Myeloid Cells in Health and Disease: A Synthesis
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Role of the Cytoskeleton in Myeloid Cell Function

INTRODUCTION

The intracellular cytoskeleton, consisting of filamentous actin (F-actin), microtubules (MTs), and intermediate filaments, makes up a network of dynamic polymeric structures that regulate cell shape and function (1). Chemotaxis and phagocytosis, two essential myeloid cell functions that enable them to defend the host against harmful opportunistic microorganisms, rely heavily on a highly dynamic and multifunctional cytoskeletal network (2–4). The importance of the cytoskeleton in myeloid cells is emphasized by human innate immune dysfunction syndromes that arise from defects in cytoskeletal proteins or the proteins that regulate cytoskeletal function (5–7). Although most of our knowledge of cytoskeletal structure and function comes from fibroblasts and other cell types, myeloid cells are valuable model systems that have been exploited to study the diverse roles and functions of the cytoskeleton. In this chapter, we will review the current knowledge with respect to cytoskeletal regulation of myeloid cell function, with an emphasis on neutrophils and macrophages.

ACTIN

The actin cytoskeleton is intricately involved in many fundamental aspects of eukaryotic cell biology. Myeloid cells, as important protective cells of the innate immune system, have many unique and diverse applications of common cellular functions, which require distinct regulation of the actin cytoskeleton compared to other cell types, such as fibroblasts. Myeloid cell functions that require unique regulation of the actin cytoskeleton include phagocytosis of pathogens, the ability to squeeze into tight spaces, and regulation of cell morphology and adhesion to facilitate fast cell migration. The dynamic regulation of the actin cytoskeleton is an essential component of membrane receptor-initiated myeloid cell responses including changes in cell shape (8, 9), rapid cell motility (10), phagocytosis (11, 12), transepithelial migration (9), and signal transduction and transcriptional regulation (13, 14). The actin cytoskeleton is composed of a network of actin filaments (F-actin), which arise from the polymerization of actin monomers. Six highly homologous actin monomer isoforms are expressed in humans. Four of the six actin isoforms ($\alpha_{skeletal}$-actin, $\alpha_{cardiac}$-actin, α_{smooth}-actin, and γ_{smooth}-actin) are specific to muscle tissues, while B_{cyto}-actin and γ_{cyto}-actin are ubiquitously expressed in all cells, including those of myeloid origin (13). Due to the intrinsic polarity of actin monomers, actin polymers are characterized by a distinct barbed (+) end and a pointed (–) end. Actin monomers actively assemble at

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barbed ends and disassemble at pointed ends in a process known as F-actin treadmilling. Although this linear model of polymerization and depolymerization suitably describes actin dynamics in a cell-free system, actin regulation is much more complex *in vivo* (15). Living cells organize their actin filaments into complex three-dimensional networks, bundles, and gels via interaction with a variety of actin-binding proteins. These include proteins that promote *de novo* nucleation of actin filaments (e.g., Arp2/3 complex, formins, Cobl, Spire, and leiomodin), proteins that sever actin filaments and generate new barbed ends and thus promote F-actin remodeling (e.g., actin-depolymerizing factor [ADF]/cofilins, gelsolin, and adseverin), proteins that sequester actin monomers and retard actin filament polymerization (e.g., profilin, twinfilin, and β-thymosins), proteins that limit F-actin polymerization by capping the barbed end of F-actin strands (e.g., CapG, Eps8, and Ena/vasodilator-stimulated phosphoprotein [VASP]), and finally actin-binding proteins that bundle and cross-link preexisting actin filaments (e.g., α-actinin and fascin) (reviewed in 15–17). Together, these actin-binding proteins mediate the length, flexibility, and viscosity of the actin network, altering its emergent properties, to orchestrate changes in cell morphology and function.

Rho GTPases in Actin Assembly

Leukocytes recognize and respond to bacterial by-products and endogenous proinflammatory mediators, aggregate at sites of inflammation, and eliminate invading pathogens (18). The coordination of these events requires the recognition of an extracellular stimulus, the transduction of the signal across the plasma membrane, and an appropriate change in cell state. Rho family small GTPases are critical signaling nodes that interpret extracellular stimuli and orchestrate a wide range of cellular responses (19–21), including dynamic regulation of the actin cytoskeleton (22, 23). In leukocytes, they are important regulators of chemotaxis (24), phagocytosis (2), degranulation (25), and production of reactive oxygen species (ROS) (26, 27). Their activity is regulated by cycling between an inactive, GDP-bound form and an active, GTP-bound form. Loading of the small GTPases with GTP by guanine nucleotide exchange factors (GEFs) induces a conformational change that promotes the activation of downstream effector molecules. The prototypical Rho family GTPases are RhoA, Rac1, and Cdc42. In crawling fibroblasts, Rac1 and Cdc42 are responsible for protrusive F-actin-based structures at the leading edge during migration (28), and RhoA is required for formation of F-actin stress fibers and integrin-based focal adhesions (29–31). These factors play somewhat analogous roles in myeloid cells (32); however, the cytoskeletal structures formed are different. For example, in macrophages the small GTPases generate distinct focal complexes and fine actin cables (33). Important downstream effector molecules of the Rho GTPases include serine/threonine kinases, such as p21-activated kinase (PAK) (34) and Rho-associated protein kinase (ROCK) (35); actin nucleating proteins such as the formin mDia (mammalian homolog of *Drosophila* diaphanous) (36); and members of the WASP (Wiskott–Aldrich syndrome protein)/WAVE (WASP-family verprolin homologous protein) family that promote nucleation through Arp2/3 (37). Mutations in WASP cause Wiskott-Aldrich syndrome, a severe human immunodeficiency syndrome characterized by myeloid cell defects (38–40).

During neutrophil chemotaxis, Rac GTPases promote F-actin formation at the leading edge and coordinate directional migration (41–43). Rac2 is the predominant Rac isoform in neutrophils, making up 80 to 95% of total Rac protein (44). Although Rac1 and Rac2 have >90% homology, they have nonredundant roles in neutrophil function (45). Rac1-deficient neutrophils fail to properly orient in a chemotactic gradient, accompanied by the formation of multiple randomly oriented lamellipodia (45, 46), while Rac2-deficient cells show defective F-actin assembly and reduced cell migration speed; however, they are still able to orient in a chemotactic gradient (47). Rac1 controls the uncapping of existing F-actin barbed ends, whereas Rac2 plays a major role in the extension of actin filaments via cofilin and Arp2/3-dependent mechanisms (48). Distinct activation kinetics and different roles for Rac1 and Rac2 have been demonstrated in response to low versus high concentrations of the formylated tripeptide chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (42). In the low range of stimulatory fMLP concentrations, Rac1 is activated and initiates cell spreading. At high stimulatory fMLP concentrations, Rac1 is first activated to initiate the formation of a lamellipod at the leading edge, but Rac2 is further required for continuous expansion of the lamellipod, which drives effective migration. In addition, Rac1 and Rac2, through their roles as part of the NADPH oxidase complex, are both required to produce ROS at the neutrophil leading edge, which is essential for directional migration through a redox-mediated feedback loop to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (49).

Several Rac GEFs have been implicated in stimulation of F-actin at the leading edge in neutrophils. The Rac GEF P-Rex1 is activated downstream of G protein-

coupled receptors (GPCRs) and promotes neutrophil polarization and leading edge F-actin formation (50). This occurs through stimulation of RhoG, which activates another Rac GEF, DOCK2, to stimulate Rac1 activity at the leading edge. DOCK2 was shown to localize at the leading edge and trigger neutrophil migration and polarity in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (41). Also, the adaptor protein 3BP2 was shown to be required for localization of P-Rex1 and another Rac GEF, Vav1, at the leading edge to stimulate Rac2 activity, neutrophil activation, and F-actin polymerization (51).

RhoA promotes cell contractility through ROCK and stimulates actin polymerization through mDia. ROCK phosphorylates LIM kinase, MLC phosphatase, and the myosin light chain (MLC), leading to the stabilization of actin filaments and formation of actomyosin bundles, whereas mDia nucleates actin polymerization (52). Stimulation of cell surface cytokine and chemokine GPCRs stimulates Rho-dependent actin polymerization and actomyosin contractility to orchestrate cell polarization, adhesion, and protrusion during migration (53). In neutrophils stimulated with fMLP, active RhoA localizes at the sides and back (54), where it promotes uropod contractility through actomyosin. Inhibition of RhoA causes tail retraction defects in monocytes (55), neutrophils (46, 56, 57), eosinophils (58), and lymphocytes (59). In addition to deadhesion at the rear, RhoA-dependent contractility also strengthens integrin-based adhesions in neutrophils (60), lymphocytes (61), and fibroblasts (62). In resting neutrophils, RhoA deficiency caused random migration, F-actin cap formation, elongated uropods, increased granule exocytosis, and elevated ROS production (63). The authors show that in the absence of stimulation RhoA acts to limit neutrophil priming and hyperresponsiveness through formins.

Inhibition of the RhoA pathway may represent a novel target for immunosuppression and antirejection therapies through effects on neutrophil migration. Modulation of the cytoskeleton through specific activation of RhoA and Rac1 is thought to be a critical mechanism of action of the small-molecule anti-inflammatory drug pomalidomide (64). The Rho kinase inhibitor fasudil has therapeutic efficacy in a number of human conditions (65, 66) and may also have immunosuppressive effects.

THE ROLE OF ACTIN IN MYELOID CELL RECRUITMENT

The recruitment of leukocytes from the bloodstream to a site of infection, inflammation, or injury entails multiple bidirectional signaling and adhesive interactions between the leukocytes and vascular endothelial cells, resulting in attachment of leukocytes to the endothelium near the affected area, rolling and arrest, crawling, and transmigration of the leukocyte across the endothelium (2, 67). Each of these processes relies on intricate regulation of the actin cytoskeleton, as discussed below.

Rolling

Nonpolarized neutrophils in the circulation typically have a robust cortical-actin cytoskeleton. The ability of rolling neutrophils to be captured by the endothelial surface depends on deformability of the cell. Computer simulations and experimental evidence suggest that loss of cell deformability limits rolling interactions and cell capture under shear stress (68–70). Treatment with cytochalasin, an inhibitor of actin polymerization, increases neutrophil deformability (71), resulting in a larger contact area with the endothelium and consequently slower and more stable rolling. This suggests that changes in the polymerization state of leukocyte cortical actin can raise or lower the threshold at which stable rolling can occur. Neutrophils isolated from the wounds and peripheral blood of burn victims display increased polymerized actin content, increased actin stability, and reduced random motility and chemotaxis (72). The increase in actin stability in neutrophils of burn patients results in increased neutrophil stiffness, decreased deformation, and hindered passage through distal capillaries (73).

Shear Stress

Leukocytes are well adapted to mechanical shear forces that they experience in the circulation. Leukocytes need to adhere and migrate on the endothelial surface only when the appropriate stimuli are detected, and these responses have evolved in the presence of, and are closely regulated by, shear stress due to blood flow. Fluid shear stress in the range of 1 to 10 dyn/cm^2 promotes leukocyte crawling and diapedesis under inflammatory conditions (74–76). The mechanisms and pathways that contribute to the mechanosensory response of leukocytes to shear stress are likely to be initiated by physical perturbation of load-bearing subcellular structures including integrin-based adhesions and the cytoskeleton (77). Human leukocytes undergo myosin- and F-actin-dependent cell stiffening within seconds of exposure to shear stress (78) and show pseudopod projection and spreading 5 min after exposure to shear stress (79). In contrast, promyelocytic HL-60 cells, differentiated into neutrophils, and mouse blood neutrophils had a cell rounding response to shear stress (80). The shear

stress-induced cell retraction response of HL-60 cells was accompanied by a transient increase in RhoA activity, while active Rac1 and Rac2 levels were reduced. When HL-60s were prestimulated with fMLP, cell rounding and reduced Rac1 and Rac2 activity were reversed. This is suggestive of a mechanism whereby Rac activity, and thereby neutrophil pseudopod projection, is inhibited by neutrophil exposure to shear stress in the absence of stimulation, limiting unsolicited recruitment (81), while shear stress enhances transmigratory responses in the presence of proinflammatory signals.

Neutrophils rolling on the endothelial surface are captured through an integrin catch-bond mechanism, and adhesions are reinforced by F-actin. RhoA promotes neutrophil adhesion through F-actin-dependent regulation of integrin function (60, 82–84), facilitating spreading and the formation of F-actin-rich podosomes. In one study, monocytes plated on physiological ligands formed F-actin-based structures at $\alpha_4\beta_1$ integrin adhesion sites in response to shear stress (85). These F-actin-based anchors formed as a result of a signaling cascade that depended on Rap1, PI3Kγ, and Rac, but not Cdc42, and are thought to help leukocytes brace themselves against the force of flowing blood. Also, *in vivo* studies of the inflamed mouse microvasculature showed that the Rac GEF Vav1 was required to induce neutrophil crawling in the direction perpendicular to the flow of blood (86). Since endothelial cells elongate parallel to the direction of flow, this directional crawling ensures the shortest distance of travel to the closest endothelial junction when leukocytes are exposed to shear stress on the vascular surface.

Polarization

In order to crawl on a two-dimensional surface or within the tissue matrix, leukocytes undergo polarization by creating a morphologically distinct leading edge as well as a trailing edge. The leading edge is characterized by F-actin-rich membrane extensions, while the trailing uropod region is rich in contractile actomyosin

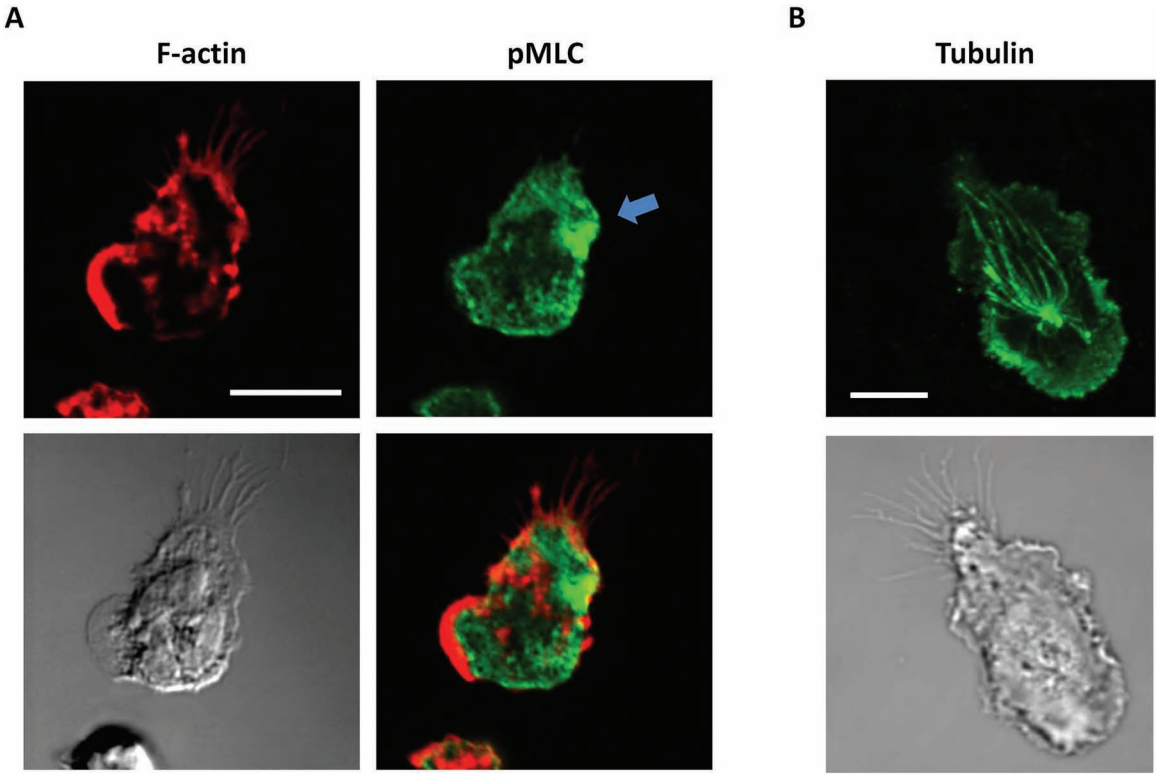

Figure 1 The neutrophil F-actin and MT cytoskeletons. (**A**) Mouse bone marrow neutrophils were plated on ICAM-1 (Intercellular Adhesion Molecule-1)-coated glass and treated with 1 M fMLP for 10 min. Cells were fixed with paraformaldehyde and labeled for F-actin (red) and phosphorylated MLC (Thr18/Ser19) (green). An arrow indicates the position of the uropod. (**B**) Cells were treated as above, fixed with glutaraldehyde as in reference 120 and labeled for MTs (green). Bars, 10 m.

fibers (Fig. 1A). Unique signaling modules have been identified that designate cell "frontness" and "backness," dictating an axis of symmetry that can be oriented in a chemotactic gradient to facilitate directional homing. The neutrophil axis of polarity is stable, as demonstrated by their ability to reorient toward a chemotactic stimulus that is repositioned (87).

GPCRs, membrane phosphoinositides, and small GTPases are important regulators of cell polarity and the chemotactic compass. PIP_3 stabilizes polarity by locally enhancing Rac activity at the leading edge and by stimulating the activation of Cdc42, which promotes RhoA-dependent backness at the trailing edge. Inhibition of PI3K results in the formation of multiple weak and transient pseudopods culminating in reduced directionality of migration (88). Furthermore, the internal cellular gradient of PIP_3 was impaired when cells were treated with the F-actin inhibitors latrunculin or jasplakinolide, indicating that F-actin plays a reciprocal role in stabilizing membrane phosphoinositide distribution and the chemotactic compass (88). Inhibitor studies suggest that initial stimulation of neutrophil polarity with fMLP, characterized by protrusive F-actin, occurs through two distinct pathways (89): a PI3Kγ-dependent pathway, which also involves protein kinase B (Akt/PKB) and protein kinase C-ζ (PKC-ζ); and a PI3Kγ-independent pathway, which depends on RhoA, ROCK, Src family kinases, and NADPH. Once established, a positive feedback loop linking PIP_3, Rac, and F-actin maintains a robust pseudopod at the leading edge (88, 90, 91), while RhoA and ROCK maintain backness, associated with actomyosin-based contractility in the uropod (87, 92). Cross talk between the Rho GTPases helps to establish and maintain polarity. Leading edge signals locally inhibit myosin activity at the leading edge, which effectively limits the domain of Rho-induced contractile activity to the uropod (93). Cdc42 activation at the leading edge induces RhoA-mediated contractility in the uropod at a distance (94) and maintains polar alignment of the MT cytoskeleton (95). Contractility in the uropod in turn generates membrane tension that inhibits frontness. Establishing and maintaining these mutually exclusive domains serves to coordinate and promote a stable axis of polarity during neutrophil chemotaxis (46, 54).

PAK kinases are important downstream effectors of Rac and Cdc42 that facilitate cross talk between Rho family GTPases to establish frontness and limit Rho-dependent signals to the posterior end of the neutrophil. PAK1 phosphorylates numerous cytoskeletal regulators and promotes the formation of protrusive F-actin and inhibition of contractile actomyosin at the leading edge (96). Activated PAK2 also accumulates at the neutrophil leading edge in response to fMLP to support Rac/Cdc42-mediated actin dynamics (97). PAK inhibition alters the subcellular localization of active RhoA and induces aberrant formation of vinculin-rich complexes, increased spreading, decreased migration speed, and loss of directionality.

Crawling

Resting leukocytes possess an actin-myosin-rich cortex that runs parallel to the plasma membrane. This actin-myosin cortex is important in determining cell shape and regulating cortical tension and undergoes dynamic reorganization to allow for adhesion and migration (98). The kinetics of cortical F-actin polymerization/depolymerization determines changes in cell morphology and is the driving force for cell migration. Unstimulated neutrophils lack motility, and treatment with cytochalasin, a toxin that binds to barbed ends and inhibits actin polymerization, of resting neutrophils does not decrease basal F-actin levels (99, 100), suggesting limited dynamics of the F-actin cytoskeleton under these conditions. The polymerization state of actin filaments is subject to regulation by extracellular signals and in this way coupled to cellular responses to environmental stimuli. In neutrophils, stimulation with a variety of chemoattractants and cytokines triggers actin polymerization at the leading edge (101, 102). The number of actin filaments per cell doubles 90 s after stimulation of resting neutrophils with fMLP, which can be reversed by addition of cytochalasin (103). The force generated by polymerization of actin is sufficient to produce a membrane extension at the leading edge. F-actin-based structures in the lamellipod, in association with integrin-based adhesions, generate force and traction at the leading edge to facilitate crawling (104). Rapid cell migration is thought to depend on a dynamic balance between actomyosin-based contraction and integrin-based adhesions (105). Furthermore, integrin-based adhesions are themselves subject to regulation by actomyosin contractility. Remodeling of the F-actin cytoskeleton is necessary to promote stable integrin-based adhesions in monocytes, and the RhoA effector ROCK limits adhesion formation by phosphorylating and inactivating cofilin through LIM kinase (92). Monocytes that were treated with the ROCK inhibitor Y-27632 were strongly adherent and could not undergo productive migration (92).

In addition to pulling forces at the leading edge, crawling neutrophils are propelled by actomyosin-based pushing forces at the rear. Contractile forces that concentrate in the uropod under the control of RhoA

facilitate deadhesion and tail retraction of crawling leukocytes (55, 58). The neutrophil uropod is formed by segregation of detergent-insoluble membrane rafts into functional domains that are enriched for flotilins and phosphorylated ezrin/radixin/moesin (pERM) proteins (106–108) and phosphorylated MLC. Uropods are functional repositories of adhesion molecules where rearward tractional stresses are generated during migration (109). Uropods are also thought to be important sites for immunological interactions due to the abundance of receptors and coreceptors in these domains (110). Contractile forces in the uropod are important for an amoeboid mode of migration in a three-dimensional matrix that has been demonstrated in neutrophils and dendritic cells (111).

Transmigration

Prior to actively participating in the elimination of invasive pathogens, leukocytes must exit the bloodstream and enter infected tissues through transendothelial migration (112). Actin polymerization at the leading edge initiates neutrophil transmigration through the endothelial cell-cell junctions. Neutrophils failed to undergo transendothelial migration when actin polymerization or depolymerization (113) was inhibited by latrunculin-A or jasplakinolide, respectively. The lack of transendothelial migration by latrunculin-A-treated neutrophils also indicates that neutrophil transmigration is an active process that requires more than a decrease in cellular stiffness (114) and "oozing" of cells through endothelial cell junctions.

THE ROLE OF MTs IN MYELOID CELL MIGRATION

The MT cytoskeleton is a dynamic intracellular structure that regulates diverse cellular phenomena. MTs enable vesicle trafficking; regulate cell polarity and migration; are the basic structural elements of cilia and flagella; and form the mitotic spindle, essential for cellular division. MTs act as a platform for intracellular transport through interaction with the molecular motors dynein and kinesin. The MT array is formed by polymerization of α- and β-tubulin dimers and has inherent polarity, growing through polymerization at the plus end, with the minus end generally nucleated at a microtubule organizing center (MTOC). MTs display dynamic instability with steady-state levels depending on the balance between polymerization and depolymerization. MTs undergo posttranslational modifications including acetylation and detyrosination (115) that can influence cytoskeletal function (116). Detyrosination is a marker of a distinct subset of stable MTs that are thought to be important for generating asymmetries associated with directional cell migration (117, 118).

Myeloid cells and other leukocytes are among the fastest-moving cells in the body and are quickly recruited from the circulation to sites of inflammation. As discussed previously, myeloid cells must undergo complex morphological changes as they transition from rolling to crawling cells, cross the endothelial barrier, and navigate the three-dimensional tissue matrix. Irrespective of the role of MTs in cell polarity during migration, myeloid cells require a highly labile and dynamic cytoskeleton in order to undergo the morphological contortions necessary during extravasation and to navigate the three-dimensional tissue matrix. Given this, it is not surprising that the MT array is much more dynamic in monocytes (119) and neutrophils (120) relative to other cell types. When human neutrophils and monocytes are treated with nocodazole, an inhibitor of MT polymerization, the MT array is lost with a half-life of ~30 s (120), 10 to 40 times faster than for most mammalian cells. Regrowth of the MTs begins as early as 1 min after washout of nocodazole and is completed in between 5 and 10 min in neutrophils and monocytes (119, 120).

There are some striking differences in the organization of the MT array in neutrophils compared to other cell types. In contrast to most cell types, including macrophages (Fig. 2), where the MTs grow into the leading lamellipod, the MT array extends backwards from the MTOC into the uropod in polarized neutrophils (121) (Fig. 1B). Upon stimulation with chemotactic peptides, the MTs, which initially have a radial appearance, reorient toward the uropod of the newly polarized cell in a Cdc42/WASP-dependent manner (95). Cdc42 promotes polarity and orients the MTOC in front of the nucleus in fibroblasts and astrocytes (122, 123). In neutrophils, Cdc42 also promotes polarity; however, it does so through a unique "at a distance" mechanism of action, through signaling to the uropod (124). When neutrophils from $Cdc42^{-/-}$ mice were exposed to a gradient of fMLP, they lost uropod sequestration of the MT array, developed multiple F-actin-rich protrusions, and turned frequently (95). It was demonstrated that the Cdc42 effector protein, WASP, promotes sequestration of CD11b in uropods, which in turn recruits the MT end-binding protein EB1 to capture and stabilize the MT array. Cdc42, WASP, and CD11b were all necessary for proper orientation of the MT array toward the uropod in polarized neutrophils. It is unclear whether MT polarization actively promotes contractility and membrane tension at the rear of the cell,

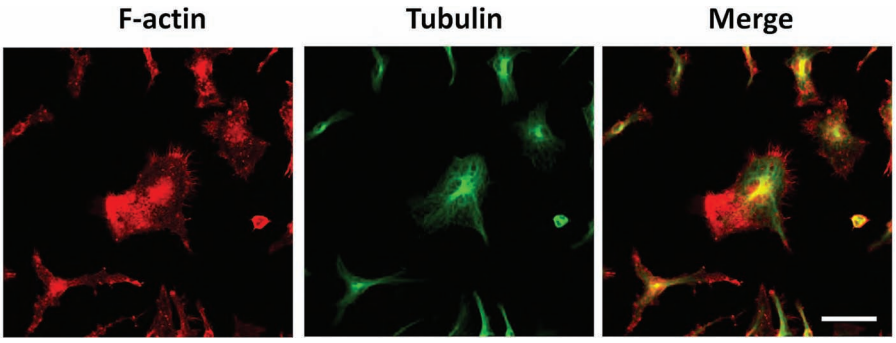

Figure 2 The macrophage F-actin and MT cytoskeletons. Colony-stimulating factor-1-cultured mouse bone marrow macrophages were fixed and labeled for F-actin (red) and MTs (green). Bar, 50 m.

through cross talk to the Rho pathway, for example, or is simply a by-product of cell polarization.

In addition to the unique orientation of the MT array toward the uropod, neutrophils also respond differently from other cells to depolymerization of the MT array. While MT depolymerization promotes Rho-dependent stress fiber formation and cellular contractility in fibroblasts and macrophages, in neutrophils it induces cell polarity and random migration (107, 125–127). Therefore, if sequestration of the MT array toward the uropod is necessary to maintain neutrophil polarity, the complete loss of polymerized MTs also produces neutrophil polarity. Induction of neutrophil polarity by MT-depolymerizing agents is characterized by formation of an F-actin-rich leading edge and a uropod at the posterior of the cell (128); however, the mechanism of action is different from that of chemoattractant-mediated polarization. Based on the observed migration of neutrophils upon MT depolymerization, early studies concluded that the MT array serves to limit cell migration in the absence of chemokine (129). Indeed, when neutrophils with a depleted MT array were stimulated with chemotactic peptide, a subpopulation of cells became multipolar (121), suggesting that the MTs serve to maintain a single axis of polarization. However, more-recent results have demonstrated that MT depolymerization increased the speed of differentiated HL-60 cell migration toward a gradient of fMLP, while simultaneously impairing purposeful and directional migration due to excessive activation of Rho, which inhibits fMLP-induced frontness (130). Although it has been assumed that the MT array maintains polarity in migrating neutrophils, as it does in many other cell types (123, 131, 132), these MT inhibitor studies demonstrate that the MT array may act simply to limit excessive Rho activity and therefore to allow directional migration. Consistent with inhibition of directional migration *in vitro*, *in vivo* imaging using zebrafish showed that recruitment of neutrophils was inhibited by nocodazole (133). Although nocodazole induced random migration, increased Rho and Rac activity, and increased polar F-actin levels in live zebrafish, leading edge PI3K and purposeful migration was defective. Similar to neutrophils, macrophages treated with MT-depolymerizing agents form multiple opposing lamellipodia with a consequent loss of random migration (134), and nocodazole inhibited recruitment of macrophages to a wound in live zebrafish (135). Interestingly, normal *in vivo* wound recruitment was restored by the ROCK inhibitor Y-27632, suggesting that macrophages can chemotax normally in the complete absence of MTs, as long as excessive Rho activation is blocked. It would be interesting to see if Y-27632 has a similar effect on neutrophils *in vivo*.

The asymmetrical distribution of the MT array toward the uropod is thought to reinforce neutrophil polarity during migration by buffering front-to-back signaling, so that the uropod is stable despite fluctuations at the leading edge (136). However, since the leading edge orientation of the MT array accomplishes a similar task in other cells, it is not clear what additional benefits are afforded to neutrophils by this innovation. One possibility is that neutrophils rely more heavily on a Rho-dependent amoeboid form of migration that puts unique emphasis on uropod regulation, and that this is somehow accomplished by sequestering the MTs, and associated factors, in the uropod. Also, uropod sequestration of MTs could be related to the unique ability of neutrophils to establish stable self-organizing polarity that is not dependent on the presence of a gradient of chemoattractant (54, 87). An alternative interpretation is that the MT array acts to sequester factors that might otherwise elicit excessive Rho activity and tightly regulates their activation in a

localized manner. One potential activator of Rho signaling in response to MT depolymerization is the MT-associated GEF GEF-H1, which has been shown to trigger RhoA-induced contractility in response to MT depolymerization in other cell types (137, 138).

In addition to their role in cell migration, MTs play an important role in intracellular trafficking. MTs are necessary for macrophage secretion of MMP-9 (139), a matrix metalloproteinase important for degradation of extracellular matrix by migrating cells. In one study, lipopolysaccharide was shown to induce MT acetylation in macrophages and increased secretion of the anti-inflammatory cytokine interleukin-10. Interleukin-10 secretion could also be induced by treatment of macrophages with the MT-stabilizing agent paclitaxel (140).

CROSS TALK

Although MT and actin cytoskeletons are distinct polymeric structures that independently influence cell shape, polarity, and movement, they also collaborate. Evidence indicates that there is direct interaction and signaling between these two systems (141). Formins, which nucleate actin polymerization, can also bind to and stabilize MTs (142, 143). This promotes alignment of MTs along actin filaments and the reorientation of a subset of MTs into the leading edge of migrating cells.

In fibroblasts, depolymerization of the MT network triggers polymerization of the actin cytoskeleton and increased actomyosin contractility through stimulation of RhoA (144, 145), indicating that these two systems are fundamentally coupled. This phenomenon is mediated by activation of the RhoA-specific MT-associated GEF GEF-H1 (137, 138). As mentioned previously, neutrophils also undergo actomyosin-mediated contractility and random migration in response to MT depolymerization (126, 127); however, it is not yet known whether GEF-H1 is responsible for this effect or what is the function of this biochemical feedback pathway under normal physiological conditions.

PHAGOCYTOSIS

Phagocytosis is a specialized form of endocytosis in which large particles such as pathogenic organisms, dead cells, mineral particles, and neutrophil extracellular traps (NETs) are recognized via specific tags, internalized, and destroyed after fusion with lysosomes (146). Phagocytic cells recognize their prey through the surface chemistry of their targets, either through pattern recognition receptor interaction with pathogen-associated molecular patterns or through FcR or complement receptor (CR) interaction with host molecules (opsonins) on the surface of pathogens. Although FcR- and CR-mediated phagocytosis occur through distinct mechanisms (24), they both require dynamic regulation of the actin cytoskeleton and actomyosin contractility (4).

The Role of F-Actin in Phagocytosis

The fundamentals of actin polymerization/depolymerization during phagocytosis remain constant, but the function of F-actin, the protein interactions, and the signaling pathways differ depending on the type of phagocytosis. Recognition of a particle by Fc receptors triggers a signaling cascade that leads to activation of Src and Syk family serine/threonine kinases and Rho GTPases. Actin assembly at this site is dually regulated: (i) phosphatidylinositol 4,5-bisphosphate and Cdc42 signaling activates WASP, which in turn binds and activates the Arp2/3 complex (147, 148); and (ii) Fc-mediated Syk signaling stimulates Vav1, which in turn actives Cdc42 and Rac to stimulate actin polymerization through PAK1 (148–152). The Arp2/3 complex induces branching of actin filaments, leading to an increase in the number of uncapped ends and to isotropic growth of the actin network, which forms a phagocytic cup around the target particle (153). The developing phagocyte is enriched in gelsolin (154, 155) and cofilin (156). Further, gene targeting approaches in macrophages have demonstrated the importance of the F-actin capping protein CapG in regulating phagocytosis (157).

Phagocytes stained with fluorescein isothiocyanate (FITC)-phalloidin show a dense band of F-actin progressing from the bottom toward the top of the phagocytic cup during pseudopod extension (Fig. 3). Experiments based on fluorescent speckle microscopy show that actin polymerization does not directly push the membrane outwards but prevents the membrane from moving backwards by acting as a ratchet and making the ligand-receptor bonds effectively irreversible (158, 159). In FcR-mediated phagocytosis, advancing membrane-particle tethering occurs through a zipper model, with F-actin contributing structural stability.

In CR-mediated phagocytosis, particles "sink" into phagocytes without formation of a protrusive structure. Internalization occurs through F-actin and contractility-dependent pinching of particles. The Rho effector mDia is recruited early on during CR-mediated phagocytosis and colocalizes with polymerized actin in the phagocytic cup (160). Inhibiting mDia activity has a negative effect on CR-mediated phagocytosis but not FcR-mediated phagocytosis. Although myosin-mediated contractility is necessary for particle internalization in both

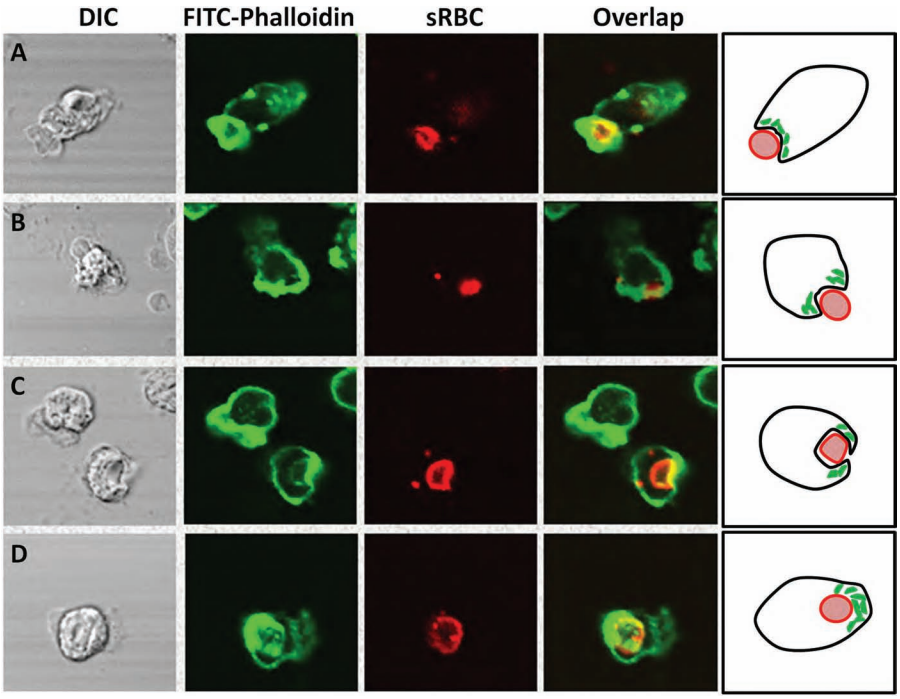

Figure 3 F-actin localization during Fc-mediated phagocytosis in neutrophils. Mouse bone marrow-derived neutrophils were incubated with IgG-tagged sheep red blood cells (sRBCs) at 37°C. Cells were fixed during phagocytosis, labeled, and visualized by confocal microscopy. F-actin was labeled with FITC-phalloidin and RBCs were labeled with anti-IgG Alexa-568. (**A**) F-actin concentrates on the site of recognition of the IgG-labeled RBC. (**B**) It then extends toward the tips of the nascent phagosome, creating protrusions at the site. (**C**) Consequently, cell membrane is pushed forward and receptor-ligand binding ensures enwrapping of the particle completely. (**D**) Lastly, the tips of the protrusions fuse due to being pushed into close proximity and the particle is internalized (S. Khaliq, unpublished data).

types of phagocytosis, the MLC kinase inhibitor ML-7 blocks actin filament assembly and phagocytic cup formation in CR-mediated phagocytosis but not in FcγR-mediated phagocytosis (161).

Maturation of phagosomes requires Rac in both Fc- and CR-mediated phagocytosis. Macrophages lacking Rac1 and -2 exhibited defective phagosome maturation (162), and macrophages that lacked β_1 integrin expression had reduced F-actin accumulation in the periphagosomal region, reduced maturation of the phagosome, and impaired bactericidal activity. Defective phagosome maturation in β_1 integrin-deficient macrophages could be rescued by ectopic expression of Rac1 but not Cdc42 (162). The oral pathogen *Treponema denticola* evades phagocytosis by inhibiting neutrophil Rac1 (163).

Localized PIP_3 production at the phagosome triggers downstream Rac and PKC signaling, which regulates actin assembly. It has been shown that PKC-α localizes to nascent phagosomes in macrophages (164). Myristoylated, alanine-rich C-kinase substrate (MARCKS), a major substrate of PKC-α, is rapidly phosphorylated during particle uptake and recruited to the nascent phagosome (164). MARCKS cross-links F-actin, and this activity is prevented by PKC-dependent phosphorylation (165). PKC inhibitors block recruitment of MARCKS and F-actin to the site of bound particles and prevent phagocytosis (164). Another PIP_3 effector molecule, phospholipase D (PLD), is also important for actin dynamics during phagocytosis. Lack of PLD1 or PLD2 in macrophages causes isoform-specific actin cytoskeleton abnormalities leading to decreased phagocytosis (166).

In addition to the roles of actin in cell crawling and phagocytosis, recent results have highlighted its importance in other effector functions of neutrophils, including NET formation. NET formation is a highly regulated terminal process in which neutrophils entrap and kill microorganisms through the release of relaxed chromatin strands, coated with antimicrobial compounds, into the extracellular space (167). Following signal transduction of appropriate cues from the microenvironment, neutrophils initiate DNA breakdown followed by the

dissolution of the nuclear membrane and plasma membrane and release of NETs. Neutrophil elastase released from granules prior to NET formation degrades actin polymers, which triggers the release of chromatin from neutrophils (168). These results indicate that F-actin may act as a barrier to NET formation and release. Furthermore, it has been demonstrated that the MT cytoskeleton as well as active polymerization of actin are both critical for lipopolysaccharide-induced NET formation (169), since depolymerization of MTs with nocodazole or inhibition of actin polymerization with cytochalasin D limited nuclear breakdown and NET release. Furthermore, resolution of inflammation depends on active ingestion of NETs by macrophages in an F-actin-dependent endocytic process (146).

The Role of MTs in Myeloid Effector Function

The MT array has a specific role in CR-mediated phagocytosis, since disruption of MTs inhibits CR-mediated but not Fc receptor-mediated phagocytosis (170, 171). This is thought to be due to the ability of MTs to regulate β_2 integrin mobility and clustering rather than through direct effects on phagosome closure (172).

The MT array is also required for maturation of phagosomes due to the vesicle trafficking function of MTs, which facilitates vesicle fusion. This is confirmed by the observation that nocodazole-treated macrophages show defects in phagosome maturation (173, 174). Early-stage phagosomes have a strong preference for MT plus ends, whereas late-stage phagosomes do not. This plus-end affinity has been attributed to the presence of MT-associated proteins (175). Most phagosomes show centripetal movement toward the MTOC, where phagosome-lysosome fusion frequently occurs. Both dynein and dynactin are required for this minus-end-directed movement along the MTs. Although some phagosomes move to the plus end along the MTs using kinesin, the significance of this centrifugal movement is unknown (176).

In neutrophils, primary (azurophil) granules, which are lysosomal in nature, are exocytosed near the site of phagosome formation by focal exocytosis (177, 178). MTs contribute to the vectorial nature of the response, since the localized delivery of primary granules can be disrupted by colchicine (177). However, since the exocytosis of secondary granules is less polarized than that of primary granules, the transport of secondary granules may not be MT dependent. Similarly, macrophages treated with colchicine fail to localize antigens to the trans-Golgi network, indicating a requirement for MT in antigen presentation in these cells (179).

CONCLUSION

Myeloid cells perform unique cell biological functions related to pathogen killing, processes that require distinctive cytoskeletal responses. The importance of the cytoskeleton in myeloid function is underlined by mutations in cytoskeletal components that lead to myeloid dysfunction and human disease. Defects in actin polymerization and actin-dependent neutrophil function including chemotaxis, phagocytosis, and degranulation have been noted in a number of pathological conditions including chronic myeloid leukemia (180), localized aggressive periodontitis (181), neutrophil actin dysfunction disorder (182), and as a result of burn trauma (72). Mutations in WASP (183, 184) and Rac2 (6, 185, 186) cause defects in myeloid cell chemotaxis that lead to severe infections, and a mutation in the human β-actin gene is associated with recurrent infections due to dysfunctional neutrophil migration and ROS production (5, 187, 188). Furthermore, several strains of mice predisposed to spontaneous autoimmune disease all show reduced RhoA activation and dysregulation of F-actin in serum-treated macrophages (189, 190). These macrophages also display elevated adhesion and an abnormal morphology due to altered regulation of integrin dynamics resulting from the Rho and F-actin defects. In addition to primary defects of the cytoskeleton and cytoskeletal regulation, a number of pathogenic organisms have learned to subvert the cytoskeleton in order to overcome destruction by phagocytes (191–194). A thorough understanding of cytoskeleton function in myeloid cells could lead to development of novel antiphlogistic approaches and other therapeutic interventions.

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