Regulation of Phagocyte Immunity by Lipid Signals

by

Daniel Schlam

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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Doctor of Philosophy
Institute of Medical Science
University of Toronto
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Abstract

Phagocytosis, the process by which relatively large particles are internalized into a membrane-bound compartment known as the phagosome, is paramount to both fighting infection and maintaining tissue homeostasis. Professional phagocytes routinely contend with a large assortment of targets, ranging from small pathogenic bacteria to large fungal networks. Non-professional and professional phagocytes also cooperate in the daunting yet critical task of clearing hundreds of billions of apoptotic cells on a daily basis. By promptly removing effete cells, phagocytes prevent secondary necrosis and the onset of autoimmunity, aid in tissue remodelling during wound healing, and orchestrate morphogenesis throughout development. To carry out these functions, phagocytes must be able to effectively find, engulf and dispose of their prey. In this dissertation, I focus on the phagocytic response from the conceptual stages of: 1) capture, 2) uptake and 3) degradation—all presented as self-contained chapters—while placing particular emphasis on the mechanisms by which glycerophospholipids and diacylglycerols regulate these processes. I provide evidence that phosphatidic acid, which is uniquely enriched in the plasmalemma of professional phagocytes, facilitates the continuous membrane ruffling that underlies the uptake of particulate and fluid-phase antigen. Similarly, I argue that phosphatidylinositol-3,4,5-trisphosphate
biosynthesis signals actin remodelling during the uptake of large particles, thereby mediating the orderly progression of pseudopodia around large targets. I then discuss the role of diacylglycerol kinases in controlling deployment of the NADPH oxidase, thus fine-tuning the compromise between eliciting effective antimicrobial responses and preventing oxidative damage to the host. Notably, phosphoinositide signalling is often subverted by invading microorganisms in order to avoid phagocyte recognition. The final study in this thesis, also a self-contained chapter, describes a phosphoinositide-centric mechanism by which gliotoxin, an *Aspergillus fumigatus* mycotoxin, precludes macrophage immune defences.
Acknowledgments

Being able to express—genuinely and in only a few pages—gratitude towards those that made a positive difference in one’s journey through life seems to me like a pretty difficult task. While these words may seem superficial, they are meant to sincerely thank those that dramatically changed the way that I think about life, conduct science and relate to the world.

I first want to thank those closest to me: members of my family, including Samantha, my parents, as well as my twin and older sisters. Having myself experienced the difficulties of leaving my family behind in Mexico in order to pursue science in Canada, I can only imagine how difficult my absence must have been for them as well. However, they were always loving, supportive and encouraging of my dreams, regardless of how different these were from theirs. Samantha was particularly strong, tolerating my consistent lateness and obsession with research. I am forever grateful for her love.

Second, I want to thank Sergio for almost 10 years of continued guidance and support. He was my first contact in Canada when I arrived here in 2006, confused and disoriented. Sergio was the covert driving force behind my pursuit of molecular biology as an undergraduate at McMaster University. Later on, he was the reason why I was able to so thoroughly enjoy my doctoral studies in cell biology. Anyone who has met Sergio would agree that he is a truly unique individual; not only does he have one of the sharpest minds I will ever encounter, he is also deeply humanistic, very hard working and has a great sense of humour. I can only hope that some of his attributes are contagious, and that I have absorbed some of his skills and knowledge during the past years.

Third, I want to thank Dr. Gregory Fairn for his unconditional supervision and unbelievable mentoring skills. Greg always found time for teaching, coaching and simplifying concepts for me, despite his busy schedule. I will always think of him as a role model, mentor and friend.

Fourth, I want to acknowledge the members of my Program Advisory Committee: Dr. Bebhinn Treanor, Dr. Michael Glogauer and Dr. Myron Cybulsky. They are exceptional
scientists who acted as my informal supervisors, providing valuable feedback, asking provocative questions and offering constructive criticism. Similarly, I want to thank Dr. Kodi Ravichandran for agreeing to act as my external examiner, appraising this dissertation, and missioning all the way to Toronto from Virginia to attend my Ph. D. defence.

Finally, I want to express my gratitude to every one of my lab members, past and present. They were not only outstanding friends and mentors, but also empathetic, smart, helpful, creative, funny and supportive individuals who I will always remember with a smile.
Contributions

I was responsible for executing the great majority of experiments described throughout this dissertation. Dr. Sergio Grinstein, Dr. Gregory Fairn and I designed these experiments, as well as analyzed the resulting data. For all chapters, Dr. Grinstein and Dr. Fairn played instrumental roles in the conception and interpretation of experiments. They also greatly assisted in the editing of all manuscripts resulting from this body of work.

Chapter 1. General introduction: Lipid signals in phagocytosis and macropinocytosis.

Roni Levin reviewed and collated the primary literature, wrote part of the manuscript and assisted with the making of figures.

Chapter 2. Phosphatidic acid is required for the constitutive ruffling and macropinocytosis of phagocytes.

Dr. Michal Bohdanowicz, a primary co-author in this published chapter, was critical for several aspects; he designed and performed a large number of experiments, as well as prepared various figures. Dr. Martin Hermansson collected the mass spectrometry data. David Rizzuti assisted with the preparation of primary dendritic cell cultures. Dr. Takehiko Ueyama provided the constructs encoding diacylglycerol kinases. Dr. Guangwei Du developed the phosphatidic acid biosensor.

Chapter 3. Phosphoinositide 3-kinase enables phagocytosis of large particles by coordinating actin disassembly through Rac/Cdc42 GTPase-activating proteins.

Dr. Richard Bagshaw cloned the fluorescent RhoGAP library. Dr. Spencer Freeman devised critical experiments, helped troubleshooting and edited the manuscript. Richard Collins assisted with site-directed mutagenesis. Dr. Tony Pawson was instrumental for the scientific framework behind the cloning of the GAP constructs.
Chapter 4. Diacylglycerol kinases terminate the respiratory burst and establish heterogeneity in phagosomal NADPH oxidase activation.

Dr. Michal Bohdanowicz assisted with troubleshooting and the optimization of numerous experiments. Dr. Alexandros Chatgilialoglu helped devise the assay for superoxide production by individual phagosomes. Dr. Takehiko Ueyama provided the constructs encoding diacylglycerol kinases. Dr. Guangwei Du developed the phosphatidic acid biosensor.

Chapter 5. Gliotoxin supresses macrophage immune function by subverting PtdIns(3,4,5)P3 homeostasis.

Dr. Johnathan Canton helped with the flow cytometry assays and time-lapse microscopy determinations. Marvin Carreño performed experiments relating to F-actin quantification and phosphoinositide distribution. Hannah Kopinski assessed the effect of gliotoxin exposure on the macrophage phagocytic capacity. Dr. Spencer Freeman conceived critical experiments and helped troubleshooting.
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<tr>
<td>2PABD</td>
<td>Tandem phosphatidic acid-binding domain of Spo20p</td>
</tr>
<tr>
<td>Alexa 555-SE</td>
<td>Alexa Fluor 555-succinimidyl ester</td>
</tr>
<tr>
<td>APPL1</td>
<td>Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1</td>
</tr>
<tr>
<td>Arp</td>
<td>Actin related protein</td>
</tr>
<tr>
<td>BAI1</td>
<td>Brain-specific angiogenesis inhibitor 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine-serum albumin</td>
</tr>
<tr>
<td>CalDAG-GEFI</td>
<td>Calcium and DAG-regulated guanine nucleotide exchange factor I</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CTB</td>
<td>Clostridium difficile toxin B</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DGK</td>
<td>Diacylglycerol kinase</td>
</tr>
<tr>
<td>DGKi I</td>
<td>Diacylglycerol kinase inhibitor I; R59022</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOCK2</td>
<td>Dedicator of cytokinesis protein 2</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyleneiodonium</td>
</tr>
<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, radixin, moesin</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>EYFP</td>
<td>Enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FIPI</td>
<td>5-fluoro-2-indolyl des-chlorohalopemide</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506-binding protein</td>
</tr>
<tr>
<td>FRB</td>
<td>FKBP-rapamycin-binding domain</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDI</td>
<td>GDP-dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IA</td>
<td>Invasive aspergillosis</td>
</tr>
<tr>
<td>iDC</td>
<td>Immature dendritic cell</td>
</tr>
<tr>
<td>IgG-E</td>
<td>IgG-coated erythrocyte</td>
</tr>
<tr>
<td>IgG-RBC</td>
<td>IgG-opsonized red blood cell</td>
</tr>
<tr>
<td>IgG-zymo-Alexa</td>
<td>IgG-opsonized zymosan conjugated to Alexa Fluor 555</td>
</tr>
<tr>
<td>ILV</td>
<td>Intraluminal vesicle</td>
</tr>
<tr>
<td>INPP4</td>
<td>Inositol polyphosphate 4'-phosphatase</td>
</tr>
<tr>
<td>INPP5</td>
<td>Inositol polyphosphate 5'-phosphatase</td>
</tr>
<tr>
<td>Ins(1,4,5)P₃</td>
<td>Inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-associated membrane protein</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for activation of T-cells family member 1</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>Mac-1</td>
<td>Macrophage-1 antigen (integrin α₃β₂ or CR3)</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe-associated molecular pattern</td>
</tr>
<tr>
<td>ManLAM</td>
<td>Mannose-capped lipoarabinomannan</td>
</tr>
<tr>
<td>mRFP</td>
<td>Monomeric red fluorescent protein</td>
</tr>
<tr>
<td>MTM</td>
<td>Myotubularin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular body</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>NOX</td>
<td>NAPDH oxidase</td>
</tr>
<tr>
<td>NOX2</td>
<td>NADPH oxidase 2 (gp91phox)</td>
</tr>
<tr>
<td>OCRL</td>
<td>Oculocerebrorenal syndrome of Lowe</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAK(PBD)</td>
<td>p21-binding domain of p21-activated kinase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIKfyve</td>
<td>Phosphatidylinositol-3-phosphate 5-kinase</td>
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<tr>
<td>PIP5K</td>
<td>Phosphatidylinositol-4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PM</td>
<td>Palmitoylated-myristoylated</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>-----------</td>
<td>---------------------------------------------------------------------------</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PtdCho</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
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<td>PtdIns(3,4,5)P_3</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
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<td>PtdIns(4,5)P_2</td>
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<td>PtdSer</td>
<td>Phosphatidylserine</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<td>PX</td>
<td>Phox homology</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species.</td>
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<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
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<tr>
<td>SCV</td>
<td>Salmonella-containing vacuole</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy; standard error of the mean</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinases</td>
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<tr>
<td>SH2</td>
<td>Src-homology 2</td>
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<tr>
<td>SHIP</td>
<td>SH2 domain-containing inositol phosphatase</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SNX</td>
<td>Sorting nexin</td>
</tr>
<tr>
<td>SOZ</td>
<td>Serum-opsonized zymosan</td>
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<td>STAB2</td>
<td>Stabilin-2</td>
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<td>STIM1</td>
<td>Stromal interaction molecule 1</td>
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<td>T3SS</td>
<td>Type III secretion system</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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<td>TGN</td>
<td>Trans-Golgi network</td>
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<td>T-cell lymphoma invasion and metastasis-inducing protein 1</td>
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<tr>
<td>TIM</td>
<td>T-cell immunoglobulin mucin</td>
</tr>
<tr>
<td>Tir</td>
<td>Translocated intimin receptor</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TMR</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar adenosine triphosphatase</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>WASp</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Chapter 1

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1 General introduction: Lipid signals in phagocytosis and macropinocytosis

1.1 Summary

Professional phagocytes provide immunoprotection and aid in the maintenance of tissue homeostasis. They perform these tasks by recognizing, engulfing and eliminating pathogens and endogenous cell debris. In this introductory chapter, we examine the paramount role played by signalling lipids in phagocytosis and macropinocytosis, two major endocytic routes that mediate the uptake of particulate and fluid matter, respectively. We analyze accumulating literature describing the molecular mechanisms whereby glycerophospholipids and diacylglycerols translate environmental cues into the complex, sophisticated responses that underlie the formation, internalization and maturation of phagosomes and macropinosomes. Placing particular emphasis on the subcellular distribution of phosphoinositides as well as their mechanisms of biosynthesis and degradation, we detail the physiological implications of acute membrane remodelling for the orchestration of phagocytic and macropinocytic events. In addition, we exemplify virulence strategies involving modulation of host cell phosphoinositide signalling that are employed by bacteria to undermine immunity.
1.2 Introduction

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

In addition to its immunoprotective role in infection, phagocytosis is also pivotal for important housekeeping tasks. In particular, professional and non-professional phagocytes (like fibroblasts and epithelial cells) support the turnover of billions of apoptotic cells every day. The internalization of effete cells by phagocytosis—also known as efferocytosis—has important ramifications for wound healing, development and morphogenesis, all processes in which timely removal of cell debris is critical for homeostasis.

Macrophages and dendritic cells continuously sample and probe their environment in the lookout for foreign antigens and danger signals (Bohdanowicz et al. 2013). While these cells internalize particulate matter by phagocytosis, they also take up fluid-phase material and soluble antigens by macropinocytosis. Phagocytosis and macropinocytosis give rise to large vacuoles known as phagosomes and macropinosomes, respectively. Such vacuoles, which are initially generated by invagination of the plasma membrane, endure a radical metamorphosis in order to become microbicidal and degradative organelles conducive to pathogen elimination, antigen presentation and ultimate disposal of the vacuolar contents. This makeover, known as the maturation process, requires a carefully orchestrated sequence of membrane fusion and fission events with multiple components of the endocytic pathway. Phagocytosis and macropinocytosis can therefore be conceptually bisected into two phases: one consisting of involution and scission of a plasmalemma-derived vacuole (formation), and the other its progression through the endocytic pathway (maturation).
Although their internalized cargo clearly differs, similar molecular machinery is engaged for the biogenesis of phagocytic and macropinocytic compartments. Both the formation and maturation of phagosomes/macropinosomes entail sophisticated signalling cascades, as well as extensive cytoskeletal reorganization and membrane remodelling. Specifically, the capture of phagocytic prey (Flannagan et al. 2010) and the formation of macropinosomes (Bohdanowicz et al. 2013) are both preceded by Rho GTPase activation (West et al. 2000a; Beemiller et al. 2010) and the extension of actin-driven membrane protrusions. Moreover, both phagocytosis and macropinocytosis require large-scale membrane remodelling, as well as activation of phosphoinositide 3-kinases (PI3K) (Araki, Johnson, and Swanson 1996).

Unlike macropinocytosis, phagocytosis is triggered upon the engagement of particulates by cellular receptors. When phagocytic targets are initially encountered, extracellular signals are conveyed across the plasma membrane in order to initiate the complex cellular behaviours that culminate in phagocytosis. It is becoming increasingly apparent that phosphoinositides play a prominent role in relaying this information. Interestingly, both the detection of ligands by transmembrane phagocytic receptors and the ruffling of membranes observed during macropinocytosis are accompanied by local changes in phosphoinositide composition. Similarly, phosphoinositides coordinate membrane fusion and fission events that lead to the acquisition of lysosomal properties during the course of maturation (Vieira, Botelho, and Grinstein 2002). This is accomplished primarily via recruitment of effector proteins by a combination of stereochemical and electrostatic interactions. Thus, phosphoinositides are much more than mere building blocks or structural bystanders of cellular membranes; instead, they fine-tune signal transduction pathways (Di Paolo and De Camilli 2006), help specify organelle identity (Kutateladze 2010), and direct membrane traffic (Simonsen et al. 2001).

Given its pivotal role in phagocyte function, phosphoinositide metabolism is often subverted by invasive pathogens as a colonizing strategy. By undermining or hijacking phosphoinositide homeostasis, intracellular bacteria alter membrane dynamics and gain entry into their host (Ham, Sreelatha, and Orth 2011). Maturation of the bacterium-containing vacuole into an organelle with lysosomal characteristics is similarly affected by the subversion of phosphoinositide signalling.
The intent of this introductory chapter is to collate the available information regarding the involvement of phosphoinositides in phagocytosis and macropinocytosis, while stressing the current gaps in our knowledge. The current chapter will also feature selected examples of the molecular mechanisms by which bacterial pathogens commandeer phosphoinositide homeostasis in an attempt to undermine phagocytic defences.

1.3 Phagocytosis: A primer on phagosome formation and maturation

Unlike macropinocytosis, phagocytosis is a receptor-mediated process; initiation of phagocytosis is strictly dependent on the engagement of specific receptors by a multivalent, particulate ligand (Grinstein 2012). The variety of distinct phagocytic targets that can be recognized by phagocytic receptors is extensive. These range from foreign bodies—including microbial pathogens, such as bacteria, fungi and other parasites—to endogenous apoptotic corpses, cellular debris and old erythrocytes (Flannagan, Jaumouillé, and Grinstein 2012). The ability of phagocytes to engage such a wide array of particles is attributable to their vast repertoire of receptors, which recognize a large number of ligands. Multiple types of receptors co-exist in mammalian macrophages, and each of these recognizes not one but often a number of different ligands (Botelho, Scott, and Grinstein 2004). Under physiological conditions, several types of receptors are engaged simultaneously and likely cooperate in the development of a complex phagocytic response (Flannagan, Jaumouillé, and Grinstein 2012). Of note, the signal transduction pathways and molecular mechanisms whereby distinct phagocytic receptors prompt particle internalization can be substantially different (Grinstein 2012). Thus, considering phagocytosis as a single biological process is overly simplistic; phagocytosis should instead be thought of as an umbrella term that describes a family of processes that, while phenotypically related, are ultimately distinct at the molecular level.

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

1.3.1 Phagosome formation

Phagosome formation can be conceptually divided into three stages: 1) binding of phagocytic targets; 2) extension of pseudopodia around the particle and; 3) sealing of the phagosome as pseudopodia meet and fuse (Hoppe and Swanson 2004). Particle binding is dependent on the affinity of the receptor(s) for their cognate ligands, as well as on the surface density of both of ligands (on the particle surface) and receptors (on the phagocyte membrane). What follows is a brief description of some of the most thoroughly characterized phagocytic receptors, their respective ligands and the signalling pathways through which they operate.

1.3.1.1 Phagocytic receptors and particle recognition

The recognition of microbes by cell-surface receptors can occur directly or indirectly. Indirect binding entails an intermediary opsonizing molecule that coats the microorganism and bridges its association with phagocytic receptors. In turn, direct interactions involve recognition of microbe-associated molecular patterns (MAMPs), such as peptidoglycan or surface carbohydrates, by pattern recognition receptors (PRRs). MAMPs represent regular arrays of molecular structure that are present in microbes, but are absent in metazoans. PRRs are germline-encoded cell-surface proteins that quickly initiate immune responses upon recognition of MAMP signatures. Prototypical PRRs include scavenger receptors, C-type lectin receptors of the Dectin family, the mannose receptor and Toll-like receptors (TLRs). All of these receptor types act as microbial sensors and function in different capacities to prime or initiate the phagocytic response. Some of these non-opsonic receptors, like Dectin-1 that
recognizes β-glucans on fungal cell walls, are sufficient to initiate phagocytosis (Herre et al. 2004) as well as the secretion of inflammatory cytokines (Rogers et al. 2005).

However, not all PRRs are inherently capable of triggering phagocytosis: TLRs, for instance, may serve a tethering function by latching onto their ligands on the surface of incoming targets, without initiating particle engulfment. While not themselves phagocytic, TLRs have been reported to enhance antimicrobial responses and to influence the course of phagosome maturation (Blander and Medzhitov 2004; Kagan and Iwasaki 2012).

In contrast to the engulfment of microbial pathogens during occasional infection, clearance of apoptotic corpses must occur continuously. Indeed, between 1 and 5 million cells are turned over by efferocytosis every second in healthy human subjects (Ravichandran 2010), a truly remarkable feat. Failure to quickly and efficiently dispose of effete cells can result in tissue abnormalities (Henson and Hume 2006), including secondary necrosis, which is in turn associated with recurrent inflammation and the development of autoimmunity (Nagata, Hanayama, and Kawane 2010). Interestingly, recognition and processing of apoptotic bodies result in the secretion of anti-inflammatory cytokines and tolerogenic mediators by professional phagocytes. Thus, recognition of phagocytic targets as ‘self’ allows for the establishment of an immunologically silent microenvironment (Savill and Fadok 2000).

Exofacial phosphatidylserine (PtdSer) exposure, the most distinctive marker of apoptosis, is also a universal ‘eat me’ signal. In healthy cells, PtdSer is abundant in the plasmalemmal inner leaflet, but becomes scrambled and exposed to the outer leaflet as part of the apoptotic program. Multiple distinct receptors can recognize PtdSer on the surface of apoptotic corpses, either directly or indirectly. Receptors that bind PtdSer directly include members of the T-cell immunoglobulin mucin (TIM) family, as well as brain-specific angiogenesis inhibitor 1 (BAI1) (Park et al. 2007) and Stabilin-2 (STAB2) (Park et al. 2008). TIM4, a phagocyte-restricted transmembrane protein, engages PtdSer directly while concomitantly associating with integrins, which serve as ancillary receptors that promote engulfment of the apoptotic corpses (Flannagan et al. 2014). Alternatively, PtsSer can also be bridged to surface receptors by soluble extracellular
molecules. For instance, the protein lactadherin (MFG-E8) links PtdSer and the integrin \(\alpha_\nu\beta_3\) (Hanayama et al. 2002), which in turn initiates phagocytosis.

In contrast to receptors that directly sense patterns on microbial surfaces or “eat me” signals, opsonic receptors detect intermediary molecules (opsonins) that have deposited on foreign bodies (Flannagan, Jaumouillé, and Grinstein 2012). Opsonins are soluble molecules found in the circulation and extracellular space, where they recognize and coat their ligands. The most widely studied opsonins include complement fragments (e.g. iC3b) and immunoglobulins (e.g. IgG). iC3b- and IgG-coated particles are recognized by the \(\alpha_M\beta_2\) integrin (Mac-1) and Fcg receptors (Fc\(\gamma\)R), respectively. Fc\(\gamma\)R are activated when clustered by the Fc portion of IgG on the surface of a multivalent ligand. Fc\(\gamma\)R is by far the most thoroughly characterized phagocytic receptor type and will therefore be prominently featured throughout this chapter.

1.3.1.2 Signals mediating pseudopod extension and engulfment of IgG-coated particles

As is the case for most other immunoreceptors, aggregation of Fc\(\gamma\) receptors in the plane of the membrane leads to their activation (Jones, Nusbacher, and Anderson 1985). The receptors cluster laterally upon engagement of their multivalent ligand, effectively bringing their cytosolic domains into close apposition. Fc\(\gamma\) receptors carry within their cytosolic tails a conserved signalling sequence, known as the immunoreceptor tyrosine-based activation motif (ITAM), which becomes phosphorylated by non-receptor tyrosine kinases of the Src family upon receptor coalescence (Ghazizadeh, Bolen, and Fleit 1994). These kinases include Hck and Lyn (Carréno et al. 2002; Wang, Scholl, and Geha 1994). ITAM sequences are characterized by a tandem YXXI/L motif, and phosphorylation of both of these tyrosines allows for optimal phagocytosis (Ibarrola et al. 1997). Amplification of the phosphorylation cascade involves the kinase activity of Syk, which carries two Src-homology 2 (SH2) domains in tandem and associates with the doubly-phosphorylated ITAM (Greenberg et al. 1996). This sequence of events—ligand binding, receptor clustering and initiation of phosphorylation signalling—is iterated multiple times as pseudopods grow around the
surface of the phagocytic target. Pseudopods surround incoming targets in a zipper-like fashion until they eventually meet and fuse at the particle’s distal end.

Upon Syk recruitment and phosphorylation, numerous adaptors and signalling proteins translocate to sites of particle engagement. Many of these become anchored at the newly formed phosphotyrosines, where they act as signalling platforms and recruit additional effectors. Specifically, Syk binds to and phosphorylates LAT, a transmembrane adaptor that becomes highly tyrosine-phosphorylated, resulting in the recruitment of additional SH2 domain-containing proteins (Greenberg et al. 1996). LAT phosphorylation precipitates translocation of the adaptor Grb2, which in turn recruits Gab2. The latter is also tyrosine-phosphorylated by Syk, which leads to its association with p85, the regulatory subunit of class I PI3K (Gu et al. 2003). Therefore, Gab2 provides an important connection between phosphotyrosine- and phosphoinositide-mediated signalling.

Phosphorylation of LAT also leads to the recruitment of another phosphoinositide-modifying protein, phospholipase C gamma (PLCγ) (Tridandapani et al. 2000). PLCγ is a hydrolytic enzyme with particularly high activity towards PtdIns(4,5)P2. The phosphoinositides metabolized by these and other enzymes play key roles in orchestrating the phagocytic and macropinocytic responses; for this reason, entire sections of this chapter are devoted to individual phosphoinositide species.

In conjunction with the early signalling events described above, the levels of PtdIns(4,5)P2 rapidly and transiently increase at the nascent phagosome during the stages of particle binding and pseudopod extension (Botelho et al. 2000). Such elevations in PtdIns(4,5)P2 synergize with the concomitant activation of Rho family GTPases, including Rac1, Rac2 and Cdc42 (Hoppe and Swanson 2004), in the formation of filamentous actin networks during phagocytosis (Saarikangas, Zhao, and Lappalainen 2010). More specifically, PtdIns(4,5)P2 stabilizes critical components of the actin polymerization machinery, including nucleation-promoting factors such as WASp (Rohatgi et al. 2001; Rohatgi, Ho, and Kirschner 2000a). At rest, WASp exists in an autoinhibited, inactive state. However, simultaneous association with active Cdc42 and PtdIns(4,5)P2 releases this intramolecular inhibition, allowing for WASp-mediated
activation of the Arp2/3 complex, a multi-subunit effector that catalyzes the nucleation of branched actin filaments (May et al. 2000).

That Rho GTPases play an indispensable role in phagocytosis is supported by multiple loss-of-function studies; inactivation of Cdc42 by silencing (Park and Cox 2009) or through dominant-negative approaches (Caron and Hall 1998; May et al. 2000) impairs actin assembly and inhibits phagocytosis. Similarly, the phagocytic ability of macrophages from Rac1−/− and Rac2−/− double-knockout mice is impaired (Koh et al. 2005).

The Rho-family GTPases are activated with distinct kinetics and appear to execute non-redundant functions during phagocytosis (Hoppe and Swanson 2004). Single-cell based determinations suggest that Cdc42 activation occurs first and that this is restricted to the leading margin of pseudopodia. A wave of Rac1 and Rac2 activation follows soon after. However, while sites of active Rac1 extend throughout the nascent phagosome, Rac2 seems to be confined to the base of the phagocytic cup. Of note, inactivation of Rho GTPases may be as important as their activation, as cells expressing a constitutively active form of Cdc42 display a marked phagocytic impairment (Beemiller et al. 2010). This suggests that the cycling of Rho GTPases between active and inactive states is essential to sustain actin dynamics during phagocytosis.

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

As a final requirement for particle internalization, the nascent vacuole must seal. This entails fusion between plasmalemmal pseudopods. Remarkably, virtually nothing is known about the molecular machinery that catalyzes the fusion of exofacial leaflets. Myosin-driven contractility might facilitate this process. Supporting this notion, myosin IC (Swanson et al. 1999) and myosin X (Cox et al. 2002) are recruited to sites of
phagocytosis and have been implicated in phagosome closure. Myosin X interacts with phagosomal PtdIns(3,4,5)P3 through a PH domain (Isakoff et al. 1998), suggesting that PI3K could be indirectly involved in the generation of contractile forces that promote the tight apposition of pseudopods that precedes phagosome sealing.

1.3.2 Phagosome Maturation

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

Interestingly, the fate of phagosomes varies depending on the nature of their cargo. The luminal composition of phagosomes harbouring infectious agents fosters the preservation of antigenic determinants, which can be presented to lymphocytes (Savina and Amigorena 2007). Conversely, antigens derived from endogenous apoptotic corpses are quickly degraded in an immunologically silent manner in order to prevent the development of autoimmunity (Ravichandran 2010). Similarly, FcγR-mediated phagocytosis is accompanied by the secretion of pro-inflammatory cytokines, such as tumour necrosis factor-α, whereas internalization of apoptotic bodies leads to the secretion of anti-inflammatory mediators (Flannagan, Jaumouillé, and Grinstein 2012; Scholl, Ahern, and Geha 1992).

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

Nascent phagosomes show a predisposition to fuse with early and recycling endosomes (Mayorga, Bertini, and Stahl 1991), but not with lysosomes (Desjardins et al. 1997). The coalescence of phagosomes and early endosomes yields a hybrid organelle with low hydrolytic activity and a slightly acidified lumen (pH 6.1-6.5) (Flannagan, Cosío, and Grinstein 2009).

Rab family proteins are instrumental in the maturation process, as they direct vesicular traffic to and from the phagosome (Kinchen and Ravichandran 2008). In particular, early phagosomes accumulate Rab5, which regulates traffic by coordinating tethering and fusion of endomembranes (Bucci et al. 1992). As with other GTPases, activation of Rab5 requires nucleotide exchange. During phagocytosis of apoptotic bodies, Rab5 is activated by GAPex-5, a GEF that is delivered to sites of phagocytosis through the microtubular network (Kitano et al. 2008). Tellingly, phagosomes fail to acquire endolysosomal characteristics in cells that have been transfected with a dominant-negative form of Rab5 (Vieira et al. 2003).

Having undergone repeated cycles of fusion with endocytic compartments, maturing phagosomes would be expected to increase in size. However, their surface area remains constant throughout the maturation process. A concomitant removal of membrane accounts for this seemingly paradoxical observation. Specifically, membrane is recycled to the plasmalemma, allowing for the retrieval of bystander membrane components that were unintentionally internalized with the phagocytic particle (Driskell et al. 2007). Rab4 and Rab11 associate with the early phagosome, controlling the recycling back to the plasma membrane (Cox et al. 2000; Damiani et al. 2004). In addition, the retromer—a complex consisting of Vps26-Vps29-Vps35 (a trimer that provides cargo selectivity), plus a sorting nexin (SNX) dimer (consisting of either SNX1/SNX2 and SNX5/SNX6)—diverts phagosomal components to the trans-Golgi
network (TGN) and recycling endosomes. Interestingly, deficiencies in retromer levels or in its assembly have recently been linked to the pathophysiology of Alzheimer’s disease; microglial cells (the primary phagocytes of the central nervous system) that do not recruit the retromer complex efficiently to nascent phagosomes display impaired recycling of receptors to the plasmalemma, which could affect recognition of debris or effete cells in the brain (Lucin et al. 2013).

Phagosomes also undergo invagination of their own limiting membrane, resulting in the formation of intraluminal vesicles (ILVs) (Lee et al. 2005). The process of phagosomal membrane deformation and scission is likely similar to that responsible for the formation of multivesicular bodies (MVBs). As opposed to budding outward, intended for retrieval of salvageable phagosomal components, inward budding diverts membrane-associated cargo for degradation. ILVs are formed through the combined action of a group of protein complexes, known as endosomal sorting complex required for transport (ESCRT) (Wollert and Hurley 2010). The different complexes that comprise the ESCRT machinery recognize phagosomal membrane proteins that have been tagged for degradation by ubiquitylation, and force their extrusion into luminal vesicles (Muzioł et al. 2006).

**1.3.2.2 The late phagosome**

The membrane and luminal composition of the maturing phagosome continue to change as it progresses into the next discernible stage: the late phagosome. This stage is characterized by the accumulation of proteases and proton-pumping vacuolar ATPases (V-ATPases) that render the lumen degradative and hostile (Desjardins et al. 1994). The pH of the late phagosomes drops to 5.5-6.0.

The acquisition of active Rab7 and the concomitant dissociation of Rab5 mark the conversion to late phagosomes. This Rab5-to-Rab7 transition is orchestrated by the protein SAND-1 (the ortholog of mammalian Mon1a and Mon1b) (Poteryaev et al. 2010; Kinchen and Ravichandran 2010). Mon1 interacts directly with active Rab5 and promotes the recruitment of an additional effector, Ccz-1. Through the tethering function
of Ccz-1, the Rab5-GTP/Mon1 complex engages Rab7-GDP, and indirectly promotes its conversion to the active, GTP-bound form (Kinchen and Ravichandran 2010). Mon1 seems to serve a dual role, as it has been reported to displace Rabex-5 (a Rab5 GEF) from the phagosomal membrane, thereby facilitating Rab5 inactivation (Poteryaev et al. 2010).

The late phagosome also acquires lysosomal-associated membrane proteins (LAMPs), integral membrane glycoproteins normally enriched in late endosomes and lysosomes. The role of LAMPs was long presumed to be restricted to providing structural support to lysosomal membranes and protecting them from the harsh environment of the lysosomal lumen. However, recent reports suggest that these glycoproteins are required for the recruitment of Rab7 (Huynh et al. 2007) and therefore for phagolysosome biogenesis and microbicidal competence (Binker et al. 2007).

Rab7 is instrumental for the maturation of the phagocytic vacuole; expression of dominant-negative Rab7 depresses phagolysosome formation and acidification (Harrison et al. 2003). Two Rab7 effectors have been implicated in this process: ORPL1 and RILP (Johansson et al. 2007). These two proteins associate with Rab7-GTP on late degradative compartments, where they bind to the dynein-dynactin complex. Therefore, by promoting tethering of late phagosomes to minus end-directed motors, Rab7 and its effectors direct the centripetal movement of phagosomes that is required for efficient fusion with lysosomes (Harrison et al. 2003).

Phagosomal membrane remodelling via recycling and ILV formation, a process that commenced in sorting endosomes, persists in late phagosomes. Indeed, retrograde transport of transmembrane cargo to the TGN is facilitated by the association of retromer components with Rab7 (Rojas et al. 2008). Having sorted cargo for recycling or degradation, the late phagosome continues to develop by fusing with lysosomes, giving rise to the terminal station in the maturation process: the phagolysosome.
1.3.2.3 The phagolysosome

Rab7-dependent fusion of phagosomes with lysosomes results in the generation of fully competent microbicidal organelles. As with endosomes (Ward et al. 2000), fusion of lysosomes with phagosomal membranes involves the formation of a SNARE hairpin between VAMP7 and syntaxin 7 (Collins et al. 2002), and likely requires Ca\(^{2+}\) release from the lumen of lysosomes. Once formed, phagolysosomes can be distinguished biochemically from earlier endocytic compartments by a paucity of PtdIns(3)P in their internal membranes, the lack of mannose-6-phosphate receptors, an enrichment in acid hydrolases of the cathepsin family, and the pronounced acidity of its lumen (pH 4.5-5.0) (Griffiths et al. 1988; Carraro-Lacroix et al. 2011; Huynh and Grinstein 2007).

The acidic nature of the phagolysosome renders its lumen effectively microbicidal and degradative for a number of reasons. First, the lytic activity of the hydrolases is favoured by acidification (Turk et al. 1993). Second, low phagosomal pH directly interferes with bacterial growth by directly impairing microbial metabolism. Moreover, the transmembrane H\(^+\) gradient drives the extrusion of luminal divalent cations that are critical for bacterial survival (Jabado et al. 2000). Third, the positive voltage generated by the V-ATPase facilitates the formation of microbicidal superoxide anions by the electrogenic NADPH oxidase. Fourth, acidification of the phagosome seems to be a requirement for (and not only a consequence of) the maturation process. This is borne out by experiments where prevention of acidification caused a block in phagolysosome biogenesis (Gordon, Hart, and Young 1980).

In addition to acidification and generation of highly reactive oxygen species, phagolysosomes accumulate a collection of antibiotic compounds that compromise the integrity of the ingested microbes. These include heavy metal scavengers like lactoferrin (Masson, Heremans, and Schonne 1969), lytic cationic peptides like defensins and cathelicidins (Zanetti 2004; Ganz 2003), as well as assorted hydrolases such as lysozyme and phospholipase A2 (Pillay, Elliott, and Dennison 2002).
1.4 Glycerophospholipid and diacylglycerol signalling during phagosome formation

1.4.1 Phosphatidylinositol-4,5-bisphosphate and diacylglycerol in phagocytosis

The primary location of PtdIns(4,5)P$_2$ in resting phagocytes is the inner leaflet of the plasmalemma, where it encompasses approximately 2 mol % of the phospholipid content (McLaughlin and Murray 2005). A small fraction of PtdIns(4,5)P$_2$ is also detectable in the Golgi network (Godi et al. 1999; Czech 2000). PtdIns(4,5)P$_2$ is primarily generated by type I phosphatidylinositol phosphate kinases (PIPKI or PIP5K), which phosphorylate PtdIns(4)P at the D5 position of the inositol moiety. Although to a lesser extent, PtdIns(4,5)P$_2$ is also synthesized through the phosphorylation of PtdIns(5)P$_2$ at the D4 position by type II PIPK (Bohdanowicz and Grinstein 2013). Dephosphorylation of PtdIns(3,4,5)P$_3$ by phosphatase and tensin homolog (PTEN) also represents a source for the resting pool of PtdIns(4,5)P$_2$ (Mondal et al. 2011), but the relative contribution of this pathway is not well established.

The PIP5K family is comprised of 3 isoforms: PIP5K$_\alpha$, $\beta$ and $\gamma$. Though PIP5K moderately associates with the Golgi complex and tubular lysosomes (Brown et al. 2001; Hammond, Schiavo, and Irvine 2009), it predominantly distributes to the plasma membrane—the most negatively charged compartment in the cell—through a polycationic region located on its surface (Fairn et al. 2009). Rho (Weernink et al. 2004; Tolias, Cantley, and Carpenter 1995) and Arf (Brown et al. 2001) GTPases positively regulate PIP5K activity, thereby integrating diverse processes such as cytoskeletal remodelling and membrane traffic with PtdIns(4,5)P$_2$ synthesis. The relationship between PIP5K and small GTPases appears to be reciprocal; not only do Rho GTPases control PIP5K activation (Weernink et al. 2004), but actin polymerization driven by these GTPases is also dependent on PIP5K (Tolias et al. 2000). PIP5K is also activated by Arf6, which colocalizes with PIP5K at sites of PtdIns(4,5)P$_2$ enrichment, including membrane ruffles (Brown et al. 2001; Honda et al. 1999). Of note, Arf6-mediated activation of PIP5K is
strictly dependent on the presence of anionic phospholipids, such as phosphatidic acid (PA) (Honda et al. 1999). PtdIns(4,5)P$_2$ and PA likely engage in a positive feedback loop, as PtdIns(4,5) P$_2$ is a cofactor necessary for the optimal activity of phospholipase D (PLD) (Divecha et al. 2000), an enzyme that forms PA by hydrolysing phosphatidylcholine (PtdCho). The top panel of Figure 2 illustrates the signalling pathways leading to PIP5K activation and the consequent formation of phagosomal PtdIns(4,5)P$_2$.

Counter-balancing the anabolic pathways described above are at least 3 independent mechanisms that break down PtdIns(4,5)P$_2$. Class I PI3K catalyzes the phosphorylation of PtdIns(4,5)P$_2$, converting it to PtdIns(3,4,5)P$_3$. A wide number 5-phosphatases, including OCRL (Mehta, Pietka, and Lowe 2014), INPP5B (Bohdanowicz et al. 2012) and the synaptojanins (Voronov et al. 2008), promote its degradation to PtdIns(4)P. PLC$_\gamma$ hydrolyzes the PtdIns(4,5)P$_2$ head-group, thus forming plasmalemmal DAG and releasing Ins(1,4,5)P$_3$ into the cytosol. The role of these enzymes in phagocytosis is detailed below.

PtdIns(4,5)P$_2$ exerts many important functions at the membrane. It binds to proteins with polybasic motifs or with PH, FERM or ENTH domains (McLaughlin and Murray 2005), and can also be converted to second messengers, such as PtdIns(3,4,5)P$_3$, DAG and Ins(1,4,5)P$_3$, all of which play crucial roles in the formation and maturation of phagosomes, as detailed in the sections below.

PtdIns(4,5)P$_2$ and its metabolites control a remarkable number of events in phagocytosis. These include rearrangement of the actin cytoskeleton (Rohatgi, Ho, and Kirschner 2000) and the accompanying changes in phagocytic receptor mobility (Jaumouillé and Grinstein 2011), integrin activation (Martel et al. 2001), membrane traffic (Simonsen et al. 2001; Paolo et al. 2004), plasmalemmal-cytoskeletal linkages (Hamada et al. 2000), and ion channel activity (Suh and Hille 2005). Notably, each of these events takes place in a discrete cellular location and at a particular time during the course of phagocytosis. The latter raises a recurrent question: how can a single phosphoinositide that is present throughout the plasma membrane orchestrate such a diverse range of spatiotemporally restricted phenomena? Part of the answer is that
PtdIns(4,5)P$_2$ levels change only locally during phagocytosis (Kutateladze 2010). Microscopy-based determinations in single macrophages expressing a fluorescent chimera of the PH domain of PLCδ [a PtdIns(4,5)P$_2$ reporter], revealed biphasic changes in the membranes of forming phagosomes (Botelho et al. 2000). In contrast, PtdIns(4,5)P$_2$ levels remain steady during the course of phagocytosis in unengaged aspects of the plasmalemma (Botelho et al. 2000). Figure 1 illustrates that, while there is a noticeable accumulation of PtdIns(4,5)P$_2$ in emerging pseudopods during the early stages of phagosome formation, the concentration of the phosphoinositide drops at the base of the phagocytic cup as pseudopodia extend. Following phagosome sealing and severing, phagosomal PtdIns(4,5)P$_2$ decreases precipitously and is no longer detectable by fluorescence microscopy (Botelho et al. 2000).

In principle, the localized initial accumulation of PtdIns(4,5)P$_2$ at pseudopods could result from an upsurge in synthesis, a decline in consumption, or both. Two lines of evidence point towards an increase in synthesis. First, activation of PLD has been detected during phagocytosis (Lee et al. 2002; Iyer et al. 2004; Kusner, Hall, and Jackson 1999), and as mentioned before, this enzyme promotes the recruitment and activation of PIP5K (Divecha et al. 2000). The simultaneous activation of Arf6 and PLD are likely to stimulate PIP5K, thereby promoting PtdIns(4,5)P$_2$ formation. Second, all 3 PIP5K isoforms transiently accumulate at the phagosomal membrane during the early phase of phagocytosis, and detach during the later stages (Fairn et al. 2009), correlating with the biphasic nature of phagosomal PtdIns(4,5)P$_2$ levels.

In turn, as shown in Figure 2B, the disappearance of PtdIns(4,5)P$_2$ from the base of phagocytic cups and eventually from the membrane of sealed phagosomes is mediated by a combination of kinases, phosphatases and lipases. Specifically, at later stages of phagosome formation PI3K promotes phosphorylation of PtdIns(4,5)P$_2$ into PtdIns(3,4,5)P$_3$, which also serves as a signal for the recruitment of PLCγ (Falasca et al. 1998). Indeed, having being recruited to sites of PtdIns(3,4,5)P$_3$ production, PLCγ becomes largely responsible for the disappearance of PtdIns(4,5)P$_2$ (Azzoni et al. 1992; Liao, Shin, and Rhee 1992). The inositol 5-phosphatases OCRL and INPP5B also contribute to degradation by converting PtdIns(4,5)P$_2$ into PtdIns(4)P.
In addition to the catabolic pathways described above, detachment of PIP5K from internalized phagosomes facilitates the exclusion of PtdIns(4,5)P2 from these compartments. The dissociation of PIP5K has been attributed to a localized and acute drop in phagosomal surface charge (Yeung, Terebiznik, et al. 2006). The release of PIP5K from sealed phagosomal membranes terminates PtdIns(4,5)P2 synthesis and further promotes the disappearance of this lipid at sites where degradation is already ongoing.

In phagocytes, as in many other cellular systems, sites of PtdIns(4,5)P2 formation serve as signalling platforms that trigger robust actin polymerization. PtdIns(4,5)P2 promotes the activation of a number of actin-regulatory proteins that are responsible for filament assembly, while inhibiting those in charge of disassembly (Saarikangas, Zhao, and Lappalainen 2010) (Figure 2A). Proteins that directly bind to actin and dictate the equilibrium between its monomeric and filamentous form include profilin (Chaudhary et al. 1998), coflin (Gorbatyuk et al. 2006), gelsolin (Janmey and Stossel 1987) and capping protein (Cooper and Sept 2008). In addition to increasing the number of barbed ends, PtdIns(4,5)P2 induces de novo actin nucleation by activating nucleation-promoting factors (Miki, Miura, and Takenawa 1996). Lastly, ezrin/radixin/moesin (ERM), which directly link the cytoskeleton to the plasmalemma, are also well established PtdIns(4,5)P2 effectors (Bretscher, Edwards, and Fehon 2002). Because of these effects, the local increase in PtdIns(4,5)P2 biosynthesis that ensues upon engagement of phagocytic receptors triggers a large-scale reorganization of the actin cytoskeleton, driving the extension of pseudopodia around the surface of phagocytic targets (Coppolino et al. 2002). This claim is supported by studies in which expression of a kinase-dead PIP5K that impairs the formation of phagosomal PtdIns(4,5)P2 precluded accumulation of F-actin in nascent phagocytic cups while depressing phagocytic capacity (Coppolino et al. 2002).

Although PIP5Kα, β and γ are all recruited to nascent phagosomes, different isoforms have been reported to mediate distinct, non-redundant roles during phagocytosis (Mao et al. 2009). PIP5Kα activity has been implicated in the activation of the nucleation promoting factor WASp (Mao et al. 2009), which catalyzes actin polymerization and pseudopod extension through the Arp2/3 complex (Park and Cox 2009). In contrast,
PIP5Kγ seems to control the mobility of phagocytic receptors in the plane of the membrane, presumably by modulating the density of cortical actin. Subsequently, PIP5Kα catalyzes the emission of pseudopodia by promoting WASp activity (Mao et al. 2009). To reconcile the fact that these isoforms have divergent effects, it has been postulated that PIP5Kγ is subjected to post-translational control by Syk, thereby restricting its activity to a particular region and time (Mao et al. 2009).

While expansion of the actin skeleton and its anchorage to the plasmalemma drive formation of pseudopodia at early phases of phagocytosis, phagosome scission is accompanied by the disappearance of actin from the base of the phagocytic cup (Scott et al. 2005). Indeed, actin clearance is a requirement for completion of phagocytosis, especially of large phagocytic targets (Beemiller et al. 2010; Cox et al. 1999). Abortive phagocytic cups develop when the disintegration of the phagosomal actin meshwork is prevented either by expressing constitutively-active Rho GTPases (Beemiller et al. 2010) or by inhibiting PI3K (Araki, Johnson, and Swanson 1996; Cox et al. 1999). The role of PtdIns(3,4,5)P₃ in actin disassembly is discussed in the next section and in chapter 4.

The catabolism of PtdIns(4,5)P₂ coincides in space and time with the breakdown of actin (Scott et al. 2005). Moreover, the loss of phagosomal actin occurs asymmetrically after phagosomal sealing, with depolymerization arising initially at the base of the phagocytic cup. The dynamics of actin disassembly strongly resemble the pattern of PtdIns(4,5)P₂ disappearance. Of note, dismantling of actin at the base of the cup and particle internalization are blocked if high PtdIns(4,5)P₂ levels are artificially sustained by promoting PIP5K-mediated synthesis or by inhibiting PLCγ-driven degradation (Scott et al. 2005). It thus appears that PtdIns(4,5)P₂ metabolism is intimately linked to actin disassembly, which is in turn required for completion of phagocytosis.

In addition to the implications of PtdIns(4,5)P₂ metabolism for actin cytoskeletal dynamics, breakdown of the phosphoinositide to secondary metabolites also has important ramifications (Figure 2C). PLCγ-mediated hydrolysis of PtdIns(4,5)P₂ leads to the formation of DAG and Ins(1,4,5)P₃. The kinetics and spatial distribution of DAG formation during phagocytosis have been measured with a genetically encoded
fluorescent chimera of the C1 domain of PKCδ (Botelho et al. 2000), which selectively associates with DAG (Oancea et al. 1998). Consistent with the role of PLCγ in phagocytosis, appearance of DAG coincides in space and time with the disappearance of PtdIns(4,5)P$_2$. Unexpectedly, though neither DAG nor Ins(1,4,5)P$_3$ are essential for particle engulfment, inhibition of PLCγ blocks the phagocytic response (Botelho et al. 2000; Scott et al. 2005). It thus appears that disappearance of PtdIns(4,5)P$_2$, rather than the formation of its metabolites, is essential for completion of phagocytosis.

While DAG and Ins(1,4,5)P$_3$ are not required for particle internalization, recruitment of conventional and novel PKC isoforms by DAG, as well as Ca$^{2+}$ mobilization by Ins(1,4,5)P$_3$, play significant roles in other stages of phagocytosis (Ueyama et al. 2004; Schlam et al. 2013; Nunes et al. 2012; Bengtsson et al. 1993). Recruitment and activation of PKC by DAG affect the elimination of internalized pathogens, as PKC phosphorylates and activates p47$^{phox}$, a regulatory subunit of the NADPH oxidase (NOX) (He et al. 2004; Cheng et al. 2007). Underscoring the significance of DAG formation is the observation that NOX activation is precluded if individual phagosomes fail to reach a critical concentration of DAG (Schlam et al. 2013).

As shown in Figure 2C, PtdIns(4,5)P$_2$ hydrolysis is accompanied by the release of Ins(1,4,5)P$_3$ into the cytosol and its diffusion to the endoplasmic reticulum (ER), where it induces Ca$^{2+}$ release by binding to the Ins(1,4,5)P$_3$ receptor (Nunes and Demaurex 2010). Depletion of Ca$^{2+}$ from intracellular stores is sensed by STIM1, a transmembrane ER-resident protein. A recent study demonstrated that, upon depletion of calcium from the ER lumen, STIM1 recruits ER cisternae to nascent phagosomes and promotes opening of store-operated calcium entry channels present at the phagosomal membrane (Nunes et al. 2012). Earlier studies implicated an increase in free cytosolic Ca$^{2+}$ in the fusion of secondary granules with the plasma membrane of neutrophils (Jaconi et al. 1990). Ca$^{2+}$ influx may play a similar role in the focal delivery of endomembranes to sites of phagocytosis and/or to maturing phagosomes.

Comparatively little is known about the regulation of phosphatases during phagocytosis and their contribution to PtdIns(4,5)P$_2$ removal. Nonetheless, evidence pointing to a role for OCRL and INPP5B has begun to emerge (Bohdanowicz et al. 2012). These inositol
5'-phosphatases are Rab5 effectors, and associate with nascent phagosomes through an adaptor protein called APPL1, also a Rab5 effector (Bohdanowicz et al. 2012). Silencing of either APPL1 or Rab5 prolonged the accumulation of both PtdIns(4,5)P$_2$ and filamentous actin on phagosomal membranes (Bohdanowicz et al. 2012). These observations are consistent with previous studies in Dictyostelium, where inactivation of Dd5P4 (a homolog of OCRL), resulted in phagocytic impairment (Loovers et al. 2007). Remarkably, although phagocytic cups did form in Dd5P4-null cells, these did not manage to seal and remained at an abortive stage (Loovers et al. 2007). Recent experimental findings have implicated a trimeric complex consisting of Bcl10 and the clathrin adaptors AP1 and EpsinR in ferrying OCRL to nascent phagosomes (Marion et al. 2012). In these studies, depletion of Bcl10 resulted in the formation of unproductive phagocytic cups, rich in PtdIns(4,5)P$_2$, Cdc42 and F-actin (Marion et al. 2012).

It is evident that PtdIns(4,5)P$_2$ metabolism—and consequently that of the secondary messengers DAG and Ca$^{2+}$—has profound implications for the formation and maturation of phagosomes. Thus, it is not surprising that microbes often subvert PtdIns(4,5)P$_2$ signalling in order to colonize their host. In an evolutionary arms race, a number of intracellular microbes have developed the ability to hijack host actin polymerization, allowing them to gain entry and displace within host cells (Frischknecht and Way 2001). Active modulation of plasmalemmal PtdIns(4,5)P$_2$ homeostasis by secreted effectors is one of the strategies used by invasive bacteria to co-opt the host actin-regulatory machinery. This is particularly important because most of the tension of the plasma membrane ($\approx$ 75%) is thought to result from its coupling to cortical actin (Sheetz 2001), which is in turn dependent on PtdIns(4,5)P$_2$. By promoting breakdown of PtdIns(4,5)P$_2$, intracellular pathogens weaken cytoskeletal support to the membrane and reduce membrane rigidity, thereby facilitating cellular entry (Terebiznik et al. 2002).

Salmonella spp., facultative intracellular pathogens, have developed a number of virulence mechanisms that enable their entry into the host (Fu and Galán 1999). These bacteria deliver effector proteins into host cells via a type III secretion system (T3SS), a specialized needle-like molecular machine (Fu and Galán 1999), causing massive reorganization of the host’s actin network. In conjunction to SopE and SopE2—two effectors that act as GEF mimics for Rho GTPases—Salmonella translocates a
phosphoinositide phosphatase, SopB, that rapidly eliminates PtdIns(4,5)P$_2$ from the invaginating regions of membrane ruffles (Terebiznik et al. 2002). The disappearance of PtdIns(4,5)P$_2$ facilitates membrane deformation, enabling both extension of ruffles as well as the scission of the *Salmonella*-containing vacuoles (SCV). Remarkably, SopB-mediated invasion is not restricted to the subversion of cytoskeletal dynamics; this protein also diverts phagosome maturation to a non-lytic compartment by reducing the surface charge of the SCV (Bakowski et al. 2010). By reducing the levels of anionic phospholipids, SopB precludes association of specific Rab GTPases that carry polycationic tails with the SCV. These proteins rely on electrostatic forces for their localization, and their displacement interferes with proper endocytic traffic and phagolysosome formation (Hamada et al. 2000).

Similar invasive strategies have evolved independently in other intracellular pathogens, such as *Vibrio parahaemolyticus* and *Shigella flexneri*, causative agents of bacillary dysentery and gastroenteritis in humans, respectively. Like *Salmonella*, these microbes use T3SS to inject PtdIns(4,5)P$_2$ phosphatases that compromise the membrane integrity of their host and disrupt tethering of actin-binding proteins to the inner aspect of the plasmalemma (Niebuhr et al. 2000; Broberg et al. 2010).

1.4.2 Phosphatidic acid in phagocytosis

Like most of the phospholipids described in this chapter, PA plays a role both as a biosynthetic and signalling intermediate. In fact, because of its head-group's simple structure (a single phosphate moiety), PA serves as the precursor for every triglyceride and glycerophospholipid in the cell. In turn, the biosynthesis of PA itself is under control of ER-resident acyltransferases that catalyze the dual transfer of acyl chains from acyl-CoA to glycerol-3-phosphate (Mok and McMurray 1990). Another major source of plasmalemmal PA is the phosphorylation of DAG by diacylglycerol kinase (Bohdanowicz and Grinstein 2013), of which 10 isoforms are endogenously expressed by macrophages (Schlam et al. 2013). While these various isoforms distribute to distinct subcellular compartments, diacylglycerol kinase β localizes exclusively to the plasmalemma, potentially implicating it in the constitutive biosynthesis of PA that drives
ruffling in professional phagocytes. Moreover, by quickly metabolizing DAG into PA, diacylglycerol kinase β has been suggested to terminate the respiratory burst, thereby establishing heterogeneity in NOX responsiveness (Schlam et al. 2013). Another pathway leading to PA biosynthesis from pre-existing lipids is the hydrolysis of phosphatidylcholine by PLD (Peng and Frohman 2012). While 2 PLD isoforms (PLD1 and PLD2) have been identified, only PLD2 distributes to the plasmalemma and endocytic membranes (Du et al. 2004). In turn, PLD1 mainly localizes to juxtanuclear compartments and the Golgi (Brown et al. 1998).

Given that 1 of its 2 dissociable protons has a pKa of 6.5-8.0, the anionic form of PA (phosphatidate) is most prevalent at physiological pH (Moncelli, Becucci, and Guidelli 1994); it is this anionic nature that allows PA to exert many of its effector functions, including directly associating with the polycationic tail of Rac (Chae et al. 2008) as well as with the Rac GEF DOCK2 (Nishikimi et al. 2009). Interestingly, many of these interactions seem to be strictly electrostatic, with no conserved PA-binding domains having been identified in these proteins so far.

In contrast to non-professional phagocytes (e.g., epithelial or endothelial cells), macrophages and immature dendritic cells display relatively high levels of PA at their plasma membrane. Biosynthesis of this simple phospholipid is particularly important for some of the dedicated immune functions of these cells (Bohdanowicz et al. 2013). Notably, in their restless lookout for foreign particles and soluble antigen, phagocytes constitutively extend membrane ruffles, which allow them to survey the environment. This incessant ruffling precedes both phagocytosis and macropinocytosis, and is driven by the ongoing formation of PA and the consequent recruitment of the Rac1 GEF TIAM1 to the plasmalemma (Bohdanowicz et al. 2013). TIAM1-dependent activation of Rac1 then leads to nucleation of actin filaments and the extension of membrane protrusions that capture and engulf phagocytic targets.

In addition to its central role in coordinating actin cytoskeletal dynamics, it has been suggested that PA directly influences membrane curvature by virtue of its inverted conical geometry (McMahon and Gallop 2005). However, given that it only constitutes
1-2% of all lipids in the cell (Vance and Steenbergen 2005), it is not perfectly intuitive how PA would manage to alter membrane curvature to a noticeable extent.

1.4.3 Phosphatidylinositol-3,4,5-trisphosphate in phagocytosis

Like other 3’-polyphosphoinositides, PtdIns(3,4,5)P₃ levels are scarce in unstimulated cells. While its levels are minute at rest (less than 0.2% of all inositol-containing lipids) (Rameh and Cantley 1999), PtdIns(3,4,5)P₃ is quickly generated upon engagement of immune receptors. The metabolism of PtdIns(3,4,5)P₃ is strictly and dynamically regulated, and in general restricted to the cytosolic leaflet of the plasmalemma (Palmieri et al. 2010). Generation of PtdIns(3,4,5)P₃ occurs mainly via phosphorylation of PtdIns(4,5)P₂ by the family of class I PI3K, which localize to the plasma membrane and use PtdIns(4,5)P₂ as a substrate for PtdIns(3,4,5)P₃ biosynthesis (Botelho, Scott, and Grinstein 2004). The class I PI3K holoenzymes are comprised of a regulatory subunit (either p85 or p101) and a catalytic p110 subunit (Vanhaesebroeck and Waterfield 1999). While the p85 regulatory subunit acts downstream of receptor tyrosine kinases, the p101 subunit responds to G protein-coupled receptors (GPCRs).

The breakdown of PtdIns(3,4,5)P₃ occurs mainly through the action of 3’- and 5’-phosphatases; PTEN dephosphorylates PtdIns(3,4,5)P₃ at the D3 position, generating PtdIns(4,5)P₂ (Maehama and Dixon 1998), while SHIP hydrolyzes the D5 position, producing PtdIns(3,4)P₂ (McCrea and De Camilli 2009).

The spatiotemporal dynamics of PtdIns(3,4,5)P₃ synthesis during phagocytosis mirror those of PtdIns(4,5)P₂ disappearance, consistent with a role for class I PI3K in mediating the conversion of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃. Indeed, PI3K is recruited to and activated at sites of phagocytosis (Marshall et al. 2001); upon particle engagement, tyrosine kinases recruit p85, the regulatory subunit of class I PI3K, initiating PtdIns(3,4,5)P₃ formation (Kwiatkowska and Sobota 1999). Synthesis of phagosomal PtdIns(3,4,5)P₃ is detectable shortly after phagocytic targets are engaged, and the phosphoinositide continues to accumulate as the phagocytic cup progresses. While PtdIns(3,4,5)P₃ is still detectable after sealing, its presence in the phagosomal compartment is short-lived and its concentration declines sharply within 1-2 minutes of sealing. Notably, SHIP accumulates at the phagosomal membrane (Marshall et al. 2001).
2001), where it likely promotes the breakdown of PtdIns(3,4,5)P$_3$ to PtdIns(3,4)P$_2$ (Marshall et al. 2001; Kamen, Levinsohn, and Swanson 2007). Figure 3 shows a diagrammatic representation of the spatiotemporal dynamics of PtdIns(3,4,5)P$_3$ during phagocytosis.

PtdIns(3,4,5)P$_3$ plays a critical and pleiotropic role during phagocytosis. Accordingly, a profound impairment of phagocytosis is observed in macrophages treated with PI3K inhibitors (Cox et al. 1999). Tellingly, treatment with the PI3K inhibitors wortmannin or LY294002 results in the formation of abortive cups that do not extend fully around the particle’s circumference. However, the polymerization of actin and the initial extension of pseudopodia persist in cells treated with these inhibitors. Thus, PI3K is dispensable for the initial stages of actin polymerization, but is necessary for later stages of pseudopodial progression, and perhaps even for phagosome sealing. Interestingly, the dependency of phagocytosis on PI3K seems to be a size-dependent phenomenon, as the uptake of large particles is much more affected by PI3K inhibitors than that of small ones (Cox et al. 1999).

It is worth emphasizing that the unproductive phagocytic cups that form when PI3K is inhibited stall at a stage where filamentous actin is richly accumulated at the base of the cup. The latter observation suggests that PtdIns(3,4,5)P$_3$ may be necessary for mediating actin breakdown, perhaps by allowing for actin, or determinants of its polymerization, to be recycled to the tips of advancing pseudopods. Orchestration of actin clearance by PI3K likely arises as a combined effect of PtdIns(4,5)P$_2$ removal and the inactivation of Rho GTPases. Consistent with this notion, it has been suggested that PI3K negatively regulates Cdc42 at later stages of phagocytosis (Beemiller et al. 2010).

Formation of 3’-polyphosphoinositides also seems to be necessary for the delivery of endomembranes to nascent phagosomes. Although most of the phagosomal membrane is of plasmalemmal origin, several compartments of the endocytic pathway are known to be focally delivered to the nascent phagosome, including recycling (Bajno et al. 2000) and late (Braun et al. 2004) endosomes. Interestingly, fusion of
endomembranes seems to be dependent on the concomitant increase in 3'-polyphosphoinositides and intracellular calcium (Dewitt, Tian, and Hallett 2006).

Like other phosphoinositides, PtdIns(3,4,5)P$_3$ orchestrates its many cellular functions by recruiting effectors that carry domains that specifically recognize its head-group. Several of these effectors carry PH domains, including myosin X, an unconventional motor protein that has been implicated in pseudopodial extension and phagosome closure (Cox et al. 2002). Treatment with wortmannin blocks myosin X enrichment at the phagocytic cup, and the expression of a truncated form of this motor reduces the ability of macrophages to carry out FcγR-dependent phagocytosis. In this regard, it is interesting that inhibition of myosin X activity prevented spreading but not adhesion of macrophages on IgG-coated substrates, and inhibited phagocytosis of large particles (Cox et al. 2002). Thus, it has been suggested that the dependency of phagocytosis of large particles on PI3K activity may be attributable to the recruitment of myosin X. Figure 4 illustrates the practical implications of PtdIns(3,4,5)P$_3$ metabolism for phagosome formation.

PtdIns(3,4,5)P$_3$ signalling is also often hijacked by intracellular pathogens as part of their colonizing strategy. Enteropathogenic *Escherichia coli* (EPEC) invades the intestinal epithelium by inducing the formation of F-actin-rich pedestals by a process that relies on the subversion of PtdIns(3,4,5)P$_3$ homeostasis (Sason et al. 2009). These actin-driven structures facilitate colonization and increase pathogenicity by allowing the bacteria to adhere tightly to intestinal surfaces (Frischknecht and Way 2001). Using a T3SS, EPEC inject a protein called translocated intimin receptor (Tir). The extracellular region of Tir acts as a receptor for intimin, a bacterial adhesin that operates as an ‘attach and efface’ virulence factor. Engagement of intimin by Tir leads to the clustering of the translocated bacterial receptors on the plane of the host cell membrane, which in turn recruit PI3K and trigger a cascade of actin rearrangement events that require PtdIns(3,4,5)P$_3$ (Sason et al. 2009).
1.4.4 Phosphatidylinositol-3,5-bisphosphate in phagocytosis

The cellular abundance of PtdIns(3,5)P₂ is very low. For a sense of scale, it has been estimated that there are about 100 PtdIns(4,5)P₂ molecules and 10 PtdIns(3)P molecules for every PtdIns(3,5)P₂ molecule (Li et al. 2013). PtdIns(3,5)P₂ is synthesized via phosphorylation of PtdIns(3)P at the D₅ position of the inositol ring by phosphatidylinositol 3-phosphate 5-kinase (PIKfyve). Both PtdIns(3,5)P₂ and PIKfyve preferentially distribute to late endosomes and the lysosomal network (Samie et al. 2013). The converse reaction, dephosphorylation of the D₅ position in PtdIns(3,5)P₂, is carried by the SAC domain-containing phosphatase FIG4 (McCartney, Zhang, and Weisman 2014). The myotubularin (MTM) family of phosphatases catalyze an alternative mode of PtdIns(3,5)P₂ breakdown, consisting of dephosphorylation of the D₃ position, leading to PtdIns(5)P formation (Berger et al. 2003).

Until recently, the precise spatial and temporal dynamics of PtdIns(3,5)P₂ during phagocytosis had remained obscure, mostly because of a paucity in methods to detect the phosphoinositide in living cells. However, a genetically encoded probe for this lipid was recently developed, consisting of the ML1N domain of TRPML1, a PtdIns(3,5)P₂-regulated ion channel (Li et al. 2013). Expression of fluorescently conjugated ML1N in phagocytes revealed that particle engagement triggers synthesis of lysosomal PtdIns(3,5)P₂, likely through PIKfyve (Samie et al. 2013). Interestingly, ML1N also translocated to sites of phagocytosis, presumably because of an increase in PtdIns(3,5)P₂ at the cup, brought about by the fusion of lysosomal membranes with nascent phagosomes. PtdIns(3,5)P₂ accumulation was evident when 6-µm, but not 3-µm, latex beads were used as phagocytic targets (Samie et al. 2013), suggesting a role for lysosome fusion with the forming phagosome only when large particles are engaged.

Acquisition of PtdIns(3,5)P₂ occurs at early stages of phagocytosis and precedes Rab5 recruitment; it may therefore play a role in directing maturation. In this regard, PtdIns(3,5)P₂-mediated activation of TRPML1 channels has been implicated in calcium-dependent fusion of endomembranes with phagosomes. This notion is supported by the observation that, in contrast to wild-type controls, phagosomes formed in TRPML1
knock-out macrophages fail to acquire lysosomal markers such as LAMP-1 (Samie et al. 2013).

1.5 Glycerophospholipid signalling during phagosome maturation

1.5.1 Phosphatidylinositol-3-phosphate in phagocytosis

Though its cellular concentration is comparatively low, PtdIns(3)P is critically involved in the maturation of phagosomes. In mammalian cells, PtdIns(3)P is found mainly at the cytoplasmic leaflet of early endosomes and in ILVs of multivesicular bodies (Gillooly et al. 2000). The predominant source of this inositide is class III PI3K (Vps34), which phosphorylates the D3 position of PtdIns (Stephens et al. 1994; Backer 2008). Vps34 localizes to early endosomes (Yan and Backer 2007), and its inhibition by wortmannin (Stephens et al. 1994) or by specific anti-Vps34 antibodies (Vieira et al. 2001) quickly eliminates PtdIns(3)P from these compartments. Though quantitatively less predominant, other sources of PtdIns(3)P also exist: phosphorylation of PtdIns by class II PI3K (MacDougall, Domin, and Waterfield 1995) and dephosphorylation of bisphosphorylated species by inositol polyphosphate phosphatases (Ferron and Vacher 2006).

In principle, three possible mechanisms could account for disappearance of PtdIns(3)P: phosphorylation, dephosphorylation and hydrolysis. The precise relative contribution of these pathways is not clear. However, enzymes that can potentially carry each of these functions have been identified. PIKfyve eliminates PtdIns(3)P by phosphorylating its D5 position, generating PtdIns(3,5)P₂ (Burd and Emr 1998). Conversely, PtdIns(3)P can be broken down by the 3′-phosphatase MTM1, a member of the myotubularin family. In addition to its hydrolytic activity, MTM1 directly interacts with Vps34 and competitively displaces it from endosomal membranes, thereby preventing it from engaging Rab5 or Rab7 (Yan and Backer 2007). Lastly, PtdIns(3)P could be removed by lysosomal
phospholipases, which gain access to the inositide as ILVs form upon ESCRT-mediated invagination of the limiting membrane (Ching et al. 1999).

When phagocytes encounter a target, PtdIns(3)P is initially absent from pseudopodia and the neighbouring (unengaged) plasmalemma. However, sealing of the phagosome and its internalization is followed by a striking and transient accumulation of the phosphoinositide, which lasts for about 10 min and coincides with the centripetal movement of the phagosomal vacuole (Vieira et al. 2001). The disappearance of phagosomal PtdIns(3)P is predicted to involve a combination of phosphorylating and hydrolytic reactions, as well as inward budding. Figure 5 depicts the dynamic changes in the subcellular distribution of PtdIns(3)P throughout the different stages of phagocytosis, as measured by a fluorescent biosensor that specifically recognizes this phosphoinositide.

Its spatiotemporal dynamics suggest that PtdIns(3)P is dispensable for pseudopod formation but that its function is related to phagosome maturation. This notion has been amply validated by a number of studies where phagosome maturation was precluded by pharmacological inhibition of PI3K. In these experiments phagocytes were treated with wortmannin, an inhibitor of both class I and III PI3K, prior to being challenged with small (3 μm) particles (Vieira et al. 2001). Small particles were utilized in the study because, as discussed above, inhibition of class I PI3K impairs phagocytosis of large particles, while internalization of small particles is only slightly affected (Cox et al. 1999). Under these conditions, phagosomes formed but did not acquire PtdIns(3)P. More importantly, these phagosomes arrested at an immature stage that had a markedly reduced content of lysosomal markers such as LAMP-1. Similar results were obtained when class III PI3K was neutralized by the injection of specific antibodies (Vieira et al. 2001). Thus, class I and class III PI3K play distinct roles in the phagocytic process; class I PI3K is responsible for the synthesis of 3’-polyphosphoinositides that control pseudopod extension and sealing, while class III PI3K orchestrates phagosome maturation by catalyzing PtdIns(3)P formation in early phagocytic compartments.

Rab5 and Rab7 are critical regulators of membrane traffic and phagolysosome biogenesis. However, their activation at phagosomal membranes alone is insufficient to
drive phagosome maturation to completion; inhibition of PI3K blocks the progression of phagosomes, even though the arrested vacuoles retain active Rab7 (Vieira et al. 2003). The functions of Rab5 and 7 in maturation seem to be heavily dependent on their ability to associate with p150, a myristoylated Serine/Threonine protein kinase and critical binding partner of Vps34 (Murray et al. 2002; Stein et al. 2003). Both recruitment of Vps34 to membranes and its catalytic activity are augmented by binding to p150 (Yan and Backer 2007). Thus, orchestration of membrane traffic by Rab5 and Rab7 requires Vps34 activation and the consequent accumulation of PtdIns(3)P in early phagosomal compartments.

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

PtdIns(3)P exerts its effects by recruiting a number of effectors that possess PX or FYVE domains. Notably, PtdIns(3)P is the phosphoinositide with the largest collection of specific binding partners: the human genome encodes 42 PX domain- and 30 FYVE domain-containing proteins, most of which selectively recognize PtdIns(3)P (Lemmon 2003). A prototypical example is the tethering molecule EEA1, which carries a FYVE domain in its C-terminus that recognizes PtdIns(3)P (Simonsen et al. 1998) and binds to early endosomes (He et al. 2009). EEA1 is also recognized by active Rab5 (Mishra et al. 2010). Thus, Rab5 and its downstream target Vps34 synergise to recruit EEA1 to the early phagosomal membrane. EEA1 is crucial for phagosome maturation, as it mediates fusion with components of the endocytic pathway by interacting with syntaxins 6 and 13 (Simonsen et al. 1999; Collins et al. 2002); these SNAREs catalyze membrane fusion during phagocytosis (Collins et al. 2002). For these reasons, neutralization of EEA1 function by introduction of inhibitory antibodies results in a blockade in phagosome maturation, much like that observed in wortmannin-treated cells (Fratti et al. 2001).
In addition to orchestrating fusogenic events between phagosomes and the early endosomal system, PtdIns(3)P is central for sorting phagosomal contents to degradative compartments. As illustrated in Figure 6, phagosomal membrane proteins destined for lysosomal destruction undergo mono- or poly-ubiquitylation and are subsequently internalized through invagination of the phagosome limiting membrane, leading to the formation of ILVs. The activated phagocytic receptor FcγRIIA is one such protein (Lee et al. 2005).

As discussed above, ESCRT proteins are responsible for the generation of ILVs. Most relevant to this review is Hrs, a subunit of ESCRT-0 that carries a FYVE domain and interacts with phagosomal PtdIns(3)P in a highly specific fashion (Vieira et al. 2004). Indeed, inhibition of PtdIns(3)P synthesis with wortmannin prevents Hrs recruitment to the phagosome. More importantly, silencing of Hrs arrests maturation at an early stage, with retention of markers of early (sorting) endosomes on the phagosomal membrane (Vieira et al. 2004).

Certain phagosomal components, such as acid hydrolase receptors, are not destined for degradation to the lysosome and instead are retrogradely ferried to the TGN by the retromer complex. Notably, the SNX subunits of retromer carry a PX domain, which mediates their tethering to phagosomal PtdIns(3)P (Cozier et al. 2002). By binding to PtdIns(3)P through their PX domain while concomitantly facilitating membrane curvature through their BAR domain, the SNX subunits of retromer mediate tubule and vesicle formation for the purposes of retrograde transport (Figure 6D).

Formation of a complex between class III PI3K and the autophagy related protein Beclin-1 appears to be necessary for retromer function. Mutations in Vps30 (the yeast ortholog of Beclin-1) lead to sorting and maturation defects, as well as decreased PtdIns(3)P levels (Burda et al. 2002). The failure to attain suitable PtdIns(3)P levels precludes association of the SNX1/2-SNX5/6 dimer with endosomal membranes (Burda et al. 2002). In *C. elegans*, clearance of apoptotic corpses is defective when *bec-1* (the Beclin-1 ortholog) is mutated, suggesting that retromer-dependent transport is a component of efferocytosis. Also, deficiencies in retromer levels or in its assembly have been linked to phagocytic defects and amyloid-beta removal in the brain; beclin-1
mutant microglia do not recruit retromer efficiently to nascent phagosomes, making them incapable of properly recycling receptors such as CD36 to the plasmalemma (Lucin et al. 2013). Neurodegenerative consequences may ensue due to the depletion of phagocytic receptors for apoptotic bodies or cell debris.

An important component of the phagosomal microbicidal arsenal is the NADPH oxidase (NOX), an electrogenic complex that generates reactive oxygen species (ROS) in the phagosomal lumen. As shown in panel B of Figure 6, NOX is a multicomponent system, comprised of two membrane proteins (gp91phox and p22phox), three soluble regulatory proteins (p40phox, p47phox and p67phox) (Dupré-Crochet, Erard, and Nüße 2013) and either Rac1 or Rac2 (Roepstorff et al. 2008; Ambruso et al. 2000). The membrane-associated subunits form a heterodimeric flavocytochrome that is responsible for the catalytic activity of the complex, generating superoxide from NADPH and oxygen. While NOX is inactive in unstimulated cells, phagocytic signals recruit the ternary complex to sites of particle engagement, where the flavocytochrome is activated (Nunes, Demaurex, and Dinauer 2013). Sustained association of the regulatory subunits of the oxidase with sealed phagosomes is facilitated by p40phox, which carries a PtdIns(3)P-binding PX domain (Ueyama et al. 2007; Ueyama et al. 2008). Indeed, retention of p40phox on the membrane of sealed phagosomes is prevented when PI3K is inhibited; importantly, sustained stimulation of NOX is absent when p40phox is knocked out or when its PX domain is mutated (Tian et al. 2008).

Because of its multifunctional role in transforming the phagosome into a microbicidal machine, PtdIns(3)P constitutes an attractive target for invasive organisms whose pathogenicity rests on preventing phagolysosome biogenesis. One such pathogen is Mycobacterium tuberculosis, an intracellular bacterium that manages to survive within the protected confines of the early phagosome by halting its maturation (Pethe et al. 2004). This arrest in phagosome progression has been attributed to the exclusion of PtdIns(3)P from the limiting membrane of the Mycobacterium-containing vacuole (Vergne, Chua, and Deretic 2003; Fratti et al. 2001; Fratti et al. 2003). To this end, the bacterium sheds mannose-capped lipoarabinomannan (ManLAM), a glycosylated PtdIns and major component of its cell wall that purportedly interferes with Vps34 activity (Vergne, Chua, and Deretic 2003). Mechanistically, it has been proposed that
ManLAM blocks the surge in cytosolic Ca\(^{2+}\) that normally accompanies phagocytosis, ostensibly interfering with Ca\(^{2+}\)/calmodulin-dependent activation of Vps34 (Vergne, Chua, and Deretic 2003). In a synergistic manner, \textit{M. tuberculosis} secretes SapM, a phosphatase that hydrolyzes PtdIns(3)P (Flannagan, Jaumouillé, and Grinstein 2012; Scholl, Ahern, and Geha 1992). Depletion of PtdIns(3)P from the mycobacterial phagosome by this combined strategy prevents the acquisition of critical mediators of vesicular traffic, such as EEA1 (Fratti et al. 2001) and Hrs (Vieira et al. 2004), thus hindering phagosome maturation.

1.6 Phosphoinositide signalling during macropinocytosis

Macropinocytosis is a dedicated endocytic pathway that mediates fluid-phase uptake of nutrients and other molecules (Lim and Gleeson 2011). It is characterized by the comparatively large size of the vesicles formed—known as macropinosomes—that range from 0.2 to 5 µm in diameter (Swanson 2008). Macropinocytosis is thus the mechanism responsible for the uptake of solutes that are excluded from other endocytic processes because of their large size. This form of endocytosis is also linked to immunity and infection, as it is a major pathway used by antigen-presenting cells for delivery of antigens to the class II major histocompatibility complex (Swanson 1989). Remarkably, some bacteria and viruses co-opt macropinocytosis as a means of entering the host cell (Swanson 2008; Lim and Gleeson 2011). These pathogens are capable of activating signalling pathways that initiate the formation of macropinosomes (Mercer and Helenius 2009).

Macropinocytic events are driven by regulated membrane ruffling. The ruffling processes leading to macropinocytosis can be stimulated by growth factors such as epidermal growth factor (EGF) and macrophage colony-stimulating factor (M-CSF) (Racoosin and Swanson 1989). However, several other molecules can promote this phenomenon by receptor independent means; these include Ras proteins (Bar-Sagi and Feramisco 1986), phorbol esters (Swanson 1989) and cationic membrane-permeant peptides (Futaki et al. 2007). In some cell types ruffling can also occur “spontaneously” or “constitutively” (Swanson 2008). It is not clear, however, whether such activity is truly
spontaneous or results from the autocrine or paracrine stimulation by unidentified factors.

It is important to appreciate that, while phagocytes constantly ruffle their membranes, not every ruffling event results in macropinosome formation (Swanson 1989); several steps are involved, and all must be finalized for macropinocytosis to reach completion. In order to initiate macropinocytosis, ruffles must form and curve to form a crater-shape structure known as the macropinocytic cup. Subsequently, protrusions need to extend to ensure cup closure, a process akin to phagosome formation. These events require substantial actin remodelling. The final stage of macropinosome formation is its scission from the plasma membrane, resulting in a sealed macropinocytic vacuole (Lim and Gleeson 2011).

As in phagocytosis, enzymes responsible for phospholipid synthesis and breakdown are activated during the course of macropinosome formation and maturation (Swanson 2008). The following sections review the similarities and differences between the roles of specific phosphoinositides in macropinocytosis and phagocytosis.

1.6.1 Phosphatidylinositol-4,5-bisphosphate in macropinocytosis

The metabolism of PtdIns(4,5)P$_2$ shows striking similarities in macropinocytosis and phagocytosis. The PtdIns(4,5)P$_2$ accumulation noted at early stages of phagocytosis is recapitulated when A431 cells are stimulated by EGF. The concentration of PtdIns(4,5)P$_2$ increases in ruffling membranes, compared to the rest of the plasma membrane, as revealed by accumulation of the PLC$\delta$-PH probe. Such PtdIns(4,5)P$_2$ accumulation during macropinocytosis is thought to occur by localized activation of PIP5K. The levels of this phosphoinositide reach a maximum just before macropinosome closure and decrease drastically thereafter (Araki et al. 2006). As in phagocytosis, PtdIns(4,5)P$_2$ is hydrolyzed by PLC$\gamma$, giving rise to Ins(3,4,5)P$_3$ and DAG.
On the other hand, simultaneous visualization of PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$ in macropinosomes by co-expressing fluorescent biosensors for each of these phosphoinositides has provided evidence that phosphorylation by PI3K contributes to the secondary disappearance of PtdIns(4,5)P$_2$ (Araki et al. 2006). It is important to note that PLC$\gamma$ is recruited to sites of PtdIns(3,4,5)P$_3$ accumulation and, as such, PI3K could promote PtdIns(4,5)P$_2$ disappearance by two synergistic mechanisms during macropinocytosis.

Local production of PtdIns(4,5)P$_2$ in the early stages of macropinosome development is partly responsible for the remodelling of the actin cytoskeleton, and is essential for the formation of ruffles that accompany cup formation (Swanson 2008). For the sake of brevity, the actin-remodelling proteins capable of interacting with PtdIns(4,5)P$_2$ will not be discussed in this section, as they were described in the sections describing phagocytosis.

The phase of PtdIns(4,5)P$_2$ disappearance is also critical for macropinosome formation; by reducing the thickness of cortical actin and consequently decreasing the rigidity of the plasma membrane, PtdIns(4,5)P$_2$ breakdown could foster membrane invagination and macropinosome sealing (Scott et al. 2005). The products of the reactions mediating conversion of PtdIns(4,5)P$_2$ are also important: Ins(3,4,5)P$_3$ and DAG are likely to function in macropinosome formation or maturation, although their roles have not been thoroughly investigated. The role of phosphoinositide phosphatases in macropinocytosis has not been explored to an extensive degree, and remains an interesting topic in need of study.

1.6.2 Phosphatidylinositol-3,4,5-trisphosphate in macropinocytosis

The concentration of PtdIns(3,4,5)P$_3$ is considerably greater in membrane ruffles compared to the bulk, resting plasma membrane. When visualized using fluorescent reporters, the levels of the phosphoinositide fluctuate in membrane protrusions (Swanson 2008), but increase noticeably in defined macropinocytic cups (Araki et al. 2007). At this stage, PtdIns(3,4,5)P$_3$ is mostly confined to the semi-circular section of
the cup (S. Yoshida et al. 2009) and its concentration further increases upon macropinosome closure (Welliver and Swanson 2012). After this transient spike, PtdIns(3,4,5)P$_3$ concentration decreases to negligible levels, coinciding with an increase in the concentration of PtdIns(3,4)P$_2$ (Welliver and Swanson 2012; Maekawa et al. 2014).

The fluctuations of PtdIns(3,4,5)P$_3$ reported to occur in membrane ruffles are most likely due to the opposing enzymatic activities of kinases (PI3K) and phosphatases (PTEN and/or SHIP). As in the case of phagocytosis, available evidence indicates that the sharp increase of PtdIns(3,4,5)P$_3$ is due to increased PI3KI activity at the ruffles and cup. The fluctuations and eventual termination of the PtdIns(3,4,5)P$_3$ burst may be partly due to depletion of its substrate, PtdIns(4,5)P$_2$ (Welliver and Swanson 2012), but phosphatases should also be considered as important contributors. The concomitant appearance of PtdIns(3,4)P$_2$ suggests the involvement of 5′-phosphatases and, consistently, knockdown of SHIP2 greatly prolongs the lifetime of PtdIns(3,4,5)P$_3$ in the macropinocytic cup while ablating the surge of PtdIns(3,4,5)P$_3$ (Maekawa et al. 2014). Interestingly, SHIP2 appears to be recruited to sites of macropinosome formation by SH3YL1, a bridging protein that binds the phosphatase while also associating with PtdIns(3,4,5)P$_3$ through its SYLF domain (Hasegawa et al. 2011).

The consequences of PtdIns(3,4,5)P$_3$ accumulation at forming macropinosomes are similar to those described for phagocytosis. By recruiting adaptors, RhoGEFs and RhoGAPs that contain PtdIns(3,4,5)P$_3$-interacting PH domains, the macropinocytic membrane orchestrates the activity of Rho GTPases that direct the polymerization of actin required for the extension of circular ruffles and macropinosome closure. In the case of macropinocytosis, however, Rab5 is also involved in actin remodelling (Lanzetti et al. 2004), albeit in a poorly understood manner. Rab5 has been reported to accumulate at the macropinosome following the observed increase in PtdIns(3,4,5)P$_3$ (S. Yoshida et al. 2009), and it has been suggested that the two events are causally linked. However, because detection of Rab5 is only convincing when macropinosomes have sealed, and its levels continue to increase well after PtdIns(3,4,5)P$_3$ has disappeared (Welliver and Swanson 2012), it is difficult to establish whether recruitment
of Rab5 by the phosphoinositide in fact precedes and is required for the formation of macropinosomes.

Regardless of the precise time of Rab5 recruitment, it is clear that the activity of PI3K is crucial for macropinosome formation. Indeed, sensitivity of the process to PI3K inhibitors is one of the few distinctive hallmarks of macropinocytosis. When formation of PtdIns(3,4,5)P3 is precluded by wortmannin or LY294002, primordial macropinocytic cups are formed but promptly recede towards the cytosol, unable to extend and seal (Araki, Johnson, and Swanson 1996; Clague, Thorpe, and Jones 1995; Amyere et al. 2000), a feature strongly reminiscent of phagocytosis of large particles.

1.6.3 Phosphatidylinositol-3,4-bisphosphate in macropinocytosis

A couple of recent studies have documented the presence of PtdIns(3,4)P2 in macropinosomes. The use of a Tapp1 PH domain-containing probe, which binds PtdIns(3,4)P2 with high specificity (Dowler et al. 2000), revealed a transient spike of this phosphoinositide during the early stages of macropinocytosis (Welliver and Swanson 2012; Maekawa et al. 2014). This sharp increase occurred during cup closure, coinciding with the decrease in PtdIns(3,4,5)P3 levels.

PtdIns(3,4)P2 generation during macropinocytosis presumably results from the breakdown of PtdIns(3,4,5)P3 by SHIP2. As mentioned earlier, silencing this phosphatase abolishes the production of PtdIns(3,4)P2. The same effect is seen when SH3YL1, the binding partner of SHIP2, is knocked down (Hasegawa et al. 2011). After its drastic increase, PtdIns(3,4)P2 is broken down by INPP4B, a 4'-phosphatase; when this phosphatase was knocked down, PtdIns(3,4)P2 lingered in closed macropinosomes for an extended period of time (Maekawa et al. 2014). Because of its transient existence on macropinosomes and its susceptibility to degradation by 4'-phosphatases, it is conceivable that PtdIns(3,4)P2 may be merely an intermediate in the conversion of PtdIns(3,4,5)P3 to PtdIns(3)P. However, to the extent that some proteins bear PH domains that recognize and sometimes prefer PtdIns(3,4)P2 to other phosphoinositides, PtdIns(3,4)P2 may be more than a metabolic intermediary. Tapp1, for instance, has
been proposed to be a target of PtdIns(3,4)P$_2$. When exogenously expressed, Tapp1 localizes to dorsal ruffles and depletion of endogenous Tapp1 suppresses the formation of dorsal ruffles (Hasegawa et al. 2011). Thus, PtdIns(3,4)P$_2$ may have a function of its own during macropinocytosis.

Other evidence is consistent with a role of PtdIns(3,4)P$_2$ in macropinocytosis. Knocking down INPP4B was found to impair macropinosome cup closure (Maekawa et al. 2014), which is also blocked when SHIP2 is silenced (Hasegawa et al. 2011). However, these results should be interpreted with caution, as in either case the inhibitory effects may have arisen from a failure to generate PtdIns(3)P, or even from excessive accumulation of PtdIns(3,4,5)P$_3$.

1.6.4 Phosphatidylinositol-3-phosphate in macropinocytosis

The appearance of PtdIns(3)P on macropinosomes was first visualized using the 2XFYVE domain probe. Remarkably, unlike phagosomes where it disappears within 15 minutes of sealing, PtdIns(3)P persists in macropinosomes formed by EGF-stimulated A431 cells for nearly 60 minutes. In fact, it has been suggested that high levels of PtdIns(3)P are maintained as long as the macropinosome exists (Araki et al. 2006). However, subsequent studies found the presence of PtdIns(3)P to be transient in both myeloid and A431 cells (S. Yoshida et al. 2009).

PtdIns(3)P is classically thought to be generated by Vps34 following cup closure and acquisition of Rab5 by the macropinosome (Stephens et al. 1994; Backer 2008). However, this notion has been recently disputed, as evidence now suggests that the initial PtdIns(3)P spike observed during ruffling and cup formation is generated by a cascade of sequential hydrolysis reactions that convert PtdIns(3,4,5)P$_3$ to PtdIns(3,4)P$_2$ and eventually PtdIns(3)P. These modifications are presumably catalyzed by SHIP and INNP4B, respectively (Maekawa et al. 2014).

Disappearance of PtdIns(3)P can occur by its dephosphorylation to PtdIns or by its phosphorylation to PtdIns(3,5)P$_2$. Phosphatases of the myotubularin family and PIKfyve, respectively, mediate these reactions. In this regard, it is noteworthy that
depletion of myotubularin-related protein 6 (MTMR6) was accompanied by accumulation of PtdIns(3)P in membrane ruffles, where the inositide is not normally detected (Maekawa et al. 2014).

Unlike phagocytosis, where PtdIns(3)P is believed to be important for vacuolar maturation, in macropinocytosis PtdIns(3)P has been proposed to participate in vacuolar formation. Inhibition of its synthesis impairs macropinocytosis, as suggested by experiments where INPP4B was knocked down (Maekawa et al. 2014). This puzzling observation may be related to ion fluxes. KCa3.1 is a Ca\(^{2+}\)-activated K\(^+\) channel shown to be stimulated by PtdIns(3)P (Srivastava et al. 2006). Depletion of KCa3.1 by gene silencing or its pharmacological inactivation by TAM-34, a channel-specific inhibitor, impairs macropinocytosis. Moreover, expression of a mutant KCa3.1 that can not be activated by PtdIns(3)P severely impaired macropinosome formation (Maekawa et al. 2014). Why and how K\(^+\) efflux through KCa3.1 is required for macropinocytosis remains unresolved.

1.7 Are diffusional barriers involved in the differential distribution of signalling lipids in cellular membranes?

Phagocytosis and macropinocytosis are spatially (and temporally) restricted phenomena. The large-scale cytoskeletal and membrane remodelling involved in these processes are guided by specific spatial coordinates, which are in part provided by the confined distribution of signalling lipids, most notably phosphoinositides. The ability of phosphoinositides to accumulate in the correct area is therefore instrumental for the establishment and progression of these polarized endocytic processes. However, in light of the fluid mosaic model of biological membranes, newly synthesized phosphoinositides are anticipated to quickly diffuse laterally and distribute in the plane of the plasmalemma within seconds. Considering the comparatively slow development of phagocytosis and macropinocytosis (in the order of minutes), a great deal of lipid diffusion and homogenization would be expected to occur before sealing is completed (Morrot et al. 1986). It is thus remarkable that, despite the apparent physical continuity of the nascent phagosomal membrane with the neighbouring bulk plasmalemma, the
accumulation of PtdIns(3,4,5)P$_3$ or DAG and the disappearance of PtdIns(4,5)P$_2$ are spatially restricted to the nascent phagosomal compartment (Marshall et al. 2001; Dewitt, Tian, and Hallett 2006). Two models, which are not mutually exclusive, have been proposed in order to account for this confined distribution.

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

A second model is based on the possible existence of a physical diffusional barrier located at the periphery of nascent phagosomes that prevents, or at least limits, the lateral diffusion of lipids in and out of the forming vacuole. This hypothesis is supported by the drastic decrease in lipid mobility observed when macrophages engage IgG-coated surfaces (Marshall et al. 2001). The possibility of a fence-mediated confinement is also supported by analyses of the mobility of phagosomal PtdIns(4,5)P$_2$, studied using a fluorescent derivative of the phosphoinositide (Golebiewska et al. 2011). Using fluorescence correlation spectroscopy, these experiments confirmed that PtdIns(4,5)P$_2$ diffuses rapidly both within the phagosomal membrane and the unengaged plasmalemma, but not across these different compartments. Despite this unrestricted mobility, fluorescence recovery after photobleaching experiments showed no recovery in PtdIns(4,5)P$_2$ fluorescence after the cup was photobleached. In contrast, PtdIns(4,5)P$_2$ fluorescence recovered within seconds when the unengaged plasmalemma was photobleached (Golebiewska et al. 2011). Together, these observations argue in favour of a fence that limits phosphoinositide diffusion in and out of the forming phagosome.
1.8 Figure legends

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

Figure 2. Functional implications of PtdIns(4,5)P₂ metabolism for phagocytosis. A) Pathways leading to PtdIns(4,5)P₂ biosynthesis (main panel) and consequent stabilization of F-actin networks (lower panels) at the phagocytic cup. Membranes are coloured-coded as in Figure 1. Rac1 and Arf6 activate PIP5K in a PA-dependent fashion. PA can be synthesized through the phosphorylation of DAG by DGK or through hydrolysis of PtdCho by PLD. PA recruits Rac GEFs (e.g. TIAM1) to phagocyte membranes, where Rac is activated. In turn, PIP5K associates with the plasma membrane through a positively charged surface, where it catalyzes the conversion of PtdIns(4)P to PtdIns(4,5)P₂. PtdIns(4,5)P₂ mediates linkage of actin networks to integral plasmalemmal proteins through intermediary ERM proteins, as well as actin
polymerization by nucleation-promoting factors such as WASp. In addition, actin-binding proteins that antagonize filament formation, such as capping protein and the severing factor cofilin, are inhibited by PtdIns(4,5)P_2. B) Depletion of PtdIns(4,5)P_2 from the base of the cup leads to actin filament removal. PtdIns(4,5)P_2 is converted by kinases (PI3K), phosphatases (OCRL) and phospholipases (PLCγ). Disappearance of the filamentous actin barrier facilitates the delivery of membranes from endolysosomal compartments to the phagocytic cup, releasing membrane tension. C) PLCγ-mediated hydrolysis of PtdIns(4,5)P_2 results in the formation of the bioactive molecules DAG and Ins(1,4,5)P_3 (IP_3). Regulatory subunits of the NADPH oxidase are activated by PKC, which is recruited to phagosomal membranes by DAG. Release of Ca^{2+} from intracellular stores is promoted by IP_3.

**Figure 3. Dynamic changes in PtdIns(3,4,5)P_3 abundance during phagosome formation.** The phagocytic response is broken down into the same stages defined in Figure 1. *Left*) Time-lapse fluorescence images of a RAW 264.7 macrophage expressing Akt(PH)-GFP, a fluorescent probe that detects PtdIns(3,4,5)P_3 [and also PtdIns(3,4)P_2]. While virtually absent from the bulk plasmalemma, engagement of phagocytic receptors triggers a transient yet marked accumulation of PtdIns(3,4,5)P_3 in the membrane of the nascent phagosome. This increase persists until phagosome sealing, and PtdIns(3,4,5)P_3 is depleted from the phagosomal membrane shortly (1-2 min) after internalization. Phagocytic particles are denoted with a star. Scale bar, 5 μm. *Right*) Schematic representation of the local changes in PtdIns(3,4,5)P_3 concentration at sites of phagocytosis, corresponding to the experimental data obtained with Akt(PH)-GFP. The colour code of the membrane is indicative of the relative abundance of PtdIns(3,4,5)P_3, ranging from grey (lowest), through light red (intermediate), to dark red (highest). Other details as in Figure 1.
Figure 4. Role of PtdIns(3,4,5)P₃ synthesis in phagosome formation. A) Signal transduction pathways leading to PI3K activation at the nascent phagosome. Membranes are coloured-coded as in Figure 3. Engagement of FcγR by an IgG-coated target triggers receptor clustering in the plane of the membrane, promoting phosphorylation on ITAM motifs by Src family kinases (SFK). Doubly phosphorylated ITAMs are sensed by a tandem SH2 domain on the non-receptor tyrosine kinase Syk, which binds to and directly activates the adaptor LAT (not illustrated). LAT stimulates docking of additional, proteins, such as PLCγ and Grb2. The former catalyzes PtdIns(4,5)P₂ hydrolysis, while the latter acts as an adaptor protein for Gab2. Once recruited to active phagocytic receptors, Gab2 is phosphorylated by Syk and subsequently induces recruitment of p85, the regulatory subunit of PI3KI. The class I PI3K holoenzyme then phosphorylates PtdIns(4,5)P₂, generating PtdIns(3,4,5)P₃. Note that Gab2 is stabilized at the phagosomal membrane by PtdIns(3,4,5)P₃ produced by PI3KI, thereby amplifying the PtdIns(3,4,5)P₃ signal. B) Orchestration of pseudopodia progression by direct PtdIns(3,4,5)P₃ effectors. PLCγ is recruited to the phagocytic cup by a PtdIns(3,4,5)P₃-interacting PH domain, promoting PtdIns(4,5)P₂ breakdown. Disappearance of PtdIns(4,5)P₂ results in the removal of F-actin from the base of the cup. Termination of polymerization is reinforced by the PtdIns(3,4,5)P₃-mediated recruitment of RhoGAPs and the consequent deactivation of Rho GTPases. Myosin motors (e.g. myosin X) also translocate to the cup in a PtdIns(3,4,5)P₃ dependent manner, where they facilitate phagosome sealing by exerting contractile forces.

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

Figure 6. Orchestration of phagosome maturation and membrane traffic by PtdIns(3)P. A) The tethering protein EEA1 utilizes its FYVE domain to engage PtdIns(3)P in both phagosomal and endosomal membranes, where it also binds to active Rab5. EEA1 interacts directly with syntaxins 6 and 13, SNARE proteins that facilitate fusion between phagosomes and early endosomes (EE). B) PtdIns(3)P promotes NADPH oxidase (NOX) activity and ROS production. p40phox, a cytosolic component of NOX, is recruited to the maturing phagosome through a PX domain that recognizes PtdIns(3)P. p40phox interacts with other subunits of the oxidase, stabilizing the complex on the phagosomal membrane and sustaining ROS generation. C) Role of PtdIns(3)P in ESCRT-mediated sorting of phagosomal membrane proteins. The ESCRT-0 subunit Hrs utilizes a FYVE domain to associate with PtdIns(3)P-rich membranes, where the complex recognizes ubiquitylated cargo, such as FcγRIIA. ESCRT-0 then triggers the sequential recruitment of ESCRT complexes I, II and III, culminating in the invagination of the limiting membrane. D) PtdIns(3)P in the retrieval of phagosomal membrane proteins by the retromer complex. Retromer, consisting of a cargo-recognition Vps trimer and a sorting nexin (SNX) dimer, is recruited to maturing phagosomes, where it promotes recycling of membrane proteins aided by actin-driven membrane tubulation. The SNX proteins of retromer carry PX and BAR domains, which they utilize for the recognition of PtdIns(3)P-rich membranes and the stabilization of membrane curvature within tubules, respectively.
1.9 Figures

Figure 1. Distribution of PtdIns(4,5)P2 during the early stages of phagocytosis

Ai

Bi

Ci

Di

Aii

Bii

Cii

Dii

PLCδ(PH)-GFP

 Binding

 Extension

 Sealing/uptake

 Maturation (early phagosome)
Figure 2. Functional implications of PtdIns(4,5)P$_2$ metabolism for phagocytosis
Figure 3. Dynamic changes in PtdIns(3,4,5)P$_3$ abundance during phagosome formation.

- **Ai**
- **Bi**
- **Ci**
- **Di**

**Aii**
- Binding

**Bii**
- Extension

**Cii**
- Sealing/uptake

**Dii**
- Maturation (early phagosome)
Figure 4. Role of PtdIns(3,4,5)P₃ synthesis in phagosome formation

A. Particle binding

- PtdIns(3,4,5)P₃
- Particle binding
- [PtdIns(3,4,5)P₃]
- Syk
- Grb2
- Gab2
- p110
- p85
- PI(4,5)P₂
- (PI3KI)
- PIP₃

B. Pseudopod progression

- PI(4,5)P₂ breakdown
- PLCγ
- PL(4,5)P₂
- Rac1
- Rho GAP
- PI₃KI
- DAG + IP₃
- Termination of actin polymerization
- Myosin-driven contractility
Figure 5. Localized PtdIns(3)P synthesis during early stages of phagosome maturation

Ai

Aii

Binding

Bi

Bii

Extension

Ci

Cii

Maturation (early phagosome)

Di

Dii

Maturation (late phagosome)
Figure 6. Orchestration of phagosome maturation and membrane traffic by PtdIns(3)P

A) Endosomal fusion

B) ROS production

C) MVB biogenesis and receptor sorting

D) Retrograde traffic

EARLY PHAGOSOME

INTERMEDIATE PHAGOSOME

To lysosome for degradation

To TGN/recycling endosomes
Chapter 2

2 Thesis aims and hypotheses

2.1 First study: Signals orchestrating the constitutive membrane rearrangements of professional phagocytes

Professional phagocytes survey their environment on an ongoing basis by ruffling their membranes. These actin-driven membrane extensions precede the capture of phagocytic targets, and also underlie the formation macropinosomes. Thus, membrane ruffling is central to the ability of professional phagocytes to gather particulate antigen by phagocytosis and soluble antigen by macropinocytosis. While our knowledge on the upstream signals responsible for this constitutive membrane ruffling is incomplete, some of my preliminary observations point to phosphatidic acid (PA) as an attractive candidate: macrophages transfected with diacylglycerol kinase β, which synthesizes PA at the plasmalemma, ruffle their membranes significantly more than those transfected with an empty vector. The notion that PA could signal actin polymerization and membrane ruffling is also supported by previous findings: PA triggers dissociation of Rac from its GDI (Abramovici et al. 2009); facilitates the electrostatic interaction of Rac with the plasmalemma (Yueqiang Zhang and Du 2009); and promotes the recruitment of the Rac GEF DOCK2 (Nishikimi et al. 2009). In addition to inducing Rac signalling at the plasma membrane, PA has been implicated in the activation of PIP5K and therefore in PtdIns(4,5)P₂ biosynthesis (Roach et al. 2012). In turn, PtdIns(4,5)P₂ binds to a large array of actin-binding proteins that catalyze actin polymerization (e.g., nucleation-promoting factors), and inhibits those that promote filament disassembly (e.g., severing factors and barbed-end capping proteins) (Scott et al. 2005).

The above observations and preliminary results lead me to hypothesize that a constitutive pathway of PA biosynthesis is responsible for the ongoing membrane ruffling that characterizes professional phagocytes. This hypothesis will be examined by addressing the following specific aims:
1. Analyze PA abundance in professional phagocytes by enzymatic, mass spectrometric and microscopy-based determinations. For the latter, employ a genetically encoded PA biosensor. Also using this biosensor, compare the levels and subcellular distribution of PA in actively ruffling cells (macrophages and immature dendritic cells) vs. epithelial cells.

2. Delineate the molecular pathway, including ligand(s), upstream receptor(s) as well as biosynthetic enzymes and intermediates that are responsible for the continuous production of plasmalemmal PA. Likely mechanisms include hydrolysis of the phosphatidylcholine head-group by PLD and the phosphorylation of diacylglycerol by diacylglycerol kinase.

2.2 Second study: Phosphoinositide 3-kinase, actin remodelling and phagocytosis completion

Accumulating evidence suggests that ongoing cycles of actin assembly and disassembly are required for the effective implementation of actin-driven processes. For instance, locomoting keratocytes shift their entire body length and turn over their whole actin network within 1 min (Pollard and Borisy 2003). Likewise, the severing factor cofilin facilitates rocketing of *Listeria monocytogenes* on actin comet tails by providing a renewable source of monomeric actin (Carlier et al. 1997). Breakdown of submembranous F-actin in lymphocytes is necessary for the cytoskeletal reorganization that drives their spreading across the surface of antigen presenting cells during formation of the immune synapse (Treanor et al. 2011; Freeman et al. 2011). Given the similarly demanding nature of phagocytosis on polymerization-ready actin, it is conceivable that actin monomers or determinants of actin polymerization become limiting as pseudopods progress around incoming targets. Thus, the continued polymerization of actin at the tips of pseudopodia may be contingent on the breakdown of actin filaments at the base of the phagocytic cup. Moreover, it is plausible that disassembly of the actin meshwork is necessary for releasing those components of the actin machinery that have become limiting.
Interestingly, phagocytes treated with conventional PI3K inhibitors such as LY294002 or wortmannin are unable to complete phagocytosis of large particles, and extend actin-rich pseudopods that extend only halfway around the incoming particle’s circumference. Thus, PI3K activity seems to be dispensable for the initial polymerization of actin that drives the launching of pseudopodia, but necessary for actin depolymerization and pseudopodia progression—especially in the case of large particles. Because of their prominent role in catalyzing actin polymerization at sites of phagocytosis, it is possible that Rho GTPases are inactivated at the base of the phagocytic cup, allowing for actin breakdown and recycling.

Therefore, in the context of the above information, I suggest that products of PI3K mediate actin turnover during phagocytosis of large particles by signalling Rho GTPase inactivation. Testing of this hypothesis will require addressing the following specific aims:

3. Test the dependency of phagocytosis completion on Rho GTPase inactivation by transfecting constructs encoding constitutively active Rac/Cdc42 and challenging transfectants with large phagocytic targets. In parallel, investigate the role of PI3K in controlling Rho GTPase activity by using pharmacological inhibitors of this enzyme in combination with biosensors of Rho GTPase activity. In both instances, visualize the actin cytoskeleton in living cells with a genetically encoded probe for F-actin.

4. Assess whether formation of 3’-phosphoinositides results in either the recruitment of RhoGAPs of the displacement of RhoGEFs. Identify putative GAP/GEF candidates by transfecting constructs encoding fluorescently tagged chimeras of these proteins and visualize their translocation (or lack thereof) to sites of phagocytosis. Investigate the dependency of these factors on 3’-phosphoinositide biosynthesis by treatment with PI3K inhibitors, and validate their functional role in phagocytosis by silencing and overexpression.
2.3 Third study: Heterogeneity in NADPH oxidase responsiveness during the respiratory burst

The phagosome is a licensed-to-kill organelle that undergoes extensive membrane remodelling and acquires a multifaceted antimicrobial armamentarium rapidly after being internalized. Microbicidal molecules of the phagosome include vacuolar ATPases that acidify its lumen, as well as hydrolases that directly degrade the biological macromolecules of cargo. Maturing phagosomes also become competent to produce reactive oxygen species. The latter are generated by the phagocyte NADPH oxidase (NOX), a multicomponent system comprised of an integral membrane flavocytochrome, 3 cytosolic regulators, and either Rac1 or Rac2. A number of membrane phospholipids have been implicated in the stabilization of the active NOX complex: PtdIns(3,4,5)P$_3$ and phosphatidic acid bind in a cooperative manner to the cytosolic NOX subunit p47$^{\text{phox}}$ (Yaffe 2002), while PtdIns(3)P selectively binds to the accessory NOX subunit p40$^{\text{phox}}$ (Suh et al. 2006), thereby promoting NOX activity at nascent phagosomal membranes. Likewise, DAG production stimulates PKC activity, in turn leading to the phosphorylation and activation of the cytosolic NOX subunit p47$^{\text{phox}}$ (Ueyama et al. 2011).

Although much progress has been made in the characterization of the molecular signals lying upstream of NOX activation, the vast majority of these studies have relied on cell population-based assays. Such analyses obscure potential heterogeneity in the oxidative response of different phagosomes that often coexist in single cells. However, compelling evidence now suggests that each phagosome is a discrete, independent compartment (Griffiths 2004). I therefore hypothesize that superoxide production is heterogeneous within the phagosomal pool, and that said heterogeneity is established by variability in signals leading to NOX activation at the phagosomal membrane. These hypotheses will be addressed through the following specific aims:

1. Devise a dynamic assay with the spatial and temporal resolution to quantitatively monitor NOX activity in single phagosomes during the course of maturation.
2. If found to exist, analyze the source of said heterogeneity while placing particular emphasis on the lipid signals leading to NOX activation. Employ genetically encoded biosensors to evaluate the relative abundance and subcellular distribution of key phospholipid mediators of the oxidative response, and contrast these determinations with the potential variability in NOX responsiveness.

2.4 Fourth study: Molecular mechanisms of gliotoxin toxicity in macrophages

The fungal pathogen *Aspergillus fumigatus* represents a growing medical problem, particularly for individuals with an underlying immunosuppression or respiratory condition. This ubiquitous mold spreads in the environment by releasing copious quantities of airborne spores that can enter the small alveolar airways of human hosts. If left unchallenged, the conidia germinate into filamentous fungi that invade the lung parenchyma. Under physiological circumstances, the transition of dormant spores into metabolically active mycelia is prevented by lung-resident (alveolar) macrophages, which eliminate the inhaled conidia by phagocytosis. Dynamic remodelling of actin filaments and a coordinated integrin-activation response are indispensable for the effective capture and internalization of phagocytic targets by macrophages.

It is puzzling that, despite the vast arsenal of antimicrobial strategies with which alveolar macrophages are equipped, *A. fumigatus* manages to escape immune eradication. A possible explanation is that this pathogen deploys virulence factors that directly sabotage phagocytic defenses. One such factor is gliotoxin, a hydrophobic mycotoxin found in the circulation of patients with invasive aspergillosis. Indeed, some of my preliminary observations revealed a pronounced inability of gliotoxin-treated human macrophages to phagocytose fungal particles. Moreover, time-lapse microscopy studies showed that gliotoxin rapidly interferes both with cell morphology, spreading and the ability of extend dynamic membrane protrusions.
The dependency of ruffling and phagocytosis on actin remodelling, in conjunction with my preliminary results, lead me to hypothesize that **gliotoxin impairs phagocyte defences by interfering with the dynamic actin networks that drive membrane ruffling and pseudopodia extension.** Validation of this hypothesis will involve fulfillment of the following **specific aims:**

1. Through a combination of confocal, differential interference contrast and scanning electron microscopy, investigate whether actin-driven processes are affected by gliotoxin. Likewise, assess if the levels of filamentous actin are altered in gliotoxin-treated cells, and whether this is causally related to the activation of actin-severing factors.

2. Pinpoint the specific actin regulators targeted by gliotoxin. As potential candidates, assess the activation state of critical Rho GTPases by biochemical means. In addition, employ genetically encoded fluorescent biosensors to monitor key phosphoinositide species.

The above predictions will be tested using human monocyte-derived macrophages. These cells are amenable to electroporation, and will be polarized to an alternative (M2) lineage to more closely resemble lung-resident macrophages.
Chapter 3

This chapter has been modified from the following: Michal Bohdanowicz, Daniel Schlam, Martin Hermansson, David Rizzuti, Gregory D Fairn, Takehiko Ueyama, Pentti Somerharju, Guangwei Du, and Sergio Grinstein. “Phosphatidic Acid Is Required for the Constitutive Ruffling and Macropinocytosis of Phagocytes.” *Molecular Biology of the Cell, April 2013.*

3 Capture: Phosphatidic acid is required for the constitutive ruffling and macropinocytosis of phagocytes

3.1 Abstract

Macrophages and immature dendritic cells continuously survey their environment in the search for foreign particles and soluble antigen. Such surveillance involves the ongoing extension of actin-rich protrusions and the consequent formation of phagosomes and macropinosomes. The signals inducing this constitutive cytoskeletal remodelling have not been defined. We report that, unlike non-phagocytic cells, macrophages and immature dendritic cells have a high concentration of phosphatidic acid (PA) in their plasma membrane. The plasmalemmal PA is synthesized by phosphorylation of diacylglycerol, which is in turn generated by a G protein-stimulated phospholipase C. Inhibition of diacylglycerol kinase activity results in the detachment of TIAM1—a Rac GEF—from the plasma membrane, thereby depressing plasmalemmal Rac activity and abolishing the constitutive ruffling and macropinocytosis that characterize macrophages and immature dendritic cells. Accumulation of PA and binding of TIAM1 to the plasma membrane require the activity of phosphoinositide 3-kinase (PI3K). Thus, a distinctive and constitutive pathway of PA biosynthesis promotes the actin remodelling that is required for immune surveillance by professional phagocytes.
3.2 Introduction

Macrophages and dendritic cells are professional phagocytes and antigen-presenting cells. Through a number of dedicated mechanisms, professional phagocytes provide immune surveillance and bridge the innate and adaptive immune systems. To these ends, they constantly probe and sample the extracellular milieu for antigens. Particulate antigens are engulfed by phagocytosis, while soluble ones are internalized by macropinocytosis. Both processes are driven by actin polymerization, in turn initiated by activation of Rho-family GTPases. The membrane ruffling that underlies macropinosome formation occurs continuously and is particularly vigorous in immature dendritic cells (iDCs). Phagocytosis, by contrast, is thought to be a receptor-initiated process. However, recent evidence indicates that both macrophages and dendritic cells probe their surroundings for particulate targets by emitting extensions even prior to receptor engagement (West et al. 2000; Flannagan et al. 2010). Like phagocytosis itself, this spontaneous probing process is also actin-mediated.

What triggers the ongoing extension of ruffles and filopodia in unstimulated phagocytes was hitherto not clear. We considered the possible involvement of signalling phospholipids, which play a crucial role in controlling actin remodelling and undergo uniquely active conversions in phagocytic cells (Yeung and Grinstein 2007). In particular, we investigated the role of PA, which promotes actin polymerization by several means (Yueqiang Zhang and Du 2009): it induces the dissociation of Rac from its Rho-specific GDP-dissociation inhibitor (GDI) (Abramovici et al. 2009); aids in recruiting Rac to the plasma membrane (Stace and Ktistakis 2006); and activates the Rac guanine exchange factor (GEF), DOCK2 (Nishikimi et al. 2009). In addition, PA contributes to the recruitment and activation of phosphatidylinositol-4-phosphate 5-kinase (PIP5K) (Roach et al. 2012). Phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P$_2$], the product of this PIP5K, facilitates actin polymerization by controlling the activity of a large battery of actin-binding proteins, including those in charge of severing F-actin (Zhang et al. 2012).
Although genetically encoded fluorescent probes have been successfully used to monitor the dynamics of other phospholipids, detection of PA in live cells has been uniquely challenging, perhaps because it is a minor, rapidly interconvertible species. A probe based on the PA-binding domain of Raf1 has been used with some success (Rizzo et al. 2000), but appears to lack the sensitivity needed to detect low levels of the phospholipid. Another protein domain, identified in the yeast protein Spo20p, also binds PA selectively (Nakanishi, de los Santos, and Neiman 2004; Kassas et al. 2012). Here, we used a construct consisting of a tandem repeat of the Spo20p domain fused to a nuclear export signal to visualize PA in macrophages and iDC. Microscopy studies performed using this probe, in combination with mass spectrometric and enzymatic determinations, revealed that phagocytes are uniquely rich in plasmalemmal PA. Phosphorylation of diacylglycerol (DAG) by DGK(s) is primarily responsible for the formation of this basal PA, which is necessary for the constitutive ruffling that underlies macropinocytosis.

3.3 Methods

3.3.1 Cell culture, plasmids, transfection and reagents

RAW 264.7, J774, HeLa and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Roswell Park Memorial Institute medium supplemented with 5% heat-inactivated fetal bovine serum (Wisent; St. Bruno, QC). Cells seeded on glass coverslips were transfected with Fugene HD (Roche; Mississauga, ON) according to the manufacturer’s instructions. In brief, each well of a 12-well plate was treated with 1 µg plasmid cDNA and 3 µl Fugene HD. Cells were used 24 h after transfection. Bone marrow-derived iDCs were generated from 6-8 week old female, wild-type C57BL/6 mice, according to Lutz et al., 1999. These primary cells were electroporated with 1 µg plasmid cDNA using the Ingenio electroporation solution (Mirus; Madison, WI) and used 8 hours after electroporation with a nucleofector I (Amaxa; Allendale, NJ). RAW 264.7 macrophages stably expressing GPI-linked GFP were described earlier (Flannagan et al., 2010). The plasmid encoding GFP-2PABD consisted of GFP fused to a tandem repeat of the PA-binding domain of Spo20p.
reported before (Zeniou-Meyer et al. 2007; Du and Frohman 2009), which was modified by adding at the N-terminus a nuclear export sequence derived from protein kinase A inhibitor-α. Plasmids encoding HA-PLD1, HA-PLD2, HA-PLD1(K898R), and HA-PLD2(K758R) were from Dr. John Brumell (Hospital for Sick Children, Toronto, ON). PLCβ1-GFP and PLCβ3-CFP were gifts from Theresa Filtz (Oregon State University, Corvallis, OR) (Yong Zhang et al. 2006). The rapamycin heterodimerization constructs (LDR, phosphatase-FKBP-RFP and phosphatase-dead-FKBP-RFP) were from Gilbert Di Paolo (Columbia University, New York, NY) (Chang-Ileto et al. 2011). The recruitable form of TIAM1 was obtained from Addgene (plasmid 20154). PM-RFP, mCherry-C1 domain (PKCδ), PAK(PBD)-YFP, PLCγ1-GFP, PLCγ2-GFP, TIAM1-GFP, GFP-R-pre, DGKα-GFP, DGKβ-GFP, DGKγ-GFP, DGKδ-GFP, DGKɛ-GFP, and DGKζ-GFP were described earlier (Flannagan et al. 2010; Flannagan et al. 2012; Shindo et al. 2003; Yeung, Terebiznik, et al. 2006). PLCγ1 (2822) and PLCγ2 (3872) antibodies were from Cell Signalling (Boston, MA) and used to probe cells fixed in 4% paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA). DGKı I (R59022; 30 μM in ethanol) and PTX (0.1 μg/mL, used overnight) were from Enzo Biochem (Farmingdale, NY). FIPI (750 nM), rapamycin (1μM), U73122 (2 μM), and latrunculin B (5 μM) were from Sigma-Aldrich (St. Louis, MO). Insulin was from Eli Lilly (Toronto, ON). PMA (0.1 μM) was from Bioshop (Burlington, ON). CTB (50 ng/ml in serum-free media for 1 hour) was from Techlab (Blacksburg, VA). Rhodamine phalloidin and 70kDa TMR-dextran were from Molecular Probes (Eugene, OR). Ionomycin (0.1 μM) was from Calbiochem (San Diego, CA). PA (L-α-phosphatidic acid; 840074) and DAG (1,2-dioctanoyl-sn-glycerol; 800800) were from Avanti (Alabaster, AL); they were dried of chloroform under a stream of N2 and made up to 100 μM with media containing 4 mg of essentially-fatty-acid-free albumin (Sigma-Aldrich). All other chemicals were from Sigma-Aldrich, including NaF (30 mM) and AlCl3 (50 μM), which were added to media to produce AlF₃.

3.3.2 Reverse transcription-polymerase chain reaction (RT-PCR)
To detect expression of the ten DGKs and the two PLCβ isoforms of interest, total cellular RNA was purified from RAW 264.7 macrophages with the RNeasy Mini kit
(Qiagen; Toronto, ON), as instructed by the manufacturer. Purified total RNA was subsequently reverse transcribed and exponentially amplified using the OneStep RT-PCR kit (Qiagen). RNA was isolated from 8x10⁵ RAW 264.7 macrophages, and 1µg of purified RNA was used as template for each reverse transcription reaction. We utilized isoform-specific primers (listed below) for cDNA generation and allowed the reaction to proceed for 30 minutes at 55°C. Reverse transcriptase was inactivated and Taq polymerase was activated by increasing the reaction temperature to 94°C for 4 min. PCR amplification was performed with the same isoform-specific primers as those employed for reverse transcription at denaturing, annealing and extension temperatures of 94°C (30 seconds), 50°C (30 seconds) and 68°C (1 minute), respectively. The PCR cycle was iterated 33 times for every sample excluding DGKβ, which underwent 40 cycles.

DGKα: *Fwd:* AAGGAAGCGTTGACAGCTGGAAGC *Rev:* TTCTGGCCGGCCACCTTCTAGG

DGKβ: *Fwd:* CATCACCTACACCATTGACAAAACCAGG *Rev:* CATTCCAGGTACTCTGCACGTCGTGC

DGKγ: *Fwd:* GCGCAACCAAGTGTTCATGTTGTG *Rev:* AGACATTTGGCTACTACTGCTGGC

DGKδ: *Fwd:* CAAGAGGAGGTCTTAAAGGCTCGAGG *Rev:* AATAGGTTGCGGTGACTGG

DGKε: *Fwd:* TTCTGCAGGCTACTTTAAGCTTCAGG *Rev:* CATTACCTGTTCCCAGAAGGTACCCG

DGKζ: *Fwd:* GACCAAGCGGGCGCTTTCC *Rev:* CAGCTGATGGCTACGATCTCCTTG

DGKη: *Fwd:* GCAAAACCAGCTCTTTCCAAGGGTGCG *Rev:* GCAGTGTTGTGGCCCTCAGTGCG

DGKθ: *Fwd:* AGTGCCCTGAACGGTTGACTCCAGGACCC *Rev:* AGGCGTACCATGGAGCGTAGACG

DGKi: *Fwd:* GAGAATGCTGTGAATGGGGAGCAC *Rev:* CCTTAATGATCCAGGTGGC

DGKκ: *Fwd:* ACAATTGATCTGTCTCAAGTTGTTTTGGC *Rev:* CCCTAGGGTGCTCAGTGC
PLCβ1: Fwd: ACCTGCTGGCTCAGAACATGTCC  Rev: CCGCTCAGGTAGCGCATGAATCC

PLCβ3: Fwd: ACCTGGTGACCTGCGTGTGG  Rev: CAGGACTCCAGCGCCGTCTCC

3.3.3 Analysis of phosphatidic acid by mass spectrometry

The lipids were extracted as described earlier (Folch, Lees, and Sloane Stanley 1957), but omitting the salt. For quantification of the PA species, a mixture of internal standards was added at the one-phase stage of extraction. The extract was evaporated and re-dissolved in chloroform/methanol (1:2, vol/vol) and infused (6 µL/min) into a Micromass Quattro micro triple-quadruple mass spectrometer operated as described previously (Hermansson et al. 2005). The PA species were detected by scanning for precursors of m/z 153 (Brügger et al. 1997) and then identified and quantified using LIMSA software (Haimi et al. 2006).

3.3.4 Enzymatic PA assay and Rac ELISA

PA content was measured enzymatically with a total PA assay kit (Abnova; Taipei City, Taiwan) according to manufacturer’s instructions. In summary, for each sample, lipids were isolated from a confluent T25 flask using chloroform-methanol extraction. The cellular lipids were digested with lipase. Glycerol 3-phosphate, the product of PA digestion, was selectively oxidized by glycerol 3-phosphate oxidase to produce hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide levels were measured with Amplex red in a SpectraMax Gemini EM fluorescence microplate reader (Molecular Devices; Sunnyvale, CA). Before chloroform-methanol extraction, the protein content of each sample was measured with a bicinchoninic acid assay (Thermo Scientific; Rockford, IL).

Rac activity was measured with a G-LISA kit against all three Rac isoforms (Cytoskeleton; Denver, CO) according to manufacturer’s instructions. In short, total cell lysates were equalized based on their protein concentrations and seeded on a well that bound active Rac. Cellular debris was washed away and the bound Rac was probed
with an anti-Rac antibody, followed by secondary antibody tagged with horseradish peroxidase. The signal was measured with a SpectraMax 190 absorbance microplate reader (Molecular Devices; Sunnyvale, CA) at 490 nm after incubation of the well with a detection reagent.

3.3.5 Microscopy and analysis

Cells on coverslips were transferred into HEPES-buffered Roswell Park Memorial Institute medium (Wisent). Differential interference contrast microscopy was performed on a DMIRE2 microscope (Leica; Solms, Germany) with Volocity (version 4.3.2; PerkinElmer; Waltham, MA). TIRF images were taken with a cell^TIRF system (Olympus; Tokyo, Japan) equipped with a 50-mW, 491-nm laser and a 150X (1.45 NA) objective, operated by Volocity. All other images were captured using a spinning-disk confocal microscopy system (Quorum; Guelph, ON) with a 63X (1.4 NA) objective on an Axiovert 200M microscope (Zeiss; Toronto, ON). Images were captured at 37°C with a back-thinned electron multiplier ImagEM C9100-13 camera (Hamamatsu; Hamamatsu City, Japan) and Volocity.

Images were analyzed using Volocity (version 6.0.1) and ImageJ (NIH; Bethesda, MD). The ruffling index was calculated according to previous methodology (Araki, Johnson, and Swanson 1996). Statistical tests were performed in SPSS 17 (IBM; Armonk, NY), using the Kruskal-Wallis test with a Dunn’s correction for multiple comparisons where necessary. TIRF data was analyzed using polynomial fitting in Prism (GraphPad; La Jolla, CA).

3.4 Results

3.4.1 Plasmalemmal PA in macrophages and iDC

We used a tandem repeat of the Spo20p51-91 domain fused to green fluorescent protein (GFP) as a genetically encoded probe for PA (Du and Frohman 2009; Zeniou-Meyer et al. 2007) (see Methods). When expressed in the macrophage-like RAW 264.7 or J774
cells, the probe (called GFP-2PABD hereafter) associated with the plasma membrane (Figure 1A), implying that PA is particularly abundant in this compartment; this conclusion is in agreement with recent mass spectrometric determinations in RAW 264.7 cells (Andreyev et al. 2010). A smaller fraction of the probe bound the nuclear/ER membrane, where PA serves as a precursor for glycerophospholipid synthesis. Importantly, the plasmalemmal enrichment of PA occurred also in primary myeloid cells. This was verified by introducing the plasmid encoding GFP-2PABD into bone marrow-derived murine iDCs by electroporation. In keeping with the findings using cell lines, the PA probe was found overwhelmingly at the plasma membrane (Figure 1C and Movie 1). By contrast, in non-phagocytic cells such as the epithelioid HeLa or the kidney-derived HEK293 cells, PA localized to the nuclear/ER membrane and inside the nucleus, but the majority was cytosolic (Figure 1B). Of note, little PA was detected at the plasma membrane, which was demarcated using a plasmalemmal marker, PM-RFP (Figure 1C). The failure of the probe to bind to the plasmalemma in HeLa and HEK293 cells reflects the scarcity of PA in this compartment, rather than abnormal behaviour of the probe in these cells. This was validated in HeLa cells by addition of exogenous PA, which partitioned into the plasma membrane and caused a rapid and extensive relocalization of the GFP-2PABD construct to the plasmalemma (Figure 1D and F). Moreover, the probe could readily detect metabolically generated PA at the membrane of HeLa cells, as shown in Fig. 1E. For these experiments cells were transfected with phospholipase D2 (PLD2), which localizes to the plasma membrane and displays high basal activity. Expression of this isoform recruited the PA probe to the membrane. This effect was not observed when a construct encoding a catalytically inactive mutant was transfected (Fig. 1F). Moreover, the distribution of the probe remained similarly unaffected when PLD1 was expressed. Unlike PLD2, this isoform is found mainly in endocytic and Golgi compartments, and is relatively inactive at rest (Du et al. 2003).

PA is an essential precursor for glycerophospholipid and triglyceride synthesis that occur in endomembranes (Bohdanowicz and Grinstein 2013). Because phagocytes displayed unusually high PA at the plasma membrane, in addition to the fraction detected in endomembranes, we predicted that their total PA content per cell would be higher than that of non-myeloid cells. Two methods were used to test this prediction:
mass spectrometry and an enzymatic assay where PA is metabolized to generate a fluorescent product (see Methods) (Morita, Ueda, and Kitagawa 2009). As illustrated in Figure 1G, the latter assay indicated that, when normalized per unit protein, macrophage-like cells indeed contain considerably more PA than non-myeloid cells. We also quantified PA by mass spectrometry. These determinations confirmed that the total PA, expressed per total phospholipid, was greater in phagocytic than in non-phagocytic cells (Figure 1H).

3.4.2 Plasmalemmal PA is produced by DGK

PLD activity is an acknowledged source of PA (Yeung and Grinstein 2007). We therefore assessed its contribution to the pool of PA present constitutively in the plasma membrane of phagocytes. Neither 1-butanol nor FIPI, two well-documented antagonists of PLD-mediated PA production, had a significant effect on the membrane partition of the GFP-2PABD probe (Figure 2A and B), and neither compound decreased the total content of PA, as measured using the enzymatic assay described above (Figure 2C). The effectiveness of the inhibitors was verified using HeLa cells transfected with PLD2; in this instance, both 1-butanol and FIPI displaced the GFP-2PABD probe from the plasma membrane (Figure 2D and E). Furthermore, the catalytically inactive mutants of PLD1 and PLD2, which are often used as dominant-negative antagonists of endogenous PLD, failed to decrease the plasmalemmal PA in RAW 264.7 cells (Figure 2B).

The preceding results suggest that a pathway other than PLD is responsible for PA formation in the membrane of phagocytes. We therefore next tested the role of DGKs, which catalyze the phosphorylation of DAG into PA. In stark contrast to PLD antagonists, the DGK inhibitor, DGKi I (also called R59022) prompted dissociation of the PA probe from the plasma membrane of RAW 264.7 cells (Figure 2F) and myeloid cells (Movie 1). The probe detached rapidly from the membrane following addition of DGKi I, with a half-life of approximately 80 sec (Figure 2G), implying rapid turnover of PA. Ethanol, the vehicle used to dissolve DGKi I, was without effect. Two lines of evidence indicate that the inhibitor indeed precluded DGK activity, rather than interfering
by other means with the binding of the PA probe. First in parallel with the decrease in PA, DGKι I caused plasmalemmal accumulation of DAG, monitored using the C1 domain of PKCδ (Figure 2F, bottom). Second, addition of exogenous PA to cells treated with DGKι I restored binding of GFP-2PABD to the membrane (Figure 2H). Moreover, as shown in Figure 2C, DGKι I also reduced the total PA content of the cells by 54%, measured biochemically. Together, these results suggest that DGK activity is the predominant source of constitutive plasmalemmal PA production in phagocytes.

3.4.3 Expression and localization of DGK isoforms
Ten different members of the DGK family have been described (Sakane et al. 2007). We proceeded to test whether one or more of these are expressed in macrophages, and whether they localize to the plasma membrane. Strikingly, using RT-PCR we found that messages encoding each of the 10 DGKs are all present in RAW 264.7 macrophages (Figure 3A). Expression of GFP-tagged versions of the DGKs was used to assess their subcellular distribution. Of the 6 GFP-tagged DGKs tested, DGKβ, DGKγ and DGKζ were found at the plasma membrane of otherwise untreated RAW 264.7 cells (Figures 3B-E). The expression and localization of multiple DGK isoforms supports the hypothesis that these enzymes are responsible for plasmalemmal PA production.

3.4.4 PA production depends on PLC activity
The rapid accumulation of DAG in cells treated with DGKι I suggests that ongoing DAG generation is masked by its rapid conversion to PA by DGK. Because at rest the concentration of plasmalemmal PA remains constant, this implies that PA synthesis is matched by its conversion to further products. In this dynamic steady state, inhibition of DAG production should result in a concomitant depletion of PA. Indeed, inhibition of PLC—which is most commonly responsible for DAG production at the plasma membrane—with U73122 decreased the PA content of RAW 264.7 cells (Figure 4A). Using the converse approach, addition of the PLC activator, ionomycin, to HeLa cells
caused a transient accumulation of the PA probe at the plasma membrane (Figure 4B and C; Movie 2).

To date, 13 PLC family members have been identified (Bunney and Katan 2011). Their potential redundancy made identification of the responsible enzyme(s) by siRNA-mediated silencing impractical. Instead we investigated whether any of the isoforms were located at the plasma membrane in resting macrophages. PLCγ1 and PLCγ2, which play an important role during phagocytosis, were not localized to the plasma membrane under unstimulated conditions; PLCγ1-GFP and PLCγ2-GFP were largely cytosolic and immunofluorescence determinations similarly failed to detect endogenous PLCγ1 or PLCγ2 at the membrane (Supplementary Figure 1). We next tested PLCβ isoforms. PLCβ1-GFP and PLCβ3-CFP were present in the plasma membrane of RAW 264.7 macrophages (Figure 4D and E), and RT-PCR suggested that messages for both enzymes are generated by these cells (Figure 4F). Moreover, PLCβ3 has been reported to regulate macrophage survival (Z. Wang et al. 2008).

3.4.5 Activation of PLC and PI3K by G protein-coupled receptors is required for synthesis of plasmalemmal PA in phagocytes

To analyze the possible involvement of PLCβ in DAG and PA generation, we took advantage of the fact that β isoforms of PLC are stimulated by G protein-coupled receptors (GPCRs). To this end, we used pertussis toxin (PTX), which blocks Gi, Go and Gt proteins from coupling with their cognate receptors. As illustrated in Figure 5D, PTX lowered the total PA content of RAW 264.7 cells, and caused the PA probe to dissociate from the plasma membrane (Figure 5E and F). Furthermore, PTX caused detachment of PLCβ3-CFP from the plasma membrane (Figure 5F). We also tested the effect of an activator of GPCRs. Addition of aluminum fluoride to HeLa cells caused accumulation of plasmalemmal DAG that was accompanied by recruitment of the PA probe (Figure 5B and C; Movie 3).

The pronounced effect of PTX on plasmalemmal PA biosynthesis, as well as the sensitivity of PLCβ isoforms to this toxin, led us to question whether other key lipid intermediates laying upstream of PA synthesis were also affected by PTX treatment.
We therefore examined the distribution of DAG (the substrate of DGKs), PtdIns(4,5)P$_2$ (the primary substrate of PLCβ) and PtdIns(3,4,5)P$_3$ (that regulates the targeting and activity of PLC isoforms), using fluorescently-tagged biosensors. As shown in Figure 5E, exposure to PTX had no discernible effect on the cellular localization of PH-PLCδ-GFP and GFP-C1PKC, sensors for PtdIns(4,5)P$_2$ and DAG, respectively. In contrast, the toxin caused dissociation of PH-Akt-GFP (a probe for PtdIns(3,4,5)P$_3$) from the plasma membrane, where it was found to be enriched in ruffles. Modest amounts of PtdIns(3,4,5)P$_3$ had been detected earlier in the membrane of unstimulated macrophages, which increase markedly during phagocytosis (Vieira et al. 2001).

The shared sensitivity of PA and PtdIns(3,4,5)P$_3$ formation to PTX raised the possibility that the events leading to biosynthesis of these phospholipids may interface. Indeed, PtdIns(3,4,5)P$_3$ has been shown to recruit and stimulate a variety of PLC isoforms, including PLCβ3 (Yong Zhang et al. 2009). We therefore used PI3K inhibitors to test whether PtdIns(3,4,5)P$_3$ is in fact required for the constitutive accumulation of PA at the plasma membrane of phagocytes. The effectiveness of the PI3K inhibitor LY294002 was verified by visualizing the displacement from endomembranes of the PX domain of p40phox [a PtdIns(3)P probe] (Figure 5A). In parallel with the inhibition of PI3K activity, LY294002 caused the detachment of GFP-2PABD from the plasma membrane, implying that indeed PI3K controls PA biosynthesis. Addition of exogenous PA restored the plasmalemmal localization of GFP-2PABD to the membrane, suggesting that LY294002 did not directly interfere with the ability of the probe to recognize PA (lower panels, Figure 5A). This finding also implies that the GFP-2PABD probe does not require PtdIns(3,4,5)P$_3$ to associate with the membrane. Accordingly, when we used insulin to stimulate PI3K activity in HeLa cells, we saw plasmalemmal recruitment of PH-Akt-RFP, but not of GFP-2PABD (Supplementary Figure 2A). Conversely, DGKi I caused GFP-2PABD, but not PH-Akt-RFP, to dissociate from the plasma membrane of RAW 264.7 cells (Supplementary Figure 2B). Together, these findings indicate that the GFP-2PABD probe recognizes PA, but not PtdIns(3,4,5)P$_3$, and that PI3K is partly responsible for orchestrating PA formation in the plasma membrane of professional phagocytes.
The preceding observations support the notion that constitutive production of PA at the plasma membrane depends on the activity of PI3K and PLCβ, both of which are in turn regulated by GPCRs. Though these results provide convincing evidence of the involvement of a PTX-sensitive G protein, we were unable to pinpoint the precise receptor(s) involved. Inhibitors of sphingosine-1-phosphate, lysophosphatidic acid, prostaglandin or P2Y receptors were without effect on the membrane association of the GFP-2PABD probe (data not shown). Similarly, serum starvation for six hours had no discernible effect, and we could not detect autocrine or paracrine factors by incubating HeLa cells with conditioned medium used to culture RAW 264.7 cells. It is possible that more than one GPCR or an orphan GPCR stimulate plasmalemmal DAG and PA production in phagocytic cells.

3.4.6 PA production depends on PtdIns(4,5)P₂

PtdIns(4,5)P₂ is the preferred substrate of most mammalian PLCs. To determine whether PtdIns(4,5)P₂ was required for plasmalemmal PA production, we used a rapamycin heterodimerization system to recruit to the plasma membrane the phosphatase domain of synaptojanin, which can hydrolyze PtdIns(4,5)P₂. The heterodimerization system consists of two separate polypeptides: one is soluble and includes both the phosphatase and rapamycin-binding domain. The second is associated with the plasmalemma and binds in a complementary manner to another moiety of rapamycin. Addition of rapamycin brings the phosphatase to the membrane by promoting the interaction between rapamycin-binding domains in the cytosolic and plasmalemmal constructs (Figure 6A). As shown in Figure 6B and D, recruitment of the phosphatase caused dissociation of the GFP-2PABD from the membrane, indicating that the continuous formation of PA required the presence of PtdIns(4,5)P₂. That the effect of rapamycin was due to depletion of PtdIns(4,5)P₂ was confirmed using a catalytically-inactive mutant of the phosphatase. Rapamycin-induced recruitment of this mutant had no discernible effect on PA (Figure 6C and D).
3.4.7 PA is necessary for the plasmalemmal Rac activity that underlies membrane ruffling

PA can regulate the activity of Rac (Nishikimi et al. 2009; Abramovici et al. 2009), a monomeric GTPase key to membrane ruffle formation (West et al. 2000; Flannagan et al. 2010). It thus seemed plausible that a PA-Rac axis was involved in the constitutive ruffling of phagocytes during the course of immune surveillance. This notion is borne out by the observation that plasmalemmal PA abundance is correlated with the extent of ruffling, as RAW 264.7 and iDCs—which have more plasmalemmal PA than their epithelial counterparts—ruffle much more actively than HeLa cells (Movie 4). We therefore tested the effect of impairing PA production on membrane ruffling. Notably, inhibition of DGK activity depressed both the rate and extent of ruffle formation in iDCs (Figure 7A and B). The ruffling index (Araki, Johnson, and Swanson 1996) of these cells decreased by 65%, and this was accompanied by a decline in the formation of actin-rich protrusions that underlie ruffle formation (Figure 7C). Note that the total F-actin content of the cells—measured by extracting F-actin-bound phalloidin with methanol—was unaffected by the DGK inhibitor (Supplementary Figure 3A), indicating that its effect was specifically on ruffling, and not on wholesale inhibition of actin polymerization. Indeed, some RAW 264.7 cells treated with the DGK inhibitor exhibited bundles of actin reminiscent of stress fibres (Supplementary Figure 3B), suggesting an alteration of the equilibrium between Rho and Rac activity.

We confirmed and extended the above ruffling index and phalloidin determinations using an independent method based on total internal reflection fluorescence (TIRF) microscopy. RAW 264.7 cells stably expressing glycophosphatidylinositol(GPI)-anchored GFP, an exofacial marker, were suspended and allowed to settle on a coverslip coated with BSA. Once the cells made contact with the coverslip, dynamic membrane protrusions were readily visible in the focal (TIRF) plane (Figure 7D; Movie 5). Integration of membrane fluorescence and of its rate of change over time provided a robust measure of ruffling activity. As expected, these determinations confirmed that ruffling was inhibited by latrunculin B, an inhibitor of actin polymerization. More importantly, a profound inhibition of ruffling was also recorded in cells treated with DGKi I or PTX (Figure 7D and E).
We verified that the inhibition of ruffling was caused by interference with Rac using a construct consisting of the p21-binding domain (PBD) of p21-activated kinase (PAK) tagged with YFP. PAK(PBD)-YFP is used routinely as an indicator of Rac activity (Srinivasan et al. 2003). When expressed in RAW 264.7 cells, the construct accumulated in ruffles that formed spontaneously, consistent with Rac involvement (Figure 7G). Incubation with DGKi I reduced the number of PAK(PBD)-YFP-enriched ruffles. PAK(PBD)-YFP also weakly associates with active Cdc42 (Srinivasan et al. 2003). To ensure that Rac was in fact the target of modulation by PA, we also used an enzyme-linked immunosorbent assay that detects Rac activity specifically (Figure 7F). Unstimulated macrophages displayed readily detectable Rac activity that, as expected, was virtually eliminated by Clostridium difficile toxin B (CTB). Importantly, DGKi I also markedly depressed this resting Rac activity. That Rac deactivation is accompanied by a decrease in plasmalemmal PA was verified by transiently co-transfecting constructs encoding PAK(PBD)-YFP and GFP-PABD prior to exposure of the cells to PTX. As shown in Figure 7H, PTX impaired membrane ruffling and led to displacement of PAK(PBD)-YFP and GFP-2PABD form the plasma membrane. While addition of exogenous PA rescued the association of GFP-2PABD with the plasma membrane, this treatment was not sufficient to re-activate Rac or induce de novo formation of membrane ruffles (Movie 6), indicating that PA signalling is necessary, but not sufficient to support activation of plasmalemmal Rac. Other, factors, likely including PtdIns(3,4,5)P₃, are necessary to support Rac activation and constitutive ruffling.

3.4.8 Role of PA and PtdIns(3,4,5)P₃ in the association of TIAM1 with the plasma membrane

PA was recently reported to promote the recruitment of DOCK2, a Rac GEF, by interacting with a polybasic region at its C-terminus (Nishikimi et al. 2009). Another GEF, TIAM1 (T-cell lymphoma invasion and metastasis-inducing protein 1), has been identified as an important activator of Rac in macrophages (Mizrahi et al. 2005). Like DOCK2, TIAM1 contains a polybasic domain, consisting of five consecutive arginines near its C-terminus. We therefore investigated whether PA contributes to the association of TIAM1 with the membrane. As shown in Figure 8A, the GFP-tagged
TIAM1 constitutively localizes to the plasma membrane of macrophages, and is particularly noticeable at ruffles. Addition of the DGK inhibitor caused this Rac GEF to detach from the membrane, which concomitantly terminated ruffling (Figure 8A and C; Movie 7). Addition of exogenous PA rescued TIAM1-GFP recruitment to the membrane and restored ruffling (Movie 7, panel B). In contrast, addition of exogenous DAG to DGKi I-treated cells was unable to relocate TIAM1-GFP to the membrane (Figure 8C; Movie 7, panel A). The inhibitory effect of DGKi I on TIAM1 was not due to a global effect on plasmalemmal surface charge, as indicated by the retention on the membrane of the cationic probe GFP-R-pre, described earlier (Yeung, Terebiznik, et al. 2006) (Supplementary Figure 4). These findings indicate that not only DOCK2, but also TIAM1 could account for the constitutive, PA-dependent activity of Rac on the membrane of phagocytes.

While addition of exogenous PA was sufficient to restore plasmalemmal association of TIAM1 and membrane ruffling in DGKi I-treated cells (Figure 8A and C; movie 7), it was insufficient to restore ruffling in cells exposed to PTX (Figure 7H and movie 6). Thus, while it is clear that PA is necessary for the association of TIAM1 with the plasma membrane, PTX seemingly interferes also with other components necessary for constitutive recruitment of TIAM1. Because PTX treatment led to a marked decrease in plasmalemmal PtdIns(3,4,5)P3 (Figure 5E and F), we considered the possibility that this phosphoinositide was also required for the recruitment of TIAM1 to the plasma membrane. We therefore treated RAW 264.7 macrophages co-expressing TIAM1-GFP and PX-mCherry with LY294002. As found when using the DAG kinase inhibitor (Figure 8A), PI3K inhibition resulted in the displacement of TIAM1-GFP from the plasma membrane (Figure 8B and C). Importantly, inhibition of PI3K resulted in the concomitant impairment of membrane ruffling (Movie 8). Together, these results imply that plasmalemmal PA and PtdIns(3,4,5)P3 need to be present simultaneously for optimal recruitment of TIAM1.

That TIAM1 association with the plasmalemma is coincident in space and time with actin remodelling suggests that nucleotide exchange on Rac is a determinant of membrane ruffling under the conditions analyzed above. If this is indeed the case, then expression of a recombinant exchange factor for Rac that permanently associates with
the plasma membrane should bypass the PI3K and DGK requirement for ruffling. To test this hypothesis, we expressed the GEF (tandem DH-PH) domain of TIAM1 tagged with EYFP and joined by a flexible linker to the rapamycin-binding domain FKBP (Inoue et al. 2005). A diagrammatic representation of this construct (hereafter referred to as YF-TIAM1) is shown in Figure 8D, where it is compared to the full-length TIAM1-GFP. The YF-TIAM1 construct was co-transfected with LDR, its heterodimerization partner, which targets to the plasma membrane. In the absence of the dimerizing agent (rapamycin), YF-TIAM1 was entirely cytosolic (Figure 8E). However, YF-TIAM1 translocated to the plasma membrane upon rapamycin addition, where it effected the formation of extensive dorsal ruffles, in excess over those forming spontaneously. More importantly, TIAM1 was recruited to phagocyte surfaces even when cells had been pre-incubated for 20 min with fully inhibitory doses of DGKi or LY294002, and this was accompanied by equally pronounced dorsal ruffling (Figure 8E). These data provide strong evidence that plasmalemmal association of TIAM1 (and/or other RhoGEFs) is both necessary and sufficient to induce membrane ruffling in phagocytes. They also suggest that DGK- and PI3K-mediated signals are normally required to signal nucleotide exchange on Rac by TIAM1 during membrane ruffling and macropinocytosis.

3.4.9 PA is required for macropinocytosis

Membrane ruffling underlies macropinocytosis. We therefore anticipated that inhibition of PA synthesis, and thereby of ruffling, would also impair macropinosome formation. We tested this prediction by treating the phagocytes with DGKi I. Under control conditions, iDCs internalized labelled dextran into large vacuoles, likely macropinosomes (Figure 9A). Upon DGK inhibition, the amount of dextran internalized decreased markedly and uptake was associated with smaller vesicles, presumably endosomes (Fig. 9A and B). Though less active than iDCs, resting RAW 264.7 cells also formed macropinosomes spontaneously, and their formation was impaired by both DGKi I and the PLC inhibitor U73122 (Figure 9C). These observations support the notion that PA production is necessary for constitutive macropinocytosis in macrophages and iDCs.
3.5 Discussion

Professional phagocytes represent sentinel cells of the immune system, providing early defence against noxious agents. Their constant vigilance involves probing the environment for foreign antigens and invading microorganisms. Here, we report that the actin polymerization that drives membrane ruffling and such probing requires PA. Accordingly, macrophages and iDCs were found to contain higher levels of plasmalemmal PA than those found in non-phagocytic cells, such as HeLa and HEK293.

PLD is considered to be an important source of PA. Remarkably, a variety of manipulations designed to impair PLD activity—addition of 1-butanol or FIPI, or the expression of dominant-negative alleles of PLD1 or PLD2—failed to reduce the level of plasmalemmal PA in phagocytes. These same conditions effectively suppressed PA levels induced by increased PLD activity in non-phagocytic cells (Su et al. 2009; Shen, Xu, and Foster 2001) (see also Figure 2B and D). Instead, our results indicate that phosphorylation of DAG is the primary source of the PA produced in the membrane of unstimulated macrophages and iDCs. The source of the DAG appears to be the hydrolysis of PtdIns(4,5)P_2 by PLC; not only do PLC inhibitors reduce the plasmalemmal PA, but so does the acute depletion of PtdIns(4,5)P_2 by specific, recruitable phosphatases. Furthermore, our results suggest that PLCβ but not PLCγ is responsible for the production of DAG. PLCβ isoforms are generally regulated by GPCRs, and the observation that PA formation is susceptible to PTX (a G-protein antagonist) supports the involvement of β PLCs.

DAG released by PLCs is phosphorylated by DGK. Strikingly, phagocytes express all 10 known DGKs, and 3 of these were verified to be located at the plasmalemma. Although the redundancy of DGKs prevented identification of the specific family member(s) responsible for PA production, the effects of DGKi I clearly implied a role for this lipid kinase family. Two lines of evidence indicate that the effects of the inhibitor were specific: the delocalization of the GFP-2PABD induced by the inhibitor was reversed by addition of exogenous PA and, secondly, the reduction in PA was accompanied by an
elevation in plasmalemmal DAG. The latter also implies that PA turns over rapidly; even in cells at rest PtdIns(4,5)P$_2$ must be continuously hydrolyzed by PLC and the resulting DAG converted to PA. The inhibition of PLC could thus be expected to increase PtdIns(4,5)P$_2$. Accordingly, addition of PLC inhibitors is associated with increased cortical actin (Scott et al. 2005), which is a sensitive index of PtdIns(4,5)P$_2$.

The accumulation of plasmalemmal PA seems to be essential for the constitutive ruffling of phagocytes. This conclusion is supported directly by the inhibitory effects of DGKi I and indirectly by the inhibition caused by depletion of PtdIns(4,5)P$_2$ or by inhibition of PLC. PA can modulate Rac activity and actin polymerization in a variety of synergistic ways (Yueqiang Zhang and Du 2009): it contributes to the recruitment and activation of PIP5K (Roach et al. 2012), stimulates the dissociation of Rac from its GDI (Abramovici et al. 2009), aids in the recruitment of Rac to the membrane (Stace and Ktistakis 2006), and promotes nucleotide exchange on Rac by RhoGEFs (Nishikimi et al. 2009). Yet, while necessary, PA is seemingly not sufficient to support membrane ruffling: overexpression of PLD2 induced production of PA in HeLa cells, but did not elicit ruffling. Moreover, although PA was abundant throughout the plasma membrane of macrophages and iDCs, ruffles formed only in localized areas. Clearly, other factors are permissive to the response. Interestingly, aluminum fluoride, an activator of G proteins, promoted the recruitment of the PA probe to the membrane and the simultaneous formation of ruffle-like extensions in HeLa cells (Movie 3). The GPCRs may reside sufficiently upstream in the signalling pathway, enabling them to enlist additional regulators of ruffling such as PI3K and/or Ras GTPases. Accordingly, we found that PtdIns(3,4,5)P$_3$ was detectable in the membrane of otherwise unstimulated macrophages and that its presence was also eliminated by PTX. Our data suggest that PtdIns(3,4,5)P$_3$-generated as a result of the ongoing activity of a PTX-sensitive GPCR—plays at least two distinct roles in the genesis of membrane ruffling: it appears to be required for the recruitment to the membrane of TIAM1, which has an N-terminal PH domain known to bind PtdIns(3,4,5)P$_3$ (see Figure 8 and Ceccarelli et al., 2007); and most likely also contributes to the plasmalemmal recruitment and activation of PLC$\beta$ isoforms (Zhang et al., 2006).
The formation of PA- and PtdIns(3,4,5)P₃-dependent ruffles on the surface of phagocytes fulfills several functions: it underpins the formation of macropinosomes, facilitates fluid-phase uptake, aids in probing the environment for phagocytic targets and enables phagocytes to dynamically extend their reach. The high basal level of PA may also potentiate other key functions in myeloid cells. For instance, neutrophil chemotaxis—which is similarly sensitive to PTX—requires membrane extension prior to integrin-mediated attachment to the substratum. Additionally, because exocytosis is stimulated by PA in some cells (Zeniou-Meyer et al. 2007), the secretion of cytokines and antimicrobial factors may also be facilitated in phagocytes. Lastly, because PA can regulate mTOR activity (Fang et al. 2001), it may influence the longevity of phagocytes as well as the tubulation of their lysosomal compartments for the purposes of antigen presentation (Saric et al. 2015).

In summary, we found that the basal levels of PA vary greatly amongst cell types, being exceptionally high in professional phagocytes. This high level of PA, in conjunction with the presence of a PtdIns(3,4,5)P₃ coincident signal, is critical for the continuous membrane ruffling that underlies the specialized function of macrophages and iDCs in immune surveillance.

### 3.6 Figure legends

**Figure 1. PA is elevated at the plasma membrane of macrophages and iDCs.** A) RAW 264.7 macrophage transiently transfected with GFP-2PABD; a representative optical section obtained by confocal microscopy is illustrated. B) HeLa cells transiently co-transfected with GFP-2PABD and PM-RFP were examined as in A. C) Quantification of the fluorescence intensity of GFP-2PABD at the plasma membrane relative to that of the cytosol in various cell types. Data are means ± SE of at least 3 individual experiments; a minimum of 100 cells was quantified per cell type. D) HeLa cells were transiently transfected with GFP-2PABD and images were acquired before and 10 min after addition of exogenous PA. E) Representative confocal fluorescence image of live HeLa cells transiently co-transfected with GFP-2PABD and HA-tagged PLD2. Scale bars represent 5 µm. F) Quantification of the fluorescence intensity of GFP-2PABD at
the plasma membrane relative to that in the cytosol in HeLa cells transiently transfected with the indicated PLD constructs or exposed to 100 µM exogenous PA. Data are means ± SE of at least 3 individual experiments; a minimum of 100 cells was quantified per condition. G) Quantification of the PA content of cell lysates, determined using the enzymatic assay described under Methods. Data are means ± SE of at least 3 individual experiments for each cell type. H) Quantification of PA content of cell lysates by mass spectrometry. Data are means ± SE of at least 3 individual experiments.

**Figure 2. Plasmalemmal PA is generated by DGK.** A) RAW 264.7 macrophages transiently transfected with GFP-2PABD or (D,E) HeLa cells co-transfected with GFP-2PABD and HA-PLD2 were examined by confocal microscopy immediately before and 10 min after treatment with (A,D) 0.3% 1-butanol or (E) 750 nM FPII. Insets here and elsewhere show magnifications of the area denoted by the dashed line. B) Quantification of the fluorescence intensity of GFP-2PABD at the plasma membrane of RAW 264.7 macrophages relative to that of the cytosol. Data are means ± SE of at least 3 individual experiments; a minimum of 50 cells was quantified per condition. C) Quantification of the PA content of RAW 264.7 cell lysates using the enzymatic assay described in Methods. Data are means ± SE of at least 3 individual experiments. F) RAW 264.7 macrophages co-transfected with GFP-2PABD and mCherry-C1PKC were examined by confocal microscopy before or 10 min after treatment with 30 µM DGKi I. G) Quantitation of the fluorescence intensity of GFP-2PABD at the plasma membrane of RAW 264.7 macrophages as a function of time following exposure to 30 µM DGKi I. Plasmalemmal fluorescence was normalized to the initial value, to enable comparison between experiments. Data are means ± SE of 6 independent determinations. H) RAW 264.7 macrophages transfected with GFP-2PABD were examined by confocal microscopy before, 10 min after treatment with 30 µM DGKi I, and a further 10 min after the subsequent addition of 100 µM PA. Images in A, D, F and H are representative of at least 3 experiments of each kind. Scale bars = 5 µm.

**Figure 3. Expression of DGK isoforms in macrophages.** A) Transcription of all 10 DGK isoforms was validated by RT-PCR using mRNA extracted from RAW 264.7
macrophages. B-D) RAW 264.7 macrophages were transiently transfected with (B) DGKβ-GFP, (C) DGKγ-GFP or (D) DGKζ-GFP and examined by confocal microscopy. Scale bars = 5 µm. E) Quantification of fluorescence of the different DGK isoforms at the plasma membrane relative to that in the cytosol. Data are means ± SE of at least 3 individual experiments; a minimum of 100 cells was quantified per condition.

Figure 4. **PLC isoforms localize to the plasma membrane of macrophages, where they catalyze formation of intermediates of PA biosynthesis.** A) Quantification of PA content of RAW 264.7 macrophage lysates using the enzymatic assay. Cells were pretreated with 2 µM of the PLC inhibitor U73122 for 20 min, or with vehicle (DMSO) alone. Data are means ± SE of at least 3 individual experiments. B) HeLa cells transiently transfected with GFP-2PABD and treated with ionomycin were imaged by confocal microscopy. Scale bar = 5 µm. C) Quantification of fluorescence at the plasma membrane of HeLa cells transiently transfected with GFP-2PABD and treated with DMSO alone or with 1 µM ionomycin for 2 min. Data are means ± SE of at least 3 individual experiments; a minimum of 20 cells was quantified per condition. D-E) RAW 264.7 macrophages were transiently transfected with (D) PLCβ1-GFP or (E) PLCβ3-CFP and imaged by confocal microscopy. F) RT-PCR of PLCβ1 and β3 using mRNA from RAW 264.7 macrophages.

Figure 5. **GPCR signalling and PI3K activity are required for synthesis of plasmalemmal PA in macrophages.** A) RAW 264.7 macrophages were transiently co-transfected with constructs encoding GFP-2PABD, as well as PX-mCherry (a PtdIns(3)P biosensor used to monitor PI3K activity), and examined by confocal microscopy. Macrophages were treated with solvent only (DMSO; top), 100 µM LY294002 alone (middle), or LY294002 followed by addition of exogenous PA (bottom). B) HeLa cells were transfected with GFP-2PABD and examined immediately before and 20 min after treatment with 50 µM aluminum fluoride. C) Quantification of plasmalemmal fluorescence for experiments depicted in B. D) Quantification of the PA content in RAW 264.7 cell lysates before and after exposure to pertussis toxin (PTX). Data in C and D
are means ± SE of at least 3 individual experiments; a minimum of 50 cells was quantified per condition. 

E) RAW 264.7 macrophages were transfected with constructs encoding GFP chimeras of 2PABD, the PH domain of PLCδ [a probe for PtdIns(4,5)P2], the PH domain of Akt [a probe for PtdIns(3,4,5)P3] or the C1 domain of PKCδ (a probe for DAG). The distribution of the lipids detected by these probes was monitored by confocal microscopy in otherwise untreated (Control; top) cells and in cells exposed to PTX for 16 hours (bottom). Scale bars = 5 µm.

(F) Quantification of plasma membrane fluorescence of RAW 264.7 cells transfected with the lipid biosensors or with PLCβ3-CFP. Where indicated, cells were pre-treated with 0.1 µg/mL PTX for 16 hr. Data are means of at least 3 individual experiments; a minimum of 30 cells were quantified per condition.

Figure 6. PtdIns(4,5)P2 is required for plasmalemmal PA production. A) Experimental strategy: RAW 264.7 macrophages were transiently transfected with constructs encoding two complementary rapamycin-binding domains: LDR, which includes the membrane-targeting N-terminal sequence of Lyn; and either active phosphatase-FKBP-RFP or phosphatase-dead-FKBP-RFP. Addition of rapamycin (black circle) induces translocation of the soluble FKBP domain-containing protein to the plasma membrane. The star indicates 5’-phosphatase activity at the plasma membrane. B-C) RAW 264.7 macrophages transiently co-transfected with LDR, GFP-2PABD and either (B) active phosphatase (PaseSynj1-FKBP-RFP) or (C) phosphatase-dead (PaseSynj1(D730A)-FKBP-RFP). Confocal images were acquired immediately before (left panel in B and insets in C) or 15 min after addition of 1 µM rapamycin. Scale bars = 5 µm. D) Quantification of plasmalemmal GFP-2PABD fluorescence relative to that in the cytosol for experiments depicted in B and C. Data are means ± SE of at least 3 individual experiments; a minimum of 20 cells was quantified per condition.

Figure 7. PA is required for steady state ruffling. A) Bone marrow-derived iDCs were imaged by differential interference contrast microscopy. Images were acquired
immediately before and 15 min after treatment with 30 µM DGKi I. B) Quantification of the ruffling index of iDCs treated with either 30 µM DGKi I or with solvent (EtOH; 0.3%) alone. Data are means ± SE of 3 individual experiments; a minimum of 30 cells was quantified per condition. C) RAW 264.7 macrophages treated with EtOH (left) or 30 µM DGKi I (right) were fixed, stained with rhodamine-conjugated phalloidin and imaged by confocal microscopy. D) RAW 264.7 macrophages stably expressing GPI-linked GFP were pretreated with EtOH (above) or 30 µM DGKi I (below) for 20 min and allowed to settle onto BSA-coated coverslips. Images were acquired at 40 sec intervals by TIRF microscopy. E) Cumulative fluorescence of the contact area of macrophages stably expressing GPI-linked GFP, integrated in the TIRF plane. Cells were treated with 30 µM DGKi I, 0.1 µg/mL PTX, 5 µM latrunculin B or vehicle (EtOH) only, as indicated. Data are means ± SE of at least 3 individual experiments; a minimum of 10 cells was quantified per condition. Inset shows the mean slopes ± SE. F) Quantification of active Rac detected in RAW 264.7 cell lysates using an enzyme-linked immunosorbent assay. Cells were pre-treated with either 30 µM DGKi I for 20 min, 50 ng/mL Clostridium toxin B (CTB) for 1 hr or with solvent (EtOH alone). Data are means ± SE of 5 individual experiments. G) RAW 264.7 cells transiently co-transfected with mCherry-C1PKC and PAK(PBD)-YFP were imaged by confocal microscopy immediately before and 10 min after addition of 30 µM DGKi I. Insets show corresponding differential interference contrast images. (H) RAW 264.7 macrophages were transiently transfected with either PAK(PBD)-YFP or GFP-2PABD and incubated with 0.1 µg/mL PTX overnight (middle and right columns) or left otherwise untreated (left column). Where indicated, 100 µM PA was added to the culture medium 20 min before analysis by differential interference contrast (top row) or confocal (middle and bottom rows) microscopy. Scale bars = 5 µm.

Figure 8. DGK- and PI3K-derived lipid signals are both necessary for the constitutive association of TIAM1 with the phagocyte membrane. A) RAW 264.7 macrophages transiently co-transfected with mCherry-C1PKC and TIAM1-GFP were imaged immediately before and 10 min after addition of 30 µM DGKi I. B) RAW 264.7 macrophages transiently transfected with either PX-mCherry or TIAM1-GFP were fixed immediately before or after exposure to 100 µM LY294002 and imaged by confocal
microscopy. Scale bars = 5 μm. (C) Quantification of fluorescence at the plasma membrane of macrophages transiently transfected with TIAM1-GFP and treated with 0.3% ethanol, 30 μM DGKi I alone, 30 μM DGKi I plus 100 μM PA, 30 μM DGKi I plus 100 μM DAG, 0.1% DMSO or 100 μM LY294002. Data are means ± SE of at least 3 individual experiments; a minimum of 30 cells were quantified per condition. D) Domain architecture of TIAM1-GFP (top) and YF-TIAM1 (bottom). The former is composed of the full-length, wild-type sequence of TIAM1 fused to GFP, while the latter comprises only the tandem DH-PH (GEF) domain of TIAM1 coupled to a rapamycin-binding domain (FKBP) and EYFP by a flexible linker. E) RAW 264.7 macrophages transfected with a construct encoding YF-TIAM1 were pretreated with DMSO (vehicle control), 1 μM rapamycin, or with a combination of rapamycin and 30 μM DGKi I, or rapamycin and 100 μM LY294002 before being fixed, permeabilized and stained with Alexa 647 phalloidin. Images in the top panel are single confocal sections that show redistribution of YF-TIAM1 upon exposure to rapamycin, while those in the bottom panel are Z-projections illustrating actin staining within extensive ruffles. Arrows point to macrophages transfected with YF-TIAM1. Scale bars = 10 μm.

**Figure 9. PA is required for macropinocytosis.** A) Bone marrow-derived iDCs pretreated with 0.3% EtOH (left) or 30 μM DGKi I (right) for 20 min were pulsed with tetramethylRhodamine (TMR)-dextran (70 KDa) for 1 h and imaged by confocal microscopy. Scale bars = 5 μm. B-C) Quantification of the TMR-dextran fluorescence uptake. B) iDCs were pretreated for 20 min with the indicated concentrations of DGKi I or with vehicle only, then incubated with TMR-Dextran for 1 hour followed by imaging as above. Data are means ± SE of 3 individual experiments; a minimum of 100 cells was quantified per condition. C) RAW 264.7 macrophages were pretreated for 20 min with the indicated inhibitors or the corresponding solvents only, and then incubated with TMR-Dextran for 1 hour followed by imaging as above. Data are means ± SE of 3 individual experiments; a minimum of 100 cells was quantified per condition.
Figure S1. **PL Cy1/2 do not localize to the plasma membrane in resting macrophages.** A) RAW 264.7 macrophages were transiently transfected with PLCy1-GFP (left) or PLCy2-GFP (right) and imaged by confocal microscopy. B) RAW 264.7 macrophages were fixed, permeabilized and probed with antibodies against PLCy1 (left) or PLCy2 (right). Scale bars = 5 µm.

Figure S2. **The 2PABD probe does not bind PtdIns(3,4,5)P3 in living cells.** HeLa cells (A) and RAW 264.7 macrophages (B) were transiently transfected with PH-Akt-RFP and GFP-2PABD and treated with 100 nM insulin (A) or 30 µM DGKi I (B).

Figure S3. **The total amount of F-actin does not change with DGK inhibition.** A) RAW 264.7 macrophages pretreated for 20 min with the inhibitors listed or with vehicle alone were fixed, permeabilized and stained with rhodamine phalloidin. Methanol was used to extract the rhodamine phalloidin, and its fluorescence was measured at 565 nm. B) Confocal images of RAW 264.7 macrophages pretreated with 0.3% EtOH (left) or 30 µM DGKi I (right) for 20 min and stained with rhodamine phalloidin. These are images of the ventral surface of the cells. Scale bars = 5 µm.

Figure S4. **PA does not significantly contribute to the surface charge of the plasma membrane.** A) RAW 264.7 macrophages and (B) HeLa cells were transiently transfected with a construct encoding a surface charge probe, GFP-R-pre, which localizes to highly anionic membranes. (C) RAW 264.7 macrophages transiently co-transfected with constructs encoding GFP-R-pre and mCherry-C1PKC were treated with 30 µM DGKi I and examined by confocal microscopy. (D) HeLa cells transiently transfected with RFP-R-pre and GFP-2PABD were treated with ionomycin and examined by confocal microscopy. Scale bars = 5 µm.
3.7 Figures

Figure 1. PA is elevated at the plasma membrane of macrophages and iDCs

A) GFP-2PABD
RAW264.7

B) GFP-2PABD PM-RFP
HeLa

C) GFP-2PABD Fluorescence

D) GFP-2PABD
0 min 10 min
HeLa +100 μM PA

E) GFP-2PABD + HA-PLD2
HeLa

F) GFP-2PABD Fluorescence

G) PA content
HeLa HEK293 RAW264.7 J774

H) PA content
HeLa J774
Figure 2. Plasmalemmal PA is generated by DGK
Figure 3. Expression of DGK isoforms in macrophages

Figure 4. PLC isoforms localize to the plasma membrane of macrophages, where they catalyze formation of intermediates of PA biosynthesis
Figure 5. GPCR signalling and PI3K activity are required for synthesis of plasmalemmal PA in macrophages.
Figure 6. PtdIns(4,5)P2 is required for plasmalemmal PA production

A

 lyn FRB PI(4,5)P2

 RFP FKBP 5'-Pase Rapamycin

 lyn FRB PI(4,5)P2

 B

 0 min 15 min

 PaseSynj FKBP-RFP

 GFP-2PABD + Rapamycin

  + Rapamycin

 C

 15 min

 PaseSynj(D730A) FKBP-RFP

 GFP-2PABD + Rapamycin

 D

 GFP-2PABD Fluorescence [(PM - cytoplasm)/cytoplasm] [0.4 0.8]

 Rapamycin − + − + Pase − Pase (D730A)

 * = p < 0.001
Figure 7. PA is required for steady state ruffling

A

DIC

+ DGKi I

0 min 15 min

BM iDC

B

Ruffling Index

0.3

0.2

0.1

0 min 10 min

+ DGKi I

C

Rhodamine Phalloidin

RGKi I

+ EtOH + DGKi I

D

GPI-GFP

DGKi I EtOH

0 min 1 min

RAW264.7

E

Cumulative Contact Area (µm^2)

100

75

50

25

100 200 300 400 500 600

Time (s)

F

Rac Activity (Absorbance)

2.5

2.0

1.5

1.0

0.5

0.05

0.10

0.15

Slope

DGKi I EtOH PTX Latrunculin B

CTB

0 min 1 min

EtOH DGKi I

+ EtOH

G

mCherry-C1PKC

PAK-PBD-YFP

0 min 10 min

PTX + PA

+ DGKi I

H

Control PTX PTX + PA

GFP-2PABD

PAK-PBD-YFP

GFP-2PABD

Control PTX PTX + PA

PTX + PA

+ DGKi I

PTX + PA

+ DGKi I
Figure 8. DGK- and PI3K-derived lipid signals are necessary for the constitutive association of TIAM1 with the phagocyte membrane

A

B

C

D

E

**Figure 8.** DGK- and PI3K-derived lipid signals are necessary for the constitutive association of TIAM1 with the phagocyte membrane.

**A**

Tiam1-GFP + mCherry-C1

**B**

RAW264.7 Tiam1-GFP + PX-mCherry + EtODGKi I

**C**

RAW264.7 Tiam1-GFP Fluorescence (PM - cytoplasm) / cytoplasm

**D**

Tiam1-GFP and YF-Tiam1 protein interactions highlighted.

**E**

Control + Rapamycin + Rapamycin + DGKi I + Rapamycin + LY294002

**Phalloidin 633**

**YF-Tiam1**

**RAW264.7**

**Scale bar:** 10 μm
Figure 9. PA is required for macropinocytosis

Figure S1. PLCγ1/2 do not localize to the plasma membrane in resting macrophages
Figure S2. The 2PABD probe does not bind PtdIns(3,4,5)P$_3$ in living cells

Figure S3. The total amount of F-actin does not change with DGK inhibition
Figure S4. PA does not significantly contribute to the surface charge of the plasma membrane

A

B

C

D

RAW264.7

GFP-R-pre

HeLa

GFP-R-pre

mCherry-C1PKC

RAW264.7

0 min

10 min

+ DGKi I

D

RAW264.7

GFP-2PABD

HeLa

RFP-R-pre

+ Ionomycin

+ Ionomycin

0 min

2 min

20 min
Chapter 4

This chapter has been modified from the following: Daniel Schlam, Richard D Bagshaw, Spencer A Freeman, Richard F Collins, Tony Pawson, Gregory D Fairn, and Sergio Grinstein. “Phosphoinositide 3-Kinase Enables Phagocytosis of Large Particles by Terminating Actin Assembly Through Rac/Cdc42 GTPase-Activating Proteins.” Nature Communications, October 2015.

4 Uptake: Phosphoinositide 3-kinase enables phagocytosis of large particles by coordinating actin disassembly through Rac/Cdc42 GTPase-activating proteins

4.1 Abstract

Phagocytosis is responsible for the elimination of particles of widely disparate sizes, from large fungi to effete cells to small bacteria. Though superficially similar, the molecular mechanisms involved differ: engulfment of large targets requires phosphoinositide 3-kinase (PI3K), while that of small ones does not. Here, we report that inactivation of Rac and Cdc42 at phagocytic cups is essential to complete internalization of large particles. Through a screen of 62 RhoGAP-family members, we demonstrate that ARHGAP12, ARHGAP25 and SH3BP1 are responsible for GTPase inactivation. Silencing these RhoGAPs impairs phagocytosis of large targets. The GAPs are recruited to large—but not to small—phagocytic cups by products of PI3K, where they synergistically inactivate Rac and Cdc42. Remarkably, the prominent accumulation of phosphatidylinositol-3,4,5-trisphosphate that characterizes large-phagosome formation is less evident during phagocytosis of small targets, accounting for the contrasting RhoGAP distribution and the differential requirement for PI3K during phagocytosis of dissimilarly sized particles.
4.2 Introduction

The elimination of microbial pathogens by phagocytosis is central to the innate immune response. Bacteria, fungi and other microorganisms are ingested and destroyed by professional phagocytes. Phagocytosis is also essential for the clearance of apoptotic cells, aged erythrocytes, immune complexes and cellular debris. Recognition of such disparate targets is enabled by the expression of a wide repertoire of phagocytic receptors on the surface of myeloid cells (Flannagan, Jaumouillé, and Grinstein 2012). While sharing a similar overall outcome—the sequestration and subsequent degradation of targets in an intracellular vacuole termed the phagosome—the molecular events triggered by different types of phagocytic receptors vary considerably (Underhill and Goodridge 2012). Moreover, emerging evidence suggests that large and small particles are engulfed by different mechanisms, even when the same receptor type is engaged. A striking example is phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3]: the phosphoinositide is required for the ingestion of large (≥5 µm) particles, but is dispensable for the uptake of smaller targets (Cox et al. 1999; Araki, Johnson, and Swanson 1996).

Regardless of the type of engaged receptor or the size of the target particle, a common feature of the phagocytic process is the involvement of the actin cytoskeleton (Spencer A. Freeman and Grinstein 2014). In almost every instance, actin-rich protrusions facilitate the initial capture of targets and the extension of pseudopods that ultimately surround the particle, promoting fission of the sealed vacuole (Swanson 2008). Rho-family GTPases orchestrate the remodelling of actin during phagocytosis (Park and Cox 2009; Cox et al. 1997; Hoppe and Swanson 2004; Niedergang and Chavrier 2005; Chimini and Chavrier 2000).

Of note, the progression of pseudopods and the completion of phagocytosis involve not only actin polymerization, but also the subsequent disassembly of actin filaments—particularly in the case of larger particles. The sites where targets are initially engaged and actin first assembles begin to dismantle even as the pseudopods continue to progress along the particle’s surface. It is unclear whether this coordinated disassembly
is required to recycle limiting components to the tips of the advancing pseudopods, enables membrane deformation and/or clears a path for focal exocytosis of endomembranes.

Most of the available information regarding the cytoskeletal remodelling that accompanies phagocytosis pertains to the initial stages of F-actin polymerization, while much less is known about the mechanisms of termination and disassembly. Here, we show that actin breakdown is critical for the uptake of large particles and describe a PI3K-driven mechanism whereby Rho-GTPase activity and actin polymerization are acutely terminated, enabling completion of phagocytosis. Our observations account for the differential requirement for PtdIns(3,4,5)P₃ biosynthesis during phagocytosis of small vs. large particles.

4.3 Methods

4.3.1 Reagents

Polystyrene microspheres (8.31 or 1.58 µm in diameter) functionalized with divinylbenzene (DVB) were obtained from Bangs Laboratories Inc. Sheep erythrocytes (10% suspension) were purchased from MP Biomedicals. LY294002, rapamycin, dimethyl sulfoxide (DMSO) and human-serum IgG were from Sigma-Aldrich. Fluorescent antibodies against human and rabbit IgG, including Cy5-, Cy3- and DyLight 488-conjugates were from Jackson ImmunoResearch Labs. Anti-sheep red blood cell antibodies were purchased from Cedarlane Laboratories. Paraformaldehyde (16% w/v) was from Electron Microscopy Sciences. Fluorescent phalloidin was from Molecular Probes (Life Technologies). Rabbit polyclonal antibodies against mouse ARHGAP12, ARHGAP25 and SH3BP1 were obtained from Sigma-Aldrich (product number: HPA000412), Thermo Scientific (catalogue number: PA5-24791) and Proteintech (catalogue number: 20541-1-AP), respectively. For the dilutions at which the antibodies were used, see the Immunoblotting section below.
4.3.2 Plasmids

cDNAs encoding RhoGAP proteins were selected by the presence of a RhoGAP PFAM domain (PF00620) within putative coding regions from the Ensembl database. When multiple isoforms existed for a particular RhoGAP, the isoform encompassing all known domains carried by the protein was considered the representative longest isoform, and therefore chosen as a target for cloning. The cDNAs were sourced from the mammalian gene collection or synthesized (Genscript) when a suitable full-length isoform did not exist. The cDNAs were cloned in-frame into Ascl/Pacl sites within Creator donor plasmids, and recombined into Creator expression vectors to generate N-terminal mCitrine-tagged fusion proteins upon expression. Where PDZ binding motifs were predicted (as in BCR and ABR), the cDNAs were cloned with a stop codon immediately after the coding sequence.

Active (GTP-bound) Rac and Cdc42 were detected with PAK(PBD)-YFP (Srinivasan et al. 2003), a plasmid encoding the p21-binding domain (PBD) of p21-activated kinase (PAK) fused to YFP. Polymerized actin was visualized using Lifeact-mRFP, as described earlier (Riedl et al. 2008). Rac1(Q61L)HR_{tail}, encoding constitutively-active Rac1 conjugated to GFP and modified to carry the hydrophobic tail of H-Ras in lieu of its polybasic domain, was used to induce sustained Rac1 signalling at the plasmalemma (Yeung, Terebiznik, et al. 2006). Likewise, Cdc42(G12V) was employed to induce constitutive signalling by Cdc42. This protein was encoded in the YFP-Cdc42(V12) plasmid, a gift from Dr. Joel Swanson (Addgene plasmid #11399). A construct encoding palmitoylated-myristoylated RFP (PM-RFP) (Teruel et al. 1999) was used to label the plasma membrane. The membrane-targeting component of the rapamycin-inducible heterodimerization system, consisting of the N-terminal 11 amino acids of Lyn kinase conjugated to a complementary rapamycin-biding domain (Lyn_{11}-FRB) was a generous gift of Dr. G. Di Paolo (Columbia University) (Chang-Ileto et al. 2011). Rapamycin-recruitable TIAM1 (YFP-FKBP-TIAM1) was obtained from Addgene (plasmid #20154) (Inoue et al. 2005b). The distribution of PtdIns(3,4,5)P_3 was monitored using the PH domain of Akt (Marshall et al. 2001).
4.3.3 Isolation of primary macrophages and cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers and isolated by density gradient centrifugation using Lympholyte-H (Cedarlane). Monocytes were purified by adherence to 1.8-cm glass coverslips in 12-well plates (3x10^6 PBMCs per coverslip). Cells were then cultured for 5 days at 37°C under 5% CO_2 in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% heat-inactivated fetal bovine serum (Wisent Inc.), antibiotics (Multicell) and 25 ng/mL macrophage colony-stimulating factor (M-CSF). Following this differentiation protocol, non-adherent cells were washed off the macrophage monolayer.

The murine macrophage line RAW 264.7 was obtained from the American Type Culture Collection (ATCC) and grown in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum at 37°C under 5% CO_2.

4.3.4 Transfection of plasmid DNA

For transient transfections, nearly confluent monolayers of RAW 264.7 cells were lifted by careful scraping and plated onto 1.8-cm glass coverslips at a density of 1x10^5 cells/coverslip. Macrophages were allowed to recover for 18-24 hrs and then transfected with FuGENE HD (Promega) according to the manufacturer’s instructions. Briefly, 2 µg of plasmid DNA and 6 µL of the transfection reagent were mixed in 100 µL of serum-free RPMI and allowed to sit for 15 min. The mix was then added to 500 µL of RPMI and distributed equally into 4 wells of a 12-well plate. Cells were typically used for experimentation 18 hours after transfection.

Alternatively, high levels of protein expression were achieved by electroporating plasmid DNA with the Neon Transfection System (Life Technologies) according to the manufacturer’s protocol. Briefly, adherent RAW 264.7 cells or primary macrophages were lifted by gentle scraping or by a 10-min exposure to Accutase (Innovative Cell Technologies), respectively. Cells were then counted and sedimented at 300xg for 5 min. 5x10^5 cells were then resuspended in 100 µL of buffer R and incubated with 10 µg plasmid DNA. The cell mix (100 µL) was then subjected to electroporation, using a
single 20-ms pulse of 1750 V (RAW 264.7 cells) or two sequential 30-ms pulses of 1100 V (primary macrophages). Electroporated macrophages were immediately transferred to RPMI for seeding into coverslips.

4.3.5 Gene silencing

Small interfering RNAs (siRNA) directed against SH3BP1, ARHGAP12 or ARHGAP25 were purchased from Dharmaco. For each transcript targeted, oligonucleotides were obtained as a mixture of four different siRNAs (SMARTpools).

The sequences targeted in SH3BP1 were:

1) ccucugaccucuacgauga
2) ucacugaccucucucgaca
3) ucgagggcgcugacagaa
4) gcagaggagcaggauguaa

The sequences targeted in ARHGAP12 were:

1) ggaguaugauauggauau
2) guuuaguauguugguau
3) gcugaaaacucugacaagga
4) gcaggacaagcguauauug

The sequences targeted in ARHGAP25 were:

1) caagaacucuggcaggau
2) gaaaaucagccccucgaaau
3) gaacuaugucccaagacuu
4) uaaaaggacuaacgcuu
Oligonucleotides were delivered by electroporating $5 \times 10^5$ RAW 264.7 cells with 200 pmol of the siRNA pool with the Neon system, using a single 20-ms pulse of 1750 V. Electroporated cells were allowed to recover for 48 hours before being lifted once again for a second round of electroporation. Knockdown efficiency and phagocytosis were assessed 96 hours after the initial electroporation.

### 4.3.6 Immunoblotting

RAW 264.7 cells electroporated with siRNA directed against the RhoGAPs or with a non-targeting siRNA control were washed with PBS and lysed in cold RIPA buffer (Sigma-Aldrich) supplemented with protease- and phosphatase-inhibitor tablets (Roche). Forty µg of total protein were loaded per lane of a 12% SDS-PAGE and separated by electrophoresis, before being transferred to methanol-activated polyvinylidene fluoride (PVDF) membranes. Membranes were then blocked in TBS supplemented with 0.1% Tween 20 and 5% bovine-serum albumin (BSA) for 30 min. This solution was also used to dilute the primary anti-RhoGAP and the secondary anti-rabbit HRP-conjugated antibodies, which were incubated for 2 hours and 45 min, respectively. Primary antibodies against ARHGAP12, ARHGAP25 and SH3BP1 were used at 1:250, 1:1000 and 1:1000 dilutions, respectively. All HRP-conjugated secondary antibodies were used at a 1:10,000 dilution. Membranes exposed to ECL Western blotting substrate (GE Life Sciences) were visualized using the Odyssey Fc (LI-COR) system. Brightness/contrast parameters were adjusted globally across the entire image using the LI-COR Image Studio software. Immunoblots were cropped for presentation; see Supplementary Fig. 6 for uncropped images.

### 4.3.7 Phagocytosis

For all FcγR-mediated phagocytosis assays, $\approx 1 \times 10^5$ RAW 264.7 cells were seeded onto 1.8-cm glass coverslips and cultured for 24 hours. DVB-coated polystyrene beads were diluted 10-fold in PBS and opsonized by incubating them with human IgG (final IgG concentration = 5 mg/mL) for 60 min at room temperature. Excess IgG was...
removed by washing the beads twice with 1 mL of PBS. Alternatively, sheep erythrocytes were opsonized by incubating 200 μL of a 10% suspension with 5 μL of a rabbit anti-sheep red blood cell antibody for 60 min at room temperature. Excess IgG was removed by washing the erythrocytes 3x with PBS. Beads and sheep erythrocytes were then labelled with fluorescent antibodies against human or rabbit IgG, respectively, and resuspended in 200 μL PBS. Phagocytosis was initiated by adding 15 μL or 5 μL of the bead or erythrocyte suspension, respectively, to each well of a 12-well plate and sedimenting the targets onto the macrophage monolayer by centrifugation (300xg, 30 sec). Where indicated, cells were treated with either vehicle (DMSO) or LY294002 (100 μM) for 10 min prior to initiating phagocytosis. Cells were allowed to phagocytose for the indicated times before fixing with PFA (4%). Particles that failed to be internalized were visualized by staining with secondary antibodies against IgG conjugated to a fluorophore different from the one originally used for labelling the phagocytic targets. To stain actin filaments, cells were permeabilized with Triton X-100 (0.1%) for 5 min and incubated with fluorescent phalloidin (Molecular Probes, 1:1000 dilution) for 30 min.

4.3.8 Microscopy and image analysis

All fluorescence imaging was performed with spinning-disk confocal microscopes (Quorum Technologies). The systems in use in our laboratory are based on an Axiovert 200M microscope (Carl Zeiss) equipped with a 63x oil-immersion objective (N.A. 1.4) and a 1.5x magnifying lens. These units carry a motorized XY stage (Applied Scientific Instrumentation), a Piezo Z-focus drive and diode-pumped solid-state lasers emitting at 440-, 491-, 561-, 638- and 655-nm wavelengths (Spectral Applied Research). Images were recorded with back-thinned, cooled charge-coupled device cameras (Hamamatsu Photonics) under control of the Volocity software (version 6.2.1; Perkin Elmer). Fluorescence intensity measurements and correction of brightness/contrast were performed with ImageJ (version 1.48; National Institutes of Health). Brightness and contrast parameters were adjusted across the entire image without altering the linearity of mapped pixel values.
4.3.9 Reverse transcription-polymerase chain reaction (RT-PCR)

One-step RT-PCR (Invitrogen) was used to assess whether individual RhoGAPs were transcribed endogenously in murine macrophages. For each RT-PCR reaction, RNA was purified from ~8x10^5 RAW 264.7 cells using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Reverse transcription of RhoGAP messages or of GAPDH (positive control) was performed using sequence-specific primers spanning exon-exon junctions and 1 μg of purified RNA template. The reverse transcription reaction was carried out at 55°C and allowed to proceed for 30 min before increasing the temperature to 94°C in order to inactivate reverse transcriptase and initiate exponential amplification by Taq polymerase. PCR amplification continued for 19 cycles at denaturing, annealing and extension temperatures of 94°C, 50°C and 68°C, respectively. Finally, amplicons were visualized by electrophoresis, using agarose gels prestained with ethidium bromide. An uncropped image of the agarose gel is presented in Supplementary Fig. 6a. The primer sequences utilized for cDNA synthesis were:

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<th>RhoGAP target</th>
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<th>Primer length (bp)</th>
<th>Melting Temp. (°C)</th>
<th>Amplicon length (bp)</th>
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4.3.10 Rapamycin heterodimerization system and TIAM1 translocation

Rapamycin-inducible recruitment of the GEF domain of TIAM1 to the plasma membrane was performed as described earlier (Inoue et al. 2005; Bohdanowicz and Fairn 2011; Bohdanowicz et al. 2013). In brief, the heterodimerization system consists of two separate constructs: one encodes a polypeptide with the N-terminal membrane-targeting sequence of Lyn, which associates constitutively with the plasmalemma; the other encodes the tandem DH-PH (GEF) domain of TIAM1 conjugated to YFP, and is normally cytosolic. Both proteins also carry rapamycin-binding domains that are complementary to each other. Addition of rapamycin induces the interaction between them, thereby supporting the rapid and virtually irreversible translocation of recombinant TIAM1 to the plasmalemma.

4.3.11 Statistical analysis

At least three independent experiments of each type were performed for analysis of significance, counting as many cells as indicated in the text or figures legends. A sample size of at least 20 cells was chosen so that approximately 100 phagosomes or nascent phagocytic cups could be quantified per condition. Values are reported as means of the experiments ± standard error of the mean (SEM). Statistical parameters were assessed with GraphPad Prism 5c software (GraphPad Software, Inc.). The significance of differences was determined performing unpaired t-tests, using the routines built in GraphPad Prism.

4.4 Results

4.4.1 PI3K directs F-actin disassembly at large phagocytic cups

In cells treated with PI3K inhibitors, the initial stages of phagocytosis are seemingly normal. However, if the particles are large, the engulfment process cannot be completed. We questioned whether aberrant cytoskeletal dynamics could account for the inability of cells with impaired PI3K activity to finalize uptake of large targets. To test
this experimentally, we visualized F-actin dynamics in live macrophages during the course of phagocytosis in the presence or absence of the PI3K inhibitor LY294002. RAW 264.7 macrophages transfected with Lifeact-mRFP [a fluorescent reporter of filamentous actin (Riedl et al. 2008)] were incubated with vehicle (DMSO) alone or LY294002 before being challenged with either large (8.3-µm) or small (1.6-µm) IgG-coated particles to engage Fcγ receptors. As illustrated in Figure 1a, F-actin accumulated markedly at developing cups during the early stages (≈ 1 min) of large-particle ingestion. Of note, the progression of pseudopods along the perimeter of the particle was accompanied by the disassembly of F-actin from the base of the cup (3 min panel; dashed arrow) and, once phagosomes had sealed, actin was only detectable at the site of scission (8 min panel; see Supplementary Movie 1 for the uninterrupted time-lapse sequence). The early phase of actin assembly was also observed in macrophages treated with LY294002 (Figure 1b; solid arrow). However, as reported earlier (Cox et al. 1999), the extension of pseudopods was arrested, yielding shallow abortive cups where polymerized actin persisted for extended periods (see 3 and 8 min panels in Figure 1b and also Supplementary Movie 2). These results suggest that PI3K is dispensable for the initial polymerization of actin that drives membrane deformation, but is critical for the secondary actin clearance that accompanies internalization of large particles.

Phagocytes challenged with small (1.6-µm) beads similarly accumulated actin at nascent cups (Figure 1c; 30 sec panel). However, the beads quickly became entirely surrounded by F-actin (2 min panel), and sealed before disassembly at the base of the cup was apparent (5 min panel). Interestingly, when ingesting small beads, the dynamics of actin in cells treated with LY294002 (Figure 1d) were similar to those in vehicle (DMSO)-treated controls, and phagocytosis proceeded to completion, as reported (Cox et al. 1999). The differential effect of LY294002 on actin dynamics and on the phagocytic capacity of cells challenged with small vs. large particles suggested that PI3K-dependent actin disassembly may be required for the uptake of large, but not small targets.
4.4.2 Rac/Cdc42 inactivation accompanies phagocytosis completion

Because the Rho GTPases Rac and Cdc42 are key to the assembly of filamentous actin during FcγR-mediated phagocytosis (Hall et al. 2006; Castellano, Montcourrier, and Chavrier 2000; Massol et al. 1998; Cox et al. 1997), we speculated that their inactivation would facilitate actin disassembly, and that this was mediated by PI3K. We assessed this experimentally in primary macrophages using PAK(PBD)-YFP, a fluorescent biosensor of Rac/Cdc42 activity consisting of the p21-binding domain (PBD) of p21-activated kinase (PAK) (Hoppe and Swanson 2004; Srinivasan et al. 2003). Human monocyte-derived macrophages transiently expressing PAK(PBD)-YFP were challenged with 8.3-µm IgG-opsonized beads and phagocytosis was allowed to proceed for 1, 3 or 8 min prior to fixation and staining of F-actin with phalloidin-568 (Figure 1e,f). As expected, sites of particle engagement displayed prominent PAK(PBD)-YFP enrichment (corresponding to sites of GTP-bound Rac and/or Cdc42) and colocalized with sites of intense F-actin staining (1 min time point in Figure 1e; solid arrow). Importantly, in otherwise untreated cells disassembly of F-actin from the base of the cup was coincident in space and time with the displacement of the active Rac/Cdc42 biosensor (3 min time point; dashed arrow), implying that inactivation of these small G proteins accompanied F-actin breakdown. Fully internalized phagosomes lacked both F-actin and PAK(PBD)-YFP (8 min time point; dashed arrow). In sharp contrast, cells treated with LY294002 displayed sustained Rac/Cdc42 activity at the base of an actin-rich abortive cup, as indicated by the retention of the PAK(PBD)-YFP biosensor and by the intense phalloidin staining (1-8 min time points in Figure 1f; solid arrow). A virtually identical dependency of F-actin disassembly and Rac/Cdc42 inactivation on PI3K activity was also observed in the RAW 264.7 macrophage (Supplementary Fig. 1). Together, these results are consistent with the notion that PI3K signals the termination of Rac and/or Cdc42 activity, which in turn is necessary for orchestrating the orderly disassembly of F-actin during phagocytosis of large particles.
4.4.3 Role of RhoGEF inactivation in phagocytosis completion

Two distinct mechanisms could account for the sustained Rac and/or Cdc42 signalling observed at abortive phagocytic cups in LY294002-treated cells: prolongation of the activity of guanine nucleotide exchange factors (GEFs), or impairment of GTPase-activating proteins (GAPs). To differentiate between these possibilities, we forced the association of TIAM1, a Rac1 GEF (Worthylake, Rossman, and Sondek 2000), with the plasmalemma by means of a rapamycin-inducible heterodimerization system (Inoue et al. 2005) (see Methods). We reasoned that if GEF displacement was indeed necessary for Rac1 inactivation, then the sustained association of this recombinant GEF with the membrane should lead to persistent Rac1 signalling and formation of abortive, F-actin-rich phagocytic cups akin to those shown in Figure 1b,f. RAW 264.7 cells transiently expressing the heterodimerization system and Lifeact-mRFP were acutely exposed to rapamycin, triggering translocation of TIAM1 to the plasmalemma (Supplementary Movie 3 and Figure 1i). Note the extensive membrane ruffling induced by TIAM1 mobilization. Sustained recruitment of the GEF did not, however, prevent the effective uptake of large particles, which were fully internalized as indicated by their inaccessibility to externally added secondary antibodies (Figures 1i,j). These results suggest that the displacement of RhoGEFs from the nascent phagocytic is not a necessary condition for Rho GTPase inactivation or for F-actin disassembly. Nonetheless, we cannot rule out the possibility that removal or inactivation of GEFs occurs during the normal progression of phagocytosis.

In contrast to the inability of FKBP-TIAM1 to arrest phagocytosis, uptake of IgG-coated beads was markedly reduced in macrophages transiently expressing either Rac1(Q61L)-HRtail or Cdc42(G12V), constitutively-active forms of the GTPases (Figures 1g,h,j). Of note, the number of particles associated with the cells in fact increased in Rac1(Q61L)-HRtail transfectants, suggesting that internalization, as opposed to binding, was impaired. Accordingly, formation of nascent cups with considerable accumulation of F-actin was evident in these cells (Figure 1g). Similar abortive cups subtended by rich actin networks were observed in Cdc42(G12V) transfectants (Figure 1h), as previously described (Beemiller et al. 2010). Together, these results confirm that Rac/Cdc42 inactivation is necessary for actin breakdown and completion of phagocytosis, and
suggest that recruitment and/or activation of RhoGAPs—as opposed to displacement of RhoGEFs—is required to turn off the GTPases. This notion is bolstered by the observation that, while TIAM1 remained associated with internalized phagosomes, no F-actin was detected on their membranes (Figure 1i).

4.4.4 Subcellular distribution of RhoGAPs during phagocytosis

The human genome encodes more than 60 members of the RhoGAP family (Moon and Zheng 2003). Identification of the putative RhoGAP(s) involved in phagocytosis is further compounded because the precise GTPase(s) targeted by each of the RhoGAPs are, in many cases, not known. To overcome these hurdles, we conducted an unbiased screen of the RhoGAP family, using the strategy outlined in Figure 2a. As an initial selection criterion we assessed which RhoGAP(s) translocated to sites of phagocytosis. To this end, we generated fluorescent chimeras of 62 different RhoGAPs, individually transfected them into macrophages, and assessed their presence at phagocytic cups induced by exposure to IgG-coated particles. The tagged constructs were co-transfected with PM-RFP, a fluorescent plasmalemmal marker that enabled us to normalize the accumulation of the RhoGAPs per unit membrane area. Confocal images were acquired 3 min after the macrophages were exposed to the particles, and ratios of the fluorescence of the two probes were calculated. These ratios were then used to score the degree of RhoGAP recruitment to the cup (a relative scale ranging from a minimum of 0 to a maximum of 6 was used). Supplementary Fig. 2 includes representative micrographs of the cellular distribution of all 62 RhoGAPs and provides their corresponding recruitment score. A subset of representative images are also shown in Figure 2b-f, where 4 types of distribution are differentiated: RhoGAPs that failed to translocate to the phagocytic cup were classified either as type I-N or type I-C, depending on whether they remained in nuclear or cytosolic compartments, respectively (Figure 2b,c). RhoGAPs that were present at the plasmalemma before particle engagement but did not accumulate at the phagocytic cup were designated as type II (Figure 2d). Of most interest were types III and IV, which were either cytosolic or both membrane-associated and cytosolic at rest, respectively, but accumulated prominently
at phagocytic cups (Figure 2e,f). Ten different RhoGAPs were found to clearly accumulate (score ≥4) at the phagocytic cup.

Because our screening process involved ectopic expression of tagged proteins, it was imperative to verify whether the candidate RhoGAPs were endogenously expressed in macrophages. As shown in Figure 2g, we used RT-PCR to validate their expression. Following 19 cycles of amplification, this analysis revealed that only 6 of the 10 candidate GAPs (MYO9B, ARHGAP25, ARHGAP12, SH3BP1, PIK3R1 and GRLF1) were present at moderate-to-high levels in RAW 264.7 macrophages, as judged by their corresponding levels of mRNA expression (Figure 2g).

4.4.5 PI3K-dependent translocation of RhoGAPs to phagocytic cups

Given that PI3K inhibition resulted in the formation of abortive phagocytic cups displaying prolonged Rac and Cdc42 activity (Figure 1f and Supplementary Fig. 1b), we considered the possibility that Rho-family GAPs were recruited to sites of phagocytosis by products of PI3K. To test this, we transfected RAW 264.7 cells with plasmids encoding those RhoGAPs found to be recruited to the phagocytic cup. Transfectants were treated with vehicle or LY294002 for 10 min before being challenged with IgG-coated erythrocytes and imaged by confocal microscopy after fixation (Figure 3a-d). For brevity, representative images of only two PI3K-sensitive and two PI3K-insensitive RhoGAPs are shown in Figure 3; the remaining images are included in Supplementary Fig. 3. Collectively, only 3 of the RhoGAPs that are endogenously expressed in macrophages—ARHGAP12, ARHGAP25 and SH3BP1—require PI3K activity to associate with the phagocytic cup. It is noteworthy that ARHGAP12, ARHGAP25 and SH3BP1 are known to exert their catalytic function on Rac and/or Cdc42 (Cicchetti et al. 1995; Gentile et al. 2008; Csépanyi-Kömi et al. 2012).

To assess whether ARHGAP12, ARHGAP25 and SH3BP1 behave similarly in primary cells, human monocyte-derived macrophages transiently expressing mCitrine-chimeras of these RhoGAPs were challenged with IgG-coated erythrocytes in the presence or absence of the PI3K inhibitor LY294002. As expected, all 3 GAPs noticeably
accumulated at developing phagocytic cups in control conditions, but their recruitment was precluded in the presence of LY294002 (Supplementary Fig. 4).

4.4.6 Validation of RhoGAP function during phagocytosis

Having identified ARHGAP12, ARHGAP25 and SH3BP1 through the screening process described above, we examined their functional activity in phagocytes. We hypothesized that if these RhoGAPs were indeed capable of inactivating Rac and/or Cdc42, their overexpression would hinder Rho GTPase-mediated actin nucleation and thus impair phagocytosis at an early stage. We tested this premise using electroporation, which enabled us to express high levels of mCitrine-tagged ARHGAP12, ARHGAP25 or SH3BP1 in the macrophages. Unconjugated mCitrine was used as a control to rule out untoward effects of the electroporation procedure. As illustrated in Figure 4, deliberate overexpression of the mCitrine chimeras of ARHGAP12, ARHGAP25 or SH3BP1 markedly depressed the phagocytic efficiency of RAW 264.7 cells, compared to unconjugated mCitrine. It is noteworthy that this phagocytic impairment was accompanied by reduced F-actin accumulation at sites of contact with target particles (Figure 4b), where only modest pedestals were formed instead of the elaborate phagocytic cups normally observed. These observations support the notion that ARHGAP12, ARHGAP25 and SH3BP1 can function as RhoGAPs in macrophages during phagocytosis.

That RhoGAP overexpression impairs phagocytic capacity may seem at odds with a requirement for Rac and Cdc42 inactivation for efficient engulfment. However, these observations can be readily reconciled given that both the initial activation and subsequent inactivation of these Rho GTPases are critical for the orderly extension and progression of pseudopodia. Indeed, the immature membrane pedestals that form at sites of phagocytosis in ARHGAP12, ARHGAP25 or SH3BP1 transfectants suggest that the overexpressed RhoGAPs terminate Rac and Cdc42 signalling prematurely. Thus, cycling of the GTPases between active and inactive states is instrumental for successful phagocytosis of large particles, as both hasty termination of Rho GTPase activity and
protracted signalling by these proteins are deleterious to the completion of the phagocytic response.

4.4.7 RhoGAPs cooperate in breaking down F-actin at phagocytic cups

We used RNA interference to assess the role of the PI3K-sensitive GAPs in actin disassembly during phagocytosis of both small and large particles. RAW 264.7 macrophages were electroporated with non-targeting control siRNA or with siRNA directed against ARHGAP12, ARHGAP25 or SH3BP1. Knockdown efficiency was assessed by immunoblotting, which validated both the effectiveness and specificity of the silencing strategy; the oligonucleotides designed to silence each one of RhoGAPs markedly reduced the expression of the intended target, while the protein levels of the other 2 GAPs remained unaffected in all cases (Supplementary Fig. 5a-c). To evaluate the functional contribution of each one of the GAPs, the siRNA-treated cells were challenged with small (1.6-µm) or large (8.3-µm) IgG-coated beads, and phagocytosis was allowed to proceed for 10 min before fixation. Targets that remained extracellular were then identified by addition of a Cy3-conjugated anti-IgG antibody. As shown in Figure 5a,b, electroporation with non-targeting siRNA had no discernible effect on the phagocytosis of either small or large targets, as these cells internalized the majority of bound particles. In contrast, phagocytosis of large particles was markedly depressed in cells in which SH3BP1 was silenced (Figure 5d,f). Such cells developed abortive, actin-dense phagocytic cups (indicated with an arrow in Figure 5d) that extended only partway around the large phagocytic targets. Of note, phagocytosis of small beads was not significantly affected in these cells (Figure 5c,e). A similar pattern was observed in cells electroporated with siRNA directed against ARHGAP12 and ARHGAP25; phagocytosis of large beads was inhibited to varying extents, but that of small beads was only marginally affected (see Supplementary Fig. 5d-g for representative micrographs). Quantification of the results of 3 independent phagocytosis experiments is provided in Figure 5e,f.
4.4.8 Dependency of PI3K signalling on the size of phagocytic targets

Given that expression of ARHGAP12, ARHGAP25 and SH3BP1 was required for the uptake of large particles but dispensable for that of smaller ones, it was conceivable that these GAPs were recruited to large, but not small nascent phagosomes. To address this possibility, RAW 264.7 macrophages transiently expressing fluorescent chimeras of the 3 RhoGAPs were challenged with either small (Figure 6a-c) or large (Figure 6d-f) IgG-coated beads. As expected, all 3 GAPs were robustly recruited to sites of large-particle engagement. Of interest, however, their recruitment was inconspicuous when the small particles were used. The relative absence of these RhoGAPs is consistent with the observation that they are dispensable for phagocytosis of small particles.

Since these critical RhoGAPs mobilize to large phagocytic cups in a PI3K-dependent fashion, we questioned whether their inability to translocate to small cups was attributed to diminished PtdIns(3,4,5)_3 formation when using small particles as targets. To assess this, PtdIns(3,4,5)_3 distribution was monitored using Akt(PH)-GFP, a fluorescent biosensor consisting of the PH domain of Akt. Strikingly, PtdIns(3,4,5)_3 formation was not readily apparent in response to small phagocytic targets (Figure 6g), contrasting with its much more prominent accumulation in large phagocytic cups (Figure 6h).

Together, these observations suggest that PI3K-dependent recruitment of RhoGAPs is necessary for F-actin disassembly during the internalization of large particles. Our observations also account, at least in part, for the differential requirement for PI3K in the phagocytosis of dissimilarly sized particles.

4.5 Discussion

Rapid and extensive dismantling of actin networks is routinely observed during or immediately after phagosome sealing. Removal of cortical actin is thought to enable the fusion and fission events underlying phagosome maturation (Ferrari et al. 1999). In addition, in the case of large particles, F-actin breakdown appears to be necessary for successful engulfment. Three alternative mechanisms could explain the need for F-actin disassembly: a) persistence of the cortical cytoskeleton may limit the ability of the
forming phagosomal membrane to curve around the target. We regard this possibility as unlikely, inasmuch as phagocytosis of small particles—which exhibit a higher degree of curvature than large ones—is less dependent on F-actin disassembly. A second possibility is that internalization of large particles requires significant expansion of the lining membrane. Expansion may result from elastic stretching of the pre-existing membrane or, more likely, from exocytic insertion of endomembranes at sites of phagocytosis (Braun et al. 2004; Czibener et al. 2006; Mohammadi and Isberg 2013; Samie et al. 2013). Both of these processes would be impeded if the underlying cytoskeleton persisted. A third and in our view more plausible alternative is that as actin polymerizes during the formation of large phagocytic cups, one or more determinants of the assembly process may be exhausted, limiting the rate of growth of advancing pseudopodia. Replenishment would only occur following disassembly and recycling of the components initially engaged at the base of the phagocytic cup.

Regardless of the underlying purpose, it is clear that F-actin disassembly is essential to complete the internalization of large targets—such as fungi or apoptotic bodies—and is accompanied by inactivation of the Rho GTPases that initiate the polymerization event.

The notion that completing internalization of large endocytic vacuoles is contingent on the inactivation of the Rho GTPases responsible for initiating actin polymerization is consistent with a recent study making use of a photoactivatable form of Rac1; nascent macropinosytic cups rapidly developed upon Rac1 photoactivation at the plasmalemma, but sealed only once Rac1 activation had been reversed by interrupting illumination (Fujii et al. 2013).

Our data indicate that recruitment and/or stimulation of Rho-family GAPs is critical for the disassembly of actin when large particles are ingested. Moreover, they establish a link between the synthesis of 3'-phosphoinositides and the down-regulation of Rho GTPase activity. These observations are in good agreement with the findings of Swanson and colleagues, who originally identified PI3K as an essential component of the phagocytic machinery (Araki, Johnson, and Swanson 1996) and subsequently reported that inhibition of this lipid kinase leads to sustained GTPase signalling in stalled phagocytic cups (Beemiller et al. 2010). They are also consistent with the observation that phagocytosis of large, but not small particles is impaired by
wortmannin, a PI3K antagonist (Cox et al. 1999). The present work extends and clarifies these observations by identifying active GAPs recruited to the phagocytic membrane by 3'-phosphoinositides.

It is noteworthy that not 1, but 3 separate RhoGAPs–SH3BP1, ARHGAP12 and ARHGAP25–are required for completion of phagocytosis. This reflects, in part, the fact that multiple Rho-family GTPases are activated during phagocytosis (Caron and Hall 1998; Hall et al. 2006; Park and Cox 2009). It also suggests that the complex deactivation process is dictated by the asymmetric and asynchronous nature of the distribution and activation of the Rho GTPases: Rac is activated primarily at the base of the cup while Cdc42 is active at the tips of pseudopodia, fostering membrane extension (Hoppe and Swanson 2004). In this regard, it is important to note that SH3BP1, ARHGAP12 and ARHGAP25 display varying degrees of GAP activity towards Rac or Cdc42 (Cicchetti et al. 1995; Parrini et al. 2011; Gentile et al. 2008; Csépányi-Kőmi et al. 2012). As such, one would expect them to be differentially effective in different subdomains of the nascent phagosome, contributing to the asymmetric and asynchronous pattern of activation and deactivation of the GTPases.

The observation that sustained signalling by either Rac or Cdc42 can compromise phagocytic efficiency (Figure 5f) may help explain why multiple RhoGAPs need to be engaged and why their effects are not redundant, but rather synergistic. For instance, silencing one of the GAPs may result in prolonged Cdc42 activity–even if Rac is inactivated–which would be sufficient to hinder phagocytosis. Thus, despite their functional similarities, SH3BP1, ARHGAP12 and ARHGAP25 are all required and act synergistically to disassemble actin in a timely and spatially coordinated manner.

We have shown that FcγR-mediated phagocytosis is aborted at a relatively early stage when any one of the RhoGAPs is overexpressed in macrophages (Figure 4). These findings are in agreement with a previous study reporting that phagocytosis of serum-opsonized yeast is impaired in engineered phagocytes (COSphoxFcγR cells) overexpressing ARHGAP25 (Csépányi-Kőmi et al. 2012). However, that study also reported that silencing ARHGAP25 in PLB cells and in human macrophages increases phagocytosis of serum-opsonized particles, implicating ARHGAP25 as a negative regulator of phagocytosis. This observation seems at odds with our data that
phagocytosis is hindered as a consequence of silencing ARHGAP25, as was the case also for SH3BP1 and ARHGAP12. However, the role of ARHGAP25 in the ingestion of serum-opsonized particles is in all likelihood very different to its role in FcγR-mediated phagocytosis; the complement receptor activated during phagocytosis of serum-coated targets operates via RhoA (Caron and Hall 1998; Wiedemann et al. 2006), in contrast to the Fcγ receptors engaged in our studies, which function primarily via Rac1/2 and Cdc42 (Hall et al. 2006; Cox et al. 1997; Hoppe and Swanson 2004). Indeed, no increase in phagocytic efficiency was observed in ARHGAP25-silenced cells when the yeast particles were opsonized with heat-inactivated serum (Csépányi-Kömi et al. 2012), wherein complement is inhibited.

Why is phagocytosis of small targets possible in the absence of PI3K activity and how do such phagosomes proceed to fuse with endomembranes? As discussed earlier, we believe that sealing of small phagosomes can be completed with little need for membrane expansion and without incurring exhaustion of components of the actin cytoskeletal machinery. Thus, compared to large targets, the sealing of small phagosomes would be expected to be less dependent on PI3K-driven actin disassembly. Indeed, as documented in Figure 6, small phagocytic targets trigger little PtdIns(3,4,5)P3 biosynthesis upon engagement of Fcγ receptors. As a consequence, the RhoGAPs that are normally responsive to PI3K stimulation do not translocate to small cups, and phagosomes seal while still maintaining a ring of periphagosomal actin. Once phagosomes become internalized, detachment of the cortical skeleton likely occurs as a result of the hydrolysis of PtdIns(4,5)P2 (Scott et al. 2005). This phosphoinositide not only anchors a variety of adaptors that link F-actin filaments to the membrane (Saarikangas, Zhao, and Lappalainen 2010; Levin, Grinstein, and Schlam 2014), but also electrostatically targets and retains Rho GTPases at the plasmalemma (Yeung, Terebiznik, et al. 2006; Yeung and Grinstein 2007). Thus, fission of small phagosomes is likely followed by PtdIns(4,5)P2 catabolism and consequently F-actin disassembly. Attractive candidates for the late stage of PtdIns(4,5)P2 breakdown in small phagosomes include the 5'-phosphatases OCRL and INPP5B, which translocate to sites of phagocytosis (Sarantis et al. 2012; Bohdanowicz et al. 2012; Kühbacher et al. 2012).
4.6 Figure legends

Figure 1. PI3K controls actin disassembly during phagocytosis of large targets. (a-d) Time-lapse confocal micrographs of RAW 264.7 macrophages transiently expressing Lifeact-mRFP and challenged with 8.3-µm (a,b) or 1.6-µm (c,d) IgG-opsonized beads (signalled with a star). Cells were treated with vehicle (DMSO) or the PI3K inhibitor LY294002 for 10 min prior to initiating phagocytosis. Actin dynamics were followed throughout the course of engulfment, with the 0-min time point corresponding to the initial engagement of beads. Solid and dashed arrows point to sites of F-actin accumulation and clearance, respectively. (e,f) Confocal micrographs of primary human macrophages transfected with PAK(PBD)-YFP, a biosensor for active Rac/Cdc42, during phagocytosis of 8.3-µm IgG-beads. Prior to phagocytosis, cells were treated with DMSO (e) or LY294002 (f) for 10 min. Phagocytosis was allowed to proceed for the indicated times, before fixing and staining F-actin with phalloidin. Insets (boxed regions) show magnified views of the phagocytic cup. (g-i) Confocal micrographs of RAW 264.7 macrophages transiently co-expressing Lifeact-mRFP in combination with constitutively active Rac1 (g), constitutively active Cdc42 (h) or a recruitable form of the Rac1 GEF TIAM1 (i). Addition of rapamycin triggers translocation of TIAM1 to the plasmalemma, where its rapamycin-binding domain (FKBP) interacts with a second, complementary rapamycin-binding moiety. Transfectants were challenged with 8.3-µm IgG-beads, and phagocytosis allowed proceeding for 10 min prior to fixation. All phagocytic targets were stained with a Cy5-conjugated secondary antibody (shown in blue) prior to phagocytosis. Extracellular beads were identified by staining fixed (non-permeabilized) cells with an Alexa Fluor 488-conjugated secondary antibody (shown in green). Internalized beads are indicated with a star. Scale bar, 10 µm. (j) Quantification of phagocytic indices (total number of beads associated per cell; black bars) and phagocytic efficiencies (ratio of internalized-to-total number of beads per cell; white bars) for the experiments described in (g-i). Values represent the means of 3 independent replicates ± SEM. At least 25 cells were assessed per replicate. ** p ≤ 0.005, * p ≤ 0.05, or n.s. (not significant) using Student’s two-tailed unpaired t-tests.
Figure 2. Screening for RhoGAPs responsible for orchestrating actin breakdown.

(a) Schematic outlining the experimental approach employed in this study. We generated a collection of constructs comprising 62 members of the RhoGAP family conjugated to mCitrine. Each plasmid was independently transfected into RAW 264.7 macrophages, and the subcellular distribution of the encoded RhoGAP followed by confocal microscopy during phagocytosis. The extent of accumulation at phagocytic cups was assessed by ratiometric analysis relative to PM-RFP, a marker for bulk plasma membrane. The 10 RhoGAPs that most significantly accumulated at phagocytic cups were selected for further screening, consisting of assessing endogenous expression by RT-PCR and dependency of recruitment on PI3K. The ability of the remaining candidates to function as RhoGAPs during phagocytosis was validated by overexpressing these proteins and quantifying phagocytic capacity. Lastly, gene silencing was employed to determine whether the identified RhoGAPs were necessary for coordinating actin remodelling during phagocytosis. 

(b-f) Subcellular distribution of a selective subset of RhoGAPs during phagocytosis of IgG-coated erythrocytes (IgG-E). RAW 264.7 macrophages were co-transfected with constructs encoding mCitrine-tagged RhoGAPs plus the plasmalemnal marker PM-RFP. Each panel corresponds to a representative member of 4 identifiable RhoGAP types, as determined by their recruitment (or lack thereof) to sites of phagocytosis. RhoGAPs that failed to localize to sites of particle engagement and remained nuclear (b) or cytosolic (c) were assigned a type I-N and type I-C nomenclature, respectively. RhoGAPs that constitutively localized to the plasmalemma but did not accumulate at phagocytic cups (d) were designated as type II. In contrast, RhoGAPs that were initially cytosolic but translocated to phagocytic cups (e) were labelled as type III. Lastly, type IV RhoGAPs showed both a cytosolic and plasmalemmal distribution at rest but accumulated at phagocytic cups (f). Arrows point to sites of particle engagement. Scale bar, 10 µm. At least 20 cells were assessed for each RhoGAP in the collection. 

(g) Representative image of 3 independent RT-PCR assays, employed to ascertain the endogenous expression levels of the 10 RhoGAPs that were most markedly recruited to phagocytic cups. GAPDH was used as a positive control.
Figure 3. **PI3K dependency of RhoGAP recruitment to phagocytic cups.** (a-d) RAW 264.7 macrophages were co-transfected with constructs encoding each of the 10 RhoGAPs that most markedly accumulated at phagocytic cups as well as with the plasmalemmal marker PM-RFP. Transfectants were exposed to IgG-opsonized erythrocytes (IgG-E; shown in blue) for 3 min before being fixed and imaged by confocal microscopy. Where indicated, cells were treated with either vehicle (DMSO) or the PI3K inhibitor LY294002 for 10 min prior to the addition of phagocytic targets. Only a subset of the 10 RhoGAPs that were investigated is shown, namely 2 PI3K-independent (a,b) and 2 PI3K-dependent (c,d) proteins. (See Supplementary Fig. 3 for the remaining RhoGAPs that were investigated.) Arrows point to sites of particle engagement. Scale bar, 10 µm. Micrographs are representative of 3 independent experiments. At least 20 cells were assessed per replicate for each of the RhoGAPs examined.

Figure 4. **Functional validation of candidate RhoGAPs.** (a-b) Constructs encoding mCitrine alone (a) or SH3BP1 (b) were electroporated into RAW 264.7 macrophages to yield high levels of expression. To determine whether these proteins could indeed inactivate GTPases that are instrumental for phagocytosis, electroporated cells were challenged with IgG-opsonized erythrocytes and allowed to interact with the targets for 1 min (left panels) or 8 min (right panels) prior to fixation. All phagocytic targets (shown in blue) were stained with Cy5-conjugated anti-IgG secondary antibody prior to addition to phagocytes. To determine the number of IgG-coated erythrocytes that were not internalized, fixed (non-permeabilized) cells were stained with Cy3-conjugated anti-IgG (shown in red). Cells were then washed, permeabilized and stained for F-actin with phalloidin. Insets show magnified views of nascent phagocytic cups (boxed region) at the 1 min time point. Phagosomes that had already been formed prior to addition of the Cy3-conjugated secondary antibody are indicated with a star. Scale bar, 10 µm. Micrographs are representative of 3 independent experiments. (c) Quantification of the total number of IgG-coated erythrocytes that were engaged (white bars) or internalized (black bars) per phagocyte overexpressing mCitrine alone or mCitrine-tagged ARHGAP12, ARHGAP25 or SH3BP1. Values in (c) represent the means of 3 independent replicates ± SEM. ** p ≤ 0.005, * p ≤ 0.05 using Student’s two-tailed
unpaired t-tests. At least 20 cells were assessed per replicate for each of the RhoGAPs examined.

**Figure 5. RhoGAP-mediated actin breakdown is necessary for the uptake of large targets.** (a-d) RAW 264.7 macrophages were electroporated twice (sequentially within a 48-hour interval) with non-targeting control siRNA (a, b) or with siRNA directed against *SH3BP1* (c,d), *ARHGAP12* or *ARHGAP25* and challenged with small (left panel) or large (right panel) IgG-coated beads 96 hours after the initial electroporation. Cells were allowed to phagocytose for 10 min prior to fixation. All phagocytic targets (shown in blue) were stained with a Cy5-conjugated anti-IgG secondary antibody prior to being added to phagocytes, and particles that remained extracellular (shown in red) were identified as in Figure 4. Cells were then washed, permeabilized and stained for F-actin with phalloidin. Formed phagosomes are indicated with a star. Arrows point to abortive, actin-rich phagocytic cups that develop as a result of RhoGAP silencing in cells attempting to engulf large targets. Scale bar, 10 µm. (e,f) Quantification of the experiment described in (a-d). The total number of IgG-coated beads that were engaged (white bars) or internalized (black bars) per phagocyte is plotted. Values in (e,f) represent the means of 3 independent replicates ± SEM. * p ≤ 0.001 using Student's two-tailed unpaired t-tests. At least 20 cells were assessed per replicate for each of the siRNAs examined.

**Figure 6. Role of PtdIns(3,4,5)P3 and RhoGAPs during uptake of small particles.** Representative confocal micrographs of RAW 264.7 macrophages transfected with constructs encoding mCitrine-tagged ARHGAP12 (a,d), ARHGAP25 (b,e), SH3BP1 (c,f) or with GFP-tagged Akt(PH), a probe for PtdIns(3,4,5)P3 (g,h). The distribution of these fluorescent proteins is shown in green. Cells were challenged with either small (1.6-µm; left panels) or large (8.3-µm; right panels) IgG-coated beads to initiate phagocytosis. All beads were stained with a Cy5-conjugated secondary antibody (shown in blue) prior to being sedimented onto macrophage surfaces. Phagocytosis was allowed to continue for 8 min prior to fixation and staining of F-actin with phalloidin-568 (shown in red). Insets (boxed regions) show magnified views of the phagocytic cup. Scale bars, 10 µm. Micrographs are representative of 3 independent experiments; at least 20 cells were assessed per replicate for each condition.
Figure S1. *PI3K controls Rac/Cdc42 activity and actin dynamics in murine macrophages.* Confocal micrographs of RAW 264.7 macrophages transfected with PAK(PBD)-YFP (shown in green), a biosensor for active Rac/Cdc42, during phagocytosis of 8.3-µm IgG-beads. Prior to phagocytosis, cells were treated with DMSO (a) or LY294002 (b) for 10 min. Phagocytosis was allowed to proceed for the indicated times before fixing and staining F-actin with phalloidin (shown in red). All phagocytic targets were stained with a Cy5-conjugated secondary antibody (shown in blue) prior to initiating phagocytosis. Insets (boxed regions) show magnified views of the phagocytic cup. Scale bar, 10 µm. Micrographs are representative of 3 independent experiments; at least 20 cells were assessed per replicate.

Figure S2. *Subcellular localization of the RhoGAP family during phagocytosis.* RhoGAP names and their corresponding gene IDs (National Centre for Biotechnology Information) are indicated in the first column. Names have been colour-coded to indicate the species from which the primary sequence was derived. The distribution of mCitrine-tagged RhoGAPs was followed by confocal microscopy in RAW 264.7 macrophages challenged with IgG-opsonized erythrocytes (IgG-E, shown in blue in column 4). In order to distinguish *bona fide* accumulation of the RhoGAP in question from bulk enrichment of plasma membrane at sites of phagocytosis, cells were transfected with the plasmalemmal marker PM-RFP. The relative extent of RhoGAP accumulation at phagocytic cups was assessed by ratiometric analysis relative to PM-RFP intensity. Itemized in this figure is also a description of the subcellular localization at rest and during phagocytosis of each RhoGAP in the collection. A score ranging from 0 to 6 (seventh column) was assigned to members of the RhoGAP family according to the robustness of their recruitment to the phagocytic cup. The background of each entry was colour-coded to facilitate interpretation: a red background corresponds to proteins that were absent from (score = 0) or barely detectable at (score = 1) the phagocytic cup, while a green background corresponds to proteins that were moderately recruited (score = 2-4). A blue background was assigned to proteins showing moderate-to-exceptional (score = 5) or exceptional (score = 6) translocation to sites of phagocytosis. Only RhoGAPs receiving scores ranging from 4-6 were selected for further screening and functional validation.
Figure S3. PI3K dependency of RhoGAP recruitment to phagocytic cups. (a-f) RAW 264.7 macrophages were independently co-transfected with constructs encoding the plasmalemmal marker PM-RFP and each of the 10 RhoGAPs that most markedly accumulated at phagocytic cups. Transfectants were exposed to IgG-opsonized erythrocytes (IgG-E; shown in blue) for 3 min before being fixed and imaged by confocal microscopy. Where indicated, cells were treated with either vehicle (DMSO) or the PI3K inhibitor LY294002 for 10 min prior to the addition of phagocytic targets. RhoGAPs that were recruited to sites of phagocytosis in a PI3K-independent manner are shown in the upper four panels (a-d), while those that were sensitive to PI3K inhibition are shown in the lower two panels (e,f). Arrows point to sites of particle engagement. Scale bar, 10 µm. Micrographs are representative of 3 independent experiments; at least 20 cells were assessed per replicate.

Figure S4. RhoGAPs are recruited to phagocytic cups in a PI3K-dependent manner in primary macrophages. (a-f) Human monocyte-derived macrophages were co-electroporated with constructs encoding mCitrine-tagged ARHGAP12 (a,b), ARHGAP25 (c,d) or SH3BP1 (e,f) and the plasmalemmal marker PM-RFP. The electroporated cells were treated with either vehicle (DMSO; left panels) or the PI3K inhibitor LY294002 (right panels) for 10 min prior to being challenged with IgG-opsonized erythrocytes (IgG-E; shown in blue). Phagocytosis proceeded for 3 min before fixation and imaging by confocal microscopy. Insets (boxed regions) show magnified views of the phagocytic cup. Scale bar, 10 µm. Micrographs are representative of 3 independent experiments; at least 20 cells were assessed per replicate.

Figure S5. Silencing of PI3K-sensitive RhoGAPs precludes phagocytosis of large, but not small phagocytic targets. (a-c) Representative immunoblots from 3 experiments showing protein levels of SH3BP1 (a), ARHGAP25 (b) and ARHGAP12 (c) in cells electroporated with the indicated siRNAs. (d-g) RAW 264.7 macrophages electroporated with siRNA directed against ARHGAP12 (d,e) or ARHGAP25 (f,g) were
challenged with small (left panels) or large (right panels) IgG-opsonized phagocytic particles and fixed 10 min after particle exposure. All targets were stained with a Cy5-conjugated anti-IgG secondary antibody (shown in blue) prior to being added to phagocytes. Particles that remained extracellular (shown in red) were identified by the procedure described in the legend of Figure 4. Macrophages were then permeabilized and actin filaments stained with phalloidin-488 (shown in green). Formed phagosomes are indicated with a star. Abortive phagocytic cups, evident in cells receiving siRNA directed towards ARHGAP12 or ARHGAP25 and challenged with large particles, are signalled with arrows. Scale bar, 10 µm. Micrographs are representative of 3 independent experiments; at least 20 cells were assessed per replicate.

Figure S6. Images of uncropped gels and blots. Images are shown in their original full-size form, as they appeared before being cropped for presentation purposes. Dashed red boxes demarcate the portions of the blots displayed in the final figures. (a) Full gel for figure 2g, indicating endogenous levels of expression of different RhoGAPs, as assessed by RT-PCR. The lower bands at 200 bp correspond to the positive control GAPDH; all other bands correspond to RhoGAP messages. The lanes in this gel are indicative of expression of the following transcripts: 1) MYO9B; 2) ARHGAP24; 3) OPHN1; 4) ARHGAP25; 5) BCR; 6) ARHGAP12; 7) SH3BP1; 8) ARHGAP9; 9) PIK3R1 and; 10) GRLF1. (b-d) Full immunoblots for Supplementary Fig. 5a-c. Each blot shows the relative protein levels of SH3BP1 (b), ARHGAP25 (c) or ARHGAP12 (d) following electroporation with a non-targeting siRNA (lane 1), or with siRNA directed against ARHGAP12 (lane 2), ARHGAP25 (lane 3) or SH3BP1 (lane 4). GAPDH, detected with a fluorescently conjugated secondary antibody and shown in green, was used as a loading control in all three blots.
4.7 Figures

Figure 1. PI3K controls actin disassembly during phagocytosis of large targets

- **Figures:**
  - Control (DMSO) + 8.3-μm IgG-opsonized beads
  - + LY294002
  - Control + 1.6-μm IgG-opsonized beads
  - + LY294002
  - Control
  - Cdc42(G12V) Rac1(Q61L)
  - FKBP-Tiam1
  - Rac1(Q61L) HRtail
  - FKBP-Tiam1

- **Graphs:**
  - Total number of beads associated per cell (average)
  - Phagocytic efficiency (%)

- **Notes:**
  - * p ≤ 0.05
  - ** p ≤ 0.005
  - n.s.
Figure 2. Screening for RhoGAPs responsible for orchestrating actin breakdown

(a) RhoGAP family
- Accumulation at phagocytic cups
- Endogenously expressed
- PI3K-dependent
- Functional validation
- Requirement for actin disassembly

(b) Type I-N
- ARHGAP19
- IgG-E + PM-RFP

(c) Type I-C
- SRGAP2
- IgG-E + PM-RFP

(d) Type II
- ARHGAP36
- IgG-E + PM-RFP

(e) Type III
- SH3BP1
- IgG-E + PM-RFP

(f) Type IV
- GRLF1
- IgG-E + PM-RFP

(g) RhoGAPs
- GAPDH
- (500 bp)
Figure 3. PI3K dependency of RhoGAP recruitment to phagocytic cups
Figure 4. Functional validation of candidate RhoGAPs

(a) 1.0 min

- mCitrine + IgG-E (all)
- Phalloidin

- mCitrine alone

- mCitrine + IgG-E (out)

(b) SH3BP1 + IgG-E (all)
- Phalloidin

- SH3BP1

- SH3BP1 + IgG-E (out)

(c) Number of IgG-E per cell

- Total
- Internalized

* = p ≤ 0.05
** = p ≤ 0.005
Figure 5. RhoGAP-mediated actin breakdown is necessary for the uptake of large targets

--- 1.6-μm IgG-opsonized beads ---

![Image of cell with Phalloidin-488 staining and IgG-beads]  

gRNA: Non-targeting siRNA  
Phalloidin-488

--- 8.3-μm IgG-opsonized beads ---

![Image of cell with Phalloidin-488 staining and IgG-beads]  

gRNA: Non-targeting siRNA  
ARHGAP12 siRNA  
SH3BP1 siRNA  
ARHGAP25 siRNA

**Comparisons**

- **a, b**: Non-targeting siRNA vs. SH3BP1 siRNA
- **c, d**: Non-targeting siRNA vs. Non-targeting siRNA

**Significance**

- **p ≤ 0.001**

**Graphs**

**e, f**: Comparison of total and internalized 1.6-μm and 8.3-μm beads per cell

Non-targeting siRNA  
ARHGAP12 siRNA  
ARHGAP25 siRNA  
SH3BP1 siRNA

**Significance**

- **p ≤ 0.001**
Figure 6. Role of PtdIns(3,4,5)P$_3$ and RhoGAPs during uptake of small particles

- **a** 1.6-μm IgG-opsonized beads
- **b** 1.6-μm IgG-opsonized beads
- **c** 8.3-μm IgG-opsonized beads
- **d** 8.3-μm IgG-opsonized beads

- **mCitrine-ARHGAP12**
- **mCitrine-ARHGAP25**
- **mCitrine-SH3BP1**
- **Akt(PH)-GFP**

8.3-μm IgG-opsonized beads
Figure S1. PI3K controls Rac/Cdc42 activity and actin dynamics in murine macrophages
Figure S2. Subcellular localization of the RhoGAP family during phagocytosis

<table>
<thead>
<tr>
<th>Name &amp; GeneID (human, mouse, rat, chimpanzee)</th>
<th>mCitrine-RhoGAP</th>
<th>PM-RFP</th>
<th>RhoGAP + PM-RFP + IgG-E</th>
<th>Subcellular localisation at rest</th>
<th>Distribution during phagocytosis</th>
<th>Phagocytic cup recruitment (score)</th>
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<tbody>
<tr>
<td>Inositol polyphosphate-5-phosphatase, 75kDa (INPP5B) 3633</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Ubiquitous distribution; subtle accumulation at nuclear and plasma membranes</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>None-to-subtle: 1</td>
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<tr>
<td>Oculocerebrorenal syndrome of Lowe (OCRL) 4952</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Punctuated and primarily found in perinuclear compartments. Also present at plasma membrane</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>None-to-subtle: 1</td>
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<tr>
<td>Rho GTPase activating protein 36 (ARHGAP36) 158763</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Primarily nuclear. Also cytosolic and plasma membrane bound</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>None-to-subtle: 1</td>
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<tr>
<td>Rho GTPase activating protein 36 (ARHGAP36) short 158763</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Exclusively plasma membrane</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>Subtle: 2</td>
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<td>Rho GTPase activating protein 40 (ARHGAP40) 343578</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Ubiquitous distribution</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>None-to-subtle: 1</td>
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<tr>
<td>Rho GTPase activating protein 19 (ARHGAP19) 84986</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Exclusively nuclear and and somewhat plasma membrane</td>
<td>Subcellular localisation is a compartment distant from nascent cups</td>
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<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Mainly cytosolic but also found at plasma membrane</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>None-to-subtle: 1</td>
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<tr>
<td>STAR-related lipid transfer (START) domain containing 8 (STARD8) 9754</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>Ubiquitous distribution</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>None-to-subtle: 1</td>
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<td>Protein Name</td>
<td>Localization</td>
<td>Localization Description</td>
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<td>ARHGAP21</td>
<td>Ubiquitous distribution but primarily plasma membrane and nucleus</td>
<td>Marked retention at sealed phagosome and mild accumulation at plasma membrane</td>
<td>Subtle-to-moderate: 3</td>
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<tr>
<td>Myosin IXB</td>
<td>MYO9B</td>
<td>Phagocytic cups and sealed phagosomes nearly exclusively</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>Moderate-to-exceptional 5</td>
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<td>Subtle: 2</td>
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<tr>
<td>Histocompatibility (minor) HA-1</td>
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<td>Subtle: 2</td>
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<tr>
<td>Synapse defective 1, Rho GTPase, homolog 2</td>
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<tr>
<td></td>
<td>(C. elegans)</td>
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<tr>
<td>RalA binding protein 1 (RALBP1)</td>
<td>RALBP1</td>
<td>Ubiquitous distribution</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>None-to-subtle 1</td>
<td></td>
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</tbody>
</table>


<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Subcellular Localization</th>
<th>Localization at Particle Engagement</th>
<th>Subcellular Localization Specificity</th>
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<tr>
<td>Family with sequence similarity 13, member A (FAM13A) 10144</td>
<td>Primarily nuclear but ubiquitously present</td>
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<tr>
<td>Family with sequence similarity 13, member B (FAM13B) 51306</td>
<td>Cytosolic and plasma membrane</td>
<td>Cytosolic and plasma membrane</td>
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<tr>
<td>Chimerin 2 (CHN2) 1124</td>
<td>Ubiquitous distribution + punctae</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>Subtle: 2</td>
</tr>
<tr>
<td>Chimerin 1 (CHN1) 1123</td>
<td>Primarily nuclear. Also cytosolic and plasma membrane bound</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>Subtle-to-moderate: 3</td>
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<tr>
<td>ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1 (ARAP1) 116985</td>
<td>Ubiquitous distribution + punctae</td>
<td>Present at cup but also ubiquitously distributed</td>
<td>None-to-subtle: 1</td>
</tr>
<tr>
<td>ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3 (ARAP3) 116984</td>
<td>Plasma membrane and cytosolic</td>
<td>Subtle increase at phagocytic cup but already found at plasma membrane prior to particle engagement</td>
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<td>Rho GTPase-activating protein 20 (ARHGAP20) 57569</td>
<td>Plasma membrane and cytosolic</td>
<td>Moderate increase at phagocytic cup but already found at plasma membrane prior to particle engagement</td>
<td>Subtle-to-moderate: 3</td>
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<td>Rho GTPase-activating protein 23 (ARHGAP23) 57636</td>
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<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
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<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
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<td>Rho GTPase activating protein 24 (ARHGAP24)</td>
<td>Primarily plasma membrane</td>
<td>Marked accumulation at base of phagocytic cup although already at plasma membrane</td>
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<tr>
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<td>---------------------------</td>
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<td>Oligophrenin 1 (OPHN1) 4983</td>
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<td>Recruitment to phagocytic cup exclusively</td>
<td>Exceptional 6</td>
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<td>Rho GTPase activating protein 25 (ARHGAP25) 9938</td>
<td>Plasma membrane and cytosol</td>
<td>Moderate accumulation at nascent cups. Increase is less obvious at the base of the cup than at the top</td>
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<td>Exclusively at the plasma membrane</td>
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<td><em>GEF/GAP</em> breakpoint cluster region (BCR) 613</td>
<td>Plasma membrane and cytosol</td>
<td>Robust recruitment to base of the cup</td>
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<tr>
<td><em>GEF/GAP</em> active BCR-related gene (ABR) 29</td>
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<td>Plasma membrane and cytosol</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>Subtle-to-moderate: 3</td>
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<tr>
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<td>Plasma membrane and cytosol</td>
<td>Present at cup but already found at plasma membrane prior to particle engagement</td>
<td>Moderate 4</td>
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<tr>
<td>Rho GTPase activating protein 8 (ARHGAP8) 23779</td>
<td>Cytosolic</td>
<td>No obvious recruitment; ubiquitously present throughout the cell</td>
<td>Subtle: 2</td>
</tr>
<tr>
<td>Rho GTPase activating protein 1 (ARHGAP1)</td>
<td>Ubiquitous localisation and formation of punctae.</td>
<td>No obvious recruitment; ubiquitously present throughout the cell</td>
<td>Subtle: 2</td>
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<tr>
<td>Rho GTPase activating protein 44 (ARHGAP44)</td>
<td>Cytosolic and plasma membrane</td>
<td>Slight increase at the base of the phagocytic cup, although constitutively present at the plasma membrane</td>
<td>Subtle: 2</td>
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<tr>
<td>Rho GTPase activating protein 17 (ARHGAP17)</td>
<td>Primarily nuclear. Also cytosolic and plasma membrane bound</td>
<td>No obvious recruitment; ubiquitously present throughout the cell</td>
<td>Subtle: 2</td>
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<tr>
<td>SH3-domain binding protein 1 (SH3BP1)</td>
<td>Diffuse signal throughout cytosol</td>
<td>Marked recruitment to nascent phagocytic cups</td>
<td>Moderate-to-exceptional 5</td>
</tr>
<tr>
<td>SLIT-ROBO Rho GTPase activating protein 1 (SRGAP1)</td>
<td>Plasma membrane</td>
<td>Possible recruitment to nascent cups</td>
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<tr>
<td>Rho GTPase activating protein 4, isoform 2 (ARHGAP4)</td>
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<td>No recruitment to phagocytic cups; ubiquitously present throughout the cell</td>
<td>Subtle: 2</td>
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<tr>
<td>SLIT-ROBO Rho GTPase activating protein 3, isoform a (SRGAP3)</td>
<td>Primarily nuclear but also cytosolic and plasma membrane bound</td>
<td>No recruitment to phagocytic cups; ubiquitously present throughout the cell</td>
<td>Subtle: 2</td>
</tr>
<tr>
<td>SLIT-ROBO Rho GTPase activating protein 2, isoform c (SRGAP2)</td>
<td>Ubiquitous distribution</td>
<td>No recruitment; already present at plasma membrane</td>
<td>None-to-subtle 1</td>
</tr>
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<td>Rho GTPase activating protein 26 (ARHGAP26)</td>
<td>Primarily plasma membrane and nuclear but to a lesser extent cytosolic</td>
<td>No recruitment; already present at plasma membrane</td>
<td>Subtle: 2</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Gene ID</td>
<td>Primary Membrane Location</td>
<td>Recruitment Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Rho GTPase activating protein 9 (ARHGAP9)</td>
<td>64333</td>
<td>Primarily plasma membrane and nuclear, but to a lesser extent cytosolic</td>
<td>Recruitment is more obvious in some cells, although already present at the PM prior to particle engagement</td>
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<tr>
<td>Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (PIK3R1)</td>
<td>5295</td>
<td>Diffuse signal throughout cytosol</td>
<td>Signal exclusively at the base of phagocytic cup</td>
</tr>
<tr>
<td>Phosphoinositide-3-kinase, regulatory subunit 2 (beta) (PIK3R2)</td>
<td>5296</td>
<td>Ubiquitous distribution; subtle accumulation at nuclear and plasma membranes</td>
<td>Subtle accumulation at the base of the cup</td>
</tr>
<tr>
<td>DEP domain containing 1 (DEPDC1)</td>
<td>55635</td>
<td>Low expression levels; ubiquitously distributed</td>
<td>No recruitment; already present at plasma membrane</td>
</tr>
<tr>
<td>DEP domain containing 1B (DEPDC1B)</td>
<td>55789</td>
<td>Low expression levels; ubiquitously distributed</td>
<td>Marked retention at sealed phagosome and mild accumulation at plasma membrane</td>
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<tr>
<td>Rho GTPase activating protein 39 (ARHGAP39)</td>
<td>80728</td>
<td>Primarily nuclear, but also cytosolic</td>
<td>Moderate recruitment to the cup proportionally to plasma membrane increase</td>
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<tr>
<td>Rho GTPase activating protein 35 (ARHGAP35; GRLF1)</td>
<td>2909</td>
<td>Diffuse signal throughout cytosol</td>
<td>Signal exclusively at the base of phagocytic cup</td>
</tr>
<tr>
<td>Rho GTPase activating protein 5 (ARHGAP5)</td>
<td>394</td>
<td>Diffuse signal throughout cytosol</td>
<td>Slight recruitment to the cup proportionally to plasma membrane increase</td>
</tr>
<tr>
<td>T-cell activation RhoGTPase activating protein (TAGAP)</td>
<td>117289</td>
<td>Ubiquitous distribution</td>
<td>Slight recruitment to the cup proportionally to plasma membrane increase</td>
</tr>
</tbody>
</table>
Figure S3. PI3K dependency of RhoGAP recruitment to phagocytic cups

- **Control (DMSO) + LY294002**: This condition shows the baseline activity of RhoGAPs in the absence of PI3K inhibition.

- **ARHGAP9**: In the presence of IgG-E + PM-RFP, ARHGAP9 is recruited to phagocytic cups, indicating its role in phagocytosis.

- **ARHGAP24**: Similarly, ARHGAP24 is recruited to phagocytic cups when PI3K is inhibited.

- **ARHGAP12**: This RhoGAP is also recruited to phagocytic cups under PI3K-independent conditions.

- **BCR**: BCR recruitment is observed in control conditions, suggesting its involvement in phagocytosis.

- **MYO9B**: MYO9B recruitment is enhanced in PI3K-dependent conditions, highlighting its importance in the mechanism.

- **OPHN1**: OPHN1 recruitment is also observed in PI3K-dependent conditions, indicating its role in the process.

- **PI3K-independent**: In this condition, RhoGAPs are recruited to phagocytic cups in the absence of PI3K inhibition.

- **PI3K-dependent**: Here, RhoGAPs are recruited to phagocytic cups in the presence of PI3K inhibition, showing the dependency of recruitment on PI3K activity.
Figure S4. RhoGAPs are recruited to phagocytic cups in a PI3K-dependent manner in primary macrophages

Human monocyte-derived macrophages

--- Control (DMSO) ---

a. ARHGAP12 IgG-E + PM-RFP

b. ARHGAP12 IgG-E + PM-RFP

c. ARHGAP25 IgG-E + PM-RFP

d. ARHGAP25 IgG-E + PM-RFP

e. SH3BP1 IgG-E + PM-RFP

f. SH3BP1 IgG-E + PM-RFP

--- + LY294002 ---

Human monocyte-derived macrophages
Figure S5. Silencing of PI3K-sensitive RhoGAPs precludes phagocytosis of large, but not small phagocytic targets.

(a) Western blot showing the expression of SH3BP1 and GAPDH in control cells.
(b) Western blot showing the expression of ARHGAP25 in control cells.
(c) Western blot showing the expression of ARHGAP12 in control cells.

(d) Immunofluorescence images showing phalloidin-488 staining in cells treated with ARHGAP12 siRNA and 1.6-μm IgG-opsonized beads.
(e) Immunofluorescence images showing phalloidin-488 staining in cells treated with ARHGAP12 siRNA and 8.3-μm IgG-opsonized beads.
(f) Immunofluorescence images showing phalloidin-488 staining in cells treated with ARHGAP25 siRNA.
(g) Immunofluorescence images showing phalloidin-488 staining in cells treated with SH3BP1 siRNA.

Non-targeting siRNA was used as a control.

GAPDH expression levels were quantified as follows:
- 80% decrease in ARHGAP12
- 41% decrease in ARHGAP25
- 80% decrease in SH3BP1
Figure S6. Images of uncropped gels and blots

a) Full gel for Figure 2g

b) Full blot for Supplementary Figure 5a

c) Full blot for Supplementary Figure 5b

d) Full blot for Supplementary Figure 5c
Chapter 5

This chapter has been modified from the following: Daniel Schlam, Michal Bohdanowicz, Alexandros Chatgilialoglu, Benjamin E Steinberg, Takehiko Ueyama, Guangwei Du, Sergio Grinstein, and Gregory D Fairn. “Diacylglycerol Kinases Terminate Diacylglycerol Signaling during the Respiratory Burst Leading to Heterogeneous Phagosomal NADPH Oxidase Activation.” *The Journal of Biological Chemistry* 288 (32): 23090–104. © The American Society for Biochemistry and Molecular Biology, August 2013.

5 Degradation: Diacylglycerol kinases terminate the respiratory burst and establish heterogeneity in phagosomal NADPH oxidase activation

5.1 Abstract

It is commonly assumed that phagosomes generated as a result of a given receptor-ligand interaction have virtually identical physiological properties. This assumption has remained largely unchallenged due to a paucity of methods with enough spatiotemporal resolution to distinguish the biochemical fate of individual phagosomal compartments. To address whether the phagosomal pool is in fact homogenous, we focused on the ability of single phagosomes to generate superoxide anions during their course of maturation. This required devising a novel and dynamic assay capable of detecting and quantifying NAPDH oxidase (NOX) activity in independent phagosomal compartments. Implementation of this assay revealed striking heterogeneity in the competence of single phagosomes to generate superoxide, both between and within individual cells. While phosphatidylinositol-3,4,5-trisphosphate, phosphatidylinositol-3-phosphate and phosphatidic acid have all been implicated in NOX activation, their distribution was nearly identical in all phagosomes, and therefore could not account for the marked variability in superoxide generation. In sharp contrast, diacylglycerol (DAG) was
generated unevenly across the phagosomal population and its distribution tightly correlated with NOX activity. Pharmacological, biochemical and genetic approaches to modulate DAG signalling suggested that superoxide production is precluded when phagosomes fail to maintain a critical level of DAG. Our data suggest that DAG kinases limit the extent of NOX activation by catabolizing phagosomal DAG, thereby curtailing the excessive production of reactive oxygen species. The resulting heterogeneity in NOX responsiveness could enable the survival of a fraction of invading microorganisms.

5.2 Introduction

During phagocytosis—one of the earliest components of the innate immune response—microbial pathogens and cell debris are engulfed by specialized immune cells into vacuoles known as phagosomes (Kamen, Schlessinger, and Lowell 2011). These newly formed compartments undergo rapid remodelling in order to amass an impressive arsenal of microbicidal tools (Yeung, Ozdamar, et al. 2006). This remodelling, better known as maturation, entails the acquisition of V-ATPases that render the phagosomal lumen acidic, as well as of a variety of antimicrobial peptides, proteases and lipases, all of which undermine the viability and integrity of the incoming pathogens. Phagosomes also gain the ability to produce reactive oxygen species (ROS). The latter are in part produced by the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX), a superoxide-generating, flavocytochrome-containing complex. In turn, the superoxide generated by NOX can dismutate into hydrogen peroxide and give rise to hypochlorous acid, all effective microbicidal agents (Aratani et al. 2002).

The oxidase is a multicomponent system composed of two membrane proteins, gp91<sub>phox</sub> (NOX2, the catalytic subunit) and p22<sub>phox</sub>; three cytosolic regulators, p40<sub>phox</sub>, p47<sub>phox</sub> and p67<sub>phox</sub>; and a Rho GTPase, either Rac1 or Rac2. During phagocytosis, critical inflammatory signals trigger recruitment of the cytosolic components to the plasmalemma, where they interact with the flavocytochrome, thereby inducing formation of the active NOX complex (Casbon et al. 2009).
Several membrane lipids have been implicated in the regulation of NOX activity, including diacylglycerol (DAG) and phosphatidic acid (PA) (Qualliotine-Mann et al. 1993). DAG can be generated through hydrolysis of phosphoinositides by phospholipase C (PLC), or by the combined actions of phospholipase D (PLD) and PA phosphohydrolases. DAG promotes recruitment of PKC to the phagosomal membrane and its subsequent activation, which allows for phosphorylation of p47\textsuperscript{phox} (He et al. 2004; Cheng et al. 2007) and assembly of the active oxidative complex. PA, which is produced by the hydrolytic action of PLD on phosphatidylcholine or through phosphorylation of DAG by diacylglycerol kinases (DGKs), has also been implicated as a second messenger in the activation of the oxidase (Regier et al. 1999).

Although much progress has been made in the characterization of the molecular signals lying upstream of NOX activation, the vast majority of these studies have relied on population-based assays. Such analyses inherently assume that all phagosomes respond identically, thus masking potential heterogeneity in the oxidative response. Compelling evidence now suggests that each phagosome is a discrete, independent compartment whose fate is dictated by a unique set of signalling cues originating at its membrane (Griffiths 2004).

That heterogeneity may exist in the phagosomal pool prompted us to investigate NOX activity in individual phagosomes. To this end, we devised a dynamic assay that quantitatively monitors oxidase activity in single phagosomes during the course of particle ingestion. These measurements revealed a striking variability between phagosomes, not only in different cells but also within the same cell. We next analyzed the source of this heterogeneity, placing particular emphasis on the lipid mediators of oxidase activation. Our data indicate that local fluctuations in the levels of phagosomal DAG are the main source of variability in the oxidative response, and that DGK activity is a critical determinant of DAG concentration at nascent phagosomes. These observations have important implications regarding the fate of pathogens normally confronted by professional phagocytes.
5.3 Methods

5.3.1 Reagents

Zymosan A particles (from \textit{S. cerevisiae}), human-serum IgG, nitroblue tetrazolium (NBT) and lipopolysaccharide (LPS from \textit{S. enterica} 595) were purchased from Sigma-Aldrich. Paraformaldehyde (used in PBS at 4\% v/v) was from Electron Microscopy Sciences (Hatfield, Pennsylvania). Alexa Fluor 555succinimidyl ester was from Invitrogen Molecular Probes. Cy5-conjugated and DyLight 488-conjugated donkey anti-human secondary antibody and donkey serum were from Jackson ImmunoResearch Laboratories, Inc. Compounds R59 022 (DGKi I, 30 \mu M) and R59 949 (DGKi II, 30 \mu M) were purchased from Enzo Life Sciences, Inc. Dioctanoyl ethylene glycol (100 \mu M) was from Tocris (Ellisville, Missouri). Ionomycin (1 \mu M) was from Calbiochem (San Diego, California). PA (L-\alpha-phosphatidic acid; 840074) was purchased from Avanti (Alabaster, Alabama) and dried under nitrogen gas before being resuspended in serum-free DMEM complemented with 4mg of essentially fatty acid-free BSA (Sigma-Aldrich).

5.3.2 Cell culture and transfection

RAW 264.7 macrophages and HeLa cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10\% heat-inactivated fetal bovine serum (Wisent, St. Bruno, Canada) at 37°C in a 5\% CO$_2$-regulated incubator. Cells plated on glass coverslips were transiently transfected using FuGene HD (Roche) according to the manufacturer’s protocol. Each well of a 12-well plate was treated with 1 \mu g plasmid cDNA and 3 \mu L FuGene HD. Transfected cells were typically used 18-24 hours after transfection. Where indicated, macrophages were activated by overnight treatment with LPS (100 ng/mL). For live imaging, cells were incubated in HEPES-buffered RMPI at 37°C.
5.3.3 Constructs

The DAG biosensor consisted of an N-terminal GFP fused to the C1 domain of PKCδ in the pEGFP(C1) vector. Where indicated, an mCherry fluorophore was used to replace GFP in this vector. The PA biosensor consisted of GFP fused to two PA-binding domains of the yeast protein Spo20p, described previously (Zeniou-Meyer et al. 2007; Du and Frohman 2009), arranged in tandem and modified by including the N-terminal nuclear export signal of PKI-α. Plasmids encoding HA-tagged PLD1, wild-type PLD2 and dominant negative PLD2(K758R) were provided by Dr. J. Brumell (Hospital for Sick Children, Toronto). The surface charge probe, RFP-R-pre, as well as the PtdIns(3)P and PtdIns(3,4,5)P3 probes were described earlier (Yeung, Terebiznik, et al. 2006; Flannagan and Grinstein 2010). DGK-GFP isoforms α, β, γ, δ, ε and ζ have also been reported previously (Shindo et al. 2003). DGKθ, originally encoded in the pCMV-SPORT6 vector and provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan (Ota et al. 2004), was digested with EcoRI and Xhol and directionally subcloned into the EcoRI and SalI sites of pEGFP(C3).

5.3.4 Site-directed mutagenesis of DGKβ

Inactive (kinase dead) diacylglycerol kinase β was generated by primer-directed mutagenesis of the enzyme’s ATP-binding site using the QuikChange site-directed mutagenesis kit (Stratagene). The forward and reverse mutagenic primers shown below, each carrying two complementary point mutations, were utilized to introduce a glycine-to-aspartic acid transition at residue 495. Ten ng of template DNA [wild-type DGKβ carried in the vector pEGFP(C1)] were used for PCR-based amplification (16 cycles total) of the mutant construct. PCR reactions were subsequently incubated with the restriction enzyme DpnI in order to digest the parental, wild-type vector while maintaining the integrity of unmethylated linear amplicons. Lastly, mutant vectors were transformed into XL1-Blue supercompetent cells for nick repair and plasmid replication.

DGKβ(G495D) forward: 5'-tagcatgcgtgagatgatactgtgggctggattttg-3'
DGKβ(G495D) reverse: 5'-caaaatccagccacagtcatctccaccgcatttgtgatttttg-3'
5.3.5 Reverse transcription-polymerase chain reaction

One-step RT-PCR was used to detect transcription of DGK isoforms (α through κ), using RNA from RAW 264.7 macrophages for cDNA synthesis. For each reaction, RNA was isolated from 8×10⁵ cells using the QIAGEN RNeasy Mini kit. Invitrogen’s OneStep RT-PCR kit was utilized for cDNA generation and PCR amplification. Reverse transcription was performed with 1 µg of purified RNA per reaction, using isoform-specific primers for 30 min at 55°C (primer sequences listed below). Reverse transcriptase was then inactivated and Taq polymerase activated by increasing the temperature to 94°C for 4 min. Amplification was performed for 40 cycles at denaturing, annealing and extension temperatures of 94°C, 55°C and 68°C, respectively.

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<td>β (beta)</td>
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<tr>
<td>γ (gamma)</td>
<td>Fwd: 5'-GCCGAACCAAAGTGTTGATGTTGATG</td>
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<td>κ (kappa)</td>
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</table>
5.3.6 Phagocytosis

RAW 264.7 macrophages were grown on glass coverslips placed in 12-well plates with DMEM supplemented with 10% heat-inactivated fetal bovine serum. Before the experiments, the coverslips were transferred to Leiden chambers and bathed with HEPES-buffered medium RPMI. The chamber was subsequently placed in a holder mounted on the microscope stage. To initiate phagocytosis, 80 µL of zymosan particles (original concentration = 2 mg/mL) opsonized with human IgG and/or labelled with Alexa 555-succinimidyl ester ([final] = 3 µg/mL) were added to the cells. Where indicated, phagocytes were incubated with nitroblue tetrazolium ([final] = 1 mg/mL). For opsonization, zymosan particles at a concentration of 2 mg/mL were incubated for 60 min with human IgG ([final]= 5 µg/mL). To synchronize phagocytosis, zymosan particles were sedimented by centrifugation for 1 min at 1,000 rpm. Excess particles were then removed by washing 3 times with PBS. Zymosan particles that were not internalized were identified by staining with Cy5-conjugated anti-human IgG at 4°C for 5 min. Donkey serum was diluted 10-fold in PBS and used for blocking prior to staining non-internalized targets. Where indicated, cells were treated with diacylglycerol kinase inhibitors (30 µM) for 20 min before initiation of phagocytosis.

5.3.7 Flow cytometry

In preparation for analysis by flow cytometry, zymosan particles were either opsonized with human IgG or labelled with Alexa Fluor 555-conjugated succinimidyl esters, using the opsonin and dye concentrations described for phagocytosis. Opsonized particles were subsequently stained with donkey anti-human secondary antibody conjugated to DyLight488 (Jackson ImmunoResearch Laboratories, Inc.). Homogeneity in opsonization and labelling was determined with a Becton Dickinson (BD) Canto II flow cytometer equipped with 405 nm, 488 nm and 633 nm lasers. Flow data analysis was performed with FlowJo software (Tree Star, Inc.).
5.3.8 Enzymatic assay for assessing total phosphatidic acid content
PA was measured enzymatically (Morita, Ueda, and Kitagawa 2009) using a total phosphatidic acid kit (Abnova, Taipei City, Taiwan) adhering to the manufacturer’s instructions. In brief, lipids were purified from almost confluent RAW 264.7 monolayers grown on 10-cm plates by methanol-chloroform extraction, using polypropylene centrifuge tubes. Purified lipids were subjected to lipase digestion. Subsequently, glycerol-3-phosphate oxidase was used to produce H$_2$O$_2$ from glycerol-3-phosphate (a byproduct of PA digestion by lipases). H$_2$O$_2$ was then reacted with ADHP (10-Acetyl-3,7-dihydroxyphenoxazine) in the presence of horseradish peroxidase to yield resorufin (Amplex Red). Resorufin fluorescence was detected using a SpectraMax Gemini EM microplate reader (Molecular Devices, Sunnyvale, California). PA content was normalized to unit protein, in turn determined by a bicinchoninic acid assay (Thermo Scientific, Rockford, Illinois) before lipid extraction.

5.3.9 Microscopy and image analysis
For fluorescence quenching experiments, coverslips were placed in a Leiden chamber maintained at 37°C and mounted on the stage of a microscope (model DM IRB; Leica). The cells were excited by light from an XFO X-Cite 120 lamp (XFO Life Sciences Group) transmitted through a 485 ± 10 nm excitation filter directed to the sample using a 505 nm dichroic mirror and captured by a cooled charge-coupled device camera (Cascade II; Photometrics) using 2x2 binning. The filter wheel and camera were under the control of MetaFluor software (MDS Analytical Technologies). All other fluorescence images were acquired by spinning-disc confocal microscopy. The systems in use in our laboratory (Quorum) are based on a microscope (Axiovert 200M; Carl Zeiss, Inc.) with 63x or 100x objectives and a 1.5x magnifying lens. The units are equipped with diode-pumped solid-state lasers (440, 491, 561, 638, and 655 nm; Spectral Applied Research), a motorized XY stage (Applied Scientific Instrumentation) and a Piezo focus drive. Images were acquired using back-thinned electron-multiplied, or conventional cooled charge-coupled device cameras (Hamamatsu Photonics) driven by the Volocity software (version 4.1.1; Perkin Elmer).
5.3.10 Statistical analysis

Statistical parameters were determined using GraphPad Prism 5c (GraphPad Software, Inc.). To assess significance of differences, all comparisons between means of matched groups were performed by two-tailed unpaired $t$-tests, relying on the analysis routines built in GraphPad Prism.

5.4 Results

5.4.1 Identification of superoxide production within single phagosomes

We devised a novel assay—based on the principle of fluorescence quenching—capable of continuously monitoring NADPH oxidase activity within single phagosomes (Figure 1A). In designing this methodology, we took advantage of the previously described deposition of diformazan, generated from NBT at sites of superoxide production (Baehner and Nathan 1968). However, direct measurement by brightfield microscopy of changes in light absorbance caused by diformazan deposition is complicated by the absorbance and scattering contributed by the cells themselves and by the phagocytic targets, which vary as the particles are engulfed. To circumvent these problems, zymosan particles were labelled with succinimidyl esters conjugated to Alexa 555. The resulting labelled particles (hereafter referred to as Zymo-Alexa 555) were opsonized with human IgG and used as phagocytic prey. Phagocytosis was performed in the presence of NBT, which is membrane-permeable and capable of traversing both the plasma and phagosomal membranes, effectively reaching the phagosomal lumen. Upon activation of NOX, intraphagosomal deposition of the black-purple diformazan rapidly quenches the fluorescence of the phagocytic targets, interfering with the passage of both excitation and emission light (Figure 1A and B). The amount of remaining fluorescence is therefore inversely correlated with the cumulative activity of the NAPDH oxidase. This is illustrated in Figure 1C; while the fluorescence of internalized particles decays rapidly, the fluorescence of particles that remain extracellular is largely unaffected, implying that only a minimal decrease in fluorescence is attributable to
photobleaching. Accordingly, little to no fluorescence quenching was observed when labelled particles underwent phagocytosis in the absence of NBT (Figure 1B). It is noteworthy that the Alexa 555 Fluor is stable and its fluorescence unaffected between pH 4 and 10 (Panchuk-Voloshina et al. 1999), enabling measuring superoxide production independently of phagosomal acidification. Importantly, these observations indicate that the newly described quenching can continuously measure superoxide production within individual phagosomes. This notion was validated using diphenyleneiodonium (DPI), a potent inhibitor of the NADPH oxidase. In the presence of 10 µM DPI, fluorescence quenching was significantly prevented (Figure 2A and B).

5.4.2 NADPH oxidase activation is heterogeneous across phagosomes

By implementing the assay described above, we detected overt heterogeneity in the responsiveness of individual phagosomes in RAW 264.7 macrophages (henceforth referred to as RAW). Remarkably, differences could be noted not only between different cells, but also amongst phagosomes formed within individual cells (Figure 2A, top 2 rows), regardless of the time of phagosomal sealing. After a 30-min exposure to phagocytic targets, an average of only 26 ± 1.9% of the phagosomes formed by each RAW cell displayed detectable oxidase activity (Figure 2B, white bars), while 60 ± 2.9% of the total macrophage population had at least 1 superoxide-generating phagosome (Figure 2B, black bars).

We contemplated the possibility that either uneven labelling with Alexa 555 or variability in opsonization may have artifactually led to heterogeneity in the measured response. This possibility was discounted using flow cytometry. As illustrated in Figure 2C, cytometric analyses of both zymosan labelling and opsonisation yielded sharp, unimodal distributions that could not account for the observed heterogeneity in phagosomal oxidase activity.

Even though NBT is permeable across biological membranes (Choi et al. 2006), its rate of entry could still represent a limiting factor. It was therefore conceivable that the observed heterogeneity in the NOX response reflected different amounts of NBT being accumulated in individual phagosomes at the time of sealing. To examine this
possibility, we exposed RAW cells to IgG-Zymo-Alexa 555 in the absence of NBT for 7 min, an interval that allowed for the majority of particles to be internalized. Particles that had not been fully internalized by this time were labelled by addition of extracellular anti-human IgG labelled with DyLight488, and thereby eliminated from further analysis. NBT was then added and the reaction allowed proceeding for a further 23 min (see Supplementary Figure 1A for a diagrammatic illustration of the protocol). Distinct heterogeneity in NOX activity was still observed among the phagosomes that had sealed prior to the addition of NBT (Supplementary Figure 1B). These data validate the notion that NBT can readily cross biological membranes and imply that differential trapping of NBT during sealing cannot explain the variability of the responses.

It is also worth noting that we routinely encountered nascent phagosomes that had not yet sealed. Some, but not all of these displayed diformazan precipitates at the base of the forming cup (e.g. Figure 2D). Because at this stage access to NBT is unhindered, the variable response cannot be attributed to insufficient exposure to NBT. The latter observation also rules out differential permeability of the phagosomal membrane to NBT as the source of the heterogeneity illustrated in Supplemental Figure 1B.

Finally, we considered the possibility that heterogeneity in superoxide production was a consequence of asynchrony in the phagocytic process and/or in the activation of the oxidase. To address this, RAW cells were allowed to ingest IgG-Zymo-Alexa 555 in the presence of NBT for 30 min, as in Figures 1 and 2. However, in these experiments particles that had not been internalized after the first 3 min were marked by addition of a fluorescently labelled secondary antibody (see Supplementary Figure 1C for a diagrammatic illustration of the protocol). By excluding those targets that were ingested after addition of the antibody, we were able to analyze the response of only those phagosomes formed within a narrow time window, i.e., the initial 3 min. These were then allowed to mount an oxidative response for an additional 27 min. The integrated cumulative response over this comparatively long period should have minimized the consequences of the slight phagocytic asynchrony incurred during the initial 3 min. Notably, considerable heterogeneity was nevertheless recorded under these conditions (Supplementary Figure 1D). Jointly, these experiments implied that the variable
responsiveness of the phagosomal oxidase is neither an artifact of uneven exposure to NBT nor a reflection of asynchrony in the phagocytic response. Instead, the oxidative response of phagosomes appears to be intrinsically heterogeneous.

It is widely accepted that bacterial endotoxin can robustly prime phagocytes for superoxide production (Check et al. 2010), although the underlying mechanism is still incompletely understood (Russell et al. 2009). We sought to distinguish whether LPS priming operated by increasing the production of superoxide by an unchanging number of phagosomes or by increasing the fraction of responding phagosomes. Interestingly, overnight incubation of RAW macrophages with LPS (100 ng/mL), a Toll-like receptor-4 (TLR-4) agonist, increased the fraction of phagosomes capable of mounting an effective respiratory burst (from 26 ± 1.9% to 55 ± 1.6%), as well as the number of cells with at least 1 responsive phagosome (from 60 ± 2.9% to 91 ± 2.3%) (Figures 2A and 2B). Thus, priming reduced heterogeneity in the respiratory response.

5.4.3 Role of PtdIns(3,4,5)P₃, PA and PtdIns(3)P in the genesis of heterogeneity

A number of key lipid intermediates are required for several aspects pertaining to the formation and maturation of phagosomes, including deployment of the NADPH oxidase. In particular, engulfment of large particles (Schlam et al. 2015) and initiation of the maturation response (Zhu et al. 2006) require activation of class I phosphoinositide 3-kinase. Because different degrees of receptor engagement can influence the extent of PtdIns(3,4,5)P₃ formation (Youxin Zhang, Hoppe, and Swanson 2010), we analyzed whether differential production of this phosphoinositide accounted for the variable respiratory burst response. To monitor formation of PtdIns(3,4,5)P₃ during the course of phagocytosis of IgG-opsonized zymosan, we used a genetically encoded biosensor consisting of the PH domain of Akt tagged with GFP. Spinning-disc confocal microscopy was used to monitor translocation of the biosensor to the phagosomal membrane over time. Consistent with earlier findings (Marshall et al. 2001), we readily detected recruitment of Akt(PH)-GFP to nascent and recently formed phagosomes, followed by
rapid loss of the probe after sealing (Figure 3B, top). Of note, virtually 100% of the phagosomes induced by IgG-zymosan accumulated the probe to a comparable degree (Figure 3C). Thus, failure to generate PtdIns(3,4,5)P3 cannot account for the inability of a considerable fraction of phagosomes to activate the NADPH oxidase.

A similar approach was used to evaluate the role of PA. In this instance, we used a probe comprised of two (tandem) copies of the PA-binding domain of the yeast protein Spo20p, which has been used in the past to monitor PA distribution (Zeniou-Meyer et al. 2007; Du and Frohman 2009). The PA probe (hereafter referred to as 2PABD-GFP), was optimized by adding a nuclear export signal N-terminally to the PABD domains, as the probe tends to partition to the nuclear compartment otherwise. In order to ensure that this modification did not interfere with the ligand specificity of the probe, we monitored the distribution of 2PABD-GFP in HeLa cells before and after altering their PA content by pharmacological, biochemical or enzymatic means (Supplementary Figure 2). Unlike phagocytes (Bohdanowicz et al. 2013), unstimulated HeLa cells have little PA in their plasma membrane (Supplementary Figure 2A top left). Exposure to exogenous PA (100 µM for 10 min) induced rapid association of 2PABD-GFP with the plasma membrane (Supplementary Figure 2A top middle; Supplementary Video 2), validating the responsiveness of the probe. Recruitment of 2PABD-GFP to the membrane was also observed when HeLa cells were treated with 1 µM ionomycin for 2 min (Supplementary Figure 2A top right; Supplementary Video 3); calcium entering through the ionophore activates phospholipase C, thus generating DAG, a PA precursor. Moreover, recruitment of 2PABD-GFP was also observed when HeLa cells were co-transfected with wild-type PLD2, a plasma membrane-associated phospholipase that converts phosphatidylcholine to PA. Of note, expression of neither an inactive form of PLD2 nor of PLD1 (which is not found at the membrane but instead exhibits a vesicular, punctate distribution) caused recruitment of the PA probe to the membrane. Jointly, these results provide evidence that the 2PABD biosensor utilized in this study is a suitable probe for PA.

In contrast to HeLa cells, a sizable amount of the PA probe was found to associate with the plasma membrane of unstimulated RAW macrophages. The plasmalemma-bound probe was rapidly released to the cytosol upon treatment with the DGK inhibitor R59
022 (DGKi I, 30 µM), suggesting that PA is continuously synthesized, at least in part, through DAG phosphorylation (Supplementary Figure 2B, left panels; Supplementary Video 4). Accordingly, addition of DGKi I caused a concomitant accumulation of DAG in the membrane of RAW cells (Supplementary Figure 2B, right panels; Supplementary Video 4) as well as a reduction in the total cellular content of PA, measured biochemically (Figure 5C). These observations further validate the reliability of the 2PABD biosensor.

Despite being constitutively present at the membrane of unstimulated cells, 2PABD-GFP accumulated further at the phagocytic cup (Figure 3B, middle). The accumulation dissipated rapidly after phagosome closure, with PA being only marginally detectable in the phagosomal membrane 3 min after sealing. As was the case for PtdIns(3,4,5)P3, essentially all phagosomes analyzed accumulated PA, judged by the recruitment of the Spo20p probe (Figure 3C). Therefore, the inability of a large fraction of phagosomes to form superoxide cannot be attributed to lack of PA accumulation.

PtdIns(3)P, a product of class III PI3K, was recently shown to be required for phagosomal superoxide generation, likely by recruitment the p40phox subunit of the oxidase via its PX domain (Suh et al. 2006). A probe based on the FYVE domain of EEA1 was used to quantify the fraction of phagosomes that accumulate PtdIns(3)P. As described before (Vieira et al. 2001; Ozdamar et al. 2006), PtdIns(3)P was not detectable at the phagosomal cup and instead was only observable ≥ 1 min after sealing, persisting for nearly 10 min at the phagosomal membrane. Two lines of evidence suggest that PtdIns(3)P is not responsible for heterogeneity in the respiratory burst: first, as for the two other lipids, PtdIns(3)P was found in every phagosome studied (Figure 3B, C). Moreover, as illustrated in Figure 3A and Supplementary Video 1, diformazan initially deposits at the base of the cup, preceding phagosome closure (see also Figure 2D). Even at this early stage, heterogeneity is readily apparent. This excludes PtdIns(3)P—which forms only later and is likely important for the maintenance but not the initiation of the response—as the source of the variable respiratory burst. Jointly, these results suggest that none of PtdIns(3,4,5)P3, PA or PtdIns(3)P are responsible for the heterogeneous activation of the NADPH oxidase during FcyR-mediated phagocytosis.
5.4.4 Diacylglycerol heterogeneity correlates with NADPH oxidase activity

Diacylglycerol (DAG) stimulates conventional and novel PKC isoforms (Inanami et al. 1998; Oancea and Meyer 1998), which then phosphorylate and activate the cytosolic components of the NOX complex (Perisic et al. 2004; Nauseef 2004; Brown et al. 2003). We therefore hypothesized that variations in the phagosomal content of DAG, if existing, could account for the observed fluctuations in superoxide production. To test this hypothesis, we monitored DAG dynamics using a genetically encoded biosensor consisting of the C1 domain of PKCδ fused to GFP. Remarkably, this probe was recruited to some, but not all the phagosomes (Figure 4A); heterogeneity was observed not only across cells, but also between phagosomes formed by individual cells. In otherwise untreated macrophages, only 26 ± 2.5% of the phagosomes induced by challenging with IgG-coated zymosan were visibly endowed with DAG. In contrast, overnight activation of macrophages with LPS (100 ng/mL) increased the fraction of DAG-positive phagosomes to 60 ± 2.6% (Figure 4B). Of note, the fraction of phagosomes displaying measurable NADPH oxidase activity (Figure 4C) closely correlated with the fraction of DAG-positive phagosomes, both for untreated and LPS-activated macrophages (Figure 4A, 4B).

While the correlation between DAG formation and NOX activation was striking, it did not necessarily imply a causal relationship. To more directly test whether the variability in DAG accumulation was responsible for driving the heterogeneous nature of the respiratory burst, we sought to alter the DAG content of forming phagosomes. During phagocytosis, DAG is detected only fleetingly (≤ 1 min) on the phagosomal membrane, as it is quickly metabolized (at least in part) by its conversion to PA, a reaction catalyzed by DAG-kinase (DGK) family members. Thus, in principle, inhibition of DGK activity would be expected to enhance the phagosomal accumulation of DAG. This notion was tested by pre-treating RAW macrophages with 30 µM DGKi I for 20 min prior to the initiation of phagocytosis. Exposure to DGKi increased the average number of diacylglycerol-positive phagosomes from 26 ± 2.5% to 43 ± 1.9% in otherwise untreated cells, and further increased the number of positive phagosomes to 82 ± 1.5% in LPS-activated cells (Figure 4B). Notably, the changes in DAG content were paralleled by those in NOX activity (Figure 4C); stabilization of DAG by pharmacological means
enhanced superoxide production and reduced NOX heterogeneity in both control (non-LPS treated) and LPS-activated cells. To verify that the effects of DGKi I were indeed caused by inhibition of DGK, we used two additional inhibitors of this enzyme: R59 949 (DGKi II), and the structurally unrelated dioctanoyl ethylene glycol, a non-phosphorylatable DAG analogue. Both of these compounds decreased the cellular content of PA to an extent similar to that described for DGKi I, as measured biochemically (Figure 5C). More importantly, DGKi II and dioctanoyl ethylene glycol recapitulated the enhanced NOX responsiveness induced by DGKi I in otherwise untreated and in LPS-treated macrophages (Figure 4C). These results suggest that one or more DAG kinase isoforms lessen NOX activation by terminating DAG signalling. That DAG is in fact converted to PA during phagocytosis was documented in RAW cells expressing 2PABD-GFP. In parallel with the accumulation of phagosomal DAG, inhibition of DGK activity caused a marked decrease in the PA content of the phagosomal membrane (Figure 5A, B).

5.4.5 Expression of diacylglycerol kinase isoforms in macrophages

The preceding pharmacological experiments implied that one or more DGK isoforms are present and active on the membranes of macrophages. The endogenous expression of DGKs in RAW cells was verified by RT-PCR. Messages for all isoforms (α through κ) were found to be expressed by RAW cells (Figure 6A). The subcellular distribution of representative members of all 5 DGK families was next analyzed by confocal microscopy. Because specific antibodies to all of these isoforms were not readily available, macrophages were instead transfected with constructs encoding individual GFP-tagged DGKs. The translocation of the chimeric DGKs to sites of phagocytosis was then evaluated by challenging with IgG-opsonized zymosan. The α, γ and ε isoforms were primarily cytosolic, and did not particularly accumulate at sites of phagocytosis. In turn, DGKδ-GFP appeared as punctate structures, which may have been homotypic aggregates caused by heterologous expression. The γ, θ and ζ isoforms could be detected at the plasmalemma, albeit modestly, and their concentration did not increase at sites of phagocytosis. These isoforms were not investigated further. In contrast, DGKβ was exclusively membrane-bound in unstimulated cells. Remarkably, this isoform accumulated at the base of the phagocytic...
cup and in extending pseudopods during the course of particle engulfment (Figure 6B, top row, middle panel; Supplementary Video 5).

5.4.6 Modulation of NOX responsiveness through DGKβ-mediated control of phagosomal DAG signalling

We took advantage of the preferential accumulation of DGKβ in forming phagosomes to test the notion that DAG availability limited the generation of superoxide during phagocytosis. We hypothesized that the forcible (over)expression of DGKβ would effectively deplete phagosomal DAG, and consequently diminish superoxide generation in nascent phagosomes. A representative experiment is illustrated in Figure 7 A-C, where RAW cells were co-transfected with the DAG probe PKCδ(C1)-mCherry and either wild-type or kinase dead [KD(G495D)] DGKβ-GFP. Note that neither wild-type nor kinase-dead DGKβ expression affected phagocytic efficiency. Remarkably, only a small portion of phagosomes accumulated DAG in cells expressing wild-type DGKβ (Figure 7A). Conversely, most of the phagosomes accumulated DAG in cells expressing the kinase-dead DGKβ (Figure 7B), which likely exerted a dominant-negative effect.

That DAG formation was limiting to superoxide generation was assessed by the experiments shown in Figure 7 D-F, wherein diformazan deposition was compared between controls and cells expressing either wild-type or kinase-dead DGKβ. The overexpression of wild-type DGKβ led to a reduction in superoxide production from 23 ± 2.0% to 7.0 ± 1.1% in otherwise untreated cells, and from 58 ± 1.2% to 9 ± 1.2% in LPS-primed cells (Figure 7F). In contrast, expression of kinase-dead DGKβ increased the proportion of NOX-responsive phagosomes to 49 ± 2.4% in otherwise untreated cells and to 71 ± 2.4 % in LPS-primed cells.

Cumulatively, our results indicate that the extent of DAG accumulation is controlled by phagosomal DAG kinases. Moreover, they provide evidence that the levels of phagosomal DAG vary among phagosomes, accounting for the heterogeneity in the oxidative response. Variability in NOX activation could enable the survival of a fraction of invading pathogens within the phagosomal pool.
5.5 Discussion

It is commonly assumed that all phagosomes formed by engaging a given homogeneous target particle have the same composition, trigger identical signals and undergo comparable maturation pathways (Henry et al. 2004). Nonetheless, emerging evidence now suggests that even when generated as a result of the same ligand-receptor combination, phagosomes are heterogeneous. Unappreciated variation in the density or disposition of ligands on the particle’s surface, and/or in the distribution or responsiveness of receptors on the phagocyte membrane can result in the formation of phagosomes with different properties (Zhang, Hoppe, and Swanson 2010). To understand the source and significance of this heterogeneity, it is imperative to analyze the behaviour of single phagosomes, as opposed to the more conventional population assays. Even flow-cytometric data describing the behaviour of individual cells can be insufficient, inasmuch as multiple phagosomes often coexist in a single cell.

These considerations prompted us to develop an assay that would allow us to dynamically study the development of the respiratory burst in individual phagosomes. Pre-existing methods to analyze superoxide generation in phagosomes had limitations. Those that use soluble detectors, such as luminol, C11-BODIPY or cytosolically-trapped fluorogenic probes like dihydrofluorescein are unable to distinguish oxidants generated at the plasmalemma from those formed by phagosomes, cannot detect single phagosomes and, in most cases, the nature of the oxidant detected is unclear, making the measurements liable to extraneous factors such as the presence or peroxidases (Russell et al. 2009). Reagents like OxyBURST, which can be attached covalently to the phagocytic particle, can in principle be used to monitor the behaviour of single phagosomes. However, this approach has several shortcomings: not only is the oxidation of the precursor sensitive to peroxidases, but also the resulting fluorescence is sensitive to pH and highly susceptible to photobleaching (Afzal et al. 2003). More importantly, the amount of the probe bound to the particle is finite, compromising the sensitivity and linearity of the measurements over extended periods, particularly in phagosomes with high rates of oxidant production.
We circumvented these limitations by designing a novel fluorescence-quenching assay that relies on NBT, which is converted to insoluble diformazan at sites of superoxide production. Because NBT readily traverses biological membranes and its availability is not limiting, the deposition of diformazan can in principle proceed linearly as a function of superoxide generation for indefinite periods. Indeed, NBT has been used extensively to monitor superoxide generation in a variety of systems (DiGregorio, Cilento, and Lantz 1987; Goebel and Dinauer 2002; Choi et al. 2006). However, to our knowledge, it had not been applied for the quantitative measurement of NOX activity within single phagosomes, in all likelihood because it is difficult to uncouple the absorbance of diformazan from the light absorbed or scattered by the particle or by the phagocyte itself. We overcame this constraint by using fluorescently labelled particles as phagocytic targets and then measuring quenching of their fluorescence (rather than the absorbance of NBT). Labelling the targets with a bright, photo-resistant and pH-insensitive fluorophore (Alexa Fluor-555) enabled accurate measurements over extended periods. In this manner, we were able to monitor superoxide generation dynamically and unambiguously in single phagosomes. Application of this assay led us to appreciate and quantify the inherent heterogeneity of the respiratory response of phagosomes challenged with a seemingly homogeneous particle.

Variability in the response has been noted previously between cells, as well as between phagosomes of a particular cell. Cell-to-cell heterogeneity in the respiratory burst was described earlier (DiGregorio, Cilento, and Lantz 1987), and phagosome-to-phagosome differences were reported for PLB-985 cells (Li et al. 2009). Yet the source of this variability had hitherto not been defined.

In an effort to establish the source of this heterogeneity, we turned our attention to key bioactive lipids that are critical for mounting the respiratory burst. Phosphoinositides are of particular importance to the activation of the oxidase. The PX domain of p40phox selectively binds to PtdIns(3)P, and this interaction is likely required for sustaining, superoxide production in phagosomes (Suh et al. 2006; Ueyama et al. 2008). In contrast, the PX domain of p47phox binds to both PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (Brown et al. 2003). This domain is unusual in that it contains 2 distinct lipid-binding pockets, allowing it to simultaneously and cooperatively bind to a phosphoinositide and
a second negatively charged, small lipid, such as PA (Yaffe 2002). Thus, it has been postulated that by sensing multiple inputs, p47phox integrates PI3K and PLD signalling pathways (Perisic et al. 2004). We hypothesized that variations in the metabolism of these bioactive lipids could underlie the observed heterogeneity in NOX activity across the phagosomal population. However, while only a subpopulation of the phagosomes produced detectable amounts of superoxide, virtually all of them produced PtdIns(3,4,5)P3, PtdIns(3)P and PA (Figure 3). We cannot rule out that subtle differences in the levels of these lipid mediators contribute to the observed heterogeneity, but our measurements provided no evidence to this effect. While it has been suggested that phagosomes are unevenly endowed with PtdIns(3)P (Henry et al. 2004), such heterogeneity was reported to become apparent 20 min after particle engulfment, long after the NOX complex is initially activated. Indeed, our observations indicate that the phagocytic oxidase is activated—and heterogeneously so—even prior to phagosome sealing (Figures 2D and 3A, and Supplementary Video 1).

In contrast to the consistent behaviour and relatively long-lasting presence of PA and the phosphoinositides, we found the incidence of phagosomal DAG to vary greatly, both across and within single cells. Of note, previous studies have conclusively demonstrated that DAG activates PKC signalling and therefore NOX assembly (Cheng et al. 2007). Together, these observations led us to hypothesize that modulation of DAG production in phagocytes would have reciprocal effects on the heterogeneity in NOX responsiveness. Indeed, interfering with PKC or DAG signalling has profound effects on superoxide production at the cell population level in resident macrophages of the central nervous system (microglia) (Ueyama et al. 2004). The latter observation suggests that heterogeneity in phagosomal DAG signalling is a universal phenomenon for phagocytes, and that DAG metabolism can be finely tuned according to the context of the immunological stimuli.

To elucidate whether DAG was in fact responsible for the heterogeneity in NOX activation, we utilized a combination of pharmacological and molecular biological methods. Given the redundancy in the number of DGKs and the potentially masking effects of knocking down any particular DGK isoform, we utilized pan-DGK inhibitors. Remarkably, impairment of DGK activity not only augmented overall superoxide...
generation, it also reduced the heterogeneity of the response, thus facilitating the activation of phagosomes that would otherwise remain inactive. Similar effects were noted when expressing a kinase-dead DGKβ. Conversely, overexpression of functional DGKβ diminished the content of phagosomal DAG and, more significantly, reduced the fraction of responsive phagosomes. Jointly, these findings indicate that limiting amounts of DAG can account for the variable response of the NADPH oxidase in phagosomes.

Experiments using cells primed with LPS are also consistent with the previous conclusion, as pre-incubation with bacterial endotoxin has been shown to noticeably augment NOX activity (Perry et al. 1992). We complemented these observations by demonstrating that NOX heterogeneity diminishes in parallel, as the fraction of unresponsive phagosomes was greatly reduced in LPS treated macrophages, and diminished even further upon treatment with DGKi (Figures 2 and 4). The elevated NOX responsiveness in LPS-primed cells was accompanied by an increased fraction of DAG-positive phagosomes and a more intense and prolonged recruitment of PKCδ(C1)-GFP, the DAG biosensor. The mechanism behind LPS-priming of NOX could thus be attributed, at least partly, to an increase in the levels of the second messenger DAG.

The phagosomal NADPH oxidase has been reported to remain active for approximately 30 min (Russell et al. 2009), while DAG is detectable on phagosomes only during the first minute or two following particle engagement (Scott et al. 2005). DAG therefore seems to be essential for initiation of the response, but not for its maintenance. The latter role may be served by PtdIns(3)P, which was shown to be involved in the recruitment of p40phox and in supporting superoxide formation in sealed phagosomes (Henry et al. 2004; Suh et al. 2006).

In summary, we have shown that variable DAG production is associated with and can account for the heterogeneous nature of the respiratory burst. Rather than insufficient DAG generation, it appears that rapid conversion to PA by DGK precludes the accumulation of DAG in all phagosomes. This suggests that DGKs operate as regulatory nodes in the signalling pathways leading to NOX activation. In this regard, it is interesting that DGK activity is impaired in neutrophils from patients with localized juvenile periodontitis, which therefore have elevated DAG levels and mount exaggerated oxidative responses (Tyagi et al. 1992). DGKs have also been implicated...
in a variety of immunomodulatory processes, including attenuation of cytokine production in mast cells (White and Zembryki 1989), down-regulation of T-cell receptor signalling, (Zhong et al. 2003) and the induction of anergic or tolerogenic profiles in lymphocytes (Zha et al. 2006; Zhong et al. 2008; Wattenberg and Raben 2007). Our observations indicate that DGKs play an analogous immunoregulatory role in macrophages, this time by executing the critical function of balancing effective microbicidal responses with the prevention of self-harm due to excessive ROS formation.

5.6 Figure legends

**Figure 1. Detection of superoxide production within individual phagosomes.** (A) Schematic of the experimental protocol. Zymosan particles labelled with Alexa 555-SE (i) and opsonized with human IgG (ii) were used as phagocytic targets for RAW macrophages in the presence of the membrane permeable dye NBT (iii). Uptake of opsonized, fluorescent zymosan (IgG-zymo-Alexa 555) and the ensuing activation of NOX2 result in the generation of superoxide anions, which in turn reduce NBT to diformazan crystals in the phagosomal lumen. Diformazan precipitates in the form of blue/black deposits (iv) that effectively quench the IgG-zymo-Alexa 555 fluorescence. (B) Representative differential interference contrast (left) and corresponding epifluorescence (right) images of RAW macrophages fixed 25 min after exposure to IgG-opsonized zymo-Alexa 555. Where indicated, cells were incubated with NBT prior to and throughout phagocytosis. Double arrowheads (>>) point to non-internalized particles, while diformazan-negative and -positive phagosomes are indicated by dashed (---) or solid arrows (→), respectively. Scale bar, 5 µm. (C) Quantification of fluorescence intensity as a function of time during phagocytosis. Open circles and closed diamonds correspond to external and internalized IgG-zymo-Alexa 555, respectively. Images in (B) and results in (C) are representative of at least 6 and 2 individual experiments of each kind, respectively.
Figure 2. Superoxide production during FcγR-mediated phagocytosis is heterogeneous. (A) Representative DIC (left), corresponding epifluorescence (middle) and merged (right) images of RAW macrophages fixed 25 min after exposure to IgG-zymo-Alexa 555 in the presence of NBT. The top two panels show differential diformazan deposition within phagosomes of single cells and between those of different cells. LPS-primed cells display a higher fraction of NOX2-responsive phagosomes and therefore reduced phagosomal heterogeneity, while exposure to the NOX inhibitor, diphenyleneiodonium (DPI, 10 µM), abrogates diformazan deposition and fluorescence quenching (bottom panel). The notation for arrows and arrowheads is as in Figure 1. Images are representative of at least 5 independent experiments. (B) Quantification of the relative abundance of superoxide-producing cells (left axis, black bars) and phagosomes (right axis, white bars), before and after LPS activation or in the presence of DPI, using diformazan deposition and fluorescence quenching as a proxy of NOX2 activation. Macrophages with at least 1 phagosome with diformazan deposits were classified as positive cells. Data are averages ± SEM of at least 5 individual experiments; a minimum of 80 phagosomes was counted per experiment. (C) Flow cytometry histograms showing homogeneity in Alexa 555 labelling (left, red trace) and IgG opsonization (right, green trace) of zymosan particles used as phagocytic targets. (D) Representative DIC (left), corresponding epifluorescence (middle) and merged (right) images showing differential superoxide production in two macrophages at an early stage of phagocytosis, preceding phagosomal sealing. Incompletely internalized IgG-zymo-Alexa 555 particles were labelled with a DyLight488-conjugated antibody after fixing. Diformazan-positive and -negative phagosomes that are labelled by secondary antibody—and therefore unsealed and with equal access to extracellular NBT—are indicated with a solid and a dashed arrow, respectively. Scale bars in (A) and (D), 5 µm.

Figure 3. The phagosomal population is uniformly endowed with PtdIns(3,4,5)P₃, PA and PtdIns(3)P. (A) Representative DIC images of live RAW macrophages during phagocytosis of IgG-opsonized zymosan in the presence of NBT. The phagocytosis time course has been broken down to 3 stages: pseudopod extension (minus 30 sec,
left), phagosome sealing (0-to-30 sec, middle), and phagosome maturation (plus 25 min, right).  

(B) Spinning-disk confocal fluorescence images of RAW macrophages challenged with IgG-opsonized zymosan particles labelled with Cy5-conjugated secondary antibody (blue). Cells were transfected with constructs encoding biosensors for PtdIns(3,4,5)P$_3$ [Akt(PH)-GFP, top], PA (2PABD-GFP, middle) and PtdIns(3)P (2FYVE-GFP, bottom). Recruitment of the probes to the phagosomal membrane was monitored during pseudopod extension (minus 30 sec), phagosome sealing (0-to-30 sec) and early stages of maturation (plus 150 sec). Asterisks indicate phagosomes. Note that the -30 sec image for PA was underexposed compared to the other times to avoid saturation of fluorescence at the phagocytic cup. Scale bars in A and B, 5 µm.  

(C) Quantification of the fraction of phagosomes displaying diformazan deposits and accumulation of PtdIns(3,4,5)P$_3$, PA or PtdIns(3)P. Data indicate the average fraction of positive phagosomes per cell for each condition at the following time points: Diformazan (20 min after exposure to zymosan); PtdIns(3,4,5)P$_3$ (0-to-30 sec after phagosome sealing); PA (30 sec before phagosome sealing); PtdIns(3)P (150 sec after sealing). Data are representative of at least 5 independent experiments; a minimum of 100 phagosomes was counted per experiment.

Figure 4. **Diacylglycerol is heterogeneously distributed across the phagosomal population in a manner that mirrors NADPH activity.** (A) Representative spinning-disk confocal fluorescence images of RAW cells transfected with a construct encoding a diacylglycerol biosensor and challenged with IgG-opsonized zymosan particles. Where indicated, cells were incubated with the DGK inhibitor R59 022 (DGKi I; 30 µM) for 20 min prior to phagocytosis and/or primed overnight with LPS (0.1 µg/mL). DAG-positive and -negative phagosomes are indicated with asterisks and stars, respectively. Scale bars, 5 µm.  

(B) Quantification of the relative abundance of DAG- and (C) diformazan-positive phagosomes after DAG kinase inhibition, LPS priming, or both. DAG kinases where inhibited with R59 022, R59 949 or diocanoyl ethylene glycol. Data are averages ± SEM of positive phagosomes per cell for each condition, 5 min after exposure to zymosan. Data are representative of at least five independent experiments; a minimum of 100 phagosomes was counted per experiment.
**Figure 5. The PA content of macrophages is controlled by DGKs. (A, B)**

Representative spinning-disk confocal fluorescence sections (left panels) and multi-channel Z projections (right panel) of RAW cells co-transfected with 2PABD-GFP (a PA probe) and PKCδ(C1)-mCherry (a DAG probe) during phagocytosis of IgG-opsonized zymosan particles labelled with a Cy5-conjugated secondary antibody (shown in blue). Under control conditions (A), the PA probe localizes primarily to the plasmalemma and accumulates acutely at the phagosomal membrane during phagosome sealing; the DAG probe remains primarily restricted to juxtanuclear compartments. Conversely, treatment with 30 µM DGKi I (B) induces dissociation of the PA probe from the plasma and phagosomal membranes, as well as the concomitant recruitment of the DAG probe to these compartments. Scale bars, 5 µm. (C) Quantification of total PA from cell lysates, as determined using the enzymatic assay described under “Methods”. Lysates were prepared from RAW macrophages that were either left untreated (control) or pre-incubated for 15 min with 30 µM of DGK inhibitors I or II (R59 022 or R59 949, respectively) or 100 µM of dioctanoyl ethylene glycol. Data are means ± SEM of the total cellular PA content (in nmoles per mg of protein). Three independent experiments were performed per condition.

**Figure 6. Transcription and subcellular distribution of DGK isoforms. (A)**

Endogenous transcript expression of distinct DGK isoforms (α through κ). Total mRNA was purified from RAW cells and DGK isoform expression was then detected by RT-PCR using transcript-specific primers encompassing exon-exon junctions. All amplicon sizes were as predicted by primer design. (B) Subcellular distribution of the indicated GFP-tagged DAG kinase isoforms—representing the five DGK families—during phagocytosis of IgG-opsonized zymosan (labelled with a Cy5-conjugated secondary antibody, shown in blue). Scale bars, 5 µm. Images in B are representative of at least 5 independent experiments.

**Figure 7. Modulation of NOX activity through DGKβ-mediated control of DAG. (A, B)**

Representative confocal sections (left panels) and multi-channel Z projection (right panels) of RAW cells co-transfected with PKCδ(C1)-mCherry (DAG probe) and either (A) wild-type or (B) kinase-dead (KD[G495D]) DGKβ-GFP during phagocytosis of IgG-opsonized zymosan particles (labelled with a Cy5-conjugated secondary antibody,
shown in blue). Asterisks indicate phagosomes. Scale bars, 10 µm. (C) Quantification of the portion of phagosomes endowed with DAG in RAW cells expressing either wild-type or kinase-dead DGKβ relative to control cells transfected with the plasma membrane marker PM-GFP. Data are averages ± SEM of positive phagosomes per cell for each condition, 3 min after exposure to zymosan. At least 3 independent experiments were performed and 80 phagosomes counted for each condition. (D, E) Representative epifluorescence and DIC (left panels) and merged (right panels) images of RAW cells transfected with either (D) wild-type or (E) kinase-dead DGKβ-GFP during phagocytosis of IgG-opsonized zymosan labelled with Alexa 555-SE (shown in red). RAW cells were fixed and imaged 25 min after exposure to NBT and IgG-opsonized zymo-Alexa 555. (F) Quantification of data from experiments shown in D and E. Data are averages ± SEM of diformazan-positive phagosomes per cell for each condition, 30 min after exposure to zymosan. At least three independent experiments were performed and 60 phagosomes counted for each condition.

Figure S1. Neither differential accumulation of NBT in individual phagosomes nor time-dependency of NOX can account for the observed phagosomal heterogeneity. (A) Schematic of the experimental protocol designed to determine whether NBT accessibility to phagosomal compartments is variable. RAW macrophages were challenged at 0 min with human IgG-opsonized zymosan particles labelled with Alexa 555-SE (IgG-Zymo-Alexa 555) and phagocytosis was allowed to continue for 7 min, at which point NBT was included in the reaction. A secondary 488 anti-human IgG was also added at this 7 min to mark phagocytic targets that had not been internalized at the time of first exposure of macrophages to NBT. Phagocytosis proceeded for an additional 23 min after addition of NBT, and cells were imaged fixed at 30 min. (B) Representative epifluorescence (left, middle) and DIC (right) images of the experiment described in (A). Phagocytic targets that fluoresce both in the red and green channels (indicated with an asterisk) had not yet been internalized at the time of NBT addition, while targets that emit only in the red had been engulfed prior to NBT addition. The right panel shows diformazan-positive (solid arrow) and -negative (arrowhead) targets that resided within sealed phagosomes in the same cell when the quenching dye was
added. (C) Schematic of the experimental protocol designed to determine whether time-
dependency of NOX activation could account for the observed heterogeneity. Both NBT
and IgG-Zymo-Alexa 555 were added to macrophages at 0 min, and phagocytosis
allowed to proceed for only 3 min before addition of a secondary 488 anti-human IgG to
mark the 3 min time point. Phagocytosis then continued for an additional 27 min and
cells were imaged fixed at 30 min. (D) Representative epifluorescence (left, middle) and
DIC (right) images of the experiment described in (C). Phagocytic targets that fluoresce
in the red, but not in the green, were internalized within the first 3 min window (out of a
total 30 min). The right panel shows diformazan-positive and -negative phagosomes
that were internalized within this short 3-min window and therefore had essentially
equivalent times for maturation and NOX activation before fixation. The notation for
asterisks, arrows and arrowheads is as in (A). Scale bars, 10µm.

Figure S2. The 2PABD-GFP probe is a specific biosensor of phosphatidic acid.
(A) HeLa cells were co-transfected with constructs encoding 2PABD-GFP and either the
surface charge probe RFP-R-pre (all top panels), PLD1-HA (bottom left panel), PLD2-
HA (bottom middle panel) or dominant negative PLD2-HA (bottom right panel). Cells
were then exposed to the indicated treatments, fixed and imaged by spinning-disk
confocal microscopy. In HeLa cells, the PA probe is mostly soluble under control
conditions but partitions to the plasmalemma upon addition of exogenous PA (top
middle panel) or upon activation of PLC by calcium (top right panel), which promotes
the formation of DAG, a PA precursor. Expression of wild-type PLD2 (plasma
membrane associated), but not of dominant negative PLD2 nor PLD1 (punctate,
vesicular distribution) results in the relocation of the PA probe to the plasmalemma.
Insets show the corresponding confocal sections of the charge probe, RFP-R-pre (top)
or of the distinct PLD proteins (bottom). Images are representative of at least 3
independent experiments per condition. (B) Confocal sections of RAW cells co-
transfected with the PA and DAG biosensors [2PABD-GFP and PKCδ(C1)-mCherry,
respectively] and imaged live by spinning-disk confocal microscopy immediately before
or 5 min after treatment with the DAG kinase inhibitor, R59 022 (DGKi I). DGK inhibition
led to the displacement of the PA probe and the concomitant recruitment of the DAG
probe from and to the plasma membrane. Scale bars, 10µm.
5.7 Figures

Figure 1. Detection of superoxide production within individual phagosomes

**A**
- **Labelling**: Alexa 555-SE
- **Opsonization**: IgG
- **Phagocytosis**: NBT
- **Fluorescence quenching**: Diformazan precipitate

**B**
- **DIC**
- **IgG-Zymo-Alexa 555**

**C**
- **Normalized fluorescence intensity**
- **Time (min)**
- **External**
- **Internal**

Control + NBT
Figure 2. Superoxide production during FcγR-mediated phagocytosis is heterogeneous

A

DIC IgG-Zymo-Alexa 555 Merged

NBT

NBT

NBT + LPS

NBT + LPS + DPI

B

% diformazan-positive cells

** = p < 0.005

*** = p < 0.0005

NBT

NBT + LPS

NBT + LPS + DPI

C

Particle count (% of max)

Alexa 555-SE

Alexa 555

IgG

Unlabelled

D

DIC

α-huIgG-488

α-huIgG + IgG-zymo-A555

IgG

Unopsonized
Figure 3. The phagosomal population is uniformly endowed with PtdIns(3,4,5)P₃, PA and PtdIns(3)P
Figure 4. Diacylglycerol is heterogeneously distributed across the phagosomal population in a manner that mirrors NADPH activity.

A

Control PKCδ(C1)-GFP

B

% DAG-positive phagosomes

C

% Diformazan-positive phagosomes
Figure 5. The PA content of macrophages is controlled by DGKs.

A) 2PABD + PKCε(C1) + Cy5-IgG-zymo

B) 2PABD + PKCε(C1) + Cy5-IgG-zymo

C) Total PtdOH content (nmol PtdOH/mg protein)

Control  | DGK I (R59 022) | Dioctanoyl ethylene glycol | DGK II (R59 949)

* = p < 0.05
** = p < 0.005
Figure 6. Transcription and subcellular distribution of DGK isoforms

A

500 bp

B

Type I

Type II

Type III

Type IV

Type V

DGK-GFP
Figure 7. Modulation of NOX activity through DGKβ-mediated control of DAG

A

wtDGKβ-GFP

DAG-positive phagosomes

B

KD(G495D)DGKβ

DAG-positive phagosomes

C

% DAG-positive phagosomes

Control (PM-GFP)  wtDGKβ  KD(G495D)DGKβ

* = p < 0.01
** = p < 0.005

D

wtDGKβ-GFP

DAG-positive phagosomes

E

KD(G495D)DGKβ

DAG-positive phagosomes

F

% 4,5-dimethoxy-2-nitrobenzyl-positive phagosomes

Control  wtDGKβ  KD(G495D)DGKβ  LPS-activated  LPS + wtDGKβ  LPS + KD(G495D)DGKβ

* = p < 0.01
** = p < 0.005
Figure S1. Neither differential accumulation of NBT in individual phagosomes nor time-dependency of NOX can account for the observed phagosomal heterogeneity.
Figure S2. The 2PABD-GFP probe is a specific biosensor of phosphatidic acid

A

2PABD-GFP

xz

HeLa

Control

+100 μM PA (10 min)

+1 μM ionomycin (2 min)

+ PLD1

+ PLD2wt

+ PLD2mut(K758R)

B

RAW264.7

2PABD-GFP

PKCδ(1)-mCherry

+30 μM DGKi I

+30 μM DGKi I

0 min

5 min
Chapter 6

This chapter has been modified from the following: Daniel Schlam, Johnathan Canton, Marvin Carreño, Hannah Kopinksi, Spencer A Freeman, Sergio Grinstein, and Gregory D Fairn. “Gliotoxin Suppresses Macrophage Immune Function by Subverting Phosphatidylinositol 3,4,5-Trisphosphate Homeostasis.” *mBio*, March 2016.

6 Virulence: Gliotoxin suppresses macrophage immune function by subverting PtdIns(3,4,5)P₃ homeostasis

6.1 Abstract

*Aspergillus fumigatus*, an opportunistic fungal pathogen, spreads in the environment by releasing numerous conidia that are capable of reaching the small alveolar airways of mammalian hosts. In otherwise healthy individuals, lung-resident (alveolar) macrophages are responsible for rapidly phagocytosing and eliminating these conidia, effectively curbing their germination and consequent invasion of pulmonary tissue. However, under circumstances that are not fully understood, the fungus evades phagocyte-mediated immunity and persists in the respiratory tree. Here, we report that *A. fumigatus* escapes macrophage recognition by strategically targeting phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P₃] metabolism through gliotoxin, a potent immunosuppressive mycotoxin. Time-lapse microscopy revealed that, in response to the toxin, macrophages cease to ruffle, undergo abrupt membrane retraction and their phagocytic ability is impaired; similar effects were noted in immortalized and primary phagocytes. Gliotoxin was found to prevent integrin activation and interfere with actin dynamics, both of which are instrumental for phagocytosis. Detailed studies of the underlying molecular mechanism of toxicity revealed that inhibition of phagocytosis is attributable to impaired accumulation of PtdIns(3,4,5)P₃ and the associated inactivation of downstream effectors, including Rac and/or Cdc42. Strikingly, in response to the diacylglycerol mimetic phorbol 12-myristate 13-acetate,
gliotoxin-treated macrophages reactivate beta integrins, reestablish actin dynamics and regain phagocytic capacity, despite the overt absence of plasmalemmal PtdIns(3,4,5)P\(_3\). Together, our findings identify phosphoinositide metabolism as a target of gliotoxin, and also indicate that increased diacylglycerol levels can bypass the requirement for PtdIns(3,4,5)P\(_3\) signalling during membrane ruffling and phagocytosis.

6.2 Introduction

Of the approximately 200 aspergilli species identified to date, *Aspergillus fumigatus* is the one that most prevalently causes disease in humans (Dagenais and Keller 2009). Infection with this opportunistic mold is also the primary etiology of invasive aspergillosis (IA), a devastating disease that carries an associated mortality rate of almost 90% in high-risk populations (Lin, Schranz, and Teutsch 2001). While immunosuppressed individuals—including patients receiving a bone marrow transplant or undergoing chemotherapy—are most susceptible to acquiring and succumbing to *A. fumigatus* infections (Denning 1998), this pathogen is also frequently isolated from the sputum of individuals with chronic respiratory diseases, such as cystic fibrosis (Hauser et al. 2011; Bakare et al. 2003).

Similar to other saprophytic fungi, *A. fumigatus* is virtually omnipresent in the environment as a natural occupant of soil and plants (Dagenais and Keller 2009). This ubiquitous pathogen spreads by releasing copious quantities of airborne conidia, non-motile dormant spores that infiltrate the small alveolar space. As a necessary complement to the mucociliary apparatus, lung-resident (alveolar) macrophages are in charge of recognizing, internalizing and rapidly disposing of the hundreds of *Aspergillus* conidia inhaled by the average person every day (Tashiro et al. 2011; Hospenthal, Kwon-Chung, and Bennett 1998), thereby preventing their germination and formation of pulmonary hyphal networks (A. Schaffner et al. 1983; Merkow et al. 1971; Andreas Schaffner, Douglas, and Braude 1982). Underlying immunodeficiencies and/or impaired mucociliary clearance render the macrophage system incapable of effectively contending with *Aspergillus* spores, thus markedly increasing the susceptibility to mycelial colonization and to the consequent destruction of pulmonary tissue.
The establishment of hyphal networks by *A. fumigatus* is accompanied by the secretion of several immunosuppressive mycotoxins (Gardiner, Waring, and Howlett 2005). The most abundant of these is gliotoxin (Stanzani et al. 2005), a secondary metabolite with a wide range of immunomodulating capabilities (Yamada, Kataoka, and Nagai 2000; Tsunawaki et al. 2004; Sutton et al. 1994; Kupfahl, Geginat, and Hof 2006; Pahl et al. 1996; Philip Sutton, Waring, and Müllbacher 1996) and associated with the development of IA (Gardiner, Waring, and Howlett 2005). Like other toxins of the epipolythiodioxopiperazine class, gliotoxin carries an internal disulfide bridge that is essential for its virulence activity (Müllbacher et al. 1986).

Here, we tested the hypothesis that gliotoxin interferes with some of the dedicated mechanisms employed by macrophages for recognizing, taking up and destroying foreign and dangerous particulates, such as *Aspergillus* conidia. We find that gliotoxin markedly interferes with PtdIns(3,4,5)P3 homeostasis, thereby precluding the extension actin-driven membrane protrusions that macrophages utilize to survey their environment. PtdIns(3,4,5)P3 dysregulation results in overt integrin and actin cytoskeletal defects, profoundly affecting the ability of macrophages to remain adherent to the substratum and to phagocytose prey. Interestingly, exposing gliotoxin-treated cells to the diacylglycerol analogue PMA reverses these cytoskeletal and integrin dependent abnormalities. Overall, our observations pinpoint PtdIns(3,4,5)P3 as a novel and critical target of gliotoxin in phagocytes, and suggest that diacylglycerol signalling is paramount for orchestrating cellular processes involving actin remodelling and integrin activation, including cell spreading and phagocytosis.

### 6.3 Methods

#### 6.3.1 Plasmids

PAK(PBD)-YFP, the p21-binding domain (PBD) of p21-activated kinase (PAK) conjugated to YFP (Srinivasan et al. 2003b), was used to monitor the subcellular distribution of active (GTP-bound) Rac and Cdc42. The presence of F-actin was visualized in living cells using a construct encoding Lifeact-mRFP. The latter is an
mRFP-conjugated 17-amino acid peptide derived from the *Saccharomyces cerevisiae* actin-binding protein Abp140 (Riedl et al. 2008). In turn, the distributions of PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$ were followed by constructs encoding GFP chimeras of the PH domains of PLCδ (Botelho et al. 2000) or Akt (Marshall et al. 2001), respectively.

6.3.2 Antibodies

Erythrocyte opsonization was carried out with a rabbit anti-sheep red blood cell antibody (Cedarlane laboratories). Cy5- and Cy3-conjugated antibodies against rabbit IgG (Jackson ImmunoResearch) were employed to visualize the erythrocyte targets. The levels of cell-surface phagocytic receptors were detected by immunofluorescence and flow cytometry via an FcγRII-specific rat monoclonal antibody (R&D Systems, catalogue #MAB20741) or with a rat antibody that recognizes an epitope shared by both FcγRIIb and FcγRIII (eBioscience, catalogue #14-0161). An isotype-matched rat IgG$_{2A}$ antibody (catalogue #MAB006) was obtained from R&D Systems. Anti-FcγR antibodies and the associated isotype control were all employed at a final concentration of 10 µg/ml. As a secondary antibody, we used anti-rat IgG conjugated to Alexa Fluor 488 (Cell Signaling, catalogue #4416) at a 1:500 dilution. Sites of integrin β$_2$ activation were visualized by immunofluorescence with a mouse monoclonal antibody against high-affinity human CD18 (Abcam, clone #MEM-148). As an indication of PI3K activity, we measured phosphorylation of AKT at serine 473 by immunoblotting, using a rabbit polyclonal antibody (Cell Signaling, catalogue #9271S). Likewise, we used a rabbit polyclonal antibody for detecting VASP phosphorylation at serine 157 (Cell Signalling, catalogue #3111S) as an indication of PKA activity. An anti-GAPDH mouse monoclonal antibody (EMD Millipore, catalogue #MAB374) was utilized as a loading control. Refer to the Western blotting section below for details regarding the antibody concentrations and their respective incubation times.
6.3.3 Cell culture of immortalized and primary macrophages

The RAW 264.7 murine macrophage line was obtained from the American Type Culture Collection (ATCC) and cultured at 37°C under 5% CO₂ in RPMI supplemented with 5% heat-inactivated fetal bovine serum (Wisent). For preparation of primary human macrophages, peripheral blood was obtained from healthy donors and diluted 1:1 in PBS. The diluted blood was then resuspended in lympholyte-H (Cedarlane), and peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation. PBMCs were then allowed to settle onto 1.8-cm glass coverslips at an approximate density of 3x10⁶ cells per coverslip. The cells were then cultured for 6 days (37°C, 5% CO₂) in RPMI supplemented with 10% heat-inactivated fetal bovine serum (Wisent) and penicillin-streptomycin (Multicell). To promote monocyte differentiation, macrophage colony-stimulating factor (25 ng/mL) was added to the medium at the onset of culture and every other day throughout the 6 days of culture. Non-adherent cells were washed off the macrophage monolayer at the conclusion of the differentiation period.

6.3.4 Transfection and electroporation

For transient transfections of RAW 264.7 macrophages, almost confluent monolayers were lifted by gentle scraping and plated onto 1.8-cm coverslips (1x10⁵ cells per coverslip). Approximately 18 hours after plating, cells were transfected with FuGENE HD (Promega) according to the manufacturer’s protocol. In short, 6 µl of the FuGENE HD transfection reagent and 3 µg of plasmid DNA were mixed in 100 µl of serum-free RPMI and allowed to sit for 30 min. The transfection mix was then supplemented with 500 µl of RPMI containing 10% heat-inactivated fetal bovine serum, and distributed in equal amounts to 4 wells of a 12-well plate.

In the case of human monocyte-derived macrophages, transient expression of fluorescent proteins was attained by electroporation via the Neon Transfection System (Life Technologies). Following 5-6 days of differentiation in culture, primary macrophages were lifted by exposure to Accutase (Innovative Cell Technologies) and gentle scraping. Next, 5x10⁵ macrophages were centrifuged at 300xg for 5 min before being resuspended in 100 µl of the supplied buffer R. The cell suspension was then
added to 15 µg of plasmid DNA and subjected to electroporation by 2 sequential 30-millisecond pulses of 1,100 volts each. To maximize survival, macrophages were transferred to RPMI supplemented with 10% heat-inactivated fetal bovine serum immediately after being electroporated.

6.3.5 Gliotoxin treatment and other pharmacology

Gliotoxin (Sigma-Aldrich) was diluted in DMSO and utilized at a final concentration of 500 ng/mL (1.53 µM) in all instances, except for experiments in which the toxin was serially diluted. Gliotoxin was added while cells were in serum-free media, and allowed to act for 30 min before further experimental manipulations were carried out. In the cases when gliotoxin challenge was followed by the addition of a second compound (e.g., PMA), gliotoxin was not removed from the medium. PMA and wortmannin were both used at 100 nM for the indicated time periods. The adenylate cyclase activator forskolin (Sigma-Aldrich) and the competitive inhibitor of cAMP signalling cAMPS-Rp (Tocris Bioscience) were utilized at final concentrations of 100 and 200 µM, respectively. Where indicated, cells were pre-treated with cAMPS-Rp before being exposed to either gliotoxin or forskolin.

6.3.6 Phagocytosis

FcγR-mediated phagocytosis was studied using IgG-coated erythrocytes (MP biomedicals, 10% suspension) as targets for RAW 264.7 macrophages. Opsonization was carried out by mixing 200 µl of the erythrocyte suspension with 5 µl of a rabbit anti-sheep erythrocyte IgG at room temperature for 1 hour under constant agitation. Unbound IgG was removed by washing 3 times with PBS. Opsonized erythrocytes were subsequently labelled with a Cy3-conjugated antibody against rabbit IgG, and excess antibody was removed by washing twice with PBS before resuspending in 200 µl of PBS. To initiate phagocytosis, 10 µl of the IgG-coated erythrocytes were added to 2x10^5 RAW 264.7 cells and centrifuged at 300xg for 30 sec. Phagocytosis was allowed to proceed for 30 min before fixing with 4% PFA. Extracellular targets were identified by
incubating the fixed (non-permeabilized cells) with a Cy5-conjugated anti-rabbit antibody.

Alternatively, phagocytosis of fungal particles by primary macrophages was analyzed using Zymosan A Bioparticles (Molecular probes) as phagocytic targets. Lyophilized zymosan was suspended in PBS (20 mg/mL), boiled for 1 hour and subjected to 3 cycles of vortexing and sonication. The solution was then diluted to 2 mg/mL in PBS at pH 9 and further homogenized by passing through a syringe 10 times. The particles were then simultaneously labelled with succinimidyl esters of Alexa Fluor 555 (Invitrogen) and of biotin (Thermo Fisher Scientific), according to the manufacturer’s instructions. Excess dye and biotin were removed by centrifuging and washing twice with PBS. Labelled zymosan was left unopsonized or coated with human serum. For the latter, 500 µl of the 2 mg/mL zymosan suspension was diluted 1:1 with human serum and incubated for 1 hour with constant agitation at 37°C. The primary macrophages were challenged with 1 mg/mL unopsonized or serum-opsonized zymosan (5 µl per coverslip), and phagocytosis was terminated after 30 min by bathing the cells in HBSS at 15°C. Extracellular targets were identified by exposing the macrophages to an Alexa Fluor 647 conjugate of streptavidin for 15 min at 15°C, which labelled the biotin tag on zymosan. Lastly, macrophages were fixed for 15 in 4% PFA, permeabilized with 0.1% Triton X-100 for 5 min, and their F-actin skeleton stained with Alexa Fluor 488-conjugated phalloidin for 30 min.

6.3.7 IgG-binding assays

FcγR affinity was assessed by evaluating the ability of RAW 264.7 macrophages to bind fluorescent IgG aggregates. To form the aggregates, a 10 mg/mL solution of human IgG and Cy3-conjugated donkey IgG was agitated for 30 min at 62°C in PBS with Ca²⁺ and Mg²⁺. Insoluble aggregates were removed by spinning down at 1,600xg for 10 min. The supernatant was added to macrophages grown on 1.8-cm coverslips (20 µl of supernatant/mL of culture medium), and binding was allowed to proceed for 15 min at 10°C to prevent IgG internalization. Cells were then washed twice with PBS, fixed in 4%
PFA for 20 min and imaged by confocal microscopy. Integrated fluorescence was used as an indication of binding affinity.

6.3.8 Microscopy and image analysis

Fluorescence imaging was performed by spinning-disk confocal microscopy (Quorum Technologies). Our confocal systems are based on an Axiovert 200M microscope (Carl Zeiss) and carry a 63x oil-immersion objective with a 1.4 numerical aperture and a 1.5x magnifying lens. These microscopes are equipped with a motorized XY stage (Applied Scientific Instrumentation), a Piezo Z-focus drive and diode-pumped solid-state lasers emitting at 440, 491, 561, 638 and 655 nm (Spectral Applied Research). Images were recorded with back-thinned, cooled charge-coupled device cameras (Hamamatsu Photonics) operating under control of the Volocity software (Perkin Elmer, version 6.2.1). For time-lapse imaging by differential interference contrast, coverslips were loaded onto Leiden chambers, mounted on the stage of a DM IRB Leica microscope and maintained at 37°C throughout image acquisition. Cells were illuminated via an XFO X-Cite 120 lamp (XFO Life Sciences Group) and the signal was captured by a cooled charge-coupled device camera (Cascade II; Photometrics) driven by Volocity (Perkin Elmer, version 6.2.1). In preparation for scanning electron microscopy, primary macrophages were fixed in a 2% glutaraldehyde solution buffered with sodium cacodylate. The cells were dehydrated through a graded ethanol series, and dried in a Bal-tec CPD030 critical-point dryer. Lastly, the samples were mounted on aluminum stubs and coated with gold using a Denton Desk II sputter coater. Images were acquired with an FEI XL30 scanning electron microscope. ImageJ (National Institutes of Health, software version 1.48) was used for quantifying fluorescence intensity and correcting brightness and contrast. Adjustments were made homogenously across the entire image and the linearity of mapped pixel values was not altered.
6.3.9 Flow cytometry and immunofluorescence

RAW 264.7 macrophages were treated with gliotoxin or vehicle (DMSO) alone in serum-free RPMI for 30 min. The cells were then lifted from 6-well dishes by gentle scraping, and resuspended in 15°C PBS with BSA (5%) at a density of 1x10⁷ cells/ml. Next, the cells were incubated with primary antibodies against FcγR or with an isotype-matched rat IgG₂A for 1 hour on ice. Anti-FcγR antibodies and the associated IgG isotype control were employed at a final concentration of 10 µg/ml. Excess primary antibody was removed by centrifuging and washing twice with cold PBS. Cells were then incubated with an Alexa Fluor 488-conjugated anti-rat IgG (1:500 final dilution) for 30 min at 15°C, washed with cold PBS, and fixed in 4% PFA. Lastly, labelled cells were passed through a cell strainer and ran in the flow cytometer (LSRII, BD Biosciences). Analysis was performed in FlowJo (Tree Star, Inc.). For immunofluorescence assays, approximately 1x10⁵ RAW 264.7 macrophages were plated on 1.8-cm coverslips and cultured overnight. The cells were then switched to serum-free RPMI for treatment with gliotoxin. Following 30 min of gliotoxin incubation, the cells were fixed in 4% PFA, blocked in PBS supplemented with BSA (5%), and incubated with primary and secondary antibodies used at the same concentrations as those used for flow cytometry.

6.3.10 Western blotting

For each condition, RAW 264.7 macrophages were plated on 2 wells of a 6-well plate (2.5x10⁵ cells/well) and cultured overnight. Cells were then exposed to gliotoxin or to the indicated pharmacological compounds, washed with cold PBS and lifted by scraping in cold RIPA buffer (Sigma-Aldrich) supplemented with protease- and phosphatase-inhibitor tablets (Roche). Nuclei and cell debris were pelleted by centrifugation. A total of 30 µg of protein from the supernatant were loaded into each lane of a 12% SDS-PAGE and separated by electrophoresis. The proteins were then transferred to polyvinylidene fluoride membranes and blocked for 30 min with 5% bovine serum albumin in TBS supplemented with Tween 20 (0.1%). This solution was also used to dilute the antibodies against GAPDH as well as against phospho-AKT (serine 473) and phospho-VASP (serine 157). The anti-GAPDH antibody was employed at a 1:1,000 dilution, while
those against phospho-AKT and phospho-VASP were diluted 10,000 times. In all instances, the membranes were incubated with primary antibodies for 1.5 hours and washed with TBS-Tween before being exposed to secondary antibodies (diluted 10,000 times) for 45 min. An HRP-conjugated goat anti-rabbit secondary antibody was used to detect phospho-AKT and phospho-VASP, while GAPDH was detected with an IRDye 800CW-conjugated donkey anti-mouse secondary antibody (LI-COR, Inc.), also diluted 10,000 times. Membranes were visualized digitally on the Odyssey Fc system (LI-COR, Inc.) after being treated with the ECL Western Blotting Substrate (GE Life Sciences). The intensity of independent bands was quantified using the LI-COR Image Studio software and normalized to that of GAPDH. Brightness and contrasts were adjusted evenly across the entire image.

6.3.11 Statistical analysis

Data values are presented as means of 3 independent replicates, and error bars represent the standard error of the mean (SEM). For microscopy-based determinations, sample sizes were of at least 20 cells and 100 phagosomes. The significance of differences was assessed by unpaired Student's $t$-tests with a 95% confidence interval in GraphPad Prism 5c (GraphPad Software, Inc.).

6.4 Results

6.4.1 Gliotoxin inhibits diverse phagocytic modalities in macrophages

To characterize the effect of gliotoxin exposure on macrophage phagocytic capacity, we first treated murine RAW 264.7 macrophages with vehicle (dimethyl sulfoxide; DMSO) or gliotoxin (500 ng/ml; 1.53 µM) for 30 min. Immediately thereafter, IgG-opsonized red blood cells (IgG-RBC) that had been prelabelled with a Cy3-conjugated secondary antibody were sedimented onto the treated macrophages to initiate FcγR-mediated phagocytosis, which was allowed to proceed for 10 min before fixation. Non-phagocytosed IgG-RBC were then identified by incubating the fixed, non-permeabilized
cells with an additional Cy5-conjugated anti-IgG antibody. Thus, this phagocytic assay allowed us to distinguish non-internalized (both Cy5- and Cy3-positive), from internalized targets (Cy3-positive only). Macrophages were subsequently permeabilized with Triton X-100 and stained with Alexa Fluor 488-conjugated phalloidin to visualize actin filaments. As shown in Fig. 1A,B, gliotoxin-treated macrophages exhibited a steep decline in phagocytic ability compared to resting controls; not only did the toxin preclude binding to phagocytic targets, it also hindered their subsequent internalization. As quantified in Fig. 1G, the inhibitory effect of gliotoxin on FcγR-mediated phagocytosis was dose-dependent. The gliotoxin doses employed in this assay, ranging from 62.5 to 1000 ng/ml, are in good agreement with the concentration of the toxin in the serum of patients with invasive aspergillosis (IA), which varies between ~150 and 800 ng/ml (Lewis et al. 2005). Not surprisingly, gliotoxin is present at even higher concentrations in the lung parenchyma, the primary location of invasive hyphal growth. Indeed, analyses in mice with experimentally induced IA have shown that gliotoxin is typically found in the lung at approximately 4,000 ng/g of tissue (Lewis et al. 2005).

That gliotoxin subverted phagocytosis of IgG-coated particles in RAW 264.7 cells prompted us to investigate whether the toxin had comparable effects on the engulfment of fungal particles by primary macrophages. To this end, we used either serum-coated or unopsonized zymosan as phagocytic targets for human monocyte-derived macrophages (Fig. 1C-F). Zymosan is ideally suited for the study of phagocytosis of fungal particles, as it carries surface β(1,3)-glucans that are recognized by the phagocytic receptor Dectin-1. To facilitate their visualization by microscopy, we simultaneously labelled all zymosan particles with succinimidyl esters conjugated to either Alexa Fluor 555 (shown in red in Fig. 1C-F) or biotin. Functionalized zymosan was then sedimented onto macrophages to initiate phagocytosis, which was allowed to proceed for 20 min before fixation. Non-internalized particles were identified by labelling the biotin tag with fluorescent streptavidin (shown in blue). Similar to the effects observed for FcγR-mediated phagocytosis, gliotoxin profoundly depressed both binding and engulfment of serum-coated zymosan (Fig. 1C,D), which is normally phagocytosed upon engagement of FcγR and the complement receptor Mac-1. Likewise, Dectin-1-mediated phagocytosis of unopsonized zymosan (Fig. 1E,F) by human macrophages
was virtually obliterated in response to gliotoxin. As quantified in Fig. 1H, the total number of serum-opsonized zymosan (SOZ) particles contacted by macrophages (white bars) decreased from $9.36 \pm 1.28$ to $3.06 \pm 0.72$ upon gliotoxin treatment. This effect was even more striking on the phagocytosis of unopsonized zymosan; the total number of contacted particles was reduced from $4.35 \pm 0.58$ to $0.22 \pm 0.08$. For both serum-opsonized and unopsonized zymosan, gliotoxin exposure led to a more than 4-fold drop in phagocytic efficiency (the ratio of internalized to total number of contacted particles; shown as a percentage above the bars on Fig. 1H).

6.4.2 Macrophage viability is not compromised after acute gliotoxin treatments

Prolonged gliotoxin exposure has been reported to trigger apoptosis (Sutton et al. 1994; Stanzani et al. 2005; Pardo et al. 2006). Though our treatments were comparably short (15-30 min), we nonetheless questioned whether the observed phagocytic impairment was attributable to the toxin’s interfering with macrophage viability. We addressed this by 2 independent, complementary assays (Fig. S1). First, we monitored plasmalemmal integrity in gliotoxin-treated RAW 264.7 macrophages using the membrane-impermeant dye aqua LIVE/DEAD. This fluorescent molecule exclusively stains plasmalemmal components of live cells, but gains access to the cytoplasm of necrotic cells because of their increased plasmalemmal permeability. As illustrated in Fig. S1A,B, the vast majority (93.2 ± 1.71%) of gliotoxin-treated macrophages maintained plasmalemmal integrity, even 4 hours post-gliotoxin exposure (as evident by the absence of cytoplasmic fluorescence), suggesting that these cells were not undergoing necrosis. Second, to assess whether acute gliotoxin incubations resulted in apoptosis, we monitored phosphatidylserine (PtdSer) exposure on the exofacial leaflet and nuclear condensation via Annexin V and DAPI staining, respectively. As shown in Fig. S1C,D, PtdSer asymmetry was maintained for the first 2 hours of treatment, with 87.8 ± 4.01% of cells being Annexin V-negative at this time point. Of note, the above cell death indicators were absent for a period of time greatly exceeding the one used in our phagocytic assays. Indeed, we routinely observed that the phagocytic and cytoskeletal abnormalities shown in Fig. 1 became fully apparent within 15 min of gliotoxin
treatment. Thus, a mechanism other than cell death must account for the rapid and drastic phagocytic impairments elicited by gliotoxin.

6.4.3 The cell-surface distribution and affinity of Fcγ receptors for multivalent IgG are not altered by gliotoxin

The marked reduction in phagocytic capacity observed in gliotoxin-treated macrophages led us to consider the possibility of the toxin interfering with the ability of Fcγ receptors to remain at the cell surface and/or recognize their ligand. We examined this experimentally by a combination of immunofluorescence and flow cytometry determinations. Fig. 2A-D shows representative micrographs of control (left panels) and gliotoxin-treated (right panels) RAW 264.7 cells stained with antibodies against FcγRI (Fig. 2A,B) or against an epitope shared by FcγRIIb and FcγRIII (Fig. 2C,D). As evident from these images, the levels of cell-surface Fcγ receptors were equivalent in DMSO- and gliotoxin-treated cells. The frequency of cell-surface FcγRI and FcγRIIb/FcγRIII was also quantified by flow cytometry (Fig. 2G,H), which confirmed that the distribution of these receptors did not change upon gliotoxin treatment.

While the density of Fcγ receptors at the plasmalemma was unaltered, it was still conceivable that gliotoxin reduced their ligand affinity. Thus, we compared the ability of control and gliotoxin-treated macrophages to bind fluorescent IgG aggregates (Fig. 2E,F). These assays, performed at 16°C to avoid internalization of the aggregates, revealed no significant differences between DMSO- and gliotoxin-treated macrophages in their ability to bind aggregated IgG (Fig. 2I). Together, these data indicate that neither a change in the number of cell-surface Fcγ receptors nor in their affinity for IgG can account for the subversion of phagocytosis by gliotoxin.

6.4.4 Cell adhesion and actin dynamics are profoundly affected by gliotoxin

To aid us in identifying likely targets of the toxin in phagocytes, we characterized the ultrastructure of human monocyte-derived macrophages by scanning electron microscopy (SEM), both under control and gliotoxin-treatment conditions. As the
representative micrographs in Fig. 3B,D show, macrophages exposed to gliotoxin noticeably lost adhesion to the substratum, leading to the formation of thin retraction fibers. Moreover, gliotoxin treatment prevented the elaboration of actin-driven lamellipodial extensions, which were otherwise prominent under control conditions (Fig. 3A). Importantly, these SEM determinations also revealed that gliotoxin-treated macrophages responded to serum-opsonized zymosan by developing abortive phagocytic cups, which arrested halfway around the particle (see inset in Fig. 3D). Previous studies have reported that macrophages incubated with PI3K inhibitors such as LY294002 or wortmannin arrest at similar stages of phagocytosis, generating unproductive cups or membrane pedestals akin to those shown in Fig. 3D (Dianne Cox et al. 1999b; Beemiller et al. 2010; Schlam et al. 2015).

The capacity of gliotoxin to dually interfere with homeostatic actin dynamics and cell adhesion was also observed by time-lapse differential interference contrast (DIC) microscopy; Movie S1 shows that RAW 264.7 cells withdrew their actin-driven membrane extensions and abruptly retracted in response the mycotoxin. Likewise, the phagocytic response and highly dynamic membrane protrusions of primary human macrophages (Movie S2) were virtually obliterated upon gliotoxin treatment (Movie S3).

Given its striking effect on the ability of macrophages to extend and maintain actin-driven protrusions, we next questioned whether the levels of filamentous actin (F-actin) were altered by gliotoxin. To address this, RAW 264.7 macrophages were treated with increasing concentrations of the toxin for 30 minutes. Immediately thereafter, the macrophages were fixed, permeabilized, stained with fluorescent phalloidin and imaged by confocal microscopy (Fig. 3E,F). The intensity of phalloidin fluorescence was used as a proxy of F-actin levels. As shown in Fig. 3F, gliotoxin treatment led to a dose-dependent decline in the abundance of actin filaments, resulting in an almost 2-fold reduction in total F-actin when the toxin was used at 500 ng/ml.
6.4.5 Gliotoxin-induced cytoskeletal abnormalities are accompanied by Rac/Cdc42 activation

The small Rho GTPases Rac and Cdc42 orchestrate the extension of actin-driven pseudopodia around phagocytic targets (Park and Cox 2009; Cox et al. 1997; Hoppe and Swanson 2004; Niedergang and Chavrier 2005; Chimini and Chavrier 2000). Rac also mediates the constitutive membrane ruffling of professional phagocytes (Bohdanowicz et al. 2013). Thus, we hypothesized that the marked defects in actin cytoskeletal dynamics displayed by gliotoxin-treated macrophages were underlined by the inactivation of one or both of these Rho GTPases. To address this, we co-transfected RAW 264.7 macrophages with constructs encoding PAK(PBD)-YFP and Lifeact-mRFP, fluorescent biosensors of active (GTP-loaded) Rac/Cdc42 (Hoppe and Swanson 2004; Srinivasan et al. 2003b) and F-actin (Srinivasan et al. 2003b), respectively. Transfectants were imaged live by time-lapse confocal microscopy, both under control conditions and immediately after gliotoxin exposure (see Movie S4 for the uninterrupted time-lapse sequence). As expected, control macrophages constitutively produced actin-rich membrane ruffles (Fig. 3G; top panel), which disappeared upon gliotoxin addition (Fig. 3H; top panel). More importantly, retraction of these membrane projections was coincident in space and time with the dissociation of PAK(PBD)-YFP from the sites of actin polymerization (Fig. 3G,H; bottom panels), suggesting that gliotoxin interferes with homeostatic actin dynamics by precluding activation of Rac and Cdc42.

6.4.6 Treatment with gliotoxin does not trigger cAMP signalling in macrophages

Similar to our observations in macrophages, a previous study indicated that gliotoxin induces marked alterations in the actin cytoskeleton of neutrophils (Coméra et al. 2007). In that study, the authors reported that stimulation of cAMP signalling in neutrophils via a cAMP pharmacological agonist was sufficient to recapitulate some of the gliotoxin-induced cytoskeletal abnormalities. Conversely, these actin defects were prevented if gliotoxin treatment was preceded by incubating the neutrophils with cAMPS-Rp, a competitive inhibitor of cAMP signalling (Coméra et al. 2007). To investigate whether
the profound alterations in actin dynamics that we observed in macrophages were also cAMP-dependent, we conducted two independent assays. First, we monitored phosphorylation of VASP at serine 157 by PKA, a cAMP-dependent kinase (Reinhard et al. 1992; Lawrence and Pryzwansky 2001). Surprisingly, we found the levels of phospho-VASP to be equivalent in resting and gliotoxin-treated conditions (Fig. S2A,B), implying that gliotoxin does not cause a cAMP surge in macrophages. In contrast, treatment with forskolin—a direct activator of the adenylyl cyclase (Seamon and Daly 1986; Laurenza, Sutkowski, and Seamon 1989)—led to a more than 80-fold increase in phospho-VASP levels, and this was blunted by incubating the macrophages with cAMPS-Rp prior to forskolin stimulation.

Given that our findings were not congruent with the previously reported notion of gliotoxin toxicity being cAMP-dependent, we followed cAMP levels by an additional method. This assay, consisting of measuring integrin activation, is based on the ability of cAMP-dependent Rap1 GEFs to promote inside-out integrin activation (Kawasaki et al. 1998; Shimonaka et al. 2003). Thus, if cAMP levels indeed increased as a consequence of gliotoxin exposure, then we should be able to detect an elevation in integrin activation upon gliotoxin treatment. We carried out these experiments in human monocyte-derived macrophages, which were treated or not with gliotoxin, fixed and stained with an antibody that recognizes an epitope on high-affinity integrin β2. As a positive control, integrins were activated by incubating with the phorbol ester PMA. Contrary to the consequences expected from an elevation in cAMP, gliotoxin treatment reduced integrin activation (Fig. S2C, top panels; Fig. S2D). Likewise, pre-incubation of macrophages with gliotoxin significantly precluded PMA-mediated integrin activation (Fig. S2C, bottom panels; Fig. S2D). Together, the failure of gliotoxin to increase VASP phosphorylation and the observed decline in integrin activation implied that the toxin operates through a mechanism other than triggering cAMP signalling in macrophages.

### 6.4.7 Gliotoxin markedly downregulates PtdIns(3,4,5)P3 levels

To establish the mechanistic link between gliotoxin toxicity and Rac/Cdc42 inactivation, we turned our attention to phosphoinositides, which play a paramount role in the
integration of extracellular signals with the actin machinery (Levin, Grinstein, and Schlam 2014). In particular, PtdIns(4,5)P₂ promotes the formation of actin networks and their anchoring to the plasmalemma (Raucher et al. 2000; Rohatgi, Ho, and Kirschner 2000b; Sechi and Wehland 2000; Rohatgi et al. 2001), while PtdIns(3,4,5)P₃ recruits and activates modulators of Rho GTPase activity carrying pleckstrin homology (PH) domains. For instance, the Rac1 GEFs Vav, Sos1 and TIAM1 induce Rac1 signalling in spatially restricted membrane regions enriched in PtdIns(3,4,5)P₃ (Das et al. 2000; Ceccarelli et al. 2007; Bohdanowicz et al. 2013). To investigate whether gliotoxin depressed Rac/Cdc42 activity by altering phosphoinositide metabolism, we followed the cellular distribution of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ via the PLCδ(PH)-GFP and Akt(PH)-GFP biosensors, respectively (Botelho et al. 2000; Marshall et al. 2001) (Fig. 4A-E). As expected, the localization of PLCδ(PH)-GFP was restricted to the plasmalemma—where PtdIns(4,5)P₂ is most abundant—while Akt(PH)-GFP distributed to membrane ruffles and sites of actin polymerization. Remarkably, gliotoxin addition resulted in the dissociation of both biosensors from the plasma membrane (Fig. 4B,D), implying that the concentration of both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ decreased as a consequence of gliotoxin exposure. While the reduction in PtdIns(4,5)P₂ levels were more modest, PtdIns(3,4,5)P₃ was virtually undetectable by microscopy following gliotoxin treatment (see Fig. 4E for a quantification of normalized fluorescence).

To ensure that the observed decline in PtdIns(3,4,5)P₃ was not an artifact of the toxin’s interfering with the biosensor itself, we assessed PtdIns(3,4,5)P₃ levels by an alternative means: measuring the relative levels of Akt phosphorylation at serine 473 (Burgering and Coffer 1995). As shown in the immunoblot in Fig. 4F and its quantification in Fig. 4G, Akt phosphorylation was significantly reduced in gliotoxin-treated cells when compared to controls. Note that cells exposed to gliotoxin exhibited a decline in Akt phosphorylation equivalent to those treated with the customary PI3K inhibitor wortmannin (Fig. 4F,G). These data indicate that gliotoxin interferes with PtdIns(3,4,5)P₃ homeostasis, and implicate PI3K as a possible target of the toxin. They also suggest that the rapid inactivation of Rac/Cdc42 and the ensuing actin cytoskeletal abnormalities are attributable to a reduction in plasmalemmal PtdIns(3,4,5)P₃.
6.4.8 PMA promotes PtdIns(3,4,5)P$_3$-independent ruffling and phagocytosis

While assessing the effect of gliotoxin on integrin activation (Fig. S2C,D), we came across an interesting, serendipitous observation: in response to PMA, gliotoxin-treated macrophages not only activated β$_2$ integrins to above-resting levels (Fig. S2D), they also regained their ability to ruffle their plasma membrane and spread. Fig. 5A and Movie S5 show human monocyte-derived macrophages that were pre-treated with gliotoxin for 15 min and exposed to 100 nM PMA immediately thereafter. Notably, addition of this DAG analogue effected the transformation of gliotoxin-induced retraction fibers into highly motile and dynamic peripheral membrane ruffles. Seeing that PMA allowed for gliotoxin-treated macrophages to reestablish their morphology and regain cytoskeletal dynamics, we questioned whether these cells also recovered their phagocytic capacity. To investigate this, RAW 264.7 cells that had been treated with gliotoxin for 15 min were subsequently exposed to PMA or DMSO alone for an additional 20 min, and then challenged with IgG-opsonized erythrocytes (Fig. 5C,D). As anticipated, macrophages treated with gliotoxin alone were significantly impaired in their ability to both engage and internalize IgG-coated targets. More strikingly, however, gliotoxin-treated cells that were subsequently activated with PMA contacted and internalized a number of IgG-RBC that did not differ significantly from that of cells treated with DMSO alone (Fig. 5C,D).

That PMA was capable of bypassing gliotoxin toxicity suggested that this DAG analogue activated signalling components lying downstream of PtdIns(3,4,5)P$_3$, which we have shown to be a primary gliotoxin target. If this was the case, then PMA-induced membrane ruffling and phagocytosis in gliotoxin-treated cells should occur even in the absence of plasmalemmal PtdIns(3,4,5)P$_3$. We tested this premise by electroporating human monocyte-derived macrophages with the PtdIns(3,4,5)P$_3$ biosensor Akt(PH)-GFP. Transfectants were then treated with gliotoxin and activated or not with PMA. As expected, resting macrophages elaborated extensive membrane ruffles that were rich in PtdIns(3,4,5)P$_3$, and these disappeared upon gliotoxin treatment (Fig 5B; left and middle panels). In sharp contrast, PMA not only prompted the formation of extensive ruffles in gliotoxin-treated cells, these membrane extensions were devoid of PtdIns(3,4,5)P$_3$ (Fig. 5A,B, right panel). Together, these observations bolster the notion that PMA can rescue macrophages from a gliotoxin-mediated insult on PtdIns(3,4,5)P$_3$ by triggering signalling
pathways that normally operate downstream of this phosphoinositide. Our findings also raise the interesting possibility that a fundamental role of PtdIns(3,4,5)P₃ during the formation of membrane ruffles and pseudopodia is to signal diacylglycerol biosynthesis.

6.5 Discussion

When considering possible gliotoxin targets, it is helpful to recall that this is a small, membrane-permeant molecule (Kweon et al. 2003; Salvi, Bozac, and Toninello 2004; Stanzani et al. 2005) whose toxicity is dependent on a bridged disulfide ring (Gardiner, Waring, and Howlett 2005; C. Ward et al. 1999). Indeed, gliotoxin activity is abolished in the presence of reducing reagents or by substituting its disulfide bridge with a dimethylthioether moiety (Müllbacher et al. 1986). Gliotoxin’s hydrophobicity, in conjunction with the absolute requirement of its disulfide bridge for toxicity, supports the notion that the toxin operates by modifying cytosolic host proteins that carry susceptible thiol groups by virtue of residing in a reducing environment. Moreover, the profound immunosuppressive properties elicited by gliotoxin are likely accomplished through the inactivation of more than a single target. In fact, previous reports have indicated that gliotoxin inhibits NAPDH oxidase function in neutrophils (Tsunawaki et al. 2004; L. S. Yoshida, Abe, and Tsunawaki 2000), precludes the cytotoxic properties of T lymphocytes (Yamada, Kataoka, and Nagai 2000), and prevents NF-κB-dependent transcription of cytokines in both B and T cells (Pahl et al. 1996). A recent study also suggested that gliotoxin induces cytoskeletal abnormalities in neutrophils by elevating the cellular concentration of cAMP (Coméra et al. 2007). Despite observing similar defects in the actin architecture of macrophages, we failed to detect even slight alterations in the activity of cAMP effectors in response to the toxin (Fig. S2). Consistent with our inability to detect gliotoxin-induced fluctuations in cAMP levels, it was previously indicated that the capacity of cAMP response element-binding protein (CREB) to bind to its cognate DNA sequence was not affected in gliotoxin-treated T lymphocytes (Pahl et al. 1996). Thus, the notion that gliotoxin elevates cAMP levels may be a neutrophil idiosyncrasy.
Instead of causing an increase in the cAMP content of macrophages, gliotoxin seems to subvert PtdIns(3,4,5)P$_3$ metabolism, thereby compromising a number of PI3K-dependent processes that are essential for normal phagocyte function. At the present time it is unclear whether the overt decline in PtdIns(3,4,5)P$_3$ levels that we observe arises as a consequence of inhibiting synthesis or stimulating degradation of the phosphoinositide. In either instance, however, the physiological, morphological and cytoskeletal abnormalities ensuing from gliotoxin treatment are consistent with a decrease in PtdIns(3,4,5)P$_3$ concentration. First, it is well established that 3’-phosphoinositides provide survival signals by facilitating Akt activation (Milburn et al. 2003; Thomas et al. 2002; Calleja et al. 2007; Hers, Vincent, and Tavaré 2011), and we have seen that—if protracted enough—gliotoxin exposure eventually results in apoptosis (Fig S1). Second, localized accumulation of PtdIns(3,4,5)P$_3$ is known to be necessary for assembling and organizing the actin-driven projections that underlie membrane ruffling (Flannagan et al. 2010; Bohdanowicz et al. 2013) and phagosome formation (Beemiller et al. 2010; Schlam et al. 2015). Consistently, gliotoxin profoundly affected the ability of macrophages to form peripheral membrane ruffles (Movies S1 and S4) and phagocytose prey (Fig. 1). Remarkably, the incomplete phagocytic cups developed by gliotoxin-treated cells (Fig. 3D) aborted at a stage that was morphologically equivalent to those formed in cells treated with PI3K inhibitors (Cox et al. 1999; Beemiller et al. 2010; Schlam et al. 2015). This too bolsters the notion that gliotoxin interferes with PtdIns(3,4,5)P$_3$ homeostasis. Third, earlier studies have proposed an essential role for PtdIns(3,4,5)P$_3$ during inside-out activation of β$_1$ and β$_2$ integrins in immune cells (Zell et al. 1996; Shimizu et al. 1995; Kinashi 2005; Kolanus et al. 1996), and we have provided evidence that gliotoxin prevents activation of β integrins, both in response to PMA and under otherwise resting conditions (Fig. S2C,D). All of these essential processes—cell survival, membrane ruffling, phagocytosis and integrin activation—are profoundly affected by interfering with the PtdIns(3,4,5)P$_3$ signalling node.

Integrin activation and Rho GTPase-mediated actin nucleation are two main components of the phagocytic response, and PtdIns(3,4,5)P$_3$ is instrumental for both of them. This phosphoinositide recruits modulators of Rac activity that carry PH domains, thereby orchestrating the assembly of actin networks around incoming targets (Schlam...
et al. 2015), as well as the membrane ruffles that sample the extracellular environment (Flannagan et al. 2010; Bohdanowicz et al. 2013). In addition, the failure of gliotoxin-treated macrophages to activate Rac (Fig. 3) is expected to hinder their production of microbicidal superoxide anions, as this Rho-family GTPase is critical for NADPH oxidase activation (Dinauer 2003). Interestingly, invasive aspergillosis is the primary cause of death in patients with chronic granulomatous disease (a primary immunodeficiency caused by mutations in NADPH oxidase components), underscoring the prominent role of the respiratory burst—and therefore of Rac activation—in combating this fungal pathogen. Gliotoxin could therefore exacerbate infection by interfering with the PtdIns(3,4,5)P₃-Rac-NADPH oxidase axis. It is also very likely that the mycotoxin obstructs phagocytic responses by inhibiting the activation of β integrins, which are necessary for optimal phagocytosis (Flannagan et al. 2014; Jongstra-Bilen, Harrison, and Grinstein 2003; Vachon et al. 2007). Notably, inside-out integrin activation during phagocytosis is normally dependent on PI3K activity (Jongstra-Bilen, Harrison, and Grinstein 2003; Flannagan et al. 2014, 4).

Strikingly, while PtdIns(3,4,5)P₃ is a key component for the function of Rho GTPases and β integrins, we have found that PMA treatment allowed gliotoxin-treated macrophages to ruffle their plasma membrane once again, spread and phagocytose prey in a seemingly PtdIns(3,4,5)P₃-independent manner (Fig. 5). How is this possible? A possible explanation is that, as a diacylglycerol analogue, PMA stimulates the function of CalDAG-GEFI, a diacylglycerol-regulated GEF that promotes inside-out integrin activation through the Rap1 GTPase (Kawasaki et al. 1998; Clyde-Smith et al. 2000; Ghandour et al. 2007; Crittenden et al. 2004; Pasvolsky et al. 2007). Interestingly, active Rap1 has also been shown to directly recruit the Rac GEFs TIAM1 and Vav2 to sites of active membrane protrusion (Arthur, Quilliam, and Cooper 2004). Thus, catalyzing nucleotide exchange on Rap1 by CalDAG-GEFI would not only restore integrin function during phagocytosis and spreading, it would also promote the reestablishment of actin cytoskeletal dynamics. Because CalDAG-GEFI, TIAM1 and Vav2 signal downstream of PtdIns(3,4,5)P₃, the PMA-mediated stimulation of these GEFs would bypass the subversion of PtdIns(3,4,5)P₃ by gliotoxin, thus restoring integrin avidity and reestablishing cytoskeletal dynamics.
6.6 Figure legends

Figure 1. *Gliotoxin inhibits different modalities of phagocytosis in macrophages.* RAW 264.7 cells (A,B) and human monocyte-derived macrophages (C-F) were treated with DMSO (left panels) or 500 ng/ml gliotoxin (right panels) for 30 min immediately before being challenged with IgG-opsonized red blood cells (A,B), serum-opsonized zymosan (C,D) or unopsonized zymosan (E,F). In all instances, phagocytosis proceeded for 20 min before fixation. All IgG-coated erythrocytes were labelled with a Cy3-conjugated IgG (shown in red) prior to the onset of phagocytosis. To identify erythrocytes that were not internalized, the fixed cells were stained with a Cy5-conjugated antibody (shown in blue) following phagocytosis. Likewise, all zymosan particles were simultaneously labelled with succinimidyl esters conjugated to either Alexa Fluor 555 (shown in red) or biotin prior to initiating phagocytosis. Non-phagocytosed zymosan was visualized by staining with streptavidin-Alexa Fluor 647 (shown in blue). Macrophages were then permeabilized and stained with phalloidin-Alexa Fluor 488 to visualize the actin skeleton. All images are displayed as Z-projections of confocal sections. Scale bar = 10 µm. (G) Quantification of the average number of IgG-opsonized erythrocytes internalized by RAW 264.7 cells treated with the indicated gliotoxin concentrations. (H) Quantification of the total (white bars) and internalized (black bars) number of serum-opsonized or unopsonized zymosan particles per human macrophage. The ratio of internalized-to-total number of particles is indicated as a percentage above the bars. All experiments were conducted independently at least 3 times and error bars represent the standard error of the mean.

Figure 2. *Surface density and affinity of Fcγ receptors remain unaltered after gliotoxin treatment.* (A-F) RAW 264.7 cells were treated with vehicle alone (DMSO, left panels) or 500 ng/ml gliotoxin (right panels) for 30 min immediately before being labelled with either anti-FcγRI (A,B) or anti-FcγRIIb/III (C,D) antibodies, or exposed to aggregated IgG (E,F). Surface expression of the indicated Fcγ receptors (shown in green) was visualized by staining with Alexa Fluor 488-conjugated secondary antibodies, while nuclei were counterstained with DAPI (shown in blue). For each
individual panel in (A-F), channels are shown either individually as confocal XY sections (smaller images, left side), or merged as a Z-projection (larger image, right side). Scale bar = 10 μm. (G,H) Flow cytometry histograms depicting the relative frequency of FcγRI-positive (G) and FcγRIIb/III-positive (H) RAW 264.7 cells under control (solid blue trace) and gliotoxin (solid red trace) conditions. Isotype traces, indicative of antibody-receptor interactions that occurred through the IgG2a Fc portion (as opposed to the variable region) are shown in dashed lines. The background signal (unlabelled macrophages) is depicted by the gray trace. (I) Quantification of the results shown in (E,F). Relative fluorescence intensity of Cy3-conjugated aggregated IgG at the cell surface, representative of the affinity of Fcγ receptors for their ligand, is reported for control (black bars) and gliotoxin-treated (white bars) cells. Experiments were conducted at least 3 times and error bars represent the standard error of the mean.

**Figure 3. Gliotoxin subverts actin cytoskeletal homeostasis and Rho GTPase activation.** (A-D) Ultrastructural characterization of the morphological changes induced by gliotoxin. Primary human macrophages were treated with DMSO (left panels) or gliotoxin (right panels) for 50 min before being fixed and imaged by scanning electron microscopy. Cells in (C,D) were challenged with serum-opsonized zymosan for the last 20 min of gliotoxin treatment. Insets display magnified views of serum-opsonized zymosan particles contacted by control (C) and gliotoxin-treated cells (D). (E) Visualization of the microfilament skeleton of RAW 264.7 cells treated with gliotoxin at the indicated concentrations. After 30 min of gliotoxin treatment, cells were stained with Alexa Fluor 488-conjugated phalloidin and imaged by spinning-disk confocal microscopy. Images were pseudocolored according to the lookup table shown on the bottom right. (F) Quantification of phalloidin fluorescence intensity in the cells shown in (E) as a function of gliotoxin concentration, representative of the relative levels of F-actin. Values are normalized to the untreated control condition. (G,H) Still images obtained by time-lapse fluorescence microscopy showing sites of actin polymerization and Rac/Cdc42 activation. RAW 264.7 macrophages were transiently co-transfected with constructs encoding Lifeact-mRFP (top panel) and PAK(PBD)-YFP (bottom panel), fluorescent probes for F-actin and active Rac/Cdc42, respectively. The arrows in (G)
point to membrane ruffles formed by untreated cells at sites of Rho GTPase activation. Cells in (H) were exposed to gliotoxin. Scale bar for images in (E-H) = 10 µm. Experiments were conducted at least 3 times and error bars represent the standard error of the mean. SOZ = serum-opsonized zymosan.

Figure 4. PtdIns(3,4,5)P₃ is significantly downregulated in gliotoxin-treated macrophages. (A-D) RAW 264.7 cells transiently expressing PLCδ(PH)-GFP (A,B) or Akt(PH)-GFP (C,D), biosensors for PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, respectively. Cells were treated with DMSO (left panels) or 500 ng/ml gliotoxin (right panels) for 30 min immediately before being imaged live by spinning-disk confocal microscopy. Illustrated in each panel are single XY (top) and XZ (bottom) optical slices. The dotted line along the XY plane indicates the coordinates where the XZ plane stands. Scale bar = 10 µm. (E) Quantification of plasmalemmal fluorescence intensity in DMSO- or gliotoxin-treated macrophages expressing the indicated phosphoinositide biosensors. Values were obtained by dividing the difference between plasmalemmal and cytoplasmic fluorescence by cytoplasmic fluorescence, thus accounting for differential levels of expression and photobleaching. (F) M-CSF dependent phosphorylation of Akt at serine 473 was assessed by immunoblotting as an alternative readout of PtdIns(3,4,5)P₃ levels. RAW 264.7 cells were first treated for 30 min with DMSO, gliotoxin, or the PI3K inhibitor wortmannin. Where indicated, cells were incubated with PMA following gliotoxin or wortmannin exposure for the last 15 min of treatment. For all conditions in (F), cells were activated with M-CSF 5 min prior to obtaining extracts in order to induce robust PI3K activity. (G) Quantification of the phospho-Akt signal (normalized to that of GAPDH) in the immunoblot shown in (F). Values are reported as fold changes relative to the DMSO-treated control. Experiments were conducted at least 3 times and error bars represent the standard error of the mean.
Figure 5. PtdIns(3,4,5)P3-independent membrane ruffling and phagocytosis in response to PMA. (A,B) Representative micrographs of primary human macrophages imaged live by time-lapse DIC (A) or confocal microscopy (B). Cells were imaged under resting conditions (0-5 min time point; left panels), 15 min following gliotoxin exposure (5-20 min time point; middle panels) and 20 min after treatment with PMA (20-40 min time point; right panels). Gliotoxin was not removed from the medium during the time of PMA activation. Insets in (A) show magnified views of actively ruffling regions under resting conditions (left), the retraction of these extensions upon gliotoxin treatment (middle), and their reappearance upon activation with PMA (right). The primary macrophages in (B) were transiently electroporated with a construct encoding Akt(PH)-GFP, a fluorescent reporter of PtdIns(3,4,5)P3. Arrows point to sites of PtdIns(3,4,5)P3 accumulation within extensive membrane ruffles in resting cells. (C) Representative Z-projections of RAW 264.7 cells challenged with IgG-opsonized erythrocytes. Macrophages were treated under the same conditions as the cells in (A,B), and phagocytosis was initiated immediately thereafter. Ten min following exposure to phagocytic targets, cells were fixed, permeabilized and stained with Alexa Fluor 488-conjugated phalloidin. All phagocytic particles are shown in red, while those that remained extracellular at the time of fixation are shown in blue. Arrows point to abortive phagocytic cups developed by gliotoxin-treated cells. Scale bar = 10 μm. (D) Quantification of the total (white bars) and internalized (black bars) number of IgG-opsonized targets associated per macrophage under the indicated conditions. Experiments were conducted at least 3 times and error bars represent the standard error of the mean. Gt = gliotoxin.

Figure S1. Macrophages retain membrane integrity and phosphatidylserine asymmetry following acute exposure to gliotoxin. (A,C) Representative optical sections of RAW 264.7 macrophages stained with the membrane-impermeant dye Aqua LIVE/DEAD (A) or Alexa Fluor 488-conjugated Annexin V (C). Cells were imaged by confocal microscopy immediately after treatment with gliotoxin (500 ng/ml) for the indicated times. (A) The Aqua LIVE/DEAD dye is excluded from the cytosolic compartment of living cells, leading to selective plasmalemmal staining. In turn, the
compromised membrane integrity of necrotic cells results in bright cytosolic fluorescence. Saponin was used as a positive control of membrane permeabilization (lower right). Images in (A) were pseudocolored according to the shown lookup table. (C) Exofacial exposure of phosphatidylserine, detected by Annexin V staining, was used as a marker of apoptosis. Scale bar = 10 \( \mu \text{m} \). (B,D) Quantification of the percentage of cells preserving membrane integrity (B) and phosphatidylserine asymmetry (D) as a function of time following gliotoxin exposure.

**Figure S2. Macrophage cAMP levels are not altered in response to gliotoxin.** (A) Phosphorylation of VASP at serine 157 (resulting from the cAMP-dependent activation of PKA) was assessed by immunoblotting. Cell extracts were obtained from RAW 264.7 macrophages treated with DMSO (resting); cAMPS-Rp, a competitive inhibitor of cAMP-induced PKA activation; gliotoxin; or cAMPS-Rp followed by gliotoxin. Forskolin, a cell permeable activator of the adenylate cyclase, was used as a positive control for induction of cAMP signalling. (B) Quantification of the phospho-VASP signal from the immunoblot shown in (A), normalized to that of GAPDH. (C) Integrin activation, driven by cAMP-dependent GEFS of Rap1 GTPases, was used to monitor changes in the levels of intracellular cAMP. Shown are representative immunofluorescence micrographs of primary human macrophages treated with DMSO (left panels) or 500 ng/ml gliotoxin (right panels) before being stained with an antibody recognizing \( \beta_2 \) integrins in their active conformation of. The phorbol ester PMA was used as a positive control for integrin activation. Images were pseudocolored according to the lookup table in the micrograph on the upper left. Scale bar, 10 \( \mu \text{m} \). (D) Quantification of the fluorescence intensity of the cells in (C), normalized to DMSO-treated controls and indicative of the levels of active \( \beta_2 \) integrins. Experiments were conducted at least 3 times and error bars represent the standard error of the mean.
6.7 Figures

Figure 1. Gliotoxin inhibits different modalities of phagocytosis in macrophages

A) RAW 264.7
+ IgG-RBC

B) + Gliotoxin (500 ng/mL)

C) All SOZ + external SOZ + F-actin

D) All SOZ + F-actin

E) All zymo + external zymo + F-actin

F) All IgG-RBC + external RBC + F-actin

G) Internalized RBC per cell (normalized phagocytic index)

H) Zymosan particles (Average number per cell)

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Internalized</th>
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<tbody>
<tr>
<td>SOZ</td>
<td>17.7%</td>
<td>**</td>
</tr>
<tr>
<td>unopsonized</td>
<td>22.7%</td>
<td>***</td>
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* = p < 0.05
** = p < 0.005
*** = p < 0.005
Figure 2. Surface density and affinity of Fcγ receptors remain unaltered after gliotoxin treatment
Figure 3. Gliotoxin subverts actin cytoskeletal homeostasis and Rho GTPase activation

- A: Control
- B: + Gliotoxin (500 ng/mL)
- C: Resting
- D: + SOZ
- E: Filamentous actin (phalloidin-488)
- F: Phalloidin fluorescence intensity (fold change relative to control, A.U.)
- G: Control
- H: + Gliotoxin (500 ng/mL)
Figure 4. PtdIns(3,4,5)P3 is significantly downregulated in gliotoxin-treated macrophages
Figure 5. PtdIns(3,4,5)P3-independent membrane ruffling and phagocytosis in response to PMA
Figure S1. Macrophages retain membrane integrity and phosphatidylserine asymmetry following acute exposure to gliotoxin
Figure S2. Macrophage cAMP levels are not altered in response to gliotoxin

A

pVASP (S157) →
GAPDH →

Resting
CAMPs-Rp (200 μM)
Gliotoxin (500 ng/ml)
Forskolin (100 μM)

Active β2 integrin

B

pVASP/GAPDH signal
(fold increase over resting control)

C

Active β2 integrin

Control
Gliotoxin
Resting
PMA

D

Active β2 integrin
(fluorescence fold change)

Control
Gliotoxin (500 ng/ml)
PMA
Gliotoxin - PMA

* = p < 0.05
7 Global discussion

7.1 Summary of research findings and models

7.1.1 First study: Phosphatidic acid is required for the constitutive ruffling and macropinocytosis of phagocytes

• Professional phagocytes utilize the energy of actin polymerization to continuously and dynamically ruffle their plasma membranes.

• Extension of these membrane projections facilitates surveying the extracellular milieu, enables uptake of fluid-phase antigen through macropinocytosis and promotes the capture of particulate targets that often precedes phagocytosis.

• Macrophages and immature dendritic cells display high levels of plasmalemmal PA, which is constitutively synthesized from a DAG precursor.

• Continued biosynthesis of plasmalemmal PA and PtdIns(3,4,5)P₃ is under GPCR control, and is necessary for the constitutive ruffling of professional phagocytes.

• PA facilitates membrane ruffling by 4 related means: it induces recruitment of PIP5K to the plasma membrane, thus bolstering the activity of actin-binding proteins normally regulated by PtdIns(4,5)P₂; it promotes the association of Rac1 GEFs (e.g., TIAM1) with the plasmalemma; it triggers dissociation of Rac1 from its GDI; and it sustains the presence of Rac1 at the plasma membrane.

• In professional phagocytes, plasmalemmal PA is synthesized through the phosphorylation of DAG by DGK. In turn, DAG is produced through the hydrolysis of PtdIns(4,5)P₂ by PLC.

• These studies uncover some of the fundamental molecular signals that allow actively phagocytic cells to constitutively probe their environment, thus setting them apart from their more sedentary, non-phagocytic counterparts.
Figure 1. **Constitutive ruffling in professional phagocytes is mediated by the ongoing synthesis of PA and PtdIns(3,4,5)P₃ at the plasma membrane.** (A) Upon binding to their cognate ligand, GPCRs undergo a conformational change that promotes nucleotide exchange on their coupled Gα subunit. GTP loading induces disassembly of the trimeric complex, with Gβγ dissociating from Gα-GTP. (B) Gβγ signals PtdIns(3,4,5)P₃ biosynthesis by activating PI3K, while Gα-GTP catalyzes PLC-mediated hydrolysis of PtdIns(4,5)P₂ into DAG and Ins(1,4,5)P₃. In turn, DAG is quickly phosphorylated by DGK into PA, which indirectly replenishes the PtdIns(4,5)P₂ pool by promoting recruitment of PIP5K to the plasma membrane. PA and PtdIns(3,4,5)P₃ cooperate in ruffle formation by facilitating the activation of Rac at the plasmalemma.
7.1.2 **Second study:** Phosphoinositide 3-kinase enables phagocytosis of large particles by coordinating actin disassembly through Rac/Cdc42 GTPase-activating proteins

- Phagocytosis of small vs. large particles differs drastically at the molecular level, even when triggered by the same receptor-ligand combination.

- In contrast to the uptake of small targets, phagocytosis of large particles requires PI3K activity.

- Completing phagocytosis of large targets is strictly dependent on the disassembly of actin networks from the base of the phagocytic cup.

- Actin breakdown is orchestrated by the PtdIns(3,4,5)₃-responsive RhoGAPs ARHGAP12, ARHGAP25 and SH3BP1, which coordinate the inactivation of Rac and Cdc42 in space and time with the progression of pseudopodia.

- The above RhoGAPs translocate to sites of large-particle engagement, but their accumulation at small phagocytic cups is inconspicuous.

- The increase in PtdIns(3,4,5)₃ biosynthesis that is normally observed upon engagement of large particles is significantly more modest in the case of small particles, likely accounting for the differential accumulation of RhoGAPs during phagocytosis of dissimilarly sized targets.

- These findings provide the first clues of molecular events that enable phagocytes to discern targets according to size.
Figure 2. *PI3K mediates actin breakdown during phagocytosis of large particles.*

Engagement of phagocytic receptors by multivalent ligands triggers PtdIns(4,5)P₂ biosynthesis, itself leading to localized actin polymerization at the tips of extending pseudopodia. The organized progression of pseudopodial membranes around large particles is accompanied by and requires disassembly of actin networks from the base of the phagocytic cup. In turn, actin breakdown is contingent on the inactivation of Rac and Cdc42 by PtdIns(3,4,5)P₃-dependent RhoGAPs. By consuming PtdIns(4,5)P₂ and generating PtdIns(3,4,5)P₃, PI3K controls Rho GTPase activity and the remodelling of actin filaments that is required for completing phagocytosis of large particles.
7.1.3 **Third study**: Diacylglycerol kinases terminate the respiratory burst and establish heterogeneity in phagosomal NADPH oxidase activation

- The phagosomal pool is intrinsically heterogeneous, both molecularly and physiologically. Such heterogeneity can be masked by assays that examine phagocyte function at the cell-population level.

- To investigate if phagosomal heterogeneity existed with respect to NADPH oxidase function, we devised a novel and dynamic assay capable of assessing superoxide formation at the single-phagosome level.

- Implementation of this assay revealed that phagosomal NADPH oxidase function is variable both within a single macrophage and across different macrophages.

- PtdIns(3)P, PA and PtdIns(3,4,5)P3 are produced by virtually every phagosome, and their presence (or lack of) therefore cannot account for the marked heterogeneity in superoxide formation.

- In turn, the distribution of DAG is variable across different phagosomes, and manipulations leading to increased or decreased phagosomal DAG levels result in enhanced and impaired NADPH oxidase responses, respectively.

- Heterogeneity in phagosomal DAG accumulation and superoxide generation may ultimately be attributable to variability in the recruitment and/or activity of DAG kinases, a family of enzymes responsible for DAG consumption and PA formation.

- The fine-tuning of NAPHR oxidase function by DAG kinases may be critical for limiting host damage by preventing the excessive generation of reactive oxygen species, but may also enable the survival of a fraction of invading pathogens.
Figure 3. Variability in DAG signalling across different phagosomes gives rise to heterogeneous NADPH oxidase responses. (A) Engagement of IgG-coated targets by Fcγ receptors triggers remodelling of the plasmalemmal inner leaflet by phosphoinositide-modifying enzymes. In particular, PI3K-mediated conversion of PtdIns(4,5)P2 (shown in blue) to PtdIns(3,4,5)P3 helps to orchestrate the rearrangement of actin networks that drive pseudopodia extension, as well as the recruitment of γ phospholipases that catalyze formation of the second messengers DAG (shown in green) and Ins(1,4,5)P3. (Bi) Immediately following phagosome sealing and scission, the composition of the phagosomal membrane is equivalent to that of the plasmalemma at sites of particle engagement. This includes the presence of phagosomal DAG that, in conjunction with the Ins(1,4,5)P3-mediated release of Ca^{2+} from intracellular stores, triggers PKC activation. In turn, PKC phosphorylates cytosolic subunits of the NADPH oxidase, thereby releasing them from an autoinhibited state. (Ci) Cytosolic NADPH oxidase subunits then associate with and activate p22^{phox} and gp91—the membrane-bound catalytic component of the oxidative complex—leading to the reduction of molecular oxygen to superoxide. (Bii) In a sizable fraction of phagosomes, DAG is quickly phosphorylated to PA by DGKβ. Failing to reach critical and/or sufficiently prolonged DAG signalling, these phagosomes are incapable of activating the cytosolic NADPH oxidase subunits by PKC. (Ci) Despite the formation of PtdIns(3)P and the increased levels of intracellular Ca^{2+}, insufficient and/or short-lived DAG production precludes the activation of the p22^{phox}-gp91 flavocytochrome, and therefore also superoxide generation in the phagosomal lumen.
7.1.4 **Fourth study:** Gliotoxin suppresses macrophage immune function by subverting PtdIns(3,4,5)P$_3$ homeostasis

- Amongst all aspergilli, *A. fumigatus* is the most frequent cause of human infections and predominantly accounts for the increased incidence of invasive aspergillosis.

- This opportunistic pathogen spreads from the environment by releasing large quantities of airborne conidia (spores) that infiltrate the small airways after inhalation. Alveolar macrophages represent the first line of defence against conidia.

- If the phagocyte system cannot contend effectively with *A. fumigatus* spores, these germinate into pulmonary hyphal networks capable of secreting several mycotoxins. The most abundant of these is gliotoxin, a secondary metabolite with potent immunosuppressive properties.

- Our studies show that gliotoxin undermines the ability of phagocytes to carry out their protective functions—including immune surveillance and phagocytosis—by subverting PtdIns(3,4,5)P$_3$ homeostasis in the host. The undermining of phagocyte defences by this virulence factor could lead to a more global state of immunosuppression in the lung, thereby exacerbating polymicrobial infections.

- By markedly decreasing PtdIns(3,4,5)P$_3$ levels, gliotoxin concomitantly precludes Rac/Cdc42 cycling and integrin activation. Given that both Rho-GTPase signalling and coordinated integrin activation are instrumental for pseudopodia progression, the targeting of PtdIns(3,4,5)P$_3$ by gliotoxin significantly inhibits phagocytosis. Other actin-driven processes, such as membrane ruffling, are similarly affected.

- The gliotoxin-mediated inhibition of actin dynamics is reversed by DAG analogues, suggesting that increased DAG levels can bypass the canonical role of PI3K in membrane ruffling and phagocytosis.

- Overall, this study identifies PtdIns(3,4,5)P$_3$ as a novel immune target of gliotoxin and offers a new a new perspective on the role of signalling lipids in phagocytes.
A **Resting state**

- Membrane ruffling & phagocytosis
- Integrin activation

- Rac GTP
- PIP₃
- PI3K
- TIAM1

B **Gliotoxin treatment**

- Inhibition of actin-driven membrane extensions

- Rac GDP
- Rac GTP
- PI3K
- PIP₃

C **PMA rescue**

- Restoration of membrane ruffling & phagocytosis
- Integrin re-activation

- Rap1 GTP
- PI3K
- Actin polymerization

- Gliotoxin
- PMA
**Figure 4. Disruption of PtdIns(3,4,5)P$_3$ homeostasis by gliotoxin undermines actin-driven phagocytic defences.** (A) Under resting conditions, phagocytes maintain a basal level of active integrins (right) and continuously extend actin-driven membrane projections that probe the environment (left). Both of these processes are instrumental for sustaining efficient phagocytic responses, and are in part driven by the ongoing synthesis of plasmalemmal PtdIns(3,4,5)P$_3$ (shown in green in the plasmalemmal inner leaflet). In particular, PtdIns(3,4,5)P$_3$ helps recruit the Rac GEF TIAM1 to the plasma membrane by binding to its N-terminal PH domain. Following nucleotide exchange on Rac by TIAM1, the GTPase catalyzes actin nucleation and membrane ruffling. (B) Exposure of macrophages to gliotoxin leads to the virtual depletion of PtdIns(3,4,5)P$_3$ from the plasmalemmal inner leaflet, resulting in declined levels of active integrin and a dysfunctional actin skeleton. (C) Addition of the DAG mimetic PMA to gliotoxin-treated macrophages effects the re-activation of β integrins and restores actin dynamics, effectively reversing gliotoxin toxicity. Mechanistically, PMA could promote nucleotide exchange on Rap1 by CalDAG-GEF1, thus inducing integrin activation. Active (GTP-bound) Rap1 plays the additional role of recruiting Rac GEFs to the plasma membrane, thereby restoring Rac activity and the existence of dynamic actin networks. Thus, increased DAG levels are likely to bypass the normal requirement for PI3K signals during membrane ruffling and phagocytosis.
7.2 Future directions

As is often the case in scientific research, a number of the experiments described throughout this body of work led to unforeseen findings, which in turn generated even more questions. Many of these observations are quite interesting, and warrant further analysis. What follows is a brief depiction of these potential lines of investigation, organized according to the study that they originated from.

7.2.1 First study: Phosphatidic acid and membrane rearrangements

As described in chapter 3, macrophages and immature dendritic cells display higher levels of PA in their plasma membrane than their more sedentary, epithelial counterparts. It is this plasmalemmal PA that maintains the constitutive ruffling of professional phagocytes, primarily by promoting Rac1-mediated actin polymerization at sites of membrane extension. The ongoing projection of membrane ruffles by professional phagocytes plays an important role in securing phagocytic targets, and precedes particle internalization (Flannagan et al., 2010). However, the phagocytic response is not restricted to professional phagocytes; fibroblasts, endothelial cells and even epithelial cells are all capable of phagocytosing effete cells, albeit with different kinetics (Ravichandran 2010). To the extent that it is virtually undetectable in the plasma membrane of epithelial cells, PA seems to be dispensable for phagocytosis by these cells. Thus, the specific phagocytic advantage that PA provides professional phagocytes with is not perfectly clear. One possibility is that, due to their role in combating highly motile and often lowly opsonized pathogens, professional phagocytes must themselves become ambulatory and rely on a more dynamic form of membrane extensions. In this manner, the uptake of apoptotic bodies by efferocytic epithelial/endothelial cells could be considered a more passive process than the phagocytosis of dangerous particulates by professional phagocytes. Indeed, dying cells secrete various find-me signals that serve to recruit phagocytes to sites of apoptosis (Ravichandran 2010), partially obviating the requirement for continuously probing the environment.
Also of interest is whether PA is required for membrane ruffling in other systems, such as endothelial cells. Future studies on wound healing responses could shed light on this topic. Preliminary observations in our laboratory indicate that human endothelial cells located at the wound edge extend dynamic membrane projections that are highly reminiscent of the membrane ruffles continuously projected by professional phagocytes. Expression of the PA biosensor in the endothelial monolayers would provide valuable information on whether cells at the wound edge upregulate plasmalemmal PA biosynthesis, and whether this facilitates collective cell migration and/or wound sealing.

Perhaps the most pressing, unresolved question arising from chapter 3 is the precise identity of the ligand(s) and GPCR(s) responsible for the constitutive biosynthesis of PA in professional phagocytes. Part of the answer to this may come from our observation that pertussis toxin, which ADP-ribosylates and inhibits the Gαi or Gαo subunits of heterotrimeric GPCRs (Mangmool and Kurose 2011), significantly reduces PA levels while abolishing membrane ruffling. Thus, attractive ligands include the nucleotides ATP and UTP, the chemokine fractalkine, as well as the bioactive lipids sphingosine-1-phosphate and lysophosphatidylcholine, all of which signal through Gαi- and Gαo-coupled receptors (Singh et al. 2010; Wolf et al. 2013; Kabarowski et al. 2001; van Doorn et al. 2012). However, our attempts to prevent or even slow down membrane ruffling by inhibiting nucleotide and sphingosine-1-phosphate receptors were to no avail. Thus, while find-me signals such as ATP and sphingosine-1-phosphate receptors facilitate efferocytosis by cells in the apoptotic neighbourhood, they do not seem to be required for the ongoing immune surveillance by professional phagocytes. Of note, inhibition of protein synthesis and serum starvation was also without effect.

While at the time of publication we were unable to find the identity of the upstream signal responsible for maintaining steady-state PA levels, we now have promising data suggesting that extracellular calcium may be the culprit: primary human macrophages express on their surface calcium-sensing receptors (7-transmembrane proteins couple to Gαi subunits), whose inhibition results in the ablation of membrane ruffling and macropinocytosis. Removal of extracellular calcium is equally deleterious to these responses. Remarkably, previous reports have indicated that calcium concentrations (which fluctuate considerably in extracellular fluids) are significantly elevated at sites of
cell death of infection (Olszak et al. 2000), raising the fascinating possibility that extracellular calcium acts as a chemokinetic agent that guides phagocytes to sites of inflammation. Future experiments on this topic could shed light on whether extracellular calcium dictates the proinflammatory responses of professional phagocytes, complementing the role of find-me signals in orchestrating efferocytosis within an immunologically silent context. Other studies should also address whether elevation of calcium at sites of necrosis facilitates the clearing of kamikaze phagocytes that have died while fighting infection, as well as aid in restoring epithelial barrier integrity following wounding.

7.2.2 Second study: Phosphoinositide 3-kinase and actin remodelling

According to the data presented in chapter 4, successful engulfment of large phagocytic particles is contingent on actin breakdown and the consequent recycling of cytoskeletal components, which may have become rate limiting. These results also indicate that spatiotemporal control of Rho GTPase signalling is fundamental to the organized progression of actin-driven pseudopodia; Rac/Cdc42 seem to inactivate at the base of the phagocytic cup—coinciding with the disappearance of F-actin—but remain active at the tips of pseudopodia, where actin filaments continue to polymerize. Thus, the inactivation of Rho GTPases at the initial sites of actin polymerization prevents further nucleation events, eventually leading to actin breakdown. However, the question of what catalyzes the actual dismantling of the actin meshwork still remains. Actin polymers are intrinsically stable, undergoing only slow subunit flux at steady-state (Pollard and Borisy 2003), and therefore actin-binding proteins that accelerate filament breakdown (e.g., severing factors) are necessary to allow for the rapid actin turnover observed during phagocytosis.

More studies are required in order to elucidate the molecular players that control the severing of existing actin networks. These molecules would complement the function of the RhoGAPs identified here, namely ensuring that no further filaments are nucleated. As mentioned previously, F-actin severing is mediated by members of the ADF/cofilin family, which associate with ADP-actin subunits and induce a conformational twist in the
filament that results in its disassembly. Interestingly, cofilin has been found to accumulate at the rear end of lamellipodia in migrating cells (Svitkina and Borisy 1999), as well as in actin-rich regions of phagocytic cups formed by J774.1 macrophages (Adachi, Takeuchi, and Suzuki 2002). It is likely that activated cofilin is recruited to sites of phagocytosis in order to promote severing of actin-rich regions, thereby providing new barbed ends for elongation and replenishing the monomeric actin pool.

Of Interest, Rho GTPases can indirectly control the activity of severing proteins through a number of effector proteins. For instance, GTP-bound Rac1 catalyzes the activating phosphorylation of LIM kinases by p21-activated kinase (PAK1), one the many Rac1 effectors (Edwards et al. 1999). In turn, LIM kinases inhibit the severing function of cofilin (Yang et al. 1998). Thus, active Rho GTPases increase the ratio of filamentous-to-globular actin not only by promoting actin nucleation, but also by inhibiting cofilin-mediated severing. More importantly, by inactivating Rac and Cdc42, RhoGAPs may release cofilin from LIMK-mediated inhibition, thereby facilitating actin severing at the cup. In this manner, the RhoGAPs identified in chapter 4 could act as chief regulators of actin dynamics during phagocytosis, not only by terminating polymerization of new filaments, but also because by indirectly catalyzing the dismantling of preexisting networks. Futures studies on this topic will hopefully indicate if this is in fact the case.

Other lines of investigation on RhoGAP activity at the phagosome may go beyond cytoskeletal rearrangements and onto membrane remodeling. For instance, a recent study revealed that the RhoGAP SH3BP1 is a binding partner of the exocyst (Parrini et al. 2011), an octameric complex that coordinates delivery of post-Golgi vesicles to sites of active plasmalemmal expansion (Rossé et al. 2006). The N-terminal BAR domain of SH3BP1 was necessary for its interaction with the exocyst (Parrini et al. 2011). Future experiments should be conducted to investigate whether SH3BP1 piggybacks on the exocyst in order to traffic to sites of particle engagement. Of note, PI3K-mediated signals trigger translocation of the exocyst subunit Exo70 to the plasma membrane (Dupraz et al. 2009), bolstering the notion that SH3BP1 recruitment to nascent cups relies on the exocyst. This is in contrast to the other 2 GAPs identified in this study, ARHGAP12 and ARHGAP25, which carry PH domains and may therefore translocate to phagocytic cups by anchoring to PtdIns(3,4,5)P₃ directly.
Consistent with our findings, silencing of SH3BP1 in migrating cells precluded the ability of Rac1 to cycle between active and inactive states at the leading edge, which in turn led to slower motility and aberrant morphodynamics (Parrini et al. 2011). Like the phagocytic cup, the leading edge of motile cells expands in a very rapid and explosive manner, inevitably rising membrane tension. The latter places a high demand on the delivery of endomembranes, which relieve said tension by fusing with the plasmalemma (Masters et al. 2013). Indeed, the role of SNARE-dependent delivery of endosomal and lysosomal membranes to incipient pseudopodia during phagocytosis of large particles is well established (Braun et al. 2004; Marion et al. 2012; Samie et al. 2013). Notably, a different study reported that depletion of the exocyst subunit Exo70 significantly impaired internalization of large particles (Mohammadi and Isberg 2013). Thus, due to their combined ability to promote actin breakdown and facilitate focal exocytosis through the exocyst, RhoGAPs may integrate cytoskeletal and membrane traffic machineries at sites of active membrane deformation. Future work may help determine if this is truly the case.

Lastly, existing data clearly implicates phosphoinositides and their effectors in actin-driven cellular processes that entail significant degrees of polarization, including chemotaxis, phagocytosis and immunological synapse formation (Allen et al. 1998; Yeung and Grinstein 2007; Le Floc’h et al. 2013). While we have learned a great deal about the molecular mechanisms that coordinate the extensive cytoskeletal changes behind these processes, most of our understanding is limited to activating signals, with the counteracting, inactivating responses often being neglected. For instance, previous reports indicated that a peripheral ring of F-actin forms on the T-cell surface during formation of the immunological synapse, and implicated the RhoGEF DOCK2 in this process. Provokingly, the disappearance of F-actin from the synapse’s core (giving rise to the F-actin ring), is accompanied by an annular accumulation of PtdIns(3,4,5)P3. Indeed, the morphological and cytoskeletal similarities between the immunological and phagocytic synapse (as seen during frustrated phagocytosis, which occurs on a 2-dimensional plane) are striking. Studies on whether the RhoGAPs described on chapter 4 exert an analogous function in the immunological synapse could yield fascinating insights.
7.2.3 Third study: Diacylglycerol kinases and respiratory-burst heterogeneity

As described in chapter 5, considerable heterogeneity in NADPH oxidase (NOX) activity seems to exist across different phagosomes—not only between different cells but also within an individual cell. Moreover, the respiratory burst does not appear to be a binomial, all-or-nothing response; instead, we observed different degrees of superoxide generation in NOX-responsive phagosomes. Mechanistically, our studies indicate that the variability in NOX activity is driven by heterogeneous levels of phagosomal diacylglycerol (DAG), in turn leading to differential activation of cytosolic NOX subunits. By controlling phagosomal DAG levels (and therefore PKC activity), DAG kinases seem to be ultimately responsible for the heterogeneous nature of the respiratory burst in macrophages.

What causes DAG kinases to behave differently from phagosome to phagosome? Conceivably, heterogeneity could be brought by asynchrony in the cell cycle or by loss of clonality. We regard these possibilities as unlikely, given that variable DAG biosynthesis was routinely detected in phagosomes within the same cell. A more likely explanation is that phagocytic receptors are not engaged to the same extent by all targets. While flow cytometry analysis confirmed homogeneous opsonization, we cannot rule out the possibility that the affinity, lateral mobility and overall access of phagocytic receptors to their ligands are invariable. Alternatively, it would be worth investigating whether the membrane-associated, catalytic subunits of the oxidase distribute in an equivalent manner to different phagosomes. Given that a finite number of catalytic subunits exist in the cell, NOX deployment could be triaged on a “first-come, first-served” basis. It should be noted, however, that presence does not necessarily imply activity. Thus, as long as the signals lying upstream of the oxidative complex prove insufficient, phagosomes will be unable to generate superoxide in their lumen, even if displaying NOX catalytic subunits at their membranes.

An important consequence of the respiratory burst being heterogeneous is that microbes residing in NOX-unresponsive phagosomes would be more likely to survive the onslaught normally brought by phagosome maturation. Moreover, the heterogeneity in NOX activity is likely compounded by the variable behaviour of live pathogens, even if
belonging to the same species. This is especially so because of the several virulence strategies dedicated to hijacking host cell biology, all of which may be deployed at different times and with different intensities. Nonetheless, experiments addressing the effects that modulating phagosomal DAG levels (and therefore superoxide production) have on bacterial survival would be particularly informative on this regard, and would likely complement our understanding of what allows certain pathogens—but not others—to survive phagosomal assault.

7.2.4 Fourth study: Phosphatidylinositol-3,4,5-trisphosphate and gliotoxin toxicity

In the last study of this thesis (chapter 6), I provide evidence that the Aspergillus fumigatus virulence factor gliotoxin undermines phagocyte immune defences by subverting phosphoinositide signalling. The effects of gliotoxin on the execution of actin-driven cellular processes, including phagocytosis and membrane ruffling, are rapid and striking. Indeed, gliotoxin exposure keeps macrophages from dynamically probing their environment, and essentially obliterates phagocytosis of unopsonized fungal particles. Thus, gliotoxin secretion seems to facilitate colonization of host tissue by preventing professional phagocytes from recognizing and eliminating fungal spores. Having avoided immune recognition, these spores germinate into hyphal networks that invade pulmonary tissue and go on to secrete a number of mycotoxins.

However, given that gliotoxin is only secreted by metabolically active hypha, the chronology of events would require an already ongoing Aspergillus infection in order for gliotoxin to be secreted and inhibit phagocytosis of fungal spores. In other words, as long the Aspergillus spores are kept from germinating, tissue-resident macrophages will not be subjected to gliotoxin toxicity and should, in principle, be capable of readily eliminating them. What, then, leads to the initial germination of conidia and the establishment of infection? An answer to this comes from the observation that healthy individuals are rarely susceptible to infection with A. fumigatus. Instead, this opportunistic pathogen frequently invades the lungs of immunosuppressed patients or individuals with respiratory conditions. For instance, an impaired mucociliary escalator
(as if often seen in patients with cystic fibrosis) prevents the efficient expulsion of inhaled spores, heightening the fungal burden that alveolar macrophages must contend with at all times. If macrophage defences become overwhelmed, then some of the inhaled spores germinate into metabolically active forms capable of secreting gliotoxin and other mycotoxins. The resulting immunosuppressive environment is an ideal niche for further *Aspergillus* spread and growth.

While we have restricted our observations of gliotoxin toxicity to a single fungal pathogen, it would be of interest to examine the consequences of gliotoxin exposure on the recognition, phagocytosis and degradation of other microbes. Given that the toxin interferes with the proper functioning of the actin machinery—which is a universal component of all phagocytic modalities—it is possible that other intracellular pathogens would greatly benefit from the disruption of actin networks by gliotoxin. In that respect, it is noteworthy that the cystic fibrosis lung is notoriously susceptible to polymicrobial infections (Sibley, Rabin, and Surette 2006), a condition in all likelihood worsened by the secretion of globally immunosuppressive mycotoxins.

Interestingly, a recent study reported that rapid and efficient clearance of apoptotic cells in the bronchial epithelium is critical for averting excessive inflammation and managing the course of allergic reactions (Juncadella et al. 2013). That study showed that, if apoptotic epithelial cells are prevented from being efferocytosed by the neighbouring airway epithelium, then anti-inflammatory cytokine production becomes dysregulated and inflammation exacerbates. This type of airway hypersensitivity is all-too-common in the *Aspergillus* symptomatology, and presents as allergic bronchopulmonary aspergillosis (ABPA). The observation that gliotoxin subverts PtdIns(3,4,5)P₃ metabolism could increase our understanding of the pathophysiology of ABPA, of which we know relatively little. Specifically, it would be valuable to explore whether chronic exposure to gliotoxin results in apoptosis of airway epithelial cells, and whether these corpses fail to be efferocytosed by neighbouring or professional phagocytes whose actin dynamics have been impaired by gliotoxin. Even the ciliary beating of the mucociliary escalator may be impaired by gliotoxin, further complicating the infection. Future studies may help to clarify which, if any, of these notions are true.
7.3 Concluding remarks

Phagocytosis is a complex phenomenon and a veritable compendium of molecular and cellular biology entailing receptor activation, signal transduction, cytoskeletal remodelling and membrane traffic. Signalling lipids—phosphoinositides in particular—seem to feature importantly in every one of the response components: they influence receptor distribution and responsiveness, convey signals to downstream adaptors and effectors, orchestrate cytoskeletal rearrangements and mediate membrane fusion and fission events. Signalling lipids also control the acquisition of an impressive antimicrobial armamentarium by the maturing phagosome. The identity of the evolving compartments generated by phagocytosis is itself determined and defined by phosphoinositide composition, whether by directing stereospecific interactions with ligands or by dictating the surface charge that drives electrostatic interactions with cationic proteins. The paramount nature of phosphoinositides in orchestrating phagocyte defences has made these signalling lipids an attractive target for an expansive range of microbial virulence factors, including fungal toxins.

Given their unique size, phagosomes lend themselves to the study of sub-organelar domains, which have been difficult to resolve in other endocytic and secretory compartments. The advent of super-resolution microscopy, in combination with correlative ultrastructural analysis, should make such studies feasible in the near future, yielding fascinating insights.


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