Expanding the Model of Macrophage Chemotaxis: Exploring the Connection Between the Leading Edge Chemosensory Pathway and Cytoskeletal Restructuring

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Defense Date March 30, 2021

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Acknowledgments

First and foremost, I would like to thank Dr. Joseph Falke for providing me with this opportunity to work under his lab on a literature-based research project. I am grateful for your endless support, mentorship, and the dedication you provide to each researcher involved in your lab. Even amid a pandemic, where I was unable to physically join the research group, I always felt a part of the team. This past year you have helped me develop my scientific voice and develop a critical eye for literature which will aid me in my future endeavors as a scientist. I would also like to thank the other members of the Falke lab for helping initiate my project with their previous laboratory work on macrophage chemotaxis; my project would not have its beginnings without them. Thank you to my committee, Dr. Jeffery Cameron and Dr. Pamela Harvey, for dedicating their time to read my thesis and evaluate my undergraduate research work. Thank you as well to the Biochemistry Department for allowing literature-based theses to be defended in light of the pandemic. A final thank you to my friends and family for their unwavering support in my academic career. I would not be where I am today without them and I cannot express my gratitude enough for their encouragement.

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Abstract

Macrophages are essential first responders in the innate immune system. They protect the host organism by seeking out, identifying, engulfing, and breaking down pathogens or damaged tissue. These actions are reliant on the cell's ability to migrate up a chemoattractant gradient via the process of chemotaxis. Previous studies in the Falke lab at the University of Colorado, Boulder, as well as other labs in the field, elucidated the mechanisms of early events in chemosensing at the macrophage leading edge membrane. Such chemosensing often involves both the binding of chemoattractant to a leading edge receptor tyrosine kinase (RTK) yielding phosphotyrosine production, and the presence of a leading edge Ca^{2+} signal that recruits protein kinase C-alpha (PKCα). In turn the phosphotyrosine signal and the PKCα activity together stimulate the lipid kinase phosphoinositide 3-kinase (PI3K) to produce the signaling lipid phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3). Less is known about how the chemosensory pathway and the PIP_3 lipid signal control the leading edge cytoskeletal restructuring necessary for migration. My thesis research has analyzed the current literature, enabling me to develop a circuit diagram linking the leading edge chemosensory pathway to the actin dynamics involved in chemotactic cell movement. This review summarizes evidence that the actin nucleation promoting factors, Wiskott-Aldrich syndrome protein (WASP) and WASP-family Verprolin-homologous protein 2 (WAVE2) are directly regulated by the chemosensory pathway, and in turn, control branched actin formation via actin-binding protein ⅔ (Arp ⅔). The WASP and WAVE2 proteins are each recruited to the leading edge membrane and activated by their homologous but distinct activating signals: phosphoinositides, Rho family GTPases, and BAR proteins. Additionally, the kinase mammalian target of rapamycin (mTOR) promotes actin filament stabilization and initiates a positive feedback loop to maintain chemotactic signaling.

Membrane remodeling processes accompany these pathways to decrease the membrane strain created by the expanding actin network. The relief of strain is driven by the relaxation of membrane wrinkles and the trafficking of membrane vesicles from unstimulated regions of the cell to the leading edge. Overall, my literature research has generated a circuit diagram summarizing the connections between the components of the leading edge chemosensory and cytoskeletal systems that together control macrophage-directed migration up attractant gradients. The resulting circuit diagram reveals key areas for further research directions that the Falke lab can pursue to generate a deeper mechanistic understanding of macrophage chemotaxis.

Introduction

Background and Significance. In a time of an imminent health threat amidst the looming COVID-19 crisis, a holistic understanding of the human immune system has gained increasing importance in the global community. With a growing probability of viral pandemics and the developing severity of antibiotic-resistant bacteria, human health is in growing danger (Madhav et al., 2017; Ventola, 2015). Yet, the healthy immune system has a powerful frontline defense system against these foreign pathogens in the form of leukocytes or white blood cells, which are a central component of the innate immune system (Hirayama et al., 2017). They comprise an arsenal of cell types that seek and destroy threats to the host organism including bacteria, viruses, fungi, and damaged tissue. Unlike other immune cells, leukocytes circulate throughout the host to protect from pathogens and disease. This requires leukocytes to possess highly adaptive and flexible structures for efficient movement in a variety of interstitial environments (Kameritsch et al., 2020). A particular leukocyte of interest is the macrophage: a member of the mononuclear family that matures from bone marrow monocytes. Macrophages remove pathogens and damaged tissue through phagocytosis, a process of engulfment and digestion; however, these protective actions require that the cell first migrates to the point of tissue damage or infection. This movement must occur rapidly and accurately so that the host is not left vulnerable (Bhagavan & Ha, 2015). Thus, an effective immune response is dependent on the process of chemotaxis.

Chemotaxis is the systematic movement of a cell towards higher concentrations of a specific chemical or macromolecule defined as the chemoattractant. These molecules bind to specific chemotactic receptors on the exterior surface of cell membranes and trigger a complex signaling circuit that produces localized restructuring and growth. Chemotaxing macrophages

follow gradients of extrinsic chemoattractants towards their sources in damaged tissues or sites of infection by viruses, bacteria, or fungi. Upon arrival, macrophages may release intrinsic chemoattractants into the extracellular fluid to recruit other leukocytes including other macrophages, yielding a beneficial inflammatory response or, when too strong, a toxic inflammatory response. The chemosensory circuit on the leading edge membrane of the macrophage (or another leukocytes such as a neutrophil) detects the concentration gradient of the extrinsic or intrinsic attractant and triggers a signaling cascade that expands the region of the membrane highest in the concentration gradient to grow fastest thereby guiding the cell up the gradient. This localized growth requires remodeling of the leading edge membrane and the actin cytoskeleton adjacent to the membrane, and the formation of stable contacts with the substrate to "push off" so the leading edge can expand up the gradient. The cell continues these chemotactic migrations until it no longer encounters a chemotactic gradient, but the polarized leading edge membrane can remain stable for minutes or hours to seek out new gradients (Snyderman, 1976).

Currently, much remains to be learned about the basic mechanisms linking leading edge chemosensing to cytoskeletal dynamics in macrophages and other leukocytes. Previous studies have revealed many features of the chemosensory circuit and its control of cytoskeletal remodeling. The Falke lab discovered new features of the chemosensory circuit including the leading edge Ca^{2+} signal and the mechanisms by which the Ca^{2+} signal and small G-proteins regulate the lipid kinase PI3K and production of the signaling lipid PIP_3 (Ziemba et al., 2018). These studies have used single molecule biophysics to elucidate signaling mechanisms and cell studies to test mechanistic models. The group is now poised to investigate links between chemosensing and cytoskeletal remodeling. Therefore, this literature review aims to develop connections between the Falke lab model of the leading edge signaling circuit to current

cytoskeletal and membrane remodeling pathways to fully understand the molecular components that drive macrophage chemotaxis and identify regulatory connections for future experimental mechanistic analysis. Developing a deeper understanding of macrophage chemotaxis not only has implications for the innate immune response but also cancer as well. Specifically, mutations in key proteins that increase PIP_3 signaling—further elucidated below—have been linked to excessive cell growth and metastatic cancer (Ziemba et al., 2018). If more is known about the signaling mechanisms that connect PIP_3 to downstream events, then they could also be targeted for possible treatments.

The Macrophage Leading Edge Chemosensory Pathway. As noted earlier, chemotaxis can be divided into at least three main components: chemosensory detection of the attractant gradient, remodeling of the membrane, and reshaping the cytoskeleton. My literature research has focused on the connections between the chemosensory circuit and cytoskeletal remodeling, which plays central roles in defining the cell's directionality and polarity (Rougerie et al., 2013).

The Falke Lab at the University of Colorado, Boulder has carried out live cell and *in vitro* biophysical studies of the macrophage chemosensory pathway for over a decade (Evans & Falke, 2007; Buckles et al., 2017; Ziemba et al., 2018). In live macrophage studies they have used platelet-derived growth factor (PDGF) as a representative chemoattractant. PDGF binds to PDGF receptors (PDGFR), a receptor tyrosine kinase (RTK) subtype, and is known to strongly stimulate macrophage chemotaxis (Deuel et al., 1982). RTK activation stimulates tyrosine kinase activity and phosphorylation of tyrosine (Tyr) residues, which in turn bind and displace the autoinhibitory SH2 domains of a master lipid kinase, phosphoinositide-3-kinase (PI3K), yielding PI3K lipid kinase activation. The activated kinase phosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP_2) to generate the signaling lipid phosphatidylinositol-(3,4,5)-trisphosphate

(PIP_3). The resulting PIP_3 signal governs many downstream events, including where cytoskeletal structures and related proteins localize to drive movement up the chemoattractant gradient. This produces a highly dynamic leading edge of the membrane that directs cell movement towards higher attractant concentrations, while the back end of the cell lacks these attributes, thus generating polarity and an elongated cell shape (Charest & Firtel, 2006).

PI3K exists in four isoforms: PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ; each phosphorylate PIP_2 to PIP_3 and coordinate similar downstream pathways but differ in their upstream regulation (Campa et al., 2015). Depending on the specific chemotactic factors released by the damaged tissue or foreign pathogen, either a receptor tyrosine kinase (RTK) or a G-protein coupled receptor (GPCR) will be bound and initiate PI3K activity. RTKs activate PI3Kα, PI3Kβ, and PI3Kδ, all classified as class 1A PI3Ks. GPCRs also activate PI3Kβ, as well as the class 1B PI3K enzyme PI3Kγ. The difference in regulation between the classes is due to the varying subunits that compose the isoforms. All class 1A kinases have specific regions that interact with phosphotyrosines on RTKs, while in addition PI3Kβ and PI3Kγ contain a subunit that binds to the $G_{\beta\gamma}$ complex of GPCRs (Hawkins et al., 2015). The two receptor types also vary in their timing of actin polymerization which occurs downstream of PI3K. GPCRs are observed to produce one actin wave following receptor stimulation, while RTKs have two: an early wave shortly after chemoattractant binding and a later wave following a couple of minutes after (Rougerie et al., 2013). This concept and its possible causes will be further discussed after detailing the initial membrane activities which precede chemotactic-induced receptor stimulation.

Figure 1 presents the current working model for the core section of the macrophage chemosensory pathway, where PI3K is the regulatory hub that is controlled by Ca^{2+} and receptor

signals (Ziemba & Falke, 2018). The Falke lab has shown this section of the core pathway is part of a previously established positive feedback loop believed to play a central role in the compass that controls cell migration (Ziemba et al., 2018). In a parallel activation signal that is separate from receptor activation, a Ca^{2+} influx through plasma membrane Ca^{2+} channels generates a local Ca^{2+} signal at the leading edge, which in turn activates the Ca^{2+}-regulated protein kinase C-alpha (PKCα) (Evans & Falke, 2007). Ca^{2+} binding to the C2 domain of PKCα recruits the Ca^{2+}-PKCα which is recruited to the leading edge membrane, where the protein kinase activity of the Ca^{2+}-PKCα is stimulated by the signaling lipid diacylglycerol (DAG). At the leading edge, PKCα phosphorylates myristoylated alanine-rich C kinase substrates (MARCKS), disrupting its binding to PIP_2 headgroups in the lipid bilayer. The exposed PIP_2 headgroups then recruit additional lipid kinase PI3Kα to the membrane. The PI3Kα must also be activated by phosphotyrosine residues to displace its autoinhibitory SH2 domains. When both the free PIP_2 and phosphotyrosine signals are present, the PI3Kα phosphorylates PIP_2 to generate the PIP_3 lipid signal. The PIP_3 recruits the phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB/Akt1) to the leading edge membrane via their PIP_3-specific PH domains. Once bound to the membrane, PDK1 first activates itself via autophosphorylation and subsequently activates PKB and PKCα through phosphorylation. The PDK1 may provide positive feedback by activating the upstream PKCα and is believed to play a role in stimulating downstream signals that remodel the cytoskeleton producing cell motility (Ziemba et al., 2018). The indicated pathway is drawn for attractant activation of RTK receptors that signal primarily through PI3Kα. GPCRs can also activate PI3Kα and other PI3K isoforms by indirectly activating members of the Ras small G protein family, including HRas, NRas, KRas and Rac1, again leading to a PIP_3 signal and the downstream activation of PDK1 and AKT1 (Buckles et al., 2017).

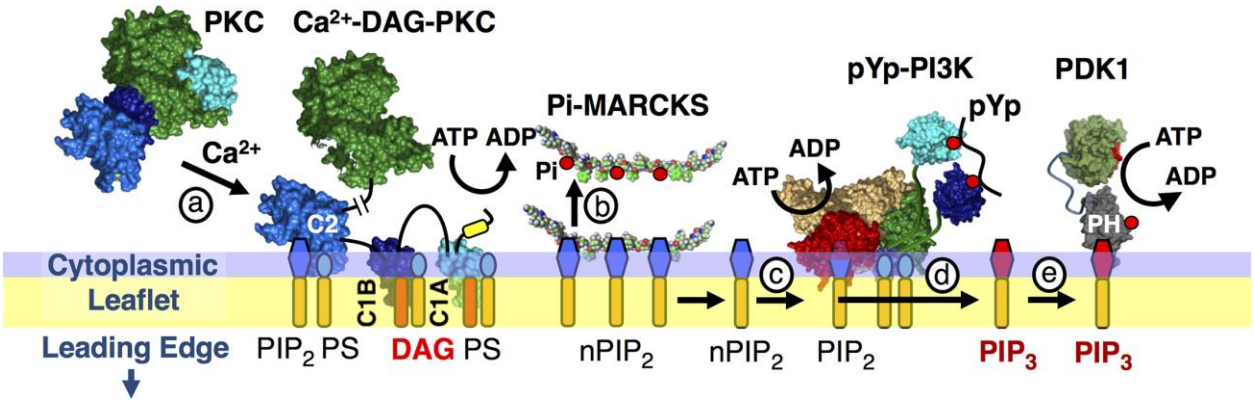


Figure 1. *Leading edge macrophage chemosensory pathway.* PKCα is activated by the leading edge Ca^{2+} signal (Evans & Falke, 2007) and phosphorylates the PIP_2-sequestering MARCKS protein, yielding free PIP_2 that in turn recruits more PI3K to the membrane. Phosphotyrosines (pYp) generated by RTK signal release SH2 autoinhibition and activate the PI3K lipid kinase domain, thereby initiating the production of the signaling lipid PIP_3 within the membrane and activation of PDK1. Not depicted is PDK1's activation of PKB/Akt1 and PKCα initiating the positive feedback loop. Reprinted from "A PKC-MARCKS-PI3K regulatory module links Ca^{2+} and PIP_3 signals at the leading edge of polarized macrophages," by Ziemba, B. P., & Falke, J. J. (2018). *Plos One, 13(5).*

The dynamics of this pathway are confined to the leading edge by the inhibitory activity of the phosphatase and tensin homolog (PTEN) protein. PTEN is somehow displaced from the the leading edge membrane but is active at all other regions of the cell membrane. Thus, PIP3 generated at the leading edge is stable unless it diffuses out of the leading edge into other membrane regions where PTEN dephosphorylates it back to PIP_2, effectively nulling any PIP_3 induced signaling outside the leading edge (Kölsch et al., 2008). If PTEN inhibition outweighs PI3K stimulation, then no leading edge is formed, and downstream actin processes fail to occur. Therefore, the mechanism's self-enhancement through the positive feedback loop is essential to prolong the effects of the chemoattractant and induce proper macrophage chemotaxis. However, to the other extreme, unchallenged PI3K phosphorylation is disadvantageous as well. Without negative regulation by PTEN outside the leading edge region, no highly concentrated area of PIP_3 emerges and the cell would not correctly polarize. Instead, a global increase of PIP_3 would

develop within the membrane resulting in a lack of region-specific actin polymerization directing cell movement (Charest & Firtel, 2006).

This delicate balance of stimulation and inhibition thus drives the macrophage's polarization and directional sensing ability so that it can accurately protect the host organism from dangerous tissues or pathogens. Yet, this model (Figure 1) does not illustrate how the macrophage restructures its cytoskeleton for movement up the chemoattractant gradient. Thus, beginning with the Falke lab model and the key regulators within it, a review of current literature has revealed the mechanisms which mediate chemotactic cell movement and stem from the PIP_3 signal at the leading edge.

Methods

Survey of Current Literature. The current model of the chemosensory leading edge developed by the Falke lab and others has been validated by multiple live cell studies that image the leading edge components and their responses to attractants and inhibitors (Evans & Falke, 2007; Ziemba et al., 2018). To find papers that hypothesize connections between the chemosensory pathway and cytoskeletal regulation, I searched *PubMed* and *Google Scholar* for papers that contain various combinations of the upstream components PI3K, PIP_3, PDK1, and Akt1 with downstream cytoskeletal components. Additional search targets included the basis of cytoskeletal and membrane remodeling and membrane processes. These searches provided links for relevant papers to their corresponding scientific journals or to the free access page provided by NIH's *PubMed Central* (PMC). Searches were refined for studies performed *in vitro* or *in vivo* with macrophages or leukocytes. Some sources used fibroblasts or cancer cells believed to

share similar or identical chemotaxis pathways with leukocytes. Publication quality and relevance was evaluated at weekly lab meetings with Dr. Falke.

Figure Development. All figures created via PowerPoint, excluding Figure 1 developed by Falke lab (Ziemba et al., 2018). Circuit diagram (Figure 2) connecting the leading edge to cytoskeletal restructuring was independently developed but based on models and results presented in literature. Mechanistic details and references for specific interactions of pathway presented in supplementary Figure 1 of appendix.

Results

Cytoskeletal Restructuring for Chemotactic Movement. The macrophage chemosensory pathway controls the downstream cytoskeletal restructuring and membrane expansion that pushes the leading edge up the attractant gradient towards the source of attractant. The cytoskeleton is composed of three major structural fibers: actin microfilaments, intermediate filaments, and microtubules; all of which play an important role in structural integrity and movement. However, actin microfilaments will be the primary focus as they are the most dynamic and are widely believed to account for the rapid changes needed in the leading edge region (Evans & Falke, 2007).

Actin Structure and Dynamics. Microfilaments consist of two twisted actin filaments (F-actin) built from globular actin monomers (G-actin) (The Cytoskeleton, 2019). Actin monomers self-polymerize into actin filaments by binding with the barbed ends of one another or an existing filament. Subsequently, the breakdown of filaments and dissociation of monomers occurs at the opposite pointed end. The relative equilibrium between actin polymerization and depolymerization processes drive actin and cell dynamics (The Cytoskeleton, 2019). Increasing

the efficiency of actin-based cell activities depends on the initiation of actin-binding proteins (ABPs) to drive polymerization and depolymerization processes. Arp ⅔, profilin, formin, and cofilin are some of the most well studied ABPs and could be downstream factors of the chemosensory pathway. Notably, profilin and cofilin contribute to the phenomenon of actin treadmilling: the simultaneous association and dissociation of G-actin monomers at opposing ends of an existing actin filament. When the two processes equalize, the filament sustains a fixed length and will progress in the direction of the added subunits producing cell movement (Merino et al., 2020). Studies of the Gelles Lab at Brandeis University have found that the function of actin-binding proteins can be enhanced through upstream regulators believed to be involved in broader signaling pathways relating to time-specific cell movement. The Wiskott-Aldrich syndrome protein (WASP) and the WASP-family Verprolin-homologous protein 2 (WAVE2) isoform have been proven to increase the activity of Arp ⅔ and are prominent factors of macrophage chemotaxis (Smith et al., 2013; Merino et al., 2020).

Actin Protrusions: Lamellipodia, Filopodia, and Podosomes. The polymerization activities of WASP and WAVE2 have been tied to the development of directional movement and actin filament protrusions at the leading edge of chemotaxing macrophages. The formation of the actin cytoskeleton at the stimulated membrane can be described as either lamellipodial, filopodial, or podosome structures. Lamellipodia are branched networks of actin extending along the leading edge membrane. Specific proteins can bundle these filaments into long linear protrusions that extend outward into the extracellular space forming filopodia (Lee & Dominguez, 2010). Filopodia are presumed to be involved in the exploratory activity of macrophages as they stretch into the extracellular space and increase the membrane's reach to interact with chemoattractants (Ridley, 2011). The wider, mesh-like lamellipodia functions for

more robust cellular movements and the significant expansion of the cell towards the chemoattractant (Kameritsch et al., 2020). WASP is believed to dominate the initial formation of filopodial protrusions, while WAVE primarily controls the latter lamellipodial formations regarding Arp ⅔ branching (Ishihara et al., 2012).

Similar to lamellipodia, podosomes are regions of branched actin networks providing structural support for cell movement. Yet, contrary to lamellipodia, podosomes have degradative and adhesive abilities to assist cell migration in various tissue environments (Goethem et al., 2010). These structures consist of metalloproteases and adhesion proteins, providing them with the ability to bind integrins and breakdown the extracellular matrix (Ridley, 2011). Macrophages that lack podosomes are observed to have reduced tissue invasiveness and disrupted chemotaxis leading to the impairment of the host organism's immune response (Tsuboi et al., 2009).

Links Between the Chemosensory Pathway and Actin Dynamics. The control of actin dynamics by the chemosensory pathway largely involves chemosensory control of WASP and WAVE2 protein activity which upregulates Arp ⅔'s binding to G-actin and increases the efficiency of nucleation for filament polymerization (Kurisu & Takenawa, 2009). It must be noted, however, that WASP and WAVE2 are not the only two proteins regulating these dynamics. Due to the limited reserves of G-actin monomers, for actin polymerization by WASP and WAVE to occur, depolymerization of existing actin filaments must occur as well. By breaking down the previous actin cytoskeleton, the macrophage not only increases its concentration of G-actin for new construction but creates free space within the cytoplasm for the new structures to form. This process is typically dominated by the actin severing protein cofilin, which is inactive when bound to PIP_2 (Janmey & Radhakrishnan, 2018). Therefore, the stimulation of the leading edge and phosphorylation of PIP_2 to PIP_3 would increase cofilin

activity, promoting the severing of actin structures. Cofilin activity is later inhibited by LIM kinase (LIMK) phosphorylation to permit extensive polymerization for cell movement (Lee & Dominguez, 2010). Without the presence of LIMK, cofilin would continue to sever filaments and contradict any polymerization activity silencing chemotactic activity.

The coordination of these various processes is represented in the circuit diagram of **Figure 2**, which depicts the protein-protein interactions at the macrophage's leading edge membrane. Specifically, the activation and involvement of WASP, WAVE2, and mammalian target of rapamycin (mTOR) will be further discussed as each has implications for continued experimental work and are the primary regulators of Arp ⅔ for chemosensory induced actin dynamics. The additional upstream processes, not addressed in this review are briefly described in supplemental **Figure 1** (see appendix).

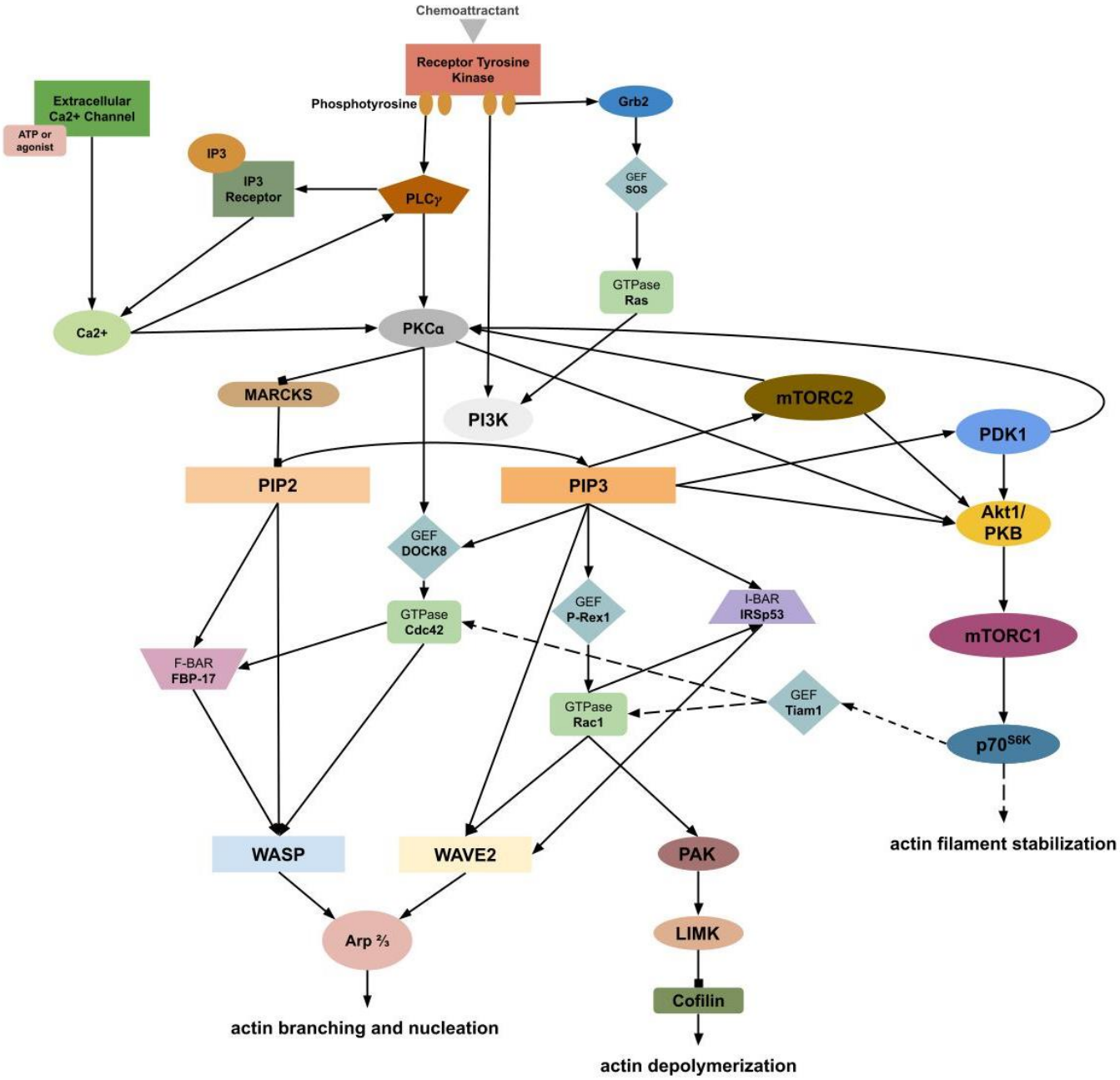


Figure 2. *Proposed circuit diagram coupling the leading edge chemosensory pathway to actin dynamics.* Signaling cascade connecting the chemosensory leading edge to the stimulation of actin dynamics for cell movement initiated by the binding of a chemoattractant to a receptor tyrosine kinase (RTK). Solid lines indicate that the protein relationship has been experimentally proven in macrophages or leukocytes, while dashed lines indicate that an interaction has yet to be demonstrated in macrophages. See supplemental Figure 1 in appendix for descriptions of pathway interactions.

Roles of the Actin Nucleation Promoting Factors WASP and WAVE2. The proteins WASP and WAVE2 are not only similar in function but similar in structure as well. Each protein consists of five distinct domains permitting their recruitment and activity for chemotaxis (Figure 3). The VCA domain, shown in Figure 3, is located at the carboxy-terminus of both WASP and WAVE2 and provides the basis of the proteins' functionality. The domain contains three specific regions to promote the binding of Arp ⅔ to G-actin monomers so that nucleation and branching can rapidly occur. The verprolin homology (V) region binds a G-actin monomer, while the central domain (C) and acidic region (A) bind the Arp 2 and Arp 3 subunits (Kurisu & Takenawa, 2009). It should be noted that Arp ⅔ and G-actin can interact independently, however, their binding would be dependent on the occurrence of combining randomly in solution. WASP and WAVE2, in essence, act as scaffolds, providing a platform for the Arp ⅔-G-actin-binding event to overall increase the probability and rate of actin filament and network construction.

Under normal cellular conditions, WASP and WAVE2 exist in a self-inhibitory, closed-loop conformation where the VCA domain is unable to bind either Arp ⅔ or G-actin monomers (Frugtniet et al., 2015). This ensures that the enhancement of Arp ⅔'s actin branching only occurs when and where necessary. Cellular and environmental conditions signal for this need by exposing, producing, or recruiting molecules for specific interactions with the other four prominent domains. The totality of these binding events leads to a conformational change, opening the loop, and exposing the VCA domain for Arp ⅔ and G-actin binding. This release of WASP and WAVE2 self-inhibition is crucial for the rapid actin polymerization driving macrophage chemotaxis; thus, determining how the molecules of the chemosensory leading edge

promote the release of the VCA domain is the core to understanding and illustrating chemotactic induced movement.

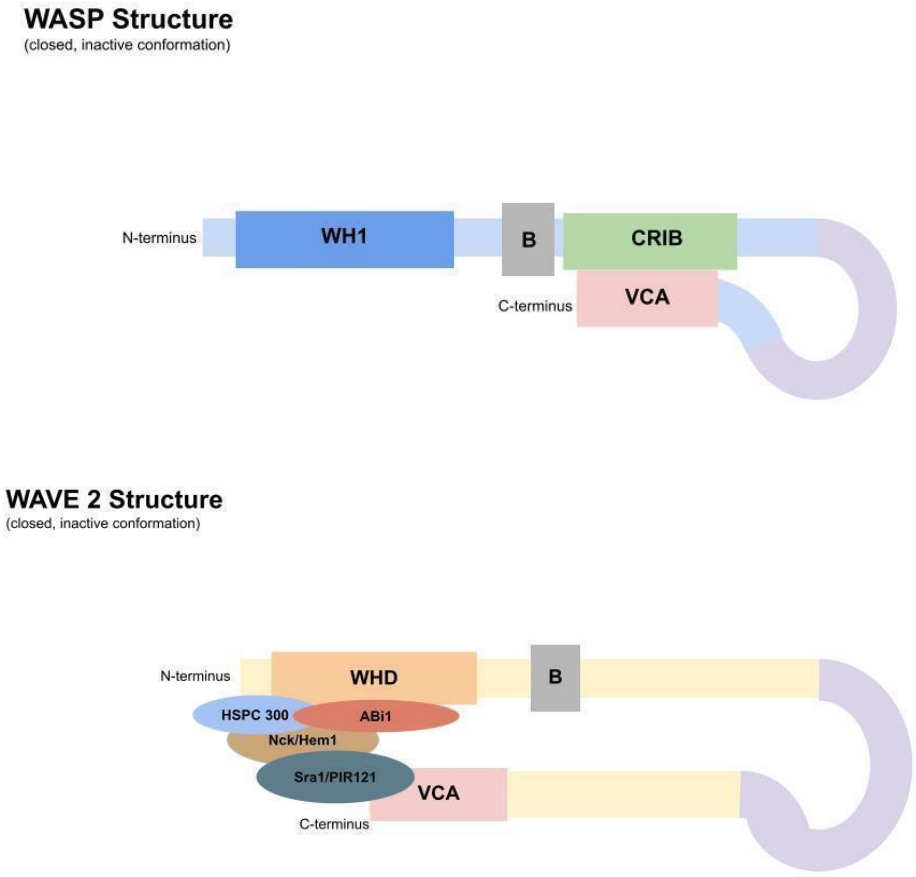


Figure 3. *WASP and WAVE2 inactive structure and protein domains.* WASP and WAVE2 exist in a closed, self-inhibitory loop during unstimulated cellular conditions. In this confirmation, the active VCA domain is sequestered and unable to bind Arp ⅔ and G-actin for stimulation. WASP has direct self-inhibition binding its VCA to the CRIB domain, while WAVE2 indirectly binds its VCA region through the additional stabilizing protein complex Sra1/PIR212-Nck/Hem1-HSPC 300-Abi1, which will be later described. The other domains include a lysine-rich basic region (B), proline-rich region (purple), Rho family GTPase binding site (CRIB and Sra1/PIR121), and either WASP-homology 1 (WH1) or WAVE-homology domain (WHD).

Unlike the VCA domain, which is fairly identical among WASP and WAVE2's structures, the other four regions have slight variance among each protein affecting their mechanisms of activation. In general, chemoattractant stimulation facilitates the release of the

self-inhibition by PKCα's phosphorylation of MARCKS leading to the exposure of PIP_2 heads and the recruitment of PI3K to produce PIP_3 in the membrane (Ziemba et al., 2018). The increased availability of PIP_2 and PIP_3 recruits WASP, WAVE2, Rho family GTPases, guanine nucleotide exchange factors (GEFs), and BAR scaffolding proteins which all interact in a manner resulting in a loss of VCA affinity thereby activating WASP and WAVE2 (Derivery & Gautreau, 2010; Rougerie et al., 2013). The exact domains and molecules involved will be specified when discussing the distinct pathways of activation for WASP and WAVE2 below.

WASP Activation. WASP is a monomeric protein that typically exists in an inactive, closed state where its active site or VCA domain is directly sequestered by the Cdc42/Rac-interactive binding (CRIB) domain. The CRIB domain is responsible for binding to Rho family GTPases, specifically Cdc42—a central activator of the protein at the leading edge. However, Cdc42 cannot independently recruit WASP to the membrane, and other regulators help concentrate their interaction in the region of chemoattractant stimulation.

Recruitment to the leading edge membrane. WASP directly localizes to the plasma membrane by binding to PIP_2 heads through a lysine-rich basic domain, illustrated as region B in Figure 3. The pH of macrophage cytosol is approximately seven and, therefore, the amino acid residue lysine holds a positive charge. This allows the region to favorably bind the negatively charged phosphoinositol heads exposed in the membrane (Kurisu & Takenawa, 2009). The relative binding affinity of WASP to PIP_2 versus PIP_3 has not been quantified in the literature; however, it has been observed that WASP regularly binds PIP_2 more frequently than PIP_3 (Senju & Lappalainen, 2019).

The recruitment of WASP to the membrane additionally requires the aid of formin binding protein 17 (FBP-17), a fes/CIP4 homology-bin/amphiphysin/rvs (F-BAR) family

protein. When bound to PIP_2 heads, FBP-17 dimerizes on the cell membrane and generates a membrane curvature (Derivery & Gautreau, 2010). This concavity is important for inducing membrane deformation for cell movement and the production of podosomes for chemotactic migration (Suman et al., 2020). However, through PIP_2 alone, FBP-17's stability on the membrane is weak. Thus, to maintain FBP-17 at the leading edge, the dimer is supported by a GTP-bound Rho family GTPase bound via the F-BAR's homology region 1 (HR1) domain (Watson et al., 2016).

Rho family GTPases are small proteins that cycle between a GDP-bound, inactive form and a GTP-bound, active form through the assistance of a guanine nucleotide exchange factor (GEF). When bound to GDP (guanosine diphosphate), the G-protein remains free in the cytosol. Once a GEF removes GDP allowing GTP (guanosine triphosphate) to bind then, the GTPase can associate with the membrane through its carboxy-terminal end. From there, it can function as an effector in different signaling pathways, such as chemotaxis (Sadok & Marshall, 2014). Within macrophages, Cdc42 is a primary GTPase of interest and found to control WASP-dependent actin dynamics during chemotactic migration. Cdc42 is stimulated by the GEF DOCK8, and though it has not been specified if Cdc42 interacts directly with phosphoinositide heads, DOCK8 does. The GEF contains a DOCK homology 1 (DHR-1) domain with an affinity for PIP_3. The recruitment of DOCK 8 to the plasma membrane is essential for the GEF's activity in signaling pathways, possibly due to the presence of PKCα at the leading edge. PKCα phosphorylates DOCK 8 to an active form and promotes its binding to GDP-bound Cdc42 (Kearney et al., 2017). Once bound, DOCK8 will transform Cdc42 into its GTP-bound form, where it can interact with the HR1 region of FBP-17 and remain localized at the leading edge. Stabilized

FBP-17 then binds the proline-rich region of WASP via its carboxy-terminal src homology 3 (SH3) domain to maintain WASP's localization at the membrane (Watson et al., 2017).

Relief of the inhibitory loop. FBP-17 colocalizes Cdc42 and WASP at the leading edge to facilitate the release of WASP's self-inhibition (Rougerie et al., 2013). Upon binding to the CRIB domain of WASP, active Cdc42 initiates a conformational change and the release of the sequestered VCA domain. This event transitions WASP from an inhibitory loop to an elongated linear conformation where Arp ⅔ and G-actin can bind the VCA stimulating actin branching and nucleation (Lane et al., 2014).

The synergistic properties of PIP_2, FBP-17, and Cdc42 involvement in WASP recruitment and activation have yet to be fully explored in the literature. Questions remain about the necessity and sufficiency of each within the pathway and if other BAR, G-proteins, or GEFs complement or inhibit the mechanism. Further experiments detailing the optimization of WASP activity *in vitro* will be crucial to fully understand the implications of this pathway during chemotaxis.

WASP regulation and filopodia formation. The actin branching induced by FBP-17 primarily drives the formation of podosomes observed *in vivo* within macrophages (Tsuboi et al., 2009; Tsujita et al., 2013). As described earlier, these structures provide the macrophage with concentrated actin matrices that can bind integrins and degrade the extracellular matrix for efficient migration to damaged tissues or pathogens (Pixley, 2012). However, while podosomes provide structural and adhesive properties, they do little to maintain the orientation of the cell toward the chemoattractant. Therefore, to sustain chemotaxis and directional cell movement, the macrophage produces filopodia or thin, elongated actin protrusions.

Filopodia formation within macrophages occurs via the regulation of WASP activity and actin-bundling by the F-BAR protein proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2)—highly expressed in monocytes and an antagonist of FBP-17 (Chitu et al., 2005; Tsujita et al., 2013). Contrary to FBP-17, PSTPIP2 does not contain an SH3 domain and therefore does not interact with WASP. However, the protein can regulate WASP-dependent actin polymerization at the leading edge by impeding FBP-17's dimerization on the membrane. PSTPIP2 competitively binds phosphoinositol heads through its F-BAR domain to block FBP-17's and other protein interactions at the leading edge (Tsujita et al., 2013). This causes a decrease in branched actin structures and podosome formation due to a lack of Arp ⅔ activity, allowing PSTPIP2 to bundle actin into long filaments protruding from the membrane (Chitu et al., 2005; Salzer et al., 2017). These filopodia structures allow the macrophage to further sense the chemoattractant gradient improving its directionality and motility (Ridley, 2011).

WAVE2 Activation. Contrary to WASP, WAVE2 exists in a heteromeric protein complex with the Abelson interactor protein (Abi1), NCK-associated protein (Nck/Hem1), hematopoietic stem/progenitor cell protein 300 (HSPC 300), and p53-inducible messenger RNA (Sra1/PIR212). Due to its heterogeneous composition, when inactive, WAVE2 remains in a self-inhibitory loop where the VCA domain is indirectly bound and sequestered to the WHD domain by the Sra1/PIR212-Nck/Hem1-Abi1 protein complex (Kurisu & Takenawa, 2009). Yet, its release follows a similar mechanism to the relief of WASP's self-inhibition with its recruitment to the membrane via phosphoinositide heads, BAR proteins, and activation via the binding of a GTP-bound G-protein (Frugtniet et al., 2015).

Recruitment to the leading edge membrane. WAVE2 localizes to the leading edge membrane following PI3K's phosphorylation of PIP_2 to PIP_3. Unlike WASP, WAVE2 has a

higher affinity for the exposed PIP_3 heads over PIP_2 and favorably binds via its lysine-rich, basic region (Kurisu & Takenawa, 2009). This ensures that WAVE2 is concentrated at the membrane where chemoattractant stimulation is occurring and allows for site-specific actin branching by Arp ⅔ facilitated by the exposed VCA domain of WAVE. However, the binding of PIP_3 alone is not sufficient to stimulate WAVE2, and thus additional scaffolding factors at the membrane are necessary for optimal activity (Suetsugu et al., 2006; Abou-Kheir et al., 2008).

The production of PIP_3 as well recruits insulin receptor substrate 53 kDa (IRSp53) to form a homodimeric complex on the membrane. Akin to the scaffolding protein FBP-17 involved in WASP activation, IRSp53 is an inverse-bin/amphiphysin/rvs (I-BAR) family protein and creates a convex deformation on the membrane to promote actin protrusions (Salzar et al., 2017). Cytosolic IRSp53 exists in a self-inhibitive conformation where its carboxy-terminal SH3 domain is sequestered by an internal CRIB domain. This restricts its ability to bind the proline-rich region of WAVE2 and promote actin dynamics at the leading edge. The relief of IRSp53's inhibition is dependent on its interaction with a GTP-bound Rho family GTPase via the CRIB domain (Carman & Dominguez, 2018). Through this region, IRSp53 can interact with both Cdc42 and Rac1. However, PIP_3-bound IRSp53 has a stronger affinity for GTP-bound Rac1 over GTP-bound Cdc42, suggesting that IRSp53 favors its involvement in the WAVE2 activation pathway (Suetsugu et al., 2006).

Rac1 can be activated from its GDP to GTP-bound form during chemotaxis by the guanine nucleotide exchange factor PIP_3-dependent Rac exchanger 1 (P-Rex1). Following the production of PIP_3 at the leading edge, P-Rex1 is recruited to the membrane through its PH-domain (Barber et al., 2007). Once active at the membrane, P-Rex1 exchanges GDP to GTP on Rac1 and the GTP-bound GTPase then binds to the CRIB domain of the IRSp53 dimer at the

leading edge. In this Rac1-bound state, IRSp53's SH3 domain relaxes from the inhibitory conformation and recruits WAVE2 to the membrane for its activation via Rac1 binding (Derivery & Gautreau, 2010). In absence of IRSp53, macrophages have impaired chemotaxis as WAVE2 cannot be sufficiently recruited and maintained at the membrane (Abou-Kheir et al., 2008). Thus, IRSp53 is a key scaffolding protein to stabilize and activate the WAVE2 complex for lamellipodia formation and efficient directional migration.

Relief of the inhibitory loop. Collectively, IRSp53 and PIP_3 both mediate the activation of WAVE2 by clustering GTP-bound Rac1 and the inhibited WAVE2 complex at the leading edge. By being brought into proximity, Rac1 can then initiate the release of WAVE2's active site or VCA domain through indirect binding. Unlike WASP, the WAVE2 monomer does not contain an internal CRIB region to independently bind GTPases. Instead, Rac1 binds Sra1/PIR212, a stabilizing protein of the WAVE2 heteromeric complex (Figure 3), subsequently bound to WAVE's VCA domain. This interaction results in the release of WAVE2's autoinhibitory confirmation and exposes the VCA domain for Arp ⅔ and G-actin binding (Rougerie et al., 2013). Once active, WAVE2 contributes to the formation of the extensive lamellipodia and actin branching organization necessary for cellular movement during macrophage chemotaxis.

Variation in P-Rex1 activation among receptor types. The circuit (Figure 2) presented in this review focuses on the downstream effectors of stimulated RTKs, and while many of the interactions preceding PI3K are downstream of GPCRs as well, mechanism variances exist among the two. One point of difference is the activation of the Rac1 GEF P-Rex1. As previously mentioned, PIP_3 recruits P-Rex1 to the leading edge, priming its catalytic GEF activity (Barber et al., 2007). This process occurs downstream of both receptor types, but GPCRs additionally

activate P-Rex1 through their $G_{\beta\gamma}$ complex (Barrows & Parsons, 2016). Upon chemosensory stimulation, GPCRs release their intracellular subunits G_{α} and $G_{\beta\gamma}$, which activate PI3K and P-Rex1. Whereas RTKs only activate PI3K directly, and thus, P-Rex1 stimulation is dependent on PIP_3 production alone at the leading edge (Jones, 2000; Welch, 2015).

This discrepancy in P-Rex1 activation with RTKs could result in a delay of Rac1 activation and recruitment to the membrane keeping WAVE2 in its inactive, autoinhibitory conformation for a prolonged period compared to GPCRs. Subsequently, GPCRs produce a single wave of actin polymerization, while RTKs produce an early and late wave of polymerization—with the early associated with WASP activity and the late associated with WAVE2 (Ishihara et al., 2012; Rougerie et al., 2013). It is plausible that the dual activation of P-Rex1 by PIP_3 and $G_{\beta\gamma}$ could account for the difference in the number of actin polymerization waves between RTKs and GPCRs; however, further investigation is required.

Involvement of mTOR Complexes. Coinciding with the activation of the nucleation promoting factors WASP and WAVE2, additional actin dynamics are implemented to achieve efficient macrophage chemotaxis. Specifically, the kinases mTOR1 and mTOR2 are involved in initiating a positive feedback loop to amplify the chemosensory signal and stabilize actin filaments (Linke et al., 2017; Aslan, 2011; Ip & Wong, 2012). Both mTOR complexes are stimulated following PIP_3 production and are concentrated at the leading edge membrane (Berven et al., 2004). PIP_3 directly stimulates mTOR2 by binding to the PH-domain of its Sin1 regulatory subunit and coupling its activation to the membrane (Yuan & Guan, 2015). mTOR1 is indirectly activated by PIP_3 through the kinase Akt1, which is stimulated by PIP_3 binding and mTOR2 or PDK1 phosphorylation. Akt1 phosphorylates and inhibits the TCS2/TSC1 regulatory complex that restricts mTOR1 activity by disrupting its G-protein activation (Linke et al., 2017).

mTOR initiation of positive feedback loop. mTOR1 and mTOR2 amplify the PIP_3 membrane signal by phosphorylating key regulators within the pathway. PIP_3-bound mTOR2 phosphorylates PCKα which is a central activator of Akt1, DOCK8, and initiates the release of MARCKS from sequestering PIP_2 in the membrane (Figure 2) (Linke et al., 2017). This allows for increased exposure of PIP_2 to PI3K phosphorylation and an increase of PIP_3 in the membrane. mTOR1 amplifies the chemosensory signal through the activity of its downstream effector p70 ribosomal S6 kinase ($p70^{S6K}$), which facilitates the activation of the GTPases Cdc42 and Rac1 presumably via the protein TIAM Rac1 associated GEF 1 (Tiam1) (Boissier & Huynh-Do, 2014). However, the $p70^{S6K}$/GTPase mechanisms are not fully understood and need to be further studied in macrophages (Linke et al., 2017; Ip & Wong, 2012; Tavares et al., 2015; Aslan, 2011).

mTOR assistance in actin stabilization. mTOR1 promotes the stabilization of actin filaments by activating actin cross-linking proteins and inhibiting filament depolymerization. These processes are primarily accomplished through the phosphorylation of $p70^{S6K}$ and its subsequent downstream effectors. Although it has yet to be demonstrated in leukocytes, $p70^{S6K}$ in cancer cells cross-links actin filaments to reinforce protrusions formed at the membrane by WASP and WAVE2 (Ip et al., 2011). This provides strength to the new filaments, so they can push against and extend the membrane outward without buckling from the force or being broken down by severing proteins. The importance of $p70^{S6K}$ in maintaining the integrity of the growing cytoskeleton in cancer cells could suggest its conservation and role in macrophage chemotaxis as well. Though, experiments would be necessary to validate this interaction in leukocytes (Ip et al., 2011).

$p70^{S6K}$ also regulates the activity of cofilin by stimulating the Rac1/PAK pathway. It is theorized that $p70^{S6K}$ promotes GDP to GTP exchange on Rac1 by acting as a scaffold on the membrane for the formation of GTP-bound Rac1 by its GEF Tiam1 (Aslan et al., 2011). As aforementioned, GTP-bound Rac1 will promote the formation of lamellipodia through WAVE2, but the GTPase also acts on the serine/threonine-protein kinase (PAK) through PAK's specific Rac1 binding domain (Weiss-Haljiti et al., 2004). PAK aids actin filament stabilization by inhibiting cofilin through a short kinase signaling cascade with LIMK (Tavares et al., 2015). This coordinated regulation of cofilin through Rac1 and PIP_3 production is necessary so that opposing actin dynamics are not occurring simultaneously at the leading edge.

Membrane Expansion at the Leading Edge. The WASP, WAVE2, and mTOR-induced actin dynamics during chemotaxis rapidly increase the internal surface area of a macrophage almost two-fold. This poses an issue for the surrounding phospholipid bilayer—deemed the cell surface-area problem (Dewitt et al., 2007; Hallett et al., 2008). Due to the necessity of the membrane to protect and contain cellular contents, the plasma membrane has little to no elasticity. Significant spreading or thinning of the organelle would decrease its structural integrity and possibly burst the cell. When membrane surface-area does not increase along with cytoskeleton growth, then actin protrusions will push against the bilayer creating growing amounts of membrane tension. At extreme levels, BAR family proteins detach from the membrane and lamellipodia breaks down, together impeding cell mobility (Pipathsouk et al., 2019; Pontes et al., 2017). Therefore, to reduce tension, the cell must have a means of substantially increasing its membrane surface-area upon chemotactic stimulation to accompany actin polymerization (Dewitt et al., 2007). This growth is dependent on the redirection of membrane reserves within the cell to sustain the phospholipid bilayer. Two main processes

contribute to this additional membrane supply: the flattening of membrane wrinkles and the manipulation of endocytosis and exocytosis through the recycling endosome (Roberts et al., 2020). Without both the remodeling of the cytoskeleton and membrane, macrophages would be unable to efficiently carry out immune functions leaving the host defenseless.

Flattening of membrane wrinkles. Membrane wrinkles are regions of gathered plasma membrane that create a ribbed morphology on the surface of the cell. These structures are formed by the scaffolding protein ezrin which cross-links a branch of the actin cytoskeleton to the phospholipid bilayer and extends an actin filament outward from the cell (Figure 4). The actin projection is less than a micrometer tall and can laterally spread around 10 to 15 micrometers. Each side of the structure, excluding the internal cytoskeletal branch point, is encompassed by the plasma membrane (Dewitt et al., 2007). These projections provide a method to store extra membrane for migration purposes, and since these wrinkles cover the macrophage, the reserves are quite substantial.

Under normal conditions, ezrin binds to both PIP_2 and F-actin, which restricts the membrane surface-area and compacts the cell. This regulation of membrane space is advantageous because sustaining a large surface-area would be energetically ill-suited and cumbersome. A smaller area requires less energy to maintain, limits unwarranted diffusion within the cytoplasm, and keeps cellular processes concentrated so that coupled reactions remain in close vicinity. Ezrin thus allows the macrophage to efficiently complete cellular processes and have the means to expand when necessary.

Referring back to Figure 1 and the Falke lab model of the chemosensory pathway, when a chemoattractant binds to its corresponding receptor, reserves of intracellular calcium ions are released. The free calcium ions then bind to PKCα and initiate the PI3K cascade, but as well

simultaneously activate the cysteine protease calpain. Activated calpain will then cleave ezrin breaking the linkage between PIP_2 and the actin filament. As a result, the actin projection will retract back into the cell, flattening the membrane wrinkle, and elongating it into a larger surface-area (Roberts et al., 2020). New membrane-cytoskeleton cross-linkages are unlikely to reform because ezrin only binds to PIP_2, so once PIP_3 forms in the membrane, ezrin can no longer act as an effective scaffolding protein. This release is the macrophage's initial solution to limit membrane tension and sustain chemotactic-induced actin polymerization.

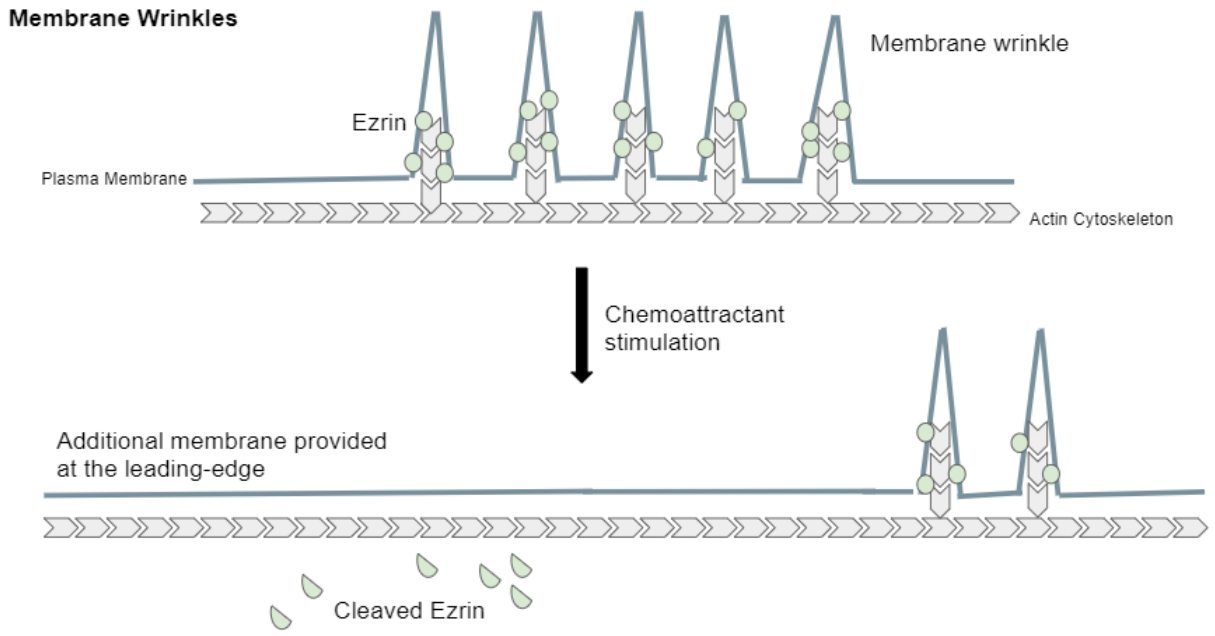


Figure 4. *Membrane wrinkles facilitate expansion at the leading edge.* Under non-stimulated conditions, ezrin binds to PIP_2 and F-actin to create small protrusions storing surface area on the membrane. Chemoattractant binding releases intracellular Ca^{2+} which activates the protease calpain that cleaves ezrin in two. The cleavage of ezrin causes the dissociation of actin from the protruding membrane leading to the membrane flattening out and providing additional phospholipid resources for expansion.

Though beneficial for providing immediate and sufficient amounts of plasma membrane during chemotaxis, the wrinkle deflation response has its limitations. Since the cleaving of ezrin and breakage of the membrane-cytoskeleton link is reliant on the activation of calpain and PI3K,

it solely localizes to the leading edge. Meaning that once all the membrane within that region smooths, membrane tension increases again. Just as before, the force of actin pushing against the phospholipid bilayer will become too much, causing actin polymerization and movement to stall, effectively stopping the chemotaxis response (Pontes et al., 2017).

Manipulation of the recycling endosome for membrane reserves. Following the exhaustion of leading edge membrane reserves, the second method to relieve membrane tension is endocytosis and exocytosis mediated by the recycling endosome. Typically, the recycling endosome facilitates autophagy during which it receives endocytic vesicles originating from the plasma membrane. These vesicles contain autophagy-related-proteins (ATGs) which flux endosomal membrane to the cytoplasm for the formation of the phagophore. The chemosensory mechanism manipulates this pathway by inhibiting ATG proteins from leaving the plasma membrane and redirecting endosomal vesicles to the leading edge. Similarly to the release of macrophage membrane wrinkles, this process is dependent on calpain and the calcium influx following chemoattractant stimulation. Calpain is believed to cleave ATG or adaptor proteins impairing the autophagic vesicle's ability to endocytose into the recycling endosome (Coly et al., 2017). Instead, membrane from the cell's side or non-stimulated areas will endocytose into the recycling endosome and then exocytose to the leading edge.

SNARE protein interactions between the recycling endosome and the leading edge control the integration of these additional membrane reserves. The R-SNARE VAMP3 on the endosome will intertwine with the Q-SNARE complex Stx4/SNAP23 on the plasma membrane and fuse the vesicle. This later addition of membrane to the leading edge is essential for lamellipodial formation and efficient chemotaxis, even though the relative amount of membrane provided is far smaller compared to the release of membrane wrinkles (Veale et al., 2010).

Discussion and Future Directions

Discussion. Macrophage chemotaxis relies on a complex interplay of cytoskeletal and membrane dynamics coordinated via the production of PIP_3 signaling lipid at the leading edge. Current literature has shown that the actin nucleation promoting factors WASP and WAVE2 are the primary drivers of macrophage chemotactic movement. By facilitating Arp ⅔ and G-actin binding at the leading edge, they stimulate the formation of branched actin structures to expand the membrane and extend the cell toward the chemoattractant of interest (Kurisu & Takenawa, 2009). Without these proteins, macrophage chemotaxis is significantly impaired, as observed in individuals with Wiskott-Aldrich syndrome—characterized by a WASP-deficiency and reduced immune function (Ishihara et al., 2012). Both WASP and WAVE2 are regulated in similar manners, where the release of their inactive conformation is dependent on the binding of a GTP-bound Rho family GTPase when coupled to the membrane via phosphoinositides and BAR scaffolding proteins (Rougerie et al., 2013). Although this review has proposed specific proteins to control the activation of the actin nucleation promoting factors, it appears plausible that redundancy is built into the system. For example, the Rho family GTPases Cdc42 and Rac1 could be activated by different GEFs present within the cell compared to what is presented in Figure 2. The involvement of different GEFs could enable regulation by different classes of receptors allowing responses to a wide array of attractants. More generally, due to the importance of macrophage chemotaxis in immune function, there are most likely redundant processes to ensure that a loss of one component is not detrimental to the pathway. Thus, understanding the necessity and sufficiency of these factors, especially in different cellular environments, will be key to continue expanding the model of macrophage chemotaxis.

The kinase complex mTOR also assists in chemotactic actin dynamics by promoting a positive feedback loop to amplify the PIP_3 signal (Linke et al., 2017; Ip & Wong, 2012; Tavares et al., 2015; Aslan, 2011). The phosphorylation of PIP_2 to PIP_3 via PI3K activates mTOR2 by binding and causing the dissociation of the inhibitory complex from the kinase active site (Yuan & Guan, 2015). Active mTOR2 then phosphorylates PKCα and blocks the inhibitory phosphorylation of Akt1/PKB, re-stimulating the downstream pathways of PKCα and Akt1/PKB (Linke et al., 2017). The mTOR complex additionally sustains actin polymerization at the leading edge by stabilizing actin filaments through mechanisms involving mTOR1. The kinase is stimulated when Akt1/PKB deactivates an inhibitor of the complex via phosphorylation (Linke et al., 2017). Active mTOR1 can then initiate $p70^{S6K}$ to cross-link actin filaments for stabilization and promote the activity of GTPases Cdc42 and Rac1 (Ip et al., 2011; Tavares et al., 2015; Aslan, 2011). GTP-bound Rac1 can further increase actin filament stabilization by acting through the PAK-LIMK signaling cascade to phosphorylate and inhibit cofilin's severing properties (Weiss-Haljiti et al., 2004). These processes involving $p70^{S6K}$ must be replicated in macrophages but have been observed in cancer and fibroblast cells (Ip et al., 2011; Aslan, 2011).

Combined with the WASP and WAVE2 pathways, these findings directed the composition of Figure 2 to detail the coordination of the mechanisms which stem from the model established by the Falke lab (Figure 1). The circuit illustrates how macrophages initiate their forward movement toward a chemoattractant gradient via the stimulation of Arp ⅔ actin branching, inhibition of cofilin, and use of actin cross-linking proteins—all regulated by PIP_3 in the membrane produced by a chemoattractant-bound RTK. The membrane remodeling processes involving ezrin and the recycling endosome are not defined in Figure 2 as they take place in various, unstimulated regions of the cell. Thus, to remain focused at the leading edge those

processes were omitted, however, that does not diminish their importance for chemotactic movement. Without the coordinated activities of all these pathways, macrophages could not effectively respond to chemoattractant signals and would remain stagnant following RTK binding. Whether these pathways are replicated downstream of other receptor types, such as GPCRs, requires further studies (Jones, 2000; Welch, 2015). While it is presumed that there are overlapping mechanisms between the receptors, differences could explain variances in F-actin polymerization rates observed following the stimulation of each (Ishihara et al., 2012; Rougerie et al., 2013).

Interestingly, these chemotactic regulators also have implications in another essential mechanism of the innate immune response—macrophage phagocytosis (Hirayama et al., 2018). Although these pathways could not be thoroughly explored due to the time constraints of this review, studies have shown that the PIP2/FBP-17/WASP pathway branch involved in expansion of the leading edge up an attractant gradient is also involved in the formation of the phagocytic cup in macrophages (Tsuboi et al., 2009**;** Rougerie et al., 2013). Dimerized FBP-17 induces membrane deformation and WASP-dependent actin polymerization to engulf diseased tissue or pathogens recognized by Fcγ receptors (Rougerie et al., 2013). Furthermore, the Falke lab has found that a strong PIP_3 signal accompanies the formation of the phagocytotic cup, suggesting that the PIP_3/IRSp53/WAVE2 pathway branch may also be involved in phagocytotic cup formation and expansion (Falke lab, unpublished). Following engulfment in the phagosome and fusion with the lysosome, the macrophage will kill and digest pathogens, thereby protecting the host from further infection (Hirayama et al., 2018). Moreover, the pathogen fragments generated by digestion are used to prime antibody production, thus the macrophages of the innate immune system play a central role in the initiation of adaptive immunity (Levin et al., 2016). With more

time, an investigation of the additional connections between the phagocytic pathway and the pathways of Figure 2 would be valuable to determine how macrophages coordinate their chemotactic movement with their degradative capabilities.

Proposed Future Studies. Though current literature has provided great insight into the interactions driving macrophage chemotactic movement, much remains to be explored about the molecular mechanisms of these processes. While Figure 2 illustrates key connections between the chemosensory stimulation of RTKs and actin polymerization, the circuit is likely not complete. Persisting questions include: What redundant processes are involved in the activation of WASP and WAVE2? Which BAR proteins have the most resilience to membrane tension? Or what other proteins could attribute to the positive feedback loops similarly seen with PKCα and mTOR2? Working to answer these questions will provide a more holistic understanding of macrophage chemotactic movement and morphology. Unfortunately, many of these questions can only be pursued in live cells where the complexity of the system makes rigorous, carefully controlled studies challenging. Thus, based on the presented circuit in Figure 2, I am proposing two main areas of study that could be researched using the well established Falke lab methods of *in vitro* pathway reconstitution followed by single molecule biophysical studies to elucidate regulatory mechanisms. These hypotheses of interest are addressed below.

Quantitative analysis of WASP and WAVE2 regulation by multiple activators. The activation of WASP and WAVE2 require multiple activators that collectively drive membrane recruitment and release of the VCA domain. Previously, it has been shown that full activity of WAVE2 requires the binding of all three regulators PIP_3, IRSp53, and GTP-Rac1 (Suetsugu et al., 2006). Yet similar *in vitro* studies regarding WASP activation by PIP_2, FBP-17, and GTP-Cdc42 have not been performed. Furthermore, for both WAVE2 and WASP activation, the

question remains of whether the interactions of the PIP_n lipid, the BAR protein, and the G protein provide simply additive activation or are able to combine synergistically to generate much higher levels of activation than expected for additivity. The Falke laboratory has the tools needed to answer these questions, as illustrated by their studies reconstituting PI3Kα activation by phosphotyrosines and the G protein HRas. These *in vitro* single molecule biophysical studies of the pathway reconstituted on supported lipid bilayers revealed that phosphotyrosines are essential for PI3Kα activation and PIP_3 production, and that HRas alone provides no activation, but phosphotyrosines and HRas together provide synergistic activation 10-fold greater than phosphotyrosines alone. Moreover, the lab found that the mechanism of HRas synergy was simply the recruitment of additional PI3Kα to the membrane (Buckles et al., 2017).

The same approach can be applied to the PIP_3/IRSp53/ GTP-Rac1/WAVE2 and the PIP_2/FBP-17/GTP-Cdc42/WASP systems. For example, the latter PIP_2/FBP-17/GTP-Cdc42/WASP pathway can be reconstituted by combining the purified components *in vitro* on a supported bilayer possessing a lipid composition designed to mimic the plasma membrane. In this reconstituted system the confounding factors present in the cell, such as complementary GEFs or F-BARs, can be eliminated. Then, the rate of WASP-induced actin polymerization can be quantified upon stimulation by PIP_2, FBP-17, or GTP-Cdc42 alone, as well as by the 3 possible pairwise combinations and by the 1 possible triple combination.

To evaluate possible crosstalk between the WASP and WAVE2 pathways, the regulators of each nucleation promoting factor should be separately incubated with the reconstituted systems as well to determine if the differing BAR proteins and GTPase have competing or augmenting activity. Notably, reconstitution of the PIP_3/IRSp53/ GTP-Rac1/WAVE2 and the PIP_2/FBP-17/GTP-Cdc42/WASP systems will be facilitated by the availability of plasmids and

expression systems for each of the protein components (Suetsugu et al., 2017; Takenawa & Suetsugu, 2007; Rougerie et al., 2013). Additionally, the proposed competitive inhibition between PSTPIP2 and FBP-17 or IRSp53 could be explored to determine if PSTPIP2 has overlapping inhibitive functions in the WAVE2 pathway as it is proposed to have in the WASP pathway.

Demonstration of $p70^{S6K}$ Pathways in Macrophages. In cancer, fibroblast, and blood platelet cells, $p70^{S6K}$ has been experimentally observed to cross-link actin filaments and activate both Cdc42 and Rac1 through direct interaction during directed cell migration (Ip et al., 2012; Tavares et al., 2015; Aslan, 2011). To test this picture, the mTOR1/ $p70^{S6K}$/Tiam1/Rac1, and mTOR1/ $p70^{S6K}$ /Cdc42 pathways can be imaged in macrophages using the same imaging approaches successful in the previously utilized cells. To evaluate the actin cross-linking ability of $p70^{S6K}$, a colocalization experiment using immunostaining or immunoprecipitation of $p70^{S6K}$ and F-actin could be performed with cell lysates from PDGF stimulated macrophages. Validating these interactions of $p70^{S6K}$ in macrophages would provide a direct test of the mechanisms of Figure 2 and would shed light on a key point of regulation for sustaining the actin dynamics driving chemotactic movement.

Closing Thoughts

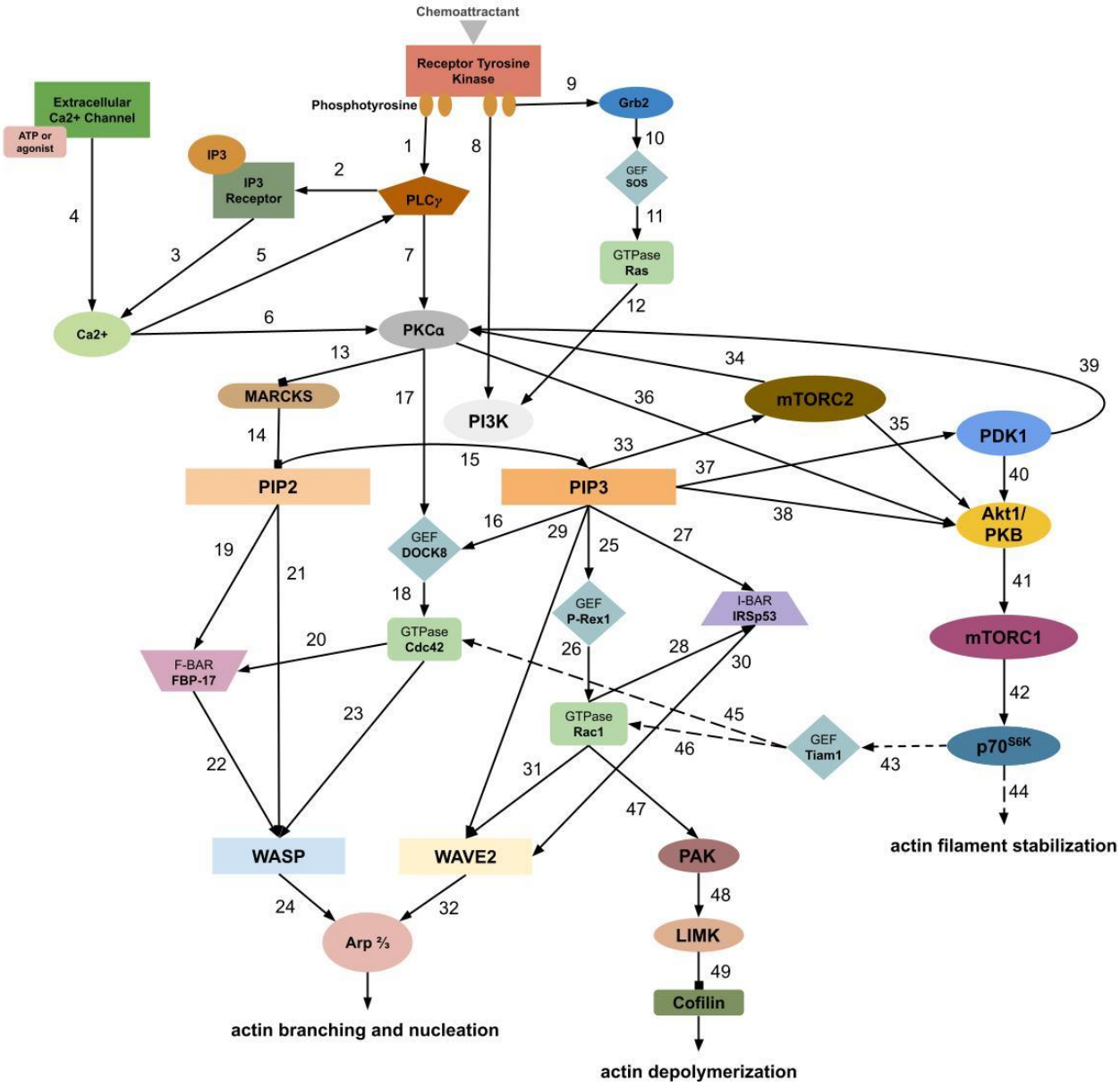
This exploration of the current literature detailing the mechanisms that underlie macrophage chemotaxis has identified that chemoattractant-bound receptors drive cytoskeletal actin dynamics by recruiting Rho family GTPases, BAR proteins, and actin nucleation promoting factors to the membrane via the signaling lipid PIP_3. This finding and the additional pathways illustrated in Figure 2 expand the Falke lab model of the chemosensory leading edge (Figure 1)

to provide a more holistic understanding of the chemotactic process and expose areas for future research. Specifically, it has been determined that the actin nucleation promoting factors WASP and WAVE2 are the primary effectors which drive actin branching for cell movement. These proteins facilitate Arp ⅔ actin branching following the release of their self-inhibitory conformations via binding to regulators recruited to the membrane by PIP_2 or PIP_3 (Kurisu & Takenawa, 2009). Additionally, the positive feedback of mTOR, PKCα, and PDK1 help amplify the PIP_3 signal to sustain chemotactic activity and implement an effective immune response even with low concentrations of chemoattractant signals (Linke et al., 2017; Ziemba et al., 2018). Yet consequently, the forward expansion of the cell due to actin polymerization places great strain on the plasma membrane and can lead to migration inhibition if not relieved. Thus, the cell dissipates this tension by providing additional membrane through the release of the macrophage's wrinkled morphology via ezrin cleaving and vesicular trafficking of membrane from unstimulated regions of the cell (Dewitt et al., 2007; Hallett et al., 2008; Roberts et al., 2020).

Collectively these processes work in a coordinated manner to stimulate the macrophage's ability to respond to chemoattractant signals and function in the innate immune response. The loss or inhibition of any of these mechanisms could have significant effects on macrophage chemotaxis resulting in health complications within the host. These issues include immunodeficiency disorders and increased viral or bacterial infections that could lead to the death of the organism (Hirayama et al., 2017). Therefore, it is imperative to continue expanding current models and investigating new mechanisms to not only strengthen the understanding of leukocyte function but to suggest possible targets for therapeutic research. The health of the

global community is dependent on improving our knowledge of these key actors in the immune system and learning how to respond when they malfunction.

Appendix



Supplemental Figure 1. *Proposed circuit diagram coupling the leading edge chemosensory pathway to actin dynamics.* Signaling cascade connecting the chemosensory leading edge to the stimulation of actin dynamics for cell movement initiated by the binding of a chemoattractant to a receptor tyrosine kinase (RTK). Phosphotyrosines produced via receptor binding activate the key pathway regulators protein kinase C-alpha (PKCα) and phosphoinositide-3-kinase (PI3K) for production of phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) signal at the membrane. PIP_3 recruits various kinase, guanine nucleotide exchange factors (GEFs), bin/amphiphysin/rvs (BAR) family proteins, and the nucleation promoting factors Wiskott-Aldrich syndrome (WASP) protein and WASP-family Verprolin-homologous protein 2 (WAVE2) protein resulting in the activation of actin related protein ⅔ (Arp ⅔) and the inhibition of cofilin. Arp ⅔ produces actin branching

and nucleation for the formation of lamellipodia and podosome structures. Filopodial structures formed via regulation of Arp ⅔ branching activity and actin bundling proteins. Positive feedback loop amplifying PIP_3 signal primarily controlled by kinases PKCα, mammalian target of rapamycin 2 (mTOR2), and phosphoinositide-dependent kinase 1 (PDK1). Solid lines indicate that the protein relationship has been experimentally proven in macrophages or leukocytes, while dashed lines indicate that an interaction has yet to be specifically demonstrated in macrophages. Specifics of protein interactions described in figure key below.

Supplemental Figure 1 Key

1. Chemoattractant-bound receptor tyrosine kinase recruits PLCγ to membrane via phosphotyrosine binding to SH2 and SH3 domains (Cocco et al., 2015)
2. PLCγ hydrolyzes PIP_2 to generate IP3 (Cocco et al., 2015)
3. Binding of IP3 triggers Influx of intracellular Ca^{2+} from ER (Zhu & Zhang, 2018)
4. Binding of ATP or intracellular agonist triggers Influx of extracellular Ca^{2+} (Zhu & Zhang, 2018)
5. Ca^{2+} recruits PLCγ to membrane via C2 domain (Cocco et al., 2015)
6. Ca^{2+} recruits PKCα to membrane via C2 domain (Ziemba et al., 2018)
7. PLCγ hydrolyzes PIP_2 to generate DAG recruiting and activating PKCα (Ziemba et al., 2018)
8. Chemoattractant-bound receptor tyrosine kinase recruits PI3K to membrane via phosphotyrosine binding to SH3 domain (Jones, 2000)
9. Chemoattractant-bound receptor tyrosine kinase recruits Grb2 to membrane via phosphotyrosine binding to SH2 domains (Lodish et al., 2000)
10. Grb2 recruits Sos GEF to membrane via SH3 domain (Lodish et al., 2000)
11. Sos exchanges GDP for GTP on Ras (Baruzzi et al., 2015)
12. GTP-bound Ras activates catalytic subunit of PI3K by binding (Charest & Firtel, 2006)
13. PKCα releases MARCKS from membrane by phosphorylation (Ziemba et al., 2018)

14. MARCKS sequesters PIP_2 in plasma membrane (Ziemba et al., 2018)
15. PI3K phosphorylates PIP_2 to PIP_3 (Ziemba et al., 2018)
16. PIP_3 recruits DOCK8 to membrane via DHR-1 domain (Kearney et al., 2017)
17. PKCα phosphorylates DOCK8 (Kearney et al., 2017)
18. DOCK8 Exchanges GDP for GTP on Cdc42 at leading edge membrane (Kearney et al., 2017)
19. PIP_2 recruits FBP-17 to membrane via F-BAR domain (Derivery & Gautreau, 2010)
20. GTP-bound Cdc42 stabilizes FBP-17 at membrane via HR1 domain (Watson et al., 2017)
21. PIP_2 recruits WASP to membrane via lysine-rich, basic region (Kurisu & Takenawa, 2009)
22. FBP-17 recruits WASP to membrane via proline rich region (Derivery & Gautreau, 2010; Watson et al., 2017)
23. GTP-bound Cdc42 releases WASP's inhibitory conformation and VCA domain via CRIB domain (Lane et al., 2014; Rougerie et al., 2013)
24. WASP recruits Arp ⅔ to membrane and stimulates actin nucleation via VCA domain (Rougerie et al., 2013)
25. PIP_3 recruits P-Rex1 to membrane via PH domain (Barber et al., 2007)
26. P-Rex1 exchanges GDP for GTP on Rac1 at membrane (Barrows et al., 2016)
27. PIP_3 recruits IRSp53 to membrane via I-BAR domain (Salzar et al., 2017)
28. GTP-bound Rac1 stabilizes and releases inhibitive conformation of IRSp53 at the membrane via CRIB domain (Carman & Dominguez, 2018)
29. PIP_3 recruits WAVE2 membrane via lysine-rich, basic region (Kurisu & Takenawa, 2009)

30. IRSp53 recruits WAVE2 to membrane via proline rich region (Derivery & Gautreau, 2010)
31. GTP-bound Rac1 releases inhibitory conformation and VCA domain via WAVE regulatory complex (Rougerie et al., 2013)
32. WAVE2 recruits Arp ⅔ to membrane and stimulates actin nucleation via VCA domain (Rougerie et al., 2013)
33. PIP_3 releases inhibitive subunit of mTORC2 via PH domain (Yuan & Guan, 2015)
34. mTORC2 phosphorylates PKCα (Linke et al., 2017)
35. mTORC2 phosphorylates Akt1/PKB (Linke et al., 2017)
36. PKCα inhibits phosphorylation of Akt1 (Ziemba et al., 2018)
37. PIP_3 recruits PDK1 to membrane via PH domain (Ziemba et al., 2018)
38. PIP_3 recruits Akt1/PKB to membrane via PH domain (Ziemba et al., 2018)
39. PDK1 phosphorylates PKCα (Ziemba et al., 2018)
40. PDK1 phosphorylates Akt1/PKB (Ziemba et al., 2018)
41. Akt1/PKB phosphorylates TSC2/TSC1 activating mTORC1 (Linke et al., 2017)
42. mTORC1 phosphorylates $p70^{S6K}$ (Linke et al., 2017)
43. $p70^{S6K}$ binds Tiam1 to promote GDP/GTP exchange on Rac1 and Cdc42 (Aslan et al., 2011; Boissier & Huynh-Do, 2014)
44. $p70^{S6K}$ cross-links actin filaments (Ip et al., 2011)
45. Tiam1 exchanges GDP for GTP on Cdc42 (Boissier & Huynh-Do, 2014)
46. Tiam1 exchanges GDP for GTP on Rac1 (Aslan et al., 2011)
47. GTP-bound Rac1 activates and recruits PAK to membrane via Rac1 specific binding domain (Weiss-Haljiti et al., 2004)

48. PAK phosphorylates LIMK (Tavares et al., 2015)

49. LIMK phosphorylates and inhibits Cofilin (Tavares et al., 2015)

Protein	Associated Lab	Plasmid	Expression Culture	Affinity Tag	Reference
Arp ⅔	Marie-France Carlier, French National Centre for Scientific Research	n/a	Purified from bovine brain	n/a	Egile & Carlier et al, 1999 J Cell. Biol. PMID: 10491394
Cdc42	Klaus Hahn, The Scripps Research Institute	pcDNA3-EGFP-Cdc42(wt)	n/a	EGFP	Nalbant & Hahn et al, 2004 Science. PMID: 15361624
Cofilin	Cytoskeleton, Inc, Denver, CO	*Product name* Cofilin 1 Protein: Human Recombinant	n/a	n/a	Ip & Wong et al, 2011 Oncogene. PMID: 21258406
DOCK8	Yoshinori Fukui, Kyushu University	pC1 (Promega)	HEK-293T	GFP	Shiraishi & Fukui et al, 2017 J. Biol. Chem PMID: 28028174
FBP-17	Tadaomi Takenawa, Kobe University	pEF-BOS-FLAG	FreeStyle-293-F	N-terminal FLAG	Tsujita & Takenawa et al, 2013 J. Cell Sci. PMID: 23525018
IRSp53	Mark Vidal, Harvard Medical School	pENTR223	HEK-293T	C-terminal GFP	Rual & Vidal, 2004 Genome Res. PMID: 15489335
LIMK1	ThermoFisher Scientific, Waltham, MA	*Product name* LIMK1 Recombinant Human Protein	Baculovirus	His	Ding & Alahari et al, 2008 Mol Cell Biol. PMID: 18332102
mTORC1	Seong Kang, Whitehead Institute for Biomedical Research	MSCV retrovirus	HEK-293T	N-terminal FLAG-raptor-M2	Yip & Kang et al, 2010 Mol Cell. PMID: 20542007
$p70^{S6K}$	John Blenis, Weill Cornell Medicine	pRK7-HA-S6K1-WT	HEK-293E	N-terminal HA	Schalm & Blenis, 2002 Curr Biol. PMID: 11967149
PAK1	Johnathan Chernoff, Fox Chase Cancer Center	pCMV6M-Pak1	n/a	N-terminal Myc	Sells & Chernoff et al, 1997 Curr Biol. PMID: 9395435
P-Rex1	Heidi Welch, Inositide Laboratory	MYC-pCMV3-PREX1	Sf9	N-terminal Myc	Welch & Stephens et al, 2002 Cell. PMID: 11955434
PSTPIP2	Tadaomi Takenawa, Kobe University	pEF-BOS-FLAG	FreeStyle-293-F	N-terminal FLAG	Tsujita & Takenawa et al, 2013 J. Cell Sci. PMID: 23525018
Rac1	Klaus Hahn, Scripps Research Institute	pcDNA3-EGFP-Cdc42(wt)	n/a	EGFP	Kryanov & Hahn et al, 2000 Science. PMID: 11030651

WASP	Katherine Siminovitch, University of Toronto	pEGFP-C3WT	K562	EGFP	McGavin & Siminovitch et al, 2001 J Exp Med. PMID: 11748279
WAVE2 Complex (WAVE2, Abi1, PIR121, and Nap1)	Tadaomi Takenawa, University of Tokyo	pFastBac-Dual	Sf9	C-terminal GST	Suetsugu & Takenawa et al, 2006 J. Cell Biol. PMID: 16702231

Plasmids and Purified Proteins. *Plasmids and Purified Proteins of WASP, WAVE2, and mTOR Systems*. Available plasmids and purified proteins of the regulators presented in WASP, WAVE2, and mTOR activation pathways. Plasmid name, tissue culture of cell expression, and protein affinity tag provided where applicable. Reference includes literature where plasmid or purified protein was constructed or used.

References

Abou-Kheir, W., Isaac, B., Yamaguchi, H., & Cox, D. (2008). Membrane Targeting of WAVE2 Is Not Sufficient for WAVE2-dependent Actin Polymerization: A Role for IRSp53 in Mediating the Interaction Between Rac and WAVE2. *Journal of Cell Science*, *121*(3), 379–390. https://doi.org/10.1242/jcs.010272

Aslan, J. E., Tormoen, G. W., Loren, C. P., Pang, J., & McCarty, O. J. T. (2011). S6K1 and mTOR Regulate Rac1-driven Platelet Activation and Aggregation. *Blood*, *118*(11), 3129–3136. https://doi.org/10.1182/blood-2011-02-331579

Barber, M. A., Donald, S., Thelen, S., Anderson, K. E., Thelen, M., & Welch, H. C. E. (2007). Membrane Translocation of P-Rex1 Is Mediated by G Protein βγ Subunits and Phosphoinositide 3-Kinase. *Journal of Biological Chemistry*, *282*(41), 29967–29976. https://doi.org/10.1074/jbc.M701877200

Barrows, D., He, J. Z., & Parsons, R. (2016). PREX1 Protein Function Is Negatively Regulated Downstream of Receptor Tyrosine Kinase Activation by p21-activated Kinases (PAKs). *Journal of Biological Chemistry*, *291*(38), 20042–20054. https://doi.org/10.1074/jbc.M116.723882

Baruzzi, A., Remelli, S., Lorenzetto, E., Sega, M., Chignola, R., & Berton, G. University of Verona, Verona, Italy. (2015). Sos1 Regulates Macrophage Podosome Assembly and Macrophage Invasive Capacity. *The Journal of Immunology*, *195*(10), 4900–4912. https://doi.org/10.4049/jimmunol.1500579

Berven, L. A., Willard, F. S., & Crouch, M. F. (2004). Role of the p70S6K Pathway in Regulating the Actin Cytoskeleton and Cell Migration. *Experimental Cell Research*, *296*(2), 183–195. https://doi.org/10.1016/j.yexcr.2003.12.032

Bhagavan, N. V., & Ha, C.-E. (2015). Chapter 33—Immunology. In N. V. Bhagavan & C.-E. Ha (Eds.), *Essentials of Medical Biochemistry* (pp. 607–636). Academic Press. https://doi.org/10.1016/B978-0-12-416687-5.00033-6

Boissier, P., & Huynh-Do, U. (2014). The guanine nucleotide exchange factor Tiam1: A Janus-faced molecule in cellular signaling. *Cellular Signalling*, *26*(3), 483–491. https://doi.org/10.1016/j.cellsig.2013.11.034

Buckles, T. C., Ziemba, B. P., Masson, G. R., Williams, R. L., & Falke, J. J. (2017). Single-Molecule Study Reveals How Receptor and Ras Synergistically Activate PI3Kα and PIP3 Signaling. *Biophysical Journal*, *113*(11), 2396–2405. https://doi.org/10.1016/j.bpj.2017.09.018

Campa, C. C., Ciraolo, E., Ghigo, A., Germena, G., & Hirsch, E. (2015). Crossroads of PI3K and Rac Pathways. *Small GTPases*, *6*(2), 71–80. https://doi.org/10.4161/21541248.2014.989789

Carman, P. J., & Dominguez, R. (2018). BAR Domain Proteins—A Linkage Between Cellular Membranes, Signaling Pathways, and the Actin Cytoskeleton. *Biophysical Reviews*, *10*(6), 1587–1604. https://doi.org/10.1007/s12551-018-0467-7

Cocco, L., Follo, M. Y., Manzoli, L., & Suh, P.-G. (2015). Phosphoinositide-specific Phospholipase C in Health and Disease. *Journal of Lipid Research*, *56*(10), 1853–1860. https://doi.org/10.1194/jlr.R057984

Charest, P. G., & Firtel, R. A. (2006). Feedback Signaling Controls Leading edge Formation During Chemotaxis. *Current Opinion in Genetics & Development*, *16*(4), 339–347. https://doi.org/10.1016/j.gde.2006.06.016

Chitu, V., Pixley, F. J., Macaluso, F., Larson, D. R., Condeelis, J., Yeung, Y.-G., & Stanley, E. R. (2005). The PCH Family Member MAYP/PSTPIP2 Directly Regulates F-Actin Bundling and Enhances Filopodia Formation and Motility in Macrophages. *Molecular Biology of the Cell*, *16*(6), 2947–2959. https://doi.org/10.1091/mbc.e04-10-0914

Coly, P.-M., Gandolfo, P., Castel, H., & Morin, F. (2017). The Autophagy Machinery: A New Player in Chemotactic Cell Migration. *Frontiers in Neuroscience, 11(78),* 1-11. https://doi.org/10.3389/fnins.2017.00078

Derivery, E., & Gautreau, A. (2010). Generation of Branched Actin Networks: Assembly and Regulation of the N-WASP and WAVE Molecular Machines. *BioEssays, 32*(2), 119–131. https://doi.org/10.1002/bies.200900123

Deuel, T. F., Senior, R. M., Huang, J. S., & Griffin, G. L. (1982). Chemotaxis of Monocytes and Neutrophils to Platelet-derived Growth Factor. *The Journal of Clinical Investigation*, *69*(4), 1046–1049. https://doi.org/10.1172/JCI110509

Dewitt, S., & Hallett, M. (2007). Leukocyte Membrane "Expansion": A Central Mechanism for Leukocyte Extravasation. *Journal of Leukocyte Biology, 81*(5), 1160–1164. https://doi.org/10.1189/jlb.1106710

Evans, J. H., & Falke, J. J. (2007). Ca2+ influx is an essential component of the positive-feedback loop that maintains leading-edge structure and activity in macrophages. *Proceedings of the National Academy of Sciences*, *104*(41), 16176–16181. https://doi.org/10.1073/pnas.0707719104

Frugtniet, B., Jiang, W. G., & Martin, T. A. (2015). Role of the WASP and WAVE family Proteins in Breast Cancer Invasion and Metastasis. *Breast Cancer: Targets and Therapy*, *7*, 99–109. https://doi.org/10.2147/BCTT.S59006

Goethem, E. V., Poincloux, R., Gauffre, F., Maridonneau-Parini, I., & Cabec, V. L. (2010). Matrix Architecture Dictates Three-Dimensional Migration Modes of Human Macrophages: Differential Involvement of Proteases and Podosome-Like Structures. *The Journal of Immunology*, *184*(2), 1049–1061. https://doi.org/10.4049/jimmunol.0902223

Hallett, M. B., von Ruhland, C. J., & Dewitt, S. (2008). Chemotaxis and the Cell Surface-area Problem. *Nature Reviews Molecular Cell Biology*, *9*(8), 662–662. https://doi.org/10.1038/nrm2419-c1

Hawkins, P. T., & Stephens, L. R. (2015). PI3K Signalling in Inflammation. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1851*(6), 882–897. https://doi.org/10.1016/j.bbalip.2014.12.006

Hirayama, D., Iida, T., & Nakase, H. (2017). The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *International Journal of Molecular Sciences, 19*(1), 1-14. https://doi.org/10.3390/ijms19010092

Ip, C. K. M., & Wong, A. S. T. (2012). P70 S6 Kinase and Actin Dynamics. *Spermatogenesis*, *2*(1), 44–52. https://doi.org/10.4161/spmg.19413

Ishihara, D., Dovas, A., Park, H., Isaac, B. M., & Cox, D. (2012). The Chemotactic Defect in Wiskott-Aldrich Syndrome Macrophages Is Due to the Reduced Persistence of Directional Protrusions. *PloS One*, *7*(1). https://doi.org/10.1371/journal.pone.0030033

Janmey, P. A., Bucki, R., & Radhakrishnan, R. (2018). Regulation of Actin Assembly by PI(4,5)P2 and Other Inositol Phospholipids: An Update on Possible Mechanisms. *Biochemical and Biophysical Research Communications*, *506*(2), 307–314. https://doi.org/10.1016/j.bbrc.2018.07.155

Jones, G. E. (2000). Cellular Signaling in Macrophage Migration and Chemotaxis. *Journal of Leukocyte Biology, 68*(5), 593–602. https://doi.org/10.1189/jlb.68.5.593

Kameritsch, P., & Renkawitz, J. (2020). Principles of Leukocyte Migration Strategies. *Trends in Cell Biology, 30*(10), 818-832. https://doi.org/10.1016/j.tcb.2020.06.007

Kearney, C. J., Randall, K. L., & Oliaro, J. (2017). DOCK8 Regulates Signal Transduction Events to Control Immunity. *Cellular & Molecular Immunology*, *14*(5), 406–411. https://doi.org/10.1038/cmi.2017.9

Kölsch, V., Charest, P. G., & Firtel, R. A. (2008). The Regulation of Cell Motility and Chemotaxis by Phospholipid Signaling. *Journal of Cell Science*, *121*(5), 551–559. https://doi.org/10.1242/jcs.023333

Kurisu, S., & Takenawa, T. (2009). The WASP and WAVE Family Proteins. *Genome Biology*, *10*(6), 1-9. https://doi.org/10.1186/gb-2009-10-6-226

Lane, J., Martin, T., Weeks, H. P., & Jiang, W. G. (2014). Structure and Role of WASP and WAVE in Rho GTPase Signalling in Cancer. *Cancer Genomics & Proteomics*, 11(3), 155-166.

Lee, S.H., & Dominguez, R. (2010). Regulation of Actin Cytoskeleton Dynamics in Cells. *Mol Cells, 29*(4), 311–325. https://doi.org/10.1007/s10059-010-0053-8

Levin, R., Grinstein, S., & Canton, J. (2016). The life cycle of phagosomes: Formation, maturation, and resolution. *Immunological Reviews*, *273*(1), 156–179. https://doi.org/10.1111/imr.12439

Linke, M., Fritsch, S. D., Sukhbaatar, N., Hengstschläger, M., & Weichhart, T. (2017). mTORC1 and mTORC2 as Regulators of Cell Metabolism in Immunity. *FEBS Letters*, *591*(19), 3089–3103. https://doi.org/10.1002/1873-3468.12711

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). Receptor Tyrosine Kinases and Ras. In *Molecular Cell Biology. 4th edition*. W. H. Freeman. https://www.ncbi.nlm.nih.gov/books/NBK21720/

Madhav, N., Oppenheim, B., Gallivan, M., Mulembakani, P., Rubin, E., & Wolfe, N. (2017). Pandemics: Risks, Impacts, and Mitigation. *Disease Control Priorities*, *9*(3), 315-345. https://doi.org/10.1596/978-1-4648-0527-1_ch17

Merino, F., Pospich, S., & Raunser, S. (2020). Towards a Structural Understanding of the Remodeling of the Actin Cytoskeleton. *Seminars in Cell & Developmental Biology*, *102*, 51-64. https://doi.org/10.1016/j.semcdb.2019.11.018

Pipathsouk, A., Brunetti, R. M., Town, J. P., Breuer, A., Pellett, P. A., Marchuk, K., Tran, N.-H. T., Krummel, M. F., Stamou, D., & Weiner, O. D. (2019). WAVE Complex Self-organization Templates Lamellipodial Formation. *BioRxiv*, 1-44. https://doi.org/10.1101/836585

Pixley, F. J. (2012). Macrophage Migration and Its Regulation by CSF-1. *International Journal of Cell Biology*, *2012*, 1-12. https://doi.org/10.1155/2012/501962

Pontes, B., Monzo, P., & Gauthier, N. C. (2017). Membrane Tsion: A Challenging But Universal Physical Parameter in Cell Biology. *Seminars in Cell & Developmental Biology*, *71*, 30–41. https://doi.org/10.1016/j.semcdb.2017.08.030

Ridley, A. J. (2011). Life at the Leading Edge. *Cell*, *145*(7), 1012–1022. https://doi.org/10.1016/j.cell.2011.06.010

Roberts, R. E., Dewitt, S., & Hallett, M. B. (2020). Membrane Tension and the Role of Ezrin During Phagocytosis. In M. B. Hallett (Ed.), *Molecular and Cellular Biology of Phagocytosis*, (pp. 83–102). Springer International Publishing. https://doi.org/10.1007/978-3-030-40406-2_6

Rougerie, P., Miskolci, V., & Cox, D. (2013). Generation of Membrane Structures During Phagocytosis and Chemotaxis of Macrophages: Role and Regulation of the Actin Cytoskeleton. *Immunological Reviews*, *256*(1), 222–239. https://doi.org/10.1111/imr.12118

Sadok, A., & Marshall, C. J. (2014). Rho GTPases: Masters of Cell Migration. *Small GTPases*, *5*(4), 1-7. https://doi.org/10.4161/sgtp.29710

Salzer, U., Kostan, J., & Djinović-Carugo, K. (2017). Deciphering the BAR Code of Membrane Modulators. *Cellular and Molecular Life Sciences*, *74*(13), 2413–2438. https://doi.org/10.1007/s00018-017-2478-0

Senju, Y., & Lappalainen, P. (2019). Regulation of actin dynamics by PI(4,5)P2 in cell migration and endocytosis. *Current Opinion in Cell Biology*, *56*, 7–13. https://doi.org/10.1016/j.ceb.2018.08.003

Smith, B. A., Daugherty-Clarke, K., Goode, B. L., & Gelles, J. (2013). Pathway of Actin Filament Branch Formation by Arp2/3 Complex Revealed by Single-molecule Imaging. *Proceedings of the National Academy of Sciences*, *110*(4), 1285-1290. https://doi.org/10.1073/pnas.121116411

Snyderman, R., & Mergenhagen, S. E. (1976). Chemotaxis of Macrophages. In D. S. Nelson, (Ed.). *Immunobiology of the Macrophage*, 323-348. https://doi.org/10.1016/b978-0-12-514550-3.50019-0

Suetsugu, S., Kurisu, S., Oikawa, T., Yamazaki, D., Oda, A., & Takenawa, T. (2006). Optimization of WAVE2 Complex–induced Actin Polymerization by Membrane-bound IRSp53, PIP3, and Rac. *Journal of Cell Biology*, *173*(4), 571–585. https://doi.org/10.1083/jcb.200509067

Suman, P., Mishra, S., & Chander, H. (2020). High Formin Binding Protein 17 (FBP17) Expression Indicates Poor Differentiation and Invasiveness of Ductal Carcinomas. *Scientific Reports*, *10*(1), 11543-11553. https://doi.org/10.1038/s41598-020-68454-9

Takenawa, T., & Suetsugu, S. (2007). The WASP–WAVE Protein Network: Connecting the Membrane to the Cytoskeleton. *Nature Reviews Molecular Cell Biology*, *8*(1), 37–48. https://doi.org/10.1038/nrm2069

Tavares, M. R., Pavan, I. C. B., Amaral, C. L., Meneguello, L., Luchessi, A. D., & Simabuco, F. M. (2015). The S6K Protein Family in Health and Disease. *Life Sciences*, *131*, 1–10. https://doi.org/10.1016/j.lfs.2015.03.001

The Cytoskeleton. (2019, April 27). LibreTexts. Retrieved October 3, 2020, from https://chem.libretexts.org/@go/page/8528

Tsuboi, S. (2007). Requirement for a Complex of Wiskott-Aldrich Syndrome Protein (WASP) with WASP Interacting Protein in Podosome Formation in Macrophages. *The Journal of Immunology*, *178*(5), 2987–2995. https://doi.org/10.4049/jimmunol.178.5.2987

Tsuboi, S., Takada, H., Hara, T., Mochizuki, N., Funyu, T., Saitoh, H., Terayama, Y., Yamaya, K., Ohyama, C., Nonoyama, S., & Ochs, H. D. (2009). FBP17 Mediates a Common Molecular Step in the Formation of Podosomes and Phagocytic Cups in Macrophages. *The Journal of Biological Chemistry*, *284*(13), 8548–8556. https://doi.org/10.1074/jbc.M805638200

Tsujita, K., Kondo, A., Kurisu, S., Hasegawa, J., Itoh, T., & Takenawa, T. (2013). Antagonistic Regulation of F-BAR Protein Assemblies Controls Actin Polymerization During

Podosome Formation. *Journal of Cell Science*, *126*(10), 2267–2278. https://doi.org/10.1242/jcs.122515

Veale, K. J., Offenhäuser, C., Whittaker, S. P., Estrella, R. P., & Murray, R. Z. (2010). Recycling Endosome Membrane Incorporation into the Leading Edge Regulates Lamellipodia Formation and Macrophage Migration. *Traffic*, *11*(10), 1370–1379. https://doi.org/10.1111/j.1600-0854.2010.01094.x

Ventola C. L. (2015). The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *P&T: A Peer-reviewed Journal for Formulary Management*, *40*(4), 277–283.

Watson, J. R., Fox, H. M., Nietlispach, D., Gallop, J. L., Owen, D., & Mott, H. R. (2016). Investigation of the Interaction between Cdc42 and Its Effector Toca1: Handover of Cdc42 to the Actin Regulator N-WASP is Facilitated by Differential Binding Affinities. *Journal of Biological Chemistry*, *291*(26), 13875–13890. https://doi.org/10.1074/jbc.M116.724294

Watson, J. R., Owen, D., & Mott, H. R. (2017). Cdc42 in Actin Dynamics: An Ordered Pathway Governed by Complex Equilibria and Directional Effector Handover. *Small GTPases*, *8*(4), 237–244. https://doi.org/10.1080/21541248.2016.1215657

Weiss-Haljiti, C., Pasquali, C., Ji, H., Gillieron, C., Chabert, C., Curchod, M.-L., Hirsch, E., Ridley, A. J., van Huijsduijnen, R. H., Camps, M., & Rommel, C. (2004). Involvement of Phosphoinositide 3-Kinase γ, Rac, and PAK Signaling in Chemokine-induced Macrophage Migration*. *Journal of Biological Chemistry*, *279*(41), 43273–43284. https://doi.org/10.1074/jbc.M402924200

Welch, H. C. (2015). Regulation and Function of P-Rex Family Rac-GEFs. *Small GTPases*, *6*(2), 49–70. https://doi.org/10.4161/21541248.2014.973770

Yuan, H.-X., & Guan, K.-L. (2015). The Sin1 PH Domain Connects mTORC2 to PI3K. *Cancer Discovery*, *5*(11), 1127–1129. https://doi.org/10.1158/2159-8290.CD-15-1125

Zhu, L., Jones, C., & Zhang, G. (2018). The Role of Phospholipase C Signaling in Macrophage-Mediated Inflammatory Response. *Journal of Immunology Research*, *2018*, 1-9. https://doi.org/10.1155/2018/5201759

Ziemba, B. P., & Falke, J. J. (2018). A PKC-MARCKS-PI3K Regulatory Module Links Ca2+ and PIP_3 Signals at the Leading Edge of Polarized Macrophages. *Plos One*, *13*(5). https://doi.org/10.1371/journal.pone.0196678