wide. They are almost entirely made up of slow-rolling leukocytes. Of all leukocytes recruited into a cluster (100%), the majority enter the cluster rolling along the endothelium and sharply reduce their velocity in the absence (59%) or presence (15%) of other leukocytes in proximity (one cell diameter). Some of the rolling leukocytes (17%) pass through the cluster without reducing their velocity. Recruitment of leukocytes from the free flow regime into a cluster is a rare event and accounts for only 7 (1.2%) of 476 leukocytes arriving in the cluster. However, of the leukocytes captured from the free flow, 6 initiated contact with a slow-rolling leukocyte rather than making direct contact with the endothelium. Our data show that leukocyte-leukocyte interactions can occur in vivo but are not important for cluster formation. This is confirmed by the observation of normal cluster formation in L-selectin–deficient mice, in which leukocyte-leukocyte interactions under flow are abolished. We conclude that leukocyte-mediated nucleation contributes little to leukocyte recruitment during inflammation in vivo. Cluster formation appears to be dominated by areas of endothelium with a higher expression of E-selectin, because cluster formation is greatly reduced in E-selectin–deficient mice. (Circ Res. 1998;82:30-38.)

Key Words: inflammation ■ E-selectin ■ rolling ■ intravital microscopy ■ knockout mouse

Selectin- and integrin-mediated capture, rolling, and adhesion of leukocytes to activated endothelium leads to leukocyte accumulation at inflammatory sites. Most reports support the notion that the selectins mediate the transient interactions underlying leukocyte capture and rolling. Integrins are responsible for firm adhesion, and selectins function in both rolling and adhesion, depending on their activation state. At the site densities of the selectins and their ligands prevailing in venules in vivo, L-selectin–dependent interactions are the most transient, leading to rolling at velocities of >100 μm/s, followed by P-selectin–mediated rolling, with characteristic rolling velocities in the range of 20 to 50 μm/s, and E-selectin–dependent rolling, which typically occurs at <10 μm/s. Since the transit time of any cell is inversely related to its rolling velocity and directly related to the local cell concentration, rolling leukocytes accumulate in venules and can reach concentrations ranging up to 100-fold higher than systemic concentrations. When observing the movement of leukocytes in venules by intravital microscopy, it is evident that the leukocyte rolling velocities and local concentrations vary with time and with position inside the microvessel. In a previous report from this laboratory, the heterogeneity of leukocyte rolling velocities in venules was analyzed and related to local differences in adhesion energy density, which reflects the density of adhesion receptor–ligand pairs formed. In the present study, we investigate the mechanisms by which leukocytes accumulate in inflamed venules.

A recent study in a cylindrical flow chamber showed that leukocytes adhere not only to endothelial cells but also to already adherent leukocytes. This interaction requires L-selectin on the interacting leukocyte but not on the leukocyte already immobilized on the wall. Further investigation of this phenomenon in a parallel-plate flow chamber has confirmed the dependence on L-selectin function and partially identified the L-selectin ligand(s) responsible for these neutrophil-neutrophil interactions.

Although L-selectin–dependent interactions between flowing and adherent neutrophils accelerate accumulation in flow chamber assays, the importance of...
this phenomenon in vivo has not yet been determined. Importantly, the geometry of small venules in vivo is very different from the geometry of both cylindrical and parallel-plate flow chambers. Flow chambers have a much smaller surface-to-volume ratio, and only a small percentage of perfused leukocytes ever comes into close contact with the substrate after sedimenting to the lower wall. In contrast, not only are leukocytes almost neutrally buoyant in blood, but small venules are only 2 to 10 times larger in diameter than the leukocytes traveling through them. Therefore, investigation of leukocyte cluster formation and accumulation in vivo is important in order to assess the potential impact of leukocyte-leukocyte interactions as a mechanism for cell accumulation during inflammation.

In the present study, we explored three questions: (1) Do leukocyte rolling and adhesion produce local clusters or strings in microvessels in vivo? (2) If so, how are leukocytes recruited into such clusters? (3) Which molecular adhesion mechanisms are responsible for cluster formation? We chose to conduct these investigations in the microvessels of the tumor necrosis factor-α (TNF-α)-treated mouse cremaster muscle because this is one of the best-described microcirculatory models of inflammation and because gene-targeted mice deficient in many of the pertinent adhesion molecules are readily available.

Materials and Methods

Mice and Reagents

Experiments were conducted in C57BL/6 wild-type mice and gene-targeted mice carrying one of the following null mutations: P-selectin, E-selectin, and L-selectin. The mutant mice were backcrossed into a C57BL/6 background for at least five generations and maintained as colonies at the University of Virginia Animal Resource Center. The mice were 8 to 12 weeks old and weighed between 20 and 35 g. All experiments were conducted under a protocol approved by the institutional review board.

Murine recombinant TNF-α was purchased from Genzyme Corp. Acridine red was obtained from Chroma.

Intravital Microscopy

After premedication with a mixture of 30 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories) and 0.1 mg/kg atropine (Elkins-Sinn, Inc) intraperitoneally, mice were anesthetized with 100 mg/kg ketamine hydrochloride IP (Ketalar, Parke-Davis). All mice were pretreated 1 to 2.5 hours before cremaster extenmization with an intracardial injection of 0.5 mg murine TNF-α in 0.30 mL isotonic saline. This treatment is known to stimulate the expression of E-selectin on the vascular endothelium of the mouse cremaster muscle.21 The trachea, carotid artery, and jugular vein were cannu-

Data Analysis

Microvessel diameter was measured from video recordings using an interactive digital image processing system. The circumferential width of leukocyte clusters was determined by measuring the radial width using the image processing system and calculating the corresponding arc length. To determine cluster stability over time, a 160-μm venule was divided into 20-μm bins, and the number of leukocytes per bin at three time points 25 seconds apart was determined. In some experiments, the velocities of many consecutive leukocytes (up to 100 per vessel) were measured from video recordings made with stroboscopic epi-illumination. This method shows both rolling and freely flowing leukocytes, which can be distinguished by virtue of their characteristic velocities. The critical velocity separates freely flowing from rolling leukocytes and corresponds to the velocity a noninteracting leukocyte would have when traveling at a clearance of 20 nm from the vessel wall. The position of each leukocyte was interactively measured with a customized imaging system at 200-millisecond intervals and stored on a personal computer for further processing. The vessel surface was divided in equally sized bins (6 μm long and 6 μm wide), and the fraction of time each bin was occupied by a leukocyte was calculated. In addition, the leukocyte velocity was calculated by dividing the distance traveled by the constant time interval (200 milliseconds). A constant time interval was chosen because previous research had indicated that varying time intervals during velocity measurements can introduce experimental artifacts.

Statistical Analysis

All statistical comparisons were performed using a one-way ANOVA followed by a post hoc Student-Newman-Keuls multiple-comparison procedure. SPSS software (SPSS, Inc) was used for all statistical analyses. Statistical significance was set at P<.05.

Results

Observation of TNF-α-treated venules in wild-type mice revealed that rolling leukocytes preferentially accumulate in circumscribed areas of venules to form clusters (Fig 1, underlined sections of venule). A leukocyte was considered to be part of a cluster when its position was within one leukocyte diameter of a neighboring leukocyte. Although leukocytes joined and left clusters, the position of the clusters in the venule remained largely unchanged over time (Fig 2). The vast majority of leukocytes (>97%) left clusters by rolling down-stream from the cluster, usually at a higher velocity than when in the cluster area (data not shown). Very few leukocytes left the cluster by detachment from the endothelium. The clusters in wild-type mice contained between 5 and 34 leukocytes, with an average of ≈12 leukocytes (Fig 3A). Owing to the variable number of participating leukocytes, the length and width of the clusters varied considerably, ranging from 19 to 50 μm along the vessel axis and from 8 to 52 μm in the circumferential direction (Fig 3B and 3C), with an average area of ≈950 μm² (Fig 3D). Although we did not systematically investigate the dependence of cluster size on wall shear rate, we...
observed clustering in venules exhibiting a wide range of shear rates between 250 and 2600 s⁻¹. Although cluster formation was regularly observed in all wild-type mice, there was no evidence of string formation, i.e., leukocytes lining up behind one another in the direction of flow. The videotapes were specifically analyzed for string formation, because this phenomenon had previously been described in parallel-plate flow chamber assays. Clusters generally had no consistently defined shape (Fig 1).

To begin to understand the cause of cluster formation, we analyzed the transit time of many leukocytes through a single venule in greater detail by recording the position of each cell as a function of time (200-millisecond time interval). This allowed the determination of individual cell velocities as a function of the position along the length of the venule. Similar to a previous study conducted in the rat mesentery, we found that the velocity of rolling leukocytes in a single venule varied systematically along the length of the venule (Fig 4A). This overall population heterogeneity can be attributed to differences in the distribution of rolling leukocyte velocities in certain sections of the venule; i.e., leukocytes shifted either to higher (Fig 4B and 4C) or lower (Fig 4D and 4E) velocities. Since we observed that clusters were two-dimensional structures with irregular shapes and did not typically occupy the whole venular circumference, we reanalyzed the data for all leukocytes traveling within the central two thirds of the top wall of a venule (refer to Fig 1B for definition of the true and observable diameters) using 6×6-μm bins (Fig 5A). These data show that certain patches of endothelium support leukocyte rolling at velocities that are high (up to 240 μm/s) but still below the critical velocity (250 μm/s in this venule), whereas other areas preferentially support slow leukocyte rolling (<10 μm/s). In order to understand the impact of local rolling velocity on cell accumulation in an inflamed venule, we reanalyzed the data for transit times through each bin and expressed the result as a percentage of the total observation time during which each bin was occupied (Fig 5B). Although it is qualitatively obvious that slower rolling causes leukocyte accumulation, it was surprising to see the extent and the variability of this accumulation. The most-favored bin was occupied by a leukocyte almost 60% of the time, whereas many other bins were not visited by a rolling leukocyte at all. The absence of bins with 100% occupation shows that no leukocyte remained adherent in the same spot throughout the 100-second observation time under the conditions used in the present study (short-term treatment with TNF-α).

These data indicate that cluster formation is a dynamic process, with leukocytes entering and leaving the cluster at all times. To analyze how leukocytes were recruited into and exited from each cluster, we formulated four working hypotheses (Fig 6). In two of the scenarios (Fig 6A and 6B), rolling cells are recruited into the cluster, either through a nucleation event using interactions with a slowly rolling (or adherent) leukocyte (Fig 6A) or through enhanced “stickiness” (e.g., increased adhesion molecule density) of the endothelial cells at a cluster location (Fig 6B). These two possibilities are predicted to lead to different characteristic shapes of the cluster, because the nucleation event would favor formation of strings or grape-shaped clusters, whereas the enhanced endothelial adhesiveness would be more likely to give rise to irregularly shaped clusters. Two other scenarios involve recruitment of leukocytes not previously in contact with the endothelium and traveling in the free flow, i.e., above critical velocity (Fig 6C and 6D). Again, a nucleation event could capture freely flowing cells to initiate rolling, which would favor string formation (Fig 6C). If capture of freely flowing cells occurred through direct leukocyte-endothelium interaction, no such string formation would be expected (Fig 6D). Finally, some leukocytes may travel through the observation area as freely flowing cells or as rolling cells without interacting with the leukocyte cluster. These null hypotheses are not depicted here.

For the purposes of this analysis, we defined any major cell velocity change (by at least a factor of 2) that occurred at less than one cell diameter away from an already adherent or slow-rolling leukocyte as a leukocyte-leukocyte event. This definition is likely to overrepresent leukocyte-leukocyte interactions, because adhesion molecules extend from the visible surface of leukocytes by much less than one cell diameter. One of the longest adhesion molecules important here is P-selectin, which extends only ~48 nm from the endothelial cell plasma membrane, a distance that cannot be resolved by the light microscope. We chose this definition to ensure that we did not miss any leukocyte-leukocyte-mediated capture events. Major...
velocity changes occurring in the absence of another nearby leukocyte were defined as direct leukocyte-endothelial events. Of the 476 cells observed in six venules, only 7 cells were recruited into the rolling and adherent pool within the field of observation from the free-flowing state (Fig 7, bottom, and refer to Fig 6C and 6D for mechanism). Of these 7 leukocytes, 6 (1.2% of all cells) were captured within one cell diameter of an already rolling leukocyte (C-C in Fig 7), and 1 (0.2% of all cells) was captured directly by the endothelium (C-E in Fig 7). These data show that the capture of free-flowing leukocytes is a very rare event. Thirty-two freely flowing leukocytes (6.9% of all cells) made no contact with the cluster area (Thru in Fig 7). The vast majority of leukocytes (437 of 476 cells, or 92% of all cells) that changed their velocities did so from a rolling position, ie, with a translational velocity that is below critical velocity (Fig 7, top, and refer to Fig 6A and 6B for mechanism). Of the leukocytes recruited from the rolling pool, 72 (15.1% of all cells) did so by reducing their rolling velocity within one cell diameter of another rolling cell (C-C), and 282 (59.2% of all cells) decelerated while rolling on the endothelium in the absence of adjacent leukocytes (C-E). The remaining 83 cells (17.4% of all cells) rolled through the respective cluster area without changing their rolling velocity significantly (Thru). These data show that most leukocytes recruited into a cluster are already rolling upstream from the cluster and reduce their velocities in most cases without contact with other leukocytes.

To understand the molecular mechanisms of cluster formation, we investigated the cluster size in selectin-deficient mice and compared the results to wild-type mice. We found that the average cluster size (Fig 8A) and percentage of observable venular surface covered by clusters (Fig 8B) was reduced in E-selectin–deficient mice compared with the other genotypes (P<.05). The venules used for this analysis are hemodynamically similar (Table). In addition to the reduced cluster area, venules with leukocyte clusters were found at least five times less frequently in E-selectin–deficient mice than in other genotypes (data not shown). These data suggest that local variation in E-selectin expression on endothelial cells along the venules is the main cause of leukocyte cluster formation in vivo. The striking reduction in cluster formation in E-selectin–deficient mice is even more impressive in view of the fact that the number of cells rolling through venules in a TNF-α–treated E-selectin–deficient mouse is, on average, about 2- to 3-fold higher than in a similarly treated wild-type mouse.7 Consistent with the data presented above showing that leukocyte-leukocyte interactions appear to play little role in in vivo cluster formation, the numbers of leukocytes per cluster

Figure 2. Leukocyte clusters in vivo exhibit dynamic stability. Leukocyte clusters observed in tumor necrosis factor-α (TNF-α)–treated wild-type mouse cremaster muscle venules remained in the same area over time. The three photomicrographs (A, B, and C) were taken 25 seconds apart. The histogram next to each photomicrograph shows the number of leukocytes in each segment of vessel at each time point. Notice that the peak in each histogram falls in the same area of the vessel at each time point, showing that the clusters are dynamically stable at steady state. Venular diameter is 43 μm. Photomicrographs were taken using a ×40 saline immersion objective.
the numbers of clusters per unit surface area are similar in mice lacking L-selectin and wild-type mice. Likewise, no deficit in cluster formation was seen in P-selectin–deficient mice.

Discussion

Leukocyte accumulation at sites of inflammation is not a uniform process but shows temporal and spatial heterogeneity. In the present study, we show that in a model of cytokine-induced inflammation caused by local injection of TNF-α, leukocytes roll at locally different velocities, which leads to the formation of dynamic leukocyte clusters. Cluster formation is diminished in gene-targeted mice homozygous for a null mutation in the E-selectin gene, suggesting that cluster formation is largely E-selectin dependent. The majority of leukocytes recruited into a cluster are already rolling upstream from the cluster and reduce their rolling velocity in the absence of detectable interaction with other leukocytes.

This finding suggests that leukocyte-leukocyte interactions recently described in various artificial in vitro flow systems do not appear to play an important role in leukocyte recruitment during inflammation in vivo. There are several significant differences between in vitro and in vivo systems that might explain this discrepancy. Flow channels used in vitro are typically several hundred micrometers high and up to several millimeters wide. Consequently, a large flow rate is necessary to maintain a realistic wall shear stress. Of the millions of cells typically perfused through a flow-chamber system, only a very small fraction (<1%) interact with the surface (roll and/or adhere). A large pool of leukocytes travel in a layer just above and within one cell diameter of the surface. These cells, although unable to interact with the chamber wall proper, can be captured by interacting with already adherent leukocytes, which protrude into the flow chamber and thus provide a surface favorable for cell adhesion. This phenomenon has been termed secondary tethering or nucleation and leads to characteristic patterns of cell accumulation in strings or grape-shaped clusters. Secondary tethering leads to leukocyte accumulation at a rate that increases with time because of the availability of more nucleating cells.

In contrast, the geometric constraints on leukocyte flow through microvessels in vivo are very different. The available vessel surface is relatively large compared with the cross-sectional area, so that most leukocytes have a chance to come into contact with the endothelium. In addition, the presence of

Figure 3. Physical characteristics of leukocyte clusters in wild-type mice. The number of leukocytes per cluster (A), cluster length (B), cluster width (C), and cluster area (D) were measured in 23 venules from 4 different wild-type mice. Cluster width in the circumferential direction was corrected for vessel curvature as described in “Materials and Methods.”

Figure 4. Leukocyte rolling velocities in a single cremaster venule show spatial heterogeneity. The rolling velocities of 40 consecutive leukocytes were measured in a cremaster muscle venule of a tumor necrosis factor-α (TNF-α)-treated wild-type mouse. The average leukocyte rolling velocity (mean ± SD) shows systematic variation along the length of the observed venule (A). This variation is also reflected in the rolling velocity distributions along the venule (panels B through E, between 350 and 540 measurements in each distribution). The percentage of leukocytes with higher velocities in each distribution changes along the length of the venule (percentages shown in panels B through E) concomitantly with the variation in average rolling velocity. Leukocytes exhibit higher rolling velocities on certain areas of the endothelium (right-shifted distributions in panels D and E). The vertical dashed line in panels B through E represents a rolling velocity of 12 μm/s, which is a reasonable cutoff for slow rolling, as shown in previous work.
red blood cells is known to promote enhanced contact between leukocytes and the vessel wall. Leukocyte rolling flux fractions, i.e., the percentage of leukocytes interacting with the vascular endothelium, are typically \( \approx 50\% \) in vivo and can range as high as \( 80\% \). Furthermore, the vascular system is composed of interconnected tubes, in which the venules are supplied with blood from capillaries whose diameter is similar or even smaller than that of an undeformed leukocyte. Early observations have suggested that leukocyte adhesion and rolling may be initiated in the smallest postcapillary venules. As a consequence, most of the leukocytes that express the appropriate receptors for rolling, mainly granulocytes and monocytes, enter the class of venules studied here already rolling.

Another difference between in vivo and in vitro systems that should be considered when assessing leukocyte-leukocyte interactions as a mechanism for leukocyte accumulation is that leukocytes flowing through a microvessel in vivo are almost neutrally buoyant in plasma and will not settle appreciably during their short transit time through a microvessel. Therefore, flowing leukocytes in vivo will only interact with adherent or slow rolling leukocytes if they are flowing in the laminar near the wall of the vessel (a small percentage of the total flowing leukocytes). In contrast, because of the lower density of the suspending media and the greater distance traveled in vitro flow chamber assays, leukocytes flowing through a flow chamber will slowly settle during their transit so that they approach the bottom wall of the flow chamber. In fact, inverting the flow chamber totally prevents new attachment of freely flowing leukocytes at any shear rate. In contrast, the number of adherent leukocyte on the “top” and “bottom” of a venule are roughly equal. As a consequence of continuous sedimentation, many more leukocytes in a flow chamber have a chance to interact with a leukocyte at the wall when the flowing cells approach the wall as they traverse the chamber. Thus, the importance of leukocyte-leukocyte interactions for leukocyte accumulation observed in the flow chamber appear to result from increased leukocyte delivery due to gravitationally induced sedimentation during transit.

Rolling is known to be a prerequisite for leukocyte recruitment into sites of inflammation and appears to strongly...
facilitate cell recruitment into clusters (the present study). We propose that a rolling leukocyte decreases its velocity when it encounters one or more endothelial cells with increased expression of adhesion molecules, which leads to formation of a dynamic cluster. Our data obtained in E-selectin–deficient mice show that local variations in E-selectin expression are the most likely explanation for the increased endothelial adhesive-ness underlying cluster formation. Many leukocytes, including neutrophils,36 monocytes,37 and some T lymphocytes,38 express ligands for E-selectin. Consequently, these cells are likely to experience a reduction in rolling velocity when they encounter an area on the endothelium with increased E-selectin expression. The small proportion of cells that pass through the cluster without a reduction in rolling velocity may be using other adhesion molecules whose expression is not increased in the endothelial area that supports the cluster. One candidate pair of adhesion molecules might be \( \alpha_4 \) integrin–vascular cell adhesion molecule-1. This receptor-ligand pair has been shown to support selectin-independent leukocyte rolling.39–41 Alternatively, these cells may be T lymphocytes expressing ligands for P-selectin but not E-selectin.42 Since cluster formation is not altered in P-selectin–deficient mice, T lymphocytes expressing ligands for P-selectin only may not participate in cluster formation.

Previous work has suggested that E-selectin ligation can trigger activation of neutrophils, resulting in \( \beta_2 \) integrin (CD18) activation.42,43 Interactions between activated or partially activated \( \beta_2 \) integrins on rolling leukocytes with endothelial ligands such as intercellular adhesion molecule-1, which is expressed in cremaster muscle venules after activation with TNF-\( \alpha \),17 could lead to a reduction in rolling velocity at cluster sites. However, the role of E-selectin ligation as a direct activator of leukocytes remains controversial. Fixed neutrophils roll just as slowly as viable neutrophils on E-selectin adsorbed to a flow chamber in vitro,45 suggesting that the rolling velocity reduction is a genuine property of the E-selectin–ligand bond. Also, recent work has shown that the activation state of neutrophils (as assessed by L-selectin shedding and Mac-1 expression) is not different when bound either to E-selectin–transfected Chinese hamster ovary cells or non-transfected cells.46 Most likely, the increased transit time afforded through E-selectin–mediated slow rolling allows exposure to effectively higher levels of activating factors present.

### Hemodynamic Parameters in Investigated Venules

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Diameter, ( \mu )m</th>
<th>Velocity, ( \mu )m/s</th>
<th>Shear Rate, s(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (5)</td>
<td>23</td>
<td>35.9±5.3</td>
<td>1817±315</td>
<td>858±165</td>
</tr>
<tr>
<td>L-Selectin deficient (4)</td>
<td>12</td>
<td>34.5±4.0</td>
<td>2027±553</td>
<td>1124±422</td>
</tr>
<tr>
<td>P-Selectin deficient (4)</td>
<td>10</td>
<td>35.0±4.4</td>
<td>1829±537</td>
<td>877±226</td>
</tr>
<tr>
<td>E-Selectin deficient (4)</td>
<td>11</td>
<td>37.3±5.4</td>
<td>1726±317</td>
<td>846±205</td>
</tr>
</tbody>
</table>

\( n \) indicates the number of venules examined. The number of mice examined is shown in parentheses after genotype. Values are mean±SEM.

Figure 7. Leukocytes are recruited into clusters in vivo mainly through leukocyte-endothelium interactions. Four hundred seventy-six leukocytes in six different wild-type venules were analyzed to determine how often a rolling (top) or free-flowing (bottom) leukocyte interacted with a slow rolling leukocyte through cell-cell (C-C) interactions, slowed down or was captured by the endothelium (C-E), or just rolled or flowed through (Thru) the field of view with no interactions. A C-C interaction was defined as a 2-fold change in velocity that occurred within one cell diameter of another leukocyte. The majority of leukocytes were recruited into clusters through direct interactions with the endothelium. *Larger percentage vs all other mechanisms (\( P<.05 \)). #Smaller percentage vs same type of interaction when cells are rolling (\( P<.05 \)). Data are presented as mean±SD.

Figure 8. Cluster formation is reduced in the absence of E-selectin. Between 10 and 23 venules in 3 to 5 mice per genotype were analyzed for the number of leukocytes per cluster (A) and the percentage of observable endothelial area covered by clusters (B). E-Selectin–deficient mice had a significant reduction in number of leukocytes per cluster (\( P<.05 \) vs all other genotypes) and cluster area (\( P<.05 \) vs all other genotypes). This suggests that E-selectin–mediated slow rolling is mainly responsible for leukocyte cluster formation in vivo. Data are presented as mean±SEM.
on the vascular endothelium, leading to partial activation of the rolling leukocyte. Therefore, it is plausible to hypothesize that integrins may be involved in slow leukocyte rolling. Of note, some clusters form in the absence of E-selectin as shown above.

The velocity of rolling leukocytes is known to depend on the quality (bond kinetics) and quantity (site density) of adhesion receptors. Although methods have been developed to measure the expression level of E-selectin and P-selectin at the organ level, no method is presently available that would quantitatively measure the site density of adhesion receptors in segments of individual microvessels. Therefore, the local site density of the endothelial selectins in vivo is unknown. However, our data suggest that local differences in E-selectin site density underlie cluster formation. E-Selectin plays a dominant role in cluster formation because E-selectin supports rolling at much lower velocities than does P-selectin at the site densities prevailing in vivo. The transit time (and hence local concentration) of leukocytes is inversely related to their velocity. Owing to this nonlinear relationship, relatively few leukocytes traveling at very small velocities can create large local concentrations. Of note, firmly adherent leukocytes were rarely observed under the conditions studied (TNF-α stimulation), showing that leukocyte clusters in vivo are highly dynamic structures.

Although E-selectin appears to be very important for cluster formation in vivo, several investigators have found that E-selectin–deficient mice have no defect in total leukocyte recruitment (ie, accumulation) into large inflammatory loci, such as thioglycolate-induced peritonitis or bacterial pneumonia. Similarly, functional blockade of E-selectin using monoclonal antibodies in C57BL/6 wild-type mice does not alter leukocyte recruitment in the same models of peritonitis. These data suggest that E-selectin–mediated cluster formation may allow for targeted accumulation of leukocytes to an inflammatory lesion. Given a fixed time for leukocyte activation, a sufficient exposure to chemotactants on the vascular endothelium and a fixed probability of extravasation after activation, an increase in the number of leukocytes rolling very slowly along the endothelium would lead to increased recruitment. However, in very large lesions like thioglycolate-induced peritonitis, targeting of leukocytes is not necessary, because the extent of the vasculature stimulated by high levels of proinflammatory cytokines and the high levels of chemotactant present in the lesion probably make the transit time of rolling leukocytes nonlimiting. Thus, a leukocyte rolling three times as fast in the absence of E-selectin will still be in contact with an ample length of activated vascular endothelium such that activation can occur. We predict that at a smaller inflammatory locus, a leukocyte rolling three times as fast may well roll out of the region of activated endothelium and not be recruited.

In conclusion, we show that leukocytes form dynamic clusters while rolling in venules in vivo. This cluster formation is not initiated by leukocyte–leukocyte interactions but requires E-selectin–mediated slow rolling. L-Selectin and P-selectin appear to be of little, if any, importance for cluster formation. Our data suggest that the local reduction in rolling velocity underlying cluster formation is caused by increased local expression of E-selectin on endothelial cells in vivo.

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