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Characterization of Efferosome Maturation and the Processing of Apoptotic Bodies

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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CHARACTERIZATION OF EFFEROSOME MATURATION AND THE PROCESSING OF APOPTOTIC BODIES

(Thesis format: Monologue)

by

Yohan Kim

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

Every day billions of cells in our bodies undergo apoptosis and are cleared through efferocytosis – a phagocytosis-like process in which phagocytes engulf and degrade apoptotic cells. Proper processing of efferosomes prevents inflammation and immunogenic presentation of antigens. In this thesis I determined that the early stages of efferosome maturation parallel that of the pro-immunogenic phagocytosis of pathogens. Mass spectrometry analysis of later maturation stages identified unique regulatory proteins on efferosomes and phagosomes. Keys among these were Rab17 and Rab45 on efferosomes versus Rab6b and PI-4-Kinase on phagosomes. The later would allow for antigen presentation from phagosomes, while the former would direct efferosome-derived antigens away from this presentation pathway. Moreover, positive regulators of MAP-kinase signaling were enriched on phagosomes, while negative regulators were enriched on efferosomes, perhaps indicating the mechanism through which efferocytosis inhibits inflammation. These regulators may account for the differences in inflammation and immunogenicity observed after efferocytosis versus phagocytosis.

Keywords

Macrophage, Efferocytosis, Efferosome, Apoptosis, Phagocytosis, Phagosome, Vesicular Trafficking, Antigen Presentation, Anti-inflammation, Cell Biology, Cell Signaling, Rab GTPases, Maturation, Eat-me Signal, Phosphatidylserine.
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Chapter 1 - Introduction

1.1 Apoptosis

Apoptosis, the programmed cell death of old, damaged or unneeded cells, is triggered by multiple factors including exogenous stimuli such as pathogen infection and engagement of pro-apoptotic receptors, and endogenous stimuli such as aging, and damage from physical or chemical stresses\(^1\). Apoptotic cells must be cleared in order to maintain an organism’s normal physiological functions and tissue homeostasis. Upon recognition by phagocytic cells, apoptotic cells are cleared promptly and efficiently. For example, neutrophils in the peripheral circulation system have a high incidence of apoptosis due to their short half-life, but the number of apoptotic neutrophils remains at a low basal level due to their efferocytic removal by macrophages in the bone marrow\(^2\). In instances where apoptotic cells are not efficiently removed, chronic inflammation and autoimmunity can occur. Apoptotic cells display many distinct hallmarks that are excluded from healthy cells such as membrane blebs on their surfaces as a result of increased intracellular hydrostatic pressure from actomyosin contractility of the cortical cytoskeleton\(^3\). This blebbing process is induced as a part of the apoptotic signaling pathway, which is driven by cysteine-dependent aspartate-driven proteases termed caspases\(^4\). Upon initiation of apoptosis by internal stress and damage (intrinsic pathway), pro-apoptotic factors induce the release of cytochrome C from mitochondria, which then complexes with the cytosolic protein apoptosis activating factor-1 (Apaf-1). Caspase 9 is recruited to this complex, forming a structure termed the “apoptosome”, which then activates caspase 3, an
executioner caspase that begins the disassembly of the cell\textsuperscript{5}. Blebbing of the apoptotic cell is initiated by activated caspase 3, which cleaves the C-terminus of Rho-associated coiled-coil-containing protein kinase 1 (ROCK1), forming a constitutively active enzyme which then phosphorylates myosin light chain kinase. The resultant activation of myosin II leads to cortical actin contraction and increased intracellular hydrostatic pressure, thus producing membrane blebs. Actin is cleared from the growing blebs, and later replaced in mature blebs through late stage actin repolymerization\textsuperscript{3}. Caspase 3 also activates other cell degrading proteins such as Caspase-activated DNAse (CAD) which fragments the nuclear DNA, which is then dispersed to the forming blebs\textsuperscript{6}. Other cellular compartments (e.g. the Golgi apparatus, endoplasmic reticulum, and mitochondria) are broken down and packaged in a similar fashion as nuclear DNA fragments, while activation of enzymes such as lipid scramblases and phospholipases lead to the exposure of “find-me” and “eat-me” signals which enable the recognition of apoptotic cells by phagocytes (discussed in Section 1.3). Once released, these blebs are termed apoptotic bodies, which are ultimately identified and removed through efferocytosis\textsuperscript{7}.

Throughout this process the apoptotic cell membrane remains intact, thus preventing the release of intracellular proteins, while the small size of the released blebs plus the exposure of “find-me” and “eat-me signals” enhances uptake by phagocytes\textsuperscript{3,8}. However, if apoptotic cells are not cleared, the membrane integrity of apoptotic bodies becomes unstable, culminating with the release of intracellular materials into the extracellular milieu through a process called secondary necrosis. This can lead to negative
immunological consequences including chronic inflammation and autoimmune diseases.\textsuperscript{1, 9, 10, 11}

1.2 Macrophage Subtypes

Macrophages, a type of professional phagocyte, have classically been regarded as leukocytes whose main function is the phagocytosis (engulfment and clearance) of pathogens. They also have minor roles in antigen presentation and regulation of the innate and adaptive immune systems through cytokine production. Recent studies revealed that macrophages have additional important roles in homeostasis and wound healing, in addition to their roles during infection. This multi-functional activity of macrophages is mediated in part by their polarization into distinct subsets, each with unique functions: 1) M1 macrophages which engage in the pro-inflammatory/pro-phagocytic activities classically assigned to macrophages, 2) M2 macrophages which drive wound healing and tissue remodeling responses, and 3) M0 (unpolarized) macrophages which patrol tissues and may engage in homeostatic processes such as efferocytosis within uninfected/undamaged tissues.\textsuperscript{12, 13}

\textit{In vitro}, each subtype can be derived from monocytes isolated from peripheral blood, which are then differentiated by the addition of either granulocyte-macrophage colony stimulating factor (GM-CSF) or macrophage colony stimulating factor (M-CSF), followed by administration of additional cytokines.\textsuperscript{14} GM-CSF plus the addition of pro-inflammatory compounds such as IFN-\(\gamma\) and LPS result in the differentiation of M1 macrophages, whereas M-CSF plus regulatory cytokines such as IL-4 or IL-13 generate
M2 macrophages. M0 macrophages are produced by culturing with M-CSF alone, thus avoiding the polarizing effects of inflammatory or regulatory cytokines\(^1\).

Jaguin et al. characterized in the patterns of cytokine production in each macrophage type\(^1\). M1 macrophages, which both produce and react to pro-inflammatory cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\), exhibit enhanced phagocytosis of pathogens compared to other macrophage subtypes. M2 cells function through the production of anti-inflammatory cytokines such as TGF-\(\beta\) and enhance following internalization of apoptotic cells or entry into wound sites\(^1\). Unpolarized M0 macrophages appear to resemble resident macrophages in healthy tissues, and display a phenotype distinct from M1 cells, but with some similarities to M2 cells\(^1\). Importantly, M0 cells can differentiate into either M1 or M2 cells, although it is not clear if M1 or M2 cells are capable of further differentiation\(^1\), \(^4\), \(^14\), \(^17\). Functional differences between M1 and M2 macrophages have been widely studied, especially in their production of reactive oxygen species production after phagocytosis; M1 cells produce high NADPH oxidase activity following phagocytosis, whereas M2-like macrophages produce minimal NADPH oxidase activity\(^1\). This may result in differences in antigen presentation and degradation of phagocytic/efferocytic cargos\(^1\). Some of these functional differences are explored later in this thesis.

### 1.3 Overview of Efferocytosis

Efferocytosis, the process through which phagocytes recognize and ingest (phagocytose) apoptotic cells, maintains tissue homeostasis by regulating cell numbers and remodels tissues in an anti-inflammatory fashion. The clearance of apoptotic cells is achieved
through the stepwise removal of apoptotic corpses by: 1) Recruitment of phagocytes to “find-me” signals secreted by apoptotic cells, 2) Display of “eat-me” signals on apoptotic cells which are then bound by efferocytic receptors on phagocytes, 3) Internalization of apoptotic cells by receptor mediated signaling pathways, and 4) Digestion of apoptotic cells by an efferosome maturation process within the phagocyte. Although efferocytosis is considered as an anti-inflammatory process, the mechanism by which apoptotic cells are degraded in a fashion which prevents inflammation and maintains immunological silence is not understood.

Phagocytes migrate to sites of apoptosis by following “find-me” signals, soluble chemotactic molecules released from apoptotic cells such as lysophosphatidylcholine (LPC). Receptors on phagocytes then bind to “eat-me” signals displayed by apoptotic corpses. Among the many “eat-me” signals, phosphatidylserine (PtdSer) is a well-characterized and widely utilized apoptotic signal. Apoptotic cells also express additional “eat-me” signals such as calreticulin and oxidized lipids on their surfaces. These are recognized by multiple surface receptors on phagocytes, which bind to apoptotic cells either directly or indirectly through soluble bridging molecules termed opsonins. Upon internalization, apoptotic corpses are retained in a membrane-sealed vacuolar structure called the efferosome, where it is processed and degraded. The resulting self-antigens are either presented without activating co-stimulatory molecules or presentation is avoided completely after the efferosome maturation process is complete. These processes which comprise efferocytosis are explored in greater detail below.
**Find-me signals**

Prior to recognition and internalization of apoptotic cells, phagocytes must be recruited to the location in which cells are undergoing apoptosis. This occurs through the release of “find-me” signals which act as chemoattractants that direct phagocytes to sites of apoptosis. The range of these “find-me” signals depends on their concentration and half-life, which may vary between tissues\(^{21}\). “Find-me” signals identified to-date include LPC, fractalkine (CXCL3), adenosine triphosphate (ATP) and uracil triphosphate (UTP) nucleotides, and sphingosine-1-phosphate (S1P)\(^{21}\).

Peter *et al.* showed that calcium-independent phospholipase-A2, activated by caspase 3, produces LPC which is then released into the extracellular matrix\(^{20}\). This soluble LPC signals through the receptor G2A, G-protein-coupled receptor found on macrophages, thus allowing macrophages to migrate towards the apoptotic cell. However, these observations are controversial as the study of Lauber *et al.* demonstrated that significantly higher concentrations of LPC were required for chemotaxis than were normally released by apoptotic cells\(^{25}\). In fact, this study showed that the membrane damage caused by production of the quantity of LPC required for chemotaxis would lead to lysis of the apoptotic cell. The role of LPC as a find-me signal is controversial for an additional two reasons; namely that it is relatively insoluble and therefore is not expected to diffuse and form gradients *in vivo*, and because it is unclear if G2A is truly a receptor for LPC\(^{26}\).
Fractalkine is a membrane tethered cytokine with dual functions as an adhesion molecule and chemokine\textsuperscript{27}. Apoptotic neurons and B cells rapidly produce and shed fractalkine, which then attracts macrophages and microglia via the fractalkine receptor CX3CR\textsuperscript{27}. Miksa \textit{et al.} showed that fractalkine also contributed to recognition and engulfment of apoptotic cells by upregulating milk fat globule EGF factor 8 (MFG-E8) expression, an opsonin which enhances efferocytosis via the MerTK efferocytic receptor\textsuperscript{27}. However, fractalkine’s role in attracting phagocytes to sites of apoptosis remains elusive. Although fractalkine enhanced MFG-E8 production in microglia, efferocytosis by microglia was not observed\textsuperscript{27}. Moreover, the role of fractalkine in attracting macrophages at sites where it is produced – e.g. Kupffer cells in the liver and alveolar macrophages in the lungs - has not been studied, meaning further research is required to elucidate fractalkine’s role as a “find-me” signal. Finally, fractalkine’s role as a find-me signal appears to be limited to immune cells undergoing apoptosis, rather than a universal find-me signal produced by all apoptotic cells.

Apoptotic cells release small amounts of ATP and UTP that induce chemotaxis of phagocytes to apoptotic cells\textsuperscript{26}. Again, the role of these nucleoside triphosphate remains controversial as ATP and UTP can also function as danger-associated molecular patterns (DAMPs) which are usually associated with necrosis and initiation of pro-inflammatory responses\textsuperscript{11}. Once ATP and UTP are present in the extracellular space, phagocytes respond via the purinergic receptor P2Y\textsubscript{2}. Again, the role of these “find-me” signals remains unclear, as these nucleotides are short-lived in the extracellular milieu as they are rapidly degraded by extracellular nucleotidases\textsuperscript{26, 28}. Thus ATP and UTP’s function as
find-me signals would be limited to attracting neighbouring phagocytes rather than cells residing in the circulation or at more distal sites.

The best characterized “find-me” signal is sphingosine-1-phosphate (S1P). Unlike the previously mentioned find-me signals, S1P appears to be produced by all apoptotic cells, is highly soluble, and has a sufficiently long half-life to recruit cells over long distances. S1P is derived from sphingomyelin, a lipid found in the plasma membrane. Once cells undergo apoptosis, sphingomyelinase processes sphingomyelin to sphingosine, followed by caspase-activated sphingosine kinases phosphorylating sphingosine to form soluble sphingosine-1-phosphate (S1P). S1P induces chemotaxis of phagocytes through the S1P receptor, with chemotaxis occurring at physiologically relevant concentrations of S1P. Moreover, S1P is anti-inflammatory, as would be expected of a “find-me” signal released during apoptosis.

How these various “find-me” signals interact to optimize in recruiting phagocytes is not completely elucidated. For example it is not yet clear whether they are synergistic or non-synergistic in attracting phagocytes, and how they interact with proposed inhibitory “don’t find-me” signals.

**Eat-me signals**

Once professional phagocytes are recruited to a site where cells are undergoing apoptosis, phagocytes must distinguish healthy cells from apoptotic cells prior to engulfment. Apoptotic cells are recognized by several “eat-me” signals present on the extrafacial surface of their plasma membrane. The plasma membrane of apoptotic cells displays
numerous changes in glycosylation patterns of proteins and lipids, changes in lipid composition and changes in surface charge, all while maintaining an intact membrane. These changes to the plasma membrane all act as “eat-me” signals which are recognized by receptors on phagocytes. Of these “eat-me” signals, phosphatidylserine (PtdSer), is the best characterized. PtdSer is normally restricted to the inner leaflet of the plasma membrane, but is flipped to the outer leaflet plasma membrane during apoptosis. This PtdSer flipping to the outer leaflet is dependent of caspase activity during apoptosis, in which the transmembrane protein 16F (TMEM16F) acts as a scramblase that randomly sorts phospholipids, including PtdSer and phosphatidylethanolamine (PtdEtn), in a calcium-dependent manner across the leaflets of the plasma membrane. The signalling that induces this process is still poorly understood, but it is established that the formation of a cytosolic Ca²⁺ gradient is required to activate TMEM16F. The same study showed that knockout of Tmem16f, using small hairpin RNA, downregulates the scrambling of PtdSer and PtdEtn during apoptosis. In addition, Scott syndrome patients have defective TMEM16F due to a splice mutation of TMEM16F and their platelets and blood cells cannot expose PtdSer and PtdEtn on the outer leaflet of the plasma membrane.

Additional “eat-me” signals include calreticulin, intracellular adhesion molecule 3 (ICAM3), and binding of serum proteins such thrombospondin and complement C1q to the cell surface. Calreticulin is a protein normally located in the endoplasmic reticulum (ER), sarcoplasmic reticulum (SR) and nucleus. However, once cells undergo apoptosis, calreticulin is transported via the secretory pathway to the plasma membrane where it becomes accessible to its receptor, low density lipoprotein receptor-related protein.
(LRP)/CD91, which is expressed on macrophages\textsuperscript{35}. Apoptotic cells also expose other molecules such as oxidized lipids and unusually glycosylated lipids and proteins, which are engaged by scavenger receptors such as CD36\textsuperscript{22}. Despite the large number of identified “eat-me” signals, only PtdSer has been studied in detail, and is the most commonly used apoptotic cell surface marker due to its exposure on apoptotic cells in abundant quantities, as well as the identification of several well characterized PtdSer receptors on phagocytes\textsuperscript{11}. It is unlikely that PtdSer on its own is sufficient for efferocytosis, as some healthy cells also display PtdSer on the outer leaflet of their plasma membrane without being targeted by phagocytes. For example, platelets expose PtdSer as part of the clotting process, while maturing B and T cells expose PtdSer in lymph nodes for an unknown purpose\textsuperscript{10, 36, 37, 38}. A part of the reason these cells may not be removed is the co-expression of CD31 and an integrin associated protein, CD47, which act as “don’t eat-me” signals. The function of CD31 is not well understood, but CD47 is known to bind to a specific receptor, signaling regulatory protein α (SIRPα), which contains an immunoreceptor tyrosine-based inhibition motif (ITIM) that inhibits phagocytosis. Thus CD31 and CD47 on healthy cells appear to prevent efferocytosis through inhibiting phagocytic signaling\textsuperscript{10, 11, 35}.

**Efferocytic receptors mediate internalization of apoptotic cells**

Once professional phagocytes recognize “eat-me” signals the phagocytes quickly internalize the apoptotic cell. This is driven by two main groups of efferocytic receptors—those that directly bind to “eat-me” signals (e.g. TIM-4, Stabilin2, CD36 and BAII1) and
those which indirectly bind to “eat-me” signals (e.g. Tyro-3-Axl-MER and αvβ5) via opsonins (e.g. Gas6, MFG-E8)10,11.

TIM-4 (Table 1) has recently been highlighted as an important apoptotic cell receptor, and is able to support the binding and internalization of apoptotic cells independent of opsonins. A recent study showed that internalization of PtdSer-coated glass beads that mimic apoptotic cells was dependent on TIM-4, with TIM-4 knockout mouse bone marrow derived macrophages (BMDM) being unable to internalize these beads. However, this paper likely over-simplified what is occurring, as other studies have shown that PtdSer on its own does not act as an “eat-me” signal, with additional signals such as the oxidation of the PtdSer being required for efferocytosis to occur22,39,40. Internalization of PtdSer-coated beads required crosslinking of TIM-4 and the β1 integrin, which led to downstream activation of Src family and focal adhesion kinases (FAK), followed by actin polymerization driven by activation of Rac1 and Rac234. Additional PtdSer receptors may also be involved in this internalization process. For example, brain-specific angiogenesis inhibitor 1(BAI1, Table 1), a G-protein coupled receptor, binds to PtdSer and cardiopilin via a binding site composed of five thrombospondin type 1 repeats (TSRs). Upon binding of PtdSer on apoptotic cells, BAI1 induces activation of the Rac1 guanine exchange factor (GEF) complex ELMO/DOCK180 to mediate Rac-activation, leading to internalization of the apoptotic cell. BAI1 knockout in primary astrocytes and J774 macrophages decreased internalization of apoptotic thymocytes and carboxylate beads that mimic apoptotic cells, supporting the concept that efficient efferocytosis requires the concordant activation of several receptors30,41.
**Table 1: Multiple efferocytic receptors are required to enhance efferocytosis**

Professional phagocytes express multiple efferocytic receptors that optimize capture and engulfment of apoptotic cells. Receptors either bind directly to PtdSer or indirectly via opsonins that connect receptors with “eat-me” signals and enhance internalization of apoptotic cells.
<table>
<thead>
<tr>
<th>Receptors</th>
<th>Opsonins</th>
<th>Eat-me signals</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIM4</td>
<td>–</td>
<td>PtdSer</td>
<td>34</td>
</tr>
<tr>
<td>BAI1</td>
<td>–</td>
<td>PtdSer</td>
<td>41</td>
</tr>
<tr>
<td>CD36</td>
<td>–</td>
<td>oxLipids (ex. oxPtdSer)</td>
<td>42</td>
</tr>
<tr>
<td>Stabilin-2</td>
<td>–</td>
<td>PtdSer</td>
<td>43</td>
</tr>
<tr>
<td>CD91</td>
<td>Calreticulin, C1q, MBL</td>
<td>Modified, Modified Glycoproteins/Glycolipids</td>
<td>44</td>
</tr>
<tr>
<td>MER</td>
<td>GAS6, Protein S, Tubby and tubby-like protein 1</td>
<td>PtdSer</td>
<td>10, 11, 45, 46</td>
</tr>
<tr>
<td>$\alpha_v\beta_{3/5}$</td>
<td>MFG-E8</td>
<td>PtdSer</td>
<td>10, 11</td>
</tr>
</tbody>
</table>
CD36 (Table 1) is a class B scavenger receptor that binds to various ligands including oxidized LDL (oxLDL) and oxidized lipids. In atherosclerosis this receptor drives the accumulation of cholesterol inside macrophages, forming foam cells. In addition to removing oxLDL, CD36 also acts as an efferocytic receptor by binding to oxidized lipids including oxidized PtdSer. Early studies revealed that the PtdSer binding site in CD36 is separate from the LDL binding site. Critically, a recent study shows CD36 to be capable of engulfing apoptotic cells in primary mouse peritoneal macrophages (MPM), with binding occurring between CD36 and oxidized PtdSer rather than normal PtdSer. Interestingly, CD36 knockout primary MPM show decreased internalization of oxidized PtdSer-coated vesicles versus increased non-oxidized PtdSer-coated vesicles, hinting that CD36 may act to both drive efferocytosis and inhibit the removal of cells displaying PtdSer in a non-apoptotic (e.g. non-oxidized) context.

Stabilin-2 (Table 1) contributes to internalization of apoptotic cells through binding PtdSer via extracellular epidermal growth factor like domain repeats (EGFrp). Stabilin-2 induces downstream signaling through the intracellular adaptor protein GULP, but the resulting signaling process remains elusive. Importantly, the integrin binding domain (FAS1) of Stabilin-2 directly interacts with integrin αvβ5 and activates ELMO/DOCK180/Rac complex to induce actin polymerization followed by internalization of apoptotic cells. This may allow this receptor to engage the classical integrin-mediated phagocytosis internalization pathway.
Previous studies by Gardai *et al.* introduced CD91 (known as a scavenger receptor and LDL-receptor-related protein, Table 1) on macrophages, which binds to calreticulin on apoptotic cells. Apoptotic cells translocate calreticulin, an ER chaperone, to the plasma membrane. Engagement of calreticulin by CD91 initiates efferocytosis. In addition to binding calreticulin, CD91 mediates efferocytosis in response to a number of other “eat-me” signals, including C1q deposited on the apoptotic cell surface, the binding of mannose binding lectin (MBL) to glycoproteins and glycolipids modified by apoptotic processes.

PtdSer and other “eat-me” signals on apoptotic cells are also bound by opsonins such as protein S, Gas6, and MFG-E8 (Table 1). These opsonins act to bridge these “eat-me” signals efferocytic receptors such as the Tyro-3-Axl-MER (TAM, Table 1) family of receptors and integrins such as αβ₃ and αvβ₅. The signaling of these receptors has not been elucidated, but they are known to play a central role in homeostasis in the eye and in preventing autoimmune disorders such as lupus. While not thoroughly understood, it is likely that these opsonin-dependent receptors cooperate with the receptors that directly bind to “eat-me” signals, culminating in fast and efficient internalization of apoptotic cells.

*Common signaling pathways driving apoptotic cell internalization*

The signaling of most efferocytic receptors remains unknown, although recent studies have provided some insights into functions of a small number of these receptors. Interestingly, many of these studies have shown that integrin-mediated signaling is a key
component of several efferocytic receptors\textsuperscript{34, 45} despite apoptotic cell binding is largely dependent on non-integrin receptors. Outside of efferocytosis, integrins play major roles in a wide range of biological activities including chemotaxis, adhesion, and phagocytosis. All integrins are heterodimers composed of $\alpha$ and $\beta$ subunits. Each subunit has a large ligand-binding extracellular domain, a single membrane spanning domain and a short lumenal domain which mediates integrin cell signaling via various tyrosine kinases and adaptor proteins\textsuperscript{48}. As described above, several apoptotic receptors interact with integrins as co-receptors, and appear to utilize the integrin signaling pathway to mediate apoptotic cell internalization. Integrins associated with efferocytosis include $\alpha_v\beta_3$ and $\alpha_v\beta_5$, with siRNA-knockout of both integrins in macrophages decreasing efferocytosis by more than 60\%\textsuperscript{34}. Addition of antagonistic RGD peptides or anti-$\alpha_v\beta_3$ antibody also reduced phagocytosis of apoptotic neutrophils by primary human macrophages to a similar degree\textsuperscript{49}. It is unclear at this time if the integrins normally associated with phagocytosis (e.g. $\alpha_M\beta_2$) are involved in efferocytosis.

Upon integrin activation, FAK is activated by auto-phosphorylation and recruits Src kinases via its Src-homology 2 (SH2) domain. Subsequent tyrosine phosphorylation on FAK by Src kinases recruits CRK2-associated substrate (CAS) and phosphatidylinositol 3-kinases (PI3-K) to FAK\textsuperscript{10, 48, 49}. Lastly CRK2 and dedicator of cytokine 180 (DOCK180) are recruited to the FAK complex. Vav, a Ras GEF, is recruited to DOCK180-ELMO1 complex to activate Rac1. Active Rac1 and CDC42 together activate Arp2/3 which then induces actin polymerization and cytoskeleton rearrangement. Ultimately, the resulting actin remodeling induces formation of membrane protrusions which envelope and
internalize the apoptotic cell\textsuperscript{10,11,34,50}. Once enveloped, Vav activates RhoA, which in turn activates ROCK followed by activation of myosin light chain kinase. This drives actin contraction through phosphorylation of the myosin light chain. The resulting actin contraction pulls the enveloped apoptotic cell into the phagocyte, resulting in formation of an efferosome. Because RhoA impairs actin polymerization it can negatively regulate apoptotic cell internalization if active during the initial stages of envelopment. However, when activated near the end of the envelopment stage, RhoA’s induction of actin contraction is key for full internalization of the apoptotic cell and its subsequent degradation\textsuperscript{11}.

1.4 Phagocytosis as a Model of Efferocytosis

The signaling molecules and vesicular fusion events that mediate the degradation of a phagocytosed apoptotic cell remain unexplored in the mammalian system. However, previous studies in the nematode \textit{Caenorhabditis elegans} suggests that the same degradative pathway is responsible for degrading both efferosomes and phagosomes\textsuperscript{51,52}. Phagocytosis is the process through which professional phagocytes internalize and destroy pathogens, and is crucial to both innate and adaptive immunity\textsuperscript{52}. Professional phagocytes (e.g. macrophages and neutrophils) recognize and internalize and destroy large particles, greater than 0.5 $\mu$m\textsuperscript{53}. In addition to removing and degrading pathogens, phagocytosis leads to the display of phagosome-derived antigens to peripheral lymphoid cells and expression of pro-inflammatory cytokines. As such phagocytosis is pro-inflammatory and immunogenic, the opposite of efferocytosis. Despite the opposite immunological outcomes, efferocytosis and phagocytosis appear to share some
similarities in the cellular processes they use for degradation, and thus we used phagocytosis as a guide for our investigations into efferocytosis.

Phagocytosis is a receptor-mediated process that can be driven by more than 20 different pathogen binding receptors which allow refined immune reactions to different types of pathogens. Indeed, some phagocytic receptors act as pattern-recognition receptors (PRRs) which detect pathogen-associated molecular patterns (PAMPs) on bacteria, fungi and parasites. For example, the phagocytic receptor dectin-1 binds β-glucans on fungal pathogens. Not all phagocytic receptors act as PRRs; some (e.g. FcγRI) instead bind to pathogens via opsonins such as immunoglobulins (e.g. IgG), which in turn bind to surface antigens on pathogens. Once the pathogen engages the phagocytic receptors on macrophages, the plasma membrane of the phagocyte is extended and envelops the receptor-engaged pathogen, eventually drawing the pathogen into an intracellular vacuolar structure that matures into a bactericidal compartment via fusion with endosomes and lysosomes. This is orchestrated by changes in membrane phosphatidylinositol (PtdIns)-derived phospholipids and recruitment of regulatory proteins that drive the step-wise maturation of nascent phagosomes into phagolysosomes. First, early endosomes are recruited to the nascent phagosome by the Rab5 GTPase, where they fuse with the nascent phagosome to form an early phagosome (Figure 1). Rab5 is then replaced by Rab7, which mediates fusion between the phagosome and late endosomes and lysosomes, forming a phagolysosome. A key component driving this process at the early phagosome stage is Vps34, a class III phosphatidylinositol 3-kinase.
Figure 1: Rab GTPases mediated phagosome maturation

Once pathogens (> 0.5 µm) are engulfed by macrophages, newly formed membrane-sealed phagosomes undergo step-wise maturation process mediated by Rab 5 and 7 GTPases, with subsequent changes in membrane phospholipids and lumenal pH. Each maturation stage is associated with acidification from endosomal and lysosomal fusion, wherein pathogens are degraded. Figure adapted from Dr. Heit.
Vps34 phosphorylates phosphatidylinositol (PI) to form phosphatidylinositol-3-phosphate (PI(3)P), which mediates further recruitment of early endosomes and their fusion to early phagosomes\textsuperscript{10}. PIKFYVE, a PI5-Kinase is recruited to, and then phosphorylates PI(3)P to form PI(3,5)P\textsubscript{2}. This triggers the exchange of Rab5 for Rab7, thus enabling fusion of the phagosome with late endosomes and lysosomes, and thus driving transition of the phagosome first to a late phagosome and then to a phagolysosome\textsuperscript{55}. It is during this later stage of phagosome maturation where the bulk of phagosome acidification occurs, thus activating the degradative enzymes delivered through lysosomal fusion. The combined effect of acidification and degradative enzyme activation kills the pathogens within the phagosome\textsuperscript{10,51,52}.

The roles of Rab GTPases in endosome trafficking and phagosome maturation

The Rab GTPases (Ras-related protein in brain) is a group of small GTPases, composed of over 60 members, that localize specifically to intracellular membranes\textsuperscript{56}. Rab GTPases cycle between active and inactive states when bound to GTP or GDP respectively\textsuperscript{10,56}. Rab GTPase activation is mediated by guanine nucleotide exchange factors (GEFs) that replace GDP for GTP, thus activating the Rab. Rab GTPases are crucial in delivery and fusion of cellular component-containing vesicles between organelles. These GTPases control traffic of these vesicular cargoes via several components: 1) SNARE complexes control the specific membrane fusion, 2) Motor proteins (e.g. dynein/dynactin) transport vesicles along the microtubular network, and 3) Cytosolic coat molecules that recruit specific molecular components to vesicles. Inactivation of Rab proteins is mediated by
GTPase-activating proteins (GAPs) that induce the hydrolysis of GTP to form GDP. An additional protein, Rab GDP dissociation inhibitor (GDI), maintains the inactive form of the GTPases, designates the delivery site of inactive Rab GTPases, and recycles inactivated Rab to the cytosol\textsuperscript{10, 56, 57, 58}.

Rab5 and 7 directly contribute to phagosomal maturation (Figure 1). The nascent phagosome undergoes fusion with early endosomes where it acquires Rab5. Active Rab5 recruits Vps34 the class III PI3K that synthesizes PI(3)P, in turn recruiting additional Rab5 effector proteins such as early endosome antigen 1 (EEA1)\textsuperscript{10}. In yeast, Rab5 contributes to the Rab5 to Rab7 transition by recruiting a Rab7 GEF, composed of Mon1 and Ccz1, but their functions in mammalian system are not well-elucidated\textsuperscript{59}. Rab7 replaces Rab5 in the transition state from early phagosomes to late phagosomes. In the end, Rab7-interacting lysosomal proteins (RILP) and the long splice-variant of oxysterol-binding protein related-protein1 (ORP1L) are recruited by Rab7 to late phagosomes where they act as Rab7 effectors. These Rab7 effectors induced linkages between microtubule proteins and phagosomes, driving the movement of the phagosome to the perinuclear area where they undergo fusion with lysosomes and late endosomes, thus forming phagolysosomes\textsuperscript{52}. Lysosome-associated membrane proteins 1 and 2 (LAMP-1 and -2) are also significant late phagosomal membrane proteins, which are critical for Rab7 recruitment and preservation of lysosomal membrane integrity\textsuperscript{52}. A recent study of Rab34 has shown that Rab34 also localizes to phagosomes where it is involved in the size-dependent delivery of cargo from late endosomes and lysosomes. Specifically, Rab34, accompanied by its effector Munc 13-2, aids in the delivery of large (>70kDa)
lysosomal contents to the maturing phagosome in Rab7 independent manner\textsuperscript{60}. In summary Rab GTPases are crucial in regulating the membrane traffic and vesicular fusion that drives phagosome maturation, and enable the step-wise maturation of the phagosome through sequential fusion with early endosomes, late endosomes and finally lysosomes.

\textit{Phagosome acidification and recruitment of degradative enzymes}

As phagosomes undergo maturation they reduce their passive proton permeability\textsuperscript{10}. At the same time Rab5 and Rab7 mediated vesicular fusions deliver vacuolar ATPases (V-ATPases) to maturing phagosomes. This V-ATPase pumps protons from the cytosol into the phagosome, leading the phagosome to acidify as it matures into a phagolysosome. The phagosomes begins with the same pH as the intracellular milieu (\textsim 7.4), drops to a pH of 6.5 in the early phagosome stage, and reaches a pH of 4.5 by the phagolysosome stage\textsuperscript{10, 61}. This thousand-fold increase in proton concentration cannot be achieved passively, and instead requires active pumping by the V-ATPase.

The resulting acidity activates many pH-dependent degradative enzymes such as cathepsins, lipases, and other lysosomal acid hydrolases, which degrade the pathogen\textsuperscript{10, 19}. This pH drop is also important for antigen presentation on MHC II molecules, as activation and loading of MHC II is driven by an acid-dependent protease that cuts the invariant chains that retains MHC II in an unloaded conformation. A recent study has shown that polarized macrophages such as M1 and M2 cells undergo different NADPH oxidase production\textsuperscript{18}. In addition, a study of dendritic cells and M0-like bone marrow
derived murine macrophages showed different superoxide production by NADPH oxidase alter the phagosomal pH through consumption of phagosomal protons to form $\text{H}_2\text{O}_2^{62,63}$. Thus it is predictable that phagosomes of M1 macrophages acidify slower and to a lesser extent than M0 or M2 macrophages due to their higher NADPH oxidase activity. Importantly, pro-inflammatory cytokines such as IFN-γ and PAMPs such as LPS not only stimulate the polarization of macrophages into M1 cells but also induce NADPH oxidase expression, suppressing the rate of acidification in the phagosomes of M1 cells. In contrast, M2-like macrophages, with similar low NADPH oxidase activity as M0-like macrophages, express fewer components and lower levels of the NADPH oxidase complex that may allow for faster acidification and a lower terminal pH$^{18,62}$. Expected prompt acidification in M2 cells may also lead to rapid cargo degradation which appears to degrade proteins before antigens can be loaded onto MHC II, preventing activation of the adaptive immune system. In contrast, the slow acidification of M1 phagosomes leads to stronger antigen presentation. While this presentation is important following the phagocytosis of a pathogen, the increased NADPH oxidase activity in M1 cells may produce oxidized self-antigens derived from apoptotic cells cargo’s, potentially leading to autoimmune antigen presentation$^{18,19}$. In summary, phagosome acidification contributes to many phagosomal functions, including not only activation of the phagosomal degradative pathways, but also in regulating antigen presentation and perhaps in the formation of autoantigens.
1.5 Known Efferocytic Maturation Mechanisms

While the efferocytic maturation process is not as well understood as the phagocytic process, some of the mechanisms have been studied in the *C. elegans* nematode model\(^5^1\). This study explored efferosome maturation in *C. elegans* and determined that it proceeds through a pathway similar to that of phagocytosis. In *C. elegans*, many cells undergo apoptosis during the nematode developmental processes. Evolutionarily, *C. elegans* and mammalian system share a common receptor-mediated signaling pathway that activates CED-10/Rac, leading to a phagocytosis-like internalization processes in which membrane ruffles and protrusions engulf apoptotic cells. Common phagosome maturation markers such as Rab5 and Rab7 GTPases were found to be recruited to *C. elegans* efferosomes in the same order and with similar time as with phagosomes\(^5^1\). The apoptotic cell containing efferosomes recruited Rab5 and Rab5 effectors such as VPS-34. Rab7 was recruited at later time points, replacing Rab5 on the efferosomal membrane without co-localization\(^5^1\).

Unlike mammalian phagocytosis, in *C. elegans* Rab5 was required for internalization, with the Rab5 knockout causing an accumulation of non-internalized apoptotic cells. In contrast, Rab7 knockout allowed for maturation to progress to the Rab5 step, but eventually the buildup of non-degraded apoptotic cells inhibited further uptake, leading to a block in apoptotic cells internalization\(^5^1\).

In contrast, our preliminary results using RAW264.7 murine macrophages showed a prolonged accumulation of Rab5 on efferosomes that contained apoptotic thymocytes and red blood cells (data not shown, by Dr. Bryan Heit), a finding also observed by other groups in dendritic cells\(^6^4,6^5\). In our experiments, PI(3)P was retained on efferosomes,
further indicating a lack of Rab5 to Rab7 transition. This suggested that the efferosome maturation process in mammalian cells was different from that used by *C. elegans*. Because of these distinct differences in efferosome maturation we became very interested in how the efferocytosis maturation pathway differed from phagocytosis, especially in how this altered processing impacts antigen presentation and inflammation in the mammalian system.

### 1.6 Efferocytic Defects and Disease

The importance of efferocytosis in maintaining homeostasis is emphasized in diseases where efferocytosis is defective. Generally apoptotic cells maintain membrane integrity and are efficiently efferocytosed by professional phagocytes without releasing any intracellular components. For example, the majority of developing thymocytes in the thymus become apoptotic, and yet it is nearly impossible to detect uncleared apoptotic thymocytes due to the high rate of efferocytosis by resident macrophages\(^66\). Deficiencies in efferocytic receptors such as CD93 lead to the accumulation of apoptotic cells in the thymus\(^67\), which would then be expected to lose membrane integrity and release their intracellular components through a process termed secondary necrosis. Some of the released cellular components can engage TLR and other pro-inflammatory receptors\(^68\). These components, termed alarmins, include proteins such as the nuclear protein high-mobility group box1 (HMGB1) as well as metabolites such as ATP\(^3\). In addition, secondary necrosis releases self-proteins which may be endocytosed by phagocytes, and due to the pro-inflammatory nature created by the simultaneous release of alarmins, may be presented as autoantigens along with immunostimulatory cytokines and co-stimulatory
molecules. Indeed, this release of self-antigens and alarmins from uncleared thymocytes in CD93 knockout mice causes an autoimmune diseases similar to systemic lupus erythematosus (SLE). Mutations in a number of efferocytic receptors are associated with the onset of SLE and other autoimmune diseases, highlighting the importance of efferocytosis in maintaining immune homeostasis.

In addition to autoimmunity, failures in efferocytosis can lead to the development of chronic inflammation such as atherosclerosis. During atherosclerosis macrophages in the arterial wall become apoptotic in response to the uptake of large quantities of cholesterol. Normally, these apoptotic macrophages are efferocytosed by neighbouring macrophages. However, for unknown reasons in some people this homeostatic process fails, leading to the accumulation of uncleared apoptotic macrophages in the vascular intima. In some patients these defects are induced by inherited mutations in efferocytic receptors and their opsonins (e.g. MerTK, MFG-E8). These uncleared apoptotic macrophages become necrotic, accumulating over time to form an atherosclerotic plaque. This structure is weak and prone to rupture - the resulting thrombus can dislodge, potentially evoking an acute myocardial infarct or ischemic stroke. Clearly, successful apoptotic cell clearance is beneficial not only to homeostasis, but also in preventing clinically important autoimmune and inflammatory diseases.

1.7 Rationale

The preliminary results from our lab, as well as the work with dendritic cells by Ackerman et al. and Jancic et al. suggest that efferocytosis proceeds through a
maturation process unique from that utilized by phagocytosis. This study aims to elucidate the maturation process of efferocytosis in mammalian cells, with a focus on the role played by the vesicular trafficking system during efferosome maturation and identifying of potential unique protein regulators found on efferosomes. I believe that a unique maturation process is required to maintain homeostasis through preventing the efferosome from maturing into the same pro-inflammatory and immunogenic compartment as the phagosome. Indeed, evidence suggest that defects in efferocytosis cause or promote several chronic inflammation and autoimmune diseases, suggesting that the normal efferocytic process is inherently non-inflammatory. In this study we will use murine and primary human macrophages to elucidate the molecular pathway regulating the maturation process of efferosomes and to identify unique protein regulators found on efferosomes versus phagosomes.

1.8 Hypothesis and Aims

Because efferocytosis and phagocytosis have different inflammatory and immunogenic outcomes, I hypothesized that phagocytic and efferocytic maturation pathways are regulated by fusions with different intracellular compartments, and that these fusion events are regulated by novel mediators recruited either to phagosomes or to efferosomes. To investigate my hypothesis, I proposed the following research aims:

Research Aim 1. Establish an in vitro macrophage model of efferocytosis.
**Rationale:** Macrophages engulf apoptotic cells in a receptor-dependent manner, but these receptors are differentially expressed on different macrophage subtypes. To ensure consistent and reproducible results, I: 1) Identified macrophage cell lines and differentiated primary human macrophages subtypes with high efferocytic capacity, and 2) Developed reproducible cell-based and bead-based efferocytic targets that were employed in later aims.

**Research Aim 2: To characterize the acquisition of common maturation markers (i.e. Rab5, Rab7, LAMP-1) on efferosomes.**

**Rationale:** The Rab GTPases Rab5 and Rab7 are critical regulators that direct remodeling phagosomes and endosomes. However, the role of these Rab proteins in efferocytosis is poorly understood in mammalian cells. By assessing the recruitment of Rab5 and Rab7, as well as the recruitment of other markers of key vesicular compartments, we can address the role of these proteins in efferosome maturation. One key marker is LAMP-1\textsuperscript{73}, a well-known marker found in late endosomes and lysosomes. Lysosomal fusion to phagosomes can be confirmed via immunostaining of LAMP-1 or by following maturation in cells transfected with fluorescent LAMP-1. Together, tracking these markers will characterize the role of these regulators on efferosome maturation.

To address this aim, I: 1) Tracked the recruitment of Rab5 and Rab7 on efferosomes. 2) Tracked LAMP-1 recruitment to efferosomes to determine if lysosomal fusion was occurring.
Research Aim 3: To identify proteins that influence efferosome maturation through an unbiased mass spectrometry based approach.

Rationale: Since we expect efferosome maturation to occur through a novel maturation pathway, it is insufficient to merely test known regulators, and as such an unbiased approach is needed to identify any novel regulatory factors. To identify these unique proteins, mass spectrometry analysis of isolated efferosomes and phagosomes was performed. To accomplish this aim I: 1) Recovered efferosomes using phospholipid-coated silica-magnetic beads as apoptotic cell mimics, and 2) Identified several novel maturation regulators on the purified efferosomes and phagosomes using mass spectrometry.
Chapter 2 – Materials and Methods

2.1 Cell Line Culture and Human Macrophage Isolation

Human Jurkat T cells (gift from Dr. Lina Dagnino, Department of Physiology and Pharmacology, Western University) and murine macrophage cell line J774.2 (ATCC) were cultured at 37 °C in 5% CO₂ in RPMI 1640 with 10% fetal bovine serum (FBS) (all from Wisnet). RAW264.7 cells were cultured in DMEM + 10% FBS. Cultures were split when they reached 80% of confluence. For phagocytosis and efferocytosis assay, 5 x 10⁵ J774.2 or RAW264.7 cells were cultured on sterile coverslips.

Human primary monocytes were isolated from blood donated by healthy donors between the ages of 18 and 65. Blood was collected in the lab under approval from Western university’s Health Science Research Ethic Board using methods meeting the guidelines of the Tri-Council Policy Statement on human research. Blood was layered over an equal volume of lympholyte poly (Cedarlane), and centrifuged at 500 x g for 35 minutes at 22 °C. The top cell layer of peripheral blood mononuclear cell (PBMC) was collected and washed with PBS. Monocytes were separated from PBMC by adhesion to glass or plastic surface - PBMC’s were resuspended in RPMI 1640 (300 µl /well). For phagocytic assays, 300 µl of suspension was added to a sterile 18mm diameter coverslips placed into a well of a 12-well tissue culture plate and incubated at 37 °C in 5% CO₂ for 1 hour. Non-adherent (non-monocyte) PBMC’s were removed by a wash with 37 °C PBS. 1ml of RPMI 1640 with cytokines (see below) was added to each well. For mass spectrometry a total of 3.6 ml PBMC suspension was added to a 1-well gelatin-covered plate. An hour
later non-adherent cells were removed by washing as above and 12ml of RPMI 1640 with cytokines (see below) was added to each plate.

2.2 Primary Human Macrophage Differentiation

Adherent monocytes were differentiated over 7 days into each subtype of macrophages through the addition of specific cytokines. M0 macrophages were differentiated by the addition of M-CSF (10 ng/µl) alone for 7 days. M1 macrophages were differentiated in GM-CSF (20 ng/µl) for 5 days followed by the addition of IFN-γ (10 ng/ml) and LPS (250 ng/ml). M2 macrophages were cultured in M-CSF (10 ng/µl) followed by the addition of IL-4 (10µg/ml) on day 5. All cells completed differentiation on day 7 and remained viable until day 1074.

2.3 Induction of Jurkat T Cell Apoptosis and PtdSer Flipping

Apoptosis was induced in Jurkat T cells by treating 2.5 mM staurosporine (STS) in RPMI 1640 and incubated at 37 °C in 5% CO2 for 2.5 hours. Jurkat T cells were then washed three times with PBS at room temperature and then resuspended to 1ml of PBS. Annexin V (1/1000, eBioscience) was added to Jurkat T cells in 1 ml of imaging buffer (25 mM HEPES, 0.8% NaCl, 5.4 mM KCl, 1.3 mM CaCl2•2H2O, and 1mM MgCl2 in 100ml ddH2O with pH 7.4) and incubated for 5 minutes, followed by three washes in PBS with 2.5 mM CaCl2 at room temperature and then resuspended in 1 ml of imaging buffer. To flip PtdSer independently of apoptosis, Jurkat T cells (no STS) were treated with 10 µM ionomycin (Fisher Scientific) and stained with annexin V as above. Untreated Jurkat T
cells were stained with annexin V (1/1000) in 1 ml of imaging buffer as above. Images of all Jurkat cells were acquired using the protocol described in section 2.8.

2.4 Induction of Neutrophil Apoptosis

The polymorphonuclear neutrophil layer (lower band of cells produced during the density gradient centrifugation in method 2.1) was collected and washed with PBS at 4 °C. PBS was aspirated gently and the neutrophils quickly and gently resuspended in 12ml ice cold water for 20 seconds to lyse remaining red blood cells. Lysis was stopped after 20 seconds by the addition of 4 ml of 0.6 M KCl solution. Neutrophils were washed with PBS a second time and resuspended in 2 ml of RPMI 1640 + 10% heat-inactivated autologous serum. Apoptosis was induced by heat-shocking the cells at 43 °C for 45 min, followed by an incubation at 37 °C in 5% CO₂ for 3 hours for recovery. Necrosis was induced by heating the neutrophils at 55°C for 1 hour. After washing with PBS twice, apoptotic/necrotic neutrophils were suspended in RPMI 1640 and labeled with eFlour670 cell proliferation dye (5 mM, eBioscience) to identify them in efferocytosis assays.

Apoptosis and necrosis were detected by co-staining of annexin V and propidium iodide in imaging buffer at 37°C for 5 min.

2.5 Preparation of Synthetic Phagocytic and Efferocytic Targets

Silica and magnetic beads were coated with lipids and IgG in order to prepare beads that function as apoptotic mimics and phagocytic targets respectively. Lipid-coated beads were prepared using silica beads (3.14 µm, Bangs Laboratories) or silica-coated magnetic beads (5µm, Bioclonel) in order to non-covalently attach a bilayer of phosphatidylserine
(PtdSer: 19.8%), phosphatidylcholine (PtdCho: 80%) and biotinylated phosphatidylethanolamine (Biotin-PtdEtn: 0.2%, all lipids from Avanti Polar Lipids). Control beads were prepared using the same mixture minus the PtdSer. The mixture of silica beads and lipids were dried under nitrogen gas for 1 hour. Lipid coated beads were then suspended with 20 mM Tris-HCl pH 7.4 and washed multiple times with PBS. For mass spectrometry, magnetic beads were coated only with PtdSer (20%) and PtdCho (80%) to prepare apoptotic targets. IgG coated beads were prepared by mixing the same silica or silica-coated magnetic beads as above with purified human IgG antibodies (Sigma). 20 µl of human IgG were coated onto 300 µl of silica/silica-coated magnetic beads in PBS. The mixture was incubated for 1 hour at room temperature, washed once with PBS, and kept at 4 °C until use.

2.6 Transfection

J774.2 and RAW264.7 cells were cultured at 5×10^5 per coverslips and incubated at 37°C with 5% CO2 overnight. 3µg of plasmid DNA (mouse pmGFP, mouse Rab5-eGFP, mouse Rab7-pmCherry, mouse Rab7-eGFP) was mixed with 100 µl of serum-free RPMI 1640 and 9 µl of FuGene-HD (Promega) transfection reagent added. This mixture was incubated for 15 min at room temperature according to the manufacturer’s manual. The mixture was added drop-wise to a single well of cells in a 12-well plate, and the cells were incubated overnight before use.
2.7 In Vitro Phagocytic and Efferocytic Assay

J774.2, RAW264.7, and ANA-1 cells were cultured on coverslips in 12-well plates and transfected with pmGFP, Rab5-eGFP, Rab7-pmCherry or Rab7-eGFP. Primary macrophages were cultured on coverslips in 12-well plates without transfection. Apoptotic neutrophils and PtdSer-coated silica beads were prepared as described in 2.4 and 2.5 and utilized as apoptotic targets. IgG-coated silica beads were prepared as described in 2.3 and utilized as phagocytic targets. $1 \times 10^6$ of neutrophils, PtdSer-coated silica beads, PtdCho-silica beads or IgG-coated silica beads in 200 µl of RPMI were added to each well of macrophages, centrifuged at 1500 rpm for 1 minute, at 4°C, and then incubated at 37°C for 10 to 30 minutes. Each well was then washed with PBS three times and fixed with 4% PFA. Wells with PtdSer-silica beads and PtdCho-silica beads were labeled with fluorescent-conjugated streptavidin (1/1000). Wells with neutrophils were blocked in blocking buffer (0.5% skim milk in PBS) for 30 minutes and then, stained with mouse anti-human CD18 antibody (1/500, Developmental Studies Hybridoma Bank) and goat-anti-mouse secondary fluorescent conjugated antibody (1/500, Jackson Laboratories) for 30 minutes. Wells with IgG-coated silica beads were stained with a fluorescent-conjugated anti-mouse Fab secondary antibody (1/500, Jackson Laboratories).

2.8 Confocal and Fluorescent Microscopy

Confocal images were captured using a Leica TCS SP5 II scanning confocal microscope (Robarts Research Institute) equipped with HeNe1/2 and Argon/2 lasers and a 60X/1.20NA objective. Widefield fluorescent images were captured using an Olympus
IX-70 microscope equipped with a 60X/0.9NA phase contrast objective running μManager software.

2.9 Calculation of Phagocytic Index

Lipid-coated or IgG coated beads were added to wells containing cultured macrophages, 1 x 10^6 beads per well in a 12-well plate, and then centrifuged at 1500 RPM for 1 minute at 4°C and then incubated at 37°C for 30 minutes. After washing with PBS twice, non-internalized beads were stained with streptavidin (1/1000) or goat-anti-mouse Fab-Cy5 (1/500) at 37°C for 5 minutes. Macrophages were washed with PBS twice, and then fixed with 4% paraformaldehyde (PFA) for 30 minutes. Phase-contrast and fluorescence images of the efferocytic assays were captured as per method 2.8. Images were analyzed with Image J (NCBI) to count internalized beads per each macrophage in the field of view. A minimum 40 macrophages were analyzed per experiment. Phagocytic index of macrophages was calculated by dividing the total number of internalized beads by total number of macrophages.

2.10 Efferosome and Phagosome Isolation

M0 macrophages were cultured on gelatin-covered tissue culture plates for 7 days as described in 2.1 and 2.2. PtdSer-magnetic beads and IgG-magnetic beads in 3ml of RPMI were added to each plate of M0 macrophages. M0 macrophages efferocytosed (PtdSer-magnetic beads) and phagocytosed (IgG-magnetic beads) for 40 min at 37 °C, then treated with ReCLIP reagent (Reverse Cross-Link Immuno-Precipitation, 1:1 mix of Dithio-bismaleimidoethane and Dithiobis[succinimidyl propionate) for 30 minutes at
room temperature. ReCLIP solution was then aspirated and 10 ml of a quenching solution (20mM of Tris-Cl pH 7.4 and 5mM of L-Cysteine) was added and incubated at room temperature for 10 min. Macrophages were then washed with PBS, placed on the ice and scrapped into 10ml of lysis buffer (Table 3). Suspended macrophages were then lysed using a nitrogen bomb, 10 minutes at 300 psi, on ice. A magnetic column was used to recover magnetic beads-containing efferosomes and phagosomes. Efferosomes and phagosomes were washed three times with 1ml of wash buffer (Table 2) and recovered using a magnetic column. Efferosomes and phagosomes were solubilized in 30 µl 1X Laemmli buffer (from 4X Laemmli buffer with 2.4 ml of 1 M Tris pH 6.8, 0.8 g of SDS stock, 4 ml of 100% Glycerol, 0.01% of Brombophenol blue, and 2.8 ml of ddH2O) with protease/phosphatase inhibitors (Fisher Scientific) and 50 mM DTT (Fisher Scientific), incubated at 37°C for 30 min to reverse the ReCLIP crosslinking, and then boiled for 5 min to denature the proteins for SDS-PAGE.

2.11 SDS-PAGE and Coomassie Staining

Phagosome and efferosome isolates from 2.10 were separated using 4-20% Mini-PROTEIN gel (Bio-Rad) for approximately 2 hours at 100 V. Gels were fixed (40% ethanol and 10% of glacial acetyl acid) followed by a quick wash with clean water, then stained with coomassie blue (Bio-Rad) for 20 hours. Gels were then destained in clean water for 3 hours. Destained protein gels were imaged on a protein gel tray of the GelDoc EZ imager (Bio-Rad) to visualize recovered proteins. Gels were stored in clean water prior to mass spectrometry.
2.12 Mass Spectrometry Analysis

Protein bands on coomassie gels were picked into 96-well plates by an Ettan Robotic Spot-picker machine located in the functional proteomics facility (Department of Biochemistry, Western University). Gel slices were destained with 50 mM ammonium bicarbonate and 50% acetonitrile, treated with 10 mM DTT and 55 mM iodoacetamide, and then digested with trypsin. Peptides were extracted by a solution of 1% formic acid and 2% acetonitrile and freeze dried. Dried peptide samples were then resuspended in a solution of 10% acetonitrile and 0.1% trifluoroacetic acid. Mass spectrometry analysis of each sample was performed using an AB Sciex 5800 TOF/TOF System, MALDI TOF/TOF (Framingham, MA, USA) by Kristina Jurcic (MALDI facility manager). Raw mass spectrometry data analysis was performed using MASCOT database search (http://www.matrixscience.com). Proteins were then divided into groups based on their protein scores.

2.13 Statistical Analysis

GraphPad prism software was used for all tests. Unless otherwise noted a one-tailed unpaired student t-test was used for analysis. Data are presented as mean ± standard error of the mean. p values ≤ 0.05 are considered to be significant and are indicated by *.
Table 2: Wash buffer used for efferosome and phagosome isolation

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (mM)</th>
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<tbody>
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<td>Sucrose</td>
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</tr>
<tr>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td>MgCl</td>
<td>3</td>
</tr>
<tr>
<td>NaVO₄</td>
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</tr>
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Table 3: Lysis buffer used for efferosome and phagosome isolation

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</tr>
</thead>
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<tr>
<td>Wash buffer (Table 1)</td>
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<tr>
<td>Protease/phosphatase inhibitors</td>
<td>1:100 dilution*</td>
</tr>
<tr>
<td>DNAse I</td>
<td>1:50 dilution*</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>200 nM</td>
</tr>
<tr>
<td>NaF</td>
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</table>

* Dilution of commercially provided stock
Chapter 3 – Results

3.1 Optimized In Vitro Efferocytosis Assay of Apoptotic Neutrophils

To ensure reproducible efferocytosis, I needed to establish efficient and reproducible methods to generate apoptotic targets. Initially I selected immortalized human Jurkat T cells, treated with staurosporine, as apoptotic targets. Jurkat T cells have been widely used because of their small size and they are easy to culture. Previous studies used staurosporine, a general inhibitor of cellular serine/threonine kinase, to induce apoptosis in these cells via a caspase 9 dependent pathway. Annexin V staining was used to confirm that PtdSer was being flipped to the outer leaflet of the apoptotic cells plasma membrane and images of apoptotic Jurkat T cells were acquired by widefield microscopy. As a positive control I used ionomycin, a Ca^{2+} ionophore, to induces PtdSer exposure independent of apoptosis. As expected, ionomycin treated Jurkat T cells stained strongly with annexin V (Figure 2A). Apoptotic Jurkat T cells also stained with annexin V (Figure 2B), whereas control Jurkat T cells were negative for annexin V staining (Figure 2C). However, staurosporine generated a low yield of apoptotic cells after 2.5 hours, and longer incubation periods were avoided in order to prevent the formation of necrotic cells. This data demonstrates that PtdSer can be used to detect apoptosis in our model systems, but staurosporine was inefficient to produce apoptotic targets.
Figure 2: Staurosporine (STS) induction of apoptosis in Jurkat T cells.

The apoptosis marker PtdSer was stained on Jurkat T-cells using fluorescent annexin V (red). A) PtdSer flipping was induced in Jurkat T cells with ionomycin. Scale bar = 20 µm. B) PtdSer flipping was observed on cells in which apoptosis was induced with STS. C) No PtdSer flipping was observed on untreated cells. Jurkat T cells are marked by arrows. Images are representative of 3 images, taken over 3 independent experiments.
I then tested an alternative method that has been used in the literature to reliably produce a large percentage of apoptotic cells without significant necrosis. This model utilizes heat-shocked human neutrophils. Human neutrophils undergo rapid apoptosis in a caspase-dependent manner by the heat-shock treatment (>40°C)\(^8^3\). Using the method of Park \textit{et al.}\(^7^5\), (details in Section 2.4) I achieved a stronger induction of apoptosis with a high yield (>40%) of apoptotic neutrophils without producing overt signs of secondary necrosis such as cell fragmentation. As expected, apoptotic neutrophils were annexin V positive and propidium iodide negative, indicating the absence of significant necrosis (Figure 3A). The positive staining control, necrotic neutrophils, stained with both annexin V and propidium iodide (Figure 3B). Because the method of Park \textit{et al.} provided high rates of apoptosis in neutrophils without necrosis it was used for all future experiments requiring apoptotic cells.

Next I assessed the efferocytic capabilities of the J774.2, RAW264.7 and ANA-1 murine macrophage cell lines. Prior to the efferocytosis assay, I transfected these cells with plasma membrane tagged GFP (pmGFP) to identify efferosomes through the formation of a GFP+ (e.g. plasma membrane-derived) intracellular vacuole\(^8^4\). These cells then became apoptotic neutrophils that were pre-labeled with the fluorescent cell tracer eFluor670. At the end of the experiment non-efferocytosed neutrophils were labeled with an anti-human CD18 antibody and Cy3-conjugated secondary antibody. Negative immunostaining of CD18 indicates that a neutrophil was completely engulfed. Images of J774.2 cells, were acquired by confocal microscopy and revealed a number of neutrophil-containing vacuoles with GFP in their bounding membrane, indicating efferocytosis had occurred.
Figure 3: Generation of apoptotic neutrophils by heat-shock.

Apoptosis and necrosis was induced in human polymorphonuclear neutrophils by heat-shock, and the cells stained with annexin V (green, merged channel) and propidium iodide (red, merged channel). A) Apoptotic neutrophils stained with annexin V but not propidium iodide. Scale bar = 10 µm. B) Necrotic cells stained with both annexin V and propidium iodide. Image is representative of 3 images, taken over 3 independent experiments.
Figure 4: J774.2 cells efferocytose apoptotic neutrophils.

J774.2 macrophages were transfected with pmGFP (green) and efferocytosed eFlour670-labeled apoptotic neutrophils (red). Non-efferocytosed neutrophils were labeled with anti-CD18 (cyan). Insert highlights an efferocytosed neutrophil which is CD18-negative, and surrounded by a pmGFP-positive bounding membrane. Image is representative of 15 images, taken over 3 independent experiments. Scale bar = 10 µm.
within 10 minute timeframe of the assay (Figure 4). All neutrophils observed in GFP+ compartments were not labelled with anti-CD18, demonstrating that J774.2 cells efficiently and completely internalized the apoptotic cells. Similar experiments were performed with ANA-1 and RAW264.7 cells, however these cells have extremely low levels of efferocytosis (less than 1 neutrophil engulfed per field of view (~20 cells), data not shown) and were not be used for future experiments.

3.2 Optimized In Vitro Efferocytosis Assay of Synthetic Apoptotic Targets

Although apoptotic human neutrophils were efferocytosed, the number of efferocytosed apoptotic cells was limited (~0.5/transfected J774.2 cell). Moreover, neutrophils were not always available due to limited blood donors. As such it was necessary to generate apoptotic cell mimics that would be consistently efferocytosed in greater numbers and which could be prepared as needed. I took a bead-based approach which is commonly used in studies of phagocytosis and efferocytosis, and which is established to generate more stable and reproducible phagocytic/efferocytic targets. Silica beads were coated with phospholipids to mimic plasma membranes of apoptotic cells by coating them with a mixture of PtdSer (20%) and PtdCho (80%). If required, 0.2% of the PtdSer could be replaced with biotinylated phosphatidylethanolamine (Biotin-PtdEtn) to allow detection of non-internalized beads with fluorescent streptavidin. To ensure that efferocytosis is PtdSer specific, control beads containing PtdCho (with or without Biotin-PtdEtn) were also prepared. pmGFP transfected J774.2 cells efficiently internalized these apoptotic
Figure 5: J774.2 cells efferocytose apoptotic cell-mimicking beads.

pmGFP-expressing J774.2 macrophages (green) were fed PtdSer/PtdCho or PtdCho (negative control) coated beads. Streptavidin (red) was used to detect non-internalized beads. **A** ) J774.2 macrophages efferocytosed apoptotic cell mimics (arrows). **B** ) J774.2 cells did not phagocytose PtdCho beads. Scale bars = 10 µm. **C** ) Phagocytic index of J774.2 cells efferocytosing apoptotic cell mimics. Data shown at means ± SEM, n=4. ***, p<0.05.
cell mimics but not the control (PtdCho) beads (Figure 5A). Efferosomes that were surrounded by a ring of GFP were not marked with streptavidin, indicating they were internalized into a membrane-bound compartment within the macrophage. Partially internalized and surface bound PtdSer-coated beads labeled with fluorophore-conjugated streptavidin (Figure 5A). The efferocytic capacity (phagocytic index) of J774.2 cells was calculated by dividing the total number of internalized beads by the total number of J774.2 cells. On average two PtdSer-coated beads were efferocytosed per J774.2, whereas no PtdCho coated beads were internalized (Figure 5B). This data demonstrates that these apoptotic mimics are a viable alternative to apoptotic neutrophils.

3.3 In Vitro Efferocytosis by Primary Human Macrophages

Because cell lines are prone to mutations that impact in downstream cellular processes, we wished to establish an efferocytosis assay using primary human macrophages. To accomplish this I quantified the efferocytosis ability of primary human macrophages differentiated to the three main subtypes of macrophages. M0 macrophages (cultured with M-CSF alone) are fully matured but non-polarized macrophages. M1 macrophages (cultured with GM-CSF, IFN-γ and LPS) and M2 macrophages (cultured with M-CSF and IL-4) are highly pro-inflammatory macrophages and anti-inflammatory/tissue healing macrophages respectively.12, 17 Each subtype was fed PtdSer-coated or PtdCho-coated beads for 30 minutes and imaged by fluorescent microscopy to calculate phagocytic index.
M0 macrophages readily internalized PtdSer-coated beads whereas no PtdCho-coated beads were engulfed (Figure 6). M1 macrophages did not internalize many beads, and internalized both PtdSer and PtdCho beads with equal efficiency (Figure 6), suggesting that this uptake was non-specific. M2 macrophages actively efferocytosed PtdSer-coated beads but to a lesser extent than M0 macrophages (Figure 6). This data suggests that M0 and M2 macrophages are effective in internalizing apoptotic cells, while M1 macrophages are not. However, as M0 cells had the highest efferocytic capacity, we selected them for use in future aims.
Figure 6: Efferocytosis by primary human macrophages

Human monocytes were differentiated into M0, M1 and M2 macrophages. Polarized macrophages were fed PtdSer-coated beads and PtdCho-only coated beads, and the phagocytic index was quantified. \( n = 4, **, p < 0.05 \) compared to PtdCho-only beads.
3.4 Efferosomes Acquire Rab5 and Rab7 Similarly to Phagosome Maturation

Using the highly effective bead-based model, I next assessed the role of Rab5 and Rab7, phagosome maturation regulators, in efferosome maturation using J774.2 cells. Specifically, I assessed whether both phagosomes and efferosomes recruited these common regulators of phagosome maturation as the *C. elegans* studies\(^5^1\), or if efferosomes only recruit Rab5 as suggested by our preliminary results and the dendritic cell studies\(^6^4, 6^5\). To this end I tracked ectopically expressed GFP- or mCherry-tagged Rab5 and Rab7, which demark early and late stages of maturation respectively\(^1^0, 5^2\), in J774.2 cells engulfed IgG-coated or PtdSer-coated beads. Phagocytosis of IgG opsonized beads was used as a positive control, and exhibited the expected pattern of Rab5/Rab7 acquisition wherein Rab5 was recruited at 10 minutes and replaced at later time points by Rab7 GTPases (Figure 7, 11A). By 20 minutes most phagosomes were Rab5 negative and had acquired Rab7, with Rab7 completely replacing Rab5 on the phagosomes by 30 minutes (Figure 7, 11A). This data confirms that J774.2 cells demonstrate normal phagosome maturation, with dynamics similar to that reported in other types of macrophages\(^5^1\).

This assay was repeated using apoptotic cell mimics (Figure 8, 11B). By 10 minutes efferosomes had recruited Rab5 GTPases, but the Rab5 began to disappear at 20 minutes, with Rab5 completely lost from the efferosomal membrane by 30 minutes (Figure 8, 11B). Rab7 began to appear on the efferosomal membrane as early as at the 10 minute
time-point, with significant recruitment observed at 20 minutes. Rab7 entirely replaced Rab5 on the efferosome membrane by 30 minutes (Figure 8, 11B).

To confirm that the observed patterns of Rab acquisition were not due to artefacts created by using lipid-coated beads we repeated these experiments using apoptotic neutrophils. Apoptotic neutrophils were readily internalized, and the resulting efferosomes positive for Rab5 by the 10 minute time-point, with the Rab5 displaced within 20 minutes (Figure 9, 11C) and replaced by Rab7 (Figure 10, 11C). Rab5 was completely supplanted from the efferosome by Rab7 after 30 minutes (Figures 9, 10, 11C). The acquisition of Rab5 and Rab7 during efferocytosis in both the bead-based and cell-based strongly suggests this is a bona fide result and not an artefact of the apoptotic bead model.
Figure 7: Tracking Rab5 and Rab7 on phagosomes

Rab5-GFP (green)/Rab7-mCherry (red) transfected J774.2 cells were fed IgG-coated and imaged at various time points. Non-internalized beads were labeled with the fluorophore-tagged secondary antibody (magenta). Image is representative of 20 images, taken over 3 independent experiments. Scale bars = 5 µm.
Figure 8: Tracking Rab5 and Rab7 on efferosomes

Rab5-GFP (green)/Rab7-mCherry (red) transfected J774.2 cells were fed PtdSer-coated beads and imaged at different time points. Non-internalized beads were labeled with streptavidin (magenta) at the end of the experiment. Image is representative of 20 images, taken over 3 independent experiments. Scale bars = 10 µm.
Figure 9: Rab5 recruitment to apoptotic-neutrophil containing efferosomes.

Rab5-GFP transfected (green) J774.2 cells efferocytosed apoptotic neutrophils labeled with eFluor670 (red) and were imaged at different time points. Non-internalized neutrophils were immunostained with anti-CD18 (cyan) at the end of the experiment. Image is representative of 16 images, taken over 3 independent experiments. Scale bar = 10 µm.
**Figure 10: Rab7 recruitment to apoptotic-neutrophil containing efferosomes.**

Rab7-GFP transfected J774.2 cells (green) efferocytosed apoptotic neutrophils labeled with eFluor670 (red) and were imaged at different time points. Non-internalized neutrophils were immunostained with anti-CD18 (cyan) at the end of the experiment. Image is representative of 16 images, taken over 3 independent experiments. Scale bar = 10 µm.
Figure 11: Quantification of Rab5 and Rab7 on phagosomes and efferosomes.

Rab GTPases mediated maturation process was elucidated by the quantification of Rab5 and Rab7 on phagosomes/efferosomes in each maturation stages. A) The presence of Rab5/7 on IgG beads containing phagosomes in each time frame was quantified by dividing numbers of Rab5 or Rab7 positive-structures by the total number of phagosomes. The presence of Rab5/7 on B) PtdSer beads and C) apoptotic neutrophils containing efferosomes in each time frame was quantified by dividing numbers of Rab5 or Rab7 positive-structures by the total number of efferosomes. Phagosomes/efferosomes were counted from two successful independent experiments.
3.5 Efferosomes Undergo Fusion with Lysosomes

Although the J774.2 murine macrophages cell line revealed that efferosomes recruited Rab5 and Rab7 in a similar manner to phagosomes, the possibility remains that this is an artefact of the cell line we are using, thereby primary macrophages were employed to assess efferosome maturation. At present we cannot transfect primary human macrophages. Thus, to assess efferosome maturation in primary human macrophages I immunostained for LAMP-1, a maturation marker that is delivered to phagosomes through Rab7-mediated fusion of the phagosome with late endosomes and lysosomes. M0 macrophages were fed IgG-coated beads (Figure 12A), PtdSer-coated beads (Figure 12B) or eFluor670 labeled apoptotic neutrophils (Figure 12C) for 30 minutes and then immunostained for LAMP-1. This time point was chosen as our previous results indicated that Rab7 completely replaced Rab5 by this time on efferosomal and phagosomal membranes (Figures 7 to 11). LAMP-1 was observed on both the phagosomal and efferosomal membranes, confirming that efferosomes were undergoing the classical Rab5-Rab7 maturation process like that utilized by phagosomes.

3.6 Identification of Regulator Proteins on Efferosomes

Although both phagosomes and efferosomes appear to utilize the same maturation process, the different immunological outcomes of phagocytosis (inflammation, immunogenic antigen presentation) and efferocytosis (homeostasis, no or tolerogenic antigen presentation) indicates that the degraded materials in efferosomes is treated differently than in phagosomes. These differences likely occur in the latest stages of the
Figure 12: M0 macrophages fuse lysosomes to efferosomes.

Human M0 macrophages phagocytosed A) IgG-coated beads marked by asterisks, B) PtdSer-coated beads marked by asterisks, or C) apoptotic neutrophils labeled with eFlour670 (red), for 30 minutes. Cells were then fixed and immunostained for LAMP-1 (green). Images are representative of 14 images, taken over 2 independent experiments. Scale bars = 10 µm.
maturation process, after lysosomal fusion. To identify these novel processes an unbiased mass spectrometry analysis was implemented using M0 macrophages and magnetic PtdSer-coated beads. A longer time frame of efferocytosis (40 minutes) was used to ensure that the majority of efferosomes reached the Rab7/LAMP-1 positive (late) stage of maturation. For comparison, phagosomes containing IgG coated magnetic beads were isolated at the same time point. To maximize protein recovery I implemented the reversible cross-link immune-precipitation (ReCLIP) technique which provided better protein recovery by using two protein cross-linking reagents, Dithiobis (succinimidyl propionate) and Dithio-bismalemidoehane, to the cells prior to lysis (data not shown)\textsuperscript{78.} Following lysis, a magnetic column was used to recover the efferosomes and phagosomes, and SDS-PAGE used to separate the recovered proteins. Coomassie staining revealed multiple unique protein bands in both the efferosome and phagosome isolates (Figure 13, arrows). Notably, the total amount of recovered proteins from efferosomes was less than recovered from phagosomes, likely due to higher levels of phagocytosis than efferocytosis in unpolarized M0 macrophages.

Bands of interest were excised from the gel and the proteins identified with mass spectrometry and MASCOT software analysis of the resulting spectra. Proteins with a MASCOT matching score greater than 40 were considered to be very high-quality hits, matching scores of 30 – 40 were considered to be high quality hits, and matching score of 20-30 considered medium-quality hits (Tables 4, 5). Proteins with scores below 20 were not considered to be significant. Several interesting proteins were recovered from efferosomes with high quality or better scores (score >30, Table 4),
Figure 13: Multiple unique proteins were recovered from efferosomes and phagosomes.

PtdSer/PtdCho-coated magnetic beads (efferosome) and IgG-coated magnetic beads (phagosome) were used as efferocytic targets and recovered from M0 primary human macrophages at 40 minutes post-internalization. Unique protein bands on each sample are marked by arrows. Image is representative of 3 independent experiments.
including regulators of vesicular traffic (Rab17\textsuperscript{85}, RASEF (Rab45)\textsuperscript{86} and VPS33B\textsuperscript{87}), an inhibitory phosphatase of MAP Kinase signaling (PP2A)\textsuperscript{88}, the efferocytic receptor CD36\textsuperscript{22}, a number of actin-regulatory proteins (WIPF3, Rac2 and Talin)\textsuperscript{49}, a negative regulator of phosphatidylinositide signaling (IMPA2)\textsuperscript{89}, and proteins involved in ubiquitination (TRIM25, USP15)\textsuperscript{90, 91}. Both MHC I and MHC II\textsuperscript{92} were identified as medium-quality hits (score of 20-30, Table 4). Phagosomes recruited a very different network of proteins. Among the high quality hits (score > 30, Tables 5) were both MHC I and MHC II\textsuperscript{92}, regulators of Golgi export (Rab6b and PI4-Kinase)\textsuperscript{93, 94}, a number of protein kinases (MKNK1, MINK1, STK4, PRKAB1)\textsuperscript{95, 96, 97, 98}, the pattern recognition receptor modulator NLRP3 (NOD3)\textsuperscript{99, 100, 101}, as well as a number regulators of calcium signaling (INPP5A and STRN3)\textsuperscript{102, 103}. Although numerous hits identified interesting proteins on both efferosomes and phagosomes, future studies using biochemical or cell based assays are required to validate these hits and test for the function of these proteins during efferosome and phagosome maturation.
Table 4: Summary of all proteins detected by mass spectrometry analysis of isolated efferosomes.

“Protein Name” and “Protein Symbol” indicate the official gene name listed in the NCBI database, and “NCBI Accession Number” indicates the specific entry in the NCBI gene catalogue corresponding to the identified peptides. “Protein Score” indicates the quality of the match, based on the Mascot software scoring system. “# peptides” indicates the total number of peptides from each protein identified. “Total residues” indicates the total number of amino acid residues identified across all peptides. “% Coverage” indicates the portion of the total protein identified by mass spectrometry. Data is the aggregate of 3 experiments.
## Very High Quality Hits (MASCOT Score > 40)

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## High Quality Hits (MASCOT Score 30 - 40)

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## Medium Quality Hits (MASCOT Score 20 – 30)

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Table 5: Summary of all proteins detected by mass spectrometry analysis of isolated phagosomes.

“Protein Name” and “Protein Symbol” indicate the official gene name listed in the NCBI database, and “NCBI Accession Number” indicates the specific entry in the NCBI gene catalogue corresponding to the identified peptides. “Protein Score” indicates the quality of the match, based on the Mascot software scoring system. “# peptides” indicates the total number of peptides from each protein identified. “Total residues” indicates the total number of amino acid residues identified across all peptides. “% Coverage” indicates the portion of the total protein identified by mass spectrometry. Data is the aggregate of 3 experiments.
### Very High Quality Hits (MASCOT Score > 40)

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Chapter 4 - Discussion

4.1 Rational of the Thesis: Efferosome Maturation Pathways and the Immune System

Billions of cells undergo programmed cell death every day in our bodies, and those apoptotic cells must be cleared by efferocytosis. If left uncleared, severe autoimmune and chronic inflammation can occur following the release of pro-inflammatory and immunogenic materials through secondary necrosis\(^6\). During efferocytosis professional phagocytes remove apoptotic cells in an anti-inflammatory manner. In contrast, phagocytosis - a part of the innate immune system - produces pro-inflammatory responses after engulfing pathogens\(^11\). Despite these differences in the immunological outcome of efferocytosis and phagocytosis, the two processes share a common degradative pathway mediated by Rab GTPases and fusion events between the forming efferosome/phagosome and vesicles from the endocytic pathway. This differential regulation of the outcomes of phagocytosis versus efferocytosis is the key for maintaining homeostasis under inflammatory versus homeostatic circumstances. Efferocytosis can be divided to three discrete but sequential stages: 1) recruitment of phagocytes to “find-me” signals secreted by apoptotic cells, 2) binding and internalization of the apoptotic cell by engagement of “eat-me” signals by phagocyte receptors, and 3) degradation of the apoptotic cell via maturation of the efferosome. The maturation process of efferocytosis has been primarily studied in *C. elegans*, and much of what is known of this process has been derived from these studies\(^51\). Many proteins involved in efferosome formation and
maturation, such as CED/Rac and Rab 5/7 are evolutionarily conserved between nematodes and mammals\textsuperscript{51}, implying that they may serve a common function in efferocytosis in both groups of organisms. However several observations suggest that efferocytosis has distinct outcomes between these organisms, suggesting that the regulation of efferocytosis - or its outcomes - has evolved differently between these two groups of organisms.

For example, Rab5 activation was inhibited by a lack of Gapex-5, a Rab5 GEF, near the site of apoptotic cell engagement in a mammalian system but not in \textit{C. elegans}\textsuperscript{104}. Moreover, efferocytosis is anti-inflammatory while phagocytosis is pro-inflammatory in a mammalian system, but both the inflammatory and antigen presentation portions of these responses do not exist in \textit{C. elegans}\textsuperscript{105,106}. In addition, recent studies of mammalian efferocytosis have identified several novel processes not observed in \textit{C. elegans}. For example, Balce \textit{et al.} revealed that alternately activated M2 mammalian macrophages showed reduced NADPH oxidase activity in efferosomes, distinct from what is observed in \textit{C. elegans} efferosomes and mammalian phagosomes\textsuperscript{18}. Indeed, the \textit{C. elegans} efferocytic pathway more closely parallels mammalian phagocytosis pathways than it does the mammalian efferocytic pathway\textsuperscript{51}. Moreover, studies of dendritic cells suggest that mammalian efferocytosis may not proceed past the Rab5 stage\textsuperscript{64,65}, although the results of this thesis (Figure 7 to 10) would suggest this is a DC-cell specific phenomenon. Combined, these studies suggest that the mammalian efferocytosis pathway is regulated differentially from efferocytosis in \textit{C. elegans} and mammalian phagocytosis,
thus allowing it to produce the cellular responses key to preventing chronic inflammation and autoimmunity downstream of apoptotic cell removal.

4.2 Hypothesis and Aims

How these molecular mechanisms controlling mammalian efferosome formation and maturation are anti-inflammatory/anti-immunogenic are not fully understood. For this thesis, because efferocytosis and phagocytosis have different inflammatory and immunogenic outcomes, I hypothesized that phagocytic and efferocytic maturation pathways are regulated by fusions with different intracellular compartments, and that these fusion events are regulated by novel mediators recruited either to phagosomes or to efferosomes. The following research aims were designed to elucidate whether efferosome maturation was unique, using cell biology and biochemical assays: 1) establish an *in vitro* macrophage model of efferocytosis, 2) characterize the acquisition of common maturation markers (i.e. Rab5, Rab7, LAMP-1) of phagocytosis to efferosomes, and 3) identify potential novel regulators on efferosomes and phagosomes by mass spectrometry.

4.3 Development of an *In Vitro* Efferocytosis Model

All aims of this study required an *in vitro* efferocytosis model that required both a consistent and reproducible efferocytic target and a cell line that exhibited strong and consistent efferocytosis. Generation of apoptotic cells is preferable for studying efferocytosis as they most closely mimic the *in vivo* environment, and several methods have been used by other researchers to induce consistent and high rates of apoptosis for
these types of assays\textsuperscript{107, 108}. Previous models have included UV exposure, anti-cancer drugs (e.g. Staurosporine), and physical stresses such as heat-shock or cold-shock treatment\textsuperscript{107, 108, 109}. Several previous studies adopted staurosporine, an inhibitor of multiple kinases, to induce apoptosis via activation of caspase 3\textsuperscript{108, 110}. However, in my hands STS did not generate a sufficient number of apoptotic Jurkat T cells for my experiments (Figure 1B). To improve on this I utilized another popular model, the heat-shocked induce apoptosis of primary human neutrophils\textsuperscript{75}. This induced high rates of apoptosis in isolated treated neutrophils without measurable necrosis (Figure 2). These apoptotic neutrophils were readily efferocytosed by J774.2 macrophages, demonstrating that they are good efferocytic targets (Figure 3).

While the neutrophil-based efferocytosis assay was successful, the availability of neutrophils was limited. Moreover, these apoptotic neutrophils would not have been suitable targets for mass spectrometry as it would not be possible to separate proteins derived from the apoptotic neutrophils from those found on the recovered efferosomes. As such I developed a bead-based efferocytosis model which provides simple and highly reproducible apoptotic targets. This method was based on of similar targets developed by other groups, but was modified to allow for detection of non-internalized versus internalized beads through the incorporation of biotinylated PtdEtn\textsuperscript{111, 34, 76}. These efferocytotic targets consisted of silica beads coated with 20\% PtdSer and 80\% PtdCho that mimic apoptotic cell membranes, while beads coated with only PtdCho were used to mimic healthy cells. When required, 0.2\% of these lipids were substituted with biotin-PtdEtn to allow for detection of non-internalized beads through the addition of
fluorescently labeled streptavidin. As expected, macrophages only efferocytosed PtdSer-coated beads, indicating these were a good model for our PtdSer specific efferocytosis studies (Figure 4).

In addition to reproducible efferocytic targets, this thesis also required the identification of macrophage cell lines or primary macrophage subtypes which consistently engage in efferocytosis. To generate reproducible in vitro model of efferocytosis I utilized J774.2 and M0 primary human macrophages, which consistent with previous studies, were determined to have high efferocytic capacities (Figures 3, 4)\(^34, 35, 74\). While the RAW264.7 cell line is the most frequently used cell line for phagocytosis studies, especially studies of phagosome maturation and vesicular trafficking\(^112, 113\), I determined that RAW264.7 and the related ANA-1 cell lines did not internalize PtdSer-coated beads (data not shown). The differences between the efferocytic capacity of the J774.2 and M0 macrophages versus the ANA-1 and RAW264.7 cell lines is unclear, but I would hypothesize that each cell line expresses different levels of efferocytic receptors. Consistent with this hypothesis are studies showing RAW264.7 cells have low expression of TIM-4\(^114\) and CD36\(^115\). In contrast, previous studies have also found J774.2 cell have a high efferocytic capacity, provided by the expression of the receptors for calreticulin and PtdSer\(^35, 116\). Importantly, Figures 4 and 5 demonstrate that macrophages recognized and internalized only PtdSer-coated beads, but not PtdCho only coated beads, indicating uptake was specific to the inclusion of the PtdSer “eat-me” signal in these beads. This specificity may have been provided by expression of the PtdSer specific receptor TIM-4 on J774.2 and M0 macrophages\(^34, 116\). Indeed, TIM-4\(^{-/-}\) bone marrow-derived
macrophages (BMDM) showed dramatically reduced internalization of PtdSer-coated beads in absence of any opsonins, while TIM-4−/− wild-type primary macrophages showed extensive uptake of PtdSer-coated beads but not PtdCho beads. Critically, this study demonstrates that the recognition of “eat-me” signals in targets containing a mixed lipid composition is specific to the presence of the eat-me signals present on the beads.

4.4 Macrophage Subtypes and Efferocytosis

Differences in the immunological responses generated by M0, M1 and M2 primary human macrophages provide insights into their efferocytic capacities. M0 macrophages resemble the macrophages which patrol healthy tissues, M1 macrophages are conventional pro-inflammatory cells found at sites of pathogen invasion, and M2 macrophages are alternatively polarized for anti-inflammatory actions such as wound healing and tissue remodeling. Figure 5 demonstrates the efferocytic capacities of each subtype. M0 and M2 macrophages could efferocytose PtdSer-coated beads, whereas M1 macrophage rarely efferocytosed PtdSer-coated beads. Moreover, the uptake of beads in M1 cells was not PtdSer-specific, suggesting the observed uptake may have been chance capture due to the high levels of membrane ruffling and spontaneous membrane enclosing observed in these cells. These results followed our predictions, as M2 cells would require higher efferocytic capacities in order to clear dying cells from wound sites, M0 macrophages would be expected to engage in efferocytosis are part of their “housekeeping” activities, while neither of these functions would be required by M1 cells attempting to restrict an infection. Unexpectedly, the highest phagocytic index (12.5) was observed in M0 macrophages, not M2 cells, suggesting that in vivo these M0 cells must
frequently encounter apoptotic cells, suggesting they play a more central role in homeostasis than previously thought. Indeed, most tissue resident macrophages are thought to have an M0 phenotype\textsuperscript{16}, suggesting that the bulk of homeostatic efferocytosis may be performed by these cells rather than by other efferocytic cell types such as epithelial cells\textsuperscript{118}.

4.5 Role of Rab5 and Rab7 in Efferosome Maturation

Previously, efferosome maturation was characterized in studies of \textit{C. elegans}, specifically assaying the removal of the apoptotic cells produced during \textit{C. elegans}’ development process\textsuperscript{51}. \textit{C. elegans} and mammals evolutionarily share many parts of the proteins regulating phagosome maturation, including the “master regulatory” Rab GTPases and phosphatidylinositol kinases that regulate much of the maturation process\textsuperscript{51}. Tracking these protein regulators during \textit{C. elegans} phagocytosis of apoptotic cell revealed a process similar to mammalian phagocytosis of pathogens – namely, Rab5 was recruited to early efferosomes and then supplanted by Rab7 in the later stages\textsuperscript{51}. Moreover, this study also demonstrated that the small GTPases CED-10 and Rac played a similar role in the internalization of apoptotic cells in both mammalian and \textit{C. elegans} phagocytes. Upon receptor engagement of apoptotic targets, CED-10 and Rac transmit downstream signals to induce membrane ruffles and protrusion, driven by actin remodeling, in order to engulf the apoptotic target. Evolutionary shared processes also regulated the exchange of Rab5 and Rab7 proteins in \textit{C. elegans} efferosomes and mammalian phagosomes, suggesting a shared process regulates both phagosome and efferosome maturation\textsuperscript{51}. The role of Rab5 and Rab7 in mammalian efferosome maturation remains controversial, as
other studies have found differences between *C. elegans* and mammalian efferosome maturation including findings indicating that the Rab5 to Rab7 transition is delayed or non-existent in mammalian cells, particularly in dendritic cells where a block in the Rab5/7 transition appears to preserve antigens until the DC cell matures\(^{64, 65}\).

In this thesis, I determined that Rab5 and Rab7 recruitment in efferosome maturation is consistent between mammalian macrophages and *C. elegans*, and that the mammalian efferosome maturation process closely paralleled phagosome maturation in the same cell type (Figures 7, 8, 9). Upon uptake of phagocytic and efferocytic targets, the engulfed particles became Rab5 positive, followed by a rapid exchange of Rab5 for Rab7. Although not tested in our experiments, this exchange was most likely driven by the Mon1a and Ccz1 proteins, which then act as a Rab7 GEF complex on efferosomes and phagosomes\(^ {52, 59}\). The presence of Rab7 was associated with fusion to late endosomes and lysosomes (Figure 7, 9), confirming that Rab7 was active on efferosomes. Late endosome and lysosome fusion to efferosomes delivers V-ATPase into the efferosomal membrane, as a result, increasing import of protons and acidifying the efferosome lumen\(^ {10, 52}\). These results ran contrary to our lab’s preliminary results from the poorly efferocytic RAW264.7 cell line, in which efferocytosis did not proceed past the Rab5 stage. The apparent conflict between the RAW264.7 cell line and the J774.2 and M0 macrophages maturation pathways may be due to the inherent M1 polarized phenotype of the RAW264.7 cells\(^ {119}\), or alternatively, due to mutations of maturation-regulating protein acquired during the immortalization of RAW264.7 cells. A third possibility is that RAW264.7 cells may have the same Rab5 blockade as that observed in dendritic cells\(^ {64, 65}\), suggesting that we may
be able to use RAW264.7 cells as a model of dendritic cell efferocytosis in future studies. Indeed, RAW264.7 cells can be differentiated into dendritic-like cells\textsuperscript{120}, suggesting that RAW264.7 cells may more closely resemble immature dendritic cells than they resemble macrophages.

While the above results suggest that a shared maturation pathway is used by both efferosomes and phagosomes, we assessed the Rab7-dependant fusion of late endosomes and lysosomes to the efferosome using a human system comprised of non-polarized (M0) primary human macrophages. This was done to confirm our results in endogenous cell lines free of any defects caused by the mutations that led to immortalization of the cell lines used in the early experiments. Because we cannot transfect primary macrophages, we instead assessed the recruitment of the lysosomal protein LAMP-1 to phagosomes (Figure 10A) and efferosomes (Figure 10B, C). The recruitment of this protein to both efferosomes and phagosomes indicates that both underwent fusion with lysosomes, suggesting that full acidification and recruitment of degradative enzymes such as cathepsins and hydrolases could occur in both compartments\textsuperscript{52}. However lysosomal cargo delivery to efferosomes was not confirmed. As such future experiments should track efferosome pH using pH-sensitive fluorescent dextran conjugates\textsuperscript{121}. Should transfection of these cells become possible, additional assays of Rab7 function such as monitoring the recruitment of fluorescently tagged Rab7 or one of its effector proteins such as RILP, can be used to better understand the dynamics of Rab7 activity efferosomes in the human system\textsuperscript{10, 52, 60}. Although this thesis determined that efferosomes in human macrophages undergo some degree of lysosomal fusion, it is still unclear whether efferosomes are
receiving the same complement of degradative proteins as phagosomes. Indeed, it has been established that other Rab proteins (e.g. Rab34) can induce the selective delivery of molecules to phagolysosomes, suggesting that other Rab proteins may act downstream of Rab7 to differentiate efferosome versus phagosomal maturation\textsuperscript{60}.

### 4.6 Novel Regulators Differentiate Efferosomal from Phagosomal Maturation

The absence of a major difference between the recruitment of Rab5 and Rab7 to phagosomes versus efferosomes, and the apparent normal fusion of late endosomes and lysosomes to both compartments, strongly suggests that there are not major differences in this portion of the phagosomal versus efferosomal maturation process. However, the different immunological outcomes of phagocytosis versus efferocytosis suggest that there must be a difference in the way that phagosomal and efferosomal cargos are trafficked\textsuperscript{19, 74, 105}. Since the maturation process appears to be the same for both types of compartments up to the phagolysosome stage, I hypothesized that this difference in cargo handling must occur within the phagolysosome itself. To test this hypothesis I implemented a mass spectrometry technique to assess the protein content of purified phagolysosomes containing apoptotic versus phagocytic targets. This successfully identified proteins selectively recruited to either types of targets, allowing us to identify putative functional differences between the efferocytosis and phagocytosis maturation pathways.

Mass spectrometry revealed three Rab GTPases that were differentially recruited to efferosomes versus phagosomes (Tables 3, 4). Efferosomes recruited Rab17 and Rab45
whereas phagosomes recruited Rab6b. Although I was unable to assess the role of these Rab GTPases in mediating maturation, it seems likely that they play a major role in the differential trafficking of efferosome- and phagosome-derived cargos. Previous studies found Rab17 on the basolateral membranes and apical tubules of adherent cells where it mediates transcytosis\textsuperscript{85}. In addition to its role in transcytosis, Rab17 also interacts with recycling endosomes and may contribute to filopodia formations\textsuperscript{122}. Rab17 appears to travel toward cell periphery, suggesting it may be carrying degraded apoptotic material for “disposal” into the extracellular milieu. Rab45 is not as well characterized and differs structurally from most other Rabs. Rab45 has two functionally distinct domains: 1) membrane trafficking regulating coiled-coil proteins in C-termini, and 2) an EF-hand domain in N-termini with unknown function. Rab45 is present on an uncharacterized membrane compartment located in the perinuclear area that is reminiscent of the recycling endosome\textsuperscript{86}. Although Rab45’s subcellular localization is unknown, it is clear that it is not present on the ER, Golgi or lysosomes, further indicating that the efferosomes are recruiting trafficking regulators not typically involved in the processing of phagocytic cargos or immunogenic antigen presentation\textsuperscript{10, 19, 56}. Indeed, the combined recruitment of Rab17 and Rab45 to efferosomes suggests that efferosomes are interacting with compartments not typically involved in antigen loading onto MHC molecules, such as the recycling endosome. Taken together, it suggests that efferosomes engages recycling endosomes and/or the transcytotic pathway, perhaps to shuttle degraded materials out of the phagocyte while avoiding the more acidic lysosomal and multi vesicular body compartment where antigen loading onto MHC II typically occurs\textsuperscript{56, 85}. 
In marked contrast, phagosomes (Table 4) recruited Rab6b along with phosphatidylinositol kinase 4 (PIK4) and GTPase of the immunity-associated protein 1 (GIMD1), all of which are involved in the trafficking of materials from the Golgi to other intracellular compartments\textsuperscript{93, 94, 123}. This suggests that the phagosome is in direct communication with the Golgi, perhaps for the purpose of delivering unloaded MHC II to the phagosome, for loading with phagosome-derived antigen and subsequent antigen presentation\textsuperscript{92}. Rab6b GTPases are typically found on the Golgi, ER membranes, and as well the ER-Golgi intermediate compartments (ERGIC)\textsuperscript{93}, with the presence of Rab6b on phagosomes suggesting that phagosomes directly fused with Golgi-derived vesicles\textsuperscript{93}. PI4K generates phosphatidylinositol 4-phosphate, a lipid central to export of vesicles and proteins from the Golgi and ER to other vesicles and the cell surface\textsuperscript{94}. GIMD1, anchored by a single C-terminal transmembrane helix, is also found on the membrane of the Golgi. While GIMD1’s function is unknown, its presence in phagosomes further supports the conclusion that there is direct trafficking between the Golgi and phagosomes\textsuperscript{123}. Taken together, the presence of these proteins on phagosomes strongly suggests that late phagosome maturation occurs through direct interactions with the Golgi, perhaps for the delivery of antigen presenting MHC II molecules.

Other than distinct vesicular trafficking differences between efferosomes and phagosomes, different signal transduction proteins were also found to be selectively recruited to efferosomes and phagosomes. Phagosomes recruited NOD3 and DOCK10 (Table 4), both important proteins in mediating inflammation via NF-kB activation, with neither protein found on efferosomes\textsuperscript{124, 125}. NOD3 is a member of the Nucleotide-
binding oligomerization domain-containing proteins family (NOD), of which NOD1 and NOD2 are the best characterized and known to activate NF-κB and MAP-Kinases (e.g. ERK1/2)\textsuperscript{99}. While NOD1 and NOD2 are known to binding to bacterial peptidoglycan, NOD3 is characterized as a critical regulatory component of caspase-1, associated in interleukin 1β (IL-1β) production in the inflammasome\textsuperscript{100,101}. Dedicator of cytokinesis 10 (DOCK10) was also recovered on phagosomes. DOCK10 has been reported to induce B cell activation and IL-4 secretion\textsuperscript{125}. Taken together, the presence of these proteins is indicative that phagosomes may be engaging or modulating signaling pathways which induce cytokine production and modulate immune cell activation. This is further supported by the differential recruitment of other proteins involved in cytokine production, discussed in detail below.

Of great interest on both phagosomes and efferosomes was the differential recruitment of MAP-Kinase pathway regulating proteins. Mass spectrometry data identified mitogen-activated protein kinase (MAPK) interacting serine/threonine kinase 1 (MKNK1) on both efferosomes and phagosomes, although the presence of MKNK1 was much more probable on phagosomes (Tables 3, 4). MKNK1 acts downstream of MAP-Kinases to enhance pro-inflammatory cytokine translation\textsuperscript{95}. Although phagosomes and efferosomes both recruited MKNK1, only phagosomes appeared to recruit additional activating components of the MAP-Kinase signaling pathway (RalBP1)\textsuperscript{126}, while efferosomes were highly enriched in the negative regulator of MAP-Kinase singling PP2A, which inhibits MAP-Kinase signaling by inhibiting Raf and MEK\textsuperscript{127}. The recruitment of these two distinct sets of MAP-Kinase regulatory proteins suggests different levels of MAP kinase
activities are derived from efferocytosis and phagocytosis. MAP kinases are critical downstream signaling transducers of pro-inflammatory receptors, often leading to the production of several pro-inflammatory cytokines by NF-κB activation\textsuperscript{128}. Upon activation of Raf, the downstream signal protein of Rac, MAP kinase kinases (MAP2K) are activated to phosphorylate MAP kinases such as p38 MAPK, Erk and Jnk\textsuperscript{88}. p38 MAPK and Erk subsequently phosphate MKNK1, which enhances cytokine production by phosphorylating EIF4E, an elongation factor that promotes cytokine translation\textsuperscript{129}. The presence of two positive regulators of MAP-kinase signaling on phagosomes, along with the low abundance of PP2A, suggests that the phagosomes likely induced signaling through MAP-Kinase cascades, accounting for the pro-inflammatory, NF-κB-dependent production of cytokines observed downstream of Fcγ-mediated phagocytosis\textsuperscript{95}. Efferosomes recruited an abundant quantity of PP2A, a serine/threonine phosphatase that antagonizes MAP-Kinase signaling through dephosphorylation of multiple members of the MAP-Kinase signalling cascade\textsuperscript{127}, suggesting that the presence of this phosphatase may be why efferocytosis does not induce NF-κB-dependent pro-inflammatory cytokines.

Another protein of interest is galectin-3, which was found on efferosomes (Table 3), as this protein is associated with pro-healing and tissue remodeling activities such as fibrogenesis and wound repair\textsuperscript{13}. In addition, recent studies have shown that galectin-3 also enhances anti-inflammatory responses and promotes the resolution of inflammation\textsuperscript{130}. Indeed, galectin-3 has been shown to be important in wound-healing in various tissues, with galectin-3 deficiency caused impaired wound healing and promoted chronic inflammation (e.g. inflammatory bowel disease)\textsuperscript{131}. Galectin-3 is actively
involved in macrophage biology, especially related to polarization into wound healing phenotypes, and its anti-inflammatory action directly leads to decreased antigen presentation to Th1 cells, although the mechanism of this reduced antigen presentation remains unknown\textsuperscript{130, 132, 133}. Efferocytosis of apoptotic hepatocytes by hepatic stellate cells increased galectin-3 production and secretion, along with TGF-β secretion, further linking galectin-3 to anti-inflammatory responses\textsuperscript{134, 135}. Other studies also revealed that galectin-3 induced fibrogenesis by myofibroblast proliferation activity in liver tissues, potentially by inducing secretion of fibroblast growth factor 2 (FGF2) by macrophages; a process known to lead to fibroblasts differentiation into myofibroblasts that form scar tissues in wound sites in other tissues\textsuperscript{13, 136, 137}. Taken together, these studies suggest that the galectin-3 found on efferosomes may play a role in the anti-inflammatory, pro-wound healing outcome of efferocytosis.

4.7 Summary and Future Aims

In summary, I have assessed the early and late steps of efferosomes maturation in the mammalian system and compared these to the equivalent stages in phagosome maturation. I determined that the early maturation stages appear to be identical between efferocytosis and phagocytosis, with Rab5 recruited early after internalization and then replaced with Rab7 and fusion with late endosomes/lysosomes at later time points. This indicates that efferosomes should undergo acidification with the subsequent activation in proteases and other degradative enzymes to process apoptotic cells\textsuperscript{51}, although I was not able to test this directly in this thesis. Mass spectrometry analysis of efferosomes and phagosomes at the phagolysosome stage of maturation identified several interesting
differences between phagosomes and lysosomes, potentially identifying the mechanism
by which efferocytic cargos induce the opposite immune response as do phagocytic
cargos. For example, different vesicular trafficking mediated by Rab17 and Rab45 may
keep efferosome-derived antigens from the antigen-loading compartment in macrophages
by directing these antigens to recycling endosomes. In contrast, phagosomes had
abundant MHC II and directly interacted with the Golgi trafficking regulators Rab6b,
PIK4 and GIMD1, indicating the phagosome was receiving MHC II directly from the
Golgi network, perhaps for efficient loading of pathogen-derived antigens onto MHC II.
Lastly, MAP kinase regulators accumulated on efferosomes and phagosomes in a pattern
suggesting that MAP Kinase activity and NF-κB activation would occur on phagosomes
but be impaired on efferosomes. These findings will lead to future studies which will
seek to validate the mass spectrometry observations, and to characterize the roles of these
proteins in mediating the differential immune responses following efferocytosis and
phagocytosis.
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