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LFA-1 and Mac-1 Define Characteristically Different Intraluminal Crawling and Emigration Patterns for Monocytes and Neutrophils In Situ

Ronen Sumagin,* Hen Prizant,* Elena Lomakina,† Richard E. Waugh,† and Ingrid H. Sarelius*

To exit blood vessels, most (~80%) of the lumenedly adhered monocytes and neutrophils crawl toward locations that support transmigration. Using intravital confocal microscopy of anesthetized mouse cremaster muscle, we separately examined the crawling and emigration patterns of monocytes and neutrophils in blood-perfused unstimulated or TNF-α-activated venules. Most of the interacting cells in microvessels are neutrophils; however, in unstimulated venules, a greater percentage of the total monocyte population is adherent compared with neutrophils (58.2 ± 6.1% versus 13.6 ± 0.9%, adhered/total interacting), and they crawl for significantly longer distances (147.3 ± 13.4 versus 61.8 ± 5.4 μm). Intriguingly, after TNF-α activation, monocytes crawled for significantly shorter distances (67.4 ± 9.6 μm), resembling neutrophil crawling. Using function-blocking Abs, we show that these different crawling patterns were due to CD11a/CD18 (LFA-1)- versus CD11b/CD18 (Mac-1)-mediated crawling. Blockade of either Mac-1 or LFA-1 revealed that both LFA-1 and Mac-1 contribute to monocyte crawling; however, the LFA-1-dependent crawling in unstimulated venules becomes Mac-1 independent upon inflammation, likely due to increased expression of Mac-1. Mac-1 alone was responsible for neutrophil crawling in both unstimulated and TNF-α-activated venules. Consistent with the role of Mac-1 in crawling, Mac-1 block (compared with LFA-1) was also significantly more efficient in blocking TNF-α-induced extravasation of both monocytes and neutrophils in cremaster tissue and the peritoneal cavity. Thus, mechanisms underlying leukocyte crawling are important in regulating the inflammatory responses by regulating the numbers of leukocytes that transmigrate. The Journal of Immunology, 2010, 185: 7057–7066.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: EC, endothelial cell; fps, frames per second; KO, knockout; TEM, transendothelial migration; WT, wild-type.

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crawling significantly decreased TEM (6, 16). Furthermore, leukocytes that were able to migrate across the endothelium migrated at nonoptimal locations and required significantly longer time (6). Due to the EC morphology, rolling leukocytes land by default on EC junctions (7, 16), but the majority immediately engage in crawling toward other junctional locations (6, 16). In most cases, crawling occurs in random directions (independent of flow direction), although this random crawling is lost in mice lacking functional Vav1, which is a major regulator of actin organization during leukocyte migration (22). Similarly, proinflammatory stimuli such as fMLP (16) induce leukocyte crawling that is parallel or perpendicular to flow but rarely against the flow direction, indicating a degree of directionality. fMLP or TNF-α activation causes rearrangement of EC surface adhesion molecules, such as ICAM-1 (16), and, in turn, blockade of ICAM-1, which is essential for leukocyte crawling (6), results in a loss of fMLP-induced directionality in the remaining fraction of crawling leukocytes (16). This suggests first that some locations are better equipped to accommodate leukocyte passage than others and second that the most likely factors to drive leukocyte crawling are the distribution of adhesion molecules and as yet unidentified chemotactic signals.

The crawling patterns of the different leukocyte subpopulations (neutrophils and monocytes) are also different. For example, the reported crawling distances for monocytes are significantly longer [up to 250 μm (15)] than those reported for neutrophils [10–40 μm (22)]. Similarly, monocytes can crawl in unstimulated venules (15) and have the ability to reverse migrate [from tissue into the blood vessels (23)], both consistent with their suggested patrolling (adhered/crawling) with the vessel wall. Although neutrophils that were able to migrate across the endothelium migrated at nonoptimal sites and as yet unidentified chemotactic signals.

In the current study, we used intravital fluorescence confocal microscopy to separately examine the crawling and emigration patterns of monocytes and neutrophils in mouse circulation under baseline and TNF-α–activated conditions was assessed using flow cytometry. To do this, mouse blood was diluted 1:6 with PBS to lyse RBC and washed twice in 10 mM Hepes-buffered physiological salt solution containing 0.1% BSA. To identify monocytes and neutrophils respectively, leukocytes were labeled with Abs against F4/80 and GR-1 conjugated to Alexa 488 (CI:A3-1 and RGB-8C5, respectively; 1.5 μg/ml; eBioscience, San Diego, CA), or DiIC9 (20 μg/ml; Molecular Probes, Eugene, OR) and the fluorescence signals were quantified. The fluorescence intensity of the cell samples was converted to numbers of binding sites from the standard solution using software provided by the manufacturer of the beads. To correct for nonspecific binding, the number of nonspecific sites identified using isotype control Ab was subtracted from the total number of sites detected using each specific Ab.

In situ immunofluorescence labeling

In separate experiments, we used either anti-F4/80 or anti–GR-1 Ag conjugated to Alexa 488 (CI:A3-1 and RGB-8C5, respectively; 1.5 μg/ml; Serotec, Raleigh, NC) together with CD11a, CD11b (20 μg/ml; eBioscience, San Diego, CA), or CD49d (20 μg/ml; Southern Biotechnology Associates, Birmingham, AL). A total of 5000 cells per sample were analyzed by flow cytometry (Guava EasyCyte Mini, Guava Technologies, Hayward, CA) as previously described (27). To estimate the number of Ab-binding sites per cell (molecules/cell), each fluorescence signal was calibrated using Quantum Simply Cellular Beads (Flow Cytometry Standards, Fishter, IN). A standard suspension of beads containing different populations was labeled with Abs used to label the cells, and the fluorescence signals were quantified. The fluorescence intensity of the cell samples was converted to numbers of binding sites from this standard solution using software provided by the manufacturer of the beads. To correct for nonspecific binding, the number of nonspecific sites identified using isotype control Ab was subtracted from the total number of sites detected using each specific Ab.
compared with the data from GR-1 labeling (Figs. 3, 4). Likewise, to confirm that labeling with F4/80 Ab detects all circulating monocytes, we compared the results obtained from these experiments to a mouse strain, CX3CR1<sup>GFP</sup>−/−, that exhibits endogenously enhanced GFP-labeled monocytes. We found no significant differences in the interacting monocyte fraction (Supplemental Fig. 4B), adhesion, and TEM (Supplemental Fig. 4A) compared with the numbers obtained with F4/80 staining (Figs. 3, 4). This confirms that under the conditions of our studies, F4/80 epitope is present on all blood monocytes and can be used for their identification. As shown in Supplemental Fig. 1, GR-1 and F4/80 Abs at the concentrations used in this work did not affect leukocyte rolling, adhesion, and TEM. We also determined that the selected concentration of 1.5 μg/mouse is optimal for both GR-1 and F4/80 labeling (Supplemental Fig. 2) and that within the 2 h time of experimental procedures, GR-1 Ab had no deleterious effect on rolling, adhesion, and crawling, all Abs were given i.v. in 50 μl saline 5 min prior to observations via a second catheter inserted into the jugular vein. To quantify TEM, all Abs were given via tail vein in 200 μl saline. For all blocking experiments, LFA-1 and Mac-1–blocking Abs (M17/4, M1/70, respectively; 50 μg/mouse; eBioscience) as well as VLA-4–blocking Ab (PS/2, Southern Biotechnology Associates) were given together with the labeling Abs either via the jugular vein or by tail vein injection.

### Leukocyte emigration into the peritoneal cavity

To quantify leukocyte transmigration into the peritoneal cavity, a separate group of mice were given an i.p. injection of TNF-α (100 ng in 50 μl saline). Four hours later, peritoneal cavities of anesthetized mice were lavaged with 3 ml PBS, and white cells were recovered and counted using a hemocytometer. Leukocyte differentials were determined on 100 μl cytospins stained with Diff-Quik (Dade Behring, Newark, DE) for all experiments; LFA-1 and Mac-1–blocking Abs (M17/4, M1/70, respectively; 50 μg/mouse) were given i.v. in 50 μl saline at least 5 min prior to observations via a second catheter inserted into the jugular vein. To quantify TEM, all Abs were given via tail vein in 200 μl saline. For all blocking experiments, LFA-1 and Mac-1–blocking Abs (M17/4, M1/70, respectively; 50 μg/mouse; eBioscience) as well as VLA-4–blocking Ab (PS/2, Southern Biotechnology Associates) were given together with the labeling Abs either via the jugular vein or by tail vein injection.

### Analysis

#### Leukocyte–EC interactions

Rolling leukocytes were defined as any leukocytes observed translocating along the vessel wall in continuous contact with the endothelium (34). Delivered leukocytes were defined as all leukocytes seen in the vicinity of the wall independently of whether they were rolling or carried in the free stream. The number of rolling leukocytes on the vessel wall was calculated by counting leukocytes rolling past a line perpendicular to the vessel axis per 40 s time interval. All leukocytes that remained stationary or did not exceed a displacement of >8 μm (one leukocyte diameter) during 30 s were considered adhered. Leukocytes that exceeded a displacement of >8 μm were considered crawling. For all treatment groups, only leukocytes that were attached to the endothelium and were able to crawl were analyzed. To quantify leukocyte crawling, each venular site was observed and recorded for 40 min at 30 fps. The original movies were time lapse to 0.33 fps for offline analysis. Crawling distances were obtained by measuring the path length of each crawling leukocyte during the 40 min observation time. The confinement ratio was defined as displacement/total path length (1 = straight line). In experiments in which LFA-1 and Mac-1–blocking Abs were used, an appropriate control isotype or a combination of isotypes was tested. Both control isotypes had no significant effect on leukocyte behavior and were not different from each other (data not shown); hence, for simplicity in Figs. 3, 4, and 7, all data are presented against IgG2b (a control isotype for Mac-1). Delivered leukocytes were defined as all leukocytes observed translating along the vessel wall in continuous contact with the endothelium (34). Delivered cells included all rolling cells and the cells that were not in direct contact with the endothelium but were translating in the extravascular tissue within 50 μm of the vessel/100-μm length vessel segments. All TEM counts were normalized to 10,000 μm².

### Statistics

Statistical significance was assessed by Student’s t test or by one way ANOVA with Newman-Keuls multiple comparison test using GraphPad Prism (version 4.0, GraphPad, San Diego, CA). Statistical significance was set at p < 0.05.

### Results

#### The majority of monocytes but not neutrophils are adhered and crawling in uninflamed venules

Leukocytes roll abundantly in unstimulated, surgically prepared venules in situ, but rarely adhere (35). As rolling is an essential step in the leukocyte recruitment cascade, we asked whether this step is equally important for both neutrophils and monocytes. Using anti-GR-1 and F4/80 Abs to identify neutrophils and monocytes, respectively, we quantified their interactions with ECs. GR-1 and F4/80 staining indicated that in control venules, ~60% of the total leukocyte population interacting with the vessel wall (rolling, adhered, and crawling) are neutrophils, and <20% are monocytes (the rest presumably are lymphocytes). Both neutrophils and monocytes were observed rolling, as summarized in Table I. Both monocytes and neutrophils maintain a round shape while rolling, but following adhesion undergo flattening to begin to crawl. The round shape of a representative rolling neutrophil and a flattening neutrophil that is transitioning from adhesion to crawling are illustrated in Fig. 1D, as captured by transmission electron microscopy (upper panels) and in situ bright field microscopy (bottom panels). Interestingly, whereas only a small fraction of interacting neutrophils (13.6 ± 0.9%; Fig. 1A) became firmly adhered (Fig. 1C, indicated by the arrows) and subsequently exhibited crawling in unstimulated venules, the majority (58.2 ± 6.1%; Fig. 1A) of interacting monocytes were found adhered (Fig. 1C, indicated by the arrows), and 47.7 ± 5.0% of these adhered cells exhibited crawling (Fig. 1B). These differences in the number of interacting monocytes and neutrophils are illustrated by the representative snapshots of the fields of view of selected venules in which monocytes were stained for F4/80 (Fig. 1C, upper panel) and neutrophils for GR-1 (Fig. 1C, bottom panel). These findings clearly indicate that in unstimulated venules, monocytes but not neutrophils are actively engaged with the vessel wall, confirming a previous report in which monocytes were suggested to exhibit patrolling behavior (random crawling) in control venules (15).

#### Monocyte crawling patterns become neutrophil-like upon TNF-α activation

TNF-α significantly increases leukocyte adhesion in situ (35, 36). We show in this study that this is true for both monocytes and neutrophils. The number of adhered neutrophils following TNF-α treatment increased ~3-fold (from 4.8 ± 0.8 to 14.4 ± 1.4 cells/field) and monocytes ~2-fold (from 2.1 ± 0.4 to 4.3 ± 0.6 cells/field; Fig. 2A). Thus, as in unstimulated venules, in TNF-α-acti-

### Table I. Leukocyte rolling in unstimulated and TNF-α-activated venules

<table>
<thead>
<tr>
<th>Condition</th>
<th>Leukocyte Subtype</th>
<th>Delivered (Cells/40 s)</th>
<th>Rolling (Cells/40 s)</th>
<th>Rolling Fraction (Percent Total)</th>
<th>Rolling Velocity (μm/s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>Neutrophils (GR-1&lt;sup&gt;+/+&lt;/sup&gt;)</td>
<td>18.2 ± 1.4</td>
<td>13.7 ± 1.7</td>
<td>73.8 ± 2.4</td>
<td>37.9 ± 1.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Monocytes (F4/80&lt;sup&gt;+/+&lt;/sup&gt;)</td>
<td>6.1 ± 0.8</td>
<td>3.9 ± 0.6</td>
<td>65.1 ± 4.5</td>
<td>48.5 ± 1.9</td>
<td>100</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Neutrophils (GR-1&lt;sup&gt;+/+&lt;/sup&gt;)</td>
<td>6.6 ± 1.1</td>
<td>5.9 ± 1.2</td>
<td>92.7 ± 5.4</td>
<td>4.1 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Monocytes (F4/80&lt;sup&gt;+/+&lt;/sup&gt;)</td>
<td>2.4 ± 1.1</td>
<td>2.2 ± 0.7</td>
<td>93.1 ± 5.5</td>
<td>4.3 ± 1.4</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are means ± SE.Neutrophils and monocytes were fluorescently labeled with GR-1 and F4/80 Abs, respectively, in unstimulated or TNF-α-activated venules. Delivered cells included all rolling cells and the cells that were not in direct contact with the endothelium but were translating in the free stream near the wall. Rolling fraction was defined as number of rolling cells/total delivered.

*Significantly different from each other (p < 0.01).

*Significantly different from appropriate unstimulated condition (p < 0.01).
The average distance that monocytes crawled became significantly
finement ratio) changed to closely resemble that of neutrophils.
monocyte crawling behavior (the crawling distance and the con-
over a 40-min time period. Intriguingly, after TNF-
field microscopy (stained for F4/80; 1.5 μg/mouse, i.v.) and neutrophils (stained for GR-1; 1.5 μg/mouse, i.v.) with the vessel wall were quantified in unstimulated venules. A, Interacting cells were defined as all cells in immediate contact with the vessel wall. Field of view is ~300 μm length. B, The fraction of leukocytes that exhibited crawling out of total number of interacting cells. For A and B, n = 11 venules, four mice. C, Representative images of the fields of view of selected venules where either monocytes (upper panel) or neutrophils (bottom panel) were immunofluorescently labeled. White arrows indicate the adhered leukocytes (confirmed from analysis of the movie from which the frame was extracted). Scale bar, 15 μm. Whereas the majority of neutrophils exhibit rolling behavior in unstimulated venules, the majority of monocytes were found adhered or crawling under these conditions. D, The round shape of a representative rolling neutrophil and a flattening neutrophil that is transitioning from adhesion to crawling, as captured by electron microscopy (upper panels, scale bar, 1.8 μm) and in situ bright field microscopy (bottom panels, scale bar, 10 μm).

FIGURE 1. The majority of monocytes but not neutrophils adhere and crawl in uninflamed venules. The interactions of monocytes (stained for F4/80; 1.5 μg/mouse, i.v.) and neutrophils (stained for GR-1; 1.5 μg/mouse, i.v.) with the vessel wall were quantified in unstimulated venules. A, Interacting cells were defined as all cells in immediate contact with the vessel wall. Field of view is ~300 μm length. B, The fraction of leukocytes that exhibited crawling out of total number of interacting cells. For A and B, n = 11 venules, four mice. C, Representative images of the fields of view of selected venules where either monocytes (upper panel) or neutrophils (bottom panel) were immunofluorescently labeled. White arrows indicate the adhered leukocytes (confirmed from analysis of the movie from which the frame was extracted). Scale bar, 15 μm. Whereas the majority of neutrophils exhibit rolling behavior in unstimulated venules, the majority of monocytes were found adhered or crawling under these conditions. D, The round shape of a representative rolling neutrophil and a flattening neutrophil that is transitioning from adhesion to crawling, as captured by electron microscopy (upper panels, scale bar, 1.8 μm) and in situ bright field microscopy (bottom panels, scale bar, 10 μm).

Interchangeable roles for LFA-1 and Mac-1 in monocyte crawling
Recent studies indicate that monocyte crawling is primarily mediated by LFA-1, whereas neutrophil crawling has been identified as a Mac-1–mediated phenomenon (6, 15). We hypothesized that the differences in monocyte crawling patterns in unstimulated versus TNF-α–activated venules could be attributed to Mac-1– versus LFA-1–mediated crawling. To test this hypothesis, we quantified the effect of blockade of LFA-1, Mac-1, or both on leukocyte crawling in control and TNF-α–activated venules. We found, as expected (6), that neutrophil crawling is mainly mediated by Mac-1 in both control and TNF-α–activated venules. However, in contrast, monocyte crawling is mediated by LFA-1 in unstimulated venules but becomes Mac-1 dependent after TNF-α activation. Blockade of Mac-1 had no significant effect on neutrophil adhesion in both control and activated venules (Fig. 3A, 3B, respectively); however, the crawling fraction (crawling/adhered) significantly decreased under both conditions (76.2 ± 3.9 IgG versus 22.4 ± 2.3% Mac-1 block in unstimulated venules and 76.4 ± 5.0 IgG versus 32.7 ± 5.8% Mac-1 block in TNF-α–activated venules; Fig. 3C, 3D). In contrast, LFA-1 block significantly attenuated neutrophil adhesion (4.3 ± 0.5 IgG versus 1.9 ± 0.5 cells/field LFA-1 block in unstimulated venules and 13.7 ± 1.1 IgG versus 3.6 ± 0.7 cells/field LFA-1 block in TNF-α–activated venules; Fig. 3F, 3G), but did not prevent the remaining fraction from crawling (Fig. 3C, 3D). A combination of anti–Mac-1 and –LFA-1 (Fig. 3C, 3D) or, alternatively, blockade of CD18 (data not shown) had no ad-

This also argues that LFA-1 indeed mediates leukocyte crawling
under these conditions without a contribution from VLA-4. Intriguingly, in TNF-α–activated vessels, VLA-4 block significantly reduced monocyte adhesion; however, its effect on the monocyte crawling fraction (Fig. 3C, 3D), the crawling distance, and the crawling velocity (Fig. 4) was not significant. Blockade of VLA-4 had no significant effect on neutrophil adhesion and crawling in both control and TNF-α–activated vessels. Together, these findings suggest that both LFA-1 and Mac-1, but not VLA-4, contribute to monocyte crawling; however, the dominant role in mediating monocyte crawling changes from LFA-1 in unstimulated venules to Mac-1 upon inflammation.

**LFA-1–mediated crawling distance is characteristically longer than that of Mac-1**

We showed that the characteristically long (147.3 ± 13.4 μm; Fig. 2D) monocyte crawling distance in unstimulated venules was LFA-1 dependent (Fig. 3C). In contrast, the significantly shorter distance crawled by monocytes in TNF-α–activated venules (67.4 ± 9.6 μm; Fig. 2D) was mainly Mac-1 dependent (Fig. 3D). Moreover, monocyte crawling distances in TNF-α–activated venules were similar to distances crawled by neutrophils in either unstimulated or TNF-α–activated vessels, in which crawling is also mediated by Mac-1. This indicates that the LFA-1–mediated crawling distance is characteristically longer than that of Mac-1. To further test this, we measured the crawling distances of monocytes and neutrophils in the presence of either LFA-1– or Mac-1–blocking Abs. In unstimulated venules following LFA-1 block, the small fraction of adhered monocytes that were able to crawl (Fig. 3A) crawled for significantly shorter distances compared with those in untreated venules or venules treated with control isotype Abs (61.0 ± 8.8 LFA-1 block versus 144.0 ± 10.2 μm IgG; Fig. 4A) and with significantly slower crawling velocities (Fig. 4C). We conclude that this crawling was likely mediated by Mac-1 because: 1) blockade of both LFA-1 and Mac-1 further decreased monocyte crawling fraction; and 2) monocyte crawling...
monocytes is to survey the unstimulated venules. Mac-1–mediated crawling distance is significantly shorter and is therefore likely to increase the number of leukocytes that transmigrate during inflammation. There were no significant differences in the crawling velocities of both monocytes and neutrophils in TNF-α–activated venules (Fig. 4D). Intriguingly, we also found that the remaining fraction of crawling neutrophils in TNF-α–activated venules following Mac-1 block (likely LFA-1 mediated) crawled for significantly longer distances compared with distances with control IgG or in untreated venules activated with TNF-α (126.6 ± 10.2 μm Mac-1 block versus 61.6 ± 8.1 μm IgG; Fig. 4B). This observation was also confirmed in Mac-1 KO mice (Supplemental Fig. 6), suggesting that in the absence of functional Mac-1, neutrophils are also able to use LFA-1 for crawling and further supports our conclusion that LFA-1–mediated crawling is characteristically longer than that mediated by Mac-1.

Expression of LFA-1 and Mac-1 on monocytes and neutrophils in situ

We hypothesized that the interchangeable roles of LFA-1 and Mac-1 in monocyte crawling would likely be reflected by changes in the expression of LFA-1 and Mac-1. Using flow cytometry, we measured the expression of LFA-1 and Mac-1 on monocytes and neutrophils isolated from control and TNF-α–treated mouse circulations. As expected (37), the expression of Mac-1 on both
monocytes and neutrophils significantly increased following TNF-α treatment (26.9 ± 1.5 unstimulated versus 50.0 ± 1.4 with TNF-α on monocytes and 33.1 ± 1.2 versus 56.7 ± 0.8 on neutrophils, × 10^4 molecules/cell; Fig. 5). The expression of LFA-1 on monocytes following TNF-α treatment was significantly lower than that in unstimulated tissue (2.9 ± 0.3 versus 9.9 ± 0.4, × 10^4 molecules/cell; Fig. 5), but remained unchanged on neutrophils (9.6 ± 0.2 TNF-α versus 8.4 ± 0.1 control, × 10^4 molecules/cell). The increased expression of Mac-1 by monocytes (together with the decreased expression of LFA-1) after TNF-α activation results in a ~1.5-fold increase in the total β2 integrin density on these cells and indicates a mechanism whereby crawling that was LFA-1 mediated could become dependent on Mac-1, as described in Fig. 3, in TNF-α–activated venules. Additionally, as shown in Fig. 3, VLA-4 block significantly decreased monocyte (but not neutrophil) adhesion in inflamed venules; thus, we also measured the expression of VLA-1 on monocytes and neutrophils from unstimulated and TNF-α–activated mice. Monocytes express VLA-4, and this expression was not significantly different in unstimulated versus activated conditions (6.9 ± 0.7 and 6.8 ± 0.7, × 10^4 molecules/cell; Fig. 5). Neutrophils expressed very low levels of VLA-4, which did not change upon activation with TNF-α (Fig. 5).

Transmigration of neutrophils and monocytes following TNF-α activation

We showed that Mac-1 plays a more prominent role in mediating both neutrophil and monocyte crawling in TNF-α–activated venules compared with LFA-1. Thus, next we asked whether Mac-1 is also more critical for leukocyte TEM into extravascular sites under these conditions. To test this, we quantified leukocyte TEM in cremaster venules and leukocyte emigration into the peritoneal cavity in response to TNF-α activation. In noninflamed, surgically prepared tissue surrounding the cremaster venules, transmigrated leukocytes are rarely observed (16); however, TNF-α treatment resulted in a robust increase in the number of leukocytes in extravascular regions (15 ± 1.0 leukocyte/10,000 μm^2; Fig. 6A). We found that whereas blocking Abs for both LFA-1 and Mac-1 (but not nonspecific IgG Abs) significantly decreased leukocyte transmigration (10 ± 0.9 and 3.7 ± 0.6 leukocyte/10,000 μm^2, respectively; Fig. 6A), a more prominent effect was observed following Mac-1 block. As Mac-1 is essential for both monocyte and leukocyte crawling in TNF-α–activated venules (Fig. 3), these findings further support the idea that getting to the transmigratory portals is critical for TEM. We also, in separate experiments, evaluated the effect of LFA-1 and Mac-1 block on monocyte and neutrophil TEM. Consistent with what we measured for the total leukocyte population (Fig. 6A), block of Mac-1 was significantly more efficient in blocking TEM of both monocytes and neutrophils (5.7 ± 0.4 versus 1.9 ± 0.2 monocytes/10,000 μm^2 and 9.3 ± 0.8 versus 1.9 ± 0.4 neutrophils/10,000 μm^2; Fig. 6A) compared with block of LFA-1 (which also significantly decreased TEM). The representative images in Fig. 6C show neutrophils (stained for GR-1, upper panels) and monocytes (stained for F4/80, bottom panels) within the blood vessels (as indicated by white lines) or in the extravascular space under the conditions specified on each panel. We further tested the effects of Mac-1– or LFA-1–blocking Abs on the emigration of all leukocytes, and specifically neutrophils, into the peritoneal cavity. Under control conditions (data not shown), the majority of leukocytes residing in the peritoneal cavity are mononuclear cells. TNF-α treatment induced leukocyte infiltration into the peritoneal cavity of WT animals (0.5 ± 0.05 control versus 1.6 ± 0.2 TNF-α, × 10^4 leukocytes/3 ml volume of lavage; Fig. 7A), primarily due to accumulation of neutrophils (7.2 ± 0.8% control versus 47.8 ± 2.3% TNF-α; Fig. 7B). Consistent with the cremaster tissue observations, blockade of Mac-1 abrogated the TNF-α–induced leukocyte emigration into the peritoneal cavity (1.6 ± 0.2 versus 0.47 ± 0.03, × 10^4 leukocyte/cavity; Fig. 7A) and decreased the fraction of emigrated neutrophils to 25.3 ± 2.1% of the emigrated cells (Fig. 7B). The decrease in leukocyte emigration following LFA-1 block was not significant in this model (Fig. 7B).

Discussion

Sequential leukocyte rolling, adhesion, and TEM constitute a complex multistep paradigm that is orchestrated by multiple adhesion molecules. Understanding of this sequence of events has been expanded with the recent recognition of postadhesion leukocyte spreading and intraluminal crawling (7) and the documentation that this additional step is essential for TEM (24). Together with the findings that regions enriched in ICAM-1 indicate portals for leukocyte TEM (16), as do less dense regions of basal lamina (38), and the possible role for ICAM-1 in guiding crawling leukocytes toward these portals, this has offered expanded paradigms concerning leukocyte recruitment during inflammation. Thus, the recognition of leukocyte crawling has not only identified a mechanism for leukocyte motility within the blood vessels, but has also revealed a physiological role for the heterogeneity of endothelial morphology and molecular composition that makes some regions better equipped to support leukocyte TEM than others.

The behavior of different leukocyte subtypes in response to inflammatory stimuli has been studied extensively, but less is known about leukocyte–EC interactions in unstimulated (physiological) conditions. Recent work has described monocyte adhesion and crawling in unstimulated venules (15). Importantly, monocyte crawling patterns under these conditions have been described as exploratory and are thought to support the unique function of monocytes to monitor healthy tissue. In this study, we extend these findings by showing that this behavior is indeed characteristic of monocytes but not neutrophils (Fig. 1). Moreover, monocyte crawling in unstimulated venules is different from that in activated vessels or from that of neutrophils, as it is for significantly longer.

**FIGURE 5.** In situ administration of TNF-α increases the expression of Mac-1 but not LFA-1 and VLA-4 on circulating monocytes and neutrophils. Flow cytometry was used to measure the surface expression of Mac-1, LFA-1, and VLA-4 on monocytes and neutrophils isolated from mouse circulation under unstimulated and TNF-α–activated conditions. Isolated leukocytes were double-labeled with either anti-F4/80 or Gr-1 (10^6 mg/ml), and the number of Ab binding sites per cell was established as defined in Materials and Methods. Both monocytes and neutrophils isolated from TNF-α–treated mice significantly increased Mac-1 expression; however, the expression of LFA-1 on monocytes was significantly lower compared with unstimulated tissue, but remained unchanged on neutrophils. Both monocytes and neutrophils expressed VLA-4 (neutrophils at very low levels that did not change following TNF-α treatment). For all groups, n = 3 mice. **Significantly different from each other (p < 0.01).
Mac-1 plays a more prominent role than LFA-1 in leukocyte TEM in cremaster venules. Leukocyte TEM in cremaster tissue in response to TNF-α treatment was quantified in the absence (−) or in the presence of a nonspecific IgG (data not shown) or LFA-1– or Mac-1–blocking Abs. Monocyte and neutrophil markers F4/80 and GR-1, respectively (1.5 μg/mouse), as well as all Ab solutions (50 μg/mouse) were administered via tail vein injection 10 min prior to the injection of TNF-α (100 ng in 50 μl saline, i.p.) and 4 h prior to observations. A, The total number of all leukocytes transmigrated into the extravascular tissue (100,000 μm²) was quantified using bright field microscopy. B, In separate experiments, transmigrated monocytes and neutrophils stained for F4/80 and GR-1, respectively, were counted in the extravascular tissue (100,000 μm²) under the specified conditions using fluorescence illumination. For all groups, n = 10–13 venules, 4–6 mice. C, Representative images (Z-stack projection of microvessels confocally scanned from the upper wall through the middle of the vessel) of neutrophils (upper panels) and monocytes (bottom panels) within the blood vessels (outlined with white lines) and in the surrounding tissue under the conditions specified on each panel. Scale bar, 15 μm. Blockade of both LFA-1 and Mac-1 significantly reduced leukocyte TEM, but Mac-1 block was more effective (as quantified in B). *Significantly different from TNF-α alone (p < 0.05); **significantly different from TNF-α alone (p < 0.01).

Total leukocyte and specifically neutrophil emigration into the peritoneal cavity is primarily Mac-1 dependent. Selected mice were injected with saline (200 μl), Mac-1–blocking Ab, LFA-1–blocking Ab, or nonspecific IgG Abs (50 μg/mouse in 200 μl saline) 10 min prior to the injection of TNF-α (100 ng in 50 μl saline, i.p.). Four hours later, peritoneal cavities of anesthetized mice were lavaged, and white cells were recovered.

A, The total number of leukocytes was counted using a hemocytometer. B, Neutrophil counts were determined on 100 μl cytospins stained with Diff-Quik and presented as percent total population. For all groups in A and B, n = three to four mice. The effects of both IgG2a (control isotope for LFA-1) and IgG2b (control isotope for Mac-1) on leukocyte adhesion and crawling were tested. As no significant differences were found between the two nonspecific IgGs for each condition, for simplicity, all data are presented against IgG2b. Consistent with the cremaster observations, Mac-1 plays a more prominent role in leukocyte TEM compared with LFA-1. *Significantly different from each other (p < 0.01).
consequently affecting leukocyte–EC interactions. We speculate that under unstimulated conditions, monocytes have a higher number of activated LFA-1 than neutrophils; however, this remains to be determined. An additional candidate likely contributing to leukocyte–EC interactions is VLA-4 (CD49d/CD29). It is abundantly expressed on monocytes (13) (Fig. 5) and is used by monocytes to adhere to the inflamed endothelium via interaction with VCAM-1 (Fig. 3), the expression of which significantly increases following TNF-α treatment (35). VLA-4 was also detected at low levels on neutrophils, but in our study, it played no significant role in neutrophil adhesion to the inflamed endothelium (Fig. 4). A previous in situ study showed a role for VLA-1 in neutrophil rolling in WT mice, as well as in supporting leukocyte adhesion in the absence of functional LFA-1 (12), suggesting that VLA-4 was able to compensate for the loss of LFA-1. Not surprisingly, we found no significant contribution of VLA-4 to both monocyte and neutrophil adhesion in unstimulated microvessels, as we (35) and others (42) have previously determined that there is very low expression of VCAM-1 on resting endothelium in microvessels. Our finding that some crawling monocytes remain following blockade of both LFA-1 and Mac-1 (Fig. 5), together with the finding that VLA-4 is not involved in monocyte crawling, suggests that additional factors other than VLA-4 are involved in this process; this remains to be determined in future work.

The expression of Mac-1 is known to increase upon leukocyte activation (43), but the constitutively expressed LFA-1 remains unchanged (44). We confirmed this by showing that activation of isolated monocytes and neutrophils with TNF-α in vitro (4 h) resulted in significant upregulation of Mac-1 expression; however, the levels of LFA-1 remained unchanged (data not shown). Similarly, both monocytes and neutrophils that were isolated from TNF-α-treated mice showed a dramatic increase in Mac-1 compared with leukocytes isolated from unstimulated mice. Surprisingly, monocytes isolated from TNF-α-treated mice showed decreased levels of LFA-1 compared with monocytes from unstimulated mice (Fig. 5). We speculate that the loss of LFA-1 might be a result of LFA-1 internalization or shedding (45, 46), or alternatively, a result of decreased adhesive function due to TNF-α treatment. Neutrophils, on the other hand, showed a dramatic increase in Mac-1 expression, which might be due to Mac-1 cross-linking with anti-Mac-1 mAb. This observation suggests that Mac-1 and their endothelial ligands, such as ICAM-1, are not rate limiting for cell migration.

Finally, LFA-1 has been shown to play an important role in leukocyte TEM (47). However, in our hands, and in agreement with other recent work (6), Mac-1 is significantly more important for this process. As shown in Figs. 6 and 7, blockade of Mac-1 was significantly more effective than blockade of LFA-1 in reducing leukocyte TEM in both cremaster venules and the peritoneal cavity. We cannot eliminate the possibility that using a mAb against Mac-1 might trigger outside in signaling, thus potentially affecting leukocyte function and preventing TEM. However, it appears unlikely that Mac-1 cross-linking would lead to an inhibition of leukocyte function, because integrin ligation will lead to leukocyte activation, resulting in increased adhesion and TEM. The finding that Mac-1 is more important in regulating leukocyte TEM than LFA-1 is not surprising as: 1) activated monocytes and neutrophils express significantly higher levels of Mac-1 compared with LFA-1; and 2) Mac-1 is predominately used by leukocytes to get to the specific venular portals where TEM takes place. In TNF-α–activated venules following blockade of Mac-1, both monocytes and neutrophils were still able to adhere to the endothelium; however, the majority of leukocytes remained stationary until they detached and washed away in the flowing blood. Due to the EC morphology, leukocytes by default land on EC junctions (7, 16), which are known to accommodate most TEM (10). Thus, although it is possible that some leukocytes landed on active portals (the specific location that is equipped to accommodate leukocyte TEM), and others were able to punch their way through the endothelium (transcellular route), as has been previously suggested (48), the current study demonstrates that intralumenal crawling is crucial for the majority of leukocyte recruitment during inflammation.

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References


