Moesin and myosin phosphatase confine neutrophil orientation in a chemotactic gradient

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Neutrophils respond to invading bacteria by adopting a polarized morphology, migrating in the correct direction, and engulfing the bacteria. How neutrophils establish and precisely orient this polarity toward pathogens remains unclear. Here we report that in resting neutrophils, the ERM (ezrin, radixin, and moesin) protein moesin in its active form (phosphorylated and membrane bound) prevented cell polarization by inhibiting the small GTPases Rac, Rho, and Cdc42. Attractant-induced activation of myosin phosphatase deactivated moesin at the prospective leading edge to break symmetry and establish polarity. Subsequent translocation of moesin to the trailing edge confined the formation of a prominent pseudopod directed toward pathogens and prevented secondary pseudopod formation in other directions. Therefore, both moesin-mediated inhibition and its localized deactivation by myosin phosphatase are essential for neutrophil polarization and effective neutrophil tracking of pathogens.

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Abbreviations used: CI, chemotaxis index; DIC, differential interference contrast; ERM, ezrin, radixin, and moesin; GEF, guanine nucleotide exchange factor; i.t., intratracheally; LSR, local Shwartzman reaction; MBS, myosin-binding subunit; MLC, myosin II light chain; MPO, myeloperoxidase; PRG, PDZ-RhoGEF; PTX, pertussis toxin; RhoGDI, Rho GDP-dissociation inhibitor.

Neutrophils are the first line of host defense against invading pathogens. To kill invading pathogens, neutrophils must attach to the blood vessel walls, transmigrate into tissues, reach the site of infection (via chemotaxis), and phagocytose pathogens (Kolaczkowska and Kubes, 2013).

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specifically implicated in the regulation of pseudopod formation (Swaney et al., 2010). Despite the importance of localized activation for cell polarization, the molecules that initiate cell polarization are still unknown (Swaney et al., 2010). Long-range inhibition is thought to be exerted by a fast diffusible global inhibitor, which is generated through localized activation and diffuses into the rest of the cell (Meinhardt, 2009). The classical function of a global inhibitor is to allow only one prominent leading edge to form (usually oriented toward the source of attractant) while preventing inefficient secondary pseudopod formation in other directions. However, after decades of intense research, there is still no experimental evidence of such a global inhibitor. Although backness signals such as RhoA and myosin II are recruited to the trailing edges and locally inhibit frontness signals, they are not the theoretical global inhibitors because they do not turn off attractant-induced backness signals (Xu et al., 2003). Instead, the activation of both the frontness and backness signals is considered to be a localized form of activation driven by receptor ligation (Narang, 2006). A recent report indicates that membrane tension, rather than diffusion-based inhibition, is responsible for long-range inhibition (Houk et al., 2012). Although both backness signals and membrane tension play important roles in preventing secondary pseudopod formation in chemokinesis, whether they play similar roles in directed cell migration (chemotaxis) remains unknown.

The ezrin, radixin, and moesin (ERM) proteins are crucial components for linking the actin cytoskeleton to the plasma membrane. Importantly they also participate in signal transduction (Bretscher et al., 2002). The ERM proteins can reciprocally regulate the small Rho GTPases through interaction with Rho guanine nucleotide exchange factors (GEFs [RhoGEFs]), Rho GTPase-activating proteins (RhoGAPs), and Rho GDP-dissociation inhibitors (RhoGDIs; Hirao et al., 1996; Takahashi et al., 1997; Tóhá et al., 2000; Hatzoglou et al., 2007; Valderrama et al., 2012). Moesin is the predominant ERM protein isoform in leukocytes such as neutrophils (Ivetic and Ridley, 2004). Moesin activity is self-inhibited by an intramolecular interaction between its N- and C-terminal domains, which upon activation bind to transmembrane proteins and the actin cytoskeleton, respectively (Reczek et al., 1997; Serrador et al., 1997; Yonemura et al., 1998). Activation of moesin is initiated by binding to PIP2 and stabilized by conserved phosphorylation at Thr558 (Hirao et al., 1996; Yoshinaga-Ohara et al., 2002). Upon attractant stimulation, neutrophils and lymphocytes polarize and migrate concurrently with the rapid dephosphorylation of moesin (Yoshinaga-Ohara et al., 2002; Brown et al., 2003; Lee et al., 2004; Martinek et al., 2013). This dephosphorylation of moesin is mediated by myosin phosphatase, which consists of a catalytic subunit (protein phosphatase 1c [PP1c]), a myosin-binding subunit (MBS), and a small subunit (Fukata et al., 1998; Kawano et al., 1999). In neutrophils, myosin phosphatase interacts with a front signaling molecule, the hematopoietic protein 1 (Hem-1; Weiner et al., 2006). How myosin phosphatase and moesin might regulate neutrophil chemotaxis remains unclear.

Here, we report that moesin was found to be required for the neutrophil chasing of invading pathogens, which allowed the formation of one prominent pseudopod extending toward the source of the attractant (e.g., bacteria) and prevented pseudopod formation in other directions. Moesin was constitutively active and membrane bound in resting cells and, thus, maintained cell symmetry by restricting the small GTPases Rac, Rho, and Cdc42 from interacting with their GEFs. To break symmetry, the cells used myosin phosphatase (activated via the G-βγHem-1 pathway) to terminate moesin-mediated inhibition at the would-be leading edges and initiated cell polarization and migration. Removal of moesin not only enhanced the activity of small Rho GTPases Rac, Rho, and Cdc42 but also induced cell protrusion in the wrong direction and thereby abolished the ability of the cells to catch bacteria. Furthermore, inhibition of these moesin-regulated GEFs fully or partially rescued the moesin-depleted phenotypes. Therefore, the inhibition–activation mechanism conferred by moesin and myosin phosphatase is critical for neutrophil polarization and orientation toward invading bacteria.

**RESULTS**

**Deletion of moesin impairs neutrophil-mediated microbial killing and inflammation**

To address the role of moesin in neutrophil-mediated microbial killing and inflammation, we monitored the killing of bacteria in mouse lungs after inducing pneumonia using *Pseudomonas aeruginosa* strain 103 (P4103) through intratracheal (i.t.) injection. We observed an augmented load of *P. aeruginosa* in moesin knockout (Msn−/−) lungs compared with WT lungs (Fig. 1 A; P < 0.01). Approximately 88% of the total 2 × 10^5 bacteria injected were killed in WT lungs, whereas only ~26% were killed in Msn−/− lungs (Fig. 1 B). To assess direct microbial killing by neutrophils, we isolated Msn−/− neutrophils and performed bacterial killing in vitro. Compared with WT neutrophils, Msn−/− neutrophils showed a significantly reduced microbial killing ability (Fig. 1 C; P < 0.01).

We also determined the function of moesin in neutrophil-mediated vascular inflammation using the modified local Shwartzman reaction (LSR; Brozna, 1990; Qian et al., 2009). The vascular inflammation induced during the LSR is mediated by neutrophils, as depletion of neutrophils prevents tissue injury (Qian et al., 2009). The dorsal skins of WT and Msn−/− mice were first injected s.c. with either 80 µg LPS (Fig. 1 D, left side of each panel) or PBS as a control (Fig. 1 D, right side of each panel). After 24 h, either 0.2 µg TNF or the same volume of PBS was injected s.c. into the site receiving LPS. We observed skin lesions resembling thrombohemorrhagic vasculitis, with visible hemorrhage and dermal tissue necrosis at the injection site (Fig. 1 D, right side of each panel). However, Msn−/− mice showed reduced vascular inflammation and injury compared with the WT (Fig. 1 D and E). Additionally, significantly decreased neutrophil accumulation at the LPS injection site was observed in Msn−/− mice compared with WT mice, as measured via tissue myeloperoxidase (MPO).
generated by the EZ-Taxiscan device, we observed that WT neutrophils migrated up the gradient with fewer turns, whereas Msn⁻/⁻ neutrophils frequently changed direction and exhibited poor directionality, showing a significantly lower chemotaxis index (CI; the ratio of net migration in the correct direction to the total migration length [Xu et al., 2005]) compared with the WT controls (0.51 vs. 0.83, P < 0.001; Fig. 1H). Thus, Msn⁻/⁻ neutrophils exhibited significantly decreased transmigration in vivo and chemotaxis in vitro.

Distinct activation pattern of moesin and myosin II
We continued to explore how moesin regulates neutrophil migration and sequestration. Although both moesin and the myosin II light chain (MLC) are localized at the trailing edges of migrating neutrophils (Yoshinaga-Ohara et al., 2002; Xu et al., 2003), we found that they exhibited different translocation and activation patterns. Moesin-YFP (stably expressed in differentiated human promyelocytic leukemia [HL60] cells) localized uniformly around the cell membrane in the resting state and dissociated from the membrane on the side of the cell where the leading edge started to form after fMLF stimulation (Fig. 2A, top; >100 cells were examined). The remaining membrane-bound moesin localized to the trailing edge and was almost absent from the leading edge (Fig. 2A, top).

activity (Fig. 1F; P < 0.01). Thus, deletion of moesin inhibited neutrophil microbial killing and neutrophil-mediated vascular inflammation.

Moesin is required for neutrophil infiltration and chemotaxis
The decreased bacterial clearance and inflammation in Msn⁻/⁻ mice described above may result from decreased neutrophil infiltration. Therefore, we studied bacterial formyl peptide fMet-Leu-Phe (fMLF; 10 nM)–induced mouse neutrophil infiltration into the peritoneal cavity in vivo. WT and Msn⁻/⁻ mice were injected with either saline or 10 nM fMLF. After 4 h, peritoneal neutrophils were recovered and counted. fMLF substantially induced neutrophil transmigration into the peritoneal cavity in WT mice (Fig. 1G). However, Msn⁻/⁻ neutrophils showed markedly reduced transmigration as compared with the WT cells (Fig. 1G).

Next, we determined whether moesin regulates neutrophil adhesion and migration, which are both required for neutrophil tissue infiltration. We isolated Msn⁻/⁻ neutrophils and tested their adhesive and chemotactic behaviors in vitro. Interestingly, deletion of moesin did not affect neutrophil adhesion to WT mouse lung vascular endothelial cells (not depicted). When applied to a chemotactic gradient generated by the EZ-Taxiscan device, we observed that WT neutrophils migrated up the gradient with fewer turns, whereas Msn⁻/⁻ neutrophils frequently changed direction and exhibited poor directionality, showing a significantly lower chemotaxis index (CI; the ratio of net migration in the correct direction to the total migration length [Xu et al., 2005]) compared with the WT controls (0.51 vs. 0.83, P < 0.001; Fig. 1H). Thus, Msn⁻/⁻ neutrophils exhibited significantly decreased transmigration in vivo and chemotaxis in vitro.
Inhibition and initiation of neutrophil migration

Activated neutrophils rapidly migrate toward sites of infection. Translocation of actin filaments from the cytosol to the cell membrane is a key event in neutrophil polarization, a process that enables neutrophils to migrate toward pathogens or in a gradient. There are multiple actin cytoskeletal proteins and regulatory mechanisms that collaborate to ensure neutrophil migration. Moesin and myosin light chain (MLC) are two such proteins that play crucial roles in neutrophil migration.

**Moesin and MLC regulate neutrophil migration.**

Moesin and MLC are both involved in neutrophil polarization and migration. Moesin is a cytoskeletal protein that promotes cell spreading and remodelling. MLC, on the other hand, is a protein that regulates the contraction of the actin cytoskeleton.

**Results:**

1. **Figure 2:** Differential activation of moesin and MLC. Attraction-induced localization of moesin at the trailing edge.

2. **Figure 3:** Disruption of actin organization by Rho kinase inhibitor Y27632.

3. **Figure 4:** Inhibition of the pseudopods by Y27632.

**Conclusion:**

Moesin and MLC are key regulators of neutrophil migration. Understanding the molecular mechanisms that govern their activity can provide insights into the regulation of neutrophil function and migration.
observed under stimulation with a different chemoattractant, IL-8 (Fig. 4 D).

Upon encountering the pathogen Candida albicans, resting control cells polarized and migrated directly toward C. albicans (Fig. 4 E, top). Stable pseudopods protruded in the correct direction (toward the pathogen) and eventually engulfed the pathogen (Fig. 4 E, top; ~79% of 91 cells tested caught the pathogen). In contrast, moesin RNAi–treated cells pro-

In contrast, moesin RNAi–treated cells were not able to form a single prominent pseudopod pointing to the pipette (Fig. 4 A, middle; and Video 3). These cells presented randomly protruding pseudopods and changed directions frequently, sometimes even migrating down the gradient. Overall, these cells traced circuitous paths and spent more time in deflective directions compared with control cells (Fig. 4 A, middle; and Video 3). Hence, their directional sensing ability was severely damaged, and they showed a significantly decreased CI (0.15 vs. 0.78, P < 0.001; Fig. 4 B) and a much slower speed compared with the controls (2.0 vs. 5.0 µm/min, P < 0.001; Fig. 4 C; speed was measured by tracking pseudopod movement). Thus, moesin and not MLC determined cell orientation in an attractant gradient. Similar results were observed under stimulation with a different chemoattractant, IL-8 (Fig. 4 D).

A small amount of ezrin is expressed in neutrophils (~10% of total ERM proteins [Ivetic and Ridley, 2004]). However, we found that the ezrin expression level was not altered in moesin-depleted cells compared with the control (Fig. 3 B).
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In contrast to the uropod localization of moesin, ezrin translocated to the pseudopod in polarized cells (Fig. 3 D). Furthermore, knockdown of ezrin had little effect on cell migration (Fig. 3, E–H). Thus, ezrin exhibited a different translocation pattern from moesin and did not determine cell orientation during directed neutrophil migration.

Constitutively active moesin inhibits cell migration

Moesin was deactivated and translocated away from pseudopod (Fig. 2), suggesting that moesin could be an inhibitor of pseudopod protrusion. We therefore predicted that enhancing moesin activity would block cell migration. To test this hypothesis, we stably expressed a YFP-tagged phosphomimetic mutant, moesin-T558D (Thr558 was mutated to Asp), in HL60 cells. Similar to WT moesin-YFP, moesin-T558D-YFP was membrane bound in the resting state but showed very little dissociation from the cell membrane after uniform application of fMLF (Fig. 5 A; >100 cells were examined). We observed that cells occasionally extended transient ruffles, but they were retracted shortly thereafter. Thus, expression of moesin-T558D inhibited cell polarization and migration.

When cells expressing moesin-T558D were exposed to an fMLF concentration gradient, the cells exhibited unstable migration with poor directionality. The CI was significantly lower in cells expressing moesin-T558D compared with WT controls (0.33 vs. 0.84, P < 0.001; Fig. 5 B). In contrast, cells expressing moesin-T558A (in which Thr558 is mutated to Ala), which cannot be phosphorylated at Thr558, showed a similar CI to the WT controls (Fig. 5 B). Thus, dephosphorylation of moesin was shown to be a prerequisite for cell migration, whereas expressing moesin-T558D inhibited cell migration.

Moesin suppresses RhoA, Rac, and Cdc42 through the inhibition of GEFs

We next examined the potential mechanisms by which moesin regulates cell migration. Moesin regulates Rho GTPase activity (Speck et al., 2003). Thus, we examined the activity of three major Rho GTPases, RhoA, Rac, and Cdc42, in both
Vav1 mediates the radixin-dependent increase in Rac activity (Valderrama et al., 2012) and is required for neutrophil activation by the integrin receptor (Cremasco et al., 2008). We found that the N terminus (aa 1–310) but not C terminus (aa 457–577) of moesin bound the DH/PH domain of Vav1 (aa 160–550), the GEF domain known to activate Rac (Fig. 6A).

To determine whether N-moesin inhibits the interaction of Rac with Vav1-DH/PH, we coexpressed FLAG-Rac with HA–Vav1-DH/PH in either the presence or absence of GFP–N-moesin. The Vav1-DH/PH fragment was pulled down with Rac in the absence of N-moesin, whereas binding was significantly inhibited in the presence of N-moesin (Fig. 6B; P < 0.05). Thus, moesin may inhibit Rac activation by reducing the interaction of Rac with the Vav1-DH/PH domain.

We further examined how moesin inhibited the small Rho GTPases. As Rho GTPase activation requires GEFs, we tested the hypothesis that moesin interferes with GEFs on the cell membrane, preventing them from activating the Rho GTPases.

control and moesin RNAi–treated cells. Attractant stimulation (100 nM fMLF, 2 min) increased RhoA-GTP (active form) levels by 1.3-fold over basal levels (Fig. 5C). In moesin RNAi–treated cells, the basal level of RhoA-GTP was also increased by ~1.5-fold compared with the control and was not further increased after fMLF stimulation (Fig. 5C). Therefore, moesin antagonized RhoA activity at the resting state. Similarly, p-MLC, Cdc42-GTP, and Rac-GTP were also substantially increased in moesin RNAi–treated cells, both before and after fMLF stimulation (Fig. 5D–F). Thus, moesin maintained basal cell symmetry by inhibiting Rac, Rho, and Cdc42 activity.

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shown to activate RhoA in HL60 cells (Wong et al., 2007). We found that N-moesin bound the DH/PH domain of PRG and prevented RhoA binding to the domain (Fig. 6, C and D). Similarly, N-moesin blocked Cdc42 binding to the DH/PH domain of α-PIX, a GEF for Cdc42 (Fig. 6, E and F). Thus, at the basal stage, membrane-bound N-moesin masked the DH/PH domain of RhoGEFs and prevented their interaction with and activation of Rho GTPases. Based on this finding, we surmised that overexpressing the N-terminal domain of moesin would prevent the formation of neutrophil polarity and cell migration. We observed that the cells expressing GFP–N-moesin exhibited severe defects in both cell polarization and migration (Fig. 5 G).

Knockdown of α-PIX restores cell migration in moesin RNAi cells

We next investigated whether increased Rho GTPase activity is responsible for the impaired polarization and migration observed in moesin knockdown cells. Moesin RNAi cells were treated with RNAi constructs specific for GEFs described above. Knockdown of α-PIX in these moesin RNAi cells reduced the elevation of Cdc42 activity and rescued the migratory defects found in moesin knockdown cells (Fig. 7, A–C).

These α-PIX and moesin double knockdown cells showed relatively stable polarity (Fig. 7 D), formed fewer multiple pseudopods (Fig. 7 D and Table S5), and exhibited a longer lifetime of pseudopods compared with moesin single knockdown cells (Fig. 7 E). Additionally, these cells migrated with fewer turns in an fMLF gradient (Fig. 7 A) and showed significantly higher CI values than moesin RNAi cells (0.84 vs. 0.35, P < 0.001), and the CI was similar to that of control cells (Fig. 7 B).

Knockdown of Vav1 also partially rescued moesin knockdown phenotypes. The Vav1 and moesin double knockdown cells exhibited better polarization and CI values compared with moesin single knockdown cells though these parameters still inferior to those in normal control cells (Fig. 7, A–E).

Myosin phosphatase mediates moesin dephosphorylation and initiates cell migration

Moesin inactivates both frontness and backness signals in resting neutrophils through the inhibition of Rho GTPases, as described above. Hence, to undergo polarization and migration, cells must deactivate moesin-mediated inhibition and thereby initiate cell migration. We next addressed the mechanism responsible for breaking the symmetry and initiating cell migration. We focused on the role of myosin phosphatase,
which dephosphorylates both moesin and MLC (Fukata et al., 1998). Both the catalytic subunit (PP1c) and the MBS of myosin phosphatase are communoprecipitated with the Hem-1 complex, which organizes the neutrophil’s leading edge (Weiner et al., 2006). We first confirmed PP1c localization to the leading edge by expressing YFP-tagged PP1c in HL60 cells. PP1c-YFP localized to both the cytosol and the nucleus in the basal stage. A uniform concentration of fMLF (100 nM) induced the recruitment of PP1c-YFP to the cell periphery and subsequently to the leading edge in polarized cells (Fig. 8 A; a time course of PP1 localization is shown in Table S2).

To determine whether PP1c recruitment to the leading edge mediates moesin dephosphorylation, we knocked down PP1c in HL60 cells via RNAi (Fig. 8 B). Knockdown of PP1c prevented the dephosphorylation of moesin but did not alter the basal level of p-moesin (Fig. 8 C). This finding indicated that PP1c was responsible for the fMLF-induced dephosphorylation of moesin. Next, we transiently expressed moesin-YFP in PP1c knockdown cells to assess moesin translocation. In PP1c-depleted cells, moesin–YFP was membrane bound and showed little dissociation from the membrane. Transient ruffle protrusion was observed, but these ruffles retracted shortly (Fig. 8 D, top). Similar results were observed in MBS (the regulatory subunit of myosin phosphatase) knockdown cells (Fig. 8, B–D).

When control cells were exposed to an fMLF gradient, they migrated up the entire gradient (Fig. 9 A). PP1c RNAi–treated cells also migrated, but with poor directionality (Fig. 9 A), and the CI was significantly lower in PP1 RNAi–treated cells compared with controls (0.26 vs. 0.72, P < 0.01; Fig. 9 B). The migration speed of PP1c RNAi–treated cells was also significantly decreased (11.5 vs. 18.4 µm/min, P < 0.01; Fig. 9 C). Similar results were obtained in MBS RNAi cells (Fig. 9, A–C). Expression of the moesin-T558A mutant, but not WT moesin, partially restored cell migration in PP1c RNAi cells (Fig. 9, D and E). Collectively, our data indicate that inhibition of myosin phosphatase prevented moesin dephosphorylation and dissociation from cell membrane, thus causing unstable cell polarity and impaired cell migration.

Myosin phosphatase is recruited to the pseudopods by front signals

We next investigated how myosin phosphatase is activated and recruited to the leading edges during cell polarization. Previous studies have shown that the interaction of PP1c with MBS significantly enhances the myosin phosphatase activity
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We performed an immunoprecipitation analysis of the interaction between PP1c and MBS before and after stimulation. The interaction between PP1c and MBS was enhanced after fMLF stimulation (Fig. 9 F), suggesting that myosin phosphatase was activated after attractant stimulation. As myosin phosphatase interacts with Hem-1 (Weiner et al., 2006), we knocked down Hem-1 in HL60 cells and examined PP1c translocation. We observed that the recruitment of PP1c to the front was severely impaired in Hem-1 RNAi cells (Fig. 9 G), indicating the role of Hem-1 in recruiting PP1c to the leading edge. Therefore, the recruitment of PP1c to the leading edge depends on the frontness signals. We further validated this finding by inhibiting the Gi-mediated frontness signals with pertussis toxin (PTX). We found that PP1c recruitment at the leading edge was blocked in PTX-treated cells (Fig. 9 G). Together these findings show that recruitment of PP1c to the front critically depends on Gi-mediated frontness signals.

**DISCUSSION**

We have demonstrated an essential role for moesin in regulating neutrophil polarization and directional sensing, both of which are crucial for neutrophils to locate invading pathogens. Deletion of moesin not only blocks neutrophil directionality toward invading bacteria but also diminishes neutrophil-mediated microbial killing and inflammation. These findings expand the previous frontness–backness model (Xu et al., 2003), demonstrating that moesin functions as a symmetry-maintaining molecule that inhibits both frontness and backness signals in the resting state. Myosin phosphatase disrupts this inhibition to initiate cell polarization. After cell polarization, moesin localizes to the sides and posterior of cells to maintain the correct cell orientation while cells chase invading pathogens. Therefore, the maintenance of cell symmetry and orientation requires the same inhibitory mechanism exerted by moesin. In our new model, attractants from bacteria not only activate the frontness and backness pathways that are essential for pseudopod and uropod formation but also promote myosin phosphatase to release moesin-mediated inhibition at the would-be leading edge, thus initiating cell polarization.

Previous reaction–diffusion models assume the existence of a global inhibitor, but biochemical evidence of such an inhibitor is still lacking. Hence, concerns have been raised about whether such a global inhibitor exists, and new models without a global inhibition component have been proposed (Onsum and Rao, 2007). Here, we provide evidence that moesin inhibits frontness and backness signals and determines cell orientation but functions differently from the theoretical global inhibitor. First, moesin-mediated inhibition was not generated through localized activation. Instead, moesin was constitutively active in the resting state and was deactivated via localized activation upon stimulation. Second, moesin did not require diffusion to function because moesin was already membrane bound and active in the basal stage. This finding offers an alternative explanation for the observation (Ichikawa et al., 1996; Terrak et al., 2004).
moesin and MLC did not always overlap at the trailing edges (Fig. 3 A, left). Furthermore, the existence of both activators (e.g., GEFs) and inhibitors (e.g., moesin) for RhoA/MLC in the uropod may be responsible for the previously reported fluctuation of RhoA activity and the rear traction force (Wong et al., 2006; Shin et al., 2010), which are required for the efficient rear contraction.

We found that myosin phosphatase was recruited to the cell's leading edge and the recruitment depended on Hem-1– and Gi-mediated frontness signals (Fig. 9). In this sense, myosin phosphatase is a key frontness molecule. Furthermore, our results showed that the initiation of cell polarization was mediated by the activation of myosin phosphatase. Upon attractive stimulation, myosin phosphatase translocated to the cell membrane and dephosphorylated moesin, releasing moesin-mediated inhibition and enabling pseudopod protrusion. Importantly, removal of this phosphatase abolished cell protrusion and migration. Thus, in our new model, the initiation of cell polarization is not required for long-range inhibition as reported previously (Houk et al., 2012).

We demonstrated that moesin mediated symmetry in resting neutrophil by inhibiting Rac, RhoA, and Cdc42 activity. This inhibition was caused by moesin's role in preventing the GEF activation of Rho GTPases. Moesin may also regulate Rho GTPases by interacting with RhoGDIs (Hirao et al., 1996; Takahashi et al., 1997). Therefore, moesin likely interacts with RhoGD1 and GEFs spatially and temporally to regulate Rho GTPase activities. After cell polarization, backness signals such as RhoA and MLC were activated at the trailing edge while moesin was also present. One possible mechanism that may explain this observation is that other GEFs, which are not inhibited by moesin, can mediate the activation of RhoA. For example, both p115RhoGEF and LARG GEF are regulated by G12/13 and can activate RhoA (Hart et al., 1998; Fukuhara et al., 2000). We found that moesin did not inhibit RhoA binding to p115RhoGEF (unpublished data). We also observed that moesin and MLC did not always overlap at the trailing edges (Fig. 3 A, left). Furthermore, the existence of both activators (e.g., GEFs) and inhibitors (e.g., moesin) for RhoA/MLC in the uropod may be responsible for the previously reported fluctuation of RhoA activity and the rear traction force (Wong et al., 2006; Shin et al., 2010), which are required for the efficient rear contraction.

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Migration is a function of counteracting moesin-mediated basal inhibition in resting neutrophils.

Although moesin is a crucial inhibitory signal responsible for cell migration, other inhibitory signals have also been reported. For example, the Erk-GRK2 signaling pathway can negatively regulate neutrophil migration (Liu et al., 2012). In other studies, blocking the activities of signaling agents such as the microtubule cytoskeleton (Xu et al., 2005) and calpain (Lokuta et al., 2003) was shown to enhance basal migration. Interestingly, inhibition of calpain increases Rac and Cdc42 activities (Lokuta et al., 2003), and removal of microtubules enhances RhoA activity (Xu et al., 2005), whereas inhibition of moesin increased the activity of all three Rho GTPases. Furthermore, microtubules and upstream regulators such as GSK3β also contribute to directional sensing in neutrophils (Xu et al., 2005, 2007). Therefore, it is possible that in a well-conserved response such as directed neutrophil migration, multiple inhibitors functioning together to provide the global inhibition is necessary for the correct cell orientation and location of pathogens. Together, our data suggest that moesin and myosin phosphatase mediate a novel regulatory pathway that is essential for the innate immune response of neutrophils, which may provide novel therapeutic targets for enhancing neutrophil migration and improving their bactericidal function in inflammatory diseases.

**MATERIALS AND METHODS**

**Antibodies, reagents, and mice.** Mouse monoclonal antibody against moesin and rabbit polyclonal antibodies against moesin IIA were from Sigma-Aldrich. Rabbit polyclonal antibodies against phosphorylated moesin light chain (Ser19) and moesin were purchased from Cell Signaling Technology. Rabbit polyclonal antibody against RhoA was purchased from Thermo Fisher Scientific. Mouse monoclonal antibody against GAPDH was from Proteintech. Rabbit polyclonal antibodies against HA were purchased from Signalway Antibody. Mouse monoclonal antibody against Flag and light chain (Ser19) and moesin were purchased from Cell Signaling Technology. Antibodies, reagents, and mice.

WT C57BL/6 mice were obtained from Charles River, and Msn−/− mice were described previously (Do et al., 1999). These genotypes have been backcrossed onto a C57BL/6 background for more than eight generations. The moesin gene is located on the X chromosome. Male hemizygous (Msn−/Y) and littermate WT (Msn+/+) mice, 8–10 wk of age, were used for experiments. Mice were bred and housed in pathogen-free conditions with access to food and water ad libitum in the Animal Care Facility. All experiments were performed 4–6 h after transfection.

**Modified LSR model and bacterial killing.** Age-matched 8–10-wk-old WT and Msn−/− mice were anesthetized by i.p. injection of 100 mg/kg ketamine and 10 mg/kg xylazine, and the dorsal skin was shaved. 80 µg LPS (O555:BS; Sigma-Aldrich) in 80 µl PBS was injected into the right dorsum. As a negative control, 80 µl PBS was injected into the left dorsum. 24 h later, 0.2 µg TNF in 80 µl PBS was injected into the same point on the right dorsum and 80 µl PBS into the left. 24 h after TNF injection, the mice were sacrificed, and the interior of the dorsal skin was exposed for microscopic examination. The tissues were either fixed in 10% formalin for histological analysis with hematoxylin/eosin staining or frozen at −80°C for MPO activity assay.

**Cell culture, transfection, and isolation of mouse neutrophils.** Procedures for cultivation and differentiation of HL60 have been described previously (Xu et al., 2003). For transient transfections, differentiated HL60 cells (on day 6 after addition of DMSO) were washed once in RPMI-Hepes and suspended in the same medium to a final concentration of 10^6/ml. DNA was then added to the cells (30 µg PPI-YFP or MLC-YFP DNA), and the cell–DNA mixture was incubated for 10 min at 25°C, transferred to electroporation cuvettes, and subjected to an electroporation pulse on ice at 310V, 1.180 µF and low resistance. Transfected cells were allowed to recover for 10 min at 25°C and then transferred to 20 ml complete medium. Subsequent assays were performed 4–6 h after transfection.

**Bone marrow neutrophil isolation, mice were sacrificed, and femurs and tibias were taken out and flushed with a 27G needle with a 10-ml syringe filled with calcium- and magnesium-free HBSS plus 0.1% BSA. Cells were then centrifuged and resuspended in HBSS. After filtering with a 40-µm strainer, cells in 3 ml HBSS were loaded onto a preprepared gradient solution (3 ml Nycodextrin on the top and 3 ml of 72% Percoll on the bottom). The samples were centrifuged at 2,400 rpm at room temperature for 20 min without break. The middle layer was collected and washed once in HBSS. To remove the red blood cells, 9 ml of sterilized distilled water was added for 22 s and after 1 ml of 10× PBS. Finally, the cells were collected and resuspended in HBSS or medium.

**Lung tissue MPO activity.** Lung tissue MPO activity was measured as described previously (Garreau et al., 2006). In brief, skin or lung tissues were flushed free of blood by PBS and homogenized in 50 mM phosphate buffer (PB), pH 6.0. The homogenates were centrifuged at 40,000 g at 4°C for 30 min. After discarding the supernatants, the pellets were resuspended in PB buffer containing 0.5% hexadecyl trimethylammonium bromide and vigorously vibrated to break up the large pellets. Then the pellets were frozen at −70°C for 30 min and thawed at 37°C. Subsequently, the pellets were homogenized and centrifuged a second time. Thereafter, the supernatants were used for MPO activity assay with a kinetics reading at 460 nm for 5 min. Neutrophil sequestration was quantified at MPO activity normalized by tissue weight, and the data were presented as V-Max value/g tissue.

**Cell migration assays.** Live cells were imaged after stimulation either with a uniform concentration of FMLF or a concentration gradient generated by an EZ-Taxiscan device or micropipette. EZ-Taxiscan assay was described previously (Liu et al., 2012). In brief, cells migrated over a 50 µg/ml fibronectin–coated cover glass on a horizontal glass surface under a silicon chip. Cells were washed with RPMI, 25 mM Hepes, pH 7.0, and 0.1% BSA and
resuspended in RPMI, 25 mM Hepes, and 0.1% BSA solution. Cells were loaded to the bottom of the chip, and chemotactrant was added to the top of the chip to generate a chemotactrant gradient. Cells migrated for 30 min, and images were recorded with the EZ-Texascan software and then analyzed in ImageJ (National Institutes of Health). For micropetette assay, the gradient was generated by a point source of chemotactrant from a micropetette containing 10 μM fMLF. Time-lapse video microscopy was performed as described previously (Xu et al., 2003). The cell migratory behaviors were recorded and analyzed in ImageJ. For neutrophil peritoneal transmigration, mice were injected with 100 μl saline or 10 nM fMLF (in 100 μl saline, i.p.). After 4 h, peritoneal cavities of anesthetized mice were lavaged, and leukocytes were recovered. The total number of leukocytes was counted using a hemocytometer, and neutrophil counts were determined on 100-μl cytopsin stained with Diff-Quik and presented as a percentage of the total population.

Pull-down assays. RhoA, Cdc42, and Rac pull-down kits were purchased from Thermo Fisher Scientific, and assays were performed according to attached protocols. In brief, HL60 cells were stimulated with or without 100 nM fMLF for 1 min and immediately lysed and centrifuged at 14,000 g for 10 min. Equal amounts of the resulting supernatant fractions were incubated with either rhodomin RBD-agarose (which binds RhoA-GTP) or PBD-agarose (which binds Rac-GTP and Cdc42-GTP) for 2 h at 4°C, followed by washing three times with lysis buffer. Proteins binding to the beads were eluted in protein loading buffer and subjected to Western blot analysis using rabbit polyclonal antibody specific to RhoA, Cdc42, or Rac.

Immunoprecipitation and immunofluorescence. Immunoprecipitation and immunofluorescence were performed as described previously (Xu et al., 2008). Densitometry of bands on autoradiograms was performed with scanned x-ray films and the ImageJ program. Results of at least three independent experiments are represented as a bar graph using arbitrary units to compare the intensity of the bands.

Statistical analysis. Statistical comparisons were made using the two-tailed Student’s t test. Experimental values are reported as the mean ± SEM. Differences in mean values were considered significant at P < 0.05.

Online supplemental material. The supplemental videos show HL60 cells left untreated (Video 1) and Y27632-treated (Video 2) or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times and distribution of moesin and MLC knockdown. Table S2 shows the percentages of polarized HL60 cells stimulated with fMLF at the indicated times and distribution of moesin and MLC knockdown. Table S3 shows the percentages of polarized HL60 cells (left untreated or left untreated or Y27632 treated) stimulated with fMLF at the indicated times and distribution of moesin and MLC knockdown. Table S2 shows the percentages of polarized HL60 cells stimulated with fMLF at the indicated times and distribution of moesin and MLC knockdown. Table S3 shows the percentages of polarized HL60 cells stimulated with fMLF at the indicated times and distribution of moesin and MLC knockdown. Table S4 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 A) and distribution of moesin or MLC in uropod in these polarized cells. Table S4 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C) and distribution of moesin or MLC in uropod in these polarized cells. Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C). Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C). Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C). Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C). Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C). Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C). Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C). Table S5 shows the distribution of MLC to uropod in control or moesin RNAi–treated HL60 cells stimulated with fMLF at the indicated times (Fig. 3 C).

In memory of Dr. Graeme K. Carnegie.

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