Characterization of F-actin flashes on phagosomes in macrophages

by

Mathieu Bisnaire Poirier

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

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Abstract

Macrophages are specialised leukocytes tasked with the engulfment and destruction of foreign particles. We are studying particle uptake by the CR3 receptor in macrophages. Post particle internalization, we have shown that F-actin localizes transiently at phagosome membranes and this event, termed F-actin flashing, often results in red blood cell deformation, lysis and occasionally fission, suggestive of a force-generating property. Putative mechanosensing properties were also proposed based on identification of certain phagosome membrane-localized proteins, as well as distinctions in temporal dynamics based on the internalized particle’s malleability. We have also reported that certain stages of maturation were delayed in phagosomes exhibiting F-actin flashing. Finally, inhibiting myosin IIA activity significantly reduced the frequency at which red blood cell-containing phagosomes became deformed simultaneously with transient F-actin accumulation. Actomyosin-driven contractions could serve to physically distort malleable particles, akin to “chewing”, which may have implications in phagosome maturation, as well as antigen presentation.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AFU</td>
<td>Arbitrary fluorescence unit</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidine-2′-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable region</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanosine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine trisphosphate</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosome-associated membrane glycoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MASP</td>
<td>Mannose-binding lectin-associated serine protease</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>NMHC</td>
<td>Non-muscle myosin heavy chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PI(3,4,5)P3</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
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<tr>
<td>PIP5K</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
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</table>
1 Introduction

1.1 Host immunity

Higher eukaryotic organisms have evolved various forms of protection from potentially harmful exogenous sources (Cooper and Alder, 2006; Parkin and Cohen, 2001). Host immunity is categorized into two major branches: the innate response and the adaptive response. The former is found in most multicellular organisms, whereas the more sophisticated adaptive immune system is found exclusively in jawed vertebrates or gnathostomes (Cooper and Alder, 2006). Physical barriers such as epithelial cells bound by tight-junctions as well as mucosal membranes coating the epithelium on internal organs, usually form the first line of defense against invading pathogens (Chaplin, 2010; Delves and Roitt, 2000). Once the pathogen finds itself inside of the organism, specific molecules on its surface will first trigger a rapid response by the innate immune system, inducing inflammation (Janeway and Medzhitov, 2002). Only if the pathogen cannot be cleared by the innate arm of the immune system will the adaptive immunity be elicited (Janeway and Medzhitov, 2002). Although slower to react, it displays much greater specificity towards the target if it had been previously encountered (Janeway and Medzhitov, 2002). In all, the immune system has evolved to address the caveats associated with more complex forms of life, where some vulnerabilities can be exploited by simpler organisms for their survival and proliferation.

1.1.1 Innate immunity

In contrast to adaptive immunity, the functions of innate immunity are encoded in the germline of organisms, giving it broad and promiscuous recognition capabilities (Chaplin, 2010). Despite this property, the innate immunity responds quickly to exogenous targets, before the engagement of the adaptive immune system. The innate immunity can make use of structures such as toll-like receptors (TLRs) to recognize PAMPs (pathogen-associated molecular patterns) to recognize molecular targets shared among a multitude of pathogens and other exogenous entities (Kawai and Akira, 2010; Takeuchi and Akira, 2010). These targets include lipids only found in bacteria such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, as well as permutations of
exogenous nucleic acids, such as viral dsDNA and unmethylated DNA (CpG motifs) (Hemmi et al., 2000; Hornung and Latz, 2010; Medzhitov, 2007; Park et al., 2009). As part of the innate immunity, the complement system initiates a cascade of proteolytic activity to effectively lyse microbes by forming pores in their cell membranes, allowing osmosis to inflict critical damage. These pores are formed by membrane attack complexes (MACs) (Carroll, 1998). The complement system has three primary activation routes: classical, alternative and the lectin pathway. Classical activation occurs by antibody-antigen binding to C1q, whereas alternative activation is induced when C3b, a cleavage product of C3, is deposited onto a target particle’s surface membrane. Finally, the lectin pathway is elicited by mannose-binding lectin (MBL)-binding of mannose or other bacteria-related carbohydrates (such as ficolins) at the surface of bacterial membranes, thereby activating MASPs, and in turn, C2 and C4 (Fujita, 2002; Merle et al., 2015; Walport, 2001). Components of the complement pathway circulate in the plasma serum, and are recruited upon activation by one of the methods described earlier. Over the course of this investigation, C3bi (in conjunction with the pentameric immunoglobulin IgM) was the main opsonin used to induce phagocytic intake.

1.1.2 Adaptive immunity

B and T lymphocytes, a subset of leukocytes originating from common lymphoid progenitor cells, represent the major effectors of the adaptive immune response. B cells are responsible for the mass production of antibodies during infections, whereas a subset of T cells (cytotoxic T cells) are endowed with properties to destroy compromised cells. Both cell types can be identified by the various receptor molecules on their surface that tend to form clusters – termed clusters of differentiation (CD) – based on exposure to select groups of monoclonal antibodies (LeBien and Tedder, 2008). One of the advantageous properties of the adaptive immunity lies in its ability to form “memories” of previously encountered pathogens. It does so by somatically rearranging genes in B-cells and T-cells coding for specific immunoglobulins and T-cell receptors (TCR) respectively (Parkin and Cohen, 2001). This allows binding of very particular epitopes of proteins expressed in invading pathogens. In the case of B cells, clonal selection ensures that the appropriate antibody is produced in high enough quantities. Clonal selection is mediated by three gene segments coding for different structural components of
immunoglobulins. The IGH segment codes for the heavy chain, whereas IGK and IGL segments code for κ and λ light chains respectively (Delves and Roitt, 2000). Each of these gene segments code for components comprising the heavy and light chains. IGH and IGK/IGL loci have V (variable), J (joining) and C (constant) genes, but only IGH has the D (diversity) segment (Delves and Roitt, 2000). Combinations of these variables allow for a very wide range of expressed immunoglobulins (D G Schatz et al., 1992; Tonegawa, 1983). The recombination of these genes is mediated by RAG-1 and RAG-2 genes, along with other nucleases and ligases (Oettinger et al., 1990; Schatz et al., 1989). If a certain antibody is produced and binds with an epitope on the target particle with great affinity, B cell proliferation will be induced, thereby generating multiple clones, each producing identical monoclonal antibodies (Delves and Roitt, 2000). These antibodies will ensure that a secondary encounter with the same pathogen will do minimal harm done by greatly increasing the immune response’s effectiveness, ultimately clearing the invader from the organism.

1.2 Macrophages

Macrophages are professional phagocytes of the innate immune system. In contrast to B and T cells, they are differentiated from myeloid progenitor cells, and subsequently monocytes by exposure to macrophage colony-stimulating factor (M-CSF). Macrophages can undergo either classic or alternative activation prior to binding and internalizing opsonized targets. Classical activation can occur by the cytokines interferon gamma (IFNγ) produced from T_{H1} lymphocytes, as well as interleukins 12 and 18 (IL-12, IL-18) from antigen-presenting cells (APCs). IFNγ serves to prime macrophages before receiving bacterial signals such as lipopolysaccharides (LPS) (Schroder et al., 2004). In turn, classically-activated or M1 (pro-inflammatory) macrophages produce various cytokines; interferon gamma (IFNγ), interleukin 6 and 12 (IL-6, IL-12), as well as tumor necrosis factor (TNF) (Benoit et al., 2008). Conversely, alternative activation of macrophages occurs by exposure to interleukin 4 and 13 (IL-4, IL-13). The resulting macrophage phenotype is termed M2 (anti-inflammatory) (Benoit et al., 2008). Both interleukins bind to the same receptor called IL-4Rα (Gordon, 2003). Furthermore, multiple phenotypical differences exist between classically- and alternatively-activated macrophages. For example, different surface receptors are expressed, including mannose receptors and MHC class
II molecules (Gordon, 2003). All effector cells (leukocytes) of the immune system originate from bone marrow. Hematopoiesis represents the differentiation process from hematopoietic stem cells (HSCs) to functionally differentiated leukocytes. Myeloid progenitor cells are differentiated into monocytes before becoming fully functional macrophages. Macrophage colony-stimulating factor (M-CSF) is the primary cytokine responsible for the progression of this differentiation pathway (Metcalf, 1986).

1.3 Phagocytosis

Phagocytosis is the engulfment of particles ≥0.5 µm in size. Its evolution can be traced back to eukaryogenesis, when archaea internalized bacterial precursors to mitochondria and chloroplasts (Yutin et al., 2009). It has functions spanning from homeostasis regulation by apoptotic cell clearance, and in the immune system, both innate and adaptive, to engulf and degrade pathogenic particles. Phagocytosis is one of many known processes that utilize actin to generate various forms of plasma membrane protrusions, catering to different scenarios. Activation of the Rho family members of GTPases to induce actin remodelling is required in phagocytosis, but not in other modes of particle internalization, such as endocytosis or pinocytosis (Etienne-Manneville and Hall, 2002).

1.3.1 FcγR-mediated phagocytosis

FcγR-mediated phagocytosis is triggered by the binding of a target particle opsonized by IgG to Fc receptor, linked to ITAMs (immuno-receptor tyrosine-based activation motifs) of Fcγ receptors. ITAMs contain YxxI/L consensus sequences Upon this binding event, proteins of Src family will phosphorylate the γ dimers on these aforementioned sequences, provoking the recruitment of Syk tyrosine kinase (Crowley et al., 1997; Greenberg and Grinstein, 2002; Aderem and Underhill, 1999). This kinase will then phosphorylate the C-terminal SH2 domain of p85 regulatory subunit of PI3K, responsible of the conversion of the phosphoinositide PI(4,5)P₂ to PI(3,4,5)P₃ (Cantley, 2002; Di Paolo and De Camilli, 2006; Gu et al., 2003; Kiefer et al., 1998; Moon et al., 2005). Syk will also activate PLC to hydrolyse PI(4,5)P₂ into IP₃ and
DAG. Small GTPases recruited to remodel actin include Rac1 and Cdc42 to form lamellipodia and filopodia respectively (Heasman and Ridley, 2008; Hoppe and Swanson, 2004; Sit and Manser, 2011).

1.3.2 Complement receptor 3-mediated phagocytosis

The main opsonin recognized in CR3-mediated phagocytosis is the inactive hydrolytic product of C3b; C3bi, formed by cleavage of C3b by serum Factor I, aided by serum Factor H (Gitlin et al., 1975; Merle et al., 2015; Nilsson and Müller-Eberhard, 1965; Weiler et al., 1976; Whaley and Ruddy, 1976; Wu et al., 2009). The pentameric structure of IgM has been shown to ease the binding of C3bi to CR3 (Bjornson and Detmers, 1995). The C3bi receptor is CR3, an αMβ2 integrin, also known as Mac-1 (macrophage-1 antigen) or CD11b/CD18 (Beller et al., 1982; Ehlers, 2000). Integrins require a priming step before their affinity can be increased enough to allow binding with the appropriate ligand (Flannagan et al., 2012). In physiological conditions, this priming step would be undertaken by diacylglycerol (DAG), a hydrolysis product of PI(4,5)P2 (Kishimoto et al., 1980; Nishizuka, 1984). DAG effectively binds and activates protein kinase Cα (PKCα) to effectively prime Mac-1 by the phosphorylation of its β2 subunit (CD18) (Buyon et al., 1990; Chatila et al., 1989; Rabb et al., 1993). Complement receptor 3 will thus change from an inactive or “bent” conformation to an active or “extended” conformation, allowing for ligand binding (Luo et al., 2007; Nishida et al., 2006; Shattil et al., 2010). Protein kinase Cα (PKCα) is a “classical” or “conventional” PKC isoform containing a C1 domain for DAG-binding, an N-terminal regulatory domain for phosphatidylinerine (PS)-binding, and finally, a C2 domain for Ca2+-dependent PI(4,5)P2-binding (Lim et al., 2015). In experimental settings, phorbol esters such as PMA (phorbol 12-myristate 13-acetate) are used to activate this kinase, and it turn, CR3 (Wright and Silverstein, 1982). As previously mentioned, C3bi is the main ligand for CR3 and it is known that the divalent cations Mg2+ and Ca2+ enhance CD11b (subunit of CR3)-binding to C3bi (Ross et al., 1983; Ueda et al., 1994; Wright and Silverstein, 1982). As a downstream effector of the Mac-1 integrin signaling pathway, the small GTPase RhoA is involved in cytoskeletal rearrangement. Unlike other members of the Rho family of GTPases such as Cdc42 and Rac1, RhoA plays an active role in CR3-mediated phagocytosis (Bishop and Hall, 2000; Caron and Hall, 1998; Mao and Finnemann, 2015). Rho-GTP levels increase when
Mac-1 integrins are engaged; this is not the case for other small GTPases such as Rac-GTP (Wiedemann et al., 2006). RhoA also has a role in the induction of the formin mDia1 through mediation of ROCK activity exclusively in CR3-mediated phagocytosis (Colucci-Guyon et al., 2005). ROCK, along with myosin II, also has a role in phagocytic cup formation in the aforementioned phagocytic pathway (Olazabal et al., 2002). The final step involves the “sinking” of the C3bi-opsonized particle in the plasma membrane (Patel and Harrison, 2008).

### 1.3.3 Phagosome maturation

Once internalized, a target particle will reside in a vesicular structure termed the phagosome. Over time, the composition of both the phagosome membrane and the phagosome lumen will be altered to create a destructive environment for the internalized particle. Phagosome maturation has only been studied in detail for FcγR-mediated phagocytosis. Multiple effectors will sequentially bind to the phagosome, as well as vesicular compartments. Included among those vesicular compartments are early and late endosomes. Rab5 and Rab7 and LAMP1 are prominent proteins on involved in phagosome maturation. LAMP1/2-knockout cells proceeded normally through phagosome maturation, until the later stages where Rab7 recruitment and lysosome fusion became inhibited (Huynh et al., 2007). Lysosomes should eventually fuse with phagosomes to form phagolysosomes (Pitt et al., 1992; Vieira et al., 2002).

### 1.4 Actin cytoskeleton

The cytoskeleton allows for cells to resist external forces such as compression or tension. The three major components of the cytoskeleton include microtubules (MTs), intermediate filaments and filamentous actin (F-actin) (Fletcher and Mullins, 2010). Remodelling of F-actin structures forms the basis for cell migration, extracellular particle engulfment, and other important processes. In this study, we have focused primarily on this cytoskeletal component due to the nature of F-actin flashing. Many intracellular structures rely on this microfilament, among them are stress fibers (actomyosin bundles), cortical actin networks, branched actin networks, and various membrane protrusions or extensions (Bezanilla et al., 2015; Blanchoin et al., 2014).
Membrane protrusions rich in F-actin, many of them crucial in phagocytosis (Swanson, 2008), include lamellipodia (Rougerie et al., 2013), filopodia (Mattila and Lappalainen, 2008), invadopodia (Murphy and Courtneidge, 2011), and finally membrane ruffles (during CR3-mediated phagocytosis) (Saarikangas et al., 2010).

1.4.1 Actin structure & dynamics

Actin is a ubiquitous protein, and is found in the form of globular actin (G-actin) in the cytosol. Three isoforms of G-actin, α, β and γ, have been described. The β and γ isoforms are found in both muscle and non-muscle cells (Dominguez and Holmes, 2011). G-actin can assemble spontaneously into F-actin, but does so inefficiently, unless the critical concentration of G-actin (around 0.1 µM), has been reached (Bezanilla et al., 2015). Typically, the cytoplasmic concentration of G-actin is ≥ 100 µM, therefore, a mechanism is required to prevent persistent formation of intracellular F-actin (Pollard et al., 2000). This can be done with the help of G-actin-binding proteins, such as profilin (Carlsson et al., 1977). Profilin partly controls G-actin cytoplasmic levels through alterations in the binding of phosphoinositides, mainly PI(4,5)P₂ (Lassing and Lindberg, 1985). PI(4,5)P₂ synthesis can be stimulated by small GTPases, such as Rac1 and RhoA, having PIP5K-binding capabilities (Sit and Manser, 2011; Weernink et al., 2004). In the case of RhoA, it can modulate actin reorganization through the disruption of the autoinhibitory domain of the mDia1 formin, allowing for stress fiber formation stabilized by ROCK activity (Watanabe et al., 1999). Actin filaments are polarized; the (+) and (-) tips are noted as barbed end and pointed end respectively (Dominguez and Holmes, 2011; Fletcher and Mullins, 2010; Wegner and Isenberg, 1983). G-actin monomers bound to ATP will bind the barbed end, hydrolyzing the latter and effectively elongate the actin filament, while the resulting ADP will dissociate from the pointed end, a process called “treadmilling” (Blanchoin et al., 2014; Pollard and Borisy, 2003). To aid in the rapid assembly of F-actin, certain nucleating agents can be recruited. Arp2/3 has a high affinity for the pointed ends of F-actin, effectively capping them, while increasing the elongation rate at barbed ends (Mullins et al., 1998). It also has the capacity to link the pointed ends of actin filaments to the sides of others at a fixed angle of 70°, creating a branched F-actin network (Mullins et al., 1998). Wiskott-Aldrich syndrome protein (WASP) is known to bind Arp2/3 through the carboxy-terminal domain of the latter’s
p21-Arc component (Machesky and Insall, 1998). It is an effector of both Cdc42, a Rho family member of GTPases, and of PI(4,5)P$_2$ (Higgs and Pollard, 2000; Symons et al., 1996).

### 1.4.2 Focal adhesions

Focal adhesion (FA) is a broad term used to describe membrane-associated protein complexes with mechanosensing properties, providing an interface between the extracellular matrix (ECM) and the cytoskeleton (Gardel et al., 2010; Parsons et al., 2010). Integrins, heterodimeric protein complexes, are a major part of the structure, as well as the signaling layer in focal adhesions, along with focal adhesion kinase (FAK) (Kanchanawong et al., 2010). Other components include talin and vinculin, which transduce mechanical forces to the cytoskeleton from the ECM (Kanchanawong et al., 2010). Talin interactions with vinculin mediate focal adhesion formation in part by the N-terminal portion of talin’s head domain binding the cytoplasmic tail of β integrins to F-actin (Calderwood et al., 1999; Humphries et al., 2007). Talin’s FERM domain also has the ability to bind PIPKIγ, generating PI(4,5)P$_2$ near focal adhesions (Di Paolo et al., 2002; Ling et al., 2002). The FERM domain of phosphorylated FAK (Tyr397) is capable of directly binding the Arp3 subunit of Arp2/3, thus influencing actin-driven membrane rearrangements, such as lamellipodia formation (Serrels et al., 2007). Due to the mechanosensing properties of focal adhesions, the substrate stiffness on which cells grow can modulate a cell’s migratory behaviour, among other parameters (Plotnikov and Waterman, 2013). Focal adhesions assembled in cells plated on stiff surfaces had normal levels of phosphotyrosine and a stable structure, whereas those on malleable substrates showed more dynamic focal adhesions with atypical morphology (Pelham and Wang, 1997). Furthermore, studies have shown that FAK-/- neutrophils had reduced phagocytic index in CR3-mediated phagocytosis, as well as impaired PI(4,5)P$_2$ signaling (Kasorn et al., 2009). FAK is normally autoinhibited by FERM domain, but is relieved from its inhibited state by PI(4,5)P$_2$, which partially opens FAK conformation to ease autophosphorylation (Cai et al., 2008; Zhou et al., 2015). This allows Src family of kinases to recruited, fully releasing FAK from autoinhibition (Calalb et al., 1995; Goñi et al., 2014). The FAT domain of FAK interacts with integrins on the plasma membrane (Zhou et al., 2015). Finally, it has been determined that mechanical forces have a direct impact on FAK activation (Plotnikov and Waterman, 2013).
1.4.3 Previous studies investigating actin on phagosome membranes

F-actin flashing was first described in Madin-Darby canine kidney (MDCK) epithelial cell phagosomes containing *Listeria monocytogenes* (Yam and Theriot, 2004). It was found that certain bacteria strains with modified endogenous proteins, such as DP-L2319 Δhly ΔplcA ΔplcB (unable to escape phagosomes), DP-L973 (reduced ActA expression), and finally DP-L3078 ΔactA (lacking cofilin homology sequence) strains (Skoble et al., 2000, 2001), did not alter the dynamics of the F-actin flashing phenotype (Yam and Theriot, 2004). Based on those observations, the authors concluded that the origin of the F-actin flashes was independent of the influence of bacteria in phagosomes. This hypothesis was further investigated by testing other mechanisms of particle entry to determine if they would illicit the same phenomenon. Neither clathrin-mediated entry, internalin(InlA)-dependent entry, nor invasin(InvA)-mediated entry (using *Escherichia coli* expressing invasin – a *Yersinia pseudotuberculosis* protein) had any impact on F-actin flashing (Yam and Theriot, 2004). This suggested that the occurrence of this phenotype was a result of a signaling cascade localized at the phagosome membrane of phagocytosed particles, as opposed to an exogenous process. It was also noted that membrane shape perturbations could induce F-actin flashing (Yam and Theriot, 2004). More recently, F-actin flashing was characterized in RAW 264.7 mouse macrophages expressing actin-GFP and internalizing IgG-opsonized latex beads (3 µm) (Liebl and Griffiths, 2009). When macrophages became overloaded with phagosomes (increased phagocytic load), a greater proportion of the phagosomes were prone to F-actin flashing, effectively reducing the rate of maturation, shown by lowered LAMP2 recruitment (Liebl and Griffiths, 2009). In addition, phagosome membrane curvatures presented a surface that was favourable for actin nucleation (Liebl and Griffiths, 2009). Finally, it was found that F-actin flashes exert forces on macropinosomes and even phagosomes, causing occasional fission of the former (Liebl and Griffiths, 2009).

1.5 Rationale and Objectives

As previously stated, other research groups have described F-actin flashes on phagosomes to a limited extent. I have set forth novel objectives in areas that were either overlooked, or less elaborated regarding F-actin flashing. Previous studies have opsonized target particles mostly
with IgG, thereby triggering FcγR-mediated phagocytosis. Furthermore, none of the studies attempted to determine if the engulfed particle’s rigidity was a factor in F-actin flashing temporal dynamics. Lastly, no clear function has been attributed to F-actin flashing on phagosomes. In this thesis, I provide some novel insight into F-actin flashing on phagosomes by: characterizing the F-actin flashing phenotype along with effectors involved specifically in CR3-mediated phagocytosis, assessing the effects of target particle rigidity on F-actin flashing, and finally, attempting to attribute a function of this phenomenon to phagosome maturation. I have given special attention to contractions seemingly due to F-actin flashing, causing occasional lysis and/or fission of red blood cells. This property was of interest due to its potential implications in either antigen presentation or increased effectiveness of enzymatic degradation. I hypothesized that forces exerted by F-actin flashes may have a role in one or both of these events during phagosome maturation – akin to chewing prior to digestion (Fig. 1). Utilizing live cell imaging and immunofluorescence, I was able to monitor and analyse F-actin flashing parameters; increasing our understanding of the mechanism and effectors behind this phenomenon.

Figure 1. Visualization of the concept behind the increase in surface area of a particle, occurring post-F-actin flashing on phagosomes. Representative three-dimensional particle before and after F-actin flashing has occurred.
2 Materials and Methods

2.1 Reagents and antibodies

Dulbecco’s Modified Eagle Medium (DMEM) as well as fetal bovine serum (FBS) were purchased from Wisent Inc. (St-Bruno, QC). GIBCO® phosphate-buffered saline (PBS) was purchased from ThermoFisher Scientific Inc. (Waltham, MA). RBC lysis buffer and macrophage colony-stimulating factor (M-CSF) were purchased from Sigma-Aldrich Canada Co. (Oakville, ON). Mouse monoclonal anti-PKCα (H-7), mouse monoclonal anti-RhoA (26C4), mouse monoclonal anti-FAK (D-1), rabbit polyclonal anti-phospho-FAK (Tyr397), mouse monoclonal anti-WASP (B-9), rat monoclonal anti-LAMP1 (1D4B), and goat polyclonal anti-EEA1 (N-19) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Rabbit polyclonal anti-p34-Arc/ARPC2 antibody was purchased from EMD Millipore Canada Ltd (Etobicoke, ON). Rabbit polyclonal anti-non-muscle myosin heavy chain II-A antibody was purchased from BioLegend® (San Diego, CA). Mouse monoclonal anti-talin antibody was purchased from Sigma-Aldrich Canada Co. (Oakville, ON). Complement C5-deficient human serum was purchased from Sigma-Aldrich Canada Co. (Oakville, ON). AffiniPure donkey polyclonal anti-rabbit, donkey anti-mouse and donkey anti-rat cyanine Cy™2, as well as AffiniPure donkey polyclonal anti-rabbit and donkey anti-human cyanine Cy™5 secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). FuGENE® HD transfection reagent was purchased from Promega Corporation (Madison, WI). Blebbistatin, ROCK inhibitor (Y-27632), 4’,6-Diamidine-2’-phenylindole dihydrochloride (DAPI), as well as phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich Canada Co. (Oakville, ON). Washed and preserved sRBCs were purchased from MP Biomedicals, LLC (Santa Ana, CA). Non-functionalized 3.87 µm crosslinked polystyrene divinylbenzene beads P(S/2%DVB) were purchased from Bangs Laboratories Inc. (Fishers, IN) through CEDARLANE® Corporation (Burlington, ON). Fluorescence mounting media was purchased from Dako, Agilent Technologies Canada Inc. (Mississauga, ON) through CEDARLANE® Corporation (Burlington, ON). Alexa Fluor® rhodamine phalloidin was purchased from ThermoFisher Scientific Inc. (Waltham, MA).
2.2 DNA Constructs and cell transfection

The GFP-C1-PLCδ-PH construct was a gift from Tobias Meyer (Addgene plasmid # 21179). RAW 264.7 macrophages stably transfected with Lifeact-CFP constructs (cerulean; a gift by Ray Truant (McMaster University, Hamilton, ON)) and those stably transfected with Lifeact-RFP constructs were produced in the lab by Dr. He Song Sun (University of Toronto Scarborough, Toronto, ON). Transformed *Escherichia coli* with GFP-C1-PLCδ-PH constructs were propagated by inoculating cells in Luria broth (LB) overnight on a shaker at 37 °C and subsequently plating agar gels, both containing 50 µg/mL of kanamycin for selection. Purification of GFP-C1-PLCδ-PH plasmids was done using PureLink® HiPure Plasmid Filter Maxiprep Kit (ThermoFisher Scientific Inc., Waltham, MA), while following the manufacturer’s instructions. Chemically competent MAX Efficiency® DH5α™ *Escherichia coli* (ThermoFisher Scientific Inc., Waltham, MA) were used for bacterial transformation to amplify GFP-C1-PLCδ-2PH. The bacterial transformation was done using the standard heat-shock procedure recommended by Addgene (Cambridge, MA).

Chemical transfections of macrophages were done with FuGENE® HD in 35 mm glass-bottom dishes. The FuGENE® HD reagent (4 µL/dish) was added to a solution containing 100 µL/dish of Opti-MEM® I Reduced Serum Medium, without phenol red, (ThermoFisher Scientific Inc., Waltham, MA), along with 2.5 µg/dish of plasmid DNA. The solution was incubated at room temperature with frequent shaking for 21 minutes before adding 100 µL of the latter to each glass-bottom dish containing RAW 264.7 macrophages (40 % confluence).

Electroporation-based transfections were performed with a Neon® transfection system (ThermoFisher Scientific Inc., Waltham, MA), following the manufacturer’s instructions. Briefly, 3 µg of LifeAct-RFP plasmid DNA was added to $1 \times 10^7$ cells/mL in suspension buffer. A single 20 ms pulse at 1680 V was applied for each loaded 10 µL Neon® pipette tips containing $1 \times 10^5$ cells. The cells were then plated on 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA).
2.3 Cell culture

RAW 264.7 mouse macrophages were purchased from American Type Culture Collection (ATCC®; Manassas, VA). The cells were incubated in complete DMEM with 10 % heat-inactivated fetal bovine serum (FBS) at 37 °C, 5 % CO₂ and 90 % relative humidity in T75 flasks (Sarstedt Inc., Montréal, QC). They were grown until ~80-90 % confluence before each passage. For passing and plating, cells were washed twice with 1X PBS and lifted using cell scrapers (Sarstedt Inc., Montréal, QC). For plating purposes, cells were counted using a Bright-Line™ hemocytometer (Sigma-Aldrich Canada Co., Oakville, ON). When plating on 35 mm dishes, $5 \times 10^5$ RAW 264.7 macrophages were plated two days prior to phagocytosis assays.

2.4 Bone marrow extraction and monocyte differentiation

Primary bone marrow-derived macrophages (BMDMs) were obtained by bone marrow extraction from the femurs and tibias of C57BL/6 mice. The RBCs contained in the isolated samples were lysed using RBC lysis buffer (Sigma-Aldrich Canada Co., Oakville, ON). Progenitor cells were incubated in DMEM and 10 % heat-inactivated FBS, along with 100 IU/mL of penicillin/streptomycin (ThermoFisher Scientific Inc., Waltham, MA) and 25 ng of M-CSF in a T75 flask (Sarstedt Inc., Montréal, QC). The next day, floating cells were transferred to a new T75 flask with 10 mL of complete DMEM while adherent cells were discarded. On the same day, the amount of M-CSF in complete DMEM was increased to 100 ng. Differentiated primary macrophages were transfected with Lifeact-RFP constructs donated by Ray Truant (McMaster University, Hamilton, ON) by electroporation using a Neon® transfection system (ThermoFisher Scientific Inc., Waltham, MA), following the manufacturer’s instructions. Briefly, 3 µg of LifeAct-RFP plasmid DNA was added to $1 \times 10^7$ cells/mL. A single 20 ms pulse (1680 V) was applied for each loaded 10 µL Neon® pipette tips containing $1 \times 10^5$ cells. The cells were then plated on 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA).
2.5 Phagocytosis assays

RAW 264.7 macrophages were incubated in serum-free DMEM at 37 °C, 5 % CO₂ and 90 % relative humidity. Prior to opsonization, 100 µL of sRBCs (10 % suspension) were washed with 500 µL of 1X PBS by centrifugation (13 200 RPM, 30 seconds at 4 °C). sRBCs were then opsonized with 2.0 mg/mL of anti-sheep rabbit IgG or IgM for 1 h on a rotator at room temperature. After opsonization, IgM-opsonized sRBCs were washed with 500 µL PBS++ (25 mg of CaCl₂ and 50 mg of MgCl₂ per 500 mL of 1X PBS). Complement C5-deficient human serum (50 µL) was then added to the sRBC solution before being vortexed and incubated in a water bath at 37 °C for 30 minutes (Fig. 2). The IgM/iC3b-opsonized sRBCs (termed C3bi-sRBCs in the thesis) were then washed twice with 500 µL of PBS++ before being used in phagocytic assays. The RAW 264.7 macrophages were activated with 150 nM of PMA in serum-free DMEM 7 minutes prior to the incubation with C3bi-sRBCs.

Polystyrene beads in aqueous suspension (200 µL of stock solution per dish, ~10 % solids, w/w) were washed with 1X PBS and subsequently opsonized with IgM from human serum (Sigma-Aldrich Canada Co., Oakville, ON), at 2.0 mg/mL for 1 h on a rotator at room temperature. Subsequently, the polystyrene beads were processed using the same procedure as the one for C3bi-sRBCs. Activated macrophages (150 nM PMA in serum-free DMEM 7 minutes prior to imaging) were challenged by 200 µL of opsonized polystyrene beads per 35 mm glass-bottom dish or well (in the case of 6-well plates).
Figure 2. Process of C3bi fragment formation and binding to IgM on a red blood cell. Serum complement factor C3b is cleaved into C3bi by the activity of serum factors I and H. C3bi subsequently binds IgM that was previously deposited on the surface of red blood cells.

2.6 Live cell imaging

Lifeact-CFP or Lifeact-RFP stably transfected RAW 264.7 macrophages were plated 2 days prior to imaging in 35 mm glass-bottom dishes (5 × 10^5 cells/dish) (Fig. 3). Serum-starvation and sRBC/polystyrene bead opsonization were done as per the cell culture protocol described earlier. RAW 264.7 macrophages were activated with 150 nM PMA in serum-free DMEM 7 minutes prior to being challenged by 50 µL of IgG- or C3b-i-sRBCs. Throughout the duration of each live imaging session, the cells were kept at 37 °C and 5 % CO2. The objectives used included a 40x lens (oil immersion) for cell population imaging and a 63x lens (oil immersion) for single-cell imaging. Four positions were defined per 35 mm glass-bottom dish, with 15-second intervals between rounds of imaging. Live cell imaging was conducted using a Zeiss AxioObserver Z1 (Carl Zeiss Canada, Toronto, ON) inverted epifluorescence microscope using AxioVision (64-bit version 4.9.1.0) software for image capture. The typical interval used between each round of imaging for each position was 15 seconds. Differential interference contrast (DIC) images were taken in addition to epifluorescence channels. An average of 240 images were taken over the course of an hour at each position on cell culture dishes.
Figure 3. Protocol for live cell imaging of RAW 264.7 macrophages challenged with sheep red blood cells opsonized with IgM and C3bi. After washing sRBC by centrifugation, they became opsonized with IgM after an hour-long incubation in 1X PBS. Incubation with C5-deficient serum occurred subsequently for 30 minutes in PBS++. During the IgM-opsonization step, the RAW 264.7 macrophages were serum-starved in serum-free DMEM for 1 hour. Mac-1 receptors on macrophages were activated by PMA treatment 7 minutes prior to incubation with red blood cells, and was sustained for the remainder of each live cell epifluorescence microscopy imaging session.
2.7 Immunostaining and fixed cell imaging

After experimentation, cells were washed three times with 1X PBS, unless noted otherwise. RAW 264.7 macrophages were plated 2 days prior to phagocytosis assays on 6-well plates containing cover slips in each well. The cells were serum-starved and the polystyrene beads or sRBCs were opsonized as previously described. Macrophages were activated (see PMA treatment in phagocytosis assay) 15 minutes before a 15-minute pulse with either 200 µL of C3bi-sRBCs polystyrene beads or 100 µL C3bi-sRBCs. Unbound polystyrene beads or sRBCs were removed by washing the cells three times with 1X PBS and then phagocytosis was allowed to proceed for 45 minutes in the “chase” period. The cells were fixed using 4 % paraformaldehyde (Canemco & Marivac, Lakefield, QC) for 20 minutes. Prior to permeabilisation, any external polystyrene beads were labelled with donkey anti-human AffiniPure Cy™5 secondary antibodies. In the case of macrophages challenged with C3bi-sRBCs, after a 45-minute chase, the external sRBCs were lysed with ddH2O for 30 sec prior to fixation. After fixation and washing, the cells were permeabilized with 0.25 % Triton X-100 for 20 minutes. Blocking was done using 10 % bovine serum albumin (BSA) for 1 h. RAW 264.7 macrophages were incubated with 100 µL of primary antibodies on parafilm for 1 h. Primary antibodies included mouse monoclonal anti-PKCα (H-7) (1:500), mouse monoclonal anti-RhoA (26C4) (1:500), mouse monoclonal anti-FAK (D-1) (1:500), rabbit polyclonal anti-phospho-FAK (Tyr397) (1:500), mouse monoclonal anti-WASP (B-9) (1:500), and rat monoclonal anti-LAMP1 (1D4B) (1:500), rabbit polyclonal anti-p34-Arc/ARPC2 (1:500), rabbit polyclonal anti-non-muscle myosin heavy chain II-A (1:250), and mouse monoclonal anti-talin (1:250). All AffiniPure Cy™2 secondary antibodies were used at a 1:500 dilution. AffiniPure Cy™2 secondary antibodies included polyclonal donkey anti-rabbit, donkey anti-rat, and donkey anti-mouse. Optical slices were taken every 0.5 µm (z-axis) to reconstruct F-actin flashes in three dimensions. Images were acquired using a Quorum WaveFX-X1 spinning disc confocal system (Quorum Technologies Inc., Guelph, ON) through MetaMorph® image acquisition software (Molecular Devices LLC, Sunnyvale, CA).
2.8 Correlative transmission electron microscopy (TEM)

To look at the ultrastructure of F-actin accumulation on C3bi-sRBC-containing phagosomes, $2 \times 10^5$ RAW 264.7 macrophages stably transfected with Lifeact-RFP were plated in 6-well plates two days before the phagocytosis assay. The opsonization protocol for C3bi-sRBCs, as well as the live cell epifluorescence imaging protocols described earlier were used before sample processing for TEM. During this period of live cell imaging, phagosomes showing F-actin recruitment were monitored before being fixed with 2 % glutaraldehyde containing 0.5 % saponin and 1 % tannic acid for 1 h. The cells were then washed three times with 0.1 M sodium cacodylate buffer, before 1 % osmium tetroxide in 1.25 % potassium ferrocyanide in cacodylate buffer was added for 1.5 h. The samples were washed three times with 0.1 M sodium cacodylate buffer, followed by two washes with ddH2O. The samples were then stained with 4 % uranyl acetate for 30 min. Prior to dehydration, the samples were washed twice with ddH2O. The dehydration itself was done using ethanol ranging from 70 % to 100 %. For the infiltration step, a 1:1 mixture of Epon and ethanol was applied for 45 min, followed by two incubations with 100 % Epon for 75 and 105 min. To embed the samples, the coverslip was inverted into a silicone mold containing Epon, which was then left overnight at 70°C. Thin sections (80 nm) were cut using a microtome before being placed on a layer of Formvar film. Finally, the processed samples were stained with uranyl acetate along with lead citrate. Images were acquired with a Hitachi H7500 transmission electron microscope (TEM) (Hitachi High Technologies Canada Inc., Rexdale, ON).

2.9 Quantification

*C3bi-sRBC deformation quantification for blebbistatin assay*

After a 12-hour treatment with 100 µM of blebbistatin of RAW 264.7 cells stably transfected with Lifeact-RFP (plated on 35 mm glass cover slips), live cell epifluorescence imaging was used to qualitatively assess the deformation of C3bi-sRBCs through visual inspection by differential interference contrast (DIC). This was done after an hour-long serum starvation and 15-minute activation period (150 nM PMA) of macrophages. A C3bi-sRBC was considered
deformed if its circularity was noticeably altered (≥10 % change by eye), and occurred synchronously with F-actin flashing. Slight fluid-like motions on the surface of phagosome membranes during F-actin flashes were not sufficient to be considered noticeable alterations of C3bi-sRBC circularity. Cells were imaged every 30 seconds for 2 hours, at multiple distinct positions on the glass cover slips. Deformation frequency (deformed C3bi-sRBCs divided by non-deformed C3bi-sRBCs) was compared between flashing and non-flashing phagosomes.

Definition of F-actin flashing on phagosomes

I have defined F-actin flashing on phagosomes as brief and symmetrical increases in F-actin polymerization at the phagosome membrane, occurring post phagocytic cup formation, while being transient, oscillatory (>1 event), stationary, and finally, often associated with target particle deformation (∴ force-generating).

Quantification of Lifeact-CFP signal during F-actin flashing on a phagosome

Using frames acquired from live epifluorescence microscopy imaging and the image-processing software Volocity (version 6.1.1, 64-bit) (Perkin Elmer, Waltham, MA), a region of interest (ROI) was defined. It included the entirety of a flashing phagosome in a RAW 264.7 macrophage that engulfed C3bi-sRBCs. This ROI did not include the plasma membrane of the phagocyte. Quantification of frames begins when the C3bi-sRBC became bound to the macrophage. The arbitrary fluorescence intensity unit (AFU) was taken for every frame in the defined ROI.

EEA1 co-localization time course

RAW 264.7 macrophages were plated in 6-well plates (5 x 10⁵ cells/well) containing glass cover slips 2 days prior to phagocytic assay. One hour prior to incubation with C3bi-beads, the cells were incubated in serum-free DMEM before a 15-minute activation with 150 nM of PMA. Opsonization of beads with C3bi was performed using the protocol described earlier (section 2.5). The RAW 264.7 macrophages were challenged with 100 µL C3bi-beads for 15 minutes, before any unbound beads were washed off with 1X PBS. From then onwards, 1 well would be fixed every 5 minutes with 4 % PFA (20-minute fixation) for a total of 60 minutes (12 wells total). Fixed cells were blocked with 10 % BSA for 30 minutes before staining external beads
with Cy5-conjugated anti-human IgG secondary antibody (1:500) for 30 minutes. Membrane permeabilisation was done with 0.25 % Triton X-100 for 20 minutes. Cells were blocked again with 10 % BSA for 1 hour before an hour-long incubation with anti-EEA1 antibody (1:250). The cells were then incubated with Cy3-conjugated anti-goat secondary antibodies (1:500) for 1 hour. F-actin was stained with Alexa Fluor® 488 phalloidin (1:500) for 1 hour. Finally, DNA was stained with DAPI (1:5000) for 10 minutes. The glass cover slips were mounted on slides and images using a spinning disk confocal microscope. EEA1-positive phagosomes were counted on both F-actin-positive phagosomes and those that did not have F-actin. The percentage of EEA1-positive phagosomes among total phagosomes over time (1 hour) was then calculated. This protocol was repeated in three independent experiments.

**LAMP1-co localization assay**

RAW 264.7 macrophages were plated in 6-well plates (5 x 10⁵ cells/well) containing glass cover slips 2 days prior to phagocytic assay. The cells were serum-starved one hour before incubation with C3bi-sRBCs. The protocol discussed earlier (section 2.5) was used to opsonize sRBCs with C3bi. The cells were activated with 150 nM of PMA for 15 minutes before a “pulse” or incubation of macrophages with 100 µL C3bi-sRBCs of the same duration. The cells were then washed with 1X PBS to remove any unbound beads and subsequently incubated for another 45 minutes. After the incubation period was over, the cells were fixed with 4 % PFA for 20 minutes. External beads were stained with Cy5-conjugated anti-human IgG secondary antibody (1:500) for 30 minutes after blocking with 10 % BSA for 30 minutes. Cell membranes were permeabilized with 0.25 % Triton X-100 for 20 minutes. Another round of blocking was done with 10 % BSA for 1 hour before an hour-long incubation with anti-LAMP1 antibody (1:250). The cells were then incubated with Cy2-conjugated anti-rat secondary antibodies (1:500) for 1 hour. F-actin was stained with rhodamine phalloidin (1:500) for 1 hour, following DNA staining with DAPI (1:5000) for 10 minutes. Images were acquired by spinning disk confocal microscopy. The number of LAMP1-positive phagosomes was determined for both flashing and non-flashing phagosomes to calculate and compare the proportion of LAMP1-positive phagosomes in both cases. Three independent experiments were performed with this protocol.
**ROCK inhibition assay**

RAW 264.7 macrophages were plated (5 x 10^5 cells/well) 2 days prior to phagocytic assay on glass cover slips inside 6-well plates (35 mm wells). Cells were treated with 10 µM of Y-27632 ROCK inhibitor for 8 hours (treatment was also maintained during the phagocytic assay). Cells were subjected to the same procedure as described previously for phagocytic assays. The same protocol for C3bi-opsonization of beads was also followed. Briefly, the macrophages were serum-starved for 1 hour in the presence of the drug, before being activated with 150 nM of PMA in serum-free DMEM for 15 minutes. The cells were then challenged with 100 µL of C3bi-sRBCs for 15 minutes. Unbound C3bi-sRBCs were washed off with 1X PBS. The cells were fixed with 4 % PFA after 45 minutes of incubation. Prior to permeabilization (0.25 % Triton X-100, 20 minutes), cells were blocked with 10 % BSA for 30 minutes. External C3bi-sRBCs were stained with Cy5-conjugated anti-rabbit secondary antibodies (1:500) for 30 minutes. Cells were blocked again with 10 % BSA for 1 hour. F-actin and DNA were stained with rhodamine phalloidin (1:500, 1 hour) and DAPI (1:5000, 10 minutes) respectively. Epifluorescence microscopy was used to image the cells at low magnification. The percentage of F-actin-positive phagosomes for each condition (treated and untreated) was determined by counting phagosomes using the “Cell Counter” plug-in for ImageJ version 1.48 (64-bit) (National Institute of Health, Bethesda, MD). Three independent experiments were conducted.

**2.10 Image analysis and preparation of figures**

Images acquired by epifluorescence microscopy were quantified using AxioVision version 4.9.1.0 (64-bit) (Carl Zeiss GmbH, Jena, Germany) and ImageJ version 1.48 (64-bit) (National Institute of Health, Bethesda, MD). Linear adjustments (such as contrast and brightness) and three-dimensional reconstructions for images acquired by spinning disk confocal microscopy was done using Volocity version 6.1.1 (64-bit) (Perkin Elmer, Waltham, MA). No non-linear adjustments, such as gamma adjustments, were made whatsoever. Images acquired by transmission electron microscopy were acquired, processed and exported using iTEM version 5.2 (Olympus Soft Imaging Solutions GmbH, Muenster, Germany). Figures were prepared using Adobe Illustrator CS6 (Adobe Systems Incorporated, San Jose, CA).
2.11 Statistical analysis

An unpaired two-tailed Student’s t-test was used to determine statistical significance between two groups (p-value < 0.05 was considered statistically significant). When comparing three groups or more, a non-parametric one-way ANOVA was used, followed by Tukey’s test for multiple comparisons. The mean ± standard error of the mean (SEM) of each group was displayed in both cases. Prism (version 5.0) was used to conduct the statistical analyses (GraphPad Software Inc., La Jolla, CA).
3 Results

3.1 Documenting the F-actin flashing phenotype

3.1.1 Assessment of F-actin flashes on phagosomes in RAW 264.7 cells

Our laboratory studies phagocytosis and we are particularly interested in CR3-mediated pathway of this process. To study F-actin dynamics during CR3-mediated phagocytosis, we stably transfected the murine macrophage cell line RAW 264.7 with Lifeact-CFP and exposed cells to C3bi-opsonized particles. Lifeact is a short peptide (17 amino acids) derived from Abp140, a conserved Saccharomyces cerevisiae-related protein, with a fluorescent protein fused at the C-terminal domain (Asakura et al., 1998; Riedl et al., 2008). It has served as a valuable tool to visualize F-actin in live cells. In cells undergo phagocytosis, Figure 4A represents the reference time course where major events were observed. The subsequent images show epifluorescent and DIC images at the specific time points using live cell epifluorescence microscopy (Figure 4B and 4C). Briefly after C3bi-sRBC contact with the cell, the Lifeact-CFP signal at the cell membrane increased dramatically, indicating the formation of an F-actin-rich phagocytic cup, as expected (Patel and Harrison, 2008). A few minutes after the signal returned to cytosolic levels, Lifeact-CFP was then recruited to the phagosome (Fig. 4B). Elevation in the Lifeact-CFP on phagosomes often occurred simultaneously with C3bi-sRBC lysis, as visualized by DIC imaging (Fig. 4C). The Lifeact-CFP signal on the phagosome would then subside to background levels before accumulating again, on the phagosome (Fig. 4B). This occurred several times during imaging and consequently we termed these fluctuations “F-actin flashes” on phagosomes. To better quantify the dynamics of the repeated Lifeact-CFP accumulations on CR3-phagosomes, I used Volocity image analysis software to measure Lifeact-CFP signal intensity over time around the selected phagosomes. A region of interest (ROI) that included the selected phagosome was defined to quantify arbitrary fluorescence units (AFU) over time (Fig. 4D).
Figure 4. F-actin flashing on phagosomes occurs on CR3-mediated phagosomes RAW 264.7 mouse macrophages. (A) Timeline of the phagocytic process divided by markers of major events in F-actin flashing on phagosomes. (B) Representative epifluorescence live cell imaging of RAW 264.7 cells stably transfected with Lifeact-CFP and challenged with C3bi-sRBCs to induce CR3-mediated phagocytosis. Images were taken every 15 seconds for 1 hour post-binding. (C) DIC channel of the cell at the time points shown in Fig. 1B. Scale bars = 10 µm. Arrowheads indicate the flashing phagosome. (D) Quantification of the Lifeact-CFP signal intensity over time inside a defined region of interest, which included the entire phagosome membrane inside the RAW 264.7 cell shown in Fig. 1B.
3.1.2 F-actin flashes on phagosomes also occurs in primary bone marrow-derived mouse macrophages (BMDM)

Next, I validated my findings from the immortalized RAW264.7 cell line using primary cells. Myeloid progenitor cells were isolated from the femurs and tibias of C57BL/6 mice before being differentiated into monocytes, and ultimately macrophages by means of murine colony stimulating factor (M-CSF) treatment. Once differentiated, the BMDMs were transiently transfected with Lifeact-RFP by electroporation. The standard live cell imaging protocol for CR3-mediated phagocytosis described in Figure 1 and Materials and Methods was followed. Figure 5A showed significant periodic increases in Lifeact-RFP signal, separated by intervals of cytosolic levels of signal. DIC images of this particular sequence depicted C3bi-sRBC lysis occurring synchronously with Lifeact-RFP recruitment at the phagosome membrane at 3.75 min.
Figure 5. F-actin flashes on phagosomes occur in primary bone marrow-derived mouse macrophages (BMDM). (A) Representative live cell epifluorescence microscopy imaging of BMDMs transiently transfected with Lifeact-RFP and challenged with C3bi-opsinized sRBCs. (B) DIC images of the cell shown in Fig. 1A. Arrowheads indicate the flashing phagosome. Scale bars = 10 µm.
3.1.3 Three-dimensional confocal reconstruction of F-actin flashes shows distinct foci of greater Lifeact-RFP signal on the F-actin coat around C3bi-bead phagosomes in RAW 264.7 cells

F-actin flashes on phagosomes were visualized in three dimensions to extract more detail. To this end, I used spinning disk confocal microscopy, and took multiple z-slices of an area where F-actin accumulation on a phagosome had occurred and was fixed in place in RAW 264.7 cells. An image was taken every 0.5 µm in the z-plane to cover the entire thickness of the cells in the field of view. Using the image analysis software Volocity, the slices were first combined to form a two-dimensional image (extended focus) before generating a three-dimensional model with adjusted opacity to minimize background signal (Fig. 6). In the extended focus image, distinct foci of greater rhodamine phalloidin signal intensity around the phagosome was observed enveloping the C3bi-beads. The F-actin that surrounded the phagosome appeared more in the form of a “cage” as opposed to a ring-like structure. A previous study suggested that these are sites of actin nucleation (Liebl and Griffiths, 2009). These foci were not visible in the three-dimensional model, where actin appeared in the form of a coat.
Figure 6. Three-dimensional reconstruction of F-actin recruitment surrounding the phagosome membrane of C3bi-beads. Fixed RAW 264.7 macrophages imaged by spinning disk confocal microscopy containing internalized C3bi-beads. Rhodamine phalloidin was used to stain F-actin, while DAPI was used to stain DNA. The extended focus image represents the combination of multiple 0.5 µm image slices, spanning the thickness of the cell. Mention the 3D reconstruction panel. Scale bar = 10 µm.

3.1.4 F-actin flashing on phagosomes is more frequent during CR3-mediated phagocytosis in macrophages

Phagocytosis can proceed through different pathways based on the receptor engaged by the various opsonins able to coat foreign particles. FcγR-mediated and CR3-mediated phagocytosis are two commonly studied phagocytic pathways that have receptors for endogenous components of the immune system (Freeman and Grinstein, 2014). Therefore, I assessed the frequency at which F-actin flashing on phagosomes occurs in different types of phagocytosis. The standard phagocytic assay and live cell imaging protocols described in Materials and Methods were used. Briefly, RAW 264.7 murine macrophages stably transfected with Lifeact-RFP were challenged with either IgG- or C3bi-opsonized sRBCs to trigger FcγR- or CR3-mediated phagocytosis respectively. Cells challenged with IgG-sRBCs were either imaged in the absence or presence of PMA. Due to a requirement for αMβ2 integrin inside-out activation I was required to use the phorbol PMA for CR3-mediated phagocytosis (Wright and Silverstein, 1982) and included it with our experiments of FcγR-mediated phagocytosis as a control. Live cells were imaged every 15 seconds for 1 hour at 40x magnification under epifluorescence microscopy. The number of F-actin flashing phagosomes were divided by the total number of phagosomes (flashing + non-
flashing) visible in each cell in each field of view. The frequency was calculated for each fields of view for a given glass-bottom dish. For each condition, the mean was obtained from 3 separate fields of view. Figure 7 revealed that sRBC opsonized with C3bi showed a significantly higher F-actin flashing frequency (29 ± 4.8 %) compared with IgG-only (2.5 ± 1.3 %) and IgG+PMA (7.6 ± 3 %).

Figure 7. F-actin flashing on phagosomes occurs at a higher frequency in CR3-mediated phagocytosis compared to FcγR-mediated phagocytosis. RAW 264.7 murine macrophages stably transfected with Lifeact-CFP were challenged with either IgG-sRBCs (in the absence or presence of PMA) or C3bi-sRBCs. The frequency of F-actin flashing on phagosomes was calculated by dividing the number of flashing phagosomes by the total number of phagosomes in each cell in the field of view. *P<0.05, **P<0.01, n = 3. Error bars represent the standard error of the mean (SEM).
3.1.5 In phagosomes coated with F-actin, deformation of C3bi-sRBCs is the most common outcome

Among macrophages engulfing C3bi-opsonized sRBCs, I assessed the frequency at which each physical change to the RBCs occurred during F-actin flashing. I divided the RBC morphological changes into three distinct groups based on visual inspect by differential interference contrast (DIC) microscopy: no event (no deformation- RBC remained intact), fission (pieces of RBC were broken off and away from the phagosome), and deformation (as defined in Materials and Methods). Briefly, the circularity of the C3bi-sRBC had to be noticeably altered by eye while the Lifeact-RFP signal simultaneously increased at the phagosome membrane. RAW 264.7 murine macrophages stably transfected with Lifeact-RFP were challenged with C3bi-sRBCs and imaged by live cell epifluorescence and DIC microscopy for 1 hour at 40x magnification. The phenotypic frequency was calculated by dividing the number of flashing phagosomes corresponding to each classification of the RBC morphologies by the total number of flashing phagosomes. The mean of multiple fields of view was obtained for each phenotype. Among flashing phagosomes, deformation of C3bi-sRBC occurred most frequently (65.7 ± 9.3 %) compared with to no deformation (“no event”) (27.7 ± 10.9 %), leaving fission as a rarer event (9.1 ± 2.7 %) (Fig. 8).
Figure 8. RBC deformation was the prominent phenotype observed in flashing C3bi-sRBC phagosomes. RAW 264.7 cells stably transfected with Lifeact-CFP were exposed to C3bi-sRBCs and imaged by live cell epifluorescence and DIC microscopy for 1 hour. The frequency of RBC deformation-related phenotypes was calculated by dividing the number of flashing phagosomes exhibiting one of the 3 phenotypes by the total number of flashing phagosomes in cells in the field of view. *P<0.05, **P<0.01, n = 3. Error bars represent the standard error of the mean (SEM).
3.2 Candidate molecules for F-actin recruitment

3.2.1 Proteins involved in branched F-actin network polymerization are localized on flashing phagosomes

Next, I was interested in how this prominent F-actin network was recruited to phagosomes. Actin nucleation factors are essential for the polymerization of globular actin (G-actin) to filamentous actin (F-actin), since spontaneous G-actin assembly is inefficient (Campellone and Welch, 2010). Arp2/3 and formins, such as mDia1, represent two major effectors of this process. They nucleate actin to form branched and unbranched networks respectively (Campellone and Welch, 2010). The F-actin coat observed in the three-dimensional reconstruction (Fig. 6) contained punctate regions of stronger Lifeact-RFP signal. These foci might indicate the presence of nucleation sites for branched actin networks that would envelop the phagosome periphery, as suggested in the F-actin flashing study mentioned earlier (Liebl and Griffiths, 2009). Consequently, I probed for the presence of Arp2/3 and the Arp2/3-binding protein Wiskott-Aldrich syndrome protein (WASP) on flashing phagosomes. In the case of Arp2/3, I specifically targeted p34-Arc, one of seven subunits of the Arp2/3 complex (two actin related proteins: Arp2, Arp3, and five Arp complex proteins: p41-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc), which has been shown to localize to the actin tails of *Listeria monocytogenes* (Welch et al., 1997). RAW 264.7 murine macrophages were challenged with C3bi-beads for 15 minutes, before washing off unbound C3bi-beads, and incubated at 37 °C and 5 % CO₂ for 45 minutes. They were fixed prior to immunostaining for any external C3bi-beads, WASP and p34. F-actin was stained using rhodamine phalloidin while DNA was stained with DAPI. The fixed cells were imaged by spinning disk confocal microscopy. Both WASP and p34 showed strong co-localization with F-actin on phagosomes in macrophages (Fig. 9A and 9B). These proteins did not co-localize to internalized C3bi-beads that did not stain with phalloidin, indicating non-flashing phagosomes or phagosomes in-between F-actin flashes (Fig. 9A and 9B).
**Figure 9. WASP and Arp2/3 (p34) localize predominantly on F-actin flashing phagosomes in macrophages.** Representative spinning disk confocal microscopy images of fixed RAW 264.7 cells processed for immunofluorescence detection of WASP and p34 and F-actin. Crosslinked polystyrene divinylbenzene beads (3.87 µm) were opsonized with C3bi. External C3bi-beads were labeled with Cy5-conjugated donkey anti-human secondary antibodies prior to cell membrane permeabilization. Arrowheads indicate phagosomes of interest. Scale bars = 10 µm.
3.2.2 Proteins involved in the complement receptor 3 (CR3) signaling pathway localize to F-actin flashing phagosomes

Upon activation as well as C3bi-binding, the complement receptor 3 (CR3) or Mac-1 integrin has the capacity to signal directly to RhoA, a small GTPase and is responsible for the recruitment and activation of actin remodelling proteins. I probed for PKCα in addition to RhoA, since active PKCα is required to prime Mac-1 integrins (Buyon et al., 1990; Chatila et al., 1989; Rabb et al., 1993). RAW 264.7 murine macrophages were challenged with C3bi-beads for 15 minutes. Unbound C3bi-beads were washed off. This was followed by a 45-minute incubation at 37 °C and 5 % CO₂. The cells were fixed at the end of the incubation period. Immunostaining was performed for external C3bi-beads, PKCα and RhoA. F-actin was stained using rhodamine phalloidin while DNA was stained with DAPI. Spinning disk confocal microscopy images show a noticeable co-localization of PKCα and RhoA with F-actin on phagosomes (Fig. 10A and 10B).

Figure 10. PKCα and RhoA strongly localize to F-actin-rich phagosomes in macrophages. RAW 264.7 cells were challenged with C3bi-beads (3.87 μm) for 15 minutes. After fixation, external beads were labeled with Cy5-conjugated anti-human secondary antibodies prior to cell membrane permeabilization and immunostaining for F-actin and PKCα (A) or RhoA (B), before representative spinning disk confocal microscopy images were taken. Downward arrows indicate flashing phagosomes, while upward arrows indicate non-flashing phagosomes. Scale bars = 10 μm.
3.2.3 Components of focal adhesions localize to the membrane of F-actin flashing phagosomes in macrophages

Since I observed prominent deformation of RBCs during F-actin flashes in macrophages, I speculated that actomyosin contractions may be mediated by mechanosensing properties of focal adhesion components. Focal adhesions are structures comprised of multiple linked proteins providing an signal integration interface between ECM cues (such as substrate density) and the cytoskeleton (Geiger et al., 2009; Kanchanawong et al., 2010; Pelham and Wang, 1997; Sun et al., 2016). First, I probed F-actin-positive phagosomes for focal adhesion kinase (FAK) and its phosphorylated form (phosphorylated at Tyr397) as well as talin by immunostaining fixed RAW 264.7 cells challenged with C3bi-sRBCs. Talin, downstream of FAK, serves as an interface between integrins and the actin cytoskeleton, and has the capacity to bind PIPKιγ, a kinase that generates PI(4,5)P₂ (Calderwood and Ginsberg, 2003; Di Paolo et al., 2002; Ling et al., 2002). Spinning disk confocal microscopy imaging revealed co-localization of FAK, P-FAK (Tyr397) and talin with F-actin on phagosomes (Fig. 11A-C).
Figure 11. FAK, P-FAK (Tyr397) and talin localize to F-actin flashing phagosomes. RAW 264.7 murine macrophages were challenged with C3bi-opsonized polystyrene beads (3.87 µm) for 15 minutes. Unbound C3bi-beads were washed from the cells. Post-wash, cells were incubated for 45 minutes before fixation. Prior to cell membrane permeabilization, external C3bi-beads were labeled with Cy5-conjugated anti-human secondary antibodies. Afterwards, representative spinning disk microscopy images were taken. Downward arrows indicate flashing phagosomes, while upward arrows indicate non-flashing phagosomes. Scale bars = 10 µm.
3.2.4 Absence of phosphatidylinositol 4,5-bisphosphate in F-actin flashing on phagosomes

Phosphatidylinositol-4,5-bisphosphte, or PI(4,5)P$_2$, is a signaling lipid found predominantly on the inner leaflet of the plasma membrane of professional phagocytes and is involved in actin recruitment during phagocytosis (Flannagan et al., 2012). The concentration of PI(4,5)P$_2$ at this location increases by PIPK$\alpha$ activity upon the entry of an engulfed particle (Botelho et al., 2000; Coppolino et al., 2002; Flannagan et al., 2012). After engulfment, phospholipase C (PLC) activity will hydrolyze PI(4,5)P$_2$ in order to allow the fusion of newly-formed phagosomes with endosomal and lysosomal vesicles (Di Paolo and De Camilli, 2006; Scott et al., 2005). I utilized a PH-PLC$\delta$-GFP construct (pleckstrin homology domain of phospholipase C$\delta$) as a probe to monitor PI(4,5)P$_2$ activity. RAW 264.7 cells stably transfected with Lifeact-RFP were transiently transfected with PH-PLC$\delta$-GFP by electroporation. Live cell epifluorescence imaging for 1 hour post-C3bi-sRBC-binding with 15-second intervals between images revealed a lack of co-localization between PI(4,5)P$_2$ and F-actin (Fig. 12A). At T2 I observed an increase in PH-PLC$\delta$-GFP signal at the location of C3bi-sRBC entry, a membrane-rich region that is likely indicative of the proper recruitment and functioning of this probe (Stauffer et al., 1998) (Fig. 12A and 12B).
Figure 12. Phosphatidylinositol 4,5-bisphosphate is absent at the membrane of flashing phagosomes. Representative live cell epifluorescence microscopy images of RAW 264.7 cells stably transfected with Lifeact-RFP and transiently transfected with PH-PLCδ-GFP. Cells were imaged every 15 seconds for 1 h post-binding of C3bi-sRBCs. (A) Lifeact-RFP and PH-PLCδ-GFP channels. (B) Lifeact-RFP and PH-PLCδ-GFP channels in addition to DIC. Arrowheads indicate phagosomes of interest. Scale bars = 10 µm.
3.2.5 Rho-associated protein kinase (ROCK) inhibition reduces the frequency of F-actin localization to phagosomes

Next, I investigated how F-actin is being recruited to C3bi-phagosomes. Induction of actin remodelling effectors in phagocytosis is dependent upon the receptors engaged. A dichotomy exists between FcγR and CR3 signaling as their respective downstream effectors activate different members of Rho family of small GTPases. Rac1 and Cdc42 remodel actin in FcγR-mediated phagocytosis, while RhoA does so during CR3-mediated phagocytosis (Caron and Hall, 1998). Rho-associated protein kinase (ROCK) is a known effector of RhoA (Leung et al., 1996). Rho-associated protein kinase (ROCK) recruits Arp2/3 exclusively in CR3-mediated phagocytosis (Amano et al., 1996; Kimura et al., 1996; Leung et al., 1996; Olazabal et al., 2002; Rayment, 1996). To see if ROCK was a major playing in F-actin recruitment to phagosomes, RAW 264.7 cells were treated with 10 µM of the ROCK inhibitor Y-27632 for 8 hours before being challenged with C3bi-beads. Cells were fixed and any extracellular C3bi-beads were stained with anti-human antibodies conjugated to Cy5 (data not shown). F-actin and nuclei were stained with rhodamine phalloidin and DAPI respectively and images were taken using epifluorescence microscopy. The number of F-actin-positive phagosomes was divided by the total number of phagosomes to obtain the F-actin flashing frequency in control and ROCK-inhibited cells. It should be noted that in fixed cells, the observed frequency of F-actin flashes on phagosomes is intrinsically lower due to fixation at a single time point (Fig. 13A). Untreated cells showed an F-actin flashing frequency of 3.1 ± 0.2 %, whereas the treated cells showed a lower frequency of 0.7 ± 0.3 %, representing a ~4.4-fold difference (Fig. 13B).
Figure 13. Inhibition of ROCK reduced the frequency of F-actin flashing. (A) Representative images of untreated and Y-27632 treated RAW 264.7 cells to inhibit ROCK, after exposure to C3bi-beads and fixation and staining with rhodamine phalloidin and DAPI. Dotted circles indicate F-actin recruitment on phagosomes. Scale bars = 10 µm. (B) Epifluorescence microscopy imaging was used to quantify the frequency of F-actin recruitment to phagosomes in fixed RAW 264.7 cells treated with 10 µM the ROCK inhibitor Y-27632 (after an 8 h incubation). **P<0.01, n = 3 (biological replicates). Error bars represent the standard error of the mean (SEM).
3.3 Impact of F-actin flashing on phagosome maturation

3.3.1 EEA1 recruitment is delayed on early F-actin flashing phagosomes compared to non-flashing phagosomes

Phagosomes fuse with both early and late endosomes, in addition to lysosomes to form phagolysosomes (Jahraus et al., 1998; Luzio et al., 2007; Mayorga et al., 1991). To detect whether F-actin flashes on phagosomes influenced fusion with components of the endocytic pathway, I immunostained cells first with an antibody to early endosome antigen 1 (EEA1). This protein was first shown to co-localize with Rab5 (Chavrier et al., 1990; Mu et al., 1995). It was later found to be a Rab5 effector implicated in endosome fusion by binding PI(3)P, generated by PI(3)K (Simonsen et al., 1998). RAW 264.7 cell populations were challenged with C3bi-sRBCs and fixed every 5 minutes post-C3bi-sRBC-binding for a total of 60 minutes. Spinning disk confocal microscopy images showed delayed recruitment of EEA1 to the phagosomes containing F-actin, detected by phalloidin staining (Fig. 14). During the first 10 minutes post-C3bi-bead-binding, there was a significant decrease in the co-localization percentage between flashing and non-flashing phagosomes. At 5 minutes post-C3bi-sRBC-binding, co-localization of EEA1 with F-actin on phagosomes occurred in 34.4 ± 3.3 % of phagosome, compared in 9.5 ± 9.5 % of phagosomes devoid of F-actin (Fig. 14). At 10 minutes post-C3bi-sRBC-binding, 33.1 ± 3 % of phagosomes enveloped with F-actin co-localized with EEA1, versus only in 5.6 ± 5.6 % of phagosomes where F-actin was absent (Fig. 14).
Figure 14. EEA1 co-localizes to phagosomes less often when F-actin is present in the first 10 minutes post-binding. (A) Representative spinning disk confocal microscopy images of RAW 264.7 cells stained for EEA1 after internalization of C3bi-beads. *P<0.05, **P<0.01, n = 3 (biological replicates) per time point, per group (non-flashing versus flashing phagosome). Arrowheads represent the flashing phagosome, and arrows represent non-flashing phagosomes. Error bars represent the standard error of the mean (SEM). (B) RAW 264.7 cells challenged with C3bi-sRBCs were fixed every 5 minutes post particle internalization for a total of 60 minutes. EEA1-positive phagosomes were counted and EEA1 co-localization with F-actin on phagosomes was determined.
3.3.2 Impact of F-actin flashing on LAMP1 recruitment during phagosome maturation

Lysosome associated protein 1 (LAMP1) has the capacity to stabilize lysosomal structures and can serve as a marker for both lysosomes and late endosomes and (Eskelinen, 2006; Eskelinen et al., 2003). Next, I probed phagosomes for LAMP recruitment to see if F-actin influenced phagolysosome formation. RAW 264.7 cells were exposed to C3bi-opsonized sRBCs for 15 minutes before washing any unbound sRBCs. The cells were then incubated for another 45 minutes before being fixed and processed for immunofluorescence. The cells were then examined with spinning disk confocal microscopy and the number of LAMP1-positive phagosomes (both F-actin flashing and non-flashing) were quantified. RAW 264.7 cells stained for LAMP1, actin, DNA, as well as C3bi-sRBCs is shown in Fig. 15A. LAMP1 co-localization is clearly visible at the phagosome membrane of the non-flashing phagosome (Fig. 15A).

Following quantification, the data revealed that 45 minutes post-C3bi-sRBC binding, non-flashing phagosomes had a significantly higher amount of LAMP1 localized to their membrane (87.3 ± 2.7 %) compared to F-actin flashing phagosomes (40.3 ± 4.8 %) (Fig. 15B). This is in agreement with previous studies by Liebl and Griffiths (Liebl and Griffiths, 2009).
Figure 15. LAMP1 is recruited to F-actin flashing phagosomes less frequently than non-flashing phagosomes. (A) Representative spinning disk confocal microscopy images of RAW 264.7 cells stained for LAMP1 and F-actin and nuclei. C3bi-sRBCs were stained with anti-rabbit IgM antibodies conjugated with Cy5 to distinguish them from other cellular structures with similar appearances through DIC. Arrows and arrowheads represent non-flashing and flashing phagosomes respectively. (B) The number of LAMP1-positive phagosomes was divided by the number of phagosomes in the case of both flashing and non-flashing phagosomes. **P<0.01, n = 3 (biological replicates). Error bars represent the standard error of the mean (SEM).
3.4 Contractile activities and the influence of myosin IIA on F-actin flashing on phagosomes in macrophages

3.4.1 Correlative transmission electron microscopy (TEM) imaging revealed deformation of C3bi-sRBC in flashing phagosome

Although epifluorescence microscopy is an adequate and practical tool to identify F-actin flashes on phagosomes (whether to image live or fixed cells), the diffraction limit of light may hide some important structural characteristics. Similarly, resolution of images by DIC imaging is limited by the wavelength of light. To inspect sRBC deformation at greater resolutions, I used correlative transmission electron microscopy (TEM) to visualize the ultrastructure of a F-actin flashing C3bi-sRBC-containing phagosome to improve the visible details of what was occurring in a flashing phagosome. RAW 264.7 macrophages stably transfected with Lifeact-RFP were on gridded glass cover slips containing etched numbers on its surface to facilitating the re-location of target cells post-embedding for EM. After PMA activation, cells were challenged with C3bi-sRBC for 15 min before washing away any unbound opsonized particles. They were subsequently imaged using live cell epifluorescence microscopy. After an F-actin flashing event was detected, the target cell was immediately fixed with glutaraldehyde before processing for TEM imaging. In the cell type of interest, three C3bi-sRBCs were taken up into a single RAW 264.7 macrophage, however only one exhibited F-actin flashing on its phagosome when viewed by epifluorescence (Fig. 16A). When the cell was located by TEM, there was a distinctively thick and lighter area surrounded this middle C3bi-sRBC (Fig. 16B). The two adjacent C3bi-sRBCs (identified by arrows) were not enveloped by such a structure. This area had a noticeable absence of vesicles, an observation noted by a previous study briefly inspecting the ultrastructure of F-actin flashes on phagosomes (Liebl and Griffiths, 2009). Interestingly, RBC within the F-actin-containing phagosome was much darker and more electron dense, indicating that it may have possibly lysed, compared to the other two C3bi-sRBCs in non-flashing phagosomes. This supports my DIC imaging results where sRBC lysis was frequently observed after particle deformation.
Figure 16. Correlative transmission electron microscopy (TEM) imaging of an F-actin flashing phagosome in macrophages. (A) Low magnification (20x) of live cell epifluorescence images of RAW 264.7 cells stably transfected with Lifeact-RFP following CR3-mediated phagocytosis induced by C3bi-sRBC incubation. Arrowheads indicate RAW 264.7 macrophage containing flashing phagosome. Scale bar = 30 µm. (B) Cells were fixed with glutaraldehyde upon detection of F-actin flashing on a C3bi-sRBC phagosome and processed for TEM analysis. Correlative TEM slice of the cell shown in Fig. 13A. The dotted boxes represent the area magnified in the image that follows. Scale bars are indicated on images.
3.5 Effects of target particle rigidity on F-actin flashing temporal dynamics in macrophages

3.5.1 Rigid opsonized particles accumulate F-actin flashes quicker than more flexible particles

Given that particle deformation often occurred after an F-actin flash on a phagosome and that the phagosome recruited focal adhesion proteins, I next wondered if the phagocyte could detect the engulfed particle’s stiffness as though it were part of the ECM. The ECM often varies in stiffness which is communicated intracellularly to the cell. If my hypothesis was true, I predicted that disparities between opsonized particles of differing stiffness would manifest itself in the form of variations in temporal dynamics of F-actin flashing of phagosomes. To assess if particle stiffness was a factor in F-actin flashing temporal dynamics, I used C3bi-sRBC and C3bi-beads of similar diameter as flexible versus rigid targets respectively. RAW 264.7 macrophages stably transfected with Lifeact-RFP were imaged using live cell epifluorescence microscopy every 15 seconds for 1 hour post-binding with either C3bi-sRBC or C3bi-beads. Individual frames were counted from the initial binding of the particle to the initial increase in Lifeact-RFP signal after phagocytic cup formation. After quantification, it was revealed that there was a significant difference in the time it took for the first F-actin flash to occur: where the malleable C3bi-sRBC phagosomes took 30.1 ± 2.9 minutes to flash, while the phagosomes containing stiff C3bi-beads only took 20.7 ± 3.2 minutes to recruit F-actin to the phagosome (Fig. 17A). I chose to evaluate several more parameters to determine the influence of particle rigidity on F-actin flashing on phagosomes. Live cell epifluorescence imaging every 15 seconds for 1 hour allowed for the quantification of other temporal parameters throughout the time course. There were no differences between C3bi-sRBCs and C3bi-beads for parameters measured in Figure 17B-C and 17E. There was no difference in F-actin flash duration between C3bi-sRBC (3.8 ± 0.3 min) and C3bi-beads (4.1 ± 0.5 min) (Fig. 17B), as well as no difference in the time between two F-actin flashes on phagosomes – C3bi-sRBC (1.8 ± 0.2 min) and C3bi-bead (2.1 ± 0.3 min) (Fig. 17C). Once F-actin flashing on phagosomes is initiated, F-actin will be recruited and disassembled in a cyclical manner. The number of F-actin remodelling cycles can vary from one phagosome to another. However, after quantifying the number of oscillations on engulfed particles of differing flexibility, C3bi-sRBC (malleable) displayed a greater number of F-actin recruitment cycles (6.4 ± 0.7) compared to the C3bi-beads (rigid) (3.1 ± 0.2) (Fig. 17D). Finally, the difference in F-
actin flashing frequency on phagosomes was not significant between C3bi-sRBC (31.2 ± 6.4 %) and C3bi-bead (19.4 ± 4.7 %) (Fig. 17E).
Figure 17. Rigid C3bi-beads phagosomes accumulate F-actin faster and have less F-actin accumulation cycles than malleable C3bi-sRBCs. C3bi-sRBC or C3bi-beads were tracked by live cell epifluorescence microscopy, in RAW264.7 macrophages stably transfected with Lifeact-RFP. (A) The time taken to exhibit F-actin recruitment from initial cell binding was calculated based on the 15-second interval between images during the imaging time course. n = 46 (C3bi-sRBC), n = 37 (C3bi-beads). (B) Duration of F-actin recruitment on phagosomes from initial increase of Lifeact-RFP signal, to decrease back to cytosolic levels from peak intensity. n = 73 (C3bi-sRBC) and n = 91 (C3bi-beads). (C) Time interval between two F-actin flashes on phagosomes. n = 69 (C3bi-sRBC) and n = 62 (C3bi-beads). (D) Number of F-actin recruitment cycles on phagosomes. n = 27 (C3bi-sRBC) and n = 45 (C3bi-bead). (E) Frequency of F-actin flashing on phagosomes. n = 6 (C3bi-sRBC) and n = 3 (C3bi-beads). *P<0.05, ***P<0.001. Error bars represent the standard error of the mean (SEM).
3.5.2 The heavy chain of non-muscle myosin IIA co-localizes with F-actin on phagosomes

I demonstrated earlier that RBC deformation is the most common phenotype observed in F-actin flashing phagosomes, which is likely to occur due to actomyosin activity at the phagosome membrane. Myosins can exert tension on actin filaments by means of ATP hydrolysis at their catalytic head domain (Vicente-Manzanares et al., 2009). Non-muscle myosin II, more specifically, the myosin IIA isoform found exclusively in hematopoietic cells, is required for the formation of the phagocytic cup during CR3-mediated phagocytosis (Maupin et al., 1994; Olazabal et al., 2002). I wanted to attempt to detect the presence of myosin IIA on F-actin-rich phagosomes. RAW 264.7 cells were challenged with C3bi-beads for 45 minutes and then processed for immunostaining for myosin II and confocal imaging. Co-localization of non-muscle heavy chain of myosin IIA (NMHC IIA) with F-actin was observed on phalloidin-positive phagosomes (Fig. 18). The RAW 264.7 cell shown in Figure 15 contained five phagosomes; only one of which displayed F-actin flashing and myosin II recruitment is prominent on this one, compared to non-flashing phagosomes.

Figure 18. Non-muscle myosin heavy chain IIA co-localizes with F-actin on phagosomes. Representative spinning disk confocal microscopy images of a fixed RAW 264.7 cell after phagocytosis of C3bi-beads and staining for NMHC IIA and phalloidin and DAPI. External beads were labeled with Cy5-conjugated donkey anti-human secondary antibodies prior to cell membrane permeabilization. Arrows indicate a non-flashing phagosome, while arrowheads indicate a flashing phagosome. Scale bars = 10 µm.
3.5.3 Role of non-muscle myosin IIA in C3bi-sRBC deformation during F-actin flashing

Finally, to determine a definitive role for myosin IIA in contractile activities in flashing phagosomes, its activity was inhibited. I then assessed the effect of this treatment on particle deformation. The pharmacological agent blebbistatin was used for this purpose. Blebbistatin has a high affinity for the myosin-ADP-Pi complex, preventing it from releasing a phosphate group, effectively disabling it from inducing actomyosin contractions without altering its actin-binding properties (Kovacs et al., 2004). RAW 264.7 macrophages stably transfected with Lifeact-RFP were treated and incubated overnight (~12 h) with 100 µM of blebbistatin. They were then challenged with C3bi-sRBCs. Figure 19A depicts the morphological changes that were observed in cells post blebbistatin treatment. The membrane extensions were visibly longer and more distorted along the edges compared to non-treated cells. Furthermore, the blebbistatin-treated cell appeared binucleated – suggestive of interrupted cytokinesis, which confirmed that the drug had an impact on cells (Guha et al., 2005; Straight et al., 2003). In the control group (DMSO vehicle control), 98.9 ± 1.1 % of flashing phagosomes exhibited sRBC deformation after DIC inspection (Fig. 19B and 19C). It is worth noting that the term “deformation” is inclusive of sRBC lysis, fission, in addition to standard morphological deformation, which partly explains the high percentage of deformation observed in F-actin flashing phagosomes in the control group. On the other hand, the sRBC deformation frequency in F-actin flashing phagosomes of blebbistatin-treated cells was quantified to be 67.6 ± 4.9 %, significantly lower than the number observed in the control group (Fig. 19B and 19C). Collectively this data suggests that F-actin flashes on phagosomes result in particle deformation in a myosin II-dependent manner.
Figure 19. Blebbistatin treatment of RAW 264.7 mouse macrophages reduces the frequency of C3bi-sRBC deformation. (A) RAW 264.7 cells stably transfected with Lifeact-RFP were treated with 100 µM of blebbistatin overnight (~12 h). Blebbistatin activity validation through differential interference contrast (DIC) inspection of cell morphology. (B) Representative live cell DIC and epifluorescent images of control and blebbistatin-treated macrophages undergoing CR3-mediated phagocytosis. Arrowheads indicate phagosomes of interest. Scale bars = 10 µm. (C) Quantification of the number of deformed phagosomes observed by DIC in F-actin-positive phagosomes. **P<0.01, n = 3 (biological replicates). Error bars represent the standard error of the mean (SEM). Scale bars = 10 µm.
4 Discussion

My investigation has revealed that F-actin flashing on phagosomes is a phenomenon that could potentially serve critical functions in clearing target particles from phagocytes, based on the characteristics of said particles. I have elucidated the temporal dynamics of F-actin flashing on phagosomes and have identified the discrepancies between particles of varying rigidity. I reported that the process of F-actin flashing on phagosomes is initiated by a signaling cascade originating from the activity of focal adhesion components, possibly including integrins bound to ligand. My model (Fig. 20) mainly depends upon the sustained presence and activation of Mac-1 integrins at the phagosome membrane to signal to RhoA GTPase, ultimately recruiting myosin IIA and actin. I speculate that a feedback mechanism plays a role in the cyclical properties of F-actin flashing progression on phagosomes. However, the mechanisms guiding F-actin shown on point A (peak) to basal levels, and point B (re-initiation of F-actin assembly) on Figure 20 are still unknown. I suspect that the mechanosensing properties of FAK bound to talin could play an important role in signaling during actomyosin contractions. PKCa, activated by either PMA or DAG, phosphorylates the β-subunit of Mac-1 integrins, increasing their affinity by conformational change to bind C3bi on opsonized particles (“inside-out” signaling). Immediately downstream of Mac-1, RhoA binds to ROCK for it to activate myosin IIA (Bhadriraju et al., 2007; Leung et al., 1996). Arp2/3 recruitment by myosin IIA proceeds to initiate F-actin polymerization. The resulting actomyosin fibers coat the phagosome and induces contractions. The signaling cascade is repeated in a cyclical manner, and is likely regulated by a mechanosensing feedback loop.
Figure 20. Model of F-actin flashing on phagosomes in CR3-mediated phagocytosis.

Phagosomes containing C3bi-opsonized particles in macrophages can exhibit a peculiar behaviour known as F-actin flashing, characterised mainly by a transient and repeated accumulation of F-actin, most often in CR3-mediated phagocytosis. Shown above is the tentative signaling cascade leading up the formation of the F-actin coat around the phagosome. F-actin disassembly and re-engagement steps have yet to be resolved.
4.1 Prevalence of F-actin flashing on phagosomes in CR3-mediated phagocytosis versus FcγR-mediated phagocytosis

F-actin flashing on phagosomes was much less prominent in FcγR-mediated phagocytosis, but was detected nonetheless. The likely explanation for this observation may rest in a study published by Jongstra-Bilen et al. that has shed light on the interactions between the activity of Fcγ receptors and complement receptor 3. They showed that IgG-binding to FcγR increases lateral diffusion of Mac-1 integrins, thereby increasing their avidity (while having no effect on their affinity) (Jongstra-Bilen et al., 2003). The authors found that the activity of effectors downstream of Fcγ receptors (including Src, PI3K, PLCγ, and novel PKC isoforms) had a significant impact on C3bi-RBC binding (Jongstra-Bilen et al., 2003). Consequently, in my experimental settings, when Fcγ receptors were engaged by either IgG-sRBCs or IgG-beads, the subpopulation of phagosomes recruiting F-actin was likely the result of activity stemming from complement receptor 3, not Fcγ receptors. In this scenario, a smaller subset of Mac-1 receptors would be activated compared to experimental conditions where C3bi-opsonized particles bind Mac-1, possibly explaining the lower frequency of F-actin flashing on phagosomes in FcγR-mediated phagocytosis.

4.2 Why does only a subset of phagosomes exhibit F-actin flashing?

My data have shown that only a portion (approximately 30%) of CR3-phagosomes exhibit F-actin flashing. A previous study by Liebl and Griffiths noted that F-actin flashing was more prevalent in particle-overloaded macrophages, meaning that the fraction of phagosomes recruiting F-actin increased proportionally with the number of phagosomes in a given macrophage (Liebl and Griffiths, 2009). They reasoned that it is possibly due to the retention of components on the plasma membrane responsible for F-actin polymerization (Liebl and Griffiths, 2009). Furthermore, it was also shown that even after a long incubation period (~5 hours), a subpopulation of phagosomes never accumulated F-actin (Liebl and Griffiths, 2009). I propose a non-mutually exclusive explanation involving variations in the degree of integrin receptor retention and activation amongst phagosomes. Some of my preliminary data indicated that Mac-1 localized at phagosomes coated with F-actin (data not shown). This would entail a
form of disruption of the integrin recycling pathway, allowing integrins to remain active at this location. They would be recycled back to the plasma membrane only after F-actin flashing on phagosome. The circulation of cytosolic Mac-1 (bidirectional integrin trafficking between the phagosome and the cell surface) is putatively due to its tyrosine residue, usually involved in clathrin-mediated endocytosis (Pearse and Robinson, 1990), on the cytoplasmic tail of the αM (CD11b) subunit (Bretscher, 1992). I therefore speculate that, based on the rate of Mac-1 circulation and the possibility of an unknown factor delaying Mac-1 recycling, only a subset of phagosomes will have active integrins and transiently accumulate F-actin. Maintaining a certain portion of Mac-1 integrins in the cytoplasm of macrophages containing multiple phagosomes suggests that they are recycled on most of these phagosomes, resulting in abrogation of the signaling cascade responsible for F-actin assembly. Finally, it is also worth mentioning the potential impact that PMA may have had on the frequency of F-actin flashing on phagosomes.

Live cell imaging of macrophages undergoing phagocytosis involved constant exposure to 150 nM of PMA present the culture medium. This could have potentially amplified the frequency of F-actin flashing on phagosomes by sustaining PKCα activation. Our data have shown that adding PMA during FcγR-mediated phagocytosis slightly increased the frequency of F-actin flashing on phagosomes, indicating that PMA is a factor, albeit not a very influential one.

4.3 Absence of phosphatidylinositol 4,5-bisphosphate on F-actin flashing phagosomes

Phagocytic cup formation relies on PI(4,5)P₂ dynamics to mediate actin assembly and dissociation (Scott, 2005). While studying actin comet tails, it was shown that the membranes of CR3 phagosomes contain PI(4,5)P₂ due to PIP5KI retention (Bohdanowicz et al., 2010). It was further suggested that talin might be behind the recruitment of PIP5KI (more specifically, the γ isoform) based on previous detection of talin on C3bi-RBCs (Lim et al., 2007). My observations regarding the absence of PI(4,5)P₂ activity contradict previous research on actin recruitment at the phagosome membrane. The Bohdanowicz et al study focused on a different manifestation of F-actin assembly at the phagosome membrane, associated with the propulsion of the phagosome across the cytoplasm, aptly named actin comet (Bohdanowicz et al., 2010). Interestingly, PI(4,5)P₂ along with Cdc42 are known to bind the widely expressed actin nucleation factor N-
WASP (Rohatgi et al., 2000). Despite not detecting PI(4,5)P₂ with F-actin at the phagosome membrane, I detected WASP at the same location. Contrary to N-WASP, its homolog WASP is expressed exclusively in hematopoietic cells (Thrasher and Burns, 2010). Therefore, based on my data, I speculate that WASP must be recruited at the phagosome membrane by alternative means. Although I did not probe for these proteins, the small GTPases Rac1 and Cdc42 both have the capability to bind WASP (Aspenström et al., 1996). Initially, it was established that F-actin recruitment was mediated by the small GTPases Rac1 and Cdc42 in FcγR-mediated phagocytosis, and RhoA in the case of CR3-mediated phagocytosis (Caron and Hall, 1998). However, more recent studies have shown compelling evidence that Rac1 also serves an important role in CR3-mediated phagocytosis. Rac GTPase was established as a downstream effector of Vav guanine nucleotide exchange factor (GEF) in CR3-mediated phagocytosis (Hall et al., 2006). The authors made their observations in Vav1/3- or Rac1/2-deficient primary murine bone marrow-derived macrophages (Hall et al., 2006). They noticed that while binding was not affected, internalization during complement-mediated phagocytosis was reduced by 82% in Rac1/2-null primary macrophages, due to nearly complete abrogation of F-actin recruitment (Hall et al., 2006). I speculate that Rac GTPase may be responsible for the WASP recruitment, shown by my data, to F-actin on C3bi-bead phagosomes.

4.4 Delayed recruitment of phagosome maturation effectors on F-actin flashing phagosomes

Phagosome maturation is dependent on the recruitment and fusion of endocytic and lysosomal compartments with phagosomes to deliver the appropriate effectors responsible for the destruction of target particles (Haas, 2007). My data indicated a delay in the recruitment of early and late effectors of phagosome maturation, EEA1 and LAMP1 respectively. I attributed this delay partly to the thickness of the F-actin coat enveloping flashing phagosomes. This became apparent upon the inspection of a flashing phagosome’s ultrastructure by correlative TEM, which was devoid of vesicular structures at the periphery of the F-actin coat. I speculate that during peak F-actin recruitment, this F-actin coat would heavily impair, if not abrogate, the fusion of early and late endosomes, as well as lysosomes with phagosomes. Only a short period exists to allow vesicular fusion during intervals between two F-actin accumulation events on phagosomes.
(~2 minutes). Despite a delay in maturation, the F-actin coat may be advantageous to macrophages harbouring hazardous phagosomal contents. The thick F-actin coat could act as a physical barrier preventing exogenous products, that would otherwise expose the macrophage to detrimental molecules, from escaping the phagosome.

Another factor that may be responsible for delaying phagosome maturation involves the engulfed particle’s flexibility. Although I did not compare the recruitment dynamics of EEA1 and LAMP1 between C3bi-sRBCs and C3bi-beads, I did however quantify the number of F-actin recruitment cycles per phagosome in the case of both particles. I presented evidence suggesting that, on average, C3bi-sRBC phagosomes exhibited twice as many cycles of F-actin accumulation when compared to C3bi-bead phagosomes. My data are in accordance with research investigating the stability of the actin recruitment machinery localized at focal adhesions on substrates of varying flexibility. A previous study demonstrated that epithelial cells grown on stiff surfaces had greater amounts of phosphotyrosine and mature focal adhesions, as opposed to those grown on flexible substrates. Weak tension applied to focal adhesions on flexible substrates would putatively reduce downstream signaling (Lo et al., 2000; Pelham and Wang, 1997). Those focal adhesions became more dynamic and unstable, increasing the rate of cell migration by means of higher receptor displacement (Lo et al., 2000; Pelham and Wang, 1997). This supports my findings that C3bi-sRBC phagosomes undergo more cycles of F-actin assembly compared to C3bi-bead phagosomes. Hence, substrate flexibility affected the average length of F-actin flashing sequences, and could consequently affect the duration of the delay in phagosome maturation. This phenomenon is possibly related to durotaxis observed in migrating cells, and may serve a specific purpose during the maturation process of phagosomes containing large particles. Durotaxis is defined as the tendency of migrating cells to move towards areas of greater stiffness by probing the flexibility of its substrate (Hoffman et al., 2011; Lo et al., 2000; Plotnikov and Waterman, 2013; Riveline et al., 2001). I suspect that the signaling cascade elicited during probing, causing migrating cells to move away from flexible substrates, is similar to the one influencing the number of F-actin assembly cycles on phagosomes. Consequently, a longer delay would be expected in malleable particles, such as C3bi-sRBCs, to first allow their mechanical deformation or “chewing” (often to the point of lysis, fission, or both). However, in the case of stiff particles such as C3bi-beads, which cannot be deformed, a shorter delay in phagosome maturation would more likely occur. In some cases of bacterial infection, delaying phagosome
maturation would be detrimental for the pathogen. The master virulence-regulating factor PhoP/PhoQ found in *Salmonella enterica* serovar Typhimurium is activated by a slightly acidic bacterial cytoplasm pH of 5.5 (Choi and Groisman, 2016; Prost et al., 2007). By delaying an increase in phagosomal lumen acidity, phagosome contractions by F-actin flashes could help eliminate the bacteria before it could execute its transcriptional program and cause disease in the organism (Miller et al., 1989). The bacterial cell wall, comprised of peptidoglycans, is a rigid structure resistant to osmotic pressures, making bacteria, especially gram-negative strains, inherently stiffer than mammalian cells (Cabeen and Jacobs-Wagner, 2005; Silhavy et al., 2010; Tommasi et al., 2015). Based on my data, I speculate that this property common to many pathogenic bacteria would induce a lower number of F-actin assembly cycles, thus a shorter delay in phagosome maturation compared to malleable targets.

4.5 Variance in temporal dynamics of F-actin flashes based on the stiffness of engulfed particles

4.5.1 Possible relationship between durotaxis and feedback mechanism behind F-actin flashing on phagosomes

I have briefly touched upon the topic of durotaxis when discussing the apparent delay in phagosome maturation induced by F-actin flashing on phagosomes. I suspect that it is central to the regulation of the oscillatory nature of the phenomenon of interest. This reasoning is based on the possibility that macrophages could potentially sense the internalized particle’s stiffness in a similar fashion to its substrate (for example, the ECM) during durotaxis. In short, migrating cells rely on probing their substrate’s rigidity to guide their displacement towards areas of greater stiffness (Pelham and Wang, 1997; Plotnikov and Waterman, 2013). This occurs by contractions of actin stress fibres tugging on individual focal adhesion proteins, in the direction of the nucleus, bound to components of the ECM (Hoffman et al., 2011). My data have shown that the temporal dynamics of F-actin flashing on phagosomes vary based on the rigidity of the engulfed particle. Certain properties of focal adhesion proteins, mainly tension sensitivity, are likely to be a factor. Based on my observations, macrophages cultured *in vitro* (on two-dimensional plastic surfaces) form neither focal adhesions nor stress fibers (data not shown). Nevertheless, I have shown that certain components of focal adhesions are exclusively localized to F-actin-positive
phagosomes in RAW 264.7 macrophages. This was indicative that these focal adhesion proteins were likely bound to integrin structures on phagosome membranes, possibly influencing F-actin recruitment at this region. For example, previous research has shown that upon the stretching of talin by stress fiber-induced tension, new (or “cryptic”) vinculin binding sites become exposed (Rio et al., 2009), allowing an even greater amount of vinculin to bind talin than the typical 11 vinculin-binding sites already present on talin rods (Gingras et al., 2005). Vinculin’s relevance in this scenario stems from its two functional domains – a head domain linked to integrin clustering as well as a tail domain involved in mechanotransduction by linking focal adhesions to the actin cytoskeleton (Humphries et al., 2007). Tension on vinculin itself is associated with focal adhesion assembly and stability (Grashoff et al., 2010). Hence, in phagosomes containing rigid particles, I would expect faster initiation of F-actin recruitment and less oscillations of F-actin assembly respectively. This is precisely the behaviour that I observed in C3bi-beads. As previously mentioned, I have revealed the presence of some focal adhesion components, mainly FAK and talin, localized with F-actin-positive phagosomes. FAK is a mechanosensor with integrin-association-dependent activity, and has a crucial role in focal adhesion turnover (Katsumi et al., 2004; Mitra et al., 2005). This is partly by phosphorylating α-actinin on Tyr12, inhibiting its crosslinking capabilities to actomyosin stress fibers, reducing its contact with focal adhesions and increasing focal adhesion turnover, ultimately increasing the fluidity of the cytoskeleton (Izaguirre et al., 2001). Autophosphorylation of FAK at Tyr397 occurs when integrins are bound to substrates (Shi and Boettiger, 2003). It was previously shown that FAK-null cells have constitutively active RhoA, which severely impacted focal adhesion turnover. In the case of talin, which has the capacity to bind integrins and actin, a 2 pN slip bond between those two components exists (Jiang et al., 2003). Tension beyond 2 pN will cause the bond to break. If integrins were to be bound to ligands on stiff surfaces, the tension may cause slip bond breakage, effectively dissociating actomyosin stress fibers and halting F-actin flashing progression. It remains to be tested to elucidate whether such tension could be generated in this way. My proposed model highlights the importance of actomyosin stress fiber tension in F-actin flashing on phagosomes (Fig. 17).

Finally, myosin may have a role in recruiting F-actin to phagosomes. The implications of myosin activity did not extend only to the contraction of phagosomes. Inhibition of the upstream myosin effector, Rho-associated protein kinase (ROCK), reduced the frequency at which F-actin flashing
on phagosomes occurred. This suggested that in addition to inducing contractions, myosin also had a distinct role in the initiation of F-actin flashes. Disruption of myosin II activation pathway through RhoA and ROCK is known to reduce accumulation of Arp2/3 and consequently actin around bound particles in CR3-mediated phagocytosis (Olazabal et al., 2002). Olazabal et al suggested a mechanism that does not involve direct recruitment of Arp2/3 by myosin (Olazabal et al., 2002). Instead, they base their model on the G-actin-binding capabilities of the S1 subfragment in the motor domain of myosin II, allowing it to mediate actin assembly, whereas Arp2/3 would be recruited after initial G-actin polymerization (Carlier et al., 1994; Eto et al., 1991; Miller et al., 1988; Olazabal et al., 2002; Toyoshima et al., 1987). Finally, there is evidence that F-actin itself is sensitive to tension as pressure applied on actin filaments increases their stiffness and adds repulsive forces during filament elongation (Galkin et al., 2012). Therefore, the counteracting force produced by compressed actin filaments could act in tandem with typical actomyosin contractions in the lysis and/or fission of C3bi-sRBCs.

4.5.2 Possible mechanism of cessation of F-actin recruitment cycles on phagosomes

F-actin flashing on phagosomes is cyclical, but not indefinite. Therefore, the feedback mechanism proposed earlier must have incorporated a function to cease F-actin assembly on phagosomes, possibly in the form of an inhibitory signal. This signal would be transduced by a certain effector required to influence actomyosin contractions after an engulfed particle has been compressed beyond a certain tension threshold sensed by focal adhesions. The effectors involved, as well as the means of signal transduction, have yet to be resolved. Nonetheless, I speculate that calpain-2 may be implicated in the cessation of F-actin assembly cycles. Calpains are a family of a cysteine proteases, and among their vast number of known substrates (Franco and Huttenlocher, 2005), are capable of cleaving both FAK and talin (Chan et al., 2010; Franco et al., 2004). Specifically, calpain-2, also known as m-calpain, is activated by millimolar concentrations of Ca$^{2+}$, the same cation used to activate PKCα to stimulate integrin affinity (Franco and Huttenlocher, 2005; Khorchid and Ikura, 2002). A previous study has highlighted calpain’s crucial role in focal adhesion turnover, focusing on the cleavage of talin as a requirement for dissociation of multiple focal adhesion components, making this process a rate-limiting step in focal adhesion turnover (Franco et al., 2004). Therefore, calpain-2 could
putatively intervene at the later stages of F-actin flashing on phagosomes in order to abrogate Mac-1 signaling by cleavage of talin and FAK. This would have significant downstream effects on RhoA GTPase as its activity requires signaling from Mac-1 ($\alpha\beta_2$) (Caron and Hall, 1998; Freeman and Grinstein, 2014; Wiedemann et al., 2006). My data show RhoA co-localizing with F-actin-positive phagosomes, confirming its recruitment to this location. The suppression of RhoA activity would effectively prevent the recruitment of myosin IIA and actin nucleation factors to the phagosomes.

4.6 Tentative functions of F-actin flashes

4.6.1 Increased efficiency of particle degradation

My data do not attribute a specific function to F-actin flashing on phagosomes. Visibly however, these flashes strongly correlate with deformation of the ingested particle. Based on this, I hypothesize that it could potentially aid in the clearance of large engulfed particles. Multiple small particles would require less exposure to proteases for degradation than a single large particle of the same volume. The resulting increase in surface area on multiple small particles would ease the degradation of the exogenous entity with a high proton concentration in the phagolysosome lumen, along with proteases such as cathepsins (Kinchen and Ravichandran, 2008; Turk et al., 2012). Macrophages tasked with engulfing opsonized particles would benefit from reducing larger particles into smaller, more manageable portions, in a process analogous to the early stages of digestion (for example, chewing). In physiological conditions, professional phagocytes are required to ingest apoptotic cells to maintain homeostasis of the organism (Arandjelovic and Ravichandran, 2015; Erwig and Henson, 2007; Kinchen and Ravichandran, 2008; Platt et al., 1998). These apoptotic bodies are typically of greater size (5-50 $\mu$m) (Kinchen and Ravichandran, 2008) than the opsonized particles used in my study (~3.87 $\mu$m), providing a greater challenge for macrophages to degrade, compared to smaller bacterial targets. I have supporting evidence of contractions occurring on phagosome membranes, which are powerful enough to lyse and cause fission in red blood cells. I identified myosin IIA, bound to F-actin, as a potential effector. While rapid F-actin polymerization alone can exert pressure on vesicles, actomyosin contractions would provide greater force to break apart malleable structures by fission (Upadhyaya and van Oudenaarden, 2004; Upadhyaya et al., 2003). Even in the case of
lysis, which occurs more frequently than fission, it would expose the contents of engulfed C3bi-sRBCs to an acidic and proteolytic environment, assisting phagosome maturation.

4.6.2 F-actin flashing role in antigen presentation

In addition to potentially increasing the efficiency of engulfed particle destruction by applying force to the phagosome, F-actin flashing may also be involved in antigen presentation. In this scenario, macrophages would be loading antigens onto MHC class II molecules after fission of engulfed particles by F-actin flashing on phagosomes. A previous study has demonstrated that B cells are sensitive to the stiffness of the antigen-presenting cell (APC) (Spillane and Tolar, 2016). The authors stipulate that stiff APCs promote a more rigorous discrimination of antigen based on affinity, due to the greater force required to extract antigen, whereas the opposite occurs on more flexible APCs (Pierobon and Lennon-Duménil, 2016; Spillane and Tolar, 2016). Therefore, if a rigid particle is engulfed and cannot be deformed by F-actin flashing, it would not be employed for antigen presentation, but rather be degraded by the progression of phagosome maturation. Conversely, flexible particles would be subject to lysis and fission by F-actin flashing on phagosomes. Their fragments would be made available for loading onto MHC class II molecules to be presented to B cells after undergoing multiple cycles of F-actin assembly at their phagosome.

4.7 Limitations of study and future experiments

One of the major limitations of this study implicates the qualitative aspects of some of the criteria followed to determine the temporal dynamics of F-actin flashing on phagosomes. An automated analysis tool that would allow identification and tracking of flashing phagosomes, along with precise measurements of their characteristics, would eliminate much of the uncertainty associated with the qualitative assays performed. Second, a more rigorous definition of malleable particle deformation would reduce the odds of improperly identifying such a phenotype. I propose the usage of a circularity index to quantitatively evaluate the circularity deviation of malleable particles from a perfect ovoid shape. It is worth mentioning the uncertainty surrounding the mechanical fragility of red blood cells, with regards to particle
malleability. Red blood cells may require some form of chemical activity to help with their fragmentation, in addition to mechanical activity from the actomyosin contractions. The observed delay in phagosome maturation poses a problem on this end. An investigation on the recruitment on certain proteolytic enzymes before or during F-actin flashing on phagosomes would be in order. Furthermore, I would require further experimentation to support some of the discussion points stipulated earlier. This would include the assessment of Arp2/3 levels on phagosomes during ROCK or myosin inhibition to link the F-actin assembly data to the myosin IIA data. To test my hypothesis regarding the increase in efficiency of phagosomal content degradation by proteases and acidic pH, I can utilize self-quenched dyes conjugated to ovalbumin as probes crosslinked to the surface of red blood cells. The fluorescence intensity emitted by the dye upon proteolysis would be quantified and compared between phagosomes lacking F-actin and flashing phagosomes. I would expect greater proteolytic activity in C3bi-sRBCs lysed by F-actin assembly on phagosomes, translating to greater fluorescence intensity.
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