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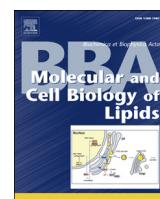
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Review

Phosphoinositides in phagocytosis and macropinocytosis

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ABSTRACT

Professional phagocytes provide immunoprotection and aid in the maintenance of tissue homeostasis. They perform these tasks by recognizing, engulfing and eliminating pathogens and endogenous cell debris. Here, we examine the paramount role played by phosphoinositides in phagocytosis and macropinocytosis, two major endocytic routes that mediate the uptake of particulate and fluid matter, respectively. We analyze accumulating literature describing the molecular mechanisms whereby phosphoinositides translate environmental cues into the complex, sophisticated responses that underlie the phagocytic and macropinocytic responses. In addition, we exemplify virulence strategies involving modulation of host cell phosphoinositide signaling that are employed by bacteria to undermine immunity. This article is part of a Special Issue entitled Phosphoinositides.

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1. Introduction

Macrophages, neutrophils and dendritic cells are professional phagocytes appointed with vital tasks such as immune surveillance and the elimination of pathogens. These myeloid cells are uniquely skilled in the recognition, engulfment and disposition of microbial intruders, thereby contributing to the first line of defense against infection by a mechanism known as phagocytosis. The latter is a dedicated, receptor-initiated mechanism that results in the internalization of particles $\geq 0.5 \mu\text{m}$ in diameter. In cases where innate defenses prove insufficient, macrophages and dendritic cells initiate adaptive immunity by presenting antigens derived from engulfed material to lymphocytes.

In addition to its immunoprotective role in infection, phagocytosis is also pivotal for important housekeeping tasks. In particular, professional and non-professional phagocytes (like fibroblasts and epithelial cells) support the turnover of billions of apoptotic cells every day. The internalization of effete cells by phagocytosis – also known as efferocytosis – has important ramifications for wound healing,

development and morphogenesis, all processes in which timely removal of cell debris is critical for homeostasis.

Macrophages and dendritic cells continuously sample and probe their environment in the lookout for foreign antigens and danger signals [1]. While these cells internalize particulate matter by phagocytosis, they also take up fluid-phase material and soluble antigens by macropinocytosis. Both phagocytosis and macropinocytosis give rise to large vacuoles known as phagosomes and macropinosomes, respectively. Such vacuoles, which are initially generated by invagination of the plasma membrane, endure a radical metamorphosis in order to become microbicidal and degradative organelles that are conducive to pathogen elimination, antigen presentation and ultimate disposal of the vacuolar contents. This makeover, known as the maturation process, requires a carefully orchestrated sequence of fusion and fission events with multiple components of the endocytic pathway. Phagocytosis and macropinocytosis can therefore be conceptually bisected into two phases: one consisting of involution and scission of a plasmalemma-derived vacuole (formation), and the other its progression through the endocytic pathway (maturation).

Although their internalized cargo clearly differs, similar molecular machinery is engaged for the biogenesis of phagocytic and macropinocytic compartments. Both the formation and maturation of phagosomes/macropinosomes entail sophisticated signaling cascades, as well as extensive cytoskeletal reorganization and membrane remodeling. Specifically, the capture of phagocytic prey [2] and the formation

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of macropinosomes [1] are both preceded by Rho GTPase activation [3, 4] and the extension of actin-driven membrane protrusions. Moreover, both phagocytosis and macropinocytosis require large-scale membrane remodeling, as well as activation of phosphoinositide 3-kinases (PI3Ks) [5].

When phagocytic targets are initially encountered, extracellular signals must be conveyed across the plasma membrane in order to initiate the complex cellular behaviors that culminate in phagocytosis. It is becoming increasingly apparent that phosphoinositides play a prominent role in relaying this information. Indeed, both the detection of ligands by transmembrane phagocytic receptors and the ruffling of membranes during macropinocytosis are accompanied by local changes in phosphoinositide composition. Similarly, phosphoinositides coordinate membrane fusion and fission events that lead to the acquisition of lysosomal properties during the course of maturation [6]. This is accomplished primarily via recruitment of effector proteins by a combination of stereochemical and electrostatic interactions. Thus, phosphoinositides are much more than mere building blocks or structural bystanders of cellular membranes. Instead, they fine-tune signal transduction pathways [7], help specify organelle identity [8], and direct membrane traffic [9].

Given its pivotal role in phagocyte function, phosphoinositide metabolism is often subverted by invasive pathogens as a colonizing strategy. By undermining or hijacking phosphoinositide homeostasis, intracellular bacteria alter membrane dynamics and gain entry into their host [10]. Maturation of the bacterium-containing vacuole into an organelle with lysosomal characteristics is similarly affected by the subversion of phosphoinositide signaling.

The intent of this review is to collate the available information regarding the involvement of phosphoinositides in phagocytosis and macropinocytosis, while stressing the gaps in our knowledge. This review will also feature selected examples of the molecular mechanisms by which bacterial pathogens commandeer phosphoinositide homeostasis, thereby undermining phagocytic defenses.

2. Phagocytosis: a primer on phagosome formation and maturation

Unlike macropinocytosis, phagocytosis is a receptor-mediated process; initiation of phagocytosis is strictly dependent on the engagement of specific receptors by a multivalent, particulate ligand [11]. The variety of distinct phagocytic targets that can be recognized by phagocytic receptors is extensive. These range from foreign bodies, including microbial pathogens such as bacteria, fungi and other parasites, to endogenous apoptotic corpses and cellular debris [12]. The ability of phagocytes to engage such a wide array of particles is attributable to their vast repertoire of receptors, which recognize a large number of ligands. Multiple types of receptors co-exist in mammalian macrophages, and each of these recognizes not one but often a number of different ligands [13]. Under physiological conditions several types of receptors are engaged simultaneously, and likely cooperate in the development of a complex phagocytic response [12]. Of note, the signal transduction pathways and molecular mechanisms whereby distinct phagocytic receptors prompt particle internalization can be substantially different [11]. Thus, considering phagocytosis as a single biological process is overly simplistic. Instead, phagocytosis should be thought of as an umbrella term that describes a family of processes that, while phenotypically related, are ultimately distinct at the molecular level.

Despite their inherent variability, different phagocytic modalities share some fundamental principles, including a dependency on membrane rearrangement and cytoskeletal remodeling, all carefully orchestrated by signal transduction pathways that involve phosphoinositides [12]. Before describing the specific role of individual phosphoinositides in phagocytosis, we describe briefly the molecular and cellular basis of phagosome formation and maturation. This should aid the reader in placing the pathways mediating phosphoinositide metabolism during phagocytosis in a more physiological context.

2.1. Phagosome formation

Phagosome formation can be conceptually divided into three stages: 1) binding of phagocytic targets; 2) extension of pseudopodia around the particle and; 3) sealing of the phagosome as pseudopodia meet and fuse [14]. Particle binding is dependent on the affinity of the receptor(s) for their cognate ligands, as well as on the surface density of both of ligands (on the particle surface) and receptors (on the phagocyte membrane). What follows is a brief description of some of the most thoroughly characterized phagocytic receptors and their respective ligands.

2.1.1. Phagocytic receptors and particle recognition

The recognition of microbial pathogens by cell surface receptors can occur directly or indirectly. Indirect binding entails an intermediary opsonizing molecule that coats the pathogen and bridges its association with phagocytic receptors. Conversely, direct interactions involve recognition of pathogen-associated molecular patterns (PAMPs), such as peptidoglycan or surface carbohydrates, by pattern recognition receptors (PRRs). PAMPs represent regular arrays of molecular structures that are present in microorganisms but are absent in metazoans. In turn, PRRs are germline-encoded cell surface proteins that quickly initiate immune responses upon recognition of these molecular signatures. Prototypical PRRs include scavenger receptors, C-type lectin receptors of the Dectin family, the mannose receptor and Toll-like receptors (TLRs). All of these receptor types act as microbial sensors and function in different capacities to prime or initiate the phagocytic response.

In contrast to the engulfment of microbial pathogens during occasional infection, clearance of apoptotic corpses must occur continuously. Indeed, between 1 and 5 million cells are turned over by efferocytosis every second in healthy human subjects [15], a truly remarkable feat. Failure to quickly and efficiently dispose of effete cells can result in tissue abnormalities [16], including secondary necrosis, which is in turn associated with recurrent inflammation and the development of autoimmune conditions [17]. Of interest, recognition and processing of apoptotic bodies result in the secretion of anti-inflammatory cytokines and tolerogenic mediators by professional phagocytes. Thus, recognition of phagocytic targets as 'self' allows for the establishment of an immunologically silent microenvironment [18].

Unlike receptors that directly sense patterns on microbial surfaces, opsonic receptors detect intermediary molecules (opsonins) that have deposited on foreign bodies [12]. Opsonins are soluble molecules found in the circulation and extracellular space, where they recognize and coat their ligands. The most widely studied opsonins include complement fragments (e.g. iC3b) and immunoglobulins (e.g. IgG). iC3b- and IgG-coated particles are recognized by the $\alpha_M\beta_2$ integrin (Mac-1) and Fc γ receptors (Fc γ R), respectively [12]. Fc γ R is by far the most thoroughly characterized phagocytic receptor type and will therefore be prominently featured throughout this review.

2.1.2. Signal transduction pathways mediating pseudopod extension and engulfment of IgG-opsonized particles

As is the case for most other immunoreceptors, aggregation of Fc γ R in the plane of the membrane leads to its activation [19]. Upon engagement of the Fc portion of IgG on the surface of a multivalent ligand, the receptors cluster laterally, effectively bringing their cytosolic domains into close apposition. Fc γ receptors carry within their cytosolic tails a conserved signaling sequence, known as the immunoreceptor tyrosine-based activation motif (ITAM), which becomes phosphorylated by non-receptor tyrosine kinases of the Src family upon receptor coalescence [20]. These kinases include Hck and Lyn [21,22]. ITAM sequences are characterized by a tandem YXXI/L motif, and phosphorylation of both of these tyrosines allows for optimal phagocytosis [23]. Amplification of the phosphorylation cascade involves the kinase activity of Syk, which carries two Src-homology 2 (SH2) domains in tandem and associates with the doubly-phosphorylated ITAM [24]. This

sequence – ligand binding, receptor clustering and initiation of phosphotyrosine signaling – is iterated multiple times as pseudopods grow around the surface of the phagocytic target. Pseudopods surround incoming targets in a zipper-like fashion until they eventually meet and fuse at the particle's distal end.

Upon Syk recruitment and phosphorylation, numerous adaptors and signaling proteins translocate to sites of particle engagement. Many of these become anchored at newly formed phosphotyrosines, where they can recruit phosphoinositide-modifying enzymes. In particular, this signaling cascade precipitates the translocation of class I phosphoinositide 3-kinase (PI3K) [25] and phospholipase C γ (PLC γ) [26]. The phosphoinositides metabolized by these and other enzymes play key roles in orchestrating the phagocytic response; for this reason, entire sections of the present review are devoted to individual phosphoinositide species.

In conjunction with the early signaling events described above, the levels of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] rapidly and transiently increase at the nascent phagosome during the stages of particle binding and pseudopod extension [27]. Such elevations in PtdIns(4,5)P₂ synergize with the concomitant activation of Rho family GTPases, including Rac1, Rac2 and Cdc42 [14], in the formation of filamentous actin networks during phagocytosis [28]. More specifically, PtdIns(4,5)P₂ stabilizes critical components of the actin polymerization machinery, including nucleation-promoting factors of the Wiskott-Aldrich syndrome protein (WASP) family, such as WAVE and WASP [29,30]. At rest, WASP exists in an autoinhibited, inactive state. However, simultaneous association with active Cdc42 and PtdIns(4,5)P₂ releases this intramolecular inhibition, allowing for WASP-mediated activation of the Arp2/3 complex, a multi-subunit effector that catalyzes the nucleation of branched actin filaments [31].

That Rho GTPases play an indispensable role in phagocytosis is supported by multiple loss-of-function studies; inactivation of Cdc42 by silencing [32] or through dominant-negative approaches [31,33] impairs actin assembly and inhibits phagocytosis. Similarly, the phagocytic ability of macrophages from Rac1^{-/-} and Rac2^{-/-} double-knockout mice is reduced [34]. Notably, the Rho-family GTPases are activated with distinct kinetics and appear to execute non-redundant functions during phagocytosis [14]. Single-cell based determinations suggest that Cdc42 activation occurs first and that this is restricted to the leading margin of pseudopodia. A wave of Rac1 and Rac2 activation follows soon after. However, while sites of active Rac1 extend throughout the nascent phagosome, Rac2 seems to be confined to the base of the phagocytic cup. Interestingly, inactivation of Rho GTPases may be as important as their activation, as cells expressing a constitutively active form of Cdc42 display a marked phagocytic impairment [4]. This suggests that the cycling of Rho GTPases between active and inactive states is essential to sustain actin dynamics during phagocytosis.

While PtdIns(4,5)P₂-driven polymerization of actin filaments is absolutely essential to drive pseudopod extension, phagosome sealing and its scission from the plasmalemma are accompanied by actin breakdown. Removal of filamentous actin networks could facilitate deformation of the curving phagosomal cup and eliminate a potential barrier for focal exocytosis and for the particle to sink into the cytoplasm.

As a final requirement for particle internalization, the nascent vacuole must seal. This entails fusion between plasmalemmal pseudopods. Remarkably, virtually nothing is known about the molecular machinery that catalyzes the fusion of exofacial leaflets. Myosin-driven contractility might facilitate this process. Supporting this notion, myosin IC [35] and myosin X [36] are recruited to sites of phagocytosis and have been implicated in phagosome closure. Myosin X interacts with phagosomal phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] through a PH domain [37], suggesting that PI3K could be indirectly involved in the generation of contractile forces that promote the tight apposition of pseudopods that precedes phagosome sealing.

2.2. Phagosome maturation

Irrespective of the initiating receptor-ligand interaction, phagocytosis results in the internalization of a membrane-bound vesicle that promptly undergoes an acute transformative process known as phagosome maturation. From the time of scission (and possibly even before), the newly formed phagosome experiences a sequence of fusion and fission reactions that remodel its membrane and luminal composition. Initially, nascent phagosomes fuse with early endosomes, giving rise to early phagosomes. Subsequently, early phagosomes fuse with late endosomes and later with lysosomes, forming late phagosomes and phagolysosomes, respectively.

Interestingly, the fate of phagosomes varies depending on the nature of their cargo. Specifically, the luminal composition of phagosomes harboring infectious agents fosters the preservation of antigenic determinants, which can be presented to lymphocytes [38]. Conversely, antigens derived from endogenous apoptotic corpses are quickly degraded in an immunologically silent manner in order to prevent the development of autoimmunity [15]. Similarly, Fc γ R-mediated phagocytosis is accompanied by the secretion of pro-inflammatory cytokines, such as tumor necrosis factor- α , whereas internalization of apoptotic bodies leads to the secretion of anti-inflammatory mediators [12,39].

Maturing phagosomes quickly attain properties of the endolysosomal compartments with which they fuse. While undergoing this transformation, phagosomes become increasingly acidic, acquire oxidative properties, and become enriched with hydrolases, all of which facilitate cargo processing and disposal. In this last introductory section, we provide a brief overview of the cellular mechanisms that orchestrate the maturation process.

2.2.1. The early phagosome

Nascent phagosomes show a predisposition to fuse with early and recycling endosomes [40], but not with lysosomes [41]. The coalescence of phagosomes and early endosomes yields a hybrid organelle with low hydrolytic activity and a slightly acidified lumen (pH 6.1–6.5) [42].

Rab family proteins are instrumental in the maturation process, as they direct vesicular traffic to and from the phagosome [43]. In particular, early phagosomes accumulate Rab5, which regulates traffic by coordinating tethering and fusion of endomembranes [44]. As with other GTPases, activation of Rab5 requires nucleotide exchange. During phagocytosis of apoptotic bodies, Rab5 is activated by GAPex-5, a GEF that is delivered to sites of phagocytosis through the microtubular network [45]. Tellingly, phagosomes fail to acquire endolysosomal characteristics in cells that have been transfected with a dominant-negative form of Rab5 [46].

Having undergone repeated cycles of fusion with endocytic compartments, maturing phagosomes would be expected to increase in size. However, their surface area remains constant throughout the maturation process. A concomitant removal of membrane accounts for this seemingly paradoxical observation. Membrane is recycled to the plasmalemma, allowing for the retrieval of bystander membrane components that were unintentionally internalized with the phagocytic particle [47]. Rab4 and Rab11 associate with the early phagosome, controlling the recycling back to the plasma membrane [48,49]. In addition, the retromer – a complex consisting of Vps26–Vps29–Vps35, a trimer that provides cargo selectivity, plus a sorting nexin (SNX) dimer, consisting of either SNX1/SNX2 and SNX5/SNX6 – diverts phagosomal components to the TGN and recycling endosomes. Interestingly, deficiencies in retromer levels or in its assembly have recently been linked to the pathophysiology of Alzheimer's disease; microglial cells (the primary phagocytes of the central nervous system) that do not recruit the retromer complex efficiently to nascent phagosomes display impaired recycling of receptors to the plasmalemma, which could affect recognition of debris or effete cells in the brain [50].

Phagosomes also undergo invagination of their own limiting membrane, resulting in the formation of intraluminal vesicles (ILVs) [51].

The process of phagosomal membrane deformation and scission is likely similar to that responsible for the formation of multivesicular bodies (MVBs). As opposed to budding outward, intended for retrieval of salvageable phagosomal components, inward budding diverts membrane-associated cargo for degradation. ILVs are formed through the combined action of a group of protein complexes, known as endosomal sorting complex required for transport (ESCRT) [52]. The different complexes that comprise the ESCRT machinery recognize phagosomal membrane proteins that have been tagged for degradation by ubiquitylation, and force their extrusion into luminal vesicles [53].

2.2.2. The late phagosome

The membrane and luminal composition of the maturing phagosome continue to change as it progresses into the next discernible stage: the late phagosome. This phase is characterized by the acquisition of active Rab7 and the concomitant dissociation of Rab5. Moreover, the late phagosome accrues proteases and proton-pumping vacuolar ATPases (V-ATPases) that render the lumen degradative and hostile [54]. The pH of the late phagosomes drops to 5.5–6.0.

Rab7 is instrumental for the maturation of the phagocytic vacuole; expression of dominant-negative Rab7 depresses phagolysosome formation and acidification [55]. Two Rab7 effectors have been implicated in this process: ORPL1 and RILP [56]. These two proteins associate with Rab7-GTP on late degradative compartments, where they bind to the dynein-dynactin complex. Therefore, by promoting tethering of late phagosomes to minus end-directed motors, Rab7 and its effectors direct the centripetal movement of phagosomes that is required for efficient fusion with lysosomes [55].

Phagosomal membrane remodeling via recycling and ILV formation, a process that commenced in sorting endosomes, persists in late phagosomes. Indeed, retrograde transport of transmembrane cargo to the TGN requires association of retromer components with Rab7 [57]. Having sorted cargo for recycling or degradation, the late phagosome continues to develop by fusing with lysosomes, giving rise to the terminal station in the maturation process: the phagolysosome.

2.2.3. The phagolysosome

Rab7-dependent fusion of phagosomes with lysosomes results in the generation of fully competent microbicidal organelles. As with endosomes [58], fusion of lysosomes with phagosomal membranes involves the formation of a SNARE hairpin between VAMP7 and syntaxin 7 [59], and likely requires Ca^{2+} release from the lumen of lysosomes. Once formed, phagolysosomes can be distinguished biochemically from earlier endocytic compartments by a paucity of phosphatidylinositol 3-phosphate [$\text{PtdIns}(3)\text{P}$] in their internal membranes, the lack of mannose-6-phosphate receptors, an enrichment in acid hydrolases of the cathepsin family, and the pronounced acidity of its lumen (pH 4.5–5.0) [60–62].

Acidification of the phagolysosomal lumen is instrumental for its microbicidal and degradative properties, for a number of reasons. First, the lytic activity of the hydrolases is favored in acidic environments [63]. Second, the low phagosomal pH interferes with bacterial growth by directly impairing bacterial metabolism. Moreover, the transmembrane H^+ gradient drives the extrusion of luminal divalent cations that are critical for bacterial survival [64]. Third, the positive voltage generated by the V-ATPase facilitates the formation of microbicidal superoxide anions by the electrogenic NADPH oxidase. Fourth, acidification of the phagosome seems to be a requirement for (and not only a consequence of) the maturation process. This is borne out by experiments where prevention of acidification caused a block in phagolysosome biogenesis [65].

In addition to acidification and generation of highly reactive oxygen species, phagolysosomes accumulate a collection of antibiotic compounds that compromise the integrity of the ingested microbes. These include heavy metal scavengers like lactoferrin [66], lytic cationic

peptides like defensins and cathelicidins [67,68], as well as assorted hydrolases such as lysozyme and phospholipase A2 [69].

3. Phosphoinositides in phagosome formation

3.1. Phosphatidylinositol 4,5-bisphosphate in phagocytosis

The primary location of $\text{PtdIns}(4,5)\text{P}_2$ in resting phagocytes is the inner leaflet of the plasmalemma, where it encompasses approximately 2 mol% of the phospholipid content [70]. A small fraction of $\text{PtdIns}(4,5)\text{P}_2$ is also detectable in the Golgi network [71,72]. $\text{PtdIns}(4,5)\text{P}_2$ is primarily generated by type I phosphatidylinositol phosphate kinases (PIP1K; PIP5K), which phosphorylate phosphatidylinositol 4-phosphate [$\text{PtdIns}(4)\text{P}$] at the D5 position of the inositol moiety. Although to a lesser extent, $\text{PtdIns}(4,5)\text{P}_2$ is also synthesized through the phosphorylation of phosphatidylinositol 5-phosphate [$\text{PtdIns}(5)\text{P}$] at the D4 position by the type II phosphatidylinositol phosphate kinases [73]. Dephosphorylation of $\text{PtdIns}(3,4,5)\text{P}_3$ by phosphatase and tensin homolog (PTEN) also represents a source for the resting pool of $\text{PtdIns}(4,5)\text{P}_2$ [74], but the relative contribution of this pathway is not well established.

The PIP5K family is comprised of three isoforms (PIP5K α , β and γ). Though PIP5K isoforms associate with the Golgi complex and tubular lysosomes [75,76], they predominantly distribute to the plasma membrane, the most negatively charged compartment in the cell, through a polycationic region located on their surface [77]. Rho [78,79] and Arf [75] GTPases positively regulate PIP5K activity, thereby integrating diverse processes – such as cytoskeletal remodeling and membrane traffic – with $\text{PtdIns}(4,5)\text{P}_2$ synthesis. The relationship between PIP5K and small GTPases appears to be reciprocal; not only do Rho GTPases control PIP5K activation [78], but actin polymerization driven by these GTPases is in turn dependent on the activity of PIP5K [80]. PIP5K is also activated by Arf6, which colocalizes with PIP5K at sites of $\text{PtdIns}(4,5)\text{P}_2$ enrichment, including membrane ruffles [75,81]. Of note, Arf6-mediated activation of PIP5K is strictly dependent on the presence of anionic phospholipids, such as phosphatidic acid (PtdOH) [81]. $\text{PtdIns}(4,5)\text{P}_2$ and PtdOH likely engage in a positive feedback loop, as $\text{PtdIns}(4,5)\text{P}_2$ is a cofactor necessary for optimal activity of phospholipase D (PLD) [82], an enzyme that forms PtdOH by hydrolyzing (phosphatidylcholine) PtdCho. The top panel of Fig. 2 illustrates the signaling pathways leading to PIP5K activation and the consequent formation of phagosomal $\text{PtdIns}(4,5)\text{P}_2$.

Counter-balancing the anabolic pathways described above are at least three independent mechanisms that break down $\text{PtdIns}(4,5)\text{P}_2$. Class I PI3K catalyzes the phosphorylation of $\text{PtdIns}(4,5)\text{P}_2$, converting it to $\text{PtdIns}(3,4,5)\text{P}_3$. A wide number of 5-phosphatases, including OCRL [83], INPP5B [84] and the synaptosomal [85], promote its degradation to $\text{PtdIns}(4)\text{P}$. PLC γ hydrolyzes the $\text{PtdIns}(4,5)\text{P}_2$ head-group, thus forming plasmalemmal DAG and releasing $\text{Ins}(1,4,5)\text{P}_3$ into the cytosol. The role of these enzymes in phagocytosis is detailed below.

$\text{PtdIns}(4,5)\text{P}_2$ exerts many important functions at the membrane. It binds proteins with polybasic motifs or with PH, FERM or ENTH domains [70], and can also be converted to second messengers, such as $\text{PtdIns}(3,4,5)\text{P}_3$, DAG and $\text{Ins}(1,4,5)\text{P}_3$, which play crucial roles in the formation and maturation of phagosomes.

$\text{PtdIns}(4,5)\text{P}_2$ and its metabolites control a remarkable number of events in phagocytosis. These include rearrangement of the actin cytoskeleton [30] and the accompanying changes in phagocytic receptor mobility [86], integrin activation [87], membrane traffic [9,88], plasmalemmal–cytoskeletal linkages [89], and ion channel activity [90]. Notably, each of these events takes place in a discrete cellular location and at a particular time during the course of phagocytosis. The latter raises a recurrent question: how can a single phosphoinositide that is present throughout the plasma membrane orchestrate such spatiotemporally restricted phenomena? Part of the answer is that $\text{PtdIns}(4,5)\text{P}_2$ levels change only locally during phagocytosis [8]. Microscopy-based

determinations in single macrophages expressing a fluorescent chimera of the PH domain of PLC δ (a PtdIns(4,5)P₂ reporter) revealed biphasic changes at the membranes of forming phagosomes [27]. In contrast, PtdIns(4,5)P₂ levels remain steady during the course of phagocytosis in the unengaged aspects of the plasmalemma [27]. Fig. 1 illustrates that, while there is a noticeable accumulation of PtdIns(4,5)P₂ in emerging pseudopods during the early stages of phagosome formation, its concentration drops at the base of the phagocytic cup as the pseudopodia extend. Following phagosome sealing and severing, phagosomal

PtdIns(4,5)P₂ decreases precipitously and is no longer detectable by fluorescence microscopy [27].

In principle, the localized initial accumulation of PtdIns(4,5)P₂ at the pseudopods could result from an upsurge in synthesis, a decline in consumption, or both. Two lines of indirect evidence point towards an increase in synthesis. First, activation of PLD has been detected during phagocytosis [91–93], and as mentioned before, this enzyme promotes the recruitment and activation of PIP5K [82]. The simultaneous activation of Arf6 and PLD is likely to stimulate PIP5K, thereby promoting

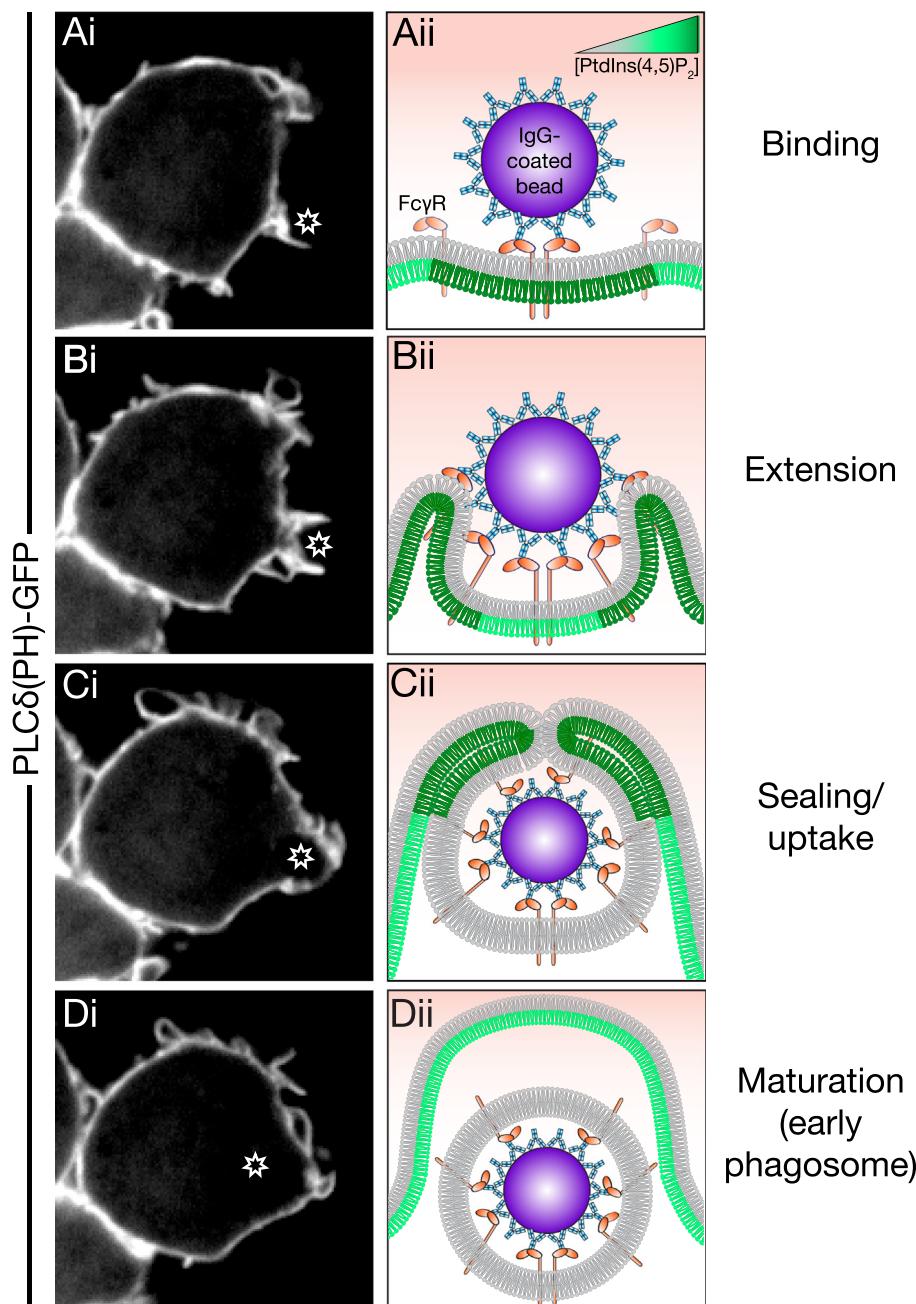


Fig. 1. Distribution of PtdIns(4,5)P₂ during the early stages of phagocytosis. The phagocytic response has been broken down into four conceptual stages: particle recognition (A); extension of pseudopodia (B); membrane fusion/particle internalization (C); and formation of an early phagosome (D). *Left*) Time-lapse fluorescence images of an RAW 264.7 macrophage expressing PLC δ (PH)-GFP, a PtdIns(4,5)P₂-specific fluorescent biosensor. Images were acquired by confocal microscopy immediately after macrophages were challenged with IgG-opsonized targets. PtdIns(4,5)P₂ is present in the inner monolayer of the plasmalemma, and its concentration increases locally at sites of particle binding and at forming pseudopods. However, the phosphoinositide is depleted from the base of phagocytic cup as pseudopodia progress and becomes undetectable at the phagosomal membrane following scission. Phagocytic particles are denoted with a star. Scale bar, 5 μ m. *Right*) Schematic representation of the local changes in PtdIns(4,5)P₂ concentration during phagocytosis, corresponding to the experimental data obtained with the PLC δ (PH)-GFP probe. The color code of the membrane is indicative of the relative abundance of PtdIns(4,5)P₂, ranging from gray (lowest) through light green (intermediate), to dark green (highest). Fc receptors are shown in orange; the opsonin, IgG, in blue and the phagocytic target in violet.

PtdIns(4,5)P₂ formation. Second, all three PIP5K isoforms transiently accumulate at the phagosomal membrane during the early phase of phagocytosis, and detach during the later stages [77], correlating with the biphasic nature of phagosomal PtdIns(4,5)P₂ levels.

In turn, as shown in Fig. 2B the disappearance of PtdIns(4,5)P₂ from the base of phagocytic cups and eventually from the membrane of

sealed phagosomes is mediated by a combination of kinases, phosphatases and lipases. Specifically, at later stages of phagosome formation PI3K promotes the phosphorylation of PtdIns(4,5)P₂ into PtdIns(3,4,5)P₃, which also serves as a signal for the recruitment of PLC γ [94]. Indeed, having been recruited to sites of PtdIns(3,4,5)P₃ production, PLC γ becomes largely responsible for the disappearance of PtdIns(4,5)P₂ [95],

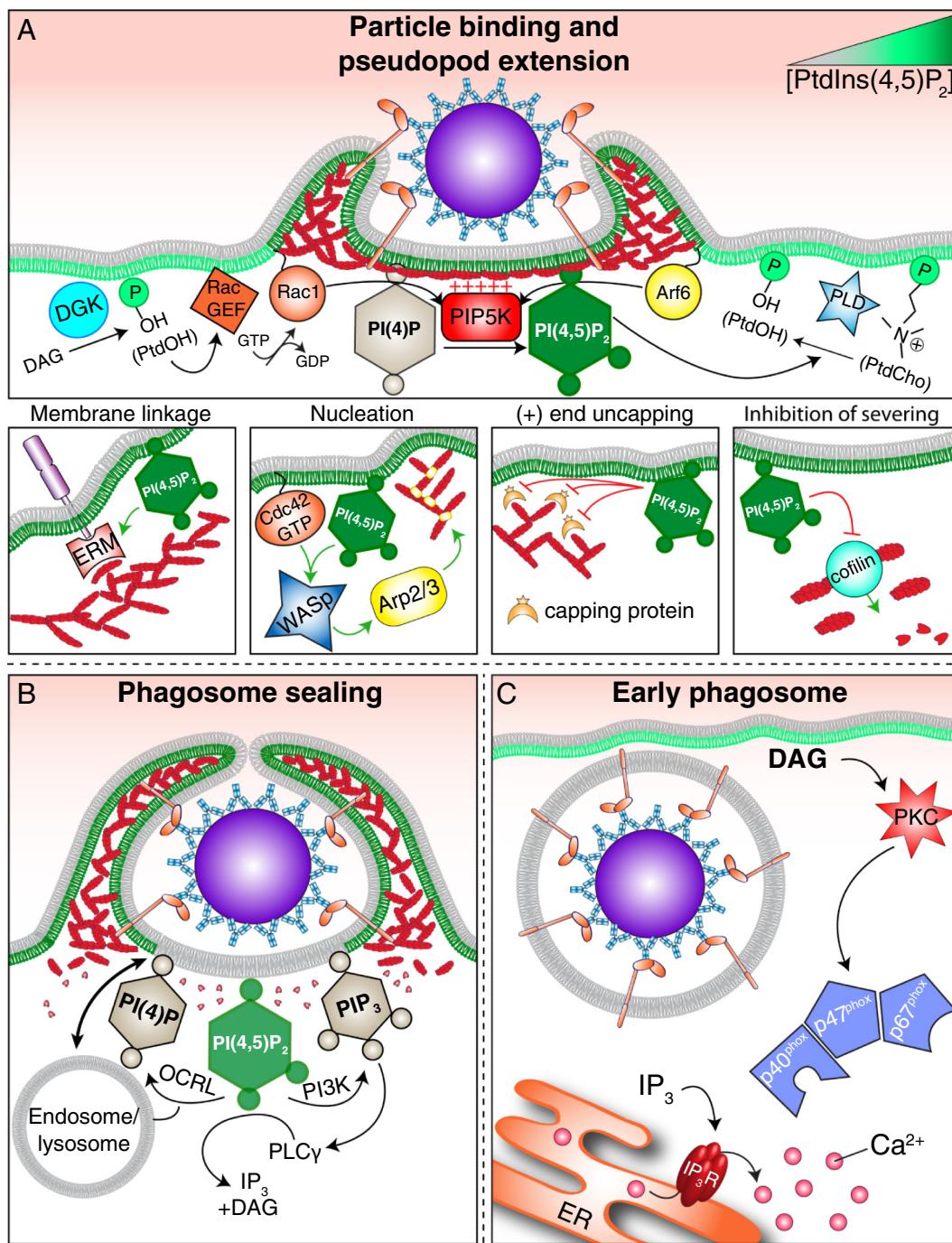


Fig. 2. Functional implications of PtdIns(4,5)P₂ metabolism for phagocytosis. A) Pathways leading to PtdIns(4,5)P₂ synthesis (main panel) and consequent stabilization of F-actin networks (lower panels) at the phagocytic cup. Membranes are colored-coded as in Fig. 1. Rac1 and Arf6 activate PIP5K in a PtdOH-dependent fashion. PtdOH can be synthesized through the phosphorylation of DAG by DGK or through hydrolysis of PtdCho by PLD. PtdOH recruits Rac GEFs (e.g. Tiam1) to phagocyte membranes, where Rac is activated. In turn, PIP5K associates with the plasma membrane through a positively charged surface, where it catalyzes the conversion of PtdIns(4)P to PtdIns(4,5)P₂. PtdIns(4,5)P₂ mediates linkage of actin networks to integral plasmalemmal proteins through intermediary ERM proteins, as well as actin polymerization by nucleation-promoting factors such as WASP. In addition, actin-biding proteins that antagonize filament formation, such as capping protein and the severing factor cofilin, are inhibited by PtdIns(4,5)P₂. B) Depletion of PtdIns(4,5)P₂ from the base of the cup leads to actin filament removal. PtdIns(4,5)P₂ is converted by kinases (PI3K), phosphatases (OCRL) and phospholipases (PLC γ). Disappearance of the filamentous actin barrier facilitates the delivery of membranes from endolysosomal compartments to the phagocytic cup, releasing membrane tension. C) PLC γ -mediated PtdIns(4,5)P₂ hydrolysis results in the formation of the bioactive molecules DAG and Ins(1,4,5)P₃ (IP₃). Regulatory subunits of the NADPH oxidase are activated by PKC, which is recruited to phagosomal membranes by DAG. Release of Ca²⁺ from intracellular stores is promoted by IP₃.

96]. The inositol 5-phosphatases OCRL and INPP5B also contribute by converting PtdIns(4,5)P₂ into PtdIns(4)P.

In addition to the catabolic pathways described above, detachment of PIP5K from internalized phagosomes facilitates the exclusion of PtdIns(4,5)P₂ from these compartments. The dissociation of PIP5K has been attributed to a localized and acute drop in phagosomal surface charge [97]. The release of PIP5K from sealed phagosomal membranes terminates PtdIns(4,5)P₂ synthesis and further promotes the disappearance of this lipid at a site where degradation is already ongoing.

In phagocytes, as in many other cellular systems, sites of PtdIns(4,5)P₂ formation serve as signaling platforms that trigger robust actin polymerization. PtdIns(4,5)P₂ promotes the activation of a number of actin-regulatory proteins that are responsible for filament assembly, while inhibiting those in charge of disassembly [28] (Fig. 2A). Proteins that directly bind to actin and dictate the equilibrium between its monomeric and filamentous form include profilin [98], cofilin [99], gelsolin [100] and capping protein [101]. In addition to increasing the number of barbed ends, PtdIns(4,5)P₂ induces *de novo* actin nucleation by activating nucleation-promoting factors [102]. Lastly, ezrin/moesin/radixin (ERM), which directly link the cytoskeleton to the plasmalemma, are also well established PtdIns(4,5)P₂ effectors [103]. Because of these effects, the local increase in PtdIns(4,5)P₂ synthesis that occurs upon engagement of receptors causes a large-scale reorganization of the actin cytoskeleton, driving the extension of pseudopodia around the surface of phagocytic targets [104]. This claim is supported by studies in which expression of a kinase-dead PIP5K that impairs the formation of phagosomal PtdIns(4,5)P₂ precluded accumulation of F-actin in nascent phagocytic cups, and depressed the phagocytic capacity of the cells [104].

Although PIP5K α , β and γ are all recruited to nascent phagosomes, different isoforms have been reported to mediate distinct, non-redundant roles during phagocytosis [105]. PIP5K α activity has been implicated in the activation of the nucleation promoting factor Wiskott–Aldrich syndrome protein homolog (WASp) [105], which catalyzes actin polymerization and pseudopod extension [32]. In contrast, PIP5K γ seems to control the mobility of phagocytic receptors in the plane of the membrane, presumably by modulating the density of cortical actin. Subsequently, PIP5K α catalyzes the emission of pseudopodia by promoting WASp activity [105]. To reconcile the fact that these isoforms have divergent effects, it has been postulated that PIP5K γ is subjected to post-translational control by Syk, thereby restricting its activity to a particular region and time [105].

While expansion of the actin skeleton and its anchorage to the plasmalemma drive formation of pseudopodia at early phases of phagocytosis, phagosome scission is accompanied by the disappearance of actin from the base of the phagocytic cup [106]. Indeed, actin clearance is a requirement for completion of phagocytosis, especially of large phagocytic targets [4,107]. Abortive phagocytic cups develop when the disintegration of the phagosomal actin meshwork is prevented either by expressing constitutively-active Rho GTPases [4] or by inhibiting PI3K [5,107]. The role of PtdIns(3,4,5)P₃ in actin disassembly is discussed in detail below.

The catabolism of PtdIns(4,5)P₂ coincides in space and time with the breakdown of actin [106]. The loss of phagosomal actin occurs asymmetrically after phagosomal sealing, with depolymerization arising initially at the base of the phagocytic cup, strongly resembling the pattern of PtdIns(4,5)P₂ disappearance. Of note, dismantling of actin at the base of the cup and particle internalization are blocked if high PtdIns(4,5)P₂ levels are sustained by promoting PIP5K-mediated synthesis or by inhibiting PLC γ -driven degradation [106]. It thus appears that loss of PtdIns(4,5)P₂ causes actin disassembly, which is in turn required for completion of phagocytosis.

In addition to the consequences that PtdIns(4,5)P₂ metabolism has on cytoskeletal dynamics, breakdown of the inositol to secondary metabolites also has important ramifications (Fig. 2C). PLC γ -mediated hydrolysis of PtdIns(4,5)P₂ leads to the formation of DAG and Ins(1,4,5)P₃.

The kinetics and spatial distribution of DAG liberation during phagocytosis have been measured with a genetically encoded fluorescent chimera of the C1 domain of PKC δ [27], which selectively associates with DAG [108]. Consistent with the role of PLC γ in phagocytosis, appearance of DAG coincides in space and time with the disappearance of PtdIns(4,5)P₂. Unexpectedly, though neither DAG nor Ins(1,4,5)P₃ is essential for particle engulfment, inhibition of PLC γ blocks the phagocytic response [27,106]. It thus appears that disappearance of PtdIns(4,5)P₂, rather than the formation of its metabolites, may be essential for completion of phagocytosis.

While DAG and Ins(1,4,5)P₃ are not required for particle internalization, recruitment of conventional and novel PKC isoforms by DAG, as well as Ca²⁺ mobilization by Ins(1,4,5)P₃, plays significant roles in other stages of phagocytosis [109–112]. Recruitment and activation of PKC by DAG during phagocytosis affect the elimination of internalized pathogens, as PKC phosphorylates and activates p47^{phox}, a regulatory subunit of the NADPH oxidase (NOX) [113,114]. Underscoring the significance of DAG formation is the observation that NOX activation is precluded if individual phagosomes fail to reach a critical DAG concentration [110].

In addition to stimulating PKC, DAG represents a source of PtdOH, which is synthesized by diacylglycerol kinases [73]. PtdOH biosynthesis is particularly important for professional phagocytes; in contrast to non-phagocytic cells, macrophages and dendritic cells display elevated levels of PtdOH at their plasma membranes [1]. Preceding phagocytosis and macropinocytosis, phagocytes continuously survey their environment in the lookout for foreign particles or soluble antigens. This incessant ruffling is strictly dependent on the constitutive conversion of DAG to PtdOH, which is in turn responsible for the recruitment of Rac1 GEFs, including TIAM1, to the plasmalemma [1]. Activation of Rac1 by TIAM1 then promotes nucleation of actin filaments and the extension of membrane protrusions that facilitate capture of phagocytic targets.

As shown in Fig. 2C, PtdIns(4,5)P₂ hydrolysis is accompanied by the release of Ins(1,4,5)P₃ into the cytosol and its diffusion to the endoplasmic reticulum (ER), where it induces Ca²⁺ release by binding to the Ins(1,4,5)P₃ receptor [115]. Depletion of Ca²⁺ from intracellular stores is sensed by STIM1, a transmembrane ER-resident protein. A recent study demonstrated that, upon depletion of calcium from the ER lumen, STIM1 recruits ER cisternae to nascent phagosomes and promotes opening of store-operated calcium entry channels present at the phagosomal membrane [111]. Earlier studies implicated an increase in free cytosolic Ca²⁺ in the fusion of secondary granules with the plasma membrane of neutrophils [116]. Ca²⁺ influx may play a similar role in the focal delivery of endomembranes to sites of phagocytosis.

Comparatively little is known about the regulation of phosphatases during phagocytosis and their contribution to PtdIns(4,5)P₂ removal. Nonetheless, evidence pointing to a role for OCRL and INPP5B has begun to emerge [84]. These inositol 5'-phosphatases are Rab5 effectors, and associate with nascent phagosomes through an adaptor protein called APPL1, also a Rab5 effector [84]. Silencing of either APPL1 or Rab5 prolonged the accumulation of both PtdIns(4,5)P₂ and filamentous actin on phagosomal membranes [84]. These observations are consistent with previous studies in *Dictyostelium*, where inactivation of Dd5P4 (a homolog of OCRL) resulted in phagocytic impairment [117]. Remarkably, although phagocytic cups did form in Dd5P4-null cells, these did not manage to seal and remained at an abortive stage [117]. Recent experimental findings have implicated a trimeric complex consisting of Bcl10 and the clathrin adaptors AP1 and EpsinR in ferrying OCRL to nascent phagosomes [118]. In these studies, depletion of Bcl10 resulted in the formation of unproductive phagocytic cups, rich in PtdIns(4,5)P₂, Cdc42 and F-actin [118].

It is evident that PtdIns(4,5)P₂ metabolism has profound implications for the formation and maturation of phagosomes. Thus, it is not surprising that microbes often subvert PtdIns(4,5)P₂ signaling in order to colonize their host. In an evolutionary arms race, a number of intracellular microbes have developed the ability to hijack the

polymerization of actin belonging to the host cells to gain entry and displace within them [119]. Active modulation of plasmalemmal PtdIns(4,5)P₂ homeostasis by secreted effectors is one of the strategies used by invasive bacteria to co-opt the host actin-regulatory machinery. This is particularly important because most of the tension of the plasma membrane ($\approx 75\%$) is thought to result from its coupling to cortical actin [120], which is in turn dependent on PtdIns(4,5)P₂. By promoting breakdown of PtdIns(4,5)P₂ intracellular pathogens weaken cytoskeletal support and reduce membrane rigidity, facilitating entry [121].

Salmonella spp., which are facultative intracellular pathogens, have evolved a number of virulence mechanisms that enable their entry into host cells [122]. These bacteria deliver effector proteins into host cells via a type III secretion system (T3SS), a specialized needle-like molecular machine [122], causing massive reorganization of the host's actin network. In conjunction to SopE and SopE2, two effectors that act as GEF mimics for Rho GTPases, *Salmonella* translocates a phosphoinositide phosphatase, SopB, that rapidly eliminates PtdIns(4,5)P₂ from the invaginating regions of membrane ruffles [121]. The disappearance of PtdIns(4,5)P₂ facilitates membrane deformation, enabling both extension of ruffles as well as the scission of the *Salmonella*-containing vacuoles (SCV). Remarkably, SopB-mediated invasion is not restricted to the subversion of cytoskeletal dynamics; this protein also diverts phagosome maturation to a non-lytic compartment by reducing the surface charge of the SCV [123]. By reducing the levels of anionic phospholipids, SopB precludes association of specific Rab GTPases that carry polycationic tails with the SCV. These proteins rely on electrostatic forces for their localization, and their displacement interferes with proper endocytic traffic and phagolysosome formation [89].

Similar invasive strategies have evolved independently in other intracellular pathogens, such as *Vibrio parahaemolyticus* and *Shigella flexneri* – causative agents of bacillary dysentery and gastroenteritis in humans, respectively. Like *Salmonella*, these microbes use T3SS to inject PtdIns(4,5)P₂ phosphatases that compromise the membrane integrity of their host and disrupt tethering of actin-binding proteins to the inner aspect of the plasmalemma [124,125]. In particular, translocation of the *Shigella* inositol phosphate phosphatase IpgD results in the marked accumulation of plasmalemmal PtdIns(5)P, in turn leading to actin rearrangements and membrane ruffling at invasive foci. The mobilization of the actin skeleton at sites of invasion has been attributed to direct interaction of the PH domain of Tiam1 – a Rac1 nucleotide exchanger – with PtdIns(5)P [126].

3.2. Phosphatidylinositol 3,4,5-trisphosphate in phagocytosis

Like other 3-polyphosphoinositides, PtdIns(3,4,5)P₃ levels are scarce in unstimulated cells. While its levels are minute at rest (less than 0.2% of all inositol-containing lipids) [127], PtdIns(3,4,5)P₃ is quickly generated upon engagement of immune receptors. The metabolism of PtdIns(3,4,5)P₃ is strictly and dynamically regulated, and in general restricted to the cytosolic leaflet of the plasmalemma [128]. Generation of PtdIns(3,4,5)P₃ occurs mainly via phosphorylation of PtdIns(4,5)P₂ by the family of class I PI3K, which localize to the plasma membrane and use PtdIns(4,5)P₂ as a substrate for PtdIns(3,4,5)P₃ biosynthesis [13]. The class I PI3K holoenzymes are comprised of a regulatory subunit (either p85 or p101) and a catalytic p110 subunit [129]. While the p85 regulatory subunit acts downstream of receptor tyrosine kinases, the p101 subunit responds to G protein-coupled receptors.

The breakdown of PtdIns(3,4,5)P₃ occurs mainly through the action of 3- and 5-phosphatases; PTEN dephosphorylates PtdIns(3,4,5)P₃ at the D3 position, generating PtdIns(4,5)P₂ [130], while SHIP hydrolyzes the D5 position, producing phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P₂] [131].

The spatiotemporal dynamics of PtdIns(3,4,5)P₃ synthesis during phagocytosis mirror those of PtdIns(4,5)P₂ disappearance, consistent with a role for class I PI3K in mediating the conversion of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃. Indeed, PI3K is recruited to and activated at sites

of phagocytosis [132]; upon particle engagement, tyrosine kinases recruit p85, the regulatory subunit of class I PI3K, initiating PtdIns(3,4,5)P₃ formation [133]. Synthesis of phagosomal PtdIns(3,4,5)P₃ is detectable shortly after phagocytic targets are engaged, and the phosphoinositide continues to accumulate as the phagocytic cup progresses. While PtdIns(3,4,5)P₃ is still detectable after sealing, its presence in the phagosomal compartment is short-lived and its concentration declines sharply within 1–2 min of sealing. Notably, SHIP accumulates at the phagosomal membrane [132], where it likely promotes the breakdown of PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂ [132,134]. Fig. 3 shows a diagrammatic representation of the spatiotemporal dynamics of PtdIns(3,4,5)P₃ during phagocytosis.

PtdIns(3,4,5)P₃ plays a critical and pleiotropic role during phagocytosis. Accordingly, a profound impairment of phagocytosis is observed in macrophages treated with PI3K inhibitors [107]. Tellingly, treatment with the PI3K inhibitors wortmannin or LY294002 results in the formation of abortive cups that do not extend fully around the particle's circumference. However, the polymerization of actin and the initial extension of pseudopodia persist in inhibitor treated cells. Thus, PI3K is dispensable for the initial stages of actin polymerization, but is necessary for later stages of pseudopodial progression, and perhaps even for phagosome sealing. Interestingly, the dependency of phagocytosis on PI3K seems to be a size-dependent phenomenon, as the uptake of large particles is much more affected by PI3K inhibitors than that of small ones [107].

It is worth emphasizing that the unproductive phagocytic cups that form when PI3K is inhibited stall at a stage where filamentous actin is richly accumulated at the base of the cup. The latter observation suggests that PtdIns(3,4,5)P₃ may be necessary for mediating actin breakdown, perhaps by allowing for actin, or determinants of its polymerization, to be recycled to the tips of advancing pseudopods. Orchestration of actin clearance by PI3K likely arises as a combined effect of PtdIns(4,5)P₂ removal and the inactivation of Rho GTPases. Consistent with this notion, it has been suggested that PI3K negatively regulates Cdc42 at later stages of phagocytosis [4].

Formation of 3-polyphosphoinositides also seems to be necessary for the delivery of endomembranes to nascent phagosomes. Although most of the phagosomal membrane is of plasmalemmal origin, several compartments of the endocytic pathway are known to be focally delivered to the nascent phagosome, including recycling [135] and late [136] endosomes. Interestingly, fusion of endomembranes seems to be dependent on the concomitant increase in 3-polyphosphoinositides and intracellular calcium [137].

Like other phosphoinositides, PtdIns(3,4,5)P₃ orchestrates its many cellular functions by recruiting effectors that carry domains that specifically recognize its head-group. Several of these effectors carry PH domains, including myosin X, an unconventional motor protein that has been implicated in pseudopodial extension and phagosome closure [36]. Treatment with wortmannin blocks myosin X enrichment at the phagocytic cup, and the expression of a truncated form of this motor reduces the ability of macrophages to carry out FcγR-dependent phagocytosis. In this regard, it is interesting that inhibition of myosin X activity prevented spreading but not adhesion of macrophages on IgG-coated substrates, and inhibited phagocytosis of large particles [36]. Thus, it has been suggested that the dependency of phagocytosis of large particles on PI3K activity may be attributable to the recruitment of myosin X. Fig. 4 illustrates the practical implications of PtdIns(3,4,5)P₃ metabolism for phagosome formation.

PtdIns(3,4,5)P₃ signaling is also often hijacked by intracellular pathogens as part of their colonizing strategy. Enteropathogenic *Escherichia coli* (EPEC) invades the intestinal epithelium by inducing the formation of F-actin-rich pedestals by a process that relies on the subversion of PtdIns(3,4,5)P₃ homeostasis [138]. These actin-driven structures facilitate colonization and increase pathogenicity by allowing the bacteria to adhere tightly to intestinal surfaces [119]. Using a T3SS, EPEC inject a protein called translocated intimin receptor (Tir). The extracellular

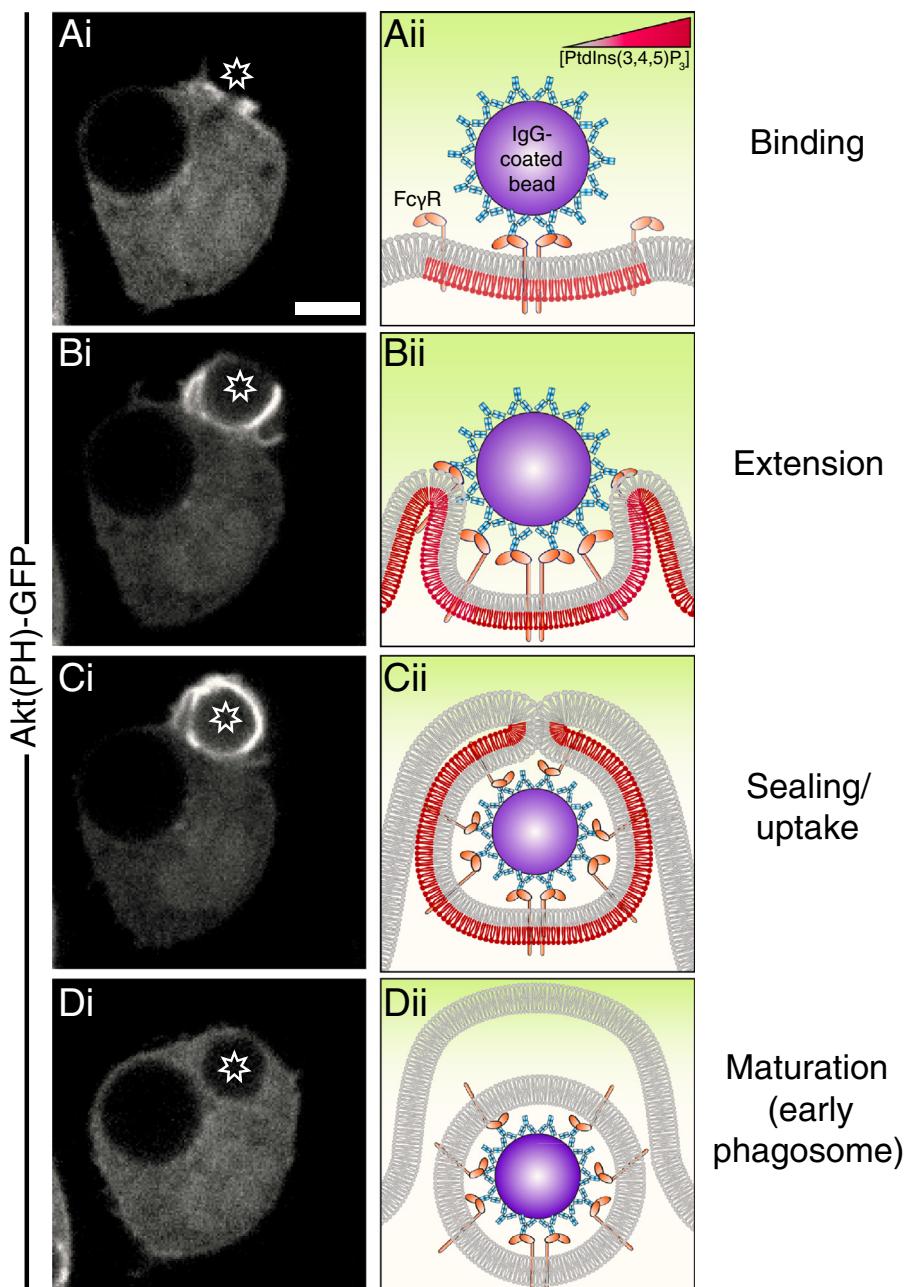


Fig. 3. Dynamic changes in PtdIns(3,4,5)P₃ abundance during phagosome formation. The phagocytic response is broken down into the same stages defined in Fig. 1. *Left*) Time-lapse fluorescence images of a RAW 264.7 macrophage expressing Akt(PH)-GFP, a fluorescent probe that detects PtdIns(3,4,5)P₃ [and also PtdIns(3,4)P₂]. While virtually absent from the bulk plasma membrane, engagement of phagocytic receptors triggers a transient yet marked accumulation of PtdIns(3,4,5)P₃ in the membrane of the nascent phagosome. This increase persists until phagosome sealing, then PtdIns(3,4,5)P₃ is depleted from the phagosomal membrane shortly (1–2 min) after internalization. Phagocytic particles are denoted with a star. Scale bar, 5 μm. *Right*) Schematic representation of the local changes in PtdIns(3,4,5)P₃ concentration at sites of phagocytosis, corresponding to the experimental data obtained with Akt(PH)-GFP. The color code of the membrane is indicative of the relative abundance of PtdIns(3,4,5)P₃, ranging from gray (lowest), through light red (intermediate), to dark red (highest). Other details as in Fig. 1.

region of Tir acts as a receptor for intimin, a bacterial adhesin that operates as an ‘attach and efface’ virulence factor. Engagement of intimin by Tir leads to the clustering of the translocated bacterial receptors on the plane of the host cell membrane, which in turn recruits PI3K and triggers a cascade of actin rearrangement events that require PtdIns(3,4,5)P₃ [138].

3.3. Phosphatidylinositol 3,5-bisphosphate in phagocytosis

The cellular abundance of PtdIns(3,5)P₂ is very low. For a sense of scale, it has been estimated that there are about 100 PtdIns(4,5)P₂ molecules and 10 PtdIns(3)P molecules for every PtdIns(3,5)P₂ molecule

[139]. PtdIns(3,5)P₂ is synthesized via phosphorylation of PtdIns(3)P at the D5 position of the inositol ring by phosphatidylinositol 3-phosphate 5-kinase (PIKfyve). Both PtdIns(3,5)P₂ and PIKfyve preferentially distribute to late endosomes and the lysosomal network [140]. The converse reaction, dephosphorylation of the D5 position in PtdIns(3,5)P₂, is carried by the SAC domain-containing phosphatase (Fig. 4) [141]. The myotubularin (MTM) family of phosphatases catalyze an alternative mode of PtdIns(3,5)P₂ breakdown, consisting of dephosphorylation of the D3 position, leading to PtdIns(5)P formation [142].

Until recently, the precise spatial and temporal dynamics of PtdIns(3,5)P₂ during phagocytosis had remained obscure, mostly because of a paucity of methods to detect the phosphoinositide in living

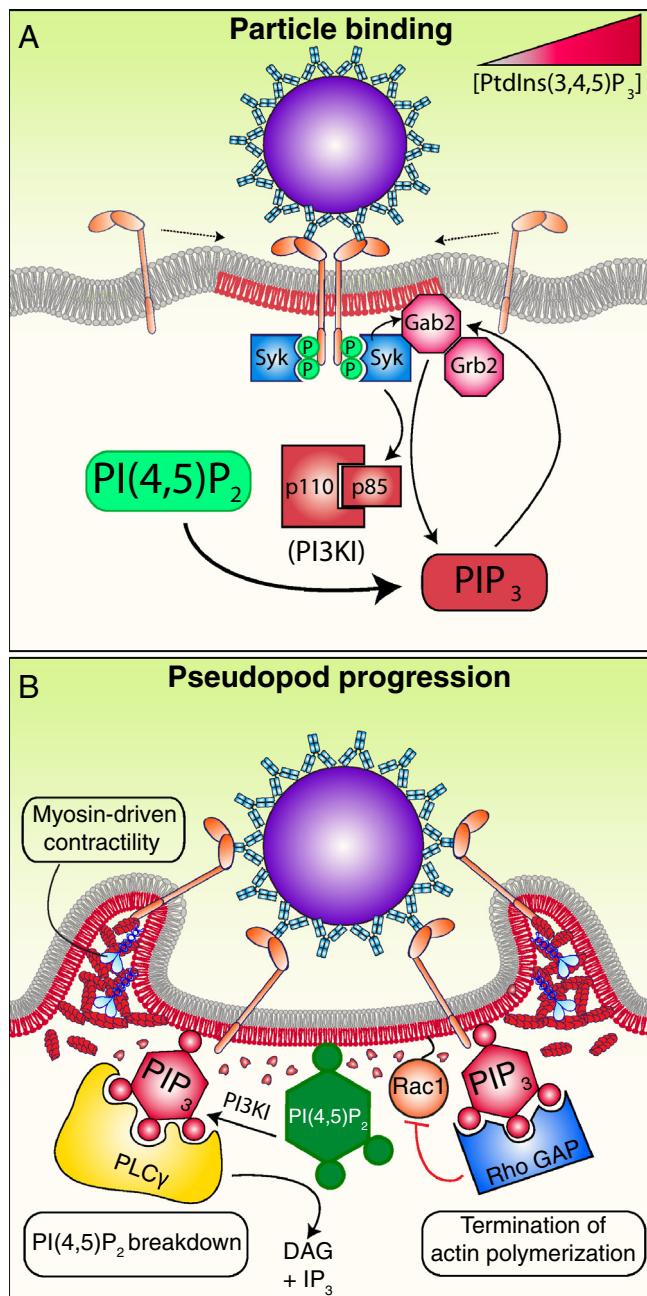


Fig. 4. Role of PtdIns(3,4,5)P₃ synthesis in phagosome formation. A) Signal transduction pathways leading to PI3K activation at the nascent phagosome. Membranes are color-coded as in Fig. 3. Engagement of Fc γ R by an IgG-coated target triggers receptor clustering in the plane of the membrane, promoting phosphorylation on ITAM motifs by Src family kinases (SFK). Doubly-phosphorylated ITAMs are sensed by a tandem SH2 domain on the non-receptor tyrosine kinase Syk, which binds to and directly activates the adaptor LAT (not illustrated). LAT stimulates docking of additional proteins, such as PLC γ and Grb2. The former catalyzes PtdIns(4,5)P₂ hydrolysis, while the latter acts as an adaptor protein for Gab2. Once recruited to active phagocytic receptors, Gab2 is phosphorylated by Syk and subsequently induces recruitment of p85, the regulatory subunit of PI3K. The class I PI3K holoenzyme then phosphorylates PtdIns(4,5)P₂, generating PtdIns(3,4,5)P₃. Note that Gab2 is stabilized at the phagosomal membrane by PtdIns(3,4,5)P₃ produced by PI3K, thereby amplifying the PtdIns(3,4,5)P₃ signal. B) Orchestration of pseudopodia progression by direct PtdIns(3,4,5)P₃ effectors. PLC γ is recruited to the phagocytic cup by a PtdIns(3,4,5)P₃-interacting PH domain, promoting PtdIns(4,5)P₂ breakdown. Disappearance of PtdIns(4,5)P₂ results in the removal of F-actin from the base of the cup. Termination of polymerization is reinforced by the PtdIns(3,4,5)P₃-mediated recruitment of Rho GTPases. Myosin motors (e.g. myosin X) also translocate to the cup in a PtdIns(3,4,5)P₃ dependent manner, where they facilitate phagosome sealing by exerting contractile forces.

cells. However, a genetically encoded probe for this lipid was recently developed, consisting of the ML1N domain of TRPML1, a PtdIns(3,5)P₂-regulated ion channel [139]. Expression of fluorescently conjugated ML1N in phagocytes revealed that particle engagement triggers synthesis of lysosomal PtdIns(3,5)P₂, likely through PIKfyve [140]. Interestingly, ML1N also translocated to sites of phagocytosis, presumably because of an increase in PtdIns(3,5)P₂ at the cup, brought about by the fusion of lysosomal membranes with nascent phagosomes. PtdIns(3,5)P₂ accumulation was evident when 6 μ m, but not 3 μ m, latex beads were used as phagocytic targets [140], perhaps suggesting a role for lysosome fusion with the forming phagosome only when large particles are engaged.

Acquisition of PtdIns(3,5)P₂ occurs at early stages of phagocytosis and precedes Rab5 recruitment; it may therefore play a role in directing maturation. In this regard, PtdIns(3,5)P₂-mediated activation of TRPML1 channels has been implicated in calcium-dependent fusion of endomembranes with phagosomes. This notion is supported by the observation that, in contrast to wild-type controls, phagosomes formed in TRPML1 knock-out macrophages fail to acquire lysosomal markers such as LAMP-1 [140].

4. Phosphoinositides in phagosome maturation

4.1. Phosphatidylinositol 3-phosphate in phagocytosis

Though its cellular concentration is comparatively low, PtdIns(3)P is critically involved in the maturation of phagosomes. In mammalian cells, PtdIns(3)P is found mainly at the cytoplasmic leaflet of early endosomes and in ILVs of multivesicular bodies [143]. The predominant source of this inositol is class III PI3K (Vps34), which phosphorylates the D3 position of PtdIns [144,145]. Vps34 localizes to early endosomes [146], and its inhibition by wortmannin [144] or by specific anti-Vps34 antibodies [147] quickly eliminates PtdIns(3)P from these compartments. Though quantitatively less predominant, other sources of PtdIns(3)P also exist: phosphorylation of PtdIns by class II PI3K [148] and dephosphorylation of bisphosphorylated species by inositol polyphosphate phosphatases [149].

In principle, three possible mechanisms could account for disappearance of PtdIns(3)P: phosphorylation, dephosphorylation and hydrolysis. The precise relative contribution of these pathways is not clear. However, enzymes that could potentially carry each of these functions have been identified. PIKfyve eliminates PtdIns(3)P by phosphorylating its D5 position, generating PtdIns(3,5)P₂ [150]. Conversely, PtdIns(3)P can be broken down by the 3-phosphatase MTM1, a member of the myotubularin family. In addition to its hydrolytic activity, MTM1 directly interacts with Vps34 and competitively displaces it from endosomal membranes, thereby preventing it from engaging Rab5 or Rab7 [146]. Lastly, PtdIns(3)P could be removed by lysosomal phospholipases, which gain access to the inositol as ILVs form upon ESCRT-mediated invagination of the limiting membrane [151].

When phagocytes encounter a target, PtdIns(3)P is initially absent from pseudopodia and the neighboring (unengaged) plasmalemma. However, sealing of the phagosome and its internalization is followed by a striking yet transient accumulation of the phosphoinositide, which lasts for about 10 min and coincides with the centripetal movement of the phagosomal vacuole [147]. The disappearance of phagosomal PtdIns(3)P is predicted to involve a combination of phosphorylating and hydrolytic reactions, as well as inward budding. Fig. 5 depicts the dynamic changes in the subcellular distribution of PtdIns(3)P throughout the different stages of phagocytosis, as measured by a fluorescent biosensor that specifically recognizes this phosphoinositide.

Its spatiotemporal dynamics suggest that PtdIns(3)P is dispensable for pseudopod formation but that its function is related to phagosome maturation. This notion has been amply validated by a number of studies where phagosome maturation was precluded by pharmacological inhibition of PI3K. In these experiments phagocytes were treated with

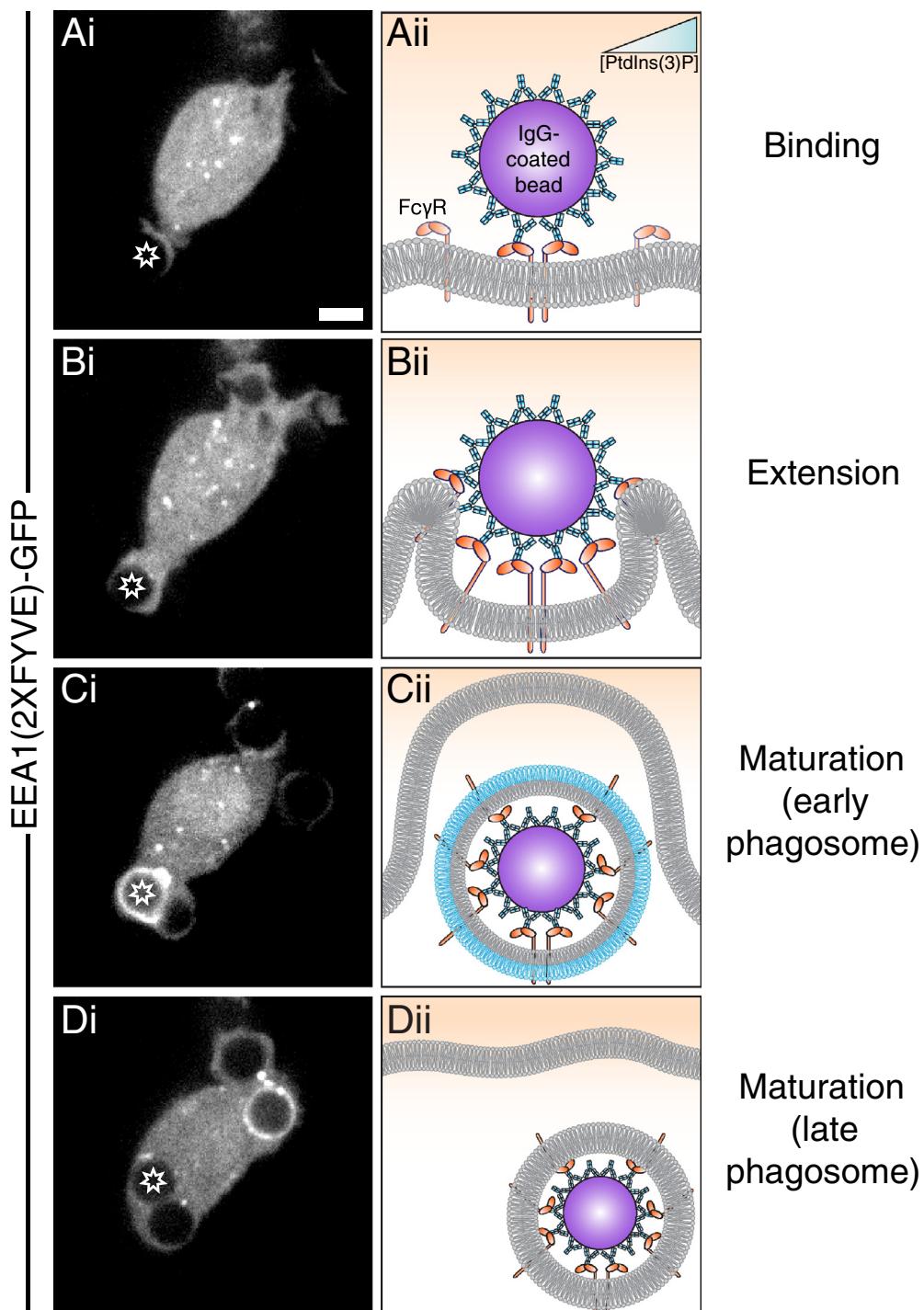


Fig. 5. Localized PtdIns(3)P synthesis during early stages of phagosome maturation. Four conceptual stages are shown: particle recognition (A); extension of pseudopodia (B); early phagosome (C); and late phagosome (D). *Left*) Time-lapse fluorescence microscopy images of a RAW 264.7 macrophage expressing EEA1(2XFYVE)-GFP, a PtdIns(3)P biosensor. PtdIns(3)P, which localizes primarily to early endosomal compartments, is undetectable at the plasmalemma. PtdIns(3)P is also absent from phagosomal membranes during particle recognition and pseudopod extension, but accumulates noticeably in the early maturing phagosome (between 1 and ≈ 10 min after sealing). However, PtdIns(3)P is lost as the phagosomal vacuole matures into a late phagosome. Phagocytic particles are denoted by a star. Scale bar, 5 μ m. *Right*) Schematic representation of the local changes in PtdIns(3)P concentration at sites of phagocytosis, corresponding to the experimental data obtained with the EEA1(2XFYVE)-GFP probe. The color code of the membrane is indicative of the relative abundance of ranging from gray (lowest), through light blue (intermediate), to dark blue (highest). Other details as in Fig. 1.

wortmannin, an inhibitor of both class I and III PI3K, prior to being challenged with small (3 μ m) particles [147]. Small particles were utilized in the study because, as discussed above, inhibition of class I PI3K impairs phagocytosis of large particles, while internalization of small particles is only slightly affected [107]. Under these conditions, phagosomes formed but did not acquire PtdIns(3)P. More importantly, these phagosomes arrested at an immature stage that had a markedly

reduced content of lysosomal markers such as LAMP-1. Similar results were obtained when class III PI3K was neutralized by the injection of specific antibodies [147]. Thus, class I and class III PI3Ks play distinct roles in the phagocytic process; class I PI3K is responsible for the synthesis of 3-polyphosphoinositides that control pseudopod extension and sealing, while class III PI3K orchestrates phagosome maturation by catalyzing PtdIns(3)P formation in early phagocytic compartments.

Rab5 and Rab7 are critical regulators of membrane traffic and phagolysosome biogenesis. However, their activation at phagosomal membranes alone is insufficient to drive phagosome maturation to completion; inhibition of PI3K blocks the progression of phagosomes, even though the arrested vacuoles retain active Rab7 [46]. The functions of Rab5 and 7 in maturation seem to be heavily dependent on their ability to associate with p150, a myristoylated Ser/Thr protein kinase and critical binding partner of Vps34 [152,153]. Both recruitment of Vps34 to membranes and its catalytic activity are augmented by binding to p150 [146]. Thus, orchestration of membrane traffic by Rab5 and Rab7 requires Vps34 activation and the consequent accumulation of PtdIns(3)P in early phagosomal compartments.

Following its synthesis during the early stages of maturation, PtdIns(3)P is responsible for carrying multiple signaling tasks. The phosphoinositide participates in endosome and phagosome progression, retrieval of membranes to the plasmalemma, sorting of membrane proteins to the TGN and targeting of cargo for degradation within ILVs. PtdIns(3)P is also partly responsible for the acquisition of bactericidal properties by the phagosome. The many versatile roles played by PtdIns(3)P in the course of phagosome maturation are illustrated in Fig. 6.

PtdIns(3)P exerts its effects by recruiting a number of effectors that possess PX or FYVE domains. Notably, PtdIns(3)P is the phosphoinositide with the largest collection of specific binding partners: the human genome encodes 42 PX domain- and 30 FYVE domain-containing proteins, most of which selectively recognize PtdIns(3)P [154]. A prototypical example is the tethering molecule EEA1, which carries a FYVE domain in its C-terminus that recognizes PtdIns(3)P [155] and binds to early endosomes [156]. EEA1 also recognizes active Rab5 [157]. Thus, Rab5 and its downstream target Vps34 synergize to recruit EEA1 to the early phagosomal membrane. EEA1 is crucial for phagosome maturation, as it mediates fusion with components of the endocytic pathway by interacting with syntaxins 6 and 13 [59,158]; these SNAREs catalyze membrane fusion during phagocytosis [59]. For these reasons, neutralization of EEA1 function by introduction of inhibitory antibodies results in a blockade in phagosome maturation, much like that observed in wortmannin-treated cells [159].

In addition to orchestrating fusogenic events between phagosomes and the early endosomal system, PtdIns(3)P is central for sorting phagosomal contents to degradative compartments. As illustrated in Fig. 6, phagosomal membrane proteins destined for lysosomal destruction undergo mono- or polyubiquitylation and are subsequently internalized through invagination of the phagosome limiting membrane, leading to the formation of ILVs. The activated phagocytic receptor Fc γ RIIA is one such protein [51].

As discussed above, ESCRT proteins are responsible for the generation of ILVs. Most relevant to this review is Hrs, a subunit of ESCRT-0 that carries a FYVE domain and interacts with phagosomal PtdIns(3)P in a highly specific fashion [160]. Indeed, inhibition of PtdIns(3)P synthesis with wortmannin prevents Hrs recruitment to the phagosome. More importantly, silencing of Hrs arrests maturation at an early stage, with retention of markers of early (sorting) endosomes on the phagosomal membrane [160].

Certain phagosomal components, such as acid hydrolase receptors, are not destined for degradation to the lysosome and instead are retrogradely ferried to the TGN by retromer. Notably, the SNX subunits of retromer carry a PX domain, which mediates their tethering to phagosomal PtdIns(3)P [161]. By binding to PtdIns(3)P through their PX domain while concomitantly facilitating membrane curvature through their BAR domain, the SNX subunits of retromer mediate tubule and vesicle formation for the purposes of retrograde transport (Fig. 6D).

Formation of a complex between class III PI3K and the autophagy related protein Beclin-1 appears to be necessary for retromer function. Mutations in Vps30 (the yeast ortholog of Beclin-1) lead to sorting and maturation defects, as well as decreased PtdIns(3)P levels [162]. The failure to attain suitable PtdIns(3)P levels precludes association of

the SNX1/2-SNX5/6 dimer with endosomal membranes [162]. In *Caenorhabditis elegans*, clearance of apoptotic corpses is defective when *bec-1* (the Beclin-1 ortholog) is mutated, suggesting that retromer-dependent transport is a component of efferocytosis. Also, deficiencies in retromer levels or in its assembly have been linked to phagocytic defects and amyloid-beta removal in the brain; *beclin-1* mutant microglia do not recruit retromer efficiently to nascent phagosomes, making them incapable of properly recycling receptors such as CD36 to the plasmalemma [50]. Neurodegenerative consequences can ensue due to the depletion of phagocytic receptors for apoptotic bodies or cell debris.

An important component of the microbicidal arsenal of phagosomes is the NADPH oxidase (NOX), an electrogenic complex that generates reactive oxygen species (ROS) in the phagosomal lumen. As shown in panel B of Fig. 6, NOX is a multicomponent system comprised of two membrane proteins ($gp91^{phox}$ and $p22^{phox}$), three soluble regulatory proteins ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) [163] and either Rac1 or Rac2 [164,165]. The membrane-associated subunits form a heterodimeric flavocytochrome that is responsible for the catalytic activity of the complex, generating superoxide from NADPH and oxygen. While NOX is inactive in unstimulated cells, phagocytic signals recruit the ternary complex to sites of particle engagement, where the flavocytochrome is activated [166]. Sustained association of the regulatory subunits of the oxidase with sealed phagosomes is facilitated by $p40^{phox}$, which carries a PtdIns(3)P-binding PX domain [167,168]. Indeed, retention of $p40^{phox}$ on the membrane of sealed phagosomes is prevented when PI3K is inhibited; importantly, sustained stimulation of NOX is absent when $p40^{phox}$ is knocked out or when its PX domain is mutated [169].

Because of its multifunctional role in transforming the phagosome into a microbicidal machine, PtdIns(3)P constitutes an attractive target for invasive organisms whose pathogenicity rests on their ability to prevent phagolysosome biogenesis. A pertinent example is *Mycobacterium tuberculosis*, an intracellular bacterium that manages to survive within the protected confines of the early phagosome by halting its progression [170]. This arrest in maturation has been attributed to the exclusion of PtdIns(3)P from the limiting membrane of the *Mycobacterium*-containing vacuole [159,171,172]. To this end, the bacterium sheds mannose-capped lipoarabinomannan (ManLAM), a glycosylated PtdIns and major component of its cell wall that purportedly interferes with Vps34 activity [171]. Mechanistically, it has been proposed that ManLAM blocks the surge in cytosolic Ca^{2+} that normally accompanies phagocytosis, ostensibly interfering with Ca^{2+} /calmodulin-dependent activation of class III PI3K [171]. In a synergistic manner, *M. tuberculosis* secretes SapM, a phosphatase that hydrolyzes PI(3)P [12,39]. Depletion of PI(3)P from the mycobacterial phagosome by this combined strategy prevents the acquisition of critical mediators of vesicular traffic, such as EEA1 [159] and Hrs [160], thus aborting phagosome maturation.

5. Phosphoinositides in macropinocytosis

Macropinocytosis is a distinctive endocytic pathway that mediates liquid phase uptake of nutrients and other molecules [173]. It is characterized by the comparatively large size of the vesicles formed, known as macropinosomes, that range from 0.2 to 5 μm in diameter [174]. Macropinocytosis is therefore the mechanism responsible for the uptake of solutes that are excluded from other endocytic processes because of their large size. It is linked to immunity and infection, as it is a major pathway used by antigen-presenting cells for delivery of antigens to the class I and class II major histocompatibility complexes [175,176]. Remarkably, some bacteria and viruses co-opt macropinocytosis as a means of entering the host cell [173,174]. These pathogens are capable of activating signaling pathways that initiate the formation of macropinosomes [177]. Of note, macropinocytosis is used by malignant cells to satisfy their unique metabolic needs [178]; as such, it is important to cancer progression.

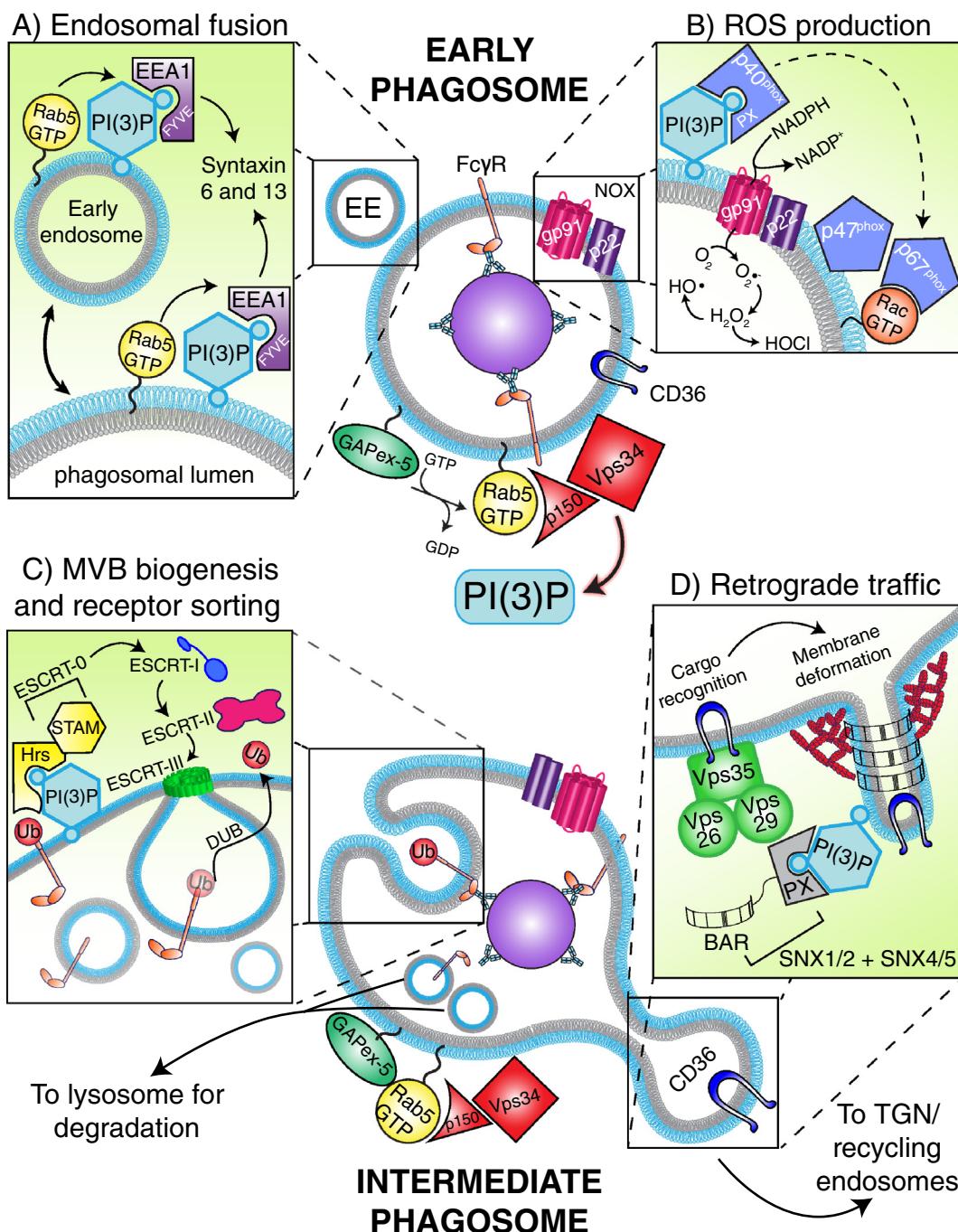


Fig. 6. Orchestration of phagosome maturation and membrane traffic by PtdIns(3)P. A) The tethering protein EEA1 utilizes its FYVE domain to engage PtdIns(3)P in both phagosomal and endosomal membranes, where it binds to active Rab5. EEA1 also interacts directly with syntaxins 6 and 13, SNARE proteins that facilitate fusion between phagosomes and early endosomes (EE). B) PtdIns(3)P promotes NADPH oxidase (NOX) activity and ROS production. p40^{phox}, a cytosolic component of NOX, is recruited to the maturing phagosome through a PX domain that recognizes PtdIns(3)P. p40^{phox} interacts with other subunits of the oxidase, stabilizing the complex on the phagosomal membrane and sustaining ROS generation. C) Role of PtdIns(3)P in ESCRT-mediated sorting of phagosomal membrane proteins. The ESCRT-0 subunit Hrs utilizes a FYVE domain to associate with PtdIns(3)P-rich membranes, where the complex recognizes ubiquitylated cargo, such as FcγRIIA. ESCRT-0 then triggers the sequential recruitment of ESCRT complexes I, II and III, culminating in the invagination of the limiting membrane. D) PtdIns(3)P in the retrieval of phagosomal membrane proteins by the retromer complex. Retromer, consisting of a cargo-recognition Vps trimer and a sorting nexin (SNX) dimer, is recruited to maturing phagosomes, where it promotes recycling of membrane proteins aided by actin-driven membrane tubulation. The SNX proteins of retromer carry PX and BAR domains, which they utilize for the recognition of PtdIns(3)P-rich membranes and the stabilization of membrane curvature within tubules, respectively.

Macropinocytic events are driven by regulated membrane ruffling. Ruffling leading to macropinocytosis can be stimulated by growth factors such as epidermal growth factor (EGF) and macrophage colony-stimulating factor (M-CSF) [179]. However, several other molecules can promote the phenomenon by receptor independent means; these include Ras proteins [180], phorbol esters [181] and cationic membrane-permeant peptides [182]. In some cell types ruffling can

also occur "spontaneously" or "constitutively" [174]; it is not clear, however, whether such activity is truly spontaneous or results from autocrine or paracrine stimulation by unidentified factors.

It is important to appreciate that, while phagocytes constantly ruffle their membranes, not every ruffling event results in macropinosome formation [181]; several steps are involved and all must be completed for macropinocytosis to reach completion. In order to initiate the

process, ruffles must form and curve to form a crater-shape structure known as the macropinocytic cup. Subsequently, protrusions need to extend to attain cup closure, a process akin to phagosome formation. These events require substantial actin remodeling. The final stage of macropinosome formation is its scission from the plasma membrane, resulting in a sealed macropinocytic vacuole [173].

As in phagocytosis, enzymes responsible for phospholipid synthesis and breakdown are activated during the course of macropinosome formation and maturation [174]. The following sections review the similarities and differences between the roles of specific phosphoinositides in macropinocytosis and phagocytosis.

5.1. Phosphatidylinositol 4,5-bisphosphate in macropinocytosis

The metabolism of PtdIns(4,5)P₂ shows striking similarities in macropinocytosis and phagocytosis. The PtdIns(4,5)P₂ accumulation noted at early stages of phagocytosis is recapitulated when A431 cells are stimulated by EGF. The concentration of PtdIns(4,5)P₂ increases in ruffling membranes, compared to the rest of the plasma membrane, as revealed by the accumulation of the PLC δ -PH probe. Such PtdIns(4,5)P₂ accumulation during macropinocytosis is thought to occur by localized activation of PIP5K. The levels of this phosphoinositide reach a maximum just before macropinosome closure and decrease drastically thereafter [183]. As in phagocytosis, PtdIns(4,5)P₂ is hydrolyzed by PLC γ , giving rise to Ins(3,4,5)P₃ and DAG [174]. On the other hand, simultaneous visualization of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ in macropinosomes by co-expressing fluorescent biosensors for each of these phosphoinositides has provided evidence that phosphorylation by PI3K contributes to the secondary disappearance of PtdIns(4,5)P₂ [183]. It is important to note that PLC γ is recruited to sites of PtdIns(3,4,5)P₃ accumulation, and, as such, PI3K could promote PtdIns(4,5)P₂ disappearance by two synergistic mechanisms during macropinocytosis.

The local production of PtdIns(4,5)P₂ in early stages of macropinosome development is partly responsible for the remodeling of the actin cytoskeleton, and is essential for the formation of ruffles that accompany cup formation [174]. For the sake of brevity, the actin-remodeling proteins capable of interacting with PtdIns(4,5)P₂ will not be discussed in this section, as they were described in the sections describing phagocytosis.

The phase of PtdIns(4,5)P₂ disappearance is also critical for macropinosome formation; by reducing the thickness of cortical actin and consequently decreasing the rigidity of the plasma membrane, PtdIns(4,5)P₂ breakdown fosters membrane invagination and macropinosome sealing [106]. The products of the reactions mediating conversion of PtdIns(4,5)P₂ are also important. Ins(3,4,5)P₃ and DAG are likely to function in macropinosome formation or maturation, although their roles have not been thoroughly investigated. PtdIns(3,4,5)P₃ is discussed below. To the best of our knowledge, the role of PtdIns(4,5)P₂-specific phosphatases in macropinocytosis has not been explored, and remains an interesting topic in need of study.

5.2. Phosphatidylinositol 3,4,5-trisphosphate in macropinocytosis

The concentration of PtdIns(3,4,5)P₃ is considerably greater in membrane ruffles compared to the bulk, resting plasma membrane. When visualized using fluorescent reporters, the levels of the phosphoinositide fluctuate dynamically in membrane protrusions [174], but increased noticeably in defined macropinocytic cups [184]. At this stage, PtdIns(3,4,5)P₃ was mostly confined to the semi-circular section of the cup [185,186]. After this transient spike, but before cup closure, PtdIns(3,4,5)P₃ concentration decreased to negligible levels, coinciding with an increase in the concentration of PtdIns(3,4)P₂ [186].

The fluctuations of PtdIns(3,4,5)P₃ reported to occur in membrane ruffles are most likely due to antagonist activities of kinases (PI3K) and phosphatases (PTEN and/or SHIP). As in the case of phagocytosis,

available evidence indicates that the sharp increase of PtdIns(3,4,5)P₃ is due to increased PI3K activity at the ruffles and cup. The fluctuations and eventual termination of the PtdIns(3,4,5)P₃ burst may be partly due to depletion of its substrate, PtdIns(4,5)P₂ [186], but phosphatases are important contributors. The concomitant appearance of PtdIns(3,4)P₂ suggests the involvement of 5-phosphatases and, consistently, knock-down of SHIP2 greatly prolongs the lifetime of PtdIns(3,4,5)P₃ in the macropinocytic cup, while ablating the surge of PtdIns(3,4)P₂ [187]. Interestingly, SHIP2 appears to be recruited to sites of macropinosome formation by SH3YL1, a bridging protein that on one hand binds the phosphatase, while also associating with PtdIns(3,4,5)P₃ through its SYLF domain [188].

The consequences of PtdIns(3,4,5)P₃ accumulation at forming macropinosomes are similar to those described for phagosomes. By recruiting adaptors, GEFs and GAPs that contain PtdIns(3,4,5)P₃-interacting PH domains, the cell is able to dictate the activity of Rho-family GTPases that direct the polymerization of actin required for the extension of circular ruffles and macropinosome closure. In the case of macropinocytosis, however, Rab5 is also involved in actin remodeling [189], albeit in a poorly understood manner. Rab5 has been reported to accumulate at the macropinosome following the observed increase in PtdIns(3,4,5)P₃ [185], and it has been suggested that the two events are causally linked [185]. However, because detection of Rab5 is only convincing when macropinosomes have sealed and its levels continue to increase well after PtdIns(3,4,5)P₃ has disappeared [186], it is difficult to establish whether recruitment of Rab5 by the inositide in fact precedes and is required for formation of the macropinosomes.

Regardless of the precise time when Rab5 is recruited, it is clear that the activity of PI3K is crucial for macropinosome formation. Indeed, sensitivity of the process to PI3K inhibitors is one of the few distinctive hallmarks of macropinocytosis. When formation of PtdIns(3,4,5)P₃ is precluded by wortmannin or LY294002, primordial macropinocytic cups are formed but promptly recede towards the cytosol, unable to extend and seal [5,190,191], a feature strongly reminiscent of phagocytosis of large particles.

5.3. Phosphatidylinositol 3,4,-bisphosphate in macropinocytosis

A couple of recent studies have documented the presence of PtdIns(3,4)P₂ in macropinosomes. The use of a Tapp1 PH domain-containing probe, which binds PtdIns(3,4)P₂ with high specificity [192], revealed a transient spike of this phosphoinositide during the early stages of macropinocytosis [186,187]. This sharp increase occurs during cup closure, coinciding with the decrease in PtdIns(3,4,5)P₃ levels.

PtdIns(3,4)P₂ generation during macropinocytosis presumably results from the breakdown of PtdIns(3,4,5)P₃ by SHIP2. As mentioned earlier, silencing this phosphatase abolishes the production of PtdIns(3,4)P₂. The same effect is seen when SH3YL1, the binding partner of SHIP2, is knocked down [188]. After its drastic increase, PtdIns(3,4)P₂ is broken down by INPP4B, a 4-phosphatase; when this phosphatase was knocked down, macropinosomes did not form and PtdIns(3,4)P₂ lingered in membrane ruffles [187]. Because of its transient existence on macropinosomes and its susceptibility to degradation by 4-phosphatases, it is conceivable that PtdIns(3,4)P₂ may be merely an intermediate in the conversion of PtdIns(3,4,5)P₃ to PtdIns(3)P. However, to the extent that some proteins bear PH domains that recognize and sometimes prefer PtdIns(3,4)P₂ to other inositides, PtdIns(3,4)P₂ may be more than a metabolic intermediary. Tapp1, for instance, has been proposed to be a target of PtdIns(3,4)P₂. When exogenously expressed, Tapp1 localizes to dorsal ruffles, which can be morphologically distinguished from the peripheral ruffles that are more classically associated with macropinocytosis. Unlike their peripheral counterpart, dorsal ruffles form on the apical surface of cells as ring-shaped structures that coalesce and contract, before subsiding. Depletion of endogenous Tapp1 suppresses the formation of dorsal ruffles [188]. Thus, PtdIns(3,4)P₂ may have a function of its own during macropinocytosis.

Other evidence is consistent with a role of PtdIns(3,4)P₂ in macropinocytosis. Knocking down INPP4B was found to impair macropinosome cup closure [187], which is also blocked when SHIP2 is silenced [188]. However, these results should be interpreted with caution, as in either case the inhibitory effects may have arisen from a failure to generate PtdIns(3)P, or even from excessive accumulation of PtdIns(3,4,5)P₃.

5.4. Phosphatidylinositol 3-phosphate in macropinocytosis

The appearance of PtdIns(3)P on macropinosomes was first visualized using the 2XFYVE domain probe. Low levels of PtdIns(3)P have been transiently detected in membrane ruffles in EGF-stimulated A431 cells [187]. After cup closure, a second burst of PtdIns(3)P is observed in the membranes of formed macropinosomes; the phosphoinositide reaches significantly higher levels in the sealed macropinosome than the ones detected in membrane ruffles. Remarkably, unlike phagosomes where it disappears within 15 min of sealing, PtdIns(3)P persists in macropinosomes for nearly 60 min. In fact, the authors of that report suggested that high levels of PtdIns(3)P are maintained as long as the macropinosome exists [183]. However, subsequent studies found the presence of PtdIns(3)P to be transient in early macropinosomes in myeloid cells [185]; in these cells macropinosomes undergo maturation into late macropinosomes, which is seemingly not the case in A431 cells.

PtdIns(3)P in macropinosomes is classically thought to be generated by Vps34 following cup closure and acquisition of Rab5 by the macropinosome [144,145]. However, the initial PtdIns(3)P spike observed during ruffling and cup formation is generated by a cascade of sequential hydrolysis reactions that convert PtdIns(3,4,5)P₃ to PtdIns(3,4)P₂ and eventually PtdIns(3)P. These modifications are presumably catalyzed by SHIP and INPP4B, respectively [187].

Disappearance of PtdIns(3)P can occur by dephosphorylation to PtdIns or by phosphorylation to PtdIns(3,5)P₂. Phosphatases of the myotubularin family and PIKfyve, respectively, mediate these reactions. In this regard, it is noteworthy that depletion of myotubularin-related protein 6 (MTMR6) was accompanied by accumulation of PtdIns(3)P in membrane ruffles, where the inositol is not normally detected [187].

Unlike phagocytosis, where PtdIns(3)P is believed to be important for vacuolar maturation, in macropinocytosis PtdIns(3)P has been proposed to participate in vacuolar formation. Inhibition of its synthesis impairs macropinocytosis, as suggested by experiments where INPP4B was knocked down [187]. This puzzling observation may be related to ion fluxes. KCa3.1 is a Ca²⁺-activated K⁺ channel that was shown to be stimulated by PtdIns(3)P [193]. Depletion of KCa3.1 by gene silencing or its pharmacological inactivation by TRAM-34, a channel-specific inhibitor, impairs macropinocytosis. Moreover, expression of a mutant KCa3.1 that cannot be activated by PtdIns(3)P severely impaired macropinosome formation [187]. Why and how K⁺ efflux through KCa3.1 is required for macropinocytosis remains unresolved.

5.5. Are diffusional barriers involved in the differential distribution of phosphoinositides in the membrane?

Phagocytosis and macropinocytosis are spatially restricted phenomena. The large-scale cytoskeletal and membrane remodeling involved in these processes are guided by specific spatial coordinates, which are in part provided by the confined distribution of phosphoinositides. The ability of phosphoinositides to accumulate in the correct area is therefore instrumental for the establishment and progression of these polarized endocytic processes. However, in the light of the fluid mosaic model of biological membranes, newly synthesized phosphoinositides are anticipated to quickly diffuse laterally and distribute in the plane of the plasmalemma within seconds. Considering the comparatively slow development of phagocytosis and macropinocytosis (in the order

of minutes), a great deal of lipid diffusion and homogenization is expected to occur before sealing is completed [194]. It is thus remarkable that, despite the apparent physical continuity of the nascent phagosomal membrane with the neighboring bulk plasmalemma, the accumulation of PtdIns(3,4,5)P₃ or DAG and the disappearance of PtdIns(4,5)P₂ are spatially restricted to the nascent phagosomal compartment [132,137]. Two models, which are not mutually exclusive, have been proposed in order to account for this confined distribution.

One model relies on a 'source-sink' mechanism, in which phosphoinositide-modifying enzymes are differentially distributed between the phagosomal membrane and its periphery. In this case, biosynthetic enzymes would localize to the nascent phagosomal membrane, while catabolic ones would be excluded from the phagocytic cup. Alternatively, degradative pathways could be equally active within the phagosomal membrane and the unengaged plasmalemma, in which case the elevation in phosphoinositide concentration would be driven solely by an upsurge in synthesis. Note that this 'source-sink' model requires the enzymes responsible for phosphoinositide metabolism to be confined, possibly by interaction with the stimulated receptors at the base of the phagocytic cup. How enzyme immobilization or selective activation/inactivation would occur in the case of macropinocytosis is less clear.

A second model is based on the possible existence of a physical diffusional barrier located at the periphery of nascent phagosomes that prevents or at least limits the lateral diffusion of lipids in and out of the forming vacuole. This hypothesis is supported by the drastic decrease in lipid mobility observed when macrophages engage IgG-coated surfaces [132]. The possibility of a fence-mediated confinement is also supported by analyses of the mobility of phagosomal PtdIns(4,5)P₂, studied using a fluorescent derivative of the phosphoinositide [195]. Using fluorescence correlation spectroscopy, these experiments confirmed that PtdIns(4,5)P₂ diffuses rapidly both within the phagosomal membrane and the unengaged plasmalemma. Despite this unrestricted mobility, fluorescence recovery after photobleaching experiments showed no recovery in PtdIns(4,5)P₂ fluorescence after the cup was photobleached. In contrast, PtdIns(4,5)P₂ fluorescence recovered within seconds when the unengaged plasmalemma was photobleached [195]. Together, these observations argue in favor of a fence that limits phosphoinositide diffusion in and out of the forming phagosome.

Restricted diffusion by virtue of a physical barrier has also been documented in the case of macropinocytosis. Using a membrane-targeted form of photoactivatable GFP, Welliver et al. demonstrated that an integral actin skeleton is required to prevent the diffusion of membrane molecules to and from circular ruffles [196]. Barriers to lateral diffusion within nascent macropinosomes could shape and potentiate the signaling events required for macropinocytosis.

6. Concluding remarks

Phagocytosis and macropinocytosis are complex phenomena, each a veritable compendium of cell biology entailing receptor activation, signal transduction, cytoskeletal remodeling and membrane traffic. Phosphoinositides seem to feature importantly in every one of these components of the response: they influence receptor distribution and responsiveness, convey signals to downstream adaptors and effectors, orchestrate cytoskeletal rearrangements and mediate membrane fusion and fission events. The identity of the evolving compartments generated by phagocytosis and macropinocytosis seems to be itself determined and defined by their phosphoinositide composition, whether by directing stereospecific interactions with ligands or by defining the surface charge that drives electrostatic interactions with cationic proteins.

Given their unique size, phagosomes and macropinosomes lend themselves to the study of sub-organellar domains, which have been difficult to resolve in other endocytic and secretory compartments. The advent of super-resolution microscopy, in combination with

correlative ultrastructural analysis, should make such studies feasible in the near future, yielding fascinating insights.

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