Cytoskeletal remodeling in leukocyte function

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Purpose of review

This review focuses on recent developments in understanding the roles and regulation of the cytoskeleton in the function of leukocytes.

Recent findings

New studies have shed light on the regulation and dynamics of actin and microtubules in leukocytes relevant both to cell motility generally and to immune function specifically. The roles of cytoskeletal dynamics in processes such as cell activation, cell migration, and phagocytosis are being elucidated. Dramatic progress has been made recently in understanding the mechanisms of leukocyte directional sensing, polarization, and chemotaxis.

Summary

Leukocytes need to be activated, polarize, change shape, move, or phagocytose in response to their environment. Leukocytes accomplish these processes by remodeling their cytoskeleton, the active musculoskeletal system of the cell that is not just the ultimate effector of motile responses but is also a dynamic framework for subcellular organization and regional signaling. Active areas of research include the direct and indirect reciprocal interactions between the cytoskeleton and the membrane and among cytoskeletal elements. The pervasive and multi-layered roles played by small GTPases of the Rho family and phosphoinositides in leukocyte function are also becoming clearer.

Keywords

cytoskeleton, Rho-family small GTPases, phosphoinositides, cell polarization and migration, chemotaxis, phagocytosis

Curr Opin Hematol 11:15–24. © 2003 Lippincott Williams & Wilkins.

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Current Opinion in Hematology 2004, 11:15–24

1065–6251

Introduction

Leukocytes are highly efficient at migration, which allows them to move rapidly and specifically to sites of infection, inflammation, or tissue damage. Even apart from their biologic function as the basis of the immune system, they are invaluable systems for understanding the signal transduction pathways and cytoskeletal dynamics underlying cell shape change and motility in a fundamental sense. They are exquisitely responsive to extracellular signals that modulate their activation, polarization, chemoattractant-stimulated directed migration (chemotaxis), or stimulated random migration (chemokinesis). Polymorphonuclear leukocytes (neutrophils) are fast migrating cells extensively used to study the signaling pathways and cytoskeletal regulation that mediate responses to chemoattractants. The other granulocytes, eosinophils and basophils, are less abundant and less well studied.

Cytoskeletal rearrangements are also critical for the migratory and other functions of other types of leukocytes. Macrophages (differentiated monocytes), like neutrophils, specialize in the phagocytosis of invading microorganisms, a process driven by cytoskeletal remodeling. Lymphocytes are a functionally diverse group evolved for antigen-specific immune functions and recruitment of other white blood cell types, and activation of T lymphocytes by antigen-presenting cells is cytoskeleton-dependent. Finally, although highly specialized for blood clotting and anucleate, blood platelets result from the cytoskeleton-mediated fragmentation of megakaryocytes, myeloid cells that share a common progenitor with leukocytes. Platelet activation is a simplified model often used to study actin cytoskeletal dynamics and remodeling relevant to cell shape change in general.

Actin dynamics and remodeling in leukocyte function

Cell shape change and migration in leukocytes and other adherent crawling cells involve the regulated assembly and cross-linking of actin filaments that support leading-edge membrane protrusion, coordinated with cycles of integrin-mediated cell-substratum attachment and detachment, actin disassembly behind the leading edge, cell body contraction, and retraction of the cell's trailing edge [1–16]. These processes are accomplished by the activities of a range of actin-binding proteins and upstream regulators of actin dynamics.

Filamentous actin (F-actin) has a fast-growing or barbed end (based on its appearance when decorated with myo-

sin head fragments) and a slow-growing or pointed end. F-actin assembly involves the polymerization of monomeric actin (G-actin). In the cell, actin polymerization only occurs from the barbed end of a filament. The means to these ends are as follows: *de novo* nucleation of new filaments by actin-nucleating proteins, the dissociation of capping proteins that normally bind the barbed ends of filaments in the resting state, or the severing of existing filaments by severing proteins to generate new barbed ends. Several routes also exist to F-actin cross-linking, bundling, and disassembly, mediated by different proteins. In addition, microtubules interact with the actin cytoskeleton in a number of ways, playing still poorly understood roles in cell polarization, migration, and other processes [17]. There are clearly multiple mechanisms to arrive at a given change in actin state in the cell. These mechanisms are not mutually exclusive, and cells may pursue different options, depending on the specific cell and situation. Despite the enormous complexity of the system, basic models of the cytoskeletal dynamics underlying cell migration have emerged to provide a source of testable hypotheses [1–17].

The migration of leukocytes, like other crawling cells, is driven by the dynamics of the actin cytoskeleton [18]. This actin-based motility is initiated by signals from the environment, orchestrating remarkably complex sequences of events. For example, migration of a leukocyte from the bloodstream to a site of infection, inflammation, or injury entails multiple bidirectional signaling and adhesive interactions between the leukocyte and vascular endothelial cells, resulting in attachment of the leukocyte to the endothelium near the affected area, rolling and arrest of the leukocyte, transient loss of cell-cell adhesion between endothelial cells, and finally transmigration of the leukocyte across the endothelium [19–21].

During activation of T lymphocytes by antigen-presenting cells, the cytoskeleton is also involved in formation and stabilization of the immunologic synapse between the antigen-presenting cell and the lymphocyte; it is required for sustained interaction and appears to provide a scaffold for signaling complexes to assemble [22–27]. Phagocytosis in neutrophils and macrophages, wherein the cells engulf and internalize microbial pathogens, is driven by actin rearrangement [18,28,29], as is endocytosis in general [30]. Furthermore, actin and microtubule dynamics are central to cell division in all eukaryotic cells [31].

Signaling to the actin cytoskeleton

A basic paradigm of cell biology is the transduction of extracellular signals across the plasma membrane and into the cytoplasm to initiate changes in the state of the cell. Pathways involving inositol phospholipids and small GTPases are of particular importance. Phosphoinositides have emerged as key modulators of the activity of actin-regulatory proteins [32]. Small GTPases of the Rho family are prenylated membrane-targeted proteins that function as critical regulators of actin cytoskeletal remodeling and other cellular responses to stimuli [33–37]. In the GTP-bound state, small GTPases are active in signaling to their effectors; they become inactive once they hydrolyze the bound GTP to GDP. Their activity is modulated by at least three classes of proteins: the guanine-nucleotide exchange factors that accelerate exchange of GDP for GTP and are therefore activating for signaling; the GTPase-activating proteins that accelerate the intrinsic rate of hydrolysis of GTP to GDP and are inactivating for signaling; and the GDP-dissociation inhibitors that prevent exchange of GDP for GTP and so are also inactivating for signaling.

Although each Rho-family member is distinct, there is considerable cross-signaling between different members of the Rho family [33–37]. Rho, the prototype member, actually comprises multiple protein isoforms that are most associated with the formation of contractile bundles of F-actin and nonmuscle myosin II (nonmuscle actomyosin), such as stress fibers, and focal adhesions. Cdc42 is involved in the formation of filopodia (finger-like membrane protrusions) and the control of cell polarity. Rac isoforms are most associated with the formation of membrane ruffles and lamellipodia (broad, sheet-like membrane protrusions). The terms *lamellipodium* and *lamella* are often used synonymously, although in many cases it is important to make a distinction between the lamellipodium as the protrusive force-generating leading-edge tip and the lamella as the rest of the protrusion back to the cell body [38]. *Pseudopod* and *pseudopodium* are terms also used to describe such protrusive structures, especially in more amoeboid cells such as leukocytes.

Wiskott-Aldrich syndrome, a disease characterized by impaired cellular and humoral immunity, results from mutation in the Wiskott-Aldrich syndrome protein (WASP). WASP is a member of a protein family that includes N-WASP and Scar/WAVE proteins, with central roles in stimulating *de novo* actin nucleation by the actin-related protein 2/3 (Arp2/3) complex; different members of the WASP family are activated by either Cdc42 or Rac [39–41]. WASP (expressed exclusively in hematopoietic cells) and N-WASP (neural WASP, which, despite its name, is ubiquitously expressed) are the best characterized mechanistically of the WASP-family proteins and link Cdc42 activation to Arp2/3 complex-dependent actin nucleation. WASP is an important regulator of actin remodeling during T cell activation [42] and other actin-dependent functions, including chemotaxis and phagocytosis, in leukocytes [43]. Its importance in leukocyte function was again recently highlighted by Jones *et al.* [44], who further characterized abnormalities in cell polarity, chemotaxis, and podosomes (cell-substratum adhesion structures similar to but distinct from focal com-

plexes, the short-lived precursors of focal adhesions) in Wiskott-Aldrich syndrome macrophages and found that induced expression of wild-type WASP restores normal morphology and behavior. Furthermore, Launey *et al.* [45•] discovered that the differentiation of promyelocytic leukemia HL-60 cells is accompanied by changes in the expression of WASP and Scar1/WAVE1, with different changes observed for differentiation into neutrophil-like *versus* monocyte/macrophage-like cells. Modulation of the expression of different members of the WASP family may be part of the differentiation program of myeloid precursors, and different members may have distinct roles in leukocyte function.

Actin and phagocytosis

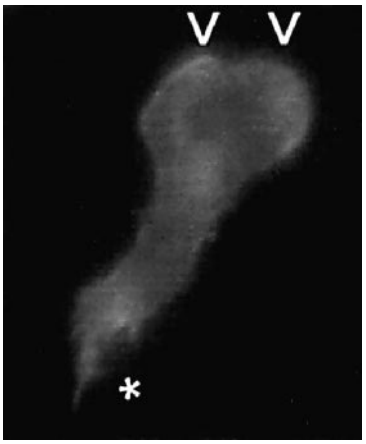
Different pathways to actin remodeling are involved in different types of phagocytosis. In macrophages, phagocytosis through complement receptor 3 (CR3) is mediated by Rho, whereas phagocytosis through Fcγ receptor (FcγR) depends on Cdc42 and Rac [46]. Expanding on these findings, Olazabal *et al.* [47•] recently found that Rho-associated kinase (Rho-kinase) and myosin II are required for phagocytic cup formation through CR3 but not FcγR, which only requires myosin II for the later step of internalization of phagocytic vesicles (phagosomes).

The subsequent fate of internalized phagosomes may also depend on the actin cytoskeleton. A general mechanism for movement of various types of intracellular vesicles, such as endosomes, lysosomes, and secretory vesicles, could be the assembly of an F-actin network behind the vesicle, which resembles a rocket or comet tail and propels the vesicle forward [48], similar to the case of the actin-based motility of certain intracellular pathogens such as *Listeria* and *Shigella* [49–51]. Such a rocket tail alone does not have a "guidance system" and is not persistently directional as is the movement of vesicles along microtubules by motor proteins; rocketing of vesicles could be tied to other cytoskeleton-dependent motor systems, or its function could be to simply augment random movement above the slow diffusion rates for large structures. Formation of short-lived actin-rich rocket tails and motility of latex bead-containing phagosomes have been observed in macrophages [52], suggesting that phagosome movement through the cytoplasm may be facilitated by actin-based propulsion. More recently, Southwick *et al.* [53•] found that phagosomes and early endosomes, induced by treatment of macrophages with the secretagogue antagonists lanthanum and zinc, assemble actin-rich rocket tails with the apparent involvement of a number of known actin-binding and actin-regulatory proteins, including the Arp2/3 complex, N-WASP, profilin, VASP, and zyxin. However, the question of whether these examples of actin-based rocketing in perturbed macrophages reflect normal modes of phagosome movement remains to be answered.

Leukocyte directional sensing, polarization, and chemotaxis

Much of our understanding of the signaling events from chemoattractant receptor occupancy to the actin cytoskeleton in leukocytes comes from studies in neutrophils or related cells such as neutrophil-differentiated HL-60 cells. Leukocytes are recruited to sites of infection, inflammation, or injury through the process of chemotaxis, which involves directed cell migration toward extracellular chemoattractants that include bacterial products such as *N*-formylated peptides (*e.g.*, formyl-Met-Leu-Phe or fMLF, but more commonly referred to as fMLP), C5a (a product of the complement cascade), products of phospholipid metabolism such as leukotriene B4, and chemokines such as interleukin-8. Once a chemoattractant binds to its cell-surface receptor, a series of membrane/cytoplasmic "directional sensing" events is triggered that results in the activation of the cytoskeletal machinery. Remodeling of the cytoskeleton then brings about transformation of the cells from a roughly spherical resting state to a polarized asymmetric shape. This morphologic polarization is characterized by a single protrusive actin-rich lamellipodium at the leading edge and a tail structure or uropod at the trailing edge (Fig. 1). Once polarized, the cell crawls in the direction of the source of chemoattractant. If the source of chemoattractant moves, the cell turns and again migrates up the gradient. Although *directional sensing*, *polarization*, and *chemotaxis* are terms often used without clear distinction between these linked processes, a recent review discusses the need to

Figure 1. Filamentous actin (F-actin) cytoskeleton of a stimulated neutrophil



Neutrophil stimulated with chemoattractant and stained with rhodamine-phalloidin, a fluorescent probe for F-actin. Note the broad lamellipodial protrusion (arrowheads) at the leading edge and the retracting tail or uropod (asterisk) at the trailing edge. Neutrophils and other leukocytes directionally migrate toward a source of chemoattractant such as bacterially derived *N*-formylated peptides by the process of chemotaxis. The dynamics of the actin cytoskeleton provide the force to drive this movement.

precisely define each and distinguish between them experimentally to build reasonable models for the overall sequence [54].

Neutrophils polarize and move directionally toward a chemoattractant source even in very shallow gradients, corresponding to as little as a 1% difference in chemoattractant concentration across the length of the cell [55]. When a source of chemoattractant forming a gradient is abruptly moved to the opposite side of the dish, the neutrophil generally does not simply extend a new membrane protrusion in the new direction and reverse in one step but rather follows the existing lamellipodium at the leading edge, which reorients gradually as it continues to displace, so that the cell appears to make a step-wise U-turn toward the source [56]. In addition, neutrophils can polarize and migrate randomly by chemokinesis even when uniform concentrations of chemoattractant are added, demonstrating that polarity can arise from self-organization in the activated neutrophil. These issues will be revisited after a discussion of recent progress on signaling to the basic machinery of migration, the actin cytoskeleton.

Rho-family small GTPases in leukocyte migration

Some of the first evidence for involvement of GTPases in the regulation of cellular actin polymerization arose from research with neutrophils [57–59]. Rac itself was first identified in a neutrophil-differentiated HL-60 cDNA library [60] and in human platelets [61], whereas Rho was first discovered in *Aplysia* [62], and Cdc42 first in yeast [63–65] and humans [66–68]. Different roles for the Rho-family GTPases in regulation of the actin cytoskeleton were subsequently clearly established [69–79]. Much progress has been made in understanding the signaling pathways involving Rho-family proteins that lead from chemoattractant receptor occupancy to F-actin assembly in neutrophils [80,81]. At least two main pathways to actin polymerization lie downstream from the fMLP receptor [82]. Both pathways depend on phosphoinositides, and both also appear to involve Cdc42 or shared factors. One pathway leads from Cdc42 to *de novo* actin nucleation through and the Arp2/3 complex. The other pathway leads to Rac-dependent actin polymerization even when Arp2/3 complex-dependent nucleation is maximally inhibited with an Arp2/3 complex-sequestering fragment derived from N-WASP. While Rac is known to initiate Arp2/3 complex-dependent actin nucleation through Scar/WAVE proteins, this is not the only pathway from activated Rac to actin polymerization. In the case of the stimulated neutrophil, the Rac-dependent mechanism appears to involve a major contribution from elongation from existing filament barbed ends generated through uncapping or severing, or an Arp2/3 complex-independent nucleation mechanism (such as through formins) [83,84].

A tale of two Racs

Rac proteins are key regulators not only of the actin cytoskeleton but also of the NADPH oxidase system in neutrophils [85]. Using Rac2-deficient mice and neutrophils from a patient with a naturally occurring mutation in Rac2, it has been demonstrated that the Rac2 isoform is a key regulator of multiple antimicrobial functions, including cell polarization and chemotaxis, granule secretion, and generation of reactive oxygen species by the NADPH oxidase complex [86]. However, the importance of Rac1 in neutrophil function has remained uncertain. The high degree of homology in the effector regions of Rac1 and Rac2 has led to the hypothesis that these two proteins function interchangeably. Using purified neutrophil membranes and recombinant Rac1 and Rac2, Heyworth *et al.* [87] demonstrated that both isoforms have equal activity in the reconstitution of superoxide production, although Rac2 was more efficient in the presence of neutrophil cytosol. In permeabilized neutrophils, dominant-negative mutants of Rac1 and Rac2 are equally effective at inhibiting fMLP-induced actin polymerization [82].

A recent study by Li *et al.* [88•] using Rac2-deficient neutrophils suggests that Rac1 and Rac2 have discrete functions inasmuch as activation and signaling profiles for each isoform in intact neutrophils are unique. The authors found that four times more Rac2 is activated compared with Rac1 in fMLP-activated murine neutrophils. In addition, they demonstrated using neutrophils from Rac2-null and Rac2-heterozygous mice that the level of activated Rac2 is rate-limiting for chemoattractant-induced actin polymerization, chemotaxis, and superoxide generation. Another study examined the role of Rac1 in regulating neutrophil functions using selective deletion of Rac1 in neutrophils [89•]. In contrast to Rac2, Rac1 was found to be required only for chemotaxis and not for NADPH oxidase function. Recent work on the unique roles of these two small GTPases demonstrates that these proteins localize to different compartments in the cell: Rac1 localizes to the actin cytoskeleton, whereas Rac2 localizes to internal membrane compartments [90,91].

A tail of Rho and Rac too

Rho-kinase activity is required for migration and tail retraction in leukocytes [92–94], for example, through phosphorylation of myosin light chain kinase [92], which would promote actomyosin bundle formation and contraction. Liu *et al.* [95] recently provided evidence that RhoA/Rho-kinase signaling also promotes de-adhesion in Jurkat T lymphoma cells and neutrophils by inhibiting actin cytoskeleton-dependent cell spreading. Worthylake and Burridge [96•] found that Rho-kinase activity is

required to restrict integrin activation and membrane protrusion to the leading edge in monocytes through a pathway in part involving actin-depolymerizing factor/cofilin. RhoA/Rho-kinase signaling outside of the leading edge appears to promote the development of a single leading edge by limiting adhesion and protrusion elsewhere. Yoshinaga-Ohara *et al.* [97] found that Rho activity is required for maintenance of polarity as well as tail retraction in neutrophils and that chemoattractant stimulation results in Rho-dependent dephosphorylation of moesin, an F-actin cross-linking protein; furthermore, inhibition of type 1 and type 2A serine/threonine protein phosphatases prevents uropod retraction.

Using a fluorescence resonance energy transfer-based biosensor, Gardiner *et al.* [98••] found that green fluorescent protein conjugates of both Rac1 and Rac2 are activated in the leading edge of migrating neutrophils. Surprisingly, however, they are also activated in the tail. Furthermore, endogenous Rac2 is recruited to the lamellipodium and to a lesser extent the tail following stimulation. Rac activation in response to stimulation with chemoattractant is greater in adherent compared with suspended cells (which also become polarized with stimulation, although still more rounded than adherent cells); adhesion is particularly important for Rac activation in the tail. Using dominant-negative mutants, these authors also found that Rac activity is functionally required for uropod retraction in addition to leading-edge extension and maintenance of polarity.

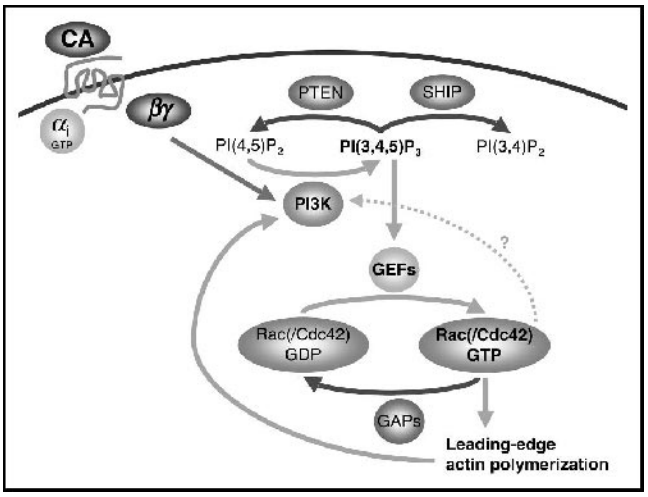
Positive feedback for polarization: phosphoinositides and Rho-family small GTPases

Intact inositol phospholipids in the plasma membrane play critical roles in the regulation of the actin cytoskeleton, and it is well established that phosphatidylinositol (PI) 4,5-bisphosphate [$PI(4,5)P_2$] in particular can bind a range of different actin-binding proteins to promote F-actin assembly and establish membrane/cytoskeleton linkages [32]. (These roles are distinct from those of $PI(4,5)P_2$ as a substrate for the well-known hydrolytic pathway catalyzed by phospholipase C.) In addition, intact PI 3,4,5-trisphosphate [$PI(3,4,5)P_3$ or PIP_3], the product of phosphorylation of $PI(4,5)P_2$ by PI 3-kinases (PI3Ks), has been implicated in cytoskeletal regulation and the control of cell polarity, with roles that appear separate from those of $PI(4,5)P_2$ [99]. PI3K activity is opposed by that of PI 3-phosphatases such as PTEN, and both play roles in the directional sensing and polarization required for chemotaxis [100]. Other phosphatidylinositol kinases and phosphatases are also involved in controlling PIP_3 levels, such as the PI 5-phosphatase SHIP, which plays roles in both hematopoiesis and mature leukocyte functions [101]. However, it is not clear which phosphatase, or phosphatases, is most responsible for setting levels and distribution of PIP_3 in neutrophils [102]. Furthermore, little is known about possible functions of other phosphoinositides, including other PI 3-phosphate lipid products of PI3K, in the regulation of the cytoskeletal dynamics and cell shape change.

Work in the last few years in neutrophils and other systems such as *Dictyostelium* has led to the concept that short-range positive feedback involving PI3K products, specifically PIP_3, and activated Rho-family GTPases leads to amplification of shallow external chemoattractant gradients to steeper internal signaling gradients that establish the "front" of the cell (Fig. 2) [102–104]. Several alternative models exist, such as the local excitation-global inhibition model [54,100], and a mature understanding may ultimately incorporate elements of different models and depend on specific cell type.

Weiner *et al.* [105••] recently provided evidence for key aspects of the positive feedback model in neutrophil-differentiated HL60 cells, showing that an increase in

Figure 2. Signaling cascade involved in neutrophil directional sensing, polarization, and chemotaxis



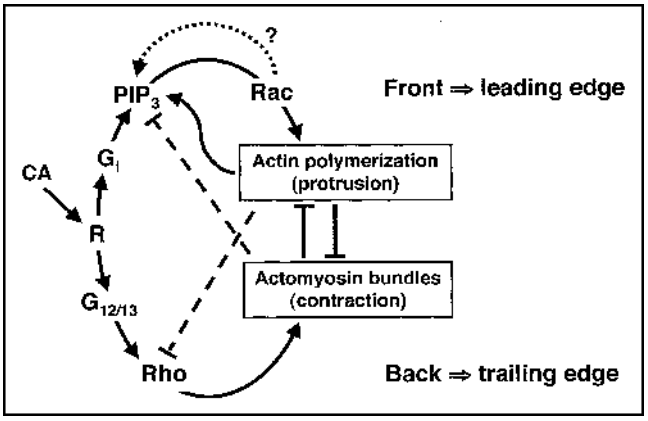
Positive signaling dominates at the leading edge (steps indicated with black arrows). Signal amplification converts a shallow external chemoattractant gradient into a steep internal signaling gradient during directional sensing and polarization, allowing chemotaxis to occur (reviewed in [102–104]). Chemoattractant first binds to a G protein-coupled receptor, which causes dissociation of the heterotrimeric G protein G_i into $G\alpha_i$ and $G\beta\gamma$ subunits. The released $G\beta\gamma$ subunits then stimulate PI3K. The resulting PIP_3, acting through specific GEFs (such as P-Rex1 [108], which is also directly stimulated by the $G\beta\gamma$ subunits), activates Rac, which stimulates PI3K, leading to further generation of PIP_3 and repetition of the cycle [105••,106••]. Rac is the dominant Rho-family small GTPase involved in this positive feedback loop [107••], while Cdc42 helps determine the location and stability of the Rac-dependent leading edge [107••,115••]. The asymmetric distribution of amplified PIP_3 strongly depends on actin dynamics [106••,107••,109••]. Abbreviations: CA, chemoattractant; $G\alpha_i$ and $G\beta\gamma$, heterotrimeric G protein subunits; PI(4,5)P2, $PI(3,4,5)P_3$ (PIP_3), and $PI(3,4)P_2$, phosphatidylinositol (PI) lipids phosphorylated at the indicated positions of the inositol ring; PI3K, PI 3-kinase; PTEN, a PI 3-phosphatase; SHIP, a PI 5-phosphatase; Rac and Cdc42, small Rho-family GTPases; GEFs, guanine-nucleotide exchange factors; GAPs, GTPase-activating proteins. Figure courtesy of O.D. Weiner, with modification; reproduced with permission [102].

PIP_3 activates a Rho-family small GTPase or GTPases, which in turn stimulates PI3K and the generation of more PIP_3. In an accompanying report, Wang *et al.* [106••] further confirmed the importance of PI3K and PIP_3 at the leading edge for polarization and chemotaxis. Moreover, the normal asymmetric distribution of amplified PIP_3 following uniform stimulation with fMLP becomes symmetric and transient when actin polymerization or depolymerization is inhibited with latrunculin B or jasplakinolide, respectively. This suggests that reciprocal interplay between PIP_3 and actin dynamics at the front/leading edge helps initiate and stabilize the internal signaling gradient required for cell polarity. Srinivasan *et al.* [107••] subsequently provided evidence that Rac is the key Rho-family small GTPase regulator of the PIP_3-dependent positive feedback loop. Relevantly, P-Rex1, a GEF for Rac discovered in neutrophils, is activated by PIP_3 and the heterotrimeric G protein subunits Gβγ [108••]. In contrast to the direct role played by Rac in the positive feedback loop, Cdc42 is critical to regulating the location and stability of the Rac-initiated leading edge [107••]. Furthermore, PIP_3 accumulation induced by expression of a constitutively active Rac mutant is sharply reduced when actin polymerization is inhibited. Combined with negative interactions that prevent a leading edge from developing elsewhere (for example, PTEN, which reduces PIP_3 levels elsewhere, or actomyosin bundle formation and contraction, which antagonizes protrusion), these mechanisms may guide orientation of the cell's axis of polarity.

An even more recent study further extends these ideas. Xu *et al.* [109••] defined divergent, opposing signals generated by different G protein-coupled receptor systems involved in polarization of neutrophil-differentiated HL60 cells (Fig. 3). "Frontness" requires the activity of heterotrimeric G_i proteins, PI3K, Rac, and F-actin assembly. "Backness," in contrast, depends on other G proteins, G_{12} and G_{13}, Rho, Rho-kinase, and myosin II. The backness pathway also leads to decreased sensitivity to chemoattractant. These two pathways lead to formation of different actin-based structures in the front (assembly of protruding lamellipodial actin networks) and back (assembly of contracting actomyosin arrays), and each negatively regulates formation of the opposite structure. These mechanisms for self-organizing polarity would provide explanations for why the leading edge is more responsive to chemoattractant stimulation than other parts of the neutrophil, how polarity is generated even in uniform concentrations of chemoattractant, and why the neutrophil turns rather than simply reverses direction when confronted with a change in the location of a chemoattractant gradient's source.

There are still many questions remaining about the precise roles of PIP_3 (and possibly other PI3K products) and different PI3K isoforms in polarization, chemotaxis, and

Figure 3. A model for how "frontness" and "backness" arise during neutrophil polarization



"Front" signals generate the leading edge/lamellipodium, whereas "back" signals generate the trailing edge/uropod (see text and [109••] for details). The positive and negative interactions indicated may establish and maintain front and back structures during polarization. The pathway to frontness involves G_i, PI3K, Rac, and F-actin assembly (which drives membrane protrusion at the leading edge), while the pathway to backness involves $G_{12/13}$, Rho, Rho-kinase, and myosin II (which forms, with F-actin, an array of actomyosin bundles that contract at the trailing edge). Frontness and backness antagonize one another. This model could account for the self-organizing nature of neutrophil polarity, explaining how asymmetry of the cell is generated even in uniform concentrations of chemoattractant and why neutrophils make U-turns when chemoattractant gradients are reversed. CA, chemoattractant; R, G protein-coupled receptor; G_i and $G_{12/13}$, heterotrimeric G proteins; Rac and Rho, small Rho-family GTPases; PIP_3, PI 3,4,5-trisphosphate. Figure courtesy of H.R. Bourne, with modification; reproduced with permission [109••].

other neutrophil functions. Deletion of PI3Kγ abates PIP_3 production following stimulation of neutrophils with fMLP or other chemoattractants and impairs normal neutrophil chemotaxis and superoxide production [110–112], although Rac activation and F-actin assembly after chemoattractant stimulation appear unaffected in suspended neutrophils [111]. PI3Kγ-null neutrophils are able to migrate randomly, albeit at a slower rate than wild-type cells; they tend to have many small protrusions around the cell body and exhibit loss of normal leading-edge colocalization of F-actin and protein kinase B/Akt [113]. Recently, Sadhu *et al.* [114•] developed a new, selective, small-molecule inhibitor of PI3Kδ and found that this PI3K isoform is also required for neutrophil polarization and chemotaxis, although again not for random migration. The authors speculated that PI3Kδ could be responsible for amplifying PIP_3 levels following an initial burst catalyzed by PI3Kγ. Collectively, these studies suggest that the primary role of PIP_3 in neutrophil chemotaxis is to control polarization and directional localization of the lamellipodium rather than movement itself.

Recent progress has been made in further defining the function of Cdc42 in leukocyte polarization. Li *et al.* [115••] described a pathway that is essential for neutrophil directional sensing, polarization, and chemotaxis in response to complement factor C5a. This pathway involves the active G protein subunits Gβγ, p21-activated kinase 1 (PAK1), the PAK-associated GEF PIXα, and

Cdc42. PAKs bind and are activated by Cdc42 and Rac [116]. In the model supported by Li *et al.* [115••], Gβγ binds PAK1, activating Cdc42 via PIXα; Cdc42 then activates PAK1. A noteworthy feature of this pathway is that PAK1 appears to function as a scaffold for activation of Cdc42 before itself being activated by Cdc42. Interestingly, while chemoattractant-induced activation of Cdc42 occurs even in PI3Kγ-deficient neutrophils, Cdc42 activation is not localized to the leading edge in these neutrophils, implying that proper localization of the Gβγ-PAK1/PIXα/Cdc42 signaling complex depends on PI3Kγ-catalyzed PIP_3 production. These authors found that this pathway to Cdc42 activation is required for excluding PTEN from and limiting new F-actin assembly to the leading edge, consistent with the role ascribed to Cdc42 in the aforementioned report by Srinivasan *et al.* [107••]. Such a mechanism may not be limited to neutrophils, as Ratner *et al.* [117] recently demonstrated a role for Cdc42 in ensuring that only a single lamellipodium is maintained in T lymphocytes.

More on membrane/cytoskeleton interactions

Other lipid components of the membrane should not be ignored, nor should consideration of the organization of the membrane. Pierini *et al.* [118] showed that depletion of plasma membrane cholesterol inhibits neutrophil polarization and chemotaxis by preventing prolonged (but not initial) activation of Rac and sustained F-actin assembly. This suggests that membrane lipid organization and structure may be important for persistent signaling and the maintenance of cell polarity. Moreover, structural interactions between the membrane and the cytoskeleton may synergize with signaling interactions. In an attempt to characterize the protein components of the neutrophil membrane skeleton (the cortical network of F-actin and associated proteins found beneath the plasma membrane), Nebl *et al.* [119•] used mass spectrometry to identify proteins from detergent-resistant membrane fractions. They found proteins already known to be associated with both membrane skeletons and lipid rafts (detergent-resistant packed lipid domains in the plasma membrane) from studies in other systems. In addition, the actin-binding protein supervillin, a component of the membrane skeleton, partially colocalizes with the G_i protein subunit $G\alpha_i$ in the cells. The authors suggest that these membrane fractions represent lipid signaling microdomains associated with the membrane skeleton. This study raises interesting questions about the relationship between the cortical cytoskeletal network and lipid rafts. Asymmetric organization of the plasma membrane into discrete lipid domains that have both signaling and structural relationships with the cytoskeleton and cytoplasm is an attractive hypothesis in trying to understand cell polarization and chemotaxis in leukocytes and other cell types [120].

What of the other cytoskeletal systems?

Microtubules and intermediate filaments constitute the two other cytoskeletal polymer systems in eukaryotic cells. While intermediate filament proteins such as vimentin are expressed in leukocytes, knowledge of their role in leukocyte function is still embryonic and mostly descriptive. Mor-Vaknin *et al.* [121] recently showed that vimentin is secreted by macrophages in response to proinflammatory signals and that extracellular vimentin may be involved in the killing of bacteria and generation of reactive oxygen species. More work has been done on the role of microtubules in leukocyte function, partly because of the availability of drugs that stabilize or destabilize microtubules. Yet the most fundamental question about the role of microtubules in leukocyte migration is still largely unanswered. What is their role? Part of the answer will come from studies of actin and its regulation. While there is no doubt that actin is the cytoskeletal element most central to cell migration, it is also clear that the actin cytoskeleton does not function in isolation from the other cytoskeletal systems. In fact, there is growing evidence that actin and microtubules engage in both indirect regulatory and direct structural interactions that are important to cell polarization and migration, as well as other cytoskeleton-dependent processes [17].

Reorientation of the microtubule-organizing center and the microtubules emanating from it occurs during activation of T lymphocytes by antigen-presenting cells, where microtubules may play roles in directed secretion and possibly also in signal transduction, as has been recently reviewed [23,26]. Microtubules also appear to be involved in regulating cell-substratum adhesion. Evans *et al.* [122••] recently showed that dynamic leading-edge podosomal adhesions in macrophages form by both *de novo* assembly/growth and fragmentation of precursor podosomes; the polarized formation and turnover of podosomes depends on microtubules, whereas actin turnover in the podosomes does not. Therefore, microtubules appear to play a role in the stabilization of podosomes at the leading edge of macrophages.

In neutrophils, Eddy *et al.* [123•] discovered that microtubules reorient toward the uropod during polarization both when cells are plated on fibronectin and when they are in suspension, and this occurs even in the presence of the microtubule-stabilizing drug Taxol, suggesting that microtubule depolymerization is not required for reorientation. Microtubules are normally excluded from the actin-rich lamellipodium; however, treatment with a myosin light chain kinase inhibitor or the F-actin-destabilizing agent cytochalasin D causes expansion of the microtubule array and penetration of the microtubules into the lamellipodium. Myosin II and the actin cytoskeleton therefore appear to be involved in the establishment or maintenance of microtubule asymmetry in the neutrophil. Moreover, pharmacological depolymerization of microtubules with nocodazole prior to

treatment with chemoattractant causes approximately 10% of the cells to lose their polarity and extend multiple lamellipodia accompanied by altered localization of lamellipodium- and uropod-specific markers. However, the authors also point out the conflicting results in the earlier literature on the effects of microtubule-destabilizing drugs on cell polarization, chemotaxis, and chemokinesis. A more recent contrasting study was published by Niggli [124•], who found that disruption of microtubules with colchicine in fact induces cell polarity and random migration in neutrophils without affecting chemotaxis. This effect of colchicine involves Rho-kinase activity and phosphorylation of myosin light chain kinase but is independent of G_i and PI3K, showing that colchicine- and chemoattractant-induced pathways to polarization and migration can be separated. The subtle roles played by microtubules in leukocyte function have yet to be fully elucidated.

Conclusion

The importance of the cytoskeleton in leukocyte function is incontrovertible. Recent progress toward understanding the roles and regulation of cytoskeletal dynamics have helped to illuminate the mechanisms of cell activation, polarization, migration, and phagocytosis. An outline is emerging to describe the way extracellular stimuli, lipids in the membrane, cytoskeletal regulators, and the cytoskeleton interact to achieve changes in cell state and shape. However, many more questions remain, making this field a fruitful one for research in the years to come.

Acknowledgments

The authors thank O.D. Weiner and J. Taunton for helpful comments.

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