Intermittent rolling is a defect of the extravasation cascade caused by Myosin1e-deficiency in neutrophils

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Neutrophil extravasation is a migratory event in response to inflammation that depends on cytoskeletal dynamics regulated by myosins. Myosin-1e (Myo1e) is a long-tailed class-I myosin that has not yet been studied in the context of neutrophil–endothelial interactions and neutrophil extravasation. Intravital microscopy of TNFα-inflamed cremaster muscles in Myo1e-deficient mice revealed that Myo1e is required for efficient neutrophil extravasation. Specifically, Myo1e deficiency caused increased rolling velocity, decreased firm adhesion, aberrant crawling, and strongly reduced transmigration. Interestingly, we observed a striking discontinuous rolling behavior termed "intermittent rolling," during which Myo1e-deficient neutrophils showed alternating rolling and jumping movements. Surprisingly, chimeric mice revealed that these effects were due to Myo1e deficiency in leukocytes. Vascular permeability was not significantly altered in Myo1e KO mice. Myo1e-deficient neutrophils showed diminished arrest, spreading, uropod formation, and chemotaxis due to defective actin polymerization and integrin activation. In conclusion, Myo1e critically regulates adhesive interactions of neutrophils with the vascular endothelium and neutrophil extravasation. Myo1e may therefore be an interesting target in chronic inflammatory diseases characterized by excessive neutrophil recruitment.

neutrophil extravasation | rolling | actin polymerization | LFA-1 activation | peritonitis

Neutrophil extravasation is a critical physiological response of the innate immune system during infection and injury (1–3). Extravasated neutrophils contribute to the elimination of the inflammatory cue and subsequent wound healing. However, given the destructive potential of neutrophil secretion products including proteases and reactive oxygen species, excessive, uncontrolled neutrophil recruitment can have severe pathological consequences. Therefore, it is critical to understand in detail the molecular processes driving neutrophil extravasation. The extravasation cascade is a multistep process in which many receptor–ligand pairs and signaling pathways are involved (4). Moreover, extravasation is a migratory event and therefore depends heavily on actin remodeling allowing the involved cells to perform the necessary morphological changes and movements (5, 6). While many receptor–ligand pairs and signaling pathways have been intensively studied, less is known about the regulatory mechanisms driving actin dynamics in both endothelial cells and neutrophils during extravasation. Talin-1 is a prominent example of actin-binding proteins (ABP) critical for neutrophil integrin activation (7). We have recently demonstrated that the ABP cortactin in endothelial cells and the cortactin-homolog HS1 in neutrophils are involved in the regulation of neutrophil extravasation by controlling the activation of small GTPases (8, 9). Another recent example of a neutrophil ABP involved in actin remodeling and integrin activation is Skap2 that regulates WASP-dependent actin polymerization (10).

By contrast, the role of myosins, actin-binding motor proteins, is poorly understood in the context of neutrophil extravasation. Myosins comprise 2 subfamilies of proteins, class I and class II myosins. Class II myosins are well-studied ABP comprising a heavy and a light chain. They cross-link and contract actin filaments to generate pulling forces that induce changes in cell morphology, and contribute to cell migration (11). For example, nonmuscle myosin IIA is required for nuclear deformations in T cells to squeeze them across endothelial monolayers (12), and it plays an important role in engraftment and brain infiltration of leukemic lymphocytes (13). After inhibition of myosin II, neutrophils fail to retract their trailing edge and complete transmigration (14). Recently, a critical role of MyH9 for neutrophil migration has been described in vivo (15). Reduced MyoH9 levels inhibited neutrophil 2D and 3D migration including extravasation due to defective cell polarization and uropod retraction.

Class I myosins are single-chain motor proteins categorized into short-tailed and long-tailed myosins (16). Long-tailed myosins (Myo1e and Myo1f) contain a glycine-rich region and an SH3 domain providing them with scaffolding abilities. Interestingly, class I myosins contain both lipid- and actin-binding regions, making them important linker proteins for actin-related processes at membranes to regulate membrane tension (17). Myo1f has recently been shown to be required for transendothelial migration of neutrophils by regulating nucleus deformation during diapedesis (18). Myo1e is involved in podocyte functions and cancer progression

Significance

Myosin1e is a protein regulating actin cytoskeleton functions, which has not been studied in the context of neutrophil recruitment during inflammation. Using intravital microscopy of inflamed tissue in WT and myosin1e-deficient mice, we show that absence of myosin1e inhibits neutrophil migration out of the blood stream into inflamed tissue. Neutrophils lacking myosin1e show defective actin cytoskeleton polymerization, integrin activation, and aberrant rolling behavior termed "intermittent rolling," suggesting that myosin1e is important for strengthening adhesive interactions of neutrophils with the vascular endothelium. Our findings are significant as they highlight myosin1e as a critical regulator of inflammatory neutrophil recruitment in vivo. Thus, myosin1e could be a therapeutic target for chronic inflammatory diseases.

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(19–21); but it has so far not been investigated in the context of neutrophil–endothelial interactions. Here, we demonstrate by intravital microscopy (IVM) of inflamed mouse cremaster muscles that Myo1e is required for establishing stable neutrophil–endothelial interactions. Surprisingly, chimeric mice revealed that the observed effects are due to Myo1e deficiency in neutrophils.

Results and Discussion

Myo1e Is Required for Efficient Neutrophil–Endothelial Interactions and Extravasation. To determine whether Myo1e is involved in the regulation of inflammatory neutrophil recruitment in vivo, we performed intravital microscopy of TNF-α–inflamed cremaster muscles in Myo1e-deficient (Myo1e-KO) and wild-type (WT) littermate mice. While neutrophil flux (*SI Appendix*, Fig. S1*A*) and the rolling flux fraction (*SI Appendix*, Fig. S1*B*) were not affected, we observed a significant increase in neutrophil rolling velocity in the absence of Myo1e (Fig. 1*A*), suggesting that initial neutrophil–endothelial adhesive interactions are weakened without Myo1e (Movies S1 and S2). Consequently, the number of firmly adherent neutrophils was strongly reduced (Fig. 1*B*), demonstrating that integrin-mediated adhesive events are also disturbed in the absence of Myo1e. Differential interference contrast (DIC) microscopy images of cremaster venules (*SI Appendix*, Fig. S1*C*) and the respective quantification (Fig. 1*C*) revealed that neutrophils transmigrated less efficiently across the vascular wall. However, the reduction in transmigration is fully reflected by the reduced number of adherent neutrophils suggesting that on top of the rolling and adhesion defects, Myo1e does not further regulate the diapedesis step. Hemodynamic parameters such as venule diameter, mean blood flow velocity, and shear stress were similar in WT and Myo1e-KO mice (*SI Appendix*, Fig. S2*A*).

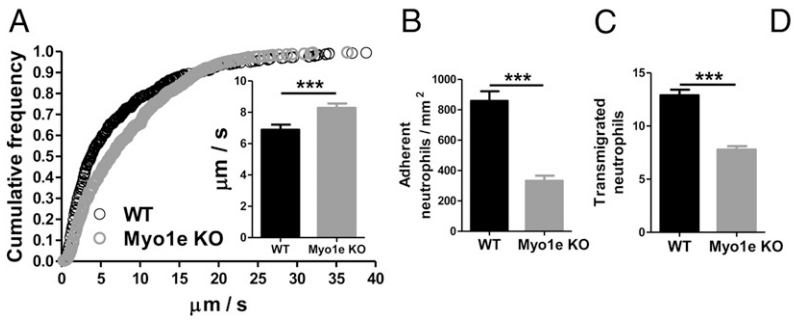
Whole-mount stainings of cremaster vessels and quantification confirmed that more than 90% of all CD45-positive extravasated cells in this model are $Gr1^{high}$ neutrophils (*SI Appendix*, Fig. S2 *B* and *C*), which is in agreement with previously published data (22). This approach also allows one to clearly discriminate between $Gr1^{high}$, round neutrophils (white arrows) and $Gr1^{low}$, elongated monocytes/macrophages. Thus, in this model we are clearly analyzing extravasation of neutrophils. These images also revealed that the overall vessel morphology including the pericyte layer is not altered in Myo1e-KO mice (*SI Appendix*, Fig. S2*B*).

Hemograms revealed that the numbers of leukocytes including neutrophils in peripheral blood were not significantly altered in Myo1e-KO mice compared to WT littermates (Fig. 1*D*). Thus, diminished neutrophil extravasation cannot be explained by reduced neutrophil numbers. However, platelet counts were twice as high in Myo1e-KO mice (Fig. 1*D*). As platelets express Myo1e and are critical players in hematological processes, it will be important to unravel in future studies whether platelets function correctly without Myo1e, and how their increased number could affect neutrophil extravasation and blood clotting in Myo1e-KO mice (23, 24).

Together, these data clearly highlight the importance of Myo1e for stable neutrophil–endothelial adhesive interactions during extravasation.

Myo1e-Deficient Neutrophils Display Intermittent Rolling Behavior. Analyzing neutrophil rolling in more detail, we found that significantly less Myo1e KO neutrophils rolled with velocities slower than 5 μm/s (Fig. 2*A*). Importantly, we consistently observed a discontinuous rolling behavior in Myo1e-KO neutrophils (Movies S3 and S4). In contrast to WT neutrophils that consistently displayed steady rolling on the endothelial surface, Myo1e-KO neutrophils rolled in a discontinuous manner, in which they frequently detached, "jumped," and then reattached to continue rolling. We termed this behavior "intermittent rolling" and propose that this aberrant rolling behavior is due to weakened adhesive interactions. Quantification revealed that only very few WT neutrophils detached and reattached in a behavior that resembled intermittent rolling, but more than 80% of all slow-rolling Myo1e-KO neutrophils showed intermittent rolling (Fig. 2*B*). Representative still images of the videos are shown in *SI Appendix*, Fig. S3*A*; and representative tracks showing rolling velocities over time for selected WT and Myo1e-KO neutrophils are shown in *SI Appendix*, Fig. S3 *B* and *C*, respectively. These data clearly show that Myo1e-KO neutrophils are unable to perform steady slow rolling. While this rolling behavior is distinct, another type of discontinuous rolling has been described before termed "skipping rolling" in neutrophils depleted for E-selectin ligand-1 (ESL-1) (25). Neutrophils lacking ESL-1 did also not establish steady rolling. In contrast to the 80% of Myo1e-KO neutrophils showing intermittent rolling, only 50% of ESL-1–depleted neutrophils displayed skipping rolling. Of note, such behavior was not observed in leukocytes lacking the selectin ligands PSGL-1 and CD44 (25). It will be interesting to unravel whether the underlying molecular mechanisms causing these discontinuous rolling behaviors are the same. Given that long-tailed class-I myosins such as Myo1e are scaffold proteins that can link F-actin to membranes (17), it is tempting to speculate that Myo1e and ESL-1 form a molecular complex important for the transition from tethering and rolling into steady slow rolling and the maintenance thereof. However, this idea needs to be tested experimentally in future studies.

Slowing down of rolling neutrophils occurs when the neutrophil integrin LFA-1 starts binding to endothelial ICAM-1, an interaction that subsequently leads to firm adhesion. To analyze the role of β2-integrins in intermittent rolling, we analyzed rolling in WT and Myo1e-KO mice after injection of blocking antibodies against LFA-1 and Mac1. As expected, we found that



D

Hemograms of WT and Myo1e KO mice

Parameter	WT, mean	SD	Myo 1e KO, mean	SD	Significance, P
WBC, x10³ cells/μl	4.14	0.82	3.84	0.65	0.5982
Neutrophils, x10³ cells/μl	0.68	0.34	0.58	0.31	0.8315
Lymphocytes, x10³ cells/μl	3.3	0.56	3.16	0.46	0.5284
Monocytes, x10³ cells/μl	0.1	0.07	0.1	0.07	0.9056
RBC, x10³ cells/μl	9.34	0.54	9.38	0.27	0.7526
Platelets, x10³ cells/μl	536.4	140.32	1130	486.06	0.0159*
Hemoglobin, g/liter	13.92	0.38	14.2	0.43	0.3443
Hematocrit, liter/liter	0.436	0.01	0.42	0.01	0.1988

Fig. 1. Myo1e regulates neutrophil rolling, adhesion, and transmigration in TNFα-inflamed mouse cremaster muscles. IVM of postcapillary venules of WT and Myo1e-KO mice after 3 h of intrascrotal administration of TNFα. (*A*) Neutrophil rolling velocity (cumulative histogram of rolling velocities and total quantification). (*B*) Number of adherent neutrophils. (*C*) Quantification of transmigrated neutrophils. (*D*) Hemograms of WT and Myo1e KO mice ($n = 8$). *$P < 0.05$; ***$P < 0.001$.

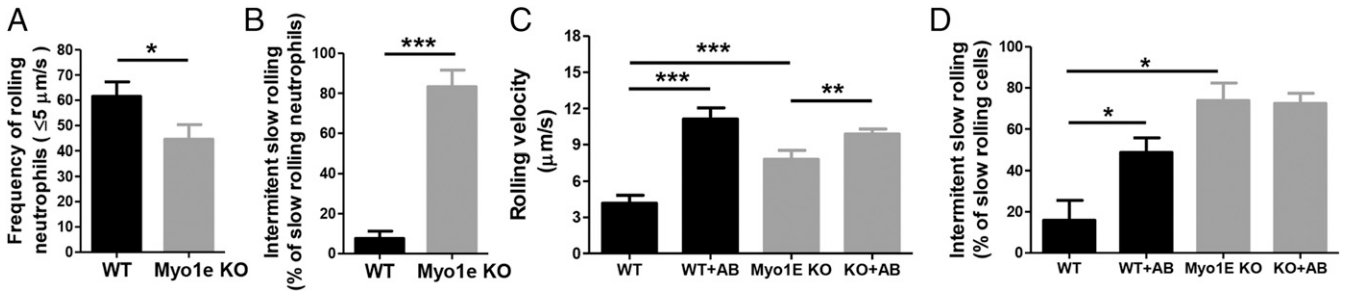


Fig. 2. Myo1e deficiency causes intermittent rolling. (*A*) Frequency of neutrophils rolling with a velocity slower than 5 µm/s. (*B*) Frequency of rolling neutrophils (≤5 µm/s) displaying intermittent rolling ($n = 7$). Neutrophil rolling velocity (*C*) and percentage of neutrophils showing intermittent rolling (*D*) as determined from IVM videos after injection of blocking antibodies against LFA-1 and Mac1 ($n = 4$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

rolling velocities increased strongly after injection of blocking antibodies in WT mice (Fig. 2*C*). Rolling velocities also increased in Myo1e-KO mice after blocking β2-integrins, but to a much lower extent than in WT mice indicating that most of the increasing effect of Myo1e deficiency on rolling velocity depends on these integrins. Of note, β2-integrin blockade induced intermittent rolling in WT mice, suggesting that this phenomenon is at least in part dependent on functional β2-integrins (Fig. 2*D*). By contrast, in Myo1e-KO mice, injection of the blocking antibodies did not affect intermittent rolling. This indicates that Myo1e deficiency causes defective integrin activation to an extent that they cannot be further inhibited by blocking antibodies, also suggesting that Myo1e-dependent intermittent rolling is entirely caused by defective integrin activation.

Blocking of L-selectin or E-selectin did not affect intermittent rolling either in WT or Myo1e-KO mice, suggesting that these selectins are not involved in the induction of intermittent rolling (*SI Appendix*, Fig. S4 *A* and *B*). These data highlight the importance of Myo1e for integrin activation leading to transition into steady slow rolling and subsequent firm adhesion.

Neutrophil Crawling and Arrest Is Reduced without Myo1e. To better characterize integrin-dependent neutrophil adhesive interactions in inflamed cremaster venules, we analyzed crawling. Of note, the number of neutrophils that crawled was strongly reduced without Myo1e (Fig. 3*A*). However, those Myo1e-KO neutrophils that did crawl only showed a tendency toward reduced crawling speed (Fig. 3*B*), suggesting that Myo1e regulates the initial spreading of neutrophils on the endothelial surface and not their subsequent displacement.

Next, we analyzed neutrophil arrest after injection of CXCL1. DIC microscopy images of cremaster venules (*SI Appendix*, Fig. S5) and quantifications of arrested neutrophils (Fig. 3*C*) showed immediate arrest of many WT neutrophils 1 min postinjection, which was reduced in Myo1e-KO mice. Of note, detachment of Myo1e-KO neutrophils occurred significantly faster, and the number of arrested Myo1e-KO neutrophils already reached basal adhesion levels after 5 min (Fig. 3*C*). By contrast, WT neutrophils continuously detached over time, and only reached basal adhesion levels after 15 min (Fig. 3*C*). These data further show that β2-integrin–mediated adhesive interactions are disturbed without Myo1e. In fact, firm adhesion and crawling have

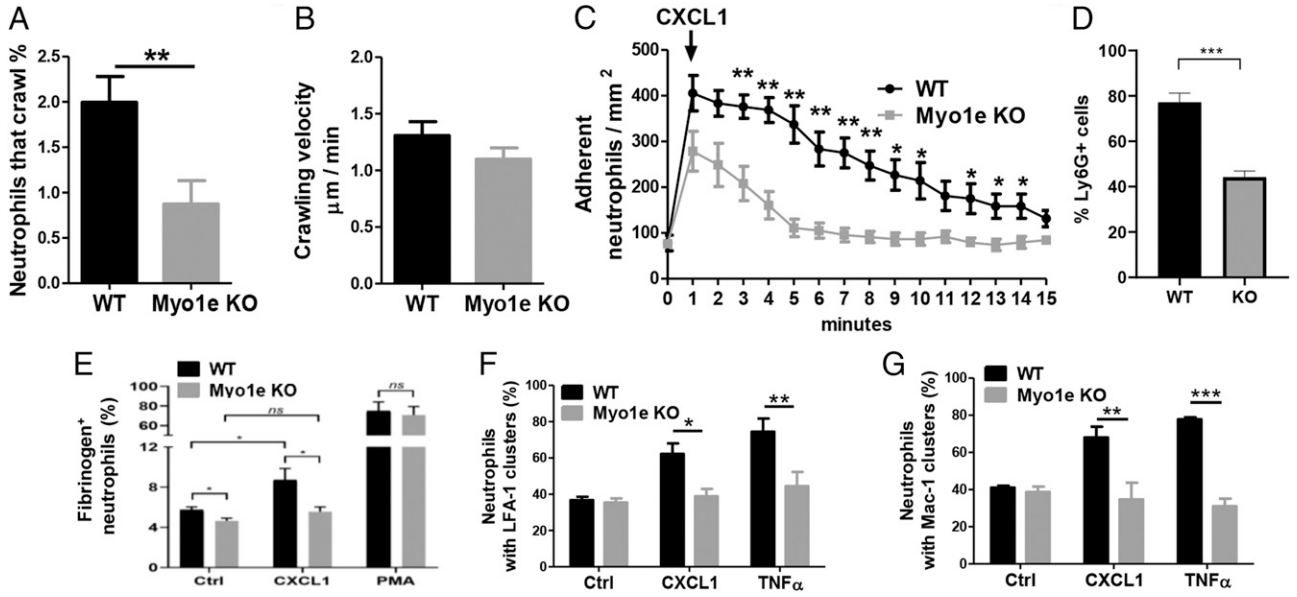


Fig. 3. Myo1e deficiency reduces chemokine-induced arrest and crawling. (*A*) Frequency of crawling cells with respect to all neutrophils observed within a vessel during 1 min. (*B*) Crawling velocity. (*C*) Quantification of adherent neutrophil detachment dynamics within cremaster venules over time ($n = 5$ WT, $n = 6$ Myo1e-KO). (*D*) Numbers of recruited Ly6G-positive neutrophils into the peritoneal cavity 2 h after injection of 3% thioglycollate ($n = 4$; $^{***}P < 0.001$). (*E*) Percentage of neutrophils positive for bound fibrinogen under basal conditions and after stimulation with CXCL1 or PMA ($n = 4$; $^*P < 0.05$). Frequency of neutrophils with clustered LFA-1 (*F*) or Mac1 (*G*) under basal or inflammatory conditions. Results are representative of 3 independent experiments per condition. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, nonsignificant.

been shown to be molecularly distinct processes, with arrest depending more on LFA-1, and crawling depending more on Mac1 interactions with endothelial ICAM-1 (26). Thus, absence of Myo1e likely affects activation of both LFA-1 and Mac1. Similar effects on arrest and crawling due to defective integrin activation have been observed in the absence of other adaptor proteins such as Skap2 (10). Thus, it will be interesting to analyze synergistic effects of different adaptor proteins to better understand the spatial-temporal control of persistent adhesion regulation.

To further corroborate the idea that Myo1e is involved in the regulation of integrin activation, we followed different experimental approaches: First, we analyzed neutrophil recruitment in peritonitis assays known to be strictly dependent on LFA-1 activation (27), and found that the number of Ly6G-positive neutrophils in the peritoneum in response to thioglycollate was significantly reduced (Fig. 3*D*). Second, soluble binding assays with fibrinogen, a ligand for Mac1, showed that Myo1e-KO neutrophils bound significantly lower amounts of fibrinogen (Fig. 3*E*). Third, LFA-1 and Mac1 clustering on neutrophils in response to both CXCL1 and TNF-α was strongly reduced in Myo1e-KO neutrophils (*SI Appendix*, Fig. S6 and Fig. 3 *F* and *G*). This is in agreement with previous studies showing that lateral integrin movement in the plasma membrane, integrin clustering, and adhesion depend on the connection of integrins to actin filaments (28, 29), and the presence of ABP such as L-plastin (30). Flow cytometry analyses showed that surface expression of L-selectin, Gr1, CXCR4, CXCR2, LFA-1, and Mac1 were not significantly changed in the absence of Myo1e under both basal and inflammatory conditions (*SI Appendix*, Fig. S7 *A* and *B*). Together, these data indicate that induction of both integrin affinity and avidity during inflammation, but not integrin expression, is defective in the absence of Myo1e.

Only Myo1e Deficiency in Neutrophils Causes the Observed Effects. Given that the Myo1e-KO mice on which we performed IVM are total KO, and the fact that Myo1e is expressed in both endothelial cells and some leukocyte subsets, we wanted to know whether the observed effects were only mediated by neutrophil Myo1e or also by endothelial Myo1e. To this end, we generated bone marrow (BM) chimeric mice (WT BM injected into lethally irradiated Myo1e-KO mice and vice versa), and analyzed them by IVM. Interestingly, we found that the observed effects were only due to neutrophil Myo1e deficiency. While neutrophil flux (Fig. 4*A*) and rolling flux fraction (Fig. 4*B*) were again similar in both chimeric mice, the percentage of slow-rolling neutrophils (Fig. 4*C*) was significantly lower and the rolling velocity (Fig. 4*D*) significantly higher in the chimeric WT mice injected with Myo1e-KO BM. Concomitantly, in these mice, the numbers of adherent (Fig. 4*E*) and transmigrated neutrophils (Fig. 4*F*) were significantly reduced when compared to the respective control mice (WT BM injected into Myo1e KO mice). Importantly, the chimeric WT mice with injected Myo1e KO BM also showed around 80% of neutrophils displaying intermittent rolling (Fig. 4*G*, compare Fig. 2*B*), clearly demonstrating that only neutrophil and not endothelial Myo1e is required for steady rolling and arrest.

These results were surprising because in neutrophils, Myo1e mRNA has been reported to be absent or only expressed at very low levels (31, 32). As neutrophils usually have low transcriptional activity, we analyzed Myo1e protein levels. Western blot analysis showed a clear specific band of Myo1e at the expected size of 127 kDa in lysates of murine BM neutrophils isolated from WT mice (Fig. 4*H*). Antibody specificity was proven by a band of the same size in murine B cells, and absence of this band in Myo1e-KO neutrophils. To confirm the purity of the neutrophil preparations from BM (by gradient centrifugation) and rule out significant contamination with B cells known to express

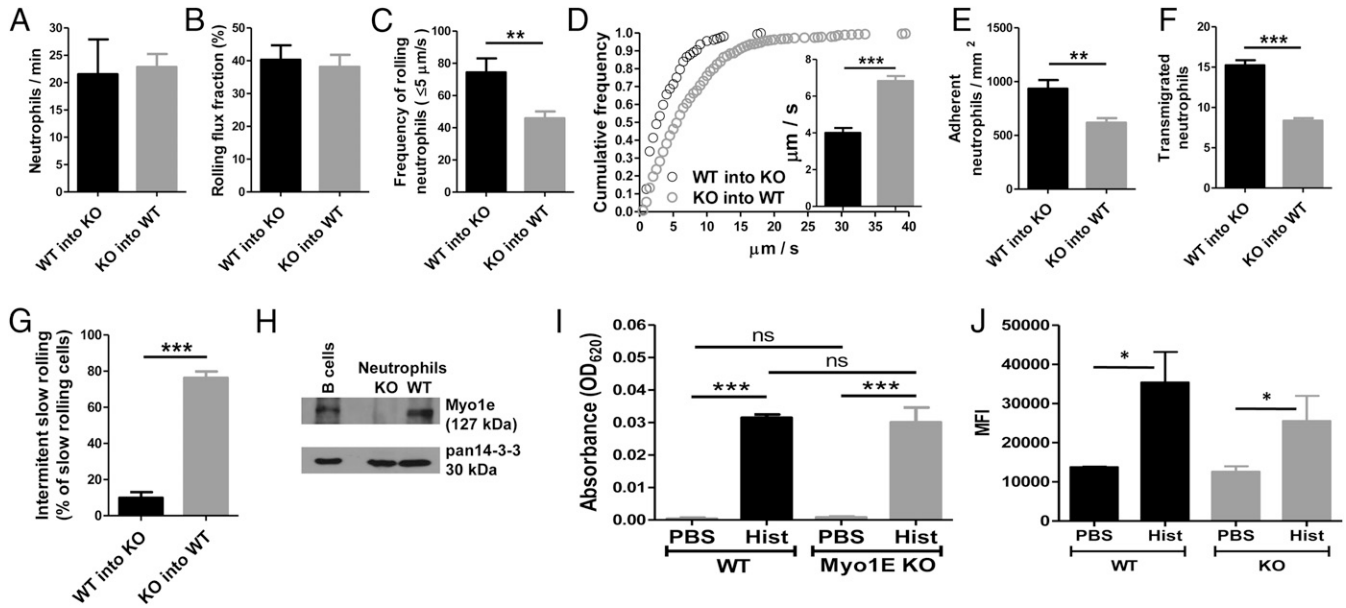


Fig. 4. Only neutrophil Myo1e is required for efficient diapedesis. (*A–G*) IVM of postcapillary venules of chimeric mice [WT BM into Myo1e-KO recipient mice (WT into KO); or Myo1e KO BM into WT recipient mice (KO into WT)] 3 h after intrascrotal administration of TNFα ($n = 4$ WT into KO; $n = 8$ KO into WT). (*A*) Neutrophil flux. (*B*) Rolling flux fraction. (*C*) Frequency of slow-rolling (<5 µm/s) neutrophils. (*D*) Neutrophil rolling velocity (cumulative histogram of rolling velocities and total quantification). (*E*) Number of adherent neutrophils. (*F*) Number of transmigrated neutrophils. (*G*) Frequency of rolling neutrophils showing intermittent rolling. Data are displayed as means ± SEM. (*H*) Representative Western blot of BM neutrophil lysates show that WT neutrophils have Myo1e protein ($n = 3$). Total lysates from B cells were used as positive control for Myo1e expression. Myo1e-KO neutrophils did not show this band, thus guaranteeing antibody specificity. Pan-14–3-3 was blotted as loading control. (*I*) Modified Miles assays to determine vascular permeability after s.c. histamine injection. Results are representative of at least 11 mice per group. (*J*) Vascular permeability for 150 kDa FITC-dextran in the cremaster muscle after intrascrotal injection of either histamine or phosphate-buffered saline (PBS) as control. No significant differences between WT and Myo1e-KO mice were observed ($n = 5$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant.

Myo1e, we analyzed our cell populations by flow cytometry (*SI Appendix*, Fig. S8). We observed higher numbers of Gr1- and Ly6G-positive neutrophils in the preparations from Myo1e KO BM (*SI Appendix*, Fig. S8*A*). Of note, contamination with B220-positive B cells was below 0.4% of total cells in the cell preparations from both WT and Myo1e KO BM (*SI Appendix*, Fig. S8*B*). Thus, it is unlikely that the strong Myo1e band shown in Fig. 4*H* was only due to contaminating B cells. These data reveal functional expression of Myo1e protein in neutrophils.

Myo1e Does Not Play a Significant Role in the Regulation of Vascular Permeability. In order to determine whether Myo1e is involved in the regulation of endothelial barrier integrity and vascular permeability, we performed permeability assays in the skin and in the cremaster. Basal vascular permeability was not significantly altered either in the skin or in the cremaster of Myo1e-KO mice (Fig. 4 *I* and *J*, respectively). Of note, histamine increased vascular permeability to the same extent in the skin and cremaster of WT and Myo1e-KO mice. Thus, Myo1e is not involved in the regulation of vascular permeability. However, it will be important to study other potential functions of Myo1e in endothelial cells.

Actin Polymerization in Neutrophils Requires Myo1e. Adhesion, migration, and integrin clustering critically depend on dynamic actin remodeling. Although it has recently been shown that the TH12 domain of Myo1e is involved in actin polymerization during podosome formation (33), an involvement of Myo1e in chemokine-induced actin polymerization has not been shown. Thus, we analyzed the content of F-actin in stimulated and unstimulated WT and Myo1e-KO neutrophils. We found that there was a nonsignificant tendency toward lower basal F-actin content in Myo1e-KO neutrophils (Fig. 5*A*). Stimulation with both CXCL1 and TNF-α induced actin polymerization in WT neutrophils, which was significantly reduced in Myo1e-KO neutrophils (Fig. 5*A*), suggesting that

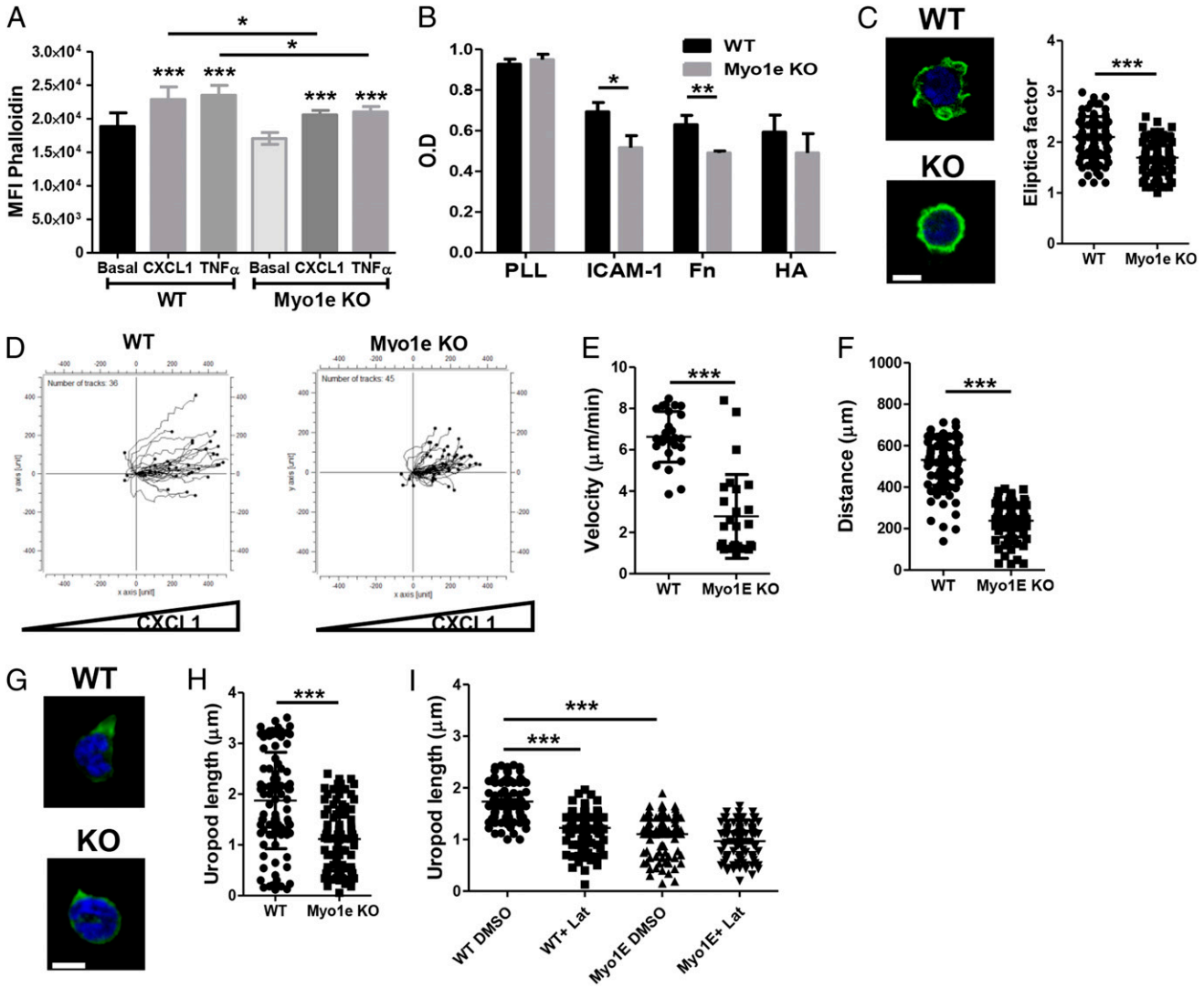


Fig. 5. Myo1e deficiency causes defective actin polymerization, adhesion, spreading, chemotaxis, and uropod formation. (*A*) Flow cytometric quantification of phalloidin stainings in unstimulated WT and Myo1e-KO neutrophils or after stimulation for 5 min with CXCL1 or TNFα. (*B*) Static adhesion of WT and Myo1e-KO neutrophils on poly-L-lysine (PLL), mICAM1-hFc, fibronectin (Fn), or hyaluronic acid (HA). (*C*) Spreading of neutrophils on mICAM1-hFc. Representative cells with phalloidin staining (*Left*); and analysis of the elliptical factor (*Right*) using ImageJ software. (*D–F*) Analysis *of* in vitro chemotaxis of WT and Myo1e-KO neutrophils on mICAM1-hFc toward a CXCL1 gradient ($n = 4$). (*D*) Representative trajectory plots of WT and Myo1e-KO neutrophils. (*E*) Migration velocity. (*F*) Accumulated distance. Uropod formation of CXCL1-treated neutrophils was analyzed by actin stainings using phalloidin (*G*) and quantification of uropod lengths (*H*) using ImageJ software. (*I*) Uropod lengths after treatment of neutrophils with latrunculin B or dimethylsulfoxide (DMSO) as vehicle control. Data shown are means ± SEM; $n = 5$ for all experiments; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

reduced chemokine/cytokine-induced actin polymerization in neutrophils contributes to weak adhesive interactions with vascular endothelial cells consequently leading to reduced neutrophil recruitment in Myo1e-KO mice.

Neutrophil Adhesion, Spreading, Chemotaxis, and Uropod Formation Is Regulated by Myo1e. Finally, we wanted to know whether Myo1e deficiency would also affect other neutrophil functions including adhesion, spreading, and chemotaxis in a 2D environment. In the case of the other long-tailed class I myosin, Myo1f, only 3D migration (such as transendothelial migration) was significantly affected in Myo1f-KO mice due to its requirement for nuclear deformation during squeezing through 3D environments, whereas 2D migration was not affected in the absence of Myo1f (18). First, we performed static adhesion assays and found that adhesion of Myo1e-KO neutrophils to ICAM-1 and fibronectin was significantly reduced (Fig. 5*B*), which is in agreement with the defective integrin activation described above. By contrast, charge-dependent adhesion on poly-L-lysine and adhesion on hyaluronic acid were not significantly affected by the absence of Myo1e (Fig. 5*B*). Second, spreading assays on ICAM-1 revealed less protrusions in Myo1e KO neutrophils when compared to WT neutrophils (Fig. 5 *C*, *Left*), and the elliptic cell factor as analyzed by ImageJ was lower in Myo1e-KO neutrophils (Fig. 5 *C*, *Right*). Third, we performed 2D chemotaxis assays on ICAM-1 using Zigmond chambers and found that the migration pattern of Myo1e-KO neutrophils was different compared to WT neutrophils (Fig. 5*D*). Migration velocity (Fig. 5*E*), and migrated distance (Fig. 5*F*) were significantly reduced in Myo1e-KO neutrophils. Moreover, we also performed chemotaxis assays on fibronectin, and found similar defects (*SI Appendix*, Fig. S9). Uropod formation is a critical event during neutrophil migration, and this was significantly reduced in Myo1e KO neutrophils migrating on ICAM-1 (Fig. 5 *G* and *H*). Thus, in contrast to Myo1f deficiency, the absence of Myo1e reduces both 2D and 3D migration of neutrophils. Although both Myo1e and Myo1f are members of the long-tailed myosin class I family, the observed functional consequences of their absence are very different, as Myo1f deficiency did not lead to reduced neutrophil rolling or adhesion on endothelial cells. Thus, it will be important to unravel what causes these functional differences.

To connect these adhesion and migration defects to the observed defect in actin polymerization, we performed uropod formation assays in the presence or absence of latrunculin, a compound known to induce depolymerization of actin filaments. As expected, latrunculin blocked uropod formation in WT neutrophils as indicated by a strong reduction in protrusion lengths (Fig. 5*I*). Importantly, latrunculin did not further reduce protrusion length in Myo1e-KO neutrophils as compared to both untreated Myo1e-KO neutrophils and latrunculin-treated WT neutrophils (Fig. 5*I*), demonstrating that the uropod defect observed in Myo1e-KO neutrophils can be fully attributed to the observed reduction in actin polymerization (Fig. 5*A*).

In summary, with Myo1e we have identified a member of the class I myosin family that is critically involved in regulating actin polymerization, integrin activation, and consequently neutrophil rolling, adhesion, migration, and recruitment. It will be interesting to test in applied studies if Myo1e may serve as a pharmaceutical target for reducing excessive leukocyte recruitment in inflammatory diseases.

Materials and Methods

Mice. Myo1e-KO mice on a C57BL/6J WT background were kindly provided by Richard Flavell (Yale School of Medicine, New Haven, CT). Male Myo1e-KO and littermate WT mice in an age range of 8–12 wk have been used for all experiments. Animals were kept under pathogen-free conditions in a barrier-type facility at CINVESTAV-IPN. All animal experiments were approved by the Institutional Animal Care and Use Committee of CINVESTAV (Mexico City, Mexico).

IVM and Chemokine-Induced Arrest. Myo1e-KO and WT control mice were anesthetized with an i.p. injection of 12.5 mg/kg xylazine and 125 mg/kg ketamine hydrochloride (Sanofi, Mexico City, Mexico) and cremaster muscles were surgically prepared as described (9). Neutrophil–endothelial interactions were monitored 3 h after injection of 500 ng TNF-α (IVM), or directly after injection of 500 ng CXCL1 via the carotid artery (chemokine-induced arrest) as described (10). Postcapillary venules (20–40 μm diameter) were recorded using an intravital upright microscope (Axioscope A1, Zeiss) with a 40 × 0.75 saline immersion objective (Zeiss). Numbers of rolling and adherent neutrophils were analyzed by transillumination video microscopy. The numbers of transmigrated neutrophils were counted by DIC microscopy. Transmigrated neutrophils were counted in an area of 75 μm on each side of a venule over a length of 100 μm. Videos and images were analyzed using ImageJ (NIH) and Zen Blue Edition (Zeiss) software.

Data Availability. All data related to this manuscript are included either in the main manuscript or *SI Appendix*. Detailed methods are described in *SI Appendix*.

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