Phagocytosis of Filamentous Bacteria: Impact of Target Morphology on Phagosomal Maturation

by

Amriya Naufer

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Department of Cell & Systems Biology
University of Toronto

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2015

Abstract

Phagocytosis has been characterized by studies that utilized spherical particles as targets. However, phagocytes encounter targets of a disparate morphology in nature. Particles with filamentous morphology can present a hurdle to phagocytosis. For example, during phagocytosis of long filamentous targets (FTs), the phagocytic cups can take more than 30 minutes to be internalized. During this time, the phagocytic cup is composed of two domains – the actin-rich domain proximal to the membrane and the internal phagocytic cup (PC), which is embedded within the cytoplasm. Here, we present evidence that early endosomal lipid regulator, PI(3)P is recruited to PCs and co-exists with lysosomal protein, LAMP1. Our results indicate that aberrant recruitment of PI(3)P is a consequence of the neutrality of the compartment on which it is maintained, acidification of PCs abrogates PI(3)P recruitment to PCs. Our findings reveal that key aspects of phagosomal maturation are conditioned by the morphology of the target.
Acknowledgements

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APPL1</td>
<td>Adaptor protein containing pleckstrin-homology domain, PTB phosphotyrosine-binding domain, and leucine zipper/bin-amphiphysin-rvs domain 1</td>
</tr>
<tr>
<td>CORVET</td>
<td>Class C core vacuole/endosomal tethering</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early Endosome Autoantigen 1</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous Actin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc Receptors</td>
</tr>
<tr>
<td>FT</td>
<td>Filamentous Target</td>
</tr>
<tr>
<td>GAPs</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GEFs</td>
<td>GDP-exchange factors</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Hrs</td>
<td>Hepatocyte growth factor-regulated tyrosine kinase substrate</td>
</tr>
<tr>
<td>HOPS</td>
<td>Homotypic fusion and vacuolar protein sorting</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosome-associated membrane protein</td>
</tr>
<tr>
<td>LBPA</td>
<td>Lysobisphatidic acid</td>
</tr>
<tr>
<td><em>Lp</em></td>
<td><em>Legionella Pneumophila</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAMPs</td>
<td>Microbe-associated molecular patterns</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule-organizing center</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>ORPL1</td>
<td>Oxysterol-binding protein-related protein 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phagocytic cup</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI(3,4,5)P3</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PI(3,5)P2</td>
<td>Phosphatiylinositol-3,5-bisphosphate</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>RFP</td>
<td>Red florescence protein</td>
</tr>
<tr>
<td>RILP</td>
<td>Rab7-interacting lysosomal protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar adenosine triphosphate</td>
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</table>
Chapter 1
Introduction

Phagocytosis— the process of a cell taking up a solid particle—is of fundamental importance to a wide variety of organisms. Phagocytosis plays a crucial role in biological processes, from simple unicellular organisms that rely on phagocytosis for the acquisition of nutrients, to complex metazoans to which phagocytosis comprises an essential branch of the immune system. In addition to providing protection against foreign, potentially pathogenic organisms and particles, phagocytosis is essential for mediating tissue homeostasis and remodelling.

Thus far, the phagocytic pathway has primarily been characterized by studies that utilized spherical particles as targets. However, phagocytes encounter targets of a disparate morphology in nature. Our knowledge of how target morphology affects phagocytosis is limited. For example, particles of a filamentous morphology are known to present a hurdle to phagocytosis. Therefore, the main focus of my thesis is to investigate how phagocytosis is altered in the face of filamentous target morphology.

1.0 Phagocytosis

Phagocytosis was first described by Élie Metchnikoff more than a hundred years ago (Metchnikoff, 1893). Since then, it has been recognized as a vital component of the innate and adaptive immune response to pathogens. This process is best defined as a receptor-mediated, actin-dependant ingestion of large particles greater than or equal to 0.5 micrometers in size (Greenberg and Silverstein, 1993).

In higher metazoans, phagocytosis plays an essential role in tissue remodelling and maintenance, by clearing cellular debris and removing billions of apoptotic bodies. This is most strikingly evident in the case of the retinal pigment epithelium that functions to breakdown and uptake shed photoreceptor membranes through phagocytosis (Schraermeyer et al., 1999). In this way, epithelial cells, endothelial cells and fibroblasts perform
phagocytosis and contribute to the clearance of billions of cells that are turned over each day (Aderem et al., 1999).

In contrast, the truly professional phagocytes are the cells of the innate immune system including the haemocytes in insects and the neutrophils, macrophages and dendritic cells of mammals. These cells act as the first line of defense and are the sentinels of the immune system. They hunt, engulf and destroy senescent or apoptotic host cells, pollutant particles, and foreign, potentially pathogenic organisms. They also play a key role in initiating the adaptive immune response; by presenting antigens derived from the degradation of the engulfed particle to lymphoid cells.

1.1 The Phagocytic Cup

1.1.1 Mechanisms of Particle Recognition and Engagement

Phagocytosis is triggered when specialized receptors on the surface of phagocytic cells engage its ligand on the target particle. These receptors either detect determinants inherent to the particle directly or through the intermediate of host serum factors (opsonins) indirectly (Shaw and Griffin, 1981). Microbes display various molecules that are not present in higher organisms. These microbe-associated molecular patterns (MAMPs) can be detected by some phagocytic receptors (Sastry and Ezekowitz, 1993). For instance, scavenger receptor A detects lipopolysaccharide (LPS), a cell-surface lipoglycan expressed by gram-negative bacteria (Peiser et al., 2000).

Foreign particles can also be recognized in the blood and interstitial fluid by opsonins such an antibodies and the complement fragment, C3bi. Antibodies recognize their cognate ligands on foreign particles with high specificity while C3bi, fixes to the carbohydrate surface of pathogens (Mantovani et al., 1972). Following their deposition on foreign surfaces through a process called opsonization, opsonins are engaged by receptors on the surface of the phagocytic cell, which allow for indirect interaction of the receptor with the particle (Janeway et al., 2001). The Fc family of receptors (FcRs) that bind to the Fc portion of the most abundant antibody isotype in serum, immunoglobulin G (IgG) and the C3bi receptor
that binds iC3b complement fragment are most effective at initiating phagocytosis (Indik et al., 1995).

1.1.2 Phagocytic Cup Formation

In addition to recognizing different ligands, phagocytic receptors differ in the downstream signalling that follows their activation. Under physiological conditions, multiple receptor types can be engaged concomitantly and several signalling cascades can be triggered at once (Shaw and Griffin, 1981). The capture and internalization of IgG-coated targets by FcγRs is by far the best-understood model of phagocytic receptor activation and signalling.

Phagocyte-mediated engagement of target particles was originally thought to result from chance encounters between the two particles. It now appears that rather than relying on random Brownian collisions, macrophages probe their microenvironment for an appropriate target by extending out dynamic membrane tentacles (Figure 1A; Flannagan et al., 2010, Mattila and Lappalainen, 2008). A particle may be captured by multiple receptors on phagocytes that are distant from each other, but for downstream signalling to commence, several receptors have to cluster in one region. For complete internalization, receptor engagement around the entire particle appears to be required, leading to a zippering phenomenon where receptors sequentially engage with ligands on the particle (May and Machesky, 2001). Zippering drives the initial extension of the membrane around the target in an actin-independent manner (Patel and Harrison, 2008; Swanson and Baer, 1995).

Following receptor engagement, lateral diffusion and clustering of FcγRs brings the cytosolic domains of these receptors into close proximity with each other (Figure 1B; Shaw and Griffin, 1981). The cytosolic domain of FcγRs comprises of a unique region known as the immunoreceptor tyrosine-based activation motif (ITAM), which is characterized by a tyrosine motif that acts as a substrate for phosphorylation by tyrosine kinases of the Src family (Abram and Lowell, 2007; Daeron et al., 1994). Following phosphorylation, the Src homology 2 (SH2) domains of spleen tyrosine kinase (Syk) bind to doubly phosphorylated ITAMs (Crowley et al., 1997; Indik et al., 1995). This second phosphorylation event leads to
**Figure 1 Phagocytic uptake of spheroidal targets.** During phagosome formation, (A) macrophages dynamically probe their microenvironment for an appropriate target by extending out F-actin-dependent membrane tentacles. These membranous projections are rich in receptors that are capable of binding targets. (B) Following receptor engagement, several receptors converge in one area, initiating downstream signaling. Intracellular signaling events lead to the remodeling of the actin-cytoskeleton and phagocytic cup formation. (C) Continued polymerization of F-actin leads to pseudopodia extension around the target, which enables receptor engagement around the entire particle, leading to a zippering phenomenon. (D) Once pseudopodia reach the apex of the particle, they meet and fuse in a contractile fashion that has been likened to the closure of a purse-string. Constriction forces reduce the circular margins of the phagosome to a narrow aperture, which enables membrane fusion and scission of the nascent phagosome from the plasma membrane. Myosin family members and PI3-Kinase products are thought to be critical for phagosome closure.
the striking recruitment of additional signalling proteins to the activated FcγR complex. The direct binding of these adaptor proteins to the tyrosine kinases, results in the formation of a platform that acts as a site for the recruitment of additional downstream signalling components (Indik et al., 1995). The intracellular signalling cascade that follows receptor binding culminates in the activation of numerous actin cytoskeleton remodelling and polymerization proteins, which work together to mediate cytoskeletal rearrangements and pseudopod extension (Figure 1C; Naik and Harrison, 2013; May et al., 2000).

1.1.3 Remodelling of Actin and membrane lipids

Receptor signalling leads to internalization of the particle into a phagosome by a complex sequence of events that requires remodelling of the actin cytoskeleton and alterations in phospholipid metabolism.

1.1.3.1 Actin: The skeleton of Phagocytosis

Phagocytosis has been known to be an actin dependant process since the seminal studies of Kaplan (1977), in which the use of a toxin that blocks F-actin polymerization, cytochalasin B, was shown to inhibit uptake of IgG-coated erythrocytes. Localized polymerization of F-actin into filaments beneath the plasma membrane through the coordinated nucleation, growth, bundling and branching of filaments allows for the extension of pseudopodia around a particle (May and Mechesky, 2001; Swanson et al., 1999). FcγR signalling leads to the recruitment of F-actin modulators which include the small GTPases of the Rho family: Cdc42, Rac1 and Rac2. Cdc4c activation precedes the subsequent activation of Rac1 and then Rac2. Together, they function as molecular switches to activate downstream proteins, which culminate in the stimulation of Arp2/3, a multi-protein complex that nucleates F-actin filaments, generating, branched filaments (Greenberg, 1995; Machesky and Gould, 1999). The ADF/cofilin family also works to mediate actin polymerization. These actin-binding proteins sever pre-existing actin filaments to produce additional barbed ends, which present as additional sites for actin polymerization (Bamburg et al., 1999). All this culminates in the
formation of a branched and complex actin network that pushes the plasma membrane further around the target.

1.1.3.2 An Early Role for Lipid Signalling

Despite making up a small proportion of cellular phospholipids, phosphoinositides play a critical role in orchestrating the signalling events that mediate phagocytosis (Figure 2). There are seven phosphoinositide species that are generated by the action of kinases and phosphatases that act upon their membrane-bound lipid substrate (Balla, 2012). The anionic nature of PIs contributes to the build-up of negative charge on the inner leaflet of the plasma membrane (Yeung et al., 2006). In a resting cell, the inner-leaflet of the plasma membrane consists of significant amounts of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2](Balla, 2012). During phagocytosis, PI(4,5)P2 undergoes a biphasic change at the region of the membrane that envelops the incoming target particle— the phagocytic cup— that mirrors the localization of F-actin accumulation (Botelho et al., 2000). There is an initial modest increase in PI(4,5)P2 concentrations but this is followed by a drastic disappearance of this PIP species. The synthesis of PI(4,5)P2 is catalyzed by phosphatidylinositol 4-phosphatase 5-kinases (PI4P-5K) and is thought to be critical in inducing F-actin polymerization (Figure 2A). Inhibition of PI4P-5K prevents PI(4,5)P2 accumulation and impairs phagocytosis (Coppolino et al., 2002). The subsequent loss of PI(4,5)P2 is essential to allow particle internalization, due to the loss of submembranous F-actin at the base of the phagocytic cup (Scott et al., 2005). Disappearance of PI(4,5)P2 is mediated by several pathways; to note is the action of phosphoinositide-specific phospholipase Cγ (PLCγ). Following phosphorylation, PLCγ is recruited to the phagocytic cup in a Syk-dependant manner; its pH domain binds PI(4,5)P2, stabilizing it at the inner leaflet. PLCγ acts to hydrolyze PI(4,5)P2 into diacylglycerol (DAG) and inositol-3,4,5-trisphosphate (IP3), two downstream players that are important for enhancing phagocytosis (Botelho et al., 2000). PI(4,5)P2 is also consumed when it is phosphorylated by Class I phosphatidylinositol 3-kinase (PI3K), producing PI(3,4,5)P3 at the phagocytic cup (Figure 2B; Ninomiya et al., 1994; Marshall et al., 2001).
**Figure 2 Phosphoinositide dynamics in phagocytosis.** During target internalization, (A) PI(4,5)P$_2$ is the first PIP species to form at the phagocytic cup. The production of PIP$_2$ by Type 1 phosphatidylinositol kinases (PIPK1) is important for promoting actin polymerization. PIP$_2$ is then hydrolyzed by phospholipase C to inositol triphosphate (IP$_3$) and diacylglycerol (DAG). (B) Loss of PIP$_2$ is also mediated by the activity of Class 1 PI3Kinases that phosphorylate the third position on the inositol ring of PIP$_2$ to produce PI(3,4,5)P$_3$. PIP$_2$ and PIP$_3$ are important for the phagocytic cup-remodeling phase of phagocytosis. (C) PIP$_3$ removal, which is mediated by 3-phosphatase PTEN and 5-phosphatase SHIP is important for bringing about the cessation of actin polymerization. (D) Once the nascent phagosome forms, active Rab5 (GTP-bound) recruits Class 3 PI3-Kinase, VPS34 which mediates the production of PI(3)P from PI. The production of PI(3)P is thought to be important for phagosomal maturation. (E) PI(3)P is eventually removed from the phagosome by a 5-kinase, PIKfyve which acts on PI(3)P to produce PI(3,5)P$_2$. Myotubularin phosphatases can also act on PI(3)P to produce PIP. PI(3,5)P$_2$ is the PIP species found predominantly on late phagosomes.
PI3K is activated by FcγRs and is required for the progression of FcγR-mediated phagocytosis. However, the dependence on PI3-Kinase products is reduced for smaller particles (≤1 µm; Araki et al., 1996; Cox et al., 1999). Pseudopod extension is thought to occur in two phases; the initial PI3-Kinase-independent phase is sufficient for the internalization of small particles. The second phase is essential for the ingestion of large particles and requires the formation of PI(3,4,5)P3. Therefore, inhibition of PI3-Kinases impairs phagocytosis of large particles (≥ 3-µm; Cox et al., 1999).

1.1.4 Phagosome closure and scission

Phagocytic uptake of a target is completed when pseudopods reach the apex of the particles, and contract in a manner similar to the closure of a purse-string (Figure 1D; Swanson et al., 1999). The constriction closes the circular margins of the phagocytic cup to a narrow aperture, which then enables membrane fusion, creating an intracellular membranous compartment. This contractility is thought to involve actin and myosins. Actin has been observed to concentrate at the distal margins of the closing phagosomes (Swanson et al., 1999). The myosin family members, myosin I, II, V and IX, are also thought to play a contractile role at the site of phagosomal closure (Laevsky and Knecht, 2003; Cox et al., 2002; Swanson et al., 1999). The convergence of pseudopods also requires PI3-kinase activity (Araki et al., 1996). Phosphorylated phosphoinositides generated by the action of PI3-kinase may play a role in positioning the contractile apparatus, either directly by binding a myosin or indirectly by organizing integral membrane proteins or by activating Rho-GTPases (Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996).

The cessation of early signalling events is critical for the completion of target uptake. The termination of stimulatory signalling such as the dephosphorylation of the phosphotyrosine residues by tyrosine phosphatase SHP as well as the elimination of PI(3,4,5)P3 by the action of phosphatidylinositol 5-phosphatase SHIP and phosphatidylinositol 3-phosphatase PTEN leads to the cessation of F-actin polymerization (Figure 2C, Kamen et al., 2007). PI(3,4,5) P3 disappears from nascent phagosomes within 1 minute of sealing (Marshall et al., 2001). In
this way, the F-actin that is polymerized extensively during cup formation is disassembled before scission occurs.

1.2 Phagosomal Maturation

Shortly after internalization, the newly denuded vacuole membrane is innocuous, as it lacks the degradative environment required to destroy foreign pathogens and digest apoptotic bodies (Muller et al., 1980). The outer membrane of the nascent phagosome very closely resembles the plasmalemma and phagosomal lumen consists of a sample of the extracellular milieu (Figure 3A,i; Swanson and Baer, 1995). However, scission is rather rapidly followed by a succession of biochemical modifications that result in substantial changes to the nascent phagosomal composition (Pitt et al., 1992; Flannagan et al., 2012). The phagosomal maturation process depends critically on the interaction of the nascent phagosome with the endocytic pathway (Figure 3A; Desjardins et al., 1994). The proposed ‘kiss and run’ hypothesis presents a model for phagosomal maturation in which phagosomes are thought to undergo multiple transient fusion-fission interactions with endocytic organelles, via a fusion-pore-like structure, in a microtubule-dependant manner. This model was proposed by Desjardins and colleagues (1995) to account for the maintenance of unique organelle identities despite fusion between distinct compartments, which would result in intermixing of non-resident molecules. The progressive maturation process ultimately yields a highly acidic and hydrolase rich phagolysosome that is capable of degrading its contents efficiently.

1.2.1 The Early Phagosome

The scission of the nascent phagosome from the plasmalemma marks the beginning of mild luminal acidification, a key event in the biochemical transformation of the phagosome (Desjardins et al, 1994). The acidification process is a consequence of proton pump recruitment to the phagosome. The pumping of protons across the membrane into the phagosomal lumen is catalyzed by the vacuolar ATPase (V-ATPase), a massive multimeric protein complex that expends ATP to function (Lukacs et al., 1990; Tapper et al., 1995). This
**A.**

1. Nascent Phagosome
2. Early Phagosome
3. Late Phagosome
4. Phagolysosome

**B.**

1. Filamentous Bacterial Target
2. External Bacteria
3. Leaking of H+ and hydrolytic enzymes
4. Late Endosome
5. Lysosome

**pH**

- **Neutral**
- **Acidic**
Figure 3 Phagosomal Maturation of spheroidal and filamentous targets. (A) Once internalized, the membrane of the nascent phagosome resembles that of the plasma membrane while the lumen resembles the extracellular environment (i). The only difference is the loss of actin and PI(4,5)P₂. In the early phagosomal stage, phagosomes fuse with early endosomes and acquire early endosomal markers (ii). This is accompanied by mild acidification of the lumen. Following this, early endosomal markers are lost and late endosomal markers are acquired in the late phagosomal stage (iii). The lumen is then further acidified. In the final stage of maturation, lysosomes fuse with phagosomes to produce a phagolyosome, where cargo degradation occurs as a result of the acidic and proteolytic environment. (iv). (B) Phagocytosis of filamentous bacterial targets proceeds through a tubular phagocytic cup stage, which can last for longer than 30 minutes (i). The phagocytic cup, which is enriched in actin near the plasma membrane, undergoes maturation by fusing with endosomes and lysosomes, despite not sealing into a phagosome (ii). The unsealed phagocytic cup causes the leakage of protons and low molecular weight hydrolytic enzymes. As such, it is a relatively neutral structure (iii). Eventually, phagocytic cups seal into phagosomes; it is only at this time that the phagosomal lumen is acidified and becomes hydrolytic (iv). The resultant antimicrobial environment allows for the complete degradation of the target (v). Differential immunostaining of filamentous bacterial targets distinguished internalized segments from external bacteria (Internal: red, External: purple).
mildly acidifies the lumen of the newly formed phagosome to a pH of around 6.5 (Gnosh et al., 1997).

Modifications to the nascent phagosome are also the result of membrane traffic to and from the phagosome, a process that is dependant primarily on the Rab GTPase family of molecular switches (Stenmark, 2009). Like other GTPase proteins, Rabs cycle between an active GTP-bound state and an inactive GDP-bound state, which is tightly regulated by their respective GDP-exchange factors (GEFs) and GTPase activating proteins (GAPs). The Rab GTPase, Rab5, plays a pivotal role in regulating maturation of the early phagosome as it mediates the recruitment of key early endosomal players to the maturing phagosome (Barbieri et al., 1996). As of yet, little is known about the mechanism that leads to the recruitment of Rab5 to the early phagosome. It is thought that signals emanating from phagocytic receptors may direct this recruitment (Vieira et al., 2002). Nevertheless, nascent phagosomes acquire Rab5 on their surface, which is activated by its cognate GEF, Rabex-5 (Fairn and Grinstein, 2012; Horiuchi et al., 1997). The ensuing activation of Rab5 then leads to the downstream recruitment of the Rab5 effector, Rabaptin-5. In a feed-forward loop, formation of the Rab5/Rabaptin-5 complex promotes further Rabex-5 GEF activity, leading to enhanced Rab5 activation (Figure 4A; Lippé et al., 2001). The activation of Rab5 is essential for promoting early endosomal fusion with the nascent phagosome. This is best demonstrated by the fact that heterologous expression of a mutated Rab5 allele that is locked in a GTP-bound state leads to the formation of giant phagosomes and early endosomes that fail to mature (Duclos et al., 2003). Therefore, Rab5 activity is critical for the recruitment of downstream effectors, including APPL1 (adaptor protein containing pleckstrin-homology domain, PTB phosphotyrosine-binding domain, and leucine zipper/bin-amphiphysin-rvs domain 1), EEA1 (Early Endosome Autoantigen 1) and the p15-/Vps34 complex.

The early endosomal adaptor, APPL1, resides on early phagosomal membranes, where in a Rab5-dependant manner, it is thought to recruit the inositol 5-phosphatases, OCRL and Inpp5b (Bohdanowicz et al., 2012). The role for phosphoinositides in phagocytosis extends beyond that of the receptor signalling and cup formation phase; phosphoinositides are also essential for phagosomal maturation. The depletion of PI(4,5)P2 from the nascent phagosome
is partly dependant on Inpp5b and OCRL (Bohdanowicz et al., 2012). Strikingly, APPL1-
dependant recruitment of these phosphatases and the ensuing depletion of 5-phosphorylated
phosphoinositides is critical for attenuating Akt-dependant signal transduction from the
phagosome (Bohdanowicz et al., 2012).

A key factor in maturation is the formation of phosphatidylinositol 3-phosphate [PI(3)P] on
the early phagosome. Class III PI3K, Vps34, is recruited to the maturing phagosome in a
Rab5-dependant manner where it catalyzes the formation of PI(3)P from phosphatidylinositol
(PI; Figure 2D; Vieira et al., 2001; Thi and Reiner, 2012). Vps34 and PI(3)P are integral to
phagosomal morphogenesis as inhibition of Vps34 function ablates phagosomal maturation
(Araki et al., 1996). The stable association of Vps34 with Vps15-like serine/threonine kinase
p150 enhances the catalytic activity of Vps34 and promotes the recruitment of the kinase
enzyme to the phagosome (Vieira et al., 2001; Murray et al., 2002). Rab5 recruitment and
activation, although independent of Vps34, is required for Vps34-dependant PI(3)P
production.

Production and accumulation of PI(3)P at the early phagosome enables the recruitment of
PI(3)P binding proteins such as Hrs (hepatocyte growth factor-regulated tyrosine kinase
substrate), the P40\text{phox} subunit of the NADPH oxidase and EEA1 (Fratti et al., 2001; Tian et
al., 2008). NADPH oxidase or NOX2 produces ROS compounds, which lethally oxidize
microbial carbohydrates, lipids and nucleic acids. The spatiotemporal distribution of the
PI(3)P binding protein, P40\text{phox} in the cell is influenced by PI(3)P (Tian et al., 2008). P40\text{phox}
is endowed with a PX lipid binding domain that recognizes PI(3)P; thus modulating local
PI(3)P production at the phagosome can have profound effects on reactive oxygen species
(ROS) production.

The PI(3)P binding protein, Early Endosomal Autoantigen 1 (EEA1) acts as a tethering
protein on the early phagosome; a single EEA1 molecule can bind PI(3)P and Rab5 through
its FYVE zinc finger and C(2)H(2) zinc finger domain respectively (Simonsen et al., 1998;
Christoforidis et al., 1999; Mishra et al., 2010). This confers on EEA1 the special ability to
bridge maturing phagosomes and endosomes decorated with Rab5 and PI(3)P to each other.
The fusogenic feature of EEA1 results from its ability to interact with syntaxin 13, a member of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family of proteins (McBride et al., 1999). SNAREs are transmembrane proteins that function by forming hairpin-like structures that drive homotypic membrane fusion of early phagosomes with early endosomes during phagosomal morphogenesis (Figure 3A,ii). SNARE function is regulated by the N-ethylmaleimide-sensitive factor (NSF) as perturbation of NSF function or of SNARE function arrests phagosomal maturation (Coppolino et al., 2001). Indeed, coordinated membrane fusion is critical for ensuring phagosomal morphogenesis.

1.2.2 Early to Late Phagosomal Transition

Continual remodelling of the early phagosome produces a more acidic late phagosome (~pH 5.5-6.0; Gnosh et al., 1997). With the delivery of endosomal membranes to maturing phagosomes continuous acquisition of the V-ATPase occurs, resulting in an increased influx of protons (Sun-Wada et al., 2009). The hallmark of early to late phagosomal transition is the acquisition of the Rab GTPase Rab7 and the accompanying loss of Rab5 (Vitelli et al., 1997). Rab7 recruits downstream effectors, which work to direct the centripetal transport of the maturing phagosome along microtubules (Harrison et al., 2003). As the preinuclear region is populated with the majority of late endosomes and lysosomes, the centripetal motion of the phagosome brings these compartments into close proximity with each other.

Some progress has been made in elucidating the molecular mechanism behind Rab5 to Rab7 transition. Although much of the work on vacuolar fusion has been done in *C. elegans* and yeast, mammalian phagosomal fusion is thought to operate analogously. In addition to its central role in early phagosomal maturation, Rab5 can also stimulate the acquisition of Rab7, albeit through indirect means (Rink et al., 2005). The Rab5 to Rab7 switch commences with the recruitment of Mon1a and its binding partner Ccz1 by Rab5 (Figure 4B; Nordmann et al., 2010). This in turn causes the displacement of the Rab5 GEF, Rabex-5, from the phagosomal membrane, which terminates the Rab5 feed-forward activation loop (Poteryaev et al., 2012). Rab5-GAPs promotes nucleotide hydrolysis and Rab5-Guanosine nucleotide
Figure 4 Rab5 to Rab7 conversion. Inactive Rab5 and Rab7 bound to GDI reside in the cytosol. (A) Rab5 activation on early endosomes/phagosomes is mediated by the Rab5-GEF, Rabex-5. Upon activation, Rab5 recruits its effector, Rabaptin-5 that further stimulates Rabex-5 activity, generating a positive feed-forward loop. (B) Active Rab5 recruits a variety of effectors, which include Mon1a and Ccz1, which is critical for the Rab5 to Rab7 transition. This leads to the displacement of Rabex-5 from the membrane, which terminates the feed-forward Rab5 activation loop. (C) Rab5GAP facilitates nucleotide hydrolysis on Rab5-GTP and Rab5-GDIs scavenge for GDP-bound forms of Rab5, thereby restoring overall balance between the active and inactive states. Recent studies carried out in the yeast model suggest that the Mon1a/Ccz1 may act as a GEF, promoting Rab7 recruitment and activation at late endosomes/phagosomes. (D) The transition of Rab5 to Rab7 is mirrored by conversion of the Rab5 effector, CORVET to the HOPS complex. CORVET and HOPS share the same core structure consisting of Vps11, Vps16, Vps18 and Vps33. Rab5 recruitment of CORVET to early endosomes/phagosomes leads to its conversion to HOPS by the removal of CORVET proteins, Vps3 and Vps8 and addition of HOPS proteins Vps39 and Vps41. (E) The Vps39 subunit of the HOPS complex is thought to act as a Rab7 GEF. Recent evidence from yeast studies suggests that the Mon1a/Ccz1 complex may also bind the HOPS complex. Therefore, by binding both Rab7 and the Mon1a/Ccz1 heterodimer, HOPS can mediate membrane fusion by promoting destabilizing cis-SNARE complexes and stabilizing trans-SNARE complexes.
disassociation inhibitors (GDIs) scavenge for GDP-bound Rab5, restoring the balance from the active to inactive state (Figure 4C).

The Rab5 to Rab7 conversion is also dependent on the activity of two Vps-C protein complexes, CORVET (class C core vacuole/endosomal tethering) and HOPS (homotypic fusion and vacuolar protein sorting) (Figure 4D). Rab5 recruits CORVET to the limiting membranes of early phagosomes where it is converted to HOPS by removal of CORVET proteins Vps3 and Vps8 and addition of HOPS proteins Vps39 and Vps41 (Peplowska et al., 2007; Plemel et al., 2011). The Vps39 protein of the HOPS complex is thought to drive Rab7 activation by acting as a Rab7 GEF (Figure 4E; Wurmser et al., 2000). Recent evidence obtained from yeast studies suggests that Mon1a may also bind the HOPs complex. Therefore, Mon1a and its multiple effectors including Ccz1 and HOPS may work together to mediate the transition of GTP-Rab5 to GTP-Rab7 on the limiting membranes of maturing phagosomes (Kinchen and Ravichandran, 2010; Nordmann et al., 2010).

1.2.3 The Late Phagosome

The increased acidity of the late phagosome is a consequence of additional protein pump recruitment to the phagosome. This produces an acidic phagosome with a pH ranging from 5.5-6.0 (Lukacs et al., 1990; Yates et al., 2005; Gnosh et al., 1997).

Recruitment of Rab7 and its multiple players are essential for the completion of phagosomal maturation (Figure 3A, iii). Active Rab7 mediates phagosomal fusion with lysosomes and yet, Rab7 activation alone is not sufficient. Inhibition of Class 3 PI3K Vps34 also ablates phagosomal maturation and phagolysosomal fusion (Vieira et al., 2003). Strikingly, obstruction of Vps34 does not prevent Rab7-GTP recruitment and activation. Hence, Rab7 must act in concert with phosphoinositide-dependant regulators of phagosome maturation to induce phagosomal maturation at the latter stage. These regulators have yet to be identified; 5-phosphoinositol kinase, PIKfyve is one known regulator. PIKfyve is essential for the conversion of PI(3)P to PI(3,5)P2 on the late phagosome (Figure 2E; Kerr et al., 2010; Hazeki et al., 2012). Ablation of PIKfyve function has been shown to delay removal of PI(3)P and reduce the acquisition of lysosomal proteins such as LAMP1 and cathepsin D.
Although phagosomes were still acidified, the microbicidal capacity of phagosomes was diminished. This may be the consequence of PIKfyve inhibition as PIKfyve appears to play a role in trafficking and recycling of the mannose-6-phosphate receptors that shuttle between late endosomes and the Golgi to deliver newly synthesized lysosomal proteases to lysosomes (Rutherford et al., 2006; de Lartigue et al., 2009). Consequently, PI(3,5)P2 synthesis via PIKfyve is essential for phagosomal morphogenesis (Kim et al., 2014).

In spite of the importance of Rab7 in phagosomal maturation, the downstream effectors of Rab7 have yet to be characterized. Two proteins that are known to associate with late phagosomes in a Rab7-dependant manner are Rab7-ineracting lysosomal protein (RILP) and oxysterol-binding protein-related protein 1 (ORPL1; Jordens et al., 2001; Johansson et al., 2007). Upon Rab7 activation, RILP and ORPL1 work together to mediate microtubule-dependant vesicular traffic of Rab7-positive compartments by coupling them to molecular motor proteins dynein/dynactin. This trafficking is essential for it brings the phagosome into closer proximity with lysosomes, at which point fusion may be facilitated in a Rab7-HOPS-dependant manner (Van der Kant et al., 2013).

Other structural components of the late phagosome that were originally thought to be unimportant are now known to be involved in phagosomal morphogenesis. Late endosomes, late phagosomes and lysosomes are enriched in the glycosylated-transmembrane proteins, lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2). These structural proteins were originally thought to maintain lysosomal integrity but recent work has provided evidence that without LAMP proteins, phagosomes lose their degradative capacity (Huynh et al., 2007; Binker et al., 2007).

1.2.4 The Phagolyosome

Through the concerted efforts of the early and late phagosomal effectors, the maturating phagosome eventually fuses with lysosomes in the final step, to yield a phagolysosome (~pH 4.5; Figure 3A, iv; Gnosh et al., 1997). The mature phagosome is the ultimate degradative and noxious organelle; the toxicity of the macrophage lysosome can be attributed to the
presence of oxidants and cationic peptides as well as to the expression of over 60 acid hydrolases that function as proteases/peptidases, lipases, amylases and glucosidases. Proteases belonging to the cathepsin family are perhaps the most extensively studied lysosomal matrix proteins (Schröder et al., 2010). The family of proteases comprises of serine, cysteine and aspartate proteases and they tend to function optimally in the low pH conditions found within the phagolysosome (Turk et al., 2012).

The acidic environment of the phagolysosome acts to directly limit microbial growth and contributes to target degradation. This acidification is the result of a reduced passive proton permeability, which allows for the retention of protons pumped into the lumen by V-ATPases (Lukacs et al., 1991), producing an optimal degradative environment.

1.3 The physical property of target morphology affects phagocytosis

Phagocytic targets such as microbes and senescent cells exhibit significant variance in their size and morphology and they often have non-uniform surfaces. In nature, phagocytes encounter targets of disparate morphology, including pathogens ranging from protozoa, filamentous moulds/yeasts and bacteria. However, most studies focused on examining the effect of particle morphology on phagocytosis employ spherical polymers as targets. The paucity of information on this subject has prompted recent studies that demonstrate that morphology can affect phagocytosis.

1.3.1 Pathogen filamentation and evasion of phagocytosis

Microorganisms have large arsenals of tools that they utilize to withstand harsh environmental pressures. Many pathogens undergo filamentation as a way of evading capture by immune cells. Recent work following the medically relevant fungus, *Candida albicans*, suggests that this opportunistic pathogen is capable of evading capture by macrophages when in filamentous form. *Candida albicans* has been observed in both yeast and filamentous forms extracellularly and within macrophages (Heinsbroek et al., 2005). The wall of *C. albicans* is rich in chitin, mannoproteins and linear/branched β-glucans that are recognized
by an array of lectins expressed by cells of the innate immune system. The phagocytic receptor, Dectin-1, a C-type lectin-like receptor, is thought to mediate recognition of β-glucans and phagocytic uptake of *C. albicans*. Gantner and colleagues have shown that exposure of β-glucans by yeasts, but not filaments, determines the Dectin-1-dependant uptake of *C. albicans* by macrophages (2005). Phagocytosis of yeasts was following by phosphorylation of specific cytosolic proteins and an initiation of a respiratory burst, which was contrary to what was observed for filaments which failed to be taken up by Dectin-1 expressing macrophages. This represents a novel immune evasion mechanism by pathogenic fungi that result from morphology switching.

A similar phenomenon has been described in bacterial biology; filamentation has been implicated in bacterial survival during exposure to environmental stressors, which include host effectors, exposure to sub-lethal doses of antibiotics and predation by protists (Justice et al., 2008). Pathogenic bacterial filaments, such as *Uropathogenic Escherichia coli* (UPEC) have been observed to subvert innate immunity in vivo. UPEC is the primary casual factor in urinary-tract infections in the mammalian bladder. This pathogen invades the epithelial cells of the bladder and undergoes a developmental programme in which cell division is inhibited, resulting in the formation of filamentous bacteria that are up to 70 µm in length (Justice et al., 2004). When the bacteria emerge from epithelial cells of the bladder they undergo attack by the host neutrophils; these cells phagocytose the bacillary forms of UPEC. The result is an enrichment of the UPEC filaments on the surface, which have been shown to evade neutrophil phagocytosis (Justice et al., 2004).

*Candida albicans* and UPEC are not the only organisms that adapt filamentous forms to evade phagocytosis; *Proteus mirabilis* can also switch to a filamentous phenotype that has been associated with increased virulence (Belas and Suivasuthi, 2005; Belas et al., 2004). Additionally, filamentous forms of *Haemophilus influenzae* that were recently isolated from biofilms, are thought to adapt this morphology to evade capture by neutrophils (Leroy et al., 2007).
1.3.2 Target morphology affects phagocytic uptake

Therapeutic drug-delivery studies focused on engineering an optimal particle shape for successful drug-delivery have provided us with useful insight into how morphology affects phagocytosis. Alveolar macrophages presented with non-spherical polystyrene particles of various shapes and sizes suggest that the orientation and morphology of the target dictates whether or not the particle will be engulfed (Champion and Mitragotri, 2006). When phagocytic cells are challenged with targets of different shapes, the local particle shape at the point of contact dictates whether macrophages initiate phagocytosis or simply spread on the particle. The local shape determines the complexity of the actin structure that is required to initiate phagocytosis; failure to create this structure inhibits true phagocytosis (Champion and Mitragotri, 2006). The resultant phenomenon is akin to that of “frustrated phagocytosis”, a term coined to describe a macrophage phagocytosing a ligand-coated flat surface or a target much larger than the cell.

Champion and colleagues used engineered elongated polymer particles and compared the phagocytosis of these particles to spherical targets (Champion and Mitragotri, 2008). Negligible phagocytosis was observed for these polymer worms compared to conventional spherical targets of equal volume. Macrophages were able to bind these targets along their long axis or their poles. However, when the particle was bound along the long axis, the actin-driven membrane extension was highly inefficient (Champion and Mitragotri, 2008). Therefore, elongated particles represent an ideal morphology that can be designed to inhibit phagocytosis of drug delivery particles.

In agreement with this, the uptake of filamentous *Escherichia coli* was observed to proceed to completion only when the bacteria were captured by the terminal poles (Möller et al., 2012). If the macrophage were to encounter the long axis of the filament, the cell would then reorient the bacteria for successful uptake through the poles (Möller et al., 2012).

Recent work from our lab following the uptake of filamentous *Legionella Pneumophila* has shown that macrophages ingest filamentous bacteria through their longitudinal axis (Figure
This engulfment occurs rather gradually and is characterized by a long-lasting phagocytic cup stage. The phagocytic cup is composed of two distinct domains— the actin-rich domain proximal to the plasma membrane and to the bacteria portion that remains engulfed, and the internal phagocytic cup, which is embedded within the cytoplasm despite its continuity with the plasma membrane. Interestingly, the phagocytic cups containing filamentous bacteria undergo maturation events typical of an enclosed phagosome (Figure 3B, ii; Prashar et al., 2013). The filamentous bacteria containing phagocytic cups sequentially fuse with early and late endosomes as well as lysosomes, but the PCs fail to acidify and acquire hydrolytic capacity, prior to their sealing (Figure 3B, iii, iv). Once they seal into phagosomes, the hydrolytic environment allows for the complete degradation of the target (Figure 3B, v). In this way, filamentous morphology influences key aspects in phagocytic cup remodeling and phagosomal maturation.

1.4 Objectives

The canonical model of phagocytosis has been established on the basis of spherical target uptake by phagocytes. In this paradigm of phagocytosis, target uptake occurs rapidly and is followed by maturation which is characterized by a distinct early phagosomal and late phagosomal stage that is marked by the presence of early endosomal and late endosomal/lysosomal proteins respectively (Vieira et al., 2003). In comparison, filamentous target (FT) phagocytosis deviates from this established model. Despite similarities in PI(4,5)P₂ and F-actin remodelling at phagocytic cups (PCs), phagocytosis of FTs differs significantly with respect to the duration required for target uptake. Additionally, prior to phagosomal scission, PCs begin to undergo maturation events typical of a sealed phagosome. As FT-containing PCs remain continuous with the plasma membrane, lysosomal content leaks out into the extracellular milieu, resulting in a neutral compartment. Maturation occurs despite a lack of phagosomal acidification, which was originally thought to be a prerequisite for these events.

As a result of the aforementioned deviations, the main focus of our work will be to explore the phagocytic maturation of filamentous bacterial targets in murine macrophages. We hope
to characterize the remodelling and maturation process at filamentous target-containing phagocytic cups, particularly the molecular mechanisms that contribute to this process. During the course of our study, we hope to elucidate the mechanism behind the transition between the early and late stages of maturation and identify key players and events required for successful phagolysosomal formation.
Chapter 2
Materials and Methods

2.1 Reagents and antibodies
Dulbecco’s modified Eagle’s medium (DMEM) and Fetal bovine serum (FBS) were purchased from Wisent Inc. (St-Bruno, Quebec). Alexa Fluor-conjugated secondary antibodies, phalloidin, LysoSensor green and tetramethylrhodamine dextrans and pHrodo red, succinimidyl ester were from Life Technologies Inc. (Burlington, Ontario). Skim milk was purchased from BioShop Canada Inc. (Burlington, Ontario) and Paraformaldehyde (PFA) was obtained from Canemco & Marivac (Lakefield, Quebec). Rat polyclonal (1D4B) anti-LAMP-1 antibodies were from Developmental Studies Hybridoma Bank (Iowa City, Iowa) while goat polyclonal anti-EEA1 (N-19) antibody was from Santa Cruz Biotechnology, Inc. (Dallas, Texas). Rabbit polyclonal anti-Legionella pneumophila (Lp) 1 was provided by C. Guyard (Ontario Agency for Health Protection and Promotion, Toronto, Ontario).

2.2 DNA constructs and preparation
2FYVE-GFP encodes two tandem copies of the FYVE domain of EEA1 fused to GFP (Gillooly et al., 2000) and PX-GFP encodes the PX domain of p40Phox fused to GFP (Kanai et al., 2001). Rab7-GFP encodes WT Rab7 cloned into pEGFP-C1 vector (Bucci et al., 2000). pEGFP-RILP-c33 encodes the C-terminal half of RILP (RILP-c33) cloned into pEGFPC1 vector (Colucci et al., 2005) while the generation of the plasmid used for the expression of wild type GFP-conjugated Rab5 is described in detail elsewhere (Roberts et al., 1999). Plasmid DNA constructs were propagated by transformation into MAX Efficiency® DH5α Competent Cells (Life Technologies Inc.) and purified using the PureLink® HiPure Plasmid Maxiprep Kit (Life Technologies Inc.).

2.3 Cell culture and transfection
The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA). All RAW cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS at 37°C and 5% CO2. RAW 264.7 cells were transfected
using FuGENE HD (Promega Corp.) according to manufacturer’s instructions. Briefly, for each glass coverslip, 1 µg of DNA was diluted in 50 µl serum-free DMEM to which 3.5 µl of FuGENE HD was added and incubated for 23 min at room temperature (RT). The suspension was added to RAW cells plated (3x10^5 cells/coverslip) the day before transfection and used at least 25 hours post addition to allow for plasmid expression. Cells overly transfected were not included; only cells of an average transfection level were imaged and quantified.

2.4 Phagocytosis assay
FTs were opsonized with 0.1 mg/ml rabbit polyclonal anti-Lp1 antibody for 1 hour at RT. RAW 264.7 cells growing on glass coverslips (4x10^5 cells/coverslip) were pre-cooled at 15°C for 5 min to slow cellular processes. To induce phagocytosis, FTs were added to cells (1 cell: 40 FTs). Attachment was synchronized by spinning FTs onto cells at 300xg for 5 min at 15°C. Unattached filaments were washed away using PBS. Cells were then moved to 37°C to allow phagocytosis to proceed to indicated time periods.

In brief, filamentous bacterial targets were prepared as follows. Red fluorescent protein (RFP)-expressing *Legionella pneumophila* strain Lp02 were grown in buffered yeast extract broth under low agitation to reach post-exponential growth phase bacteria (Prashar et al., 2013; Prashar et al., 2012). Next, bacteria (FTs) were fixed with 4% PFA. Prior to opsonization for phagocytosis, FTs were washed three times with PBS. Bacteria longer than 5 µm were considered as filamentous targets. Only filamentous targets captured by the poles were selected for analysis. Those FTs that landed on top of the cell were not imaged or quantified.

2.5 Pharmacological Inhibition
For assays using pharmacological inhibitors, phagocytosis assays were performed using filamentous FTs as described above. Prior to addition of opsonized RFP-Lp02, cells were washed three times and incubated in pre-warmed media containing the following inhibitors: 100 µM Ly294002 (Sigma-Aldrich), 1 µM Zstk474 (Selleckchem), 10 nM apilimod (pre-incubated with cells for 1 hour; Toronto Research Chemicals, Inc.), and 1µM Vps34-IN1 (Bago et al., 2014). Five minutes after synchronized attachment, cells were washed to
remove unbound FTs and pre-warmed media containing inhibitor was re-added. Internalization was allowed to proceed for an additional 25 minutes at 37°C following which the cells were fixed using 4% PFA for 20 minutes.

2.6 Preparation of pHrodo-conjugated FTs
PFA-killed filamentous Lp (FTs) were washed three times with 250 mM glycine in PBS, pH 7.2 (quench buffer) and then conjugated to pHrodo™ Red, succinimidyl ester molecular probe (Fisher Scientific) as per manufacture’s protocol. In brief, 10⁹ filaments were prepared at 20 mg/ml in sodium bicarbonate, and the pHrodo dye was prepared at 10 mM in DMSO. The dye was then diluted in the bacterial suspension to a final dye concentration of 0.5 mM. Amine labeling occurred over a 45-minute period at room temperature, following which unbound pHrodo was removed by three washes in 1xPBS in between centrifugations. FT-pHrodo was finally resuspended in 1xPBS before use in a phagocytic assay.

2.7 Fluorescence labeling
To label F-actin, cells were permeabilized after PFA-fixation with 0.1% Triton X-100 (BioShop) in PBS for 20 min, washed three times with PBS, and stained with Alexa Fluor-conjugated phalloidin/PBS (1:500) for 1 hour at RT. For immunofluorescence, cells were permeabilized as above and blocked with 5% skim milk/PBS for 1 hour before incubating with primary antibody for 1 hour at RT. Early endosomes were labeled with anti-EEA1 (1:30), while late endosomes and lysosomes were labeled with anti-LAMP-1 antibody (1:25) in solutions containing 5% skim milk/PBS. Finally, cells were incubated with the corresponding secondary antibody (1:500) in 5% skim milk for 1 hour at RT. The coverslips were washed three times with PBS after each antibody incubation and mounted onto glass slides using Fluorescence Mounting Medium (Dako, Inc.).

To label acidic organelles, 30-60 minutes after the onset of phagocytosis, RAW cells were incubated with 1 µM of LysoSensor green for 5 min in PBS supplemented with 1 mM MgCl₂ and 1 mM CaCl₂, washed, and imaged on a precooled microscope stage in FluoroBrite DMEM supplemented with 10% heat-inactivated FBS.
2.8 Live-cell Microscopy
For live-cell time-lapse imaging, phagocytosis assays were performed on stage using RAW cells transiently expressing 2FYVE-GFP. Coverslips were first mounted in a coverslip chamber and maintained at 37°C/5% CO₂ with a stage incubator (Live Cell Instrument). Opsonized FTs were added to the coverslips for 15 minutes. Following this incubation period, cells were washed with PBS and re-incubated in FlouroBrite DMEM.

Confocal images for fixed and live fluorescently labeled cell samples were acquired using a spinning disc confocal microscope (Quorum Technologies) equipped with an inverted fluorescence microscope (DMI6000B; Leica Microsystems), consisting of EM-CCD camera (Hamamatsu Photonics) and ORCA-R² cameras and spinning disc confocal scan head, a Piezo Focus Drive and an ASI motorized XY stage (Quorum Technologies). Unless indicated otherwise, images were acquired using a 63x oil immersion objective, NA 1.4 on MetaMorph acquisition software (Molecular Devices, LLC.). Fixed cells were imaged using the ORCA-R² camera; live cells were imaged using the EM-CCD cameras. Image processing, deconvolution (90% confidence interval) and analysis were performed using Volocity software (PerkinElmer Inc.). Adobe Photoshop and Illustrator (Adobe Systems, Inc.) were used to prepare all digital images.

2.9 Statistical Analysis
Data shown are mean ± SEM from three independent experiments unless otherwise stated. Fluorescence microscopy was used to perform all quantifications and statistical analysis was performed using two-tailed, Student’s $t$ test using GraphPad Prism software (GraphPad Software, Inc.). 95% confidence interval was used to determine statistical significance and $P \leq 0.05$ was considered to be statistically significant. Data was also processed and analyzed using SigmaPlot software (Systat Software Inc.). The lengths of FTs were measured using Volocity 6.1 software.
Chapter 3
Results

3.1 Phagocytic cups containing filamentous targets undergo phagosomal maturation prior to sealing

Shortly after the phagocytic cup (PC) seals around a spherical target, the nascent phagosome matures by sequentially fusing with endocytic compartments, allowing for the transfer of endosomal and lysosomal proteins to the phagosome. However, much less is known about the impact of filamentous morphology on the remodelling and morphogenesis of the maturing phagosome. In accordance with what had been observed for filamentous Escherichia coli, IgG-opsonized filamentous Legionella pneumophila (Lp) captured by the poles can be internalized by RAW264.7 macrophages (Möller et al., 2012; Prashar et al., 2013). Prashar et al. reported the gradual engulfment of filamentous bacterial targets (FTs) by pseudopodia that extended along the long axis of the target (2013). Given that the filamentous bacteria-containing phagocytic cup undergoes maturation events typical of a sealed phagosome, we sought to characterize the maturation process in greater detail by analysing the interaction/fusion of the filamentous target (FT)-containing phagocytic cup with endosomes and lysosomes.

3.1.1 Early Endosomes are recruited to phagocytic cups containing filamentous targets

According to the canonical model of phagocytosis developed with spherical targets, phagosomes begin to mature by transiently fusing with early endosomes. Immediately after sealing and separation from the plasma membrane, nascent phagosomes recruit Rab5, which is activated on the phagosomal membrane. Rab5-GTP is required for Class 3 PI3-Kinase Vps34 recruitment, which catalyzes PI(3)P production from PI. Rab5 and PI(3)P then recruit EEA1; all three work in concert to promote homotypic fusion of early endosomes to phagosomes, which is critical for the subsequent phagosomal maturation. We followed the distribution of Rab5, EEA1 and PI(3)P during the uptake of PFA-killed filamentous Lp
Figure 5 Early endosomal markers are recruited to FT-containing PCs. (A) EEA1 and F-actin distribution in cells after 5 minutes of phagocytosis. Right: magnified single plane from framed regions, EEA1 recruitment is seen in white, filamentous targets are in blue (B) EEA1 and F-actin distribution in cells after 30 minutes of phagocytosis. Right: magnified merged planes from two framed regions; EEA1 is in white. (C) Rab5-GFP recruitment around internal FT segments. RAW macrophages expressing Rab5-GFP were challenged with filamentous targets; cells were fixed 5 minutes post target attachment, permeabilized and F-actin was stained with phalloidin (red). Right: magnified merge of framed regions, Rab5 recruitment (white). (D) Rab5 recruitment around internal FT segments in Rab5-GFP transfected cells 30 minutes after phagocytosis. Right: magnified merge of two framed region; Rab5 recruitment is in white. (E) Recruitment of EEA1 and Rab5 around internal FT sections over time. Cells were either expressing Rab5-GFP or were immunolabeled for EEA1 and partially internalized FTs were scored for the recruitment of these markers. Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R2 camera. Data shown are percentages from a representative experiment out of three repeats. For the data shown, n = 50 at each time point. Scale bars = 5 µm.
RAW cells were either transfected with Rab5-GFP or immunolabeled for EEA1 to follow Rab5 and EEA1 localization respectively. During filamentous target uptake, the base of the phagocytic cup loses F-actin while the sides of the elongated PCs retain F-actin in an ‘actin jacket’ structure that is proximal to the plasma membrane (Prashar et al., 2013). As early as 5 minutes post bacterial attachment to RAW macrophages, the base of the phagocytic cup lost F-actin and the early endosomal markers Rab5 and EEA1 were recruited (Figure 5A, C).

To investigate if FT-containing phagocytic cups recruited PI(3)P, we followed the distribution of this early endosomal lipid regulator using fluorescent chimeric probes. RAW cells were transfected with a construct consisting of GFP fused to two tandem copies of the FYVE domain of EEA1; the usage of two FYVE domains increases the avidity of PI(3)P binding. In agreement with what was observed for Rab5 and EEA1, 2-FYVE-GFP labeled the phagocytic cup as early as 5 minutes post-FT attachment, thereby indicating that PI(3)P was present (Figure 6A). This was confirmed by expressing a chimeric GFP fused to the PX domain of p40phox, a subunit of the NADPH oxidase in RAWs. As seen with 2-FYVE-GFP, P40Phox-GFP localized rapidly to the base of the phagocytic cup where actin accumulation was lost, suggesting that actin dissociation primed the tips of the PC for maturation (Figure 6C). The recruitment of early endosomal markers to phagocytic cup suggested that the PCs were undergoing a similar maturation process to what had been characterized for spherical-target containing phagosomes.

### 3.1.2 PI(3)P persists at filamentous target-containing phagocytic cups

At high levels of Rab5 activation (Rab5-GTP), the fusion of endosomes to phagosomes is refractory to PI3-Kinase inhibition, presumably because Rab5 can recruit sufficient EEA1 to the phagosome independently of PI(3)P (Jones et al., 1998; Li et al., 1995). Therefore, cessation of Rab5 recruitment to the FT-containing PC would most likely result in the timely loss of EEA1 at PCs, as EEA1 docking is dependant to a greater extent on Rab5. In line with this, Rab5 and EEA1 were both only detectable at the phagocytic cup until 15 minutes following attachment of FTs; after this time, the two markers disassociated from PCs and
Figure 6 PI(3)P persists at FT-containing PCs. (A) 2-FYVE-GFP recruitment around internal FT segments. Filamentous targets were added to cells expressing 2-FYVE-GFP, cells were fixed after 5 minutes of phagocytosis, permeabilized and F-actin was stained with phallodin (red). Right: magnified merge of the framed regions. 2-FYVE recruitment is in white. (B) 2-FYVE recruitment around internal FT segments in 2-FYVE –GFP transfected cells 30 minutes after phagocytosis. Right: magnified merge of two framed regions. 2-FYVE recruitment is in white. (C) P40\textsubscript{Phox}-GFP recruitment around internal FT segments. Filamentous targets were added to cells expressing P40\textsubscript{Phox}–GFP, cells were fixed after 5 minutes of phagocytosis, permeabilized and F-actin was stained with phallodin (red). Right: magnified merge of framed regions. P40\textsubscript{Phox} recruitment is in white. (D) P40\textsubscript{Phox} recruitment around internal FT segments in P40\textsubscript{Phox}-GFP transfected cells 30 minutes after phagocytosis. Right: magnified merge of two framed regions. P40\textsubscript{Phox} recruitment is in white. (E) Recruitment of 2-FYVE and P40\textsubscript{Phox} around internal FT sections over time. Cells expressing either 2-FYVE-GFP or P40\textsubscript{Phox}-GFP were scored for the recruitment of these markers at FT-containing PCs. The filamentous targets were partially internalized. Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R\textsuperscript{2} camera. Data shown are means ± SEMs from three independent experiments (N=3). For the data shown, n = 30 at each time point. Scale bars = 5 µm.
were no longer detected (Figure 5B, D, E). We then probed for PI(3)P over a time course of 0-45 minutes using 2FYVE-GFP and P40Phox-GFP constructs. PI(3)P was maintained at the phagocytic cup at 15 minutes following attachment, but surprisingly, unlike EEA1 and Rab5, it continued to localize to this site even as late as 45 minutes post-FT attachment (Figure 6B, D). This was different from what was observed in the canonical pathway where PI(3)P was documented to persist on the phagosome for only 7-10 minutes (Vieira et al, 2001). Thus, the behaviour of PI(3)P with respect to its persistence at PCs is different from what was observed for phagosomes containing spherical targets (Figure 6E).

Next, we utilized time-lapse imaging of RAW macrophages expressing 2FYVE-GFP to follow the distribution of PI(3)P over time. As was observed for spherical target uptake, 2FYVE labelled the base of the PC for partially internalized FTs 1 minute after the onset of phagocytosis (Figure 7; Vieira et al., 2001). As the FT was internalized, 2FYVE consistently localized to the proximal portion of the PC, close to the plasma membrane (PM). At this time, 2FYVE labeled the entire length of the FT-containing PC (Figure 7, 3:00 min). Remarkably, once a threshold internalized FT length was reached, we observed that 2FYVE was no longer detectable at the most distal portion of the PC, in the interior of the cell (Figure 7: 5:01 min). Despite this loss, PI(3)P was continuously detected in the phagocytic cup sections near the PM, until phagosomal closure (Figure 7: 14:46 min). Once FT-containing PCs sealed into phagosomes, PI(3)P detection was not observed, regardless of the FT length. This anomalous result led us to investigate how PI(3)P dynamics at the phagocytic cup relates to the length of the filament.

To this end, we evaluated the kinetics of PI(3)P recruitment to PCs as a function of time and length in fixed 2FYVE and p40Phox-positive cells stained for F-actin. Given that PI(3)P only localizes to the actin-free base of PCs, we scored the length of internalized filament as the segment of the FT under the actin jacket (Figure 8A). The trends in Figure 8 show that at the early stage of uptake (5 and 15 minutes), the entire length of PCs containing filaments less than 20 µm are labeled by 2-FYVE-GFP (Figure 8B) or P40Phox-GFP (Figure 8C), indicating PI(3)P recruitment along the structure. At this time, there is a perfect lineal correlation between PC length and the length of the PC positive for 2-FYVE-GFP and
Figure 7 PI(3)P dynamics at phagocytic cups of filamentous targets. RAW macrophages expressing 2-FYVE-GFP (white) were challenged with filamentous targets (red) and imaged live. Frames from a time-lapse sequence depict the recruitment of 2-FYVE-GFP to advancing segments of FTs from the onset of FT phagocytosis. 2-FYVE is observed to fall off of internal FT-containing PCs, in the distal regions and when the phagosome seals. Insets show merged, higher magnification of framed regions. 2-FYVE recruitment is in white. T0=1 minute and 30 seconds. Confocal images were acquired using a spinning disc confocal microscope with dual acquisition EM-CCD cameras. Scale bars = 5 µm.
Figure 8 PI(3)P recruitment to PCs is dependent on time and length of FTs. (A) Schematic of how FT length internalized and FT length positive for PI(3)P was scored. As the accumulation of F-actin (actin jacket) precludes the recruitment of endosomal markers to this region, FT length internalized and positive for PI(3)P were measured from under the actin jacket. (B) FT length positive for 2-FYVE was plotted against FT length internalized from T=5 min to T=45 min. Correlation coefficient ($r^2$) was calculated. (C) FT length positive for P40Phox-GFP was plotted against FT length internalized from T=5 min to T=45 min. Correlation coefficient ($r^2$) was calculated.
P40\textsubscript{Phox}-GFP. Strikingly, once this 20 µm threshold for FT length internalized was surpassed, PI(3)P was no longer detectable at the most distal portion of the PC and the positive lineal correlation between FT length internalized and length positive for PI(3)P is lost.

Altogether, our results indicate that as filamentous target-containing PCs mature, the recruitment of PI(3)P at these structures is compartmentalized in a length-dependant manner.

### 3.1.3 Surface Potential at FT-containing PCs

Previous studies following phosphoinositide (PI) dynamics during the phagocytosis of spherical targets have shown that the surface potential of the plasma membrane is altered during target uptake. The surface charge of the plasma membrane (PM) results from the preferential accumulation of negatively charged membrane lipids, mainly at the inner leaflet of the PM, which creates an electric field that strongly attracts cationic molecules (Yeung et al., 2006). Yeung and colleagues utilized engineered polycationic fluorescent polypeptides such as R-Pre-GFP that selectively target to negatively charged regions of the PM to study surface potential. They showed that the surface potential of the inner leaflet of the PM at the phagocytic cup, decreases locally, during the uptake of spherical targets (2006). This decrease in surface charge was attributed to the hydrolysis of phosphoinositides, particularly PI(4,5)P\textsubscript{2} and displacement of phosphatidylserine (PS).

We investigated the surface potential in filamentous target-containing phagocytic cups utilizing R-pre-GFP. Strikingly, R-Pre was retained at PCs-containing partially internalized filamentous targets (**Figure 9A**) but was not detectable in sealed phagosomes (**Figure 9B**). Taken together, this suggests that negatively charged molecules accumulate at PCs but not on phagosomes. Prashar and colleagues recently reported on the hydrolysis of PI(4,5)P\textsubscript{2} from the base of FT-containing PCs (2013). Therefore, we speculate that an accumulation of the anionic lipid PI(3)P may account for the localization of R-pre at PCs and its disappearance from phagosomes may be attributed to a drop in negative charge on the surface of the phagosome.
Figure 9 Alterations in surface potential during filamentous target uptake. (A) R-Pre-GFP was recruited to internal FT segments at PCs. RAW macrophages were transfected with the polycationic polypeptide probe, R-pre-GFP (white), and fixed after 15 minutes of phagocytosis. External filamentous targets are shown in purple. Right: magnified single plane, arrowheads indicate R-pre recruitment (white), filamentous target (red). (B) R-pre recruitment around internal FT segments in a phagosome in R-Pre-GFP (white) transfected cells 15 minutes after phagocytosis. Right: magnified merge of framed regions. R-pre recruitment is in white, filamentous target is in red. Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R² camera. Scale bars = 5 µm.
3.2. Late stages of phagosomal maturation in the FT-containing PC

Following the early phagosomal stage, the maturing phagosome transiently interacts with late endosomes and lysosomes as it continues to mature. Late endosomal and lysosomal proteins such as Rab7 and LAMP1, become incorporated on phagosomes of spherical targets. PCs containing FTs also recruit late endosomal and lysosomal markers, including LAMP-1 along the actin-free base of cups (Prashar et al., 2013).

3.2.1 Late Endosomes and lysosomes fuse with phagocytic cups containing filamentous bacterial targets

To investigate the kinetics of late endosomal and lysosomal recruitment to PCs containing FTs, we followed the distribution of Rab7 and LAMP1 during the uptake of FTs. RAW cells were transfected with Rab7-GFP or immunolabeled for LAMP1 and then, stained for F-actin. In agreement with what had been previously observed, Rab7 and LAMP1 localized to FT containing PCs as early as 20 minutes and were observed to persist at this site 30-45 minutes post attachment (Figure 10 A, B).

The localization of active Rab7 (Rab7-GTP) on the phagosome is key in modulating the transition of phagosomes from the early to the late stage of maturation. Rab7-GTP recruits its effector proteins, Rab-Interacting Lysosomal Protein (RILP) and Oxysterol-binding protein-related protein 1L (ORP1L) to phagosomes, where they act as adaptors for dynein, a microtubule-associated motor protein. Dynein travels towards the minus end of microtubules, propelling the phagosomes centripetally towards the Microtubule Organizing Center (MTOC), where an abundance of late endosomes and lysosomes accumulate. In this way, by bringing the partners of the reaction together, Rab7 and its effectors favour their fusion. To determine whether Rab7 localized at the phagocytic cup is in its active, GTP-bound state, we utilized a truncated form of the Rab7 effector-protein, RILP, (GFP fused to RILP-c33-mutant: lacks N-terminal) which localizes and binds active Rab7. By 30 minutes following FT attachment, RILP-c33 was recruited to the phagocytic cup and bound active Rab7 (Figure 10C). Thus, our study following Rab7, LAMP1 and RILP-c33, provides evidence
Figure 10 Late endosomes and lysosomes fuse with FT-containing phagocytic cups.

(A) LAMP1 (red) localization around internal FT segments. Cells were challenged with filamentous targets and after 30 minutes of phagocytosis, they were fixed, permeabilized and stained for F-actin (phallodin: green) and LAMP1 (red). Down: magnified merge of two framed regions. LAMP1 recruitment is in white.

(B) Rab7 (green) recruitment around internal FT segments in Rab7-GFP transfected cells 30 minutes after phagocytosis. Phallodin: red. Down: magnified merge of two framed regions. Rab7 recruitment is in white.

(C) RILP-c33 localization (green) around internal FT segments. Filamentous targets were added to cells expressing RILP-c33-GFP, cells were fixed after 30 minutes of phagocytosis, permeabilized and F-actin was stained with phallodin (red). Right: magnified merge of two framed regions. RILP-c33 recruitment is in white.

Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R2 camera. Scale bars = 5 µm.
that late endosomal and lyosomal proteins are acquired and are in an active configuration on PCs.

In agreement with this finding, previous work utilizing dextran dyes and DQ-ovalbumin (a self-quenched protease substrate) to label the lumen of late endosomes and lysosomes in RAW cells showed that these organelles fused and discharged their contents in the PCs (Prashar et al., 2013). Taken together, these results suggest that in the presence of PI(3)P, FT containing PCs transiently fuse with late endosomes and lysosomes, prior to sealing.

### 3.2.2 Early endosomal PI(3)P persists and co-exists with lysosomal membrane protein, LAMP1 on FT containing PCs

Spherical target phagocytosis is characterized by an early and late stage of maturation. During the early stage, PI(3)P rapidly accumulates on the phagosome following which EEA1 is recruited and localizes to the phagosome. This period is thought to last for 20 minutes once the phagosome seals as EEA1 recruitment begins to secede during this time and PI(3)P is no longer detected after 10 minutes (Vieira et al, 2001). The late stage of maturation for spherical targets is delineated by the acquisition of LAMP1 on the phagosomal membrane.

In the case of filamentous targets, even prior to phagosomal sealing, endosomal and lysosomal markers localize to FT containing PCs (Prashar et al, 2013). To evaluate the role of these markers in defining an early and late phagosomal stage, we expressed 2FYVE-GFP in RAWs, performed a phagocytosis assay and probed for LAMP1 using antibodies. In agreement with what had been observed for spherical targets, PI(3)P accumulated at the PC as early as 5 minutes post attachment; LAMP1 was not detected at this time (Figure 11A). We then sought to assess the localization of PI(3)P and LAMP1 at the later stage of maturation, 45 minutes post-attachment. Remarkably, PI(3)P (detected via 2FYVE) and LAMP1 were both observed to co-exist at PCs (Figure 11B). This lengthy overlap between the early and late stage of phagosomal maturation has not been reported previously for phagosomes containing spherical targets.
Figure 11 PI(3)P coexists with lysosomal membrane protein, LAMP1 at FT-containing PCs. RAW macrophages expressing 2-FYVE-GFP were challenged with filamentous targets, fixed, permeabilized and immunostained for LAMP1. (A) 2-FYVE (green) and LAMP1 (red) localization around internal FT segments 5 minutes after phagocytosis. Right: magnified merge of framed regions. Top: 2-FYVE recruitment (white), Bottom: LAMP1 recruitment (white). (B) 2-FYVE (green) and LAMP1 (red) localization around internal FT segments 45 minutes after phagocytosis. Right: magnified merge of framed region. Top: 2-FYVE recruitment (white), Bottom: LAMP1 recruitment (white). Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R2 camera. Scale bars = 5 μm.
3.2.3 Rab7 localization to PCs overlaps with that of Rab5

In the canonical model of phagocytosis, Rab GTPases are thought to orchestrate the sequence of fusion events during phagosomal maturation, analogous to their role in the endocytic pathway (Bucci et al., 2000). Accordingly, Rab5 localizes transiently to early phagosomes while Rab7 appears downstream of this, during the later stages of maturation. Despite a growing knowledge base on the activity of Rab GTPases at the maturing phagosome, not much is known about how these proteins interact with PI3Kinase products. Nevertheless, it has been reported that PI3Kinase products are not essential for the recruitment and activation of Rab GTPases during phagosomal maturation but in the case of Rab5, removal of this GTPase is dependant on PI3K products (Vieira et al., 2003). As previous studies had focused on alterations in GTPase recruitment in the absence of PI3K products, we first sought to investigate whether the persistence of PI(3)P at PCs would affect Rab5 recruitment and activation. As previously shown, Rab5 was recruited to FT-containing PCs in a timely manner; comparable to what has been observed for spherical targets. Moreover, the presence of the PI3Kinase product, PI(3)P, ensured the timely disassociation of Rab5 from PCs.

As Rab5 seemed to have no consequence despite the persistence of PI(3)P, we then investigated the kinetics of Rab7 recruitment and activation. Remarkably, Rab7 was observed to localize at FT-containing PCs as early as 5 minutes following bacterial attachment (Figure 12C). After recruitment, Rab7 was observed to persist at PCs well into the late stage of maturation as observed in Rab7-GFP transfected RAWs (Figure 12D, 10B). This was in contrast to what had been observed for LAMP1, which was only recruited to PCs following EEA1 disassociation, during the late stage of maturation (Figure 12A, B, 10A). To determine whether the Rab7 recruited was in its active form, we then analyzed the distribution of RILP-c33 at PCs over a time course of 0-45 minutes. Strikingly, RILP-c33 only localized significantly to PCs 30 minutes following bacterial attachment (Figure 12E, F, 10C). Therefore, although Rab7 is recruited to PCs on the onset of maturation, its activation is protracted (Figure 13).
Figure 12 Rab7 recruitment to FT-containing PCs overlaps with Rab5 recruitment.

(A) LAMP1 (red) localization around internal FT segments. Cells were challenged with filamentous targets and after 5 minutes of phagocytosis, they were fixed, permeabilized and stained for F-actin (phallodin: green) and LAMP1. Down: magnified merge of framed region. LAMP1 recruitment is in white. (B) LAMP1 (red) localization around internal FT segments 15 minutes after phagocytosis. Cells were stained for F-actin (phallodin: green) and LAMP1. Right: magnified merge of framed region. LAMP1 recruitment is in white. (C) Rab7 recruitment around internal FT segments in Rab7-GFP transfected cells 5 minutes after phagocytosis. Cells were stained for F-actin with phallodin (red). Down: magnified merge of framed regions. Rab7 recruitment is in white. (D) Rab7 recruitment around internal FT segments in Rab7-GFP transfected cells 15 minutes after phagocytosis. Down, Right: magnified merge of two framed regions. Rab7 recruitment is in white. (E) RILP-c33 localization (green) around internal FT segments. Filamentous targets were added to cells expressing RILP-c33-GFP, cells were fixed after 5 minutes of phagocytosis, permeabilized and F-actin was stained with phallodin (red). Right: magnified merge of framed regions. RILP-c33 recruitment is in white. (F) RILP-c33 (green) localization around internal FT segments 15 minutes after phagocytosis. Cells were stained for F-actin with phallodin (red). Right: magnified merge of framed region. RILP-c33 recruitment is in white. Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R2 camera. Scale bars = 5 µm.
Figure 13 Kinetics of late endosomal and lysosomal marker recruitment to FT-containing PCs. Recruitment of the lysosomal protein, LAMP1, and late endosomal proteins Rab7 and RILP-c33 around internal FT sections over time. Top: Cells were immunostained for LAMP1 and scored for LAMP1 recruitment to FT-containing PCs. Middle and Bottom: Cells expressing either Rab7-GFP or RILP-c33-GFP were scored for the recruitment of these markers at FT-containing PCs. In all scored events, the filamentous target was partially internalized by a RAW macrophage. Data shown are percentages from a representative experiment out of three repeats. For the data shown, n = 40 at each time point.
Altogether these results suggest that in the presence of excess PI(3)P, Rab5 recruitment and activation at PCs is comparable to that of maturing phagosomes in the canonical pathway. Remarkably, Rab7 recruitment coincides with Rab5 and EEA1 localization to PCs, during the early stage of maturation. Yet, despite the localization of Rab7 to PCs, the activation of Rab7 was delayed significantly.

3.3 Elucidating the mechanism of PI(3)P dynamics at filamentous target-containing PCs

The plasma membrane is composed in part by phosphoinositides, which are key regulators in phagocytic signalling and remodelling events (Swanson, 2008). Based on studies utilizing spherical targets, phospholipids PI(4,5)P2 and PI(3,4,5)P3 are thought to dominate the phagocytic cup stage, prior to phagosomal formation. In the case of filamentous targets, PI(4,5)P2 is hydrolyzed into DAG, at the base of the phagocytic cup (Prashar et al., 2013). Consequently, PIP2 signalling ceases at the FT-containing PC. It is unclear, however, whether, PI(3,4,5)P3 is produced at the PC and what the kinetics of its production are.

Immediately after sealing from the plasma membrane, a high level of PI(3)P is found on phagosomal membranes of spherical targets (Ellson et al., 2001). We have shown that in the case of filamentous targets, PI(3)P localizes to the phagocytic cup prior to phagosomal sealing, and persists at this structure for extended periods. This retention of PI(3)P may be the result of excessive synthesis or aberrant degradation of PI(3)P. Although PI(3)P is characteristically thought to be produced by Class 3 PI3Kinase, Vps34, recent work has suggested that PI(3)P production may also be driven in part by the action of Class 1 PI3Kinases in concert with PI 5- and PI 4-phosphatases in a Rab5-dependant manner (Figure 14A; Shin et al., 2005). Given the continuity of FT-containing PCs with the plasma membrane, we sought to investigate by what mechanism PI(3)P is produced and why it persists at FT-containing PCs. We also investigated the downstream conversion of PI(3)P to determine how this may account for its persistence at PCs.

Firstly, we carried out a series of pharmacological inhibition studies, to determine what role PI3Kinases play on PI(3)P recruitment and retention at the PC. To this end, we applied an
A

Three possible sources for PI(3)P:

i. Class 1 PI3Kinase, 5-, 4-Phosphatase
ii. Class 3 PI3Kinase, Vps34
iii. Lack of PI(3)P conversion

B

Vehicle

30 min

2-FYVE Actin Fts

2-FYVE

2-FYVE

C

Ly294002: Blocks Class I and III PI3Kinases (i, ii)

30 min

Recruitment of PI(3)P to PCs

D

ZSTK474: Blocks Class I PI3Kinase (i)

2-FYVE Actin Fts

2-FYVE

E

Vps34-IN1: Blocks Class III PI3Kinase, Vps34 (ii)

30 min

Recruitment of PI(3)P to PCs
Figure 14 PI(3)P synthesis at FT-containing PCs is driven by Class 3 PI3-Kinase, Vps34. (A) Schematic of possible mechanisms for PI(3)P production and retention at FT-containing PCs. (i) PI(3)P production may be mediated by Class 1 PI3-Kinases in concert with PI 5- and PI 4-phosphatases. (ii) PI(3)P may be produced by Class 3 PI3-Kinase from PIP. (iii) Inhibition of downstream conversion of PI(3)P to PI(3,5)P2 via PIKFyve or PIP via Myotubularins. (B) Vehicle Control: Cells expressing 2-FYVE-GFP (green) were treated with 1 µM DMSO and challenged with filamentous target for 30 minutes after phagocytosis. Actin (phallodin): red. Right: magnified merge of framed region. 2-FYVE recruitment is in white. (C) Blocking Classes 1, 2 and 3 PI3-Kinases ablates PI(3)P recruitment at PCs. Cells expressing 2-FYVE-GFP were treated with 100 µM Ly294002 and challenged with filamentous targets for 30 minutes after phagocytosis, in the presence of Ly. Cells were then fixed, permeabilized and stained for F-actin with phallodin (red). Right: magnified merge of framed region. 2-FYVE recruitment is in white. Cells were scored for the recruitment of PI(3)P (detected via 2-FYVE) at FT-containing PCs following Ly treatment. Vehicle: In place of Ly inhibitor, cells were treated with 100 µM DMSO and scored for PI(3)P recruitment at PCs. Data shown are means ± SEMs from three independent experiments. For the data shown, n = 35 for each experiment. P < 0.05. (D) Blocking Class 1 PI3-Kinase has little to no effect on PI(3)P recruitment. Cells expressing 2-FYVE-GFP were treated with 1 µM Zstk474 and challenged with filamentous targets for 30 minutes after phagocytosis, in the presence of Zstk474. F-actin (Phallodin): red. Below: magnified merge of framed region. 2-FYVE recruitment is in white. Cells were scored for the recruitment of PI(3)P at FT-containing PCs following Zstk474 treatment. Vehicle: In place of Zstk474 inhibitor, cells were treated with 1 µM DMSO and scored for PI(3)P recruitment at PCs. Data shown are means ± SEMs from three independent experiments. For the data shown, n = 35 for each experiment. P < 0.05. (E) Inhibition of Vps34 ablates PI(3)P recruitment at PCs. Cells expressing 2-FYVE-GFP were treated with 1 µM Vps34-IN1 and challenged with filamentous targets for 30 minutes after phagocytosis, in the presence of Vps34-IN1. F-actin (Phallodin): red. Right: magnified merge of framed region. 2-FYVE recruitment is in white. Cells were scored for the recruitment of PI(3)P at FT-containing PCs following Vps34-IN1 treatment. Vehicle: In place of Vps34-IN1 inhibitor, cells were treated with 1 µM DMSO and scored for PI(3)P recruitment at PCs. Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R2 camera. Data shown are means ± SEMs from three independent experiments. For the data shown, n = 35 for each experiment. P < 0.05. Scale bars = 5 µm.
inhibitor of Class 1, 2 and 3 PI3-Kinases, Ly294002 to RAW macrophages and observed the
effects of this inhibitor on the localization pattern of PI(3)P. Following treatment of cells
with Ly294002, we observed that 2-FYVE-GFP was no longer recruited to FT-containing
PCs (Figure 14C). From this, we were able to confirm that the production of PI(3)P at PCs
was the result of catalysis by either Class 1, 2 or 3 PI3-Kinases.

Since the PC remains continuous with the plasma membrane for much of its maturation, we
were initially unable to rule out the possibility that Class 1 PI3-Kinases may play a role in
PI(3)P production at the PC. Class 1 PI3-Kinase phosphorylates PI(4,5)P2 to PI(3,4,5)P3 in
the phagocytic cup. PTEN and SHIP are resident, membrane phosphatases that are known to
hydrolyze PI(3,4,5)P3 into PI(4,5)P2 and PI(3,4)P2 respectively (Cantley and Neel, 1999;
Vanhaesebroeck et al., 2001; Mitchell et al., 2002). Since the PC is an extension of the
plasma membrane, these enzymes may also play a role in producing a certain amount of
PI(3)P. In particular, 5’-phosphatase SHIP may hydrolyze PI(3,4,5)P3 to PI(3,4)P2, which
may then be further processed by a 4’-phosphatase (4-Pase) such as INPP4B to produce
PI(3)P (Shin et al., 2005).

In support of this, studies have identified a dual role for Rab5 in PI(3)P production, firstly via
Vps34 and secondly through Class 1 PI3Kinase, PI3Kβ (Shin et al., 2005). In the latter
mechanism, Rab5 regulates the production of PI(3)P through a hierarchical enzymatic
cascade of Class 1 PI3Kβ1, PI 5-, and PI 4-phosphatases (enzymes Rab5 is known to interact
with directly; Shin et al., 2005). To investigate whether the action of Class 1 PI3-Kinase β,
SHIP 1/2 and INPP4B PtdIns Phosphatase (a 4-Pase) may sequentially contribute to the
production of PI(3)P at the PC, we utilized a pharmacological inhibitor specific for Class 1
PI3-Kinases, ZSTK474. Remarkably, following treatment with ZSTK474, 2-FYVE-GFP was
observed to continue to localize to the PC, indicative of the presence of PI(3)P (Figure 14D).
Even prolonging the treatment with Class 1 inhibitor had no effect on the localization of
2FYVE to FT-containing PCs. Altogether, these data indicate that Class 1 PI3-Kinases are
not involved in the production of PI(3)P at the PC.
In the canonical model for PIP dynamics during phagosomal maturation, PI(3)P is thought to be produced primarily by Class 3 PI3-Kinase, Vps34, which is recruited to early phagosomes by active Rab5. To elucidate a clear role for Class 3, PI3Kinase, Vps34, in the production of PI(3)P at PCs, we treated RAWs with a Vps34 specific inhibitor, Vps34-IN1, that was recently reported on in work published by Bago et al. (2014). Our results following micromolar treatment of cells with VPS34-IN1 show the loss of PI(3)P recruitment to the PC, as evidenced by lack of 2-FYVE-GFP localization to this structure (Figure 14E). This suggests that Vps34 may be the primary kinase responsible for the continuous production of PI(3)P.

Because PI(3)P persists on PCs well into the late stage of phagosomal maturation, we sought to investigate whether this phenomenon was a consequence of continued PI(3)P synthesis or whether it was the result of aberrant conversion of PI(3)P to PI(3,5)P2. To this end, we allowed phagocytosis of filamentous targets to proceed in 2FYVE transfected RAW cells before treating them with VPS3IN1, a Vps34 specific inhibitor, 20 minutes following bacterial attachment. Notably, 2FYVE localization to FT-containing PCs was lost following this treatment (Figure 15B). Thus, Vps34-catalyzed de novo synthesis of PI(3)P at PCs is critical for the maintenance of PI(3)P at this structure.

It is noteworthy that the PI(3)P population produced prior to Vps34 inhibitor addition, is not detected by 2FYVE after inhibition. Therefore, this population of PI(3)P must be acted on by kinases such as PIKfyve to produce PI(3,5)P2 or phosphatases such as myotubularins (MTM1 or MTMR2) to produce PIP (Cao et al., 2008). In order to assess the downstream conversion of PI(3)P, we pre-treated 2-FYVE-transfected RAW macrophages with apilimod, a PIKfyve specific inhibitor one hour prior to phagocytosis assays (Cai et al., 2013). We then allowed phagocytosis to proceed and PI(3)P to form, before treating cells with Vps34IN-1, 25 minutes post-bacterial attachment. After an additional 20 minutes, cells were fixed and immunostained for F-actin. We then assessed the fate of PI(3)P. Remarkably, despite the inhibition of PIKfyve, as evidenced by the enlarged hollow vacuoles present in treated cells, PI(3)P did not accumulate at FT-containing PCs (Figure 15D; Kim et al., 2014). Consequently, eventual loss of PI(3)P is not mediated primarily by PIKfyve action for if this
A Vehicle

2-FYVE Actin FTs

B Vps34-IN1

2-FYVE Actin FTs

Add Vps34 inhibitor

Fix cells

Add Vps34 inhibitor

Time (min)

10 20 45

PI(3)P forms

Vps34-IN1 and apilimod

C Apilimod

2-FYVE Actin FTs

2-FYVE

D Vps34-IN1 and apilimod

2-FYVE Actin FTs

Add Vps34 inhibitor

Fix cells

Add Vps34 inhibitor

Pretreat: apilimod (1hr)

PI(3)P forms

Time (min)

15 25 45

10 20 45

55
Figure 15 Synthesis but not conversion of PI(3)P is critical for recruitment. (A) RAW macrophages expressing 2-FYVE-GFP (green) were treated with 1 µM DMSO and challenged with filamentous target for 30 minutes after phagocytosis. Actin (phallodin): red. Right: magnified merge of framed region. 2-FYVE recruitment is in white. (B) Cells expressing 2-FYVE-GFP (green) were challenged with filamentous targets for 20 minutes before Vps34-IN1 inhibitor was added. Phagocytosis was allowed to progress for an additional 25 minutes before cells were fixed, permeabilized and stained for F-actin with phallodin (red). Below: Schematic of experimental methodology. Right: magnified merge of framed region. 2-FYVE recruitment is in white. (C) Cells expressing 2-FYVE-GFP (green) were pretreated with apilimod for 1 hour prior to the onset of phagocytosis. Cells were then challenged with FTs and after 45 minutes of phagocytosis in the presence of apilimod, cells were fixed, permeabilized and stained for F-actin (phallodin: red). Right: magnified merge of framed region. 2-FYVE recruitment is in white. (D) Cells expressing 2-FYVE-GFP (green) were pretreated with apilimod for 1 hour prior to the onset of phagocytosis. Cells were then challenged with FTs and after 25 minutes of phagocytosis in the presence of apilimod, cells were treated with Vps34-IN1. After 20 minutes of phagocytosis in the presence of apilimod and Vps34-IN1, cells were fixed, permeabilized and stained for F-actin (phallodin: red). Arrowheads indicate enlarged hollow vacuoles that result from successful PIKFyve inhibition. Below: magnified merge of two framed region. 2-FYVE recruitment is in white. Schematic of experimental methodology. Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R2 camera. Scale bars = 5 µm.
were the case, we would have observed a detectable recruitment of PI(3)P at PCs, resulting from a lack of conversion of PI(3)P. Cells treated with PIKfyve inhibitor in the absence of Vps34 inhibitor were observed to accumulate PI(3)P significantly (Figure 15C).

Taken together, our results indicate the PI(3)P is synthesized continuously at PCs by Class 3 PI3Kinase, Vps34. Furthermore, the eventual disappearance of PI(3)P is not the result of PIKfyve action, although this enzyme may play a minor role in mediating PI(3)P loss. Rather, myotubularins such as MTM1 and MTMR2 which have been reported to utilize PI(3)P as a substrate in PIP production may be responsible for the loss of PI(3)P on PCs (Cao et al., 2008). At this time, we can not rule out the possibility that other kinases and phosphatases may also play a role in mediating the loss of PI(3)P from FT-containing PCs.

3.4 Phagocytic cup acidification may play a role in mediating the loss of PI(3)P at distal regions of PCs

Thus far, it is clear that the eventual disappearance of PI(3)P from PCs does not result from PIKfyve action. Consequently, we investigated other possible mechanisms that may account for the loss of PI(3)P. A fundamental difference between the phagocytosis of spherical and filamentous targets is the pH of the maturing compartment. Although lysosomes fuse and impart their contents to PCs, these structures do not acquire hydrolytic capacity as they leak protons and lysosomal enzymes into the extracellular milieu (Prashar et al., 2013). In contrast, the phagosomal maturation of spherical targets is accompanied by acidification of the lumen. Therefore, we postulated that the lack of PC acidification may affect the processing of PI(3)P. In agreement with this possibility, we found evidence of a pH gradient that exists along phagocytic cups of long filamentous targets (≥ 20 µm) that mirrors the gradient observed for PI(3)P persistence at PCs.

We followed the accumulation of the acidotropic dye, Lysosensor™ Green in RAW macrophages phagocytosing filamentous targets. In accordance with what had been reported previously, Lysosensor dye failed to label the majority of FT-containing PCs (Figure 16A), whereas it strongly accumulated in phagosomes (Figure 16C; Prashar et al., 2013). We then
Figure 16 FT-containing PCs acidify within the tightly coiled regions of PCs. (A) RAW macrophages were challenged with filamentous targets for 5 minutes before Lysosensor™ green was added (5 minutes) and external filamentous targets were labeled (purple). Cells were then transferred to a pre-warmed stage (37°C) and imaged. Lysosensor (white) accumulation in FT-containing PCs for a filamentous target ≤ 20 µm. Below: magnified merge of framed region. Lysosensor accumulation is in white. (B) Lysosensor (white) accumulation in FT-containing PCs for a filamentous target ≥ 20 µm. Below: magnified merge of framed region. Lysosensor accumulation is in white. (C) Lysosensor (white) accumulation in FT-containing phagosomes. Below: magnified merge of framed region. Lysosensor accumulation is in white. (D) RAW macrophages were challenged with pHrodo conjugated-FTs (pHrodo-FTs) for 15 minutes before Lysosensor (white) was added (5 minutes) and external filamentous targets were labeled (blue). Cells were then transferred to a pre-warmed stage (37°C) and imaged. Lysosensor accumulation is in white. (D, i) pHrodo-FT in a RAW macrophage at T=1. Arrowheads indicate increased fluorescence of pHrodo-FTs. Color reference scale indicates level of fluorescence intensity (red: highest fluorescence). Dotted white line indicates cell boundary. Arrowheads indicate increased fluorescence intensity. Confocal images were acquired using a spinning disc confocal microscope with an ORCA-R² camera. Scale bars = 5 µm.
examined the small population of FT-containing PCs that accumulated Lysosensor dye. Remarkably, Lysosensor was observed to fluoresce in the coiled regions of PCs containing long FTs (≥ 20 µm) that had begun to coil inside the cell (Figure 16B). The tightly coiled regions of these filaments, where PI(3)P disappears from, were observed to fluoresce at a greater extent than regions of the PC closer to the plasma membrane, suggesting an accumulation of protons in the former regions. A similar phenomenon was observed in the tightly coiling regions of FT-containing PCs of RAWs that were pre-loaded with 3 kDa dextrans (Prashar, 2013, unpublished work). In addition, we utilized a molecular probe conjugated to FTs to further characterize the pH gradient present in FT-containing PCs. We assessed the fluorescence intensity of FT-pHrodo, which emits fluorescence when exposed to low pH environments. For long filaments (≥ 20 µm), pHrodo-FTs exhibited a fluorescence gradient where the coiling regions within the cell emitted more signal than external filaments (Figure 16D, i). Lysosensor was also observed to accumulate in these coiling regions of FT-containing PCs (Figure 16).

In agreement with this, lowering the pH of FT-containing phagocytic cups externally by applying an acidic buffer led to the loss of PI(3)P from PCs and early endosomes (Figure 17B). This loss was not observed in cells in which FT-containing PCs were treated with media of a neutral pH (Figure 17A). Taken together, these result suggests that luminal pH controls PI(3)P dynamics at filamentous target-containing PCs through an uncharacterized mechanism.
Figure 17 Acidifying the cellular environment leads to an abatement in 2-FYVE recruitment. (A) RAW macrophages expressing 2-FYVE-GFP (green) were challenged with filamentous targets and phagocytosis was allowed to progress for 10 minutes before cells were placed in RPMI media at pH 7.0. After 20 minutes of phagocytosis, cells were fixed, permeabilized and stained for F-actin with Phallodin (red). Right: magnified merge of twoframed region. 2-FYVE recruitment is in white. (B) Cells expressing 2-FYVE-GFP were challenged with FTs and were placed in RPMI media at pH 4.0 after 10 minutes. Cells were fixed after 20 minutes of phagocytosis, permeabilized and stained for F-actin (Phallodin: red). Right: magnified merge of framed region. 2-FYVE recruitment is in white. Scale bars = 5 µm.
Phagocytosis of filamentous targets deviates from the canonical model delineated using spherical targets. In comparison with the uptake of spheroids, phagocytosis of filamentous targets occurs through macrophage-capture of bacterial poles and requires a long-lasting phagocytic cup stage. The remodelling of the phagocytic cup mirrors that of the canonical model, with respect to the dynamics of PI(4,5)P₂ and F-actin remodelling. However, it is remarkable that the filamentous target-containing PC undergoes further remodelling processes that correspond to the maturation pathway of phagosomes. This is different from the canonical model in which phagosomal scission from the plasma membrane and acidification of the compartment is thought to be prerequisite for maturation. By changing the morphology of the target, two hallmarks of the canonical model are challenged. The phagocytic cup associated maturation in the absence of scission most likely results from the longer time required for the sealing of FT-containing PCs compared to the rapid uptake of spherical targets, that have been documented to occur in the order of seconds.

The maturation of FT-containing PCs was established by following the recruitment of endosomes and lysosomes to these structures. Early endosomal markers Rab5, EEA1 and PI(3)P were detected as early as 5 minutes following bacterial attachment. Although the kinetics of Rab5 and EEA1 appearance and disappearance from PCs is consistent with what was observed for spherical targets, here we demonstrate that PI(3)P persists at FT-containing phagocytic cups in a bacterial length-dependent manner. As a result of this, PI(3)P was observed to co-exist in time with lysosomal membrane protein, LAMP1. For filaments over ~20 μm, PI(3)P persists at regions of the FT-containing PC proximal to the plasma membrane, although it was lost from distal regions. This phenomenon was also dependant on time since the length of the internalized bacterial portion correlates well with the progression of time.

Despite continuity of the phagocytic cup with the plasma membrane, Class 1 PI3-Kinases were not involved in the synthesis of PI(3)P at PCs. In agreement with what had been
observed in the canonical model, PI(3)P retention at PCs is the result of action by Class 3 PI3-Kinase, Vps34, which catalyzes the continuous production of this early lipid regulator (Vieira et al., 2001). This was determined by employing a specific inhibitor against Vps34, that has no effect on other PI3-Kinase classes (Bago et al., 2014). The recruitment and retention of PI(3)P at PCs may result either from (i) excessive synthesis or (ii) inhibition of the downstream conversion/degradation of this phosphoinositide. However, when we inhibited the production of PI(3)P, recruitment of PI(3)P was no longer detected, suggesting that synthesis and not degradation accounts for the observed retention. Indeed, the inhibition of Vps34 depletes PI(3)P at PCs to an undetectable level. This was also evident in cells treated with PI3-Kinase inhibitor, Ly294002, where PI(3)P recruitment was severely abrogated. Consequently, once PI(3)P is synthesized, the downstream conversion of this lipid at PCs is unaltered. We then sought to investigate the mechanism responsible for the downstream conversion of PI(3)P at PCs. As phagosomes mature in the canonical pathway, PI(3)P is acted on by kinases such as PIKfyve to produce PI(3,5)P2 or phosphatases such as myotubularins to produce PIP (Cao et al., 2008). Remarkably, inhibition of PIKfyve did not rescue PI(3)P recruitment at FT-containing PCs. Therefore, other players may mediate the downstream conversion of PI(3)P. Mytotubularins including MTM1 or MTM2 are likely candidates for conversion of PI(3)P to PIP at PCs. The 3-phosphatase PTEN has also been shown the mediate removal of PI(3)P (Hazeki et al., 2012); its role in phagosomes may extend to the FT-containing PC as well.

In order to understand the region-specific disappearance of PI(3)P at PCs of long filaments, we investigated the mechanism behind the production of a PI(3)P gradient at phagocytic cups. PI(3)P disappeared from the distal portions of long PCs (≥20 µm internalized) and readily once phagosomes seal, regardless of the length of the target. The fact that the latter phenomenon correlates with the rapid acidification of the newly formed compartment and that the distal portion of PCs can acidify, prompted us to hypothesize that PI3P disappearance must be associated with the acidification of FT-containing PCs. Indeed, acidifying the cellular environment led to an abatement in 2-FYVE recruitment to FT-containing PCs and endosomes. Given that PI(3)P detection in our system was based on the observed localization of 2-FYVE, we had to confirm that an acidic cellular environment
affects PI(3)P recruitment but not 2-FYVE detection of this lipid at PCs. This possibility is unlikely since studies that have looked at 2-FYVE docking to membranes have shown that the interaction of FYVE domains with the membrane is stabilized by both FYVE-specific binding to PI(3)P and stabilizing electrostatic interactions of the FYVE domain with acidic phospholipids (Kutateladze et al., 2003). Therefore, the observed phenomenon of 2-FYVE loss when cells are acidified is most probably the result of a decrease in PI(3)P levels.

The compartmental pH may control PI3P synthesis by affecting Vps34 location and/or activity; acidified compartments may be refractory to Vps34 recruitment or activation, leading to a depletion in PI(3)P production. At present, not much is known on how pH may affect Vps34 activity or localization/targeting in the phagocytic pathway. We performed immunolabelling studies of Vps34, which were not successful; future work in collaboration with Dr. J. Backer (Albert Einstein College of Medicine) who has provided us with recombinant fluorescent constructs for Vps34 will provide us with insight into how pH may affect Vps34 recruitment and activity (Figure 18, iii, iv).

The requirement for PI(3)P recruitment, activation and timely disappearance are thought to be critical for the normal progression of phagosomal maturation of spherical targets. In cells treated with inhibitory antibodies against Vps34, blocking PI(3)P synthesis was observed to delay phagosomal maturation. This was evidenced by the preclusion of late endosomal and lysosomal proteins, lysobisphosphatidic acid (LBPA) and LAMP1 from phagosomes (Fratti et al., 2001; Vieira et al., 2001). Moreover, inhibiting PI(3)P conversion to downstream lipid, PI(3,5)P2 through PIKFyve antagonists also interfered with phagosomal maturation. In treated cells, PI(3)P disassociation from phagosomes, but not acquisition was delayed by 4-6 minutes. Strikingly, inhibition of PIKFyve led to a delay in phagosomal trafficking to the lysosome and a reduction in the degradative capacity of phagosomes (Kim et al., 2014).

Taken together, these studies reveal the importance of the timely appearance and disappearance of PI(3)P during maturation. However, here we present evidence that PI(3)P disassociation is not a prerequisite for maturation to occur. Rather, its persistence may account for other notable divergences from the canonical pathway.
Leaking of H+

Filamentous Target (≥ 20 μm)

External Bacteria

Early Endosomes

Late Endosomes/
Lysosomes

F-actin

PI(3)P

Vps34

Rab7

LAMP1

V-ATPase

PI(3)P

Nucleus

i. ii. iii. iv. v.
Figure 18 Schematic representation of cellular events at the phagocytic cup and phagosomes of filamentous targets. Phagocytosis of filamentous bacterial targets proceeds through a tubular phagocytic cup, which is enriched in F-actin near the plasma membrane. The bases of phagocytic cups lose F-actin and begin to undergo maturation events through fusion with endosomes and lysosomes. PI(3)P is acquired on phagocytic cups in this way (i). Despite persistence of PI(3)P, the unsealed phagocytic cups recruit late endosomal marker, Rab7, followed by lysosomal membrane protein LAMP1. During internalization the phagocytic cup remains open, causing the leakage of protons and low molecular weight hydrolytic enzymes. As such, it is a relatively neutral structure (ii). As filaments greater than 20 micrometers are internalized, PCs display inverted gradients for PI(3)P recruitment and acidification. The regions of PCs close to the plasma membrane recruits PI(3)P and is relatively neutral. As the coiled regions of PCs begins to acidify, PI(3)P recruitment is no longer detectable. The cessation of Vps34-catalysis of PI(3)P may be a consequence of acidification of internal PCs (iv). Eventually, phagocytic cups seal into phagosomes; the phagosomal lumen becomes acidified to a great extent and gains hydrolytic capacity. The resultant antimicrobial environment allows for the complete degradation of the target (v). Differential immunostaining of filamentous bacterial targets distinguished internalized segments from external bacteria (internal: red, external: purple).
Our assessment of alterations in surface potential during the phagocytosis of filamentous targets provides insight into phosphoinositide, and particularly PI(3)P dynamics during this process. As macrophages engulf large spherical targets (8 µm) or filamentous bacterial targets, PI(4,5)P₂ is metabolized at the base of the phagocytic cup, prior to cup sealing (Botelho et al., 2000; Prashar et al., 2013). In the case of spherical target uptake, the hydrolysis of PI(4,5)P₂ leads to a local decrease in surface potential, as detected by decreased localization of polycationic probes during phagosome formation (Yeung et al., 2006).

Remarkably, the surface potential at PCs of filamentous targets was unaltered, despite a near-total depletion of PI(4,5)P₂ at the base of PCs. It was only after complete engulfment of the filamentous target into a phagosome that surface potential decreased significantly, as has been observed for spherical targets (Yeung et al., 2006). Therefore, an alternative anionic lipid must exist at FT-containing PCs that can account for the recruitment and localization of polycationic probes during phagosomal maturation. As PI(3)P accumulates significantly at filamentous target-containing PCs, it may be the anionic lipid responsible for the unaltered surface potential observed. To investigate this possibility, we plan to inhibit Vps34 synthesis of PI(3)P at PCs and then follow the localization of polycationic probes for surface potential.

Changes in surface potential of the membrane are thought to play a role in the targeting of signalling molecules such as the F-actin modulator, Rac1 and the tyrosine kinase, c-Src to the plasma membrane (Yeung and Grinstein, 2007; Yeung et al., 2008). Lipid remodelling leads to localized changes in surface potential at the PM, which affects the electrostatic anchorage of these signalling molecules. As a decline of surface potential was not evident at FT-containing PCs, the anchoring of these key proteins may be altered. This may have consequences for the successful phagosomal maturation of filamentous targets that have yet to be elucidated.

PI(3)P is an important regulator in several pathways; other PI(3)P-dependant cellular processes may also be altered by its retention at PCs. For one, PI(3)P may mediate the overproduction of reactive oxygen species, which are a potent facet of the macrophages antimicrobial response. As PI(3)P recognition and binding of the p40Phox subunit of NADPH oxidase is required for oxidase activation, excess PI(3)P may lead to an over-activation of the
NADPH oxidase complex. Excess ROS production has been linked to pathological situations in chronic diseases where redox damage and inflammation prevails, as a result of the secretion of pro-inflammatory mediators (Alfadda and Sallam, 2012). In addition, PI(3)P also functions in the autophagy pathway, which involves the degradation of cell cytoplasm and organelles through the formation of a double membrane vesicle that fuses with lysosomes. In this conserved pathway, PI(3)P enables the formation of the signalling platform required for autophagosome biogenesis (Burman and Ktistakis, 2010). More recently, some aspects of the autophagic pathway have been shown to intersect with 'conventional’ phagocytosis, contributing to cellular immunity, in a process called LC3-Associated Phagocytosis (LAP). In this pathway, Vps34 production of PI(3)P is observed to precede recruitment of the autophagy machinery protein, LC3 (Sanjuan et al., 2007). In this way, PI(3)P retention at PCs may have implications that extend beyond phagocytosis. Our findings can contribute to our understanding of the role of PI(3)P in the described processes.

The maturation of filamentous target-containing PCs is not limited to the recruitment of early endosomal markers. Late endosomes and lysosomes fuse with and impart their contents to PCs, prior to scission from the plasma membrane (Prashar et al., 2013). As early as 30 minutes of phagocytosis, late endosomal markers, Rab7 and RILP-c33 and lysosomal membrane protein, LAMP1, localize to FT-containing PCs. Strikingly, recruitment of late endosomes and lysosomes to PCs was not excluded by the presence of PI(3)P. As was observed for spherical targets, LAMP1 was recruited to the maturing PC as soon as it became refractory to EEA1 and Rab5 recruitment. Interestingly, Rab7 was observed to behave in a manner that deviates from the canonical model. Rab7 localization to PCs was noted as early as 5 minutes following bacterial attachment. Although Rab7 recruitment is expected to precede that of LAMP1, the timing of its localization to PCs is rather premature. Regardless, the phagosomal maturation of spherical targets occurs rapidly, making it hard to decipher the order of marker recruitment to the phagosome. In our model, maturation of FTs occurs much more gradually, enabling us to elucidate events at the molecular level more clearly.

Despite being recruited early, Rab7 is only activated to a significant rate 30 minutes after the initiation of phagocytosis. It is notable that FTs greater than 20 µm in length require a
duration of 20 minutes or longer to be internalized. Therefore, it is possible that despite not having an effect on Rab7 recruitment, the removal of PI(3)P may be a prerequisite for Rab7 activation at PCs (Figure 18, iv). Studies utilizing spherical targets noted that the disappearance of PI(3)P often coincided with the centripetal displacement of the phagosome towards the nucleus (Vieira et al., 2001). Rab7 plays a key role in initiating the migration of phagosomes towards perinuclear region of the cell, through the recruitment of downstream effectors. Taken together, this suggests that delayed Rab7 activation, which occurs post-LAMP1 recruitment, may result from the persistence of PI(3)P at FT-containing PCs.

In the canonical model of phagocytosis, maturation of the phagosome is correlated with proton accumulation and acidification of the lumen. Although this is in part a consequence of maturation, it was thought that it might also be essential for fusion of endosomes with the phagosome and for maturation to proceed. This school of thought was founded on *in vitro* studies that perturbed proton pumping by pharmacological means or by alkalization using a cell permeant weak base, which impeded phagosomal maturation without affecting target engulfment (Gordon et al., 1980; Lukacs et al., 1990). Our results demonstrate that FT-containing PCs, although neutral, can fuse with late endosomes and lysosomes, indicating that acidified endosomal compartments and not necessarily phagosomes may be required for maturation.

Notably, the leaking of hydrolytic enzymes and protons from PCs provides filamentous bacteria with a non-acidic, non-proteolytic compartment. Longer bacterial filaments stand a better chance of modulating host cell processes to create an intracellular replicative niche through the secretion of bacterial effectors. Indeed, viable filamentous *L. pneumophila* was shown to escape phagosomal killing by murine macrophages in a length-dependent manner (Prashar et al., 2013). The leaking of hydrolytic enzymes and other noxious lysosomal compounds from ‘open’ PCs may also have consequences on host cells at the population level, leading to human disease. This is most evident in patients that suffer from Mucolipidosis (ML) types II and III who clinically present with developmental delay, abnormal skeletal development and restricted joint movement (Mueller et al., 1983). This family of autosomal recessive diseases is caused by reduced enzyme activity of N-
acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase; Mueller et al., 1983), which leads to the leakage of lysosomal content. GlcNAc-phosphotransferase is involved in catalyzing the final step during the production of a recognition marker found on lysosomal enzymes that is detected by the mannose-6-phosphate receptor. The targeting of newly synthesized lysosomal enzymes to the lysosome is mediated by the mannose-6-phosphate receptor, which recognizes a terminal phosphate on a sugar moiety on lysosomal enzymes (Dahms et al., 1989). As a result of reduced GlcNAc-phosphotransferase activity, the targeting of many acid hydrolases to the lysosome is impaired, resulting in leakage. Patients who suffer from mucolipidosis II and III present with elevated levels of lysosomal enzymes such as hydrolases in serum and body fluids (Otomo et al., 2009). These cellular events lead to the debilitating condition observed in patients.

In summary, our results demonstrate that the early lipid regulator PI(3)P accumulates in an anomalous manner at the phagocytic cups of filamentous targets. The persistence of PI(3)P may be a consequence of the neural pH of the phagocytic cup. Therefore, acidification of the compartment is a probable prerequisite for PI(3)P removal. As a consequence of PI(3)P retention at phagocytic cups, significant aspects of maturation are altered, evidenced by the delay in Rab7 activation. These findings reveal that key aspects of phagosomal maturation are in fact conditioned by the morphology of the target.
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