

Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade

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The prevailing view is that the β_2 -integrins Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) and LFA-1 ($\alpha_L\beta_1$, CD11a/CD18) serve similar biological functions, namely adhesion, in the leukocyte recruitment cascade. Using real-time and time-lapse intravital video-microscopy and confocal microscopy within inflamed microvessels, we systematically evaluated the function of Mac-1 and LFA-1 in the recruitment paradigm. The chemokine macrophage inflammatory protein-2 induced equivalent amounts of adhesion in wild-type and Mac-1^{-/-} mice but very little adhesion in LFA-1^{-/-} mice. Time-lapse video-microscopy within the postcapillary venules revealed that immediately upon adhesion, there is significant intraluminal crawling of all neutrophils to distant emigration sites in wild-type mice. In dramatic contrast, very few Mac-1^{-/-} neutrophils crawled with a 10-fold decrease in displacement and a 95% reduction in velocity. Therefore, Mac-1^{-/-} neutrophils initiated transmigration closer to the initial site of adhesion, which in turn led to delayed transmigration due to movement through nonoptimal emigration sites. Interestingly, the few LFA-1^{-/-} cells that did adhere crawled similarly to wild-type neutrophils. Intercellular adhesion molecule-1 but not intercellular adhesion molecule-2 mediated the Mac-1-dependent crawling. These *in vivo* results clearly delineate two fundamentally different molecular mechanisms for LFA-1 and Mac-1 *in vivo*, i.e., LFA-1-dependent adhesion followed by Mac-1-dependent crawling, and both steps ultimately contribute to efficient emigration out of the vasculature.

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The hallmark feature of acute inflammation is the recruitment of neutrophils to the afflicted site. The leukocyte recruitment cascade is a multistep process in which leukocytes tether to, roll along, and adhere to the endothelium before transmigrating out of the blood vessels (1, 2). Multiple molecules including the selectins and α_4 -integrin have been described to support rolling (3). Each of these molecules may predominate at different times, in different tissues, or in different inflammatory models (4), but there is abundant data suggesting redundancy between these molecules (5, 6). Neutrophil adhesion is mediated largely by the β_2 -integrins LFA-1 and Mac-1 (7). Much like the selectins, the prevailing view suggests

considerable redundancy between these integrins (8, 9). *In vitro* LFA-1 and Mac-1 bind the same ligand (intercellular adhesion molecule [ICAM]-1), suggesting redundancy between both molecules (10–12). An alternative explanation is that each β_2 -integrin plays a distinct and sequential role in the recruitment cascade. This begs the question of what other molecular step in the recruitment pathway, aside from adhesion, could LFA-1 or Mac-1 mediate? Schenkel et al. (13) recently used static *in vitro* conditions to demonstrate that monocytes crawl (locomotion) on endothelium to the nearest endothelial junction, a step essential for subsequent emigration. Blocking adhesion molecules, such as CD11/CD18, ICAM-1, or ICAM-2, prevented the monocytes from reaching the junctions and reduced transmigration,

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which raises the possibility that crawling was another step in the recruitment cascade.

However, several important new issues were raised. For example, shear forces provided by blood flow have been shown to allow selectins to catch leukocytes near junctions, thereby potentially eliminating any need for crawling to emigration sites (14). Shear flow also greatly expedites emigration of leukocytes (15), and under flow the architecture of endothelium is more elongated, such that the majority of neutrophils adhere near or on a junction, eliminating the need for extensive crawling (16). Finally, although most evidence suggests that leukocytes emigrate through junctions, there is some *in vivo* evidence that neutrophils may migrate not only paracellularly, but also potentially transcellularly, making the need to crawl to specific emigration sites unnecessary (17). We used time-lapse intravital microscopy and confocal microscopy in inflamed blood vessels to examine the existence and importance of neutrophil crawling *in vivo* and to delineate the importance of LFA-1 and Mac-1 in this process.

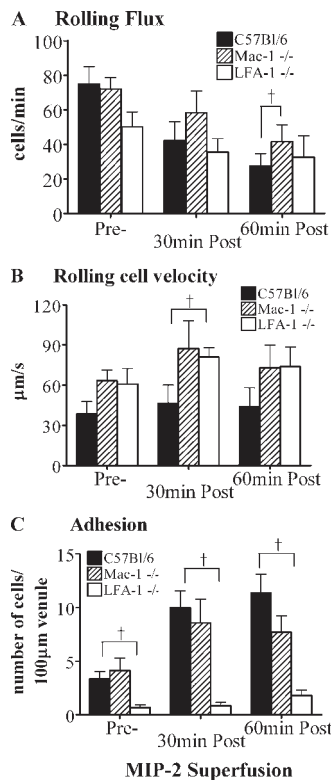


Figure 1. In vivo leukocyte-endothelial cell interactions in inflamed venules in response to MIP-2. Rolling flux (A), rolling cell velocity (B), and adhesion (C) before and after addition of MIP-2 (0.5 nM) in the superfusate. A leukocyte was considered to be adherent if it remained stationary for more than 30 s, and total leukocyte adhesion was quantified as the number of adherent cells within a 100 μ m length of venule during 5 min. All values are means of $n = 6$ or $7 \pm$ SEM. $^{\dagger}P < 0.05$ compared with C57BL/6 mice in A and B, compared with LFA-1^{-/-} mice in C.

RESULTS AND DISCUSSION

In wild-type (C57BL/6) mice ~ 60 – 70 cells min^{-1} rolled in unstimulated blood vessels (Fig. 1 A) at a rolling velocity of ~ 40 $\mu\text{m sec}^{-1}$ (Fig. 1 B). Mac-1^{-/-} and LFA-1^{-/-} mice had similar basal values for rolling flux (Fig. 1 A) and rolling velocity (Fig. 1 B). The rolling flux decreased in all three groups in response to macrophage inflammatory protein (MIP)-2 superfusion (Fig. 1 A). In wild-type mice, the velocity of rolling cells did not change over time in response to MIP-2 superfusion, whereas in Mac-1^{-/-} and LFA-1^{-/-} mice the rolling velocity was elevated (Fig. 1 B). Adhesion increased more than threefold in response to MIP-2 in wild-type and Mac-1^{-/-} mice (Fig. 1 C). LFA-1^{-/-} mice had a clear distinguishing phenotype in which LFA-1^{-/-} neutrophils had almost no basal adhesion, and this increased to only 15% wild-type adhesion after 60 min of MIP-2 superfusion. Previously, we demonstrated that $>99\%$ of cells recruited in response to MIP-2 are neutrophils (18).

To investigate whether the defects observed in the genetically modified mice could be overcome by a greater chemotactic stimulus, the dose of MIP-2 was increased 10-fold to 5 nM. Rolling flux and rolling velocity was not changed at the 5 nM of MIP-2. However, this higher concentration of MIP-2 resulted in a very large increase in the number of adherent cells (more than 30 cells/100 μ m length venule) in both wild-type and Mac-1^{-/-} mice, but the number of adhering neutrophils in LFA-1^{-/-} mice still remained very

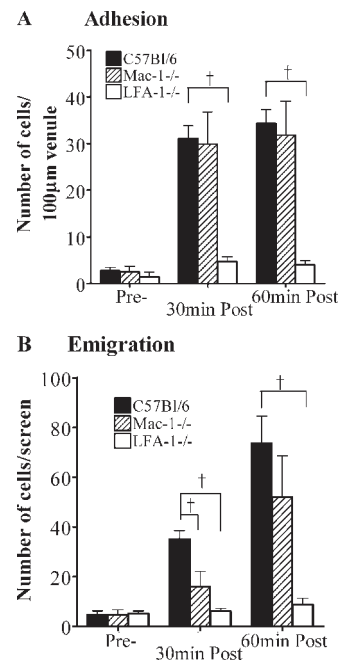


Figure 2. In vivo adhesion and emigration in response to high-dose MIP-2. Leukocyte adhesion (A) and emigration (B) before and after addition of 5 nM MIP-2 to the superfusate. Leukocyte emigration was defined as the number of cells in the extra vascular space within a 200×300 - μm area. All values are means of $n = 5 \pm$ SE. $^{\dagger}P < 0.05$ compared with C57BL/6 mice.

low (Fig. 2 A). At this high MIP-2 concentration, a robust, but delayed, emigration was noted in Mac-1^{-/-} mice at early (30 min) time points (Fig. 2 B). Emigration was much lower in LFA-1-deficient mice relative to wild-type mice (Fig. 2 B).

When time-lapse microscopy was performed over the first 60 min of MIP-2 superfusion, it became very clear that neutrophils stayed stationary for only a very brief period and then crawled equally well in all directions, including directly opposite to the flow of blood (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20060925/DC1>). Fig. 3 A, I, shows an adherent cell, which migrates perpendicular to blood flow (Fig. 3 A, II–IV), crawls with the blood flow to a site of emigration (Fig. 3 A, V–VI), transmigrates (Fig. 3 A, VII), and emerges outside the vessel (Fig. 3 A, VIII). The crawling and emigration of the same cell can be seen in Video 1 (available at

<http://www.jem.org/cgi/content/full/jem.20060925/DC1>). 100% of the wild-type neutrophils that adhered immediately began crawling along the lumen of the vessel, stopped for a brief period, then initiated emigration (Fig. 3 B). Although very few LFA-1^{-/-} neutrophils adhered, those that did behaved exactly like the wild-type neutrophils, in that all of these cells crawled in the vessel lumen (Fig. 3 B). This was also true when wild-type mice had been pretreated with a monoclonal antibody against LFA-1 (Fig. 3 B). Wild-type and LFA-1^{-/-} neutrophils crawled similar distances (Fig. 3 C) at similar velocities (Fig. 3 D). In sharp contrast, fewer than 30% of the Mac-1^{-/-} neutrophils demonstrated any ability to crawl (Fig. 3 B and Video 2, <http://www.jem.org/cgi/content/full/jem.20060925/DC1>). The 30% that did crawl, crawled or rocked back and forth over a 1–2 μm distance.

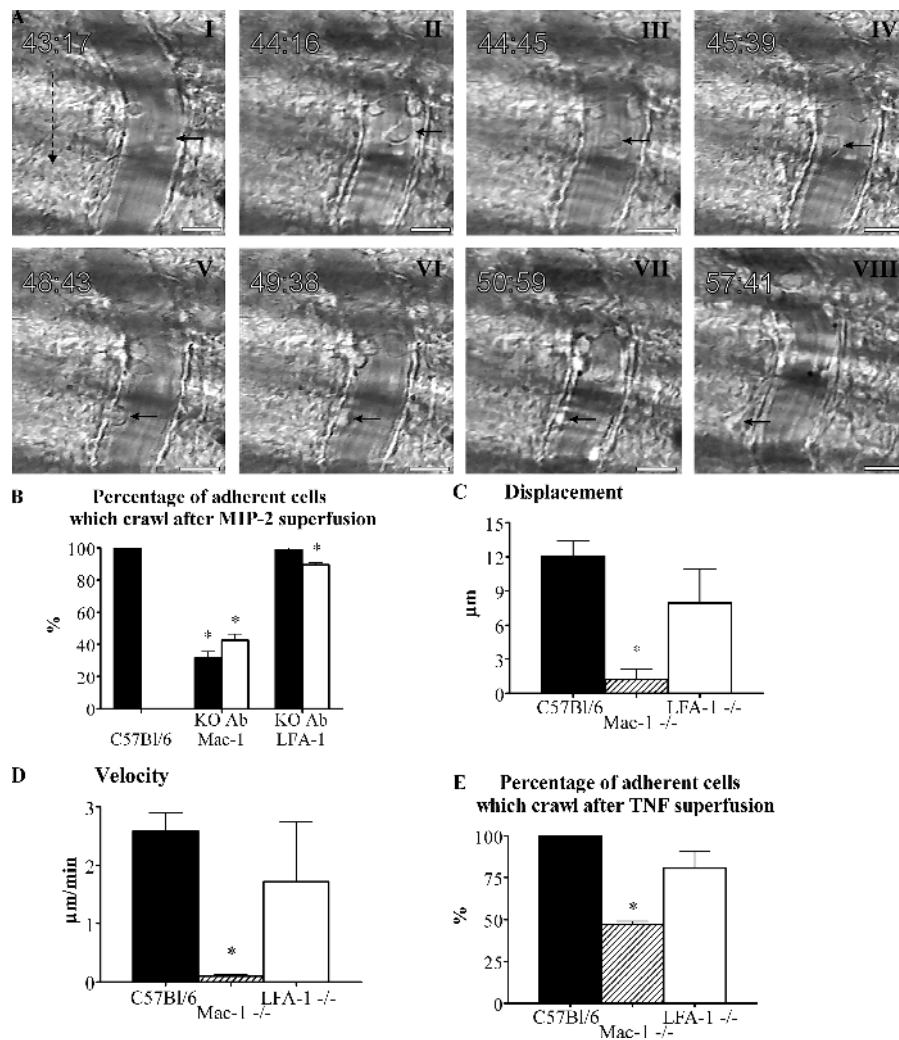


Figure 3. Intravascular crawling of leukocytes. Time-lapse microscopy of inflamed postcapillary venules allows for observation of intravascular crawling in response to MIP-2 (A). Dashed arrow indicates direction of blood flow; solid arrow indicates cell of interest. Bars, 2.5 μm . (B) The percentage of the adherent cells that crawled at the vessel wall before transmigrating in C57BL/6, Mac-1, and LFA-1 mice (KO), or C57BL/6 mice

treated with blocking antibodies (Ab). (C) The displacement of adherent neutrophils from where they first started to adhere to where transmigration took place. (D) The velocity of the displacement was calculated by dividing displacement by the time the cells were followed. (E) Percentage of adherent cells which crawl in response to TNF- α superfusion. All values are means \pm SEM. *P < 0.05 compared with C57BL/6.

A similar decrease in the number of crawling neutrophils was also observed in wild-type mice pretreated with a monoclonal antibody against Mac-1 (Fig. 3 B). In addition, the neutrophils that did crawl in Mac-1-deficient mice traveled only a short distance (Fig. 3 C) at very low velocities (Fig. 3 D). TNF- α also induced crawling in LFA-1 $^{-/-}$ and wild-type neutrophils, but not in Mac-1 $^{-/-}$ neutrophils (Fig. 3 E), similar to the MIP-2 data. Clearly this crawling phenomenon occurs in response to various stimuli (this study) and when no stimulus is used (19, 20).

When wild-type mice had been pretreated with a blocking antibody against ICAM-1, neutrophils still adhered but could no longer crawl in response to MIP-2 (Fig. 4 A). The few neutrophils that did crawl, crawled a very short distance (<2 μ m). In contrast, blocking ICAM-2 did not impair crawling of neutrophils (Fig. 4 A). It is very unlikely that neutrophils stopped crawling because they adhered to the Fc portion of the ICAM-1 antibody immobilized on endothelium, as antibodies with the same isotype that also bound endothelium, including ICAM-2 and platelet-endothelial cell adhesion molecule (PECAM)-1, did not prevent crawling.

To determine whether the impaired crawling translated into a physiologically relevant phenotype, we measured the time from initial adhesion to first appearance outside the vessel in the tissue (Fig. 4 B). The LFA-1 $^{-/-}$ neutrophils that did adhere behaved similarly to wild-type cells (Fig. 4 B). This was also true for wild-type mice treated with a monoclonal antibody against LFA-1 (Fig. 4 B). In contrast, the majority of Mac-1 $^{-/-}$ neutrophils was unable to crawl, or crawled 1–2 μ m over much longer periods of time. Fig. S2 (available at <http://www.jem.org/cgi/content/full/jem.20060925/DC1>) shows total emigration time divided into the length of time spent crawling (until extension of a pseudopod) and the length of

time spent transmigrating (until the cells appeared outside the vessel). Both the crawling/adhesion period and transmigration took longer for the Mac-1 $^{-/-}$ cells, which often moved back and forth at the initial site of adhesion and then again unflattened and sent out a pseudopod. However, it is important to note that the Mac-1 $^{-/-}$ neutrophils were nevertheless able to negotiate passage across the endothelium wherever they adhered, albeit over a much longer time frame (Fig. 4 B).

The crawling of every neutrophil after adhesion in wild-type mice would intuitively suggest that the initial adhesion sites were not optimal sites for emigration. This is further supported by the delay in emigration of neutrophils that could not crawl (Mac-1 $^{-/-}$ cells). The latter could be caused by emigration through less optimal junctional areas, or caused by emigration directly through an endothelial cell. Staining of junctions revealed that the postcapillary venular endothelium was elongated in the axial direction (up to 100 μ m in length) and quite narrow (15–20 μ m in width), such that crawling of 5 μ m would be sufficient to reach a junction (Fig. 5 A). Images of the fluorescently labeled junctions were overlaid on white light images of adherent neutrophils, which allowed us to determine where the neutrophils could be found in relation to the endothelial junctions. Adherent wild-type neutrophils were more often found at junctional sites ($77 \pm 3\%$) than Mac-1 $^{-/-}$ neutrophils ($55 \pm 3\%$), presumably as a result of crawling. These experiments were also repeated in mice where neutrophils had been stained with FITC-labeled GR-1 and the junctions with PECAM-1 antibodies. By analyzing individual sections of the z-stack of generated confocal images, we observed Mac-1 $^{-/-}$ neutrophils clearly transmigrating directly through endothelial cells (Fig. 5 D and Video 3, available at <http://www.jem.org/cgi/content/full/jem.20060925/DC1>). Fig. 5 D, I, shows the body of the neutrophil within the lumen of the blood vessel, Fig. 5 D, II, shows the transendothelial portion of the same cell in the center of the endothelium, and Fig. 5 D, III, shows the extracellular portion of the same transmigrating neutrophil. Based on this type of analysis, wild-type neutrophils emigrated most frequently through endothelial junctions (86%, Fig. 5 E). In contrast, the minority of Mac-1-deficient neutrophils emigrated at junctional sites (39%) (Fig. 5 E). We often noted that even though the neutrophils from Mac-1 $^{-/-}$ mice adhered on an endothelial junction, transmigration occurred adjacent to PECAM-1-stained junctions. This would explain why we found 55% Mac-1-deficient cells overlapping junctions but only 39% transmigrating through junctions. Because of limited z-resolution, the neutrophils emigrating at the sides of the vessels were not analyzed, as it was impossible to absolutely confirm the site of emigration. These results clearly point to a dysfunctional emigration process in Mac-1 $^{-/-}$ mice caused by lack of access to optimal, junctional emigration sites.

Based on many antibody and gene-deficient mouse studies, the prevailing view has become that both LFA-1 and Mac-1 contribute to neutrophil adhesion in the recruitment

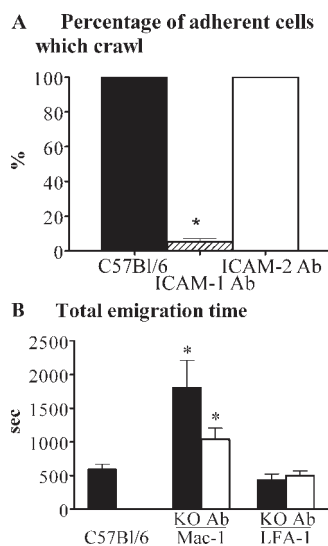


Figure 4. Crawling requires ICAM-1, and emigration is delayed in Mac-1 mice. (A) Crawling after ICAM-1/2 blockade, and (B) total emigration time of adherent knockout (KO) or antibody (Ab)-treated neutrophils. All values are means \pm SEM. * $P < 0.05$ compared with C57Bl/6.

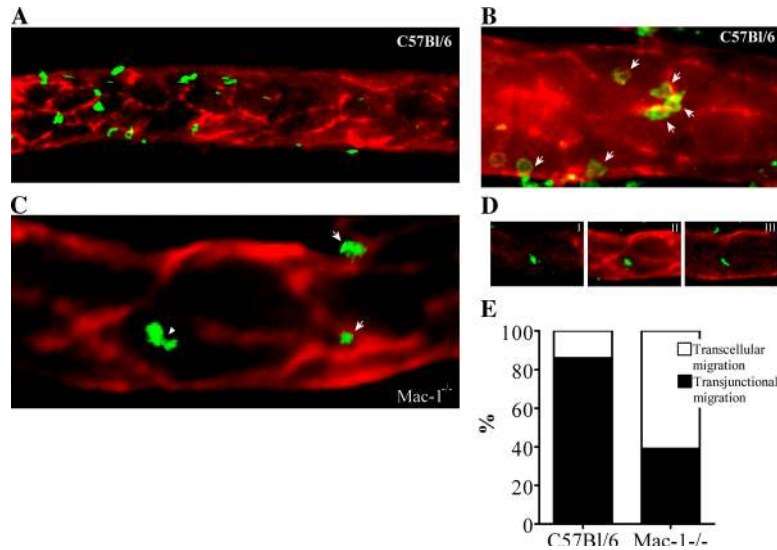


Figure 5. Confocal images of transmigrating neutrophils. Endothelial junctions are stained with anti-PECAM-1 (red), and the neutrophils are stained with anti-GR-1 (green). (A) Z-projection of a single venule demonstrating the morphology of the endothelial cells and locations of adherent and transmigrating neutrophils. (B and C) Z-projections of endothelial cells overlaid with a single z-section showing the transendothelial portion of transmigrating neutrophils in C57BL/6 (B) and Mac-1^{-/-} (C) mice. Cells are transmigrating at junctions (arrows) and undergoing transcellular migration (arrowhead). (D) Z-sequence of a transmigrating neu-

trophil moving through the middle of an endothelial cell. Each image consists of a single z-section. (I) Portion of neutrophil (green) within the lumen of the blood vessel. (II) Portion of the neutrophil extending through the endothelial cell. (III) Portion of neutrophil on the basolateral side of the endothelial cell layer. (E) Quantification of the number of transjunctional and transcellular migrating neutrophils in C57BL/6 and Mac-1^{-/-} mice. A–C is a representative sample of a minimum of 13 venules. (E) $n = 64$ (C57BL/6) and 56 (Mac-1^{-/-}) cells from 15 (C57BL/6) and 13 (Mac-1^{-/-}) venules.

process (2, 8). Our data demonstrate that these β_2 -integrins have fundamentally different functions in the multistep recruitment process. Hentzen et al. (21) first suggested sequential function when they demonstrated an early LFA-1-dependent adhesion to ICAM-1–transfected cells and then a sustained adhesion dependent on Mac-1. This certainly supports our own data that initial adhesion is LFA-1 dependent and then the cells begin to crawl via Mac-1. Visualizing the transition from adhesion to emigration using time-lapse video-microscopy, we report in vivo an additional step in the recruitment cascade, namely intraluminal crawling, comprised of pseudopod extension and uropod retraction. The crawling was not dissimilar to that previously described both in vitro (13) and in vivo in extravascular space (22) but was dependent almost exclusively on Mac-1 and ICAM-1, molecular mechanisms not thought to mediate crawling in the extravascular space (22). Our new data do suggest that there is a clear impairment in the emigration process in the absence of Mac-1 and this was due, at least in part, to an inability of the neutrophils to crawl to sites where emigration can occur in an optimal manner.

Numerous investigators have examined the route of transendothelial migration of leukocytes. Although the majority of studies have reported that the paracellular route accounts for ~90% of transmigration (references 23 and 24, and our wild-type data), some studies have proposed a more dominant role for a transcellular, nonjunctional route (17). Intuitively, since all neutrophils that adhered in vivo crawled

before initiating transmigration, it is almost certain that neutrophils did not adhere at optimal sites of emigration. Clearly, the crawling was a mechanism used to find more optimal sites of emigration. Since the Mac-1^{-/-} neutrophils did emigrate, often without crawling, this would suggest that these neutrophils emigrated in nonoptimal locations. This is evidenced physiologically as dramatically prolonged emigration time in Mac-1^{-/-} neutrophils. Although it is tempting to conclude that optimal sites of emigration are endothelial junctions, whereas nonoptimal sites might be transcellular, it may be that some junctional sites are better for emigration than other sites (i.e., tricellular junctions [23]).

Some in vitro data have shown that Mac-1 and/or LFA-1 could also contribute to the emigration process. Indeed both ligands have been shown to adhere to junctional proteins. LFA-1 has been shown to bind the junctional adhesion molecule (JAM)-A via the I domain (25). However, by separating the adhesion, crawling, and emigration, our own data do not support a role for LFA-1 in emigration. Despite the fact that the LFA-1^{-/-} cells adhered poorly, those that did manage to adhere crawled as well as wild-type cells and emigrated with equal efficiency. Neutrophils have been reported to adhere to JAM-C via Mac-1 (26). Interestingly, inhibition of Mac-1 or JAM-C had no effect on adhesion but significantly affected transmigration of leukocytes in response to monocyte chemoattractant protein-1 (26). Our own data suggest that in the absence of Mac-1, emigration was impaired at least in part via the inability to crawl to junctions. However this emigration

was greatly delayed, perhaps because of the inability of Mac-1^{-/-} neutrophils to bind JAM-C. Of course, if the Mac-1^{-/-} cells were migrating through the endothelium rather than at junctions as our data suggests, the molecular mechanisms underlying this process could be quite different and would probably not involve JAM-C. It is also important to note that different chemokines, cytokines, and proinflammatory molecules could recruit neutrophils via different adhesive mechanisms, and we cannot state that crawling occurs in all vascular beds in response to all inflammatory stimuli. However, in this study we observed Mac-1-dependent crawling in response to MIP-2 and TNF- α . Furthermore, the molecules that mediate crawling may vary for different leukocytes. For example, monocytes can use LFA-1 and ICAM-2 in addition to Mac-1 and ICAM-1, at least in vitro (13).

In summary, we would submit that in our inflammatory model crawling was extremely important for efficient transmigration. Therefore, Mac-1-dependent crawling does need to be added to the recruitment cascade as a molecularly distinct step from LFA-1-induced adhesion.

MATERIALS AND METHODS

All procedures were approved by the University of Calgary Animal Care Committee and conformed to Canadian Council for Animal Care guidelines. Male C57BL/6 mice (Jackson Laboratories) and Mac-1 and LFA-1-deficient male mice (Dr. Ballantyne, Methodist DeBakey Heart Center and Baylor College of Medicine, Houston, TX; reference 9) were anesthetized with 10 mg kg⁻¹ xylazine (Bayer, Inc.) and 200 mg kg⁻¹ ketamin hydrochloride (Biomed-MTC) i.p. Additional anesthetic was administered through a jugular vein. The cremaster muscle preparation was used to study the behavior of leukocytes in the microcirculation and adjacent tissue (27). An intravital microscope (Optiphot-2; Nikon, Inc.) with $\times 25/0.35$ (E. Leitz, Inc.) or $\times 40/0.75W$ (40/0.75W; Carl Zeiss MicroImaging, Inc.) lens with a connected video camera (5100 HS; Panasonic) was used. The number of rolling, adherent, and emigrated leukocytes in single unbranched venules (25–40 μm in diameter) was determined using video playback analysis (27).

Crawling and transmigration. Using 36 \times time-lapse recordings we monitored crawling of adherent cells within the vessels. Postcapillary venules, ranging from 18–30 μm , were imaged in the cremaster at 40 \times before and after MIP-2 (0.5 nM) or TNF- α (11 nM) superfusion. The distance, velocity, and displacement of cell crawling and the time between adhesion and emigration were recorded. For blocking experiments, monoclonal antibodies against Mac-1 (30 μg per mouse; eBioscience), LFA-1 (30 μg per mouse; eBioscience), ICAM-1 (100 μg per mouse; eBioscience), or ICAM-2 (100 μg per mouse; BD Biosciences), or isotype controls (rat IgG2a, κ and rat IgG2b, κ) were given intravenously before the experiments.

Visualization of neutrophil transmigration. At the end of an experiment, endothelial junctions were labeled with an intraarterial injection of a monoclonal anti-PECAM-1 (0.2 mg ml⁻¹; Fitzgerald Industries) conjugated to Alexa Fluor 555 (Molecular Probes). Videos were recorded with an intensified CCD camera (model C-2400-08; Hamamatsu Photonics) mounted on the microscope. To better visualize transmigrating neutrophils, z-stacks were recorded with a confocal microscope (FV300; Olympus). In addition to PECAM-1 staining, neutrophils were stained with FITC-labeled GR-1 (40 μg per mouse; eBioscience) given into the left femoral artery when the MIP-2 superfusion was started. The vessels were imaged with a 40 \times /0.8 NA water dipping objective with both fluorescence and bright field.

Statistics. All data are presented as mean \pm SEM. ANOVA, single-factor, nonrepeated measures followed by Fisher protected least significant difference

test was performed for multiple comparisons. $P < 0.05$ was deemed statistically significant.

Online supplemental material. Fig. S1 shows MIP-2-induced intravascular crawling of wild-type neutrophils. Fig. S2 displays the total time of crawling and transmigration of wild-type, LFA-1^{-/-} and Mac-1^{-/-} neutrophils in vivo. Video 1 shows the intravascular crawling of neutrophils in C57BL/6 mice after MIP-2 stimulation. Video 2 shows the lack of intravascular crawling of neutrophils in Mac-1^{-/-} mice after MIP-2 stimulation. Video 3 shows the z-stack of a Mac-1^{-/-} neutrophil transmigrating at a nonjunctional site. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20060925/DC1>.

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