THE ROLE OF THE LIPID KINASE PIKFYVE IN THE ENDOSOMAL AND PHAGOSOMAL SYSTEMS IN MACROPHAGES

by

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Abstract

THE ROLE OF THE LIPID KINASE PIKFYVE IN THE ENDOSOMAL AND PHAGOSOMAL SYSTEMS IN MACROPHAGES

Grace Ha Eun Kim, Master of Science in Molecular Science, Ryerson University, 2014

Macrophages engulf pathogens into phagosomes for degradation through a process known as phagocytosis. Nascent phagosomes progressively mature and fuse with early and late endosomes and lysosomes to form phagolysosomes, where pathogens are degraded by hydrolytic enzymes. Phosphatidylinositol-3-phosphate [PtdIns(3)P] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] are signaling lipids that recruit a unique set of effector proteins involved in distinct stages of membrane traffic to govern the function of early and late endosomes, respectively. Phagosome maturation requires the transient expression of PtdIns(3)P on early phagosomal membranes. Subsequently, PtdIns(3)P can be converted to PtdIns(3,5)P_2 by the lipid kinase PIKfyve. Thus, it remains unclear if the role of PtdIns(3)P in phagosome maturation is direct and/or indirect, through the synthesis of PtdIns(3,5)P_2. The role of PtdIns(3,5)P_2 in the endosomal system in macrophages also requires further investigation. My thesis employs a pharmacological approach to address the role of PIKfyve in macrophage biology. In general, PtdIns(3,5)P_2 appears to principally coordinate the later stages of endosome and phagosome maturation.

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List of Abbreviations

BSA	Bovine serum albumin
CI-MPR	Cation-independent mannose 6-phosphate receptor
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EEA1	Early endosome antigen 1
ESCRT	Endosomal sorting complexes required for transport
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FYVE	Fab1, YOTB, Vac1, and EEA1
GFP	Green fluorescent protein
IgG	Immunoglobulin G
LAMP1	Lysosomal-associated membrane protein 1
LB	Luria-Bertani
LDL	Low density lipoprotein
MVB	Multivesicular body
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PIKfyve	FYVE finger-containing phosphoinositide kinase
PI3K	Phosphatidylinositol 3-kinase
PtdIns	Phosphatidylinositol
PtdInsP	Phosphoinositide
PtdIns(3)P	Phosphatidylinositol 3-phosphate

$PtdIns(3,5)P_2$	Phosphatidylinositol 3,5-bisphosphate
RBC	Red blood cell
TfR	Transferrin receptor
TGN	Trans-golgi network
TRITC	Tetramethylrhodamine isothiocyanate
TRPML1	Mucolipin transient receptor potential protein 1
Vps34	Vacuolar protein sorting-associated protein 34
WIPI1	WD repeat domain containing, phosphoinositide-interacting 1

1 Introduction

1.1 The immune system

Our body is protected by the immune system, which has several lines of defense against infection. The most prominent feature of our immune system is the integumentary system, which includes the skin and chemicals that are produced by the mucous membrane lining of the gut; however, when this line of defense is penetrated, our body has an army of specialized cells that are either part of the innate (nonspecific) or adaptive (specific) immune response that further guard the body [1].

1.1.1 Innate immune response

Innate immune response is different from specific immune response, in that it is capable of mounting an immediate defense response. Inflammation typically involves the release of histamine, prostaglandins, and other "chemical alarm signals" from cells at sites of infection, causing the nearby blood vessels to dilate and become "leaky" [1].

1.1.2 Macrophages: Immune Sentinels

Our body's innate immune system is coordinated by a specialized group of professional phagocytes, among which include the macrophages. Monocytes, which are undifferentiated macrophages that circulate in the bloodstream, permeate through the walls of the leaky blood vessels to reach the infection site. There, they differentiate into macrophages that engulf and digest pathogens [1].

Macrophages also express a proinflammatory cytokine, interleukin-1 (IL-1), which promotes the expression of adhesion molecules on endothelial cells that line the inner surface of

blood vessels. This allows for a more effective migration of leukocytes from the blood to the site of tissue damage [2]. IL-1 also causes fever (an elevation of body temperature), which helps to enhance the phagocytic activity of leukocytes, thereby suppressing further spread of pathogens [2]. Nonetheless, the fundamental role of macrophages is to rid the body of foreign particles [3]. How do macrophages perform this task? Endocytosis and phagocytosis are common cellular processes that macrophages employ to protect the body from pathogen invasion.

1.2 Endocytosis

Endocytosis is a process by which cells internalize a diverse range of cargo molecules, such as fluid, solutes, plasma membrane components, extracellular–matrix components, cellular debris, bacteria and viruses [4]; however, when we discuss about endocytosis, we generally refer to the cellular uptake of smaller molecules, such as fluid, solutes, and plasma membrane components- e.g. receptor-ligand complexes.

1.2.1 Fluid-phase endocytosis

There are two distinct types of endocytosis; fluid-phase non-receptor mediated endocytosis and receptor-mediated endocytosis. During fluid-phase endocytosis, macrophages constitutively internalize surrounding fluid in a non-specific manner [5]. This can occur in a variety of ways, including clathrin-dependent and -independent endocytosis, and macropinocytosis [5]. Macropinocytosis, for example, is initiated at the ruffled border regions of the plasma membrane that are supported by actin [6,7]. It culminates with membrane ruffle closure, which leads to the formation of large endocytic vacuoles called macropinosomes filled with a large volume of extracellular fluid [6]. It is also important to note that fluid-phase endocytosis

displays bidirectional traffic, in that they are either directed into the endocytic pathway (see below) or regurgitated back to the plasma membrane. The use of such soluble markers as horseradish peroxidase (HRP) or fluorescent dextran is common while studying fluid-phase endocytosis [8].

Macrophages internalize an immense amount of fluid every hour- a volume equivalent to about 30 % of the cell volume and a fractional area equivalent to about twice the cell surface area [4,9–11]. Macrophages generally exhibit a higher endocytic activity than other cell types. For example, whereas macrophages ingest approximately 3 % of its plasma membrane each minute, the endocytic rate of fibroblasts is lower, residing at approximately 1 % per minute [12]. Thus, it is worthwhile to take a closer look at how the endosomal system functions in macrophages.

1.2.2 Receptor-mediated endocytosis

Receptor-mediated endocytosis is different from fluid-phase endocytosis, in that it engages ligand receptors to internalize target molecules. For this reason, while fluid-phase endocytosis is linearly dependent on marker concentration and incubation time, receptor-mediated endocytosis reaches its saturation point when all ligand receptors are bound to its ligand [13]. Receptor-mediated endocytosis typically requires the formation of clathrin-coated pits (CCPs) to form clathrin-coated vesicles (CCVs). Incoming receptor-ligand complexes are assembled into nascent endocytic vesicles as the plasma membrane invaginates and pinches off into the cytoplasm [14,15]. Depending on the cargo type, some internalized molecules are recycled back to the plasma membrane, while those that are destined for degradation continue on its path to the lysosomes, where they are degraded by lysosomal enzymes [16,17]. To illustrate, low density lipoprotein (LDL) is internalized into cells via receptor-mediated endocytosis [15,18]. LDL, which

carries cholesterol through the bloodstream [19], binds to LDL receptors that are heavily expressed at sites of CCP formation [20–23]. Subsequently, these CCPs pinch off to form CCVs. As CCVs uncoat within the cell, ligand reptors and target ligands are processed by different routes (see below) [18,24].

1.3 The endocytic pathway

Once cargo molecules are internalized via endocytosis, those destined for degradation are transported along the endocytic pathway. There are three main endosomal compartments in the endocytic pathway; the early and late endosomes and lysosomes [4]. Internalized molecules are first sorted into early endosomes, which has a mildly acidic luminal pH of around 6.2-6.5 [25]. Endosome and lysosome acidification is generated by a proton-pumping V-type ATPase [26], while Na^+, K^+-ATPase serves to buffer the effect of proton transport, primarily in early endosomes [27,28]. In some cases, the slightly acidic environment of early endosomes facilitates the uncoupling of ligands from their cognate receptors [29]. Dissociated ligands are sorted for degradation into lysosomes, while their receptors necessary to maintain cellular homeostasis are recycled back to the plasma membrane for further entry of ligands. Accordingly, early endosomes are largely categorized into two distinct compartments, the sorting and recycling endosomes [30].

As the pH is lowered and lysosomal enzymes are added, early endosomes mature to form late endosomes or multivesicular bodies (MVBs) [4]. The sequential recruitment of multiple endosomal sorting complexes required for transport (ESCRT) mediates the formation of MVBs. ESCRT-0 binds to ubiquitinated cargo and clusters them to the limiting membranes of early endosomes. Subsequently, ESCRT-I and –II deform these cargo-rich regions of the early

endosomal membrane to form small inward budding vesicles. These vesicles are then cleaved by ESCRT-III, giving rise to numerous intraluminal vesicles (ILVs) that reside within a larger late endosomal MVB. The successive fusion of cargo-bearing ILV-containing MVBs with lysosomes leads to the ultimate degradation of internalized molecules [31].

Lysosomes are the terminal stations in the endocytic pathway. They have a lower internal pH than early endosomes (ranging from 4.7 to 4.8 in macrophages) [32], in order to activate the lysosomal enzymes that typically have an acidic optimum pH [33].

Endosome maturation is regulated by the recruitment of various molecular markers that help to define the identity of organelles. For example, Rab GTPases are molecular switches that cycle between an active form in the GTP-bound state (catalyzed by a guanine nucleotide exchange factor; GEF) and an inactive form in the GDP-bound state (stimulated by a cognate GTPase activating protein; GAP). Rab-GTP is anchored to the cytosolic membrane of a target organelle via its carboxy-terminal two prenyl groups [34,35]. By recruiting and interacting with a host of effector proteins, Rab GTPases play important roles in a variety of endosomal functions, such as endosome tethering, fusion, and maturation. To illustrate, the recruitment of a tethering factor, early endosome antigen 1 (EEA1), by the early endosomal Rab5 in its GTP-bound state is essential for the homotypic (with other early endosomes) and heterotypic fusion (with incoming endocytic vesicles) of early endosomes. Moreover, Rab5 replacement with the late endosome- and lysosome-specific Rab7 is a key feature of endosome maturation [36].

Aside from Rab5 and EEA1, other molecular markers of early endosomes include the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which is involved in cargo sorting [37,38], syntaxin 13, which is essential for the recycling of plasma membrane proteins [39], and PtdIns(3)P, a signaling lipid (see below) [40]. LAMP1 [41] and V-type ATPase [26] are some of the

common molecular markers of late endosomes and lysosomes, whereas cation-independent mannose 6-phosphate receptor (CI-MPR) accumulation is restricted to trans-Golgi network (TGN) and late endosomes [42,43].

1.4 Phagocytosis

Ingestion of small cargo molecules occurs through a process called endocytosis; however, depending on the physical properties of cargo molecules, macrophages engage different modes of cargo entry. Phagocytosis is a distinct type of endocytosis that involves the ingestion of particles usually exceeding 0.5 μm in diameter [3,44,45]. This process plays an essential role in cell survival by enabling uptake of nutrients and eliminating cellular debris or infectious pathogens [45]. Phagocytosis is generally classified into two major groups- opsonic and non-opsonic. The latter involves the engagement of non-opsonin receptors that bind endogenous ligands on particles that are destined to be internalized. Mannose receptors and dectin1 that bind microbial polysaccharides are a few examples of such receptors [45]. Opsonin receptors, on the other hand, bind exogenous ligands, or opsonin molecules, that coat the antigen surface. To illustrate, macrophages recognize and engulf immunoglobulin G (IgG)-opsonized particles by expressing Fcγ receptors on their cell surface that interact with the Fc portion of IgG [46].

1.4.1 Fcγ receptor-mediated phagocytosis

Fcγ receptor-mediated phagocytosis requires the expression of Fcγ receptors on the surface of phagocytes and the recognition of IgG-opsonized particles by these receptors.

IgG is an antigen-recognizing antibody that is secreted by plasma B cells. It is a tetrameric protein complex, consisting of two heavy- and two light chains. The amino terminal

ends of these chains form the two variable Fab domains that recognize and bind various antigens. The one constant Fc domain that resides within the carboxy terminal ends of heavy chains adheres to Fcγ receptors that are expressed on the immune cell surface [47].

Fcγ receptor is a member of the immunoglobulin receptor superfamily, and there are currently four known classes of murine Fcγ receptors, namely FcγRI, FcγRII, FcγRIII and the more recently identified FcγRIV [3,48–50]. Each cell type of the immune system is capable of expressing multiple Fcγ receptor isoforms on its surface, which is an indication that there are distributional and functional redundancies between these receptors [49]. Depending on which cell type the receptor is localized to and what other receptor isoforms it is found in association with, there are distinct differences in how phagocytosis is mediated by the host cell.

Proper internalization of IgG-coated particles involves the clustering of multiple Fcγ receptors that recognize them. A single Fcγ receptor is incapable of maintaining its ligand-bound state. In order to securely bind a ligand to the cell surface, multiple receptors aggregate to increase their avidity for the ligand. The rapid lateral movement of the receptors along the plasma membrane appears to be an important criterion for the initial stages of Fcγ receptor-mediated phagocytosis [45]. Moreover, a more recent study revealed that the probing activity of actin-rich membrane ruffles and filopodia is required for proper binding of ligand to the Fcγ receptors [51].

As an aside, it is important to note that the size [52] and shape [53] of the target particles play inevitable roles in manipulating phagocytic activity. Their rigidity also influences the cells' phagocytic efficiency [44], further complicating our current understanding of the system.

1.5 The phagosomal pathway

Similar to that in the endosomal system, phagocytosis is followed by the successive transport of internalized particles along the phagosomal pathway. Once internalized, particles that are destined for degradation reside within a phagosome. This phagosome, which originates from the plasma membrane, progressively fuses with early and late endosomes, and eventually with lysosomes to form the phagolysosome. At this stage, the intraphagosomal pH is lowered, and various lysosomal enzymes get incorporated to complete the digestion of particles. The sequential fusion of phagosomes with various endocytic organelles depends on the stage of maturity the phagosome is at (e.g. early phagosomes with early endosomes, and late phagosomes with late endosomes and so on), and this event allows for the active transport of endosomal markers to the phagosomal membrane and lumen (Figure 1.1) [45].

It is fundamental to understand phagosome formation and maturation, in that pathogens have evolved a variety of mechanisms to circumvent their degradation by the host defense system. *Mycobacterium tuberculosis* arrest at the early stages of phagosome maturation, while *Salmonella* spp. prevent their degradation by blocking Salmonella-containing vacuole (SCV)-lysosome fusion at the late stages of phagosome maturation. Other species like *Listeria* and *Shigella* interfere with the host phagosomal system by lysing the phagosome membrane and escaping into the cytosol [45]. To address some of the underlying mechanisms of pathogen invasion, it is worthwhile to investigate how vesicular traffic is operated in macrophages.

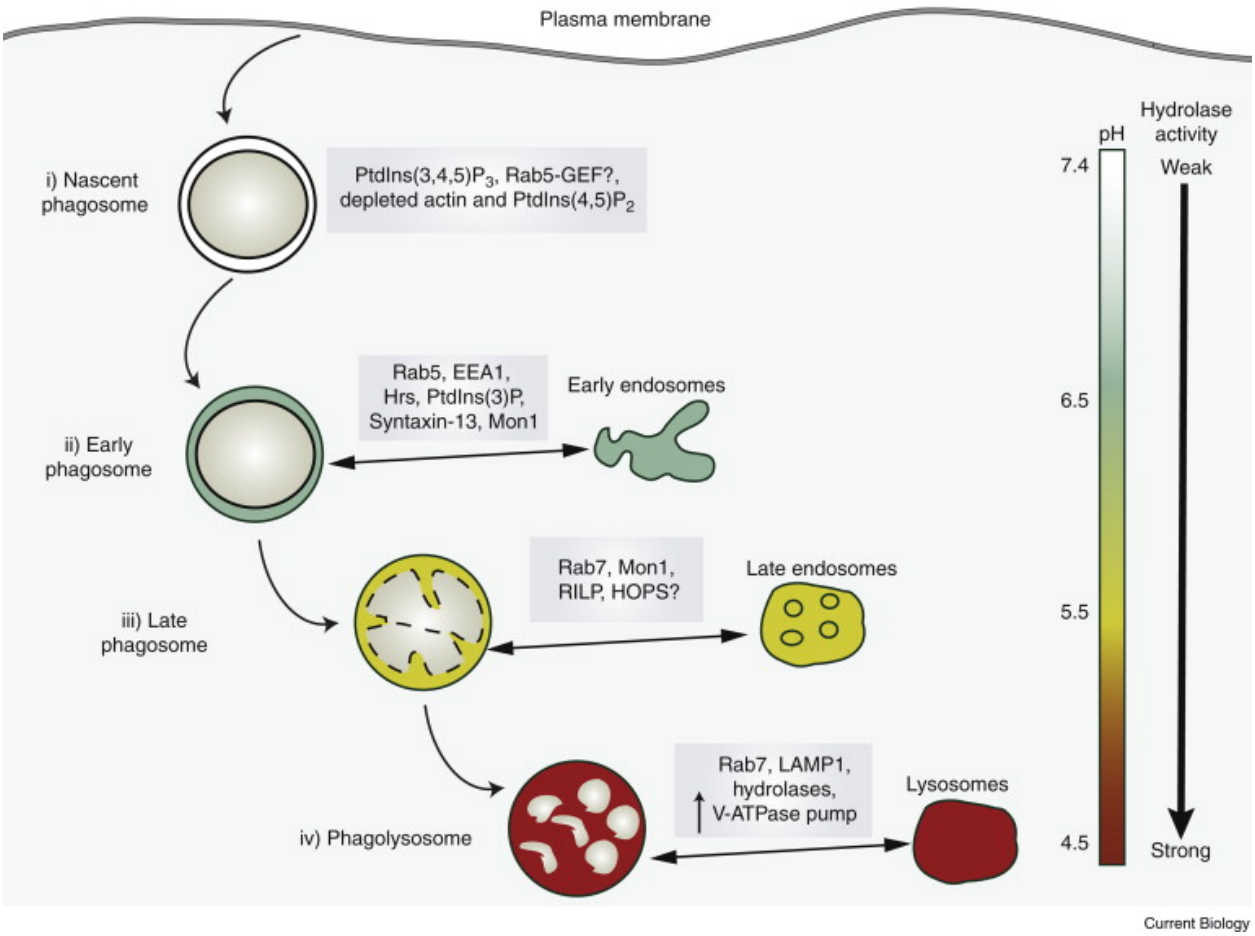

Figure 1.1 The endocytic and phagosomal pathways

Through a process known as endocytosis, cells internalize small cargo molecules into endosomes. Early endosomes mature and acidify to become late endosomes, and late endosomes are converted to lysosomes as the pH is lowered and acid hydrolase enzymes are added. Macrophages engulf pathogens into phagosomes for degradation through a process known as phagocytosis. Newly formed phagosomes sequentially fuse with early and late endosomes and lysosomes to form phagolysosomes, where pathogens are degraded by hydrolytic enzymes. [45]

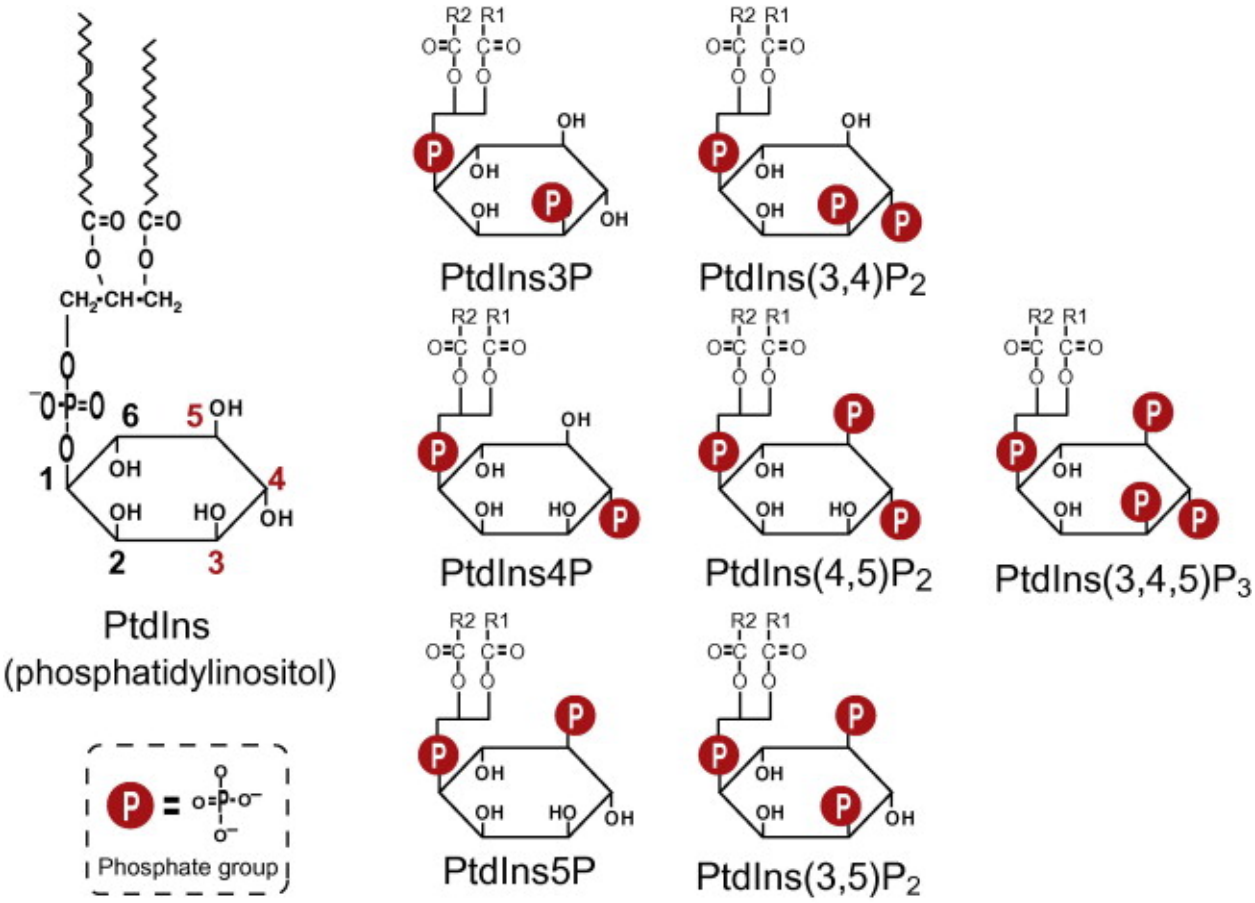

Figure 1.2 Phosphoinositides

PtdInsPs are phosphorylated derivatives of PtdIns that play important roles in many membrane trafficking events along the endocytic pathway. Some are also required for phagocytosis and phagosome maturation. Their precursor, PtdIns, is a negatively charged phospholipid, whose inositol ring can be phosphorylated on any of the hydroxyl groups of carbons 3, 4, and 5 to form seven PtdInsPs (a-b). Through their restricted distributions, PtdInsPs help to give membranes of different intracellular compartments their distinctive identity by facilitating the recruitment of separate subsets of effector proteins (see below). [54]

1.6 Phosphoinositides

Phosphoinositides (PtdInsPs) play a critical role in membrane traffic along the endocytic and phagocytic pathways. Their precursor, phosphatidylinositol (PtdIns), is a negatively charged phospholipid, whose inositol ring can be phosphorylated on any of the hydroxyl groups of carbons 3, 4, and 5 to form seven PtdInsPs (Figure 1.2) [54–56].

Through their restricted distributions, PtdInsPs help to give membranes of different intracellular compartments their distinctive identity by facilitating the recruitment of separate subsets of effector proteins. Many cytosolic proteins possess lipid-binding domains in them, such as pleckstrin homology (PH) or phox homology (PX) domains, which allows for their translocation to the membrane site via their interaction with the PtdInsPs. These effector proteins then recruit a host of other proteins and elicit an appropriate signaling response that is suited for that organelle's purpose [57,58].

1.7 Phosphatidylinositol 3-phosphate

This study focuses on the PtdInsPs, phosphatidylinositol 3-phosphate [PtdIns(3)P] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] because they confer identity to endosomal membranes. The sole member of class III PI3K, Vps34, phosphorylates PtdIns on the hydroxyl group of its third carbon to form PtdIns(3)P [59]. PtdIns(3)P ensures early endosomal identity to those membranes that carry this PtdInsP [60,61]. There are numerous lipid-binding domains that specifically recognize and bind PtdIns(3)P . Among these modules is the FYVE (Fab1, YOTB, Vac1, and EEA1) domain, which is preserved in a wide variety of proteins including EEA1 [57,62]. EEA1 is an early endosomal marker that binds directly to PtdIns(3)P. It is required for mediating early endosome tethering and fusion to incoming endocytic vesicles [63].

1.8 The role of phosphatidylinositol 3-phosphate in phagosome maturation

PtdIns(3)P is an early endosomal PtdInsP. Accordingly, PtdIns(3)P is transiently detected on the membranes of early phagosomes [40]. A construct consisting of two or more FYVE domains of EEA1 in tandem selectively binds to PtdIns(3)P in vivo [40,60]. For this reason, a green fluorescent protein (GFP) fused to this domain (2FYVE-GFP) serves as a useful probe for tracking the intracellular localization of PtdIns(3)P [40]. Fluorescent signal, which denotes the presence of PtdIns(3)P, is vividly apparent around the nascent phagosomes; however, as these phagosomes progressively mature into late phagosomes and subsequently phagolysosomes, this fluorescence is lost. As such, PtdIns(3)P acquisition is confined to early phagosomes (Figure 1.3) [40].

As confirmed by high-performance liquid chromatography (HPLC) analysis, this transient accumulation of PtdIns(3)P on early phagosomal membranes is partially accounted for by its de novo synthesis. Apart from the amount of PtdIns(3)P acquired via early endosome-early phagosome fusion, PtdIns(3)P is newly synthesized at the early phagosomal membranes. In support of this finding, it is observed that the fluorescence associated with 2FYVE-GFP that is detected around the phagosomes is markedly higher (~50% increase) in macrophages that phagocytosed IgG-opsonized latex beads for 12 min, compared to the basal amount detected prior to phagocytosis (Figure 1.3) [40].

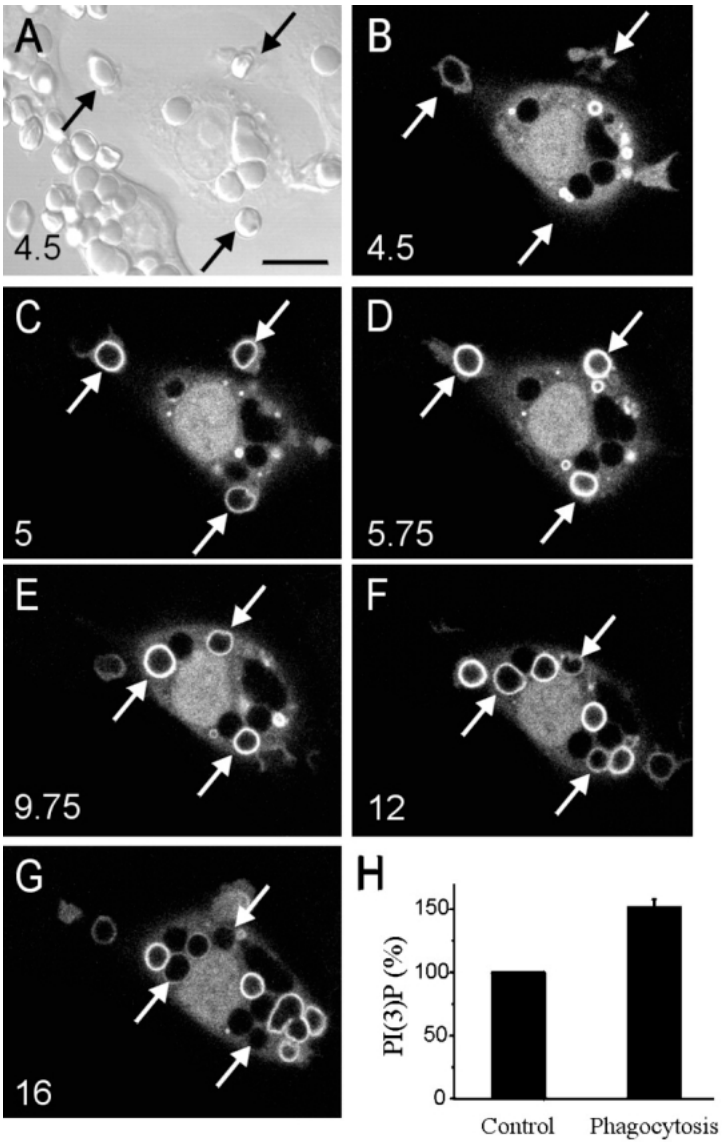

Figure 1.3 Distribution and quantification of PI(3)P during phagocytosis
RAW macrophages transfected with 2FYVE-GFP (a probe of PtdIns(3)P subcellular distribution) were allowed to phagocytose IgG-opsonized red blood cells (RBCs). The number indicated at the bottom left of each panel represents the time elapsed after addition of the RBCs. Arrows point to phagosomes formed in ~4-5 min interval. DIC image (A). Newly formed phagosomes quickly acquire PtdIns(3)P (B-F). The subsequent disappearance of PtdIns(3)P (G) indicates that PtdIns(3)P is transiently expressed on early phagosomal membranes. Bar, 10 μm. HPLC quantification of PtdIns(3)P in macrophages at basal levels (control) and 12 min after phagocytosis of IgG-opsonized beads (phagocytosis). Some PtdIns(3)P are synthesized de novo during phagocytosis (H). [40]

Wortmannin, a selective inhibitor of PI3K, is valuable in determining the distinct role of PtdIns(3)P in phagosome maturation. An early phagosome transition to phagolysosome typically involves the early phagosomal acquisition of EEA1, followed by the late phagosomal/phagolysosomal acquisition of LAMP1; however, phagosomes in macrophages pretreated with wortmannin show severely compromised EEA1 and LAMP1 acquisition. This implies that PtdIns(3)P, although an early endosomal/phagosomal PtdInsP, is critical for the complete maturation of early phagosomes to phagolysosomes (Figure 1.4) [40,64].

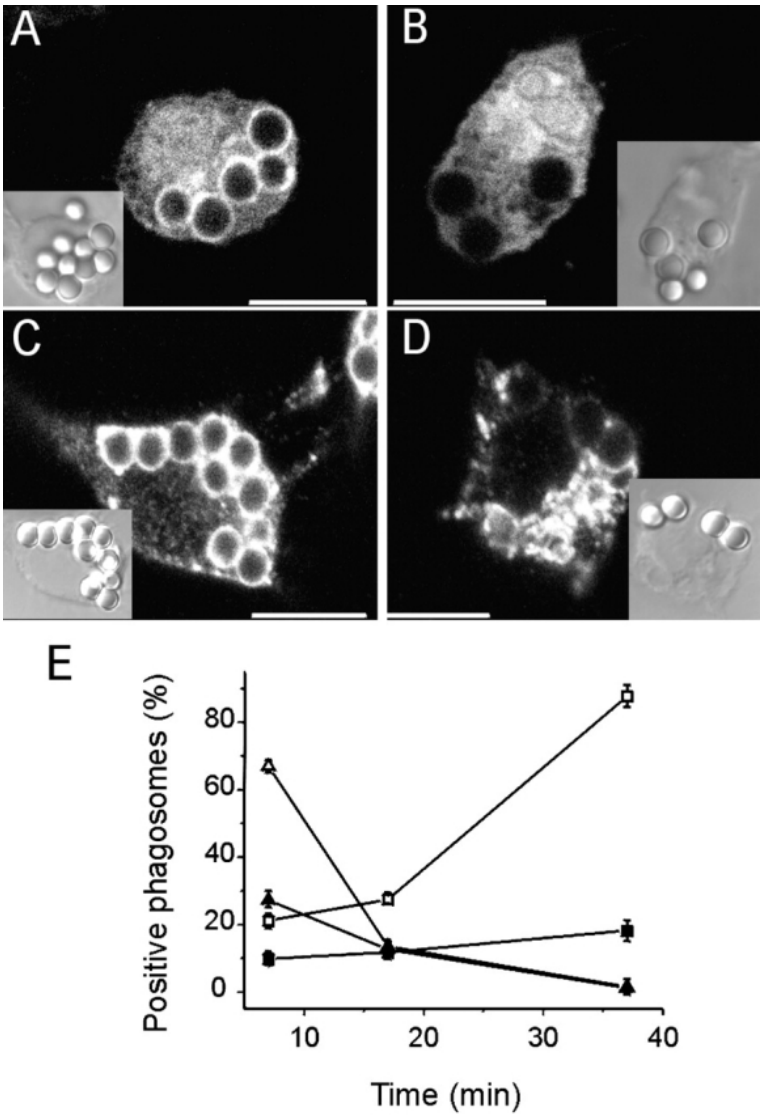

Figure 1.4 Effects of wortmannin on phagosomal acquisition of PtdIns(3)P, EEA1, and LAMP1

RAW macrophages, untreated (A, C, and open symbols in E) or pretreated with 100 nM wortmannin for 30 min (B, D, and solid symbols in E), were allowed to phagocytose IgG-opsonized beads for 7 min. The cells were transfected with 2FYVE-GFP and fixed immediately after phagocytosis (A-B) or immunostained for LAMP1 after an additional 30 min chase following phagocytosis (C-D). DIC image (inset). Bar, 10 μm. Effects of wormannin on phagosomal acquisition of EEA1 (triangles) or LAMP1(squares) were quantified after a 7 min phagocytosis pulse (as above), followed by incubation at 37℃ for the indicated chase time points. Macrophages were immunostained with antibodies to EEA1 or LAMP1 (E) [40].

PtdIns(3)P expression on early phagosomal membranes is only transient, and it is reasonable to assume that this PtdInsP is converted to other lipid products over time. There are a myriad different enzymes, lipid kinases and phosphatases, that make this happen. Members of the myotubularin family dephosphorylate PtdIns(3)P to form PtdIns [65], whereas FYVE finger-containing PtdInsP kinase (PIKfyve) promotes the conversion of PtdIns(3)P to PtdIns(3,5)P_2 [66]. In fact, Hazeki *et al*. identified the indispensable roles of PTEN and PIKfyve in elimination of PtdIns(3)P from the early phagosomal membranes [67].

1.9 Phosphatidylinositol 3,5-bisphosphate

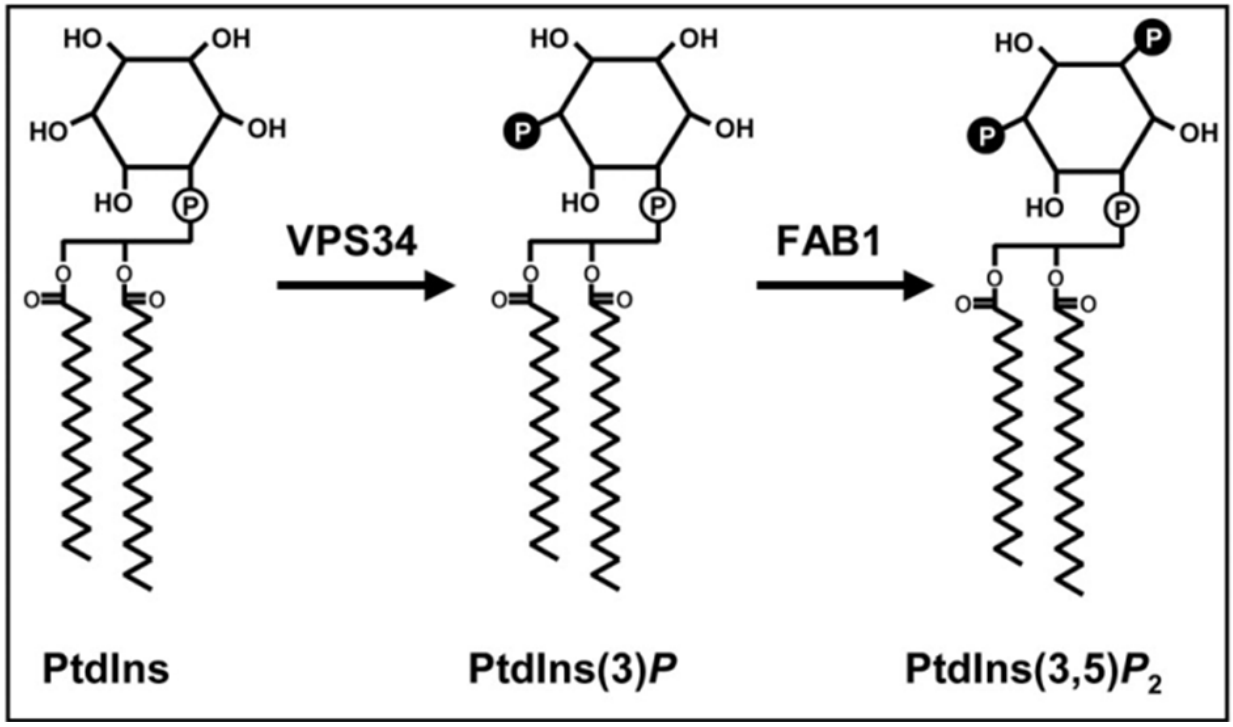

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Figure 1.5 Synthesis of PtdIns(3)P and PtdIns(3,5)P_2
The sole member of class III PI3K, Vps34, phosphorylates PtdIns on the hydroxyl group of its third carbon to form PtdIns(3)P, whereas PIKfyve (the mammalian orthologue of FAB1) promotes the conversion of PtdIns(3)P to PtdIns(3,5)P_2 (see next page). [68]

PIKfyve is a mammalian FYVE finger-containing PtdInsP kinase, which phosphorylates PtdIns(3)P at the 5-position to form PtdIns(3,5)P_2 (Figure 1.5) [66]. This multi-domain enzyme has a conserved N-terminal FYVE domain that specifically binds PtdIns(3)P [69]. In conjunction with PtdIns(3)P, this module is necessary for PIKfyve localization to late endosomes [70]. For this reason, late endosomes are thought to be the principle sites of PtdIns(3,5)P_2 accumulation [58]. Consistent with this idea, PIKfyve colocalizes with a marker that cycles between TGN and MVB, CI-MPR, whereas it is negative for such recycling endosomal markers as transferrin receptor (TfR) and glucose transporter type IV (GLUT4) [71].

Some of the more prominent morphological changes observed in PIKfyve-inhibited cells have already been established. To illustrate, PtdIns(3,5)P_2 synthesis is important to the maintenance of endolysosome size. Various cell lines transfected with a mutant form of PIKfyve that is devoid of its catalytic kinase activity (PIKfyve^{K1831E} mutant) show multiple enlarged endolysosomes that are absent in the control (Figure 1.6). These endolysosomes that are initially smaller in size, rounder in shape and cluster at perinuclear regions eventually fuse to grow bigger in size, smaller in number and spaciously distribute themselves around the whole cytoplasm [72].

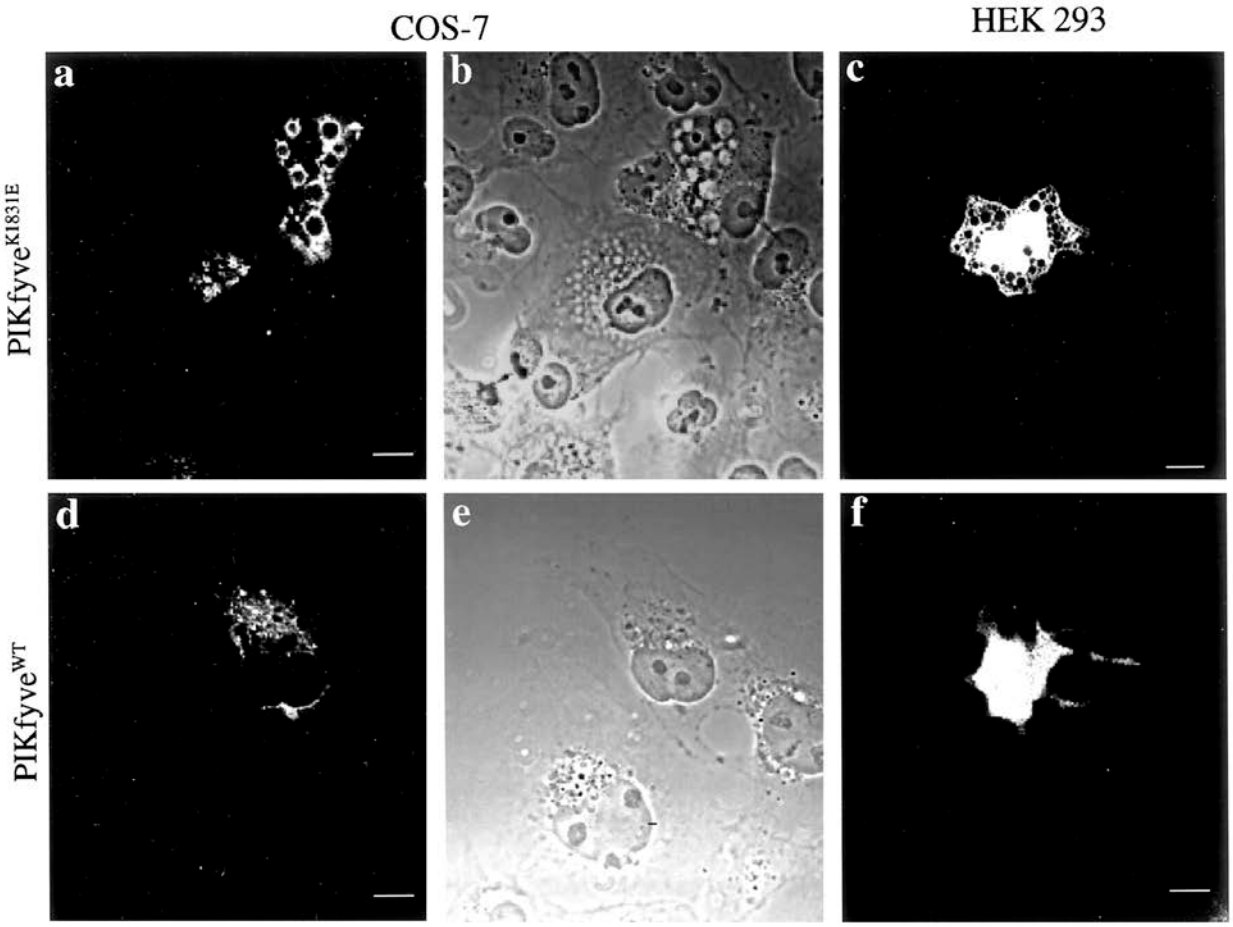

Figure 1.6 Enlarged endolysosomes in kinase-dead PIKfyve^{K1831E}-expressing COS7 and HEK293 cells

COS7 cells (a-b, d-e) and HEK293 cells (c and f) were transiently transfected with HA-PIKfyve^{K1831E} or HA-PIKfyve^{WT}. COS7 cells were fixed and immunostained with anti-HA antibody 32 h posttransfection. HEK293 cells were visualized live 48 h posttransfection. Cells transfected with PIKfyve^{K1831E} mutant show multiple enlarged endolysosomes that are absent in HA-PIKfyve^{WT}-expressing cells. Bar, 10 μm. [72]

Based on previous findings, there is much evidence to suggest that these endolysosomes are derived from late endosomes of the membrane trafficking pathway [72]; however, there are also studies that suggest these endolysosomes originate from early endosomes [73,74]. More research is

needed to unravel some of the lingering uncertainties surrounding the identity of these endolysosomes.

At present, our knowledge of PtdIns(3,5)P_2 effector proteins is quite limited. Nonetheless, a mammalian homologue of the yeast Svp1, WD repeat domain containing, phosphoinositide-interacting 1 (WIPI1) protein is among the best characterized [75,76]. WIPI1 localizes to trans-elements of the Golgi and peripheral endosomal membranes to assist in PtdIns(3,5)P_2-mediated endosome-to-TGN retrograde transport [76]. Moreover, PIKfyve also interacts with p40, a Rab9 effector protein, that is required for the retrograde transport of CI-MPR from endosomes to the TGN, further supporting the claim that PIKfyve is involved in this pathway [77].

Aside from these functions, PtdIns(3,5)P_2 pathway is selectively activated in hyper-osmotically stressed 3T3-L1 adipocytes [78]. Furthermore, the more recently discovered roles of PtdIns(3,5)P_2 in the regulation GLUT4 exocytosis [79–81], autophagy [82,83], ion transport [84–88], and transporter proteins like creatine transporter (CreaT) [89], and the excitatory amino acid transporter 4 (EAAT4) [90] further adds to the importance of studying this PtdInsP.

1.10 The role of phosphatidylinositol 3,5-bisphosphate in phagosome maturation

Up to date, the presence of PtdIns(3,5)P_2 on phagosomes remains largely unknown [91]. Phagosome maturation involves the transient expression of PtdIns(3)P on early phagosomal membranes. Subsequently, PtdIns(3)P may be converted to PtdIns(3,5)P_2 by the lipid kinase PIKfyve. Thus, it is unclear if the role of PtdIns(3)P in phagosome maturation is direct and/or indirect, through the synthesis of PtdIns(3,5)P_2. If PtdIns(3,5)P_2 has a role to play in phagosome maturation, then could it be that the role of PtdIns(3)P in phagosome maturation is to provide PtdIns(3,5)P_2? Or does it work in conjunction with PtdIns(3,5)P_2?

There are three potential models that can be devised to explain the phagosomal pathway.

1. PtdIns(3)P plays a direct role in phagosome maturation, and PtdIns(3,5)P_2 is not required for this pathway
2. The role of PtdIns(3)P in phagosome maturation is to synthesize PtdIns(3,5)P_2, which in turn, plays a direct role in phagosome maturation.
3. PtdIns(3)P and PtdIns(3,5)P_2 both take part in the transition of early phagosomes to phagolysosomes, perhaps at distinct stages of phagosomal traffic.

The present study strived to determine which one of these models best portrays the individual roles of PtdIns(3)P and PtdIns(3,5)P_2 in the phagosomal system in macrophages. The role of PtdIns(3,5)P_2 in the endosomal system in macrophages also remain largely uncharacterized and was studied in this thesis.

1.11 Hypothesis

PtdIns(3,5)P_2 confers identity to late endosomal membranes. Late endosomes fuse with late phagosomes to provide them with their properties like degradative and acidic properties to digest particles. Thus, it is likely that late phagosomes also acquire PtdIns(3,5)P_2 to coordinate the later stages of phagosome maturation. In the same way, we hypothesize that PtdIns(3,5)P_2 plays a major role in the endolysosomal system in macrophages.

1.12 Objectives

This study employs a pharmacological approach to dissect the individual roles of PtdIns(3)P and PtdIns(3,5)P_2 in the endosomal and phagosomal systems in macrophages. PIKfyve was pharmacologically inhibited with MF4. This acute inhibition of PIKfyve allowed us

to examine the direct roles of PtdIns(3,5)P_2 rather than its potential indirect roles due to the chronic loss of PIKfyve.

To determine the role of PtdIns(3,5)P_2 in the endosomal system in macrophages, we first strived to define the identity of enlarged endolysosomes induced by PIKfyve inhibition. We also examined the effects of PIKfyve inhibition on endolysosomal properties, focusing on the degradative capacity and acidic environment of lysosomes. We then characterized the effects of PIKfyve inhibition on endocytosis (both fluid-phase and receptor-mediated endocytosis), endosome recycling, and endosome maturation (by examining the rate of trafficking of aggregated IgG immune complexes to lysosomes).

Our second main objective was to determine the role of PtdIns(3,5)P_2 in the phagosomal system in macrophages. In doing so, we studied the effects of PIKfyve inhibition on phagocytosis and phagosomal acquisition of LAMP1.

2 Methods and Materials

2.1 Plasmid transformation

Plasmid deoxyribonucleic acids (DNAs) used in this study were GFP-2FYVE [40], GFP-Rab5 [92], red fluorescent protein (RFP)-Rab7 [93], and LAMP1-mcherry. The plasmids were transformed into DH5α competent cells [F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1] (Invitrogen) for DNA extraction and purification. One microliter of plasmid DNA was added to 50 µl of DH5α competent cells and incubated on ice for 30 min. Cells were heat shocked at 42 °C for 45 sec, followed by immediate incubation on ice for 2 min. One millilitre of Luria-Bertani (LB) media was added to the transformation reaction and incubated at 37 °C for 1 h. Two hundred microliters of transformed cells were plated on LB agar plates containing the appropriate antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated at 37 °C overnight.

2.2 Extraction and Purification of plasmid DNA

A single colony of bacteria (see above) was inoculated in 50 ml of LB medium containing 100 µg/ml ampicillin or 50 µg/ml kanamycin and incubated at 37 °C for 12-16 h with shaking. The overnight bacterial culture was pelleted by centrifugation at 5000 g for 10 min at room temperature. All plasmid DNAs were extracted and purified using E.Z.N.A.® Plasmid Midiprep Kit (Omega Bio-Tek) as per manufacturer's instructions.

2.3 Tissue culture

Unless indicated otherwise, RAW264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) (1X) supplemented with high glucose, L-glutamine, sodium

pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) (Gibco) at 37 °C with 5 % CO_2. Roswell Park Memorial Institute (RPMI) medium 1640 (1X) supplemented with L-glutamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (HPMI medium) (Gibco) was used in the absence of CO_2 for bead binding and live-cell imaging.

2.4 Pharmacological treatments

Prior to experiment, cells were incubated with MF4 (a gift from Dr. Kevan Shokat, University of California San Francisco, San Francisco, California, United States) [94] at 37 °C for 2 or 6 h at a final concentration of 100 nM. Preincubation with LY294002 (Promega) was done on ice for 30 min (during bead binding) at a final concentration of 100 μM. Drug incubation was kept for the whole duration of the experiment.

2.5 Transient transfection

Prior to transfection, cells were seeded onto coverslips in the wells of a 6 well plate to reach 50-60 % confluency. Plasmid DNAs were transfected into cultured cells using jetPEI®-Macrophage (Polyplus transfection) as per manufacturer's instructions and analyzed 24 h later.

2.6 Endocytosis Assays

Prior to endocytosis, cells were seeded into the wells of a 6-well plate to reach confluency. After MF4 treatment for 2 h, cells were incubated with 0.1 mg/ml fluorescein isothiocyanate (FITC)-conjugated dextran (Molecular Probes) at 37 °C for 15 min or 30 min. Subsequently, cells were washed twice with phosphate buffered saline (PBS) to remove excess dextran and fixed with 4 % paraformaldehyde (PFA) (MP Biomedicals) at room temperature for

20 min. The extent of cargo internalization was assessed using FACScalibur flow cytometer (BD Biosciences). Likewise, BODIPY FL-conjugated LDL (10 μg/ml; Molecular Probes) was added to cultured cells and incubated at 37 °C for 15 min or 30 min to assess receptor-mediated endocytosis.

The uptake of aggregated IgG was assayed by heating 10 mg/ml human IgG (Sigma-Aldrich) at 62 °C for 20 min, followed by centrifugation at 16,000 g for 10 min at room temperature to sediment insoluble complexes. Cells were incubated with 1 mg/mL soluble aggregated IgG at 37 °C for 15 min. Subsequently, cells were washed briefly with PBS to remove excess aggregated IgG. Cells were fixed with 4 % PFA for 20 min, quenched with 100 mM glycine for 20 min, and permeabilized with 0.5 % Triton X-100 for 10 min. Internalized aggregated IgG was visualized by labeling the IgG complexes with donkey anti-human Cy2, Cy3, or Cy5-conjugated antibodies (Cedarlane Laboratories) at 1:1000 dilution for 1 h. All aforementioned steps were performed at room temperature.

2.7 IgG-opsonized bead binding and phagocytosis assays

Crosslinked poly(styrene/divinylbenzene) (P[S/DVB]) polymer beads (Bangs Laboratories, Inc.), 3.87 μm in mean diameter, were opsonized with 10 mg/ml human IgG in PBS at room temperature for 1-2 h rotating. Opsonized beads were washed 3 times in PBS with centrifugation at 2000 rpm for 1 min between each wash. To synchronize phagocytosis, beads were bound to cells on ice. Beads were resuspended in PBS and added to the individual wells on ice for 20 min. Subsequently, cells were washed twice with PBS to remove any unbound beads and incubated at 37 °C for 15 min or 30 min for phagocytosis. Cells were then fixed with 4 % PFA for 20 min, quenched with 100 mM glycine for 20 min, and stained with donkey anti-

human Cy2, Cy3, or Cy5-conjugated antibodies at 1:1000 dilution for 20 min to discriminate internalized beads from externally bound beads. At least 100 phagosomes were counted per condition to determine the phagocytic index and efficiency.

$$\text{Phagocytic efficiency} = \frac{\text{number of macrophages with at least one phagosome}}{\text{total number of macrophages}}$$

$$\text{Phagocytic index} = \frac{\text{number of phagosomes}}{\text{total number of macrophages}}$$

2.8 Lysosome labeling

Lysosomes were labeled with dextran by preloading cells with 2 μM Alexa647-conjugated dextran (Molecular Probes) at 37 °C for 1 h. Cells were then washed briefly with PBS to remove excess dextran and incubated in dye-free media at 37 °C for a subsequent chase period of 2 h. For labeling of lysosomes with Lysotracker Red (Molecular Probes), cells were incubated with 1 μM Lysotracker Red at 37 °C for 5 min just prior to live-cell imaging.

2.9 Proteolysis assays

To determine the degradative capacity of lysosomes, cells were allowed to co-endocytose 10 μg/ml DQ Green BSA (Molecular Probes) and 0.1 mg/ml tetramethylrhodamine isothiocyanate (TRITC)-conjugated dextran or 2 μM Alexa647-conjugated dextran at 37 °C for 15 min. Subsequently, cells were given a chase period of 0, 30, or 60 min. Fluorophore-conjugated dextran was internalized to normalize for the possible difference in endocytosis between untreated and MF4-treated cells. Cells were then fixed with 4 % PFA for 20 min and quenched with 100 mM glycine for 20 min for microscopy. The extent of lysosomal proteolysis was further characterized using flow cytometry by gating to isolate Alexa647-conjugated

dextran-positive, endocytosis-competent macrophages, then quantifying the average ratio of the mean fluorescence of DQ Green BSA over the mean fluorescence of Alexa647-conjugated dextran for each condition.

2.10 Immunofluorescence

Endosome and phagosome trafficking assays were performed by further incubating the cells in DMEM (containing drug but free of TRITC-conjugated dextran, DQ Green BSA, aggregated IgG or beads) at 37 °C for 0, 15, 30, or 60 min after 15 min of cargo internalization at 37 °C. After the chase periods, cells were fixed with 4 % PFA for 20 min, quenched with 100 mM glycine for 20 min, and permeabilized with 0.5 % Triton X-100 for 10 min or -20 °C methanol for 5 min in the case of LAMP1 staining. Cells were washed 3 times with 0.5 % bovine serum albumin (BSA)/PBS prior to and after incubation with rat anti-LAMP1[1D4B] antibodies (Developmental Studies Hybridoma Bank; 1:100 dilution in PBS) at room temperature for 1 h. This was followed by goat anti-rat antibody (Molecular Probes) staining at 1:1000 dilution in PBS at room temperature for another hour. Finally, cells were washed and incubated with 0.5 % BSA/PBS every 5 min for 30 min. Cells were visualized for the presence of any fluorochrome labeled molecules using confocal microscopy.

2.11 Confocal microscopy and image processing

Cells were visualized on Zeiss LSM 510 META laser scanning microscope. This instrument is equipped with an Argon laser (458/477/488/514 nm), a HeNe1 laser (543 nm), and a HeNe2 laser (633 nm). Plan-Apochromat 100x/1.40 oil objective was used to collect images. In order to prevent saturated pixels during image acquisition, detector gain and amplifer offset were

adjusted by selecting *Palette/Range Indicator*. The softwares, Adobe Photoshop (v. 7.0.1, Adobe Systems Inc.) or ImageJ (v. 1.47 bundled with 64-bit Java) were used to visualize image collected from each fluorescent channel. Confocal fluorescent images obtained were pseudo-color processed where necessary in order to better categorize phagosomes as being either molecular marker-positive, partial, or negative (positive – bright, yellow fluorescent rims; partial - weak, red fluorescent rims or half-moon-shaped incomplete fluorescent rims; negative – no fluorescent rims). This was done using ImageJ by enabling "Fire" in *Image/Look up Tables.* Colocalization analyses were done using ImageJ with the plugins, JACoP (Just Another Colocalization Plugin) or Colocalization Indices under the *Plugins* function. For both plugins, automated thresholding methods were employed.

2.12 Flow cytometry

Endocytosis of fluorochrome labeled molecules was quantified using BD FACSCalibur flow cytometer. Samples of suspended cells in solution were run through the flow cytometer until 10,000 events were collected per condition. Data obtained using flow cytometry was analyzed using the softwares, Flowjo (TreeStar Inc.) or Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology, Finland). RAW macrophages were selectively gated using the forward scatter channel (FSC)/ side scatter channel (SSC) density plot. The intrinsic autofluorescence background in macrophages was set such that not more than 5 % of fluorochrome-absent macrophages would be sorted as staining positive. For endocytosis and endosome recycling assays, the mean fluorescence intensity of the macrophage population was quantified for each condition. For lysosome proteolysis assay, a two-parameter (dual-colour fluorescence) histogram was plotted with DQ Green BSA (FITC channel) in the x-axis and

Alexa647-conjugated dextran (PE channel) in the y-axis. In Alexa647-conjugated dextran-positive subset of the macrophage population, the mean fluorescence intensity of DQ Green BSA over the mean fluorescence intensity of Alexa647-conjugated dextran was quantified for each condition.

2.13 Statistical Analyses

Significance of differences was calculated in Microsoft Excel using paired samples t tests. A p value less than 0.05 was considered statistically significant.

3 Results

3.1 PIKfyve inhibition induces enlargement of endolysosomes in macrophages

As mentioned previously, PtdIns(3,5)P_2 synthesis is important to the maintenance of endolysosome size [72,95]. Up to now, the origin of the dilated endolysosomes has never been investigated in macrophages. Macrophages generally exhibit a more dynamic membrane trafficking than other cell types. Accordingly, their endolysosomal trait may be different compared to that of the previously examined cell lines.

As expected, we observed that macrophages treated with MF4 for 6 h formed multiple enlarged vacuoles that are absent in untreated cells. To define the identity of these enlarged vacuoles, we examined the intracellular localizations of various endosomal markers (early endosomal markers- 2FYVE and Rab5; late endosomal markers- LAMP1 and Rab7) in PIKfyve-inhibited macrophages using transient transfection of plasmid DNAs.

According to Figure 3.1, we observed that, in macrophages, the enlarged vacuoles induced by PIKfyve inhibition stained positive for late endosomal markers like LAMP1 and Rab7, whereas they did not acquire 2FYVE and Rab5, which are common early endosomal markers. Thus, PtdIns(3,5)P_2 synthesis is important to the maintenance of late endosome and lysosome size, but not for early endosome size in macrophages.

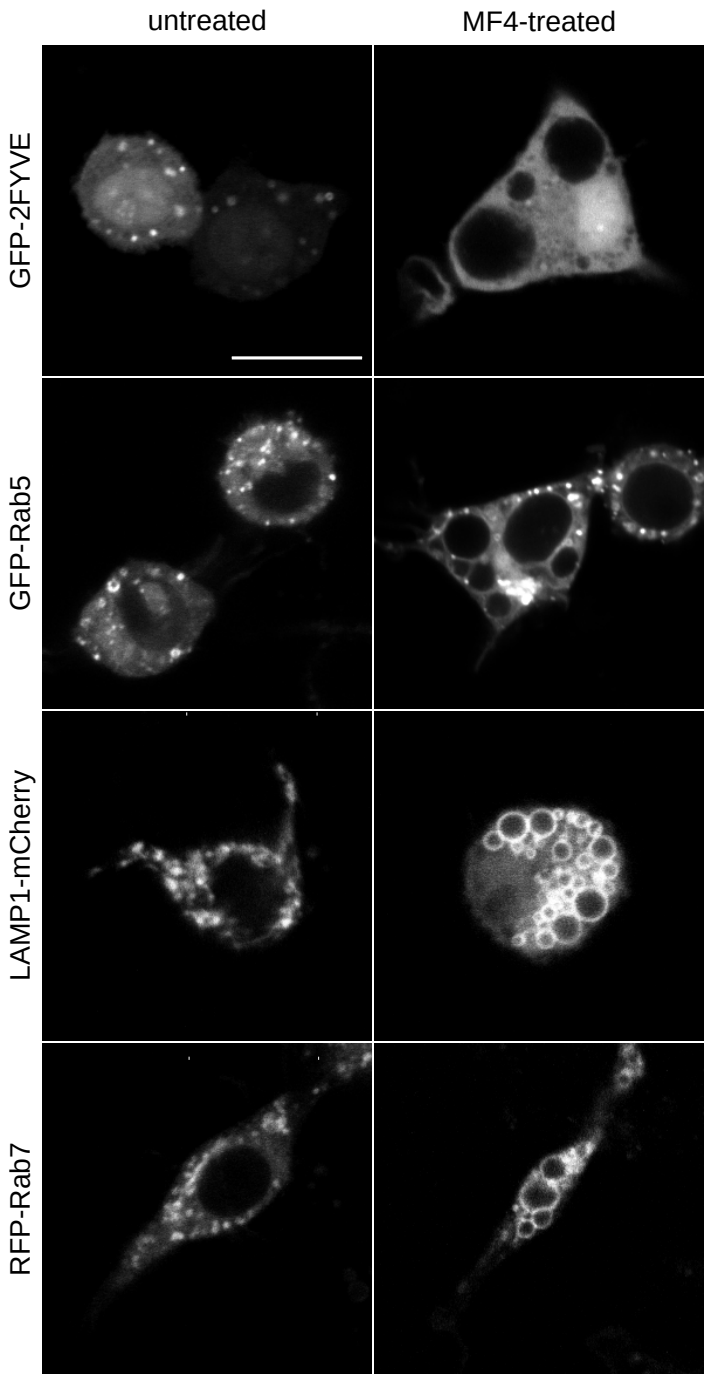

Figure 3.1 Distribution of endosomal and lysosomal markers in untreated and MF4 (6 h)-treated macrophages

RAW macrophages were transiently transfected with various fluorescence-conjugated endosomal markers (GFP-2FYVE, GFP-Rab5, LAMP1-mcherry, and RFP-Rab7). Subsequently, cells were treated with or without 100 nM MF4 for 6 h. 2FYVE and Rab5 are early endosomal markers. LAMP1 and Rab7 are late endosomal and lysosomal markers. Bar, 10 μm.

3.2 PIKfyve-inhibited macrophages display decelerated ability to degrade cargo molecules despite their lysosomes remaining acidic.

Seeing that the endolysosomes exhibit such a grossly enlarged morphology upon 6 h MF4 treatment, we further explored whether the degradative capacity and acidic environment of lysosomes were retained in PIKfyve-inhibited macrophages. To assess lysosomal acidity in PIKfyve-inhibited macrophages, a fluorescent probe called Lysotracker Red was used. Due to its fluorophore conjugation to a weak base, Lysotracker Red stains acidic organelles within the cell. Lysosomes were loaded with FITC-conjugated dextran by 1 h pulse and 2 h chase. Subsequently, cells were incubated with Lysotracker Red for 5 min just prior to live-cell imaging. Using Pearson's correlation coefficient to quantify colocalization of fluorescence derived from FITC-conjugated dextran, a marker of lysosomes, and Lysotracker Red, we conclude that lysosomes remain acidic in PIKfyve-inhibited macrophages ($r=0.519\pm0.072$; untreated macrophages $r=0.431\pm0.055$) (Figure 3.2).

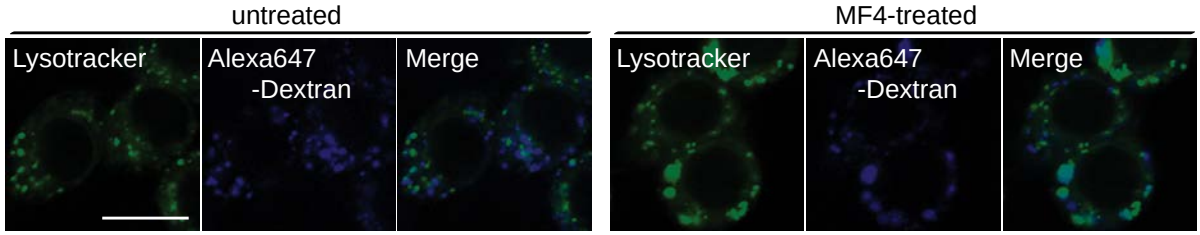

Figure 3.2 Lysosome acidification in PIKfyve-inhibited macrophages
Lysosomes in RAW macrophages, untreated or treated with 100 nM MF4 for 2 h, were loaded with Alexa647-conjugated dextran by 1 h pulse and 2 h chase. Subsequently, cells were incubated with 1 μM Lysotracker Red for 5 min just prior to live-cell imaging. Colocalization of fluorescence derived from Alexa647-conjugated dextran and Lysotracker Red was observed by confocal microscopy. Bar, 10 μm.

To assess lysosomal degradation in the absence of PtdIns(3,5)P_2 synthesis, macrophages were allowed to endocytose DQ Green BSA for 15 min. DQ Green BSA proteins, which are heavily conjugated with BODIPY FL fluorophores, are initially strongly quenched. Proteolytic digestion of the proteins by lysosomal enzymes causes the release of isolated fluorophores, which then fluoresce green. In order to ensure that a possible defect in lysosomal proteolytic activity is not confounded by altered endocytosis in PtdIns(3,5)P_2-depleted macrophages, TRITC-dextran was co-endocytosed with DQ Green BSA. Lysosomal degradation was also characterized using flow cytometry by quantifying the average ratio of the mean fluorescence of DQ Green BSA over the mean fluorescence of Alexa647-conjugated dextran in Alexa647-conjugated dextran-positive subset of macrophages for each condition.

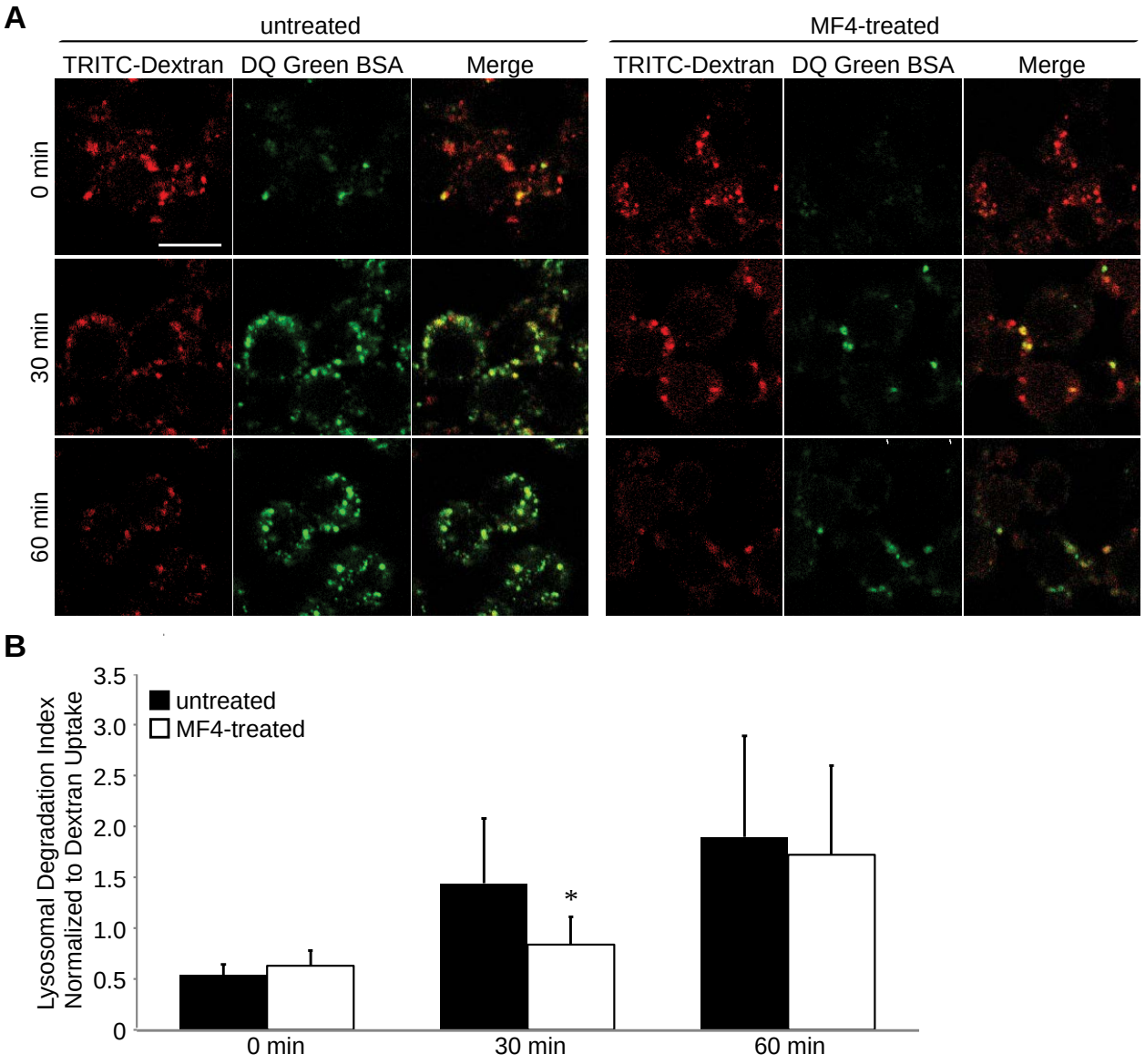

Figure 3.3 Lysosome degradation in PIKfyve-inhibited macrophages

RAW macrophages, untreated or treated with 100 nM MF4 for 2 h, were allowed to co-endocytose DQ Green BSA and TRITC-conjugated dextran (immunofluorescence) or Alexa647-conjugated dextran (flow cytometry) for 15 min, followed by the indicated chase time points. Dextran was internalized to normalize for the possible differences in fluid phase endocytosis between untreated and MF4-treated macrophages. Bar, 10 μm. (A) The average ratio of the mean fluorescence of DQ Green BSA over the mean fluorescence of Alexa647-conjugated dextran in Alexa647-conjugated dextran-positive subset of macrophages was quantified for each condition using flow cytometry. The data are means ± standard deviation of six experiments (B).

Based on our confocal microscopy data, there seemed to be a notable decline in the activity of lysosomal proteases in PIKfyve-inhibited macrophages. Typically, 30 min chase time point was sufficient for lysosomal acquisition of degradative properties. We observed that by 30 min chase time point, there was significantly less fluorescence associated with DQ Green BSA in PIKfyve-inhibited macrophages compared to control (Figure 3.3A). Accordingly, it was evident that lysosomal degradation index normalized to dextran uptake was reduced in MF4-treated macrophages at 30 min chase time point compared to that of the untreated group (n=6, p=0.0269); however, by 60 min chase time point, lysosomal degradation was comparable to control (n=6, p=0.3139) (Figure 3.3B), suggesting that PIKfyve-inhibited macrophages' decelerated ability to degrade cargo molecules is not a permanent defect and is eventually restored over time to reach normal levels. Therefore, the degradative capacity of lysosomes appears to be retarded in PtdIns(3,5)P_2 synthesis-inhibited macrophages.

3.3 PIKfyve is required for non receptor-mediated fluid-phase endocytosis, but not LDL endocytosis, one example of receptor-mediated endocytosis

Images obtained from our lysosomal degradation assay suggested that there was reduced TRITC-conjugated dextran uptake in PIKfyve-inhibited macrophages. For this reason, we further investigated the role of PtdIns(3,5)P_2 in fluid-phase endocytosis. Indeed, the notable decline in the activity of lysosomal proteases in PIKfyve-inhibited macrophages observed by confocal microscopy was later revealed to have been due to a fluid-phase endocytosis defect in these cells, which was confirmed using flow cytometry. The uptake of FITC-conjugated dextran was compromised in those macrophages treated with MF4 for 2 h, as early as 15 min after the onset of dextran incubation (n=5, p=0.0246), and this effect was even more pronounced with 30 min pulse (n=5, p=0.0081) (Figure 3.4).

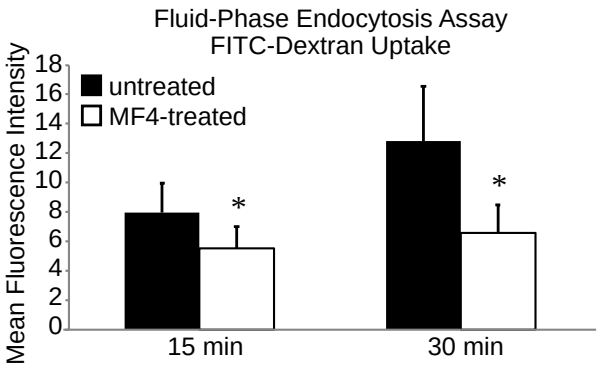

Figure 3.4 Effect of PIKfyve inhibition on fluid-phase endocytosis
RAW macrophages, untreated or treated with 100 nM MF4 for 2 h, were allowed to endocytose FITC-conjugated dextran for 15 min or 30 min. The mean fluorescence intensity was quantified for each condition by flow cytometry. The data are means ± standard deviation of five experiments.

As shown above, fluid-phase endocytosis was perturbed by PIKfyve inhibition. To determine whether or not receptor-mediated endocytosis is also affected by PIKfyve inhibition, the same endocytosis assay was performed with a receptor-engaging cargo, LDL conjugated to BODIPY FL fluorophores.

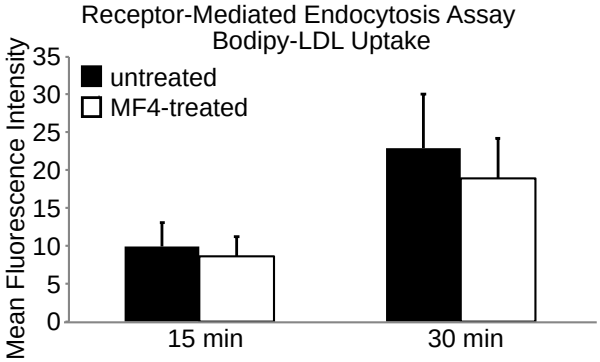

Figure 3.5 Effect of PIKfyve inhibition on LDL endocytosis, one example of receptor-mediated endocytosis
RAW macrophages, untreated or treated with 100 nM MF4 for 2 h, were allowed to endocytose BODIPY FL-conjugated LDL for 15 min or 30 min. The mean fluorescence intensity was quantified for each condition by flow cytometry. The data are means ± standard deviation of four experiments.

From Figure 3.5, we conclude that PIKfyve activity, unlike its role in fluid-phase endocytosis, is not necessary for LDL endocytosis, one example of receptor-mediated endocytosis. The intracellular accumulation of BODIPY FL-conjugated LDL was similar between untreated and MF4-treated macrophages at both 15 min and 30 min pulse times (n=4 for both pulse times, p=0.0827; p=0.0513).

3.4 Fluid-phase endocytosis defect observed in PIKfyve-inhibited macrophages does not appear to be due to a recycling defect in these cells

There are two possible ways of interpreting the apparent fluid-phase endocytosis defect in PIKfyve-inhibited macrophages. The lower accumulation of FITC-conjugated dextran may be a result of reduced endocytic activity, but it is also possible that there is accelerated efflux of FITC-conjugated dextran upon PIKfyve inhibition. To address this possibility, macrophages were allowed to endocytose FITC-conjugated dextran for 15 min, followed by 0, 5, 10, 15, and 20 min chase. The amount of FITC-conjugated dextran internalized by 0 min chase time point was normalized to 1 for each condition, and the amount recycled was measured using flow cytometry.

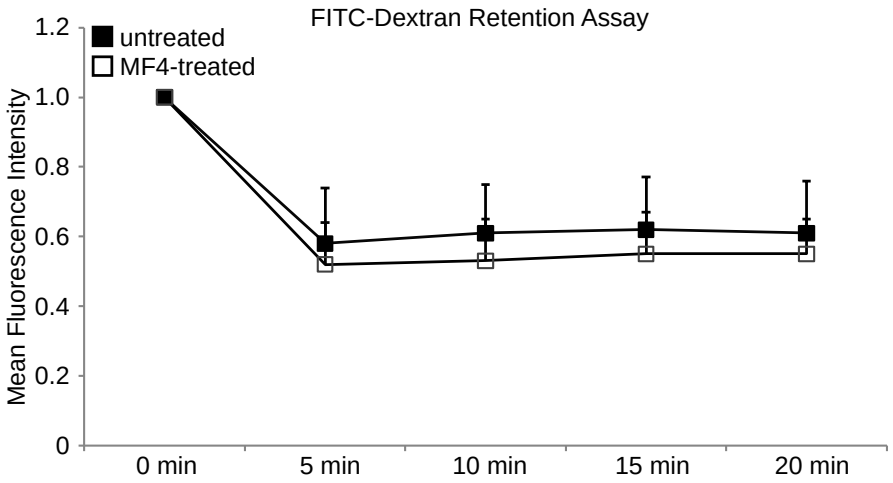

Figure 3.6 Effect of PIKfyve inhibition on FITC-conjugated dextran retention
RAW macrophages, untreated or treated with 100 nM MF4 for 2 h, were allowed to endocytose FITC-conjugated dextran for 15 min, followed by the indicated chase time points. The mean fluorescence intensity was quantified for each condition by flow cytometry. The data are means ± standard deviation of four experiments.

Our preliminary data suggested that the amount of FITC-conjugated dextran recycled was not significantly different between untreated and MF4-treated groups (n=4 for all chase time points; 5 min p=0.3151; 10 min p=0.1064; 15 min p=0.0577; 20 min p=0.0970) (Figure 3.6). Therefore, the apparent fluid-phase endocytosis defect observed in PIKfyve-inhibited macrophages is likely not due to an increase in FITC-conjugated dextran efflux. Nonetheless, it should be noted that this experiment may require additional repetitions to provide a more conclusive result.

3.5 Aggregated IgG-loaded endosome maturation is impeded in PIKfyve-inhibited macrophages

Endosome maturation and trafficking to lysosomes were assessed by quantifying the colocalization of endosomal aggregated IgG with LAMP1 staining for late endosomal or lysosomal compartments. Using untreated macrophages as a model for normal endosomal

trafficking, there appeared to be less colocalization between aggregated IgG and LAMP1 in PIKfyve-inhibited macrophages (Figure 3.7A).

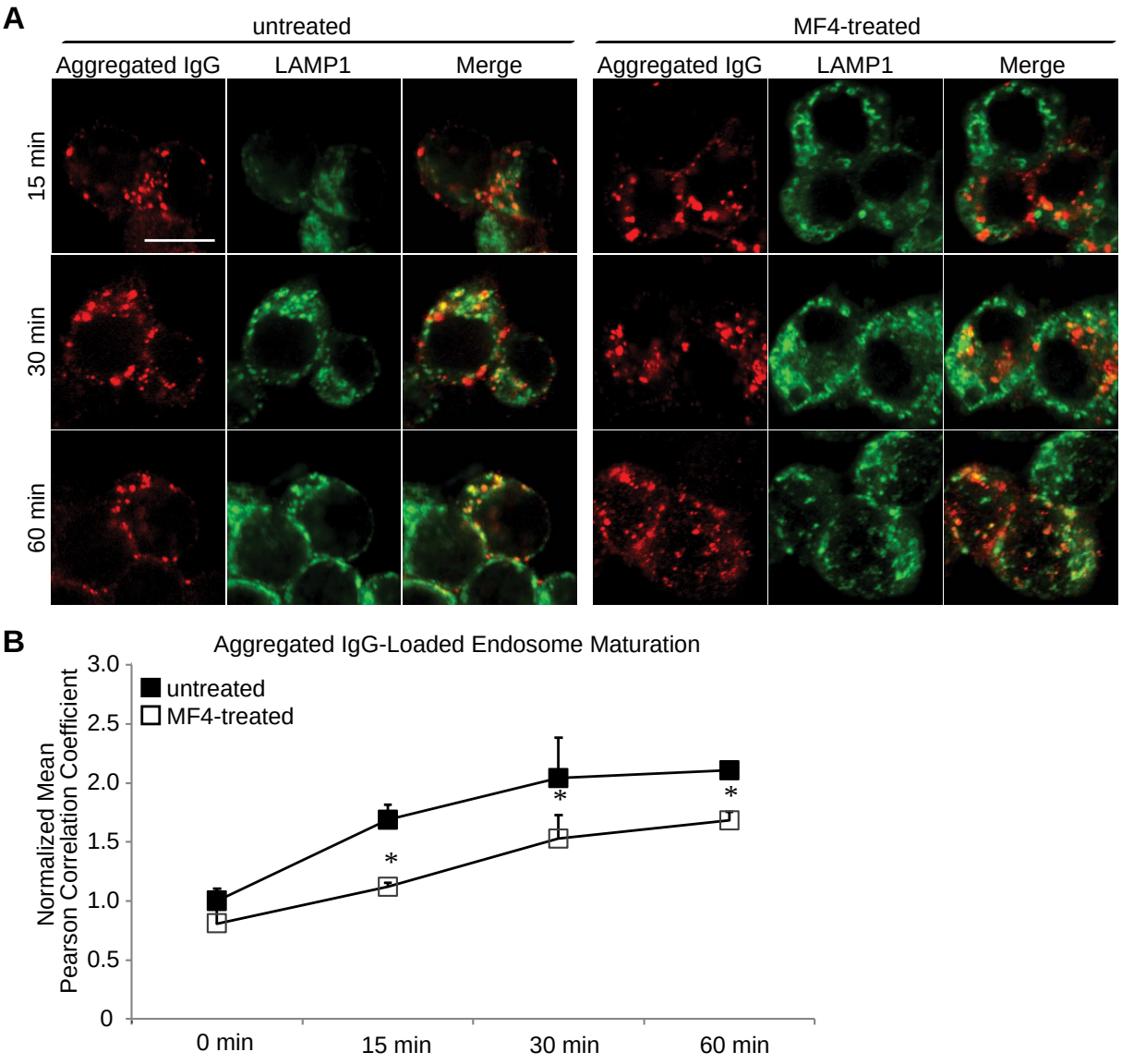

Figure 3.7 Effect of PIKfyve inhibition on trafficking of aggregated IgG immune complexes to lysosomes

(A) RAW macrophages, untreated or treated with 100 nM MF4 for 2 h, were allowed to endocytose aggregated IgG for 15 min, followed by the indicated chase time points. Subsequently, cells were fixed and immunostained for LAMP1. Bar, 10 μm. (B) Colocalization of aggregated IgG and LAMP1 was quantified by calculating the normalized mean Pearson's correlation coefficient for each condition. The data are means ± standard deviation of three experiments, at least ten cells individually analyzed per experiment.

Indeed, there was a notable decline in the rate of aggregated IgG-loaded endosome maturation in macrophages that are deficient in PtdIns(3,5)P_2. Using normalized mean Pearson's correlation coefficient as a quantitative measure, we observed that colocalization of aggregated IgG and LAMP1 was significantly reduced in PIKfyve-inhibited macrophages at 15 min, 30 min, and 60 min chase time points (n=3, p=0.0194; n=3, p=0.0280; n=2, p=0.0251) (Figure 3.7B) . These values indicate that aggregated IgG-loaded endosome maturation is impeded in PIKfyve-inhibited macrophages.

3.6 An acute inhibition of PIKfyve does not affect phagocytosis by macrophages.

Previous studies have focused primarily on the role of PtdIns(3,5)P_2 in the regulation of endosomal membrane traffic. It is now widely accepted that PtdIns(3,5)P_2 confers identity to late endosomal membranes. The expression of PtdIns(3,5)P_2 on phagosomes, however, still remains elusive. Seeing that late endosomes frequently fuse with late phagosomes to provide them their appropriate endosomal markers and PtdInsPs, we hypothesized that, these late phagosomes acquire PtdIns(3,5)P_2 and coordinate the later stages of phagosome maturation.

Consistent with this hypothesis, our findings on the role of PtdIns(3,5)P_2 in the phagosomal system suggested that even if PtdIns(3,5)P_2 was to have an effect on the phagosomal pathway, it would not intervene at the onset of phagocytosis, but rather, during the later stages of phagosome maturation (see Section 3.7).

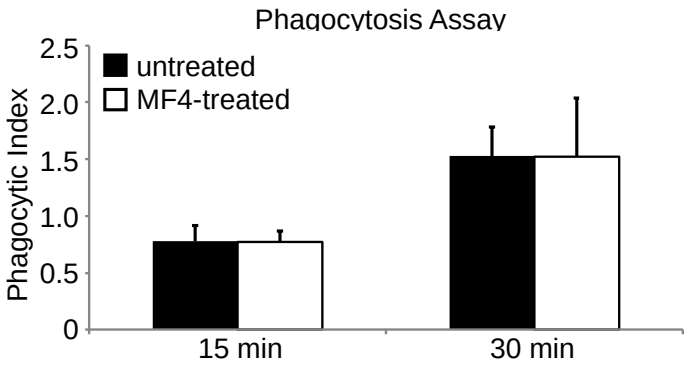

Figure 3.8 Effect of PIKfyve inhibition on phagocytosis
RAW macrophages, untreated or treated with 100 nM MF4 for 2 h, were allowed to phagocytose IgG-opsonized beads for 15 min or 30 min. Phagocytic index of each condition was calculated, each with at least 100 phagosomes counted. The data are means ± standard deviation of three experiments.

As shown in Figure 3.8, an acute inhibition of PIKfyve did not affect the phagocytic activity of macrophages, seeing that their phagocytic efficiency (data not shown) and index (n=3 for both pulse times; 15 min p=0.9013; 30 min p=0.9855) remained unperturbed by 2 h MF4 treatment.

3.7 PtdIns(3)P plays a more dominant role in phagosome maturation than PtdIns(3,5)P_2.

Typically, the conversion of nascent phagosomes into phagolysosomes involves the late phagosomal/phagolysosomal acquisition of a late endosomal or lysosomal marker, LAMP1. Previous studies have already demonstrated the requirement for PtdIns(3)P in phagosomal acquisition of LAMP1 [40]. This observation was reproduced to, once again, confirm that PtdIns(3)P is required for the sequential recruitment of various endosomal markers to phagosomes. In doing so, macrophages were pretreated with LY294002, a selective inhibitor of PI3K, for 30 min and allowed to phagocytose IgG-opsonized beads for 15 min. As described by Vieira *et al*., at 60 min chase time point, when late phagosomal/phagolysosomal acquisition of

LAMP1 peaks, we observed a severe decline in the ability of LAMP1 to associate with phagosomes in LY294002-treated macrophages compared to control (n=3, LAMP1 positive p=0.0397) .

On the other hand, most phagosomes acquired LAMP1 in MF4-treated macrophages; however, the intensity of fluorescence associated with phagosomal acquisition of LAMP1 seemed weaker in these cells. In order to better categorize phagosomes as being either molecular marker-positive, partial, or negative, confocal images obtained were pseudo-color processed using ImageJ by enabling "Fire" in *Image/Look up Tables.* Indeed, at 60 min chase time point, there were significantly fewer LAMP1 positive phagosomes in PIKfyve-inhibited macrophages (n=7, p=0.0019). Instead of acquiring bright, yellow fluorescent rims around them (strongly positive for LAMP1), phagosomes in PIKfyve-inhibited macrophages generally acquired weaker, red fluorescent rims or half-moon-shaped incomplete fluorescent rims around them, which we designated as being LAMP1 partial. From this, we conclude that PtdIns(3,5)P_2 synthesis inhibition causes a moderate defect in phagosomal acquisition of LAMP1 (Figure 3.9).

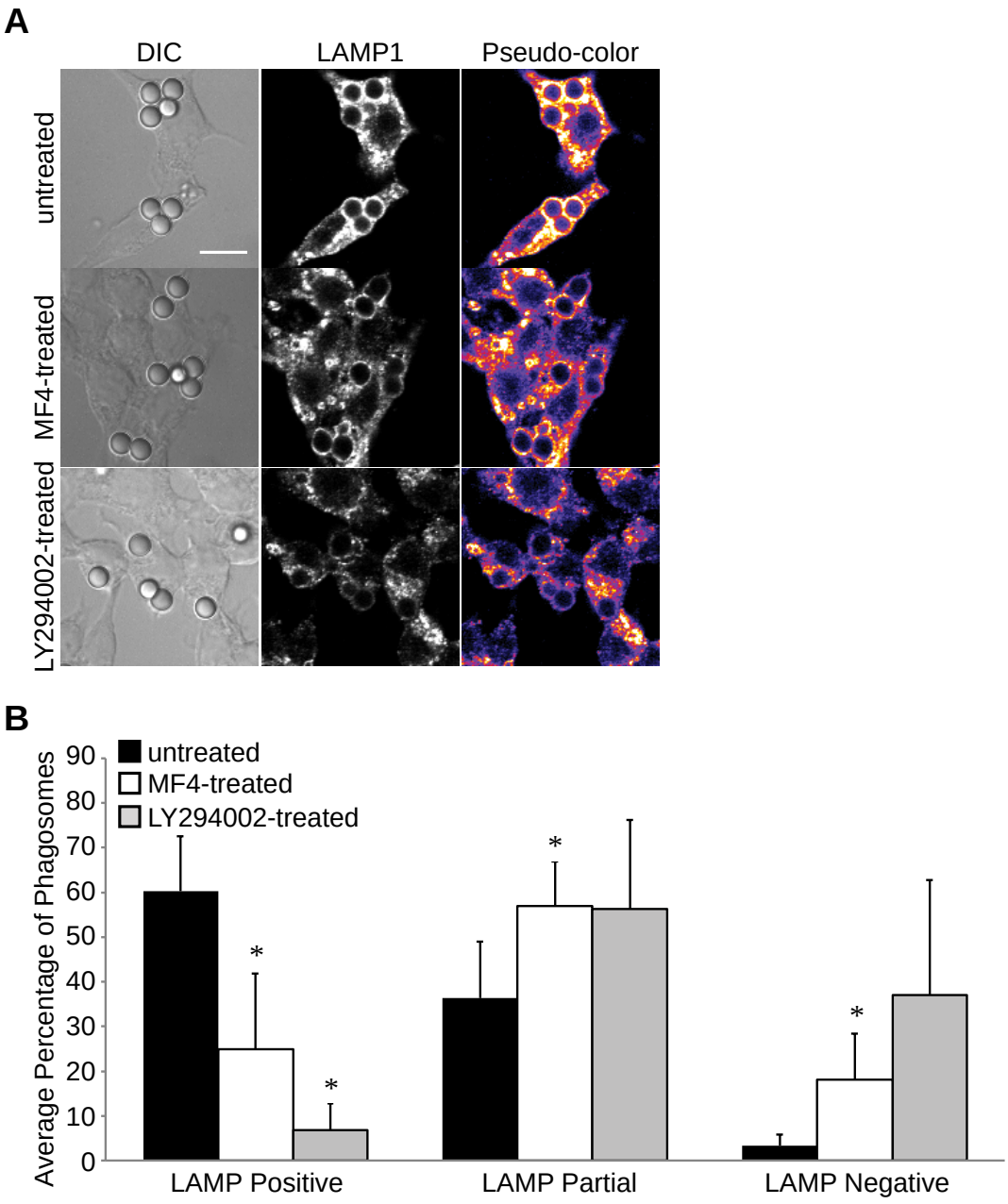

Figure 3.9 Effect of PIKfyve inhibition on phagosomal acquisition of LAMP1
(A-B) RAW macrophages, either untreated, treated with 100 nM MF4 for 2 h, or treated with 100 μM LY294002 for 30 min, were allowed to phagocytose IgG-opsonized beads for 15 min, followed by 1 h chase. Subsequently, cells were fixed and immunostained for LAMP1. Images obtained were pseudo-color processed in order to better categorize and quantify phagosomes as being strongly positive, partial, or negative for LAMP1. Bar, 10 μm. The data are means ± standard deviation of at least three experiments per treatment.

PtdIns(3)P plays an important role in phagosome maturation [40]. The role of PtdIns(3,5)P_2 in these events, however, seemed to have less of an impact, seeing that its effect was less pronounced than that of PtdIns(3)P. Nevertheless, PtdIns(3,5)P_2 also appears to partake in regulating the phagosomal pathway.

4 Discussion

4.1 The endosomal and phagosomal systems in macrophages

Macrophages protect our body from infection by invading microbes. The engulfment of foreign pathogens through processes known as endocytosis and phagocytosis is an initial step toward eliciting an appropriate cellular immune response. These pathogens are ultimately degraded in the lysosomes and phagolysosomes after a series of endosome and phagosome maturation processes.

PtdInsPs play essential roles in the traffic of membranes. For example, as described previously, PtdIns(3)P is transiently expressed on nascent phagosomes, and this event is critical for the successive maturation of these phagosomes [40]. Many microbial pathogens take advantage of the host endocytic and phagocytic pathways for their successful invasion. To illustrate, *Mycobacterium tuberculosis* blocks phagolysosome biogenesis by secreting SapM, a lipid phosphatase that hydrolyzes PtdIns(3)P, to inhibit phagosome maturation in macrophages [96].

For this reason, it is crucial to understand and appreciate the distinct roles of individual PtdInsPs (more specifically, PtdIns(3)P and PtdIns(3,5)P_2, since they confer identity to early and late endosomes, respectively) in the endosomal and phagosomal systems in macrophages and address some implications of exploiting their functions in the host immune system.

4.2 The identity of the enlarged vacuoles induced by PIKfyve inhibition

PtdIns(3,5)P_2 synthesis by the lipid kinase activity of PIKfyve is required for the maintenance of endolysosome size [72,97]. PIKfyve mutants devoid of its lipid kinase activity induce the formation of dilated endolysosomes that are absent in the wild-type. These swollen endolysosomes are important components of the endocytic pathway because we have evidence to

conclude that they are derived from late endosomal compartments. To illustrate, Ikonomov *et al*. displayed a substantial overlap between PIKfyve^{K1831E} and CI-MPR around the dilated endolysosomes in PIKfyve^{K1831E}-expressing COS-7 cells, which implied that these endolysosomes originated from the membranes of late endosomes and prelysosomal compartments [72]. In contrast, some reports suggest that early endosomes are enlarged in PIKfyve-inhibited macrophages. To illustrate, *fab1* mutant cells (Fab1p is the yeast equivalent of PIKfyve) show enlarged Hrs-positive early endosomes in *Drosophila melanogaster* [74]. For this reason, we aimed to characterize the vacuole trait in RAW264.7 macrophages.

In this study, we conclude that PIKfyve inhibition induces the enlargement of endolysosomes in macrophages (Figure 3.1). This supports the existing assumption that recycling of late endosomal and prelysosomal membranes may be defected in PIKfyve-inhibited cells [75].

4.3 The role of PtdIns(3,5)P_2 in endocytosis

The role of PtdIns(3,5)P_2 in the endosomal system in macrophages is noteworthy, in that we found that fluid-phase endocytosis is significantly decreased upon PIKfyve-inhibition (Figure 3.4). Ikonomov *et al*. demonstrated a similar observation, where prelysosomal fluid-phase endocytosis of HRP is inhibited in HEK293 cell line induced to express PIKfyve^{K1831E} mutant. Consistent with our observations, the inhibitory effect of PIKfyve inhibition on fluid-phase endocytosis becomes more pronounced over time [98].

It is interesting to note, however, that LDL endocytosis is unperturbed by PIKfyve inhibition (Figure 3.5). This suggests that bulk-flow endocytosis and receptor-mediated endocytosis engage separate driving forces to trigger cargo uptake. This is not suprising, seeing

that Ikonomov *et al*. also observed a similar phenomenon, where prelysosomal fluid-phase endocytosis of HRP is depressed, but receptor-mediated endocytosis of ^{125}I-labeled transferrin (^{125}I-Tf) is unaffected by the expression of PIKfyve^{K1831E} mutant in HEK293 cell line [98]. Likewise, the uptake of ^{125}I-labelled EGF (^{125}I-EGF) is also not significantly perturbed in PIKfyve-suppressed HeLa cells [73].

Recycling of receptor ligands, such as ^{125}I-EGF and ^{125}I-Tf , is also not dependent on PIKfyve activity [73,98], which corresponds to our finding that the fluid-phase endocytosis defect observed in PIKfyve-inhibited macrophages does not appear to be a result of the recycling defect in these cells (Figure 3.6). Although there is seemingly an inhibitory trend associated with the role of PtdIns(3,5)P_2 in recycling of fluid-phase markers, it is statistically insignificant. Nonetheless, more research is needed to confidently conclude that endosome recycling is unperturbed in PIKfyve-inhibited macrophages.

4.4 The role of PtdIns(3,5)P_2 in endosome maturation

The fluid-phase endocytosis defect in PIKfyve-inhibited macrophages is accompanied by a decelerated ability of macrophages to degrade cargo molecules (Figure 3.3) despite their lysosomes remaining acidic (Figure 3.2). It is reasonable to speculate that PtdIns(3,5)P_2 is required for the efficient degradation of cargo molecules, seeing that the activity of lysosomal hydrolases is significantly delayed upon PIKfyve inhibition. This may be due to the involvement of PtdIns(3,5)P_2 in endosome-to-TGN retrograde membrane traffic [73]. The transport of newly synthesized lysosomal hydrolases from TGN to late endosomes requires the addition of mannose 6-phosphate (M6P) groups to the N-linked oligosaccharides of these enzymes. The M6P markers are then recognized by transport receptors like CI-MPR that shuttle between TGN and

late endosomes [99]. For this reason, endosome-to-TGN retrograde transport of CI-MPR is essential for the subsequent endosomal delivery of lysosomal hydrolases that bind this receptor as means of their transport. Once in the prelysosomal compartment, these enzymes disengage from CI-MPR in order to provide an adequate degradative environment for the maturing prelysosome [100]. In PIKfyve-suppressed cells, the steady-state TGN distribution of CI-MPR that is observed under normal conditions [101], is disrupted, suggesting that the retrograde fission events may be defected in the absence of PIKfyve activity [94]. Likewise, a defect in cargo trafficking between endosomes and TGN in PIKfyve-inhibited macrophages can lead to a subsequent defect in endosomal delivery of lysosomal hydrolases, as seen by the reduction in fluorescence associated with DQ Green BSA in MF4-treated macrophages (Figure 3.3).

Lysosome acidification, however, appears to be normal in PIKfyve-inhibited macrophages (Figure 3.2). Interestingly, vacuole acidification is proposed to be abated in yeast lacking Fab1p, the yeast equivalent of PIKfyve [95]. These conflicting results may arise from the presumption that PtdIns(3,5)P_2 exhibits different functions in yeast versus higher eukaryotes. Then again, more recently, my colleague, Shannon Ho, is observing that Fab1p may not be required for yeast vacuole acidification (personal communication).

We also observe that macrophages perturbed for PIKfyve activity display retarded trafficking of aggregated IgG immune complexes to lysosomes (Figure 3.7). Perhaps the requirement for PIKfyve in MVB sorting accounts for this delay in membrane trafficking. Just as an example, the lipid kinase activity of Fab1p is necessary for sorting type II integral membrane protein carboxypeptidase S (CPS) into the vacuole lumen via the MVB pathway in yeast [102]. Likewise, PtdIns(3,5)P_2 is required for MVB inward vesiculation and storage of internal vesicles in HEK293 cells [98]; however the role of PtdIns(3,5)P_2 in MVB sorting for degradation is not as

simple as it seems, seeing that the delivery of EGFR to lysosomes and its degradation appear unaffected by PIKfyve^{K1831E} expression [98]. Further investigation is required to address why PtdIns(3,5)P_2 imposes a kinetic delay to the transport of one receptor type and not to the other, and which key effector proteins PtdIns(3,5)P_2 interacts with in order to prompt such an effect. Inconsistencies may depend on the cell model or method used.

4.5 The role of PtdIns(3,5)P_2 in phagocytosis

As mentioned previously, phagocytosis by macrophages is unaffected by an acute inhibition of PIKfyve (Figure 3.8). This is in accordance with Hazeki *et al.*'s finding, where the rate of sheep RBC uptake was unchanged in PIKfyve-deficient cells expressing shRNA against PIKfyve [67]. Typically, 15 min pulse will assess for a possible kinetic defect in phagocytosis, whereas extending this time interval to 30 min will assess whether or not there is a defect in the cells' total phagocytic capacity by looking at saturation conditions. Based on this notion, we conclude that 2 h of PIKfyve inhibition is insufficient to induce a defect in either the rate or the capacity of phagocytic activity by macrophages.

Nonetheless, upon prolonged inhibition of PIKfyve, it may not be surprising to predict a role of PtdIns(3,5)P_2 in phagocytosis. PtdIns(3,5)P_2 is required for mucolipin transient receptor potential protein 1 (TRPML1) activation. TRPML1, primarily localized to late endosomes and lysosomes [103–105], is a Ca^{2+}-permeable cation channel that releases Ca^{2+} in response to PtdIns(3,5)P_2 increase [88] and regulates membrane trafficking in the late endocytic pathway [106]. Among its many functions, TRPML1 is involved in regulating lysosomal exocytosis to provide membranes necessary for phagosome biogenesis [107]. During phagocytosis, plasmalemmal pseudopods extend and fuse at the anterior tips of target particles to form nascent phagosomes.

This leads to increased membrane tension at the site of cargo uptake [108], and TRPML1, in response to PtdIns(3,5)P_2 elevation, acts to alleviate this tension by delivering lysosomal membranes to the cell surface, allowing more phagocytosis to occur [107]. Likewise, prolonged PIKfyve inhibition may negatively regulate phagocytic activity by macrophages by preventing TRPML1 activation, which is fundamental to efficient cargo uptake.

4.6 The role of PtdIns(3,5)P_2 in phagosome maturation

Previous work supported by the present study unequivocally demonstrate that PtdIns(3)P plays an important role in phagosome maturation [40,64]. The direct role of PtdIns(3,5)P_2 in this pathway, however, seems less prominent, since LAMP1 appears partially on phagosomes in PIKfyve-inhibited cells (Figure 3.9).

While it appears that PI3K is more important for phagosome maturation than PIKfyve, PI3K inhibition deprives PIKfyve of its substrate, PtdIns(3)P; thus, a portion of the defect induced by LY294002 treatment may be, at least in part, due to impaired PIKfyve function. The additive effects of the lack of both PtdIns(3)P and PtdIns(3,5)P_2 may have caused a more dramatic effect in PtdIns(3)P-depleted macrophages. In agreement with this notion, the lack of PtdIns(3,5)P_2 also causes a moderate defect in phagosomal acquisition of LAMP1, which leads to the conclusion that PtdIns(3,5)P_2 may also play a contributing role in the phagosomal pathway.

As mentioned previously, PtdIns(3,5)P_2 is involved in a variety of endolysosomal functions along the endocytic pathway. These include such processes as endosome-to-TGN retrograde transport, MVB sorting, and TRPML1 activation in the endolysosome. TRPML1 releases Ca^{2+} in response to PtdIns(3,5)P_2 increase [88], which in turn triggers a series of membrane fission and fusion events that is necessary to mediate lysosome-lysosome homotypic

fusion [109] and late endosome-lysosome [110] and phagosome-lysosome [111] heterotypic fusions. For this reason, upon PIKfyve inhibition, the absence of PtdIns(3,5)P_2 that is crucial to bind and activate TRPML1 may decrease the level of juxtaorganellar Ca^{2+}, thereby suppress membrane fusion activities that are required for phagosome maturation. To illustrate, with the lack of lysosome-lysosome homotypic fusion and late endosome-lysosome and phagosome-lysosome heterotypic fusions, proper exchange of essential late endosomal markers between endosomal and phagosomal compartments may be perturbed, retarding the progression of phagosomes to phagolysosomes. Likewise, it is reasonable to speculate that PtdIns(3,5)P_2 recruits multiple effector proteins to perform its late phagosomal and phagolysosomal functions.

4.7 Concluding remarks

Endosomal phosphoinositides play inevitable roles in the traffic of membranes. Consistent with our hypothesis, PtdIns(3,5)P_2 appears to principally coordinate the later stages of endosome and phagosome maturation. PIKfyve inhibition causes massive swelling of late endosomes/lysosomes. This is accompanied by a decelerated ability of macrophages to degrade cargo molecules despite their lysosomes remaining acidic. We also observe that macrophages perturbed for PIKfyve activity have retarded trafficking of aggregated IgG immune complexes to lysosomes. In the phagosomal system, PIKfyve-inhibited macrophages exhibit normal phagocytic activity; however, there appears to be a moderate defect in phagosomal acquisition of LAMP1 during the later stages of the phagosomal pathway. Overall, PtdIns(3,5)P_2 plays substantial roles in the macrophage endosomal and phagosomal systems.

5 Future Studies

5.1 Acute versus chronic effects of PIKfyve inhibition

In this study, much of the work focused on exploring the direct responses of macrophages to PIKfyve inhibition. Two hours of MF4 incubation is sufficient to trigger PIKfyve inhibition; however, to examine the effects of a prolonged loss of PtdIns(3,5)P_2, different approaches are needed. One way to address this problem is to extend the duration of MF4 pre-treatment, and thereby inhibit PIKfyve for a longer period of time. Likewise, we can study the chronic effects of the lack of PtdIns(3,5)P_2 by using small interfering RNA (siRNA) to selectively eliminate PIKfyve. My colleague, Monica Dayam, is currently utilizing these techniques to examine how our current observations are modulated by prolonged inhibition of PIKfyve.

Our data suggest that prolonged inhibition of PIKfyve has an inhibitory role in phagocytosis by macrophages. Moreover, its effect on phagosomal acquisition of LAMP1 is even more profoundly perturbed, supporting the role of PtdIns(3,5)P_2 in the phagosomal system in macrophages (personal communication).

5.2 Using digestible cargo molecules to study phagosomal fission and degradation

One of the most obvious limitations of using an indigestible cargo model like polymer beads is that it is difficult to observe the full life cycle of an internalized particle. Lysosomal degradation involves a combination of multiple processes, which include particle digestion, phagosome fission, fragmentation and disappearance. It will be worthwhile to follow phagosome breakdown and degradation using a digestible cargo type. We are currently collaborating with Akriti Prashar and Dr. Mauricio Terebiznik at University of Toronto Scarborough to track these processes with digestible RBCs by conjugating them to DQ Green BSA.

5.3 Verifying the role of PtdIns(3,5)P_2 in phagosome maturation using other techniques

Moreover, one caveat in the use of a pharmacological inhibitor MF4 is that PIKfyve may catalyze the synthesis of two lipid products, PtdIns(3,5)P_2 and phosphatidylinositol 5-phosphate [PtdIns(5)P]. Accordingly, pharmacological inhibition of PIKfyve does not take into consideration the accompanied synthesis inhibition of PtdIns(5)P. At present, it is unfeasible to dissociate the effect of PtdIns(3,5)P_2 synthesis inhibition from the effect of the lack of PtdIns(5)P upon PIKfyve inhibition. Although we assume that PtdIns(3,5)P_2 synthesis is inhibited upon MF4 treatment (owing to the fact that we observe dilated endolysosomes in MF4-treated macrophages, which is an altered phenotype associated with the absence of PtdIns(3,5)P_2), it is possible that some of the findings observed in this study may due to the absence of PtdIns(5)P rather than PtdIns(3,5)P_2. It will be valuable to employ other techniques to verify the role of PtdIns(3,5)P_2 in the endosomal and phagosomal systems in macrophages.

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