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The dynamics of apoptotic cell clearance

Michael R. Elliott[1,2,*] and **Kodi S. Ravichandran**[3,4,5,*]

[1]Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA

[2]David H. Smith Center for Vaccine Biology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA

[3]Department of Microbiology, Immunology, Cancer Biology, University of Virginia, Charlottesville, VA 22908, USA

[4]Beirne B. Carter Center for Immunology Research, University of Virginia, Charlottesville, VA 22908, USA

[5]Center for Cell Clearance, University of Virginia, Charlottesville, VA 22908, USA

Summary

The phagocytic clearance of dying cells in a tissue is a highly orchestrated series of intercellular events coordinated by a complex signaling network. Recent data from genetic, biochemical, and live imaging approaches have greatly enhanced our understanding of the dynamics of cell clearance and how the process is orchestrated at the cellular and tissue levels. We discuss how networks regulating apoptotic cell clearance are integrated to enable a rapid, efficient, and high-capacity clearance system within tissues.

Keywords

Phagocytosis; Apoptosis; Efferocytosis; Chemotaxis; Imaging; Signal transduction; Immunity

Introduction

The maintenance of tissue health and function requires the constant replacement of damaged or aged cells with new ones. In fact, on a daily basis the typical healthy adult is estimated to turn over via apoptosis approximately ~150 billion cells (out of the estimated 37.2 trillion cells in the human body), or roughly 0.4% of a body's cellular mass (Bianconi et al., 2013). Removal of these apoptotic cell corpses without damaging the healthy neighboring cells via phagocytosis thus plays a decisive role in cellular homeostasis. The phagocytic clearance of apoptotic cells (a process referred to as efferocytosis) plays key roles in embryonic

[*]**Correspondence:** ravi@virginia.edu, michael_elliott@urmc.rochester.edu.

development, organogenesis, tissue repair and immunity. Moreover, aberrant interstitial cell clearance is increasingly being viewed as both a cause and consequence of the pathobiology of many diseases (Elliott and Ravichandran, 2010; Green et al., 2016; S. Nagata et al., 2010; Poon et al., 2014b; Saas et al., 2013). Accordingly, there is growing interest in understanding the regulatory networks that control this dynamic and ubiquitous biophysical process.

Even in healthy tissues where cellular turnover rates are high (e.g. intestine, lung, bone marrow and thymus), uncleared apoptotic cells are relatively rare, suggesting that homeostatic efferocytosis operates at an impressive level of efficiency (Bailey et al., 2002; Braun, 1998; De Paepe, 2004; Elliott et al., 2009; 2010; Griswold, 1998; Lee et al., 2016; Liu and Keller, 2016; Nakanishi and Shiratsuchi, 2004; Park et al., 2011; Pittet and Weissleder, 2011; Shiratsuchi et al., 1997; Sunaga et al., 2013; Surh and Sprent, 1994). The mechanistic underpinnings of this efficiency are the focal point of a number of important, unanswered questions in the field: How is cell clearance orchestrated such that diverse signaling pathways facilitate efferocytosis? What is the capacity of the clearance system? What are the biophysical and spatiotemporal constraints for corpse recognition and removal? What are the constraints and unique features of cell clearance in specific tissue/disease contexts? What are the consequences of cell clearance on the metabolomics of cells and tissues? Over the past 20 years, we have gained an impressive amount of knowledge regarding the key molecular players and signaling pathways that regulate both cell death and efferocytosis. Moreover, we have recently begun to gain insight into how this complex and seamless biological process is carried out in real-time at the cellular and tissue levels in mammals. Increasingly sensitive, tractable intravital and in vitro microscopy techniques, together with new tissue-specific genetic probes have led to a number of recent advances in our understanding of apoptotic cell clearance in vivo. This has allowed us to begin to develop an integrated view of the physical and temporal constraints on cell clearance in a tissue context, which will have implications for cell clearance both under physiological and pathological conditions. This review highlights some of these recent advances. As there are a number of excellent reviews of the relevant technical advances (Elliott et al., 2010; Fourgeaud et al., 2016; Griswold, 1998; Liu and Keller, 2016; Lu et al., 2011; Mattocks and Tropepe, 2010; Nakanishi and Shiratsuchi, 2004; Park et al., 2011; Pittet and Weissleder, 2011; Shiratsuchi et al., 1997; Sierra et al., 2010), we will specifically focus here on findings that have helped reframe our conceptual understanding of this dynamic intercellular process and discuss how newer approaches can be applied to key unanswered questions in the field. While this review is focused primarily on cell clearance in the mammalian context, we acknowledge the invaluable contribution of many elegant genetic and intravital imaging studies from the nematode *C. elegans*, fruit fly *Drosophila melanogaster*, and the zebra fish *Danio rerio* models in developing our understanding of cell clearance in vivo.

Meeting Up: How Phagocytes Find Dying Cells

The first and most obvious requirement for efficient apoptotic cell clearance is to bring the apoptotic cell and phagocyte near enough to facilitate physical interaction between the cells. This proximity is facilitated in three different ways: adjacency, phagocyte migration, and the more recently recognized concept of apoptotic cell motility. Although useful for categorization, these mechanisms are not mutually exclusive, but rather likely act in concert

to influence efficient cell clearance in the interstitium (Desch et al., 2011; Fourgeaud et al., 2016; Fujimori et al., 2015; Jenkins et al., 2011; Juncadella et al., 2012; Larson et al., 2016; Lee et al., 2016; Lu et al., 2011; Mattocks and Tropepe, 2010; Okabe and Medzhitov, 2014; Rosas et al., 2014; Sierra et al., 2010; Yang et al., 2015). Interstitial cell clearance is frequently carried out by neighboring or adjacent phagocytes that are of non-hematopoietic origin, such as epithelial cells in the lung and gut, and mesenchymal cells in the developing embryo (Juncadella et al., 2012; Lee et al., 2016; Wood et al., 2000). The efficiency and capacity of these so-called “non-professional” phagocytes to clear dying cells is typically much less than that of “professional” phagocytes of hematopoietic origin such as macrophages and dendritic cells. The roles of professional versus non-professional phagocytes in the clearance of dying cells has been discussed at length in several recent reviews (Arandjelovic and Ravichandran, 2015; Desch et al., 2011; Green et al., 2016). Here, we focus on spatiotemporal features related to motile, professional phagocytes that are important to establish the phagocyte-apoptotic cell interactions required for the highly efficient removal of dead cells.

Possible relevance of phagocyte positioning within the interstitium for apoptotic cell clearance

Most tissues are interspersed with networks of hematopoietic phagocytes, including macrophages, monocytes, and dendritic cells (Davies et al., 2013; Dzhagalov et al., 2013; H.-J. Kim et al., 2010; Okabe and Medzhitov, 2015; Perdiguero and Geissmann, 2015; Westphalen et al., 2014). These cells act as immune sentinels for infection and tissue damage and are also key mediators of dead cell clearance. However, in most tissues, professional phagocytes are greatly outnumbered by the non-phagocytic cells in the organ. Therefore, the positioning of these phagocytes within a tissue is likely important for maximizing their opportunity for interaction with dying cells. For example, in sinusoidal tissues like bone marrow, spleen, and liver, the tissue-resident macrophages are positioned either within or just exterior to the arterial sinus. While these macrophages can engulf apoptotic cells (e.g. aged neutrophils in the bone marrow and hepatocyte corpses in the liver (Arandjelovic and Ravichandran, 2015; Casanova-Acebes et al., 2013; Furze and Rankin, 2008; Juncadella et al., 2012; Suratt et al., 2004)), their primary function is thought to be the clearance of damaged or effete red blood cells (RBC). By contrast, interstitial positioning of macrophages and dendritic cells (DC) for engulfment of nucleated cells appears to be highly dependent on the nature of the cellular environment and function of the tissue. This is particularly true for lymphoid organs, where lymphocyte development, activation and subsequent contraction of immune effector cells lead to large numbers of apoptotic leukocytes (Garrod et al., 2012; Gautier et al., 2012; Klein et al., 2014; LeBien and Tedder, 2008; Okabe and Medzhitov, 2015; Perdiguero and Geissmann, 2015). In these tissues, macrophages and dendritic cells appear to be pre-positioned at locations where apoptotic cells accumulate or are likely to occur based on the nature of death stimuli in the tissue. For example, during an adaptive immune response, tingible body macrophages are located at the light/dark border of the germinal centers in the spleen and lymph nodes where they capture proliferating B cells undergoing apoptosis due to low affinity or self-reactivity (Gray and Cyster, 2012; Hanayama et al., 2004; Headland and Norling, 2015; N. D. Kim and Luster, 2015; Muñoz et al., 2015; Newson et al., 2014; Serhan, 2014; Vinuesa et al., 2009). T

lymphocyte development in the thymus results in large numbers of apoptotic T cells, where thymic macrophages, and to a lesser extent dendritic cells, are sparse in numbers (~1% of total thymic cells) but are positioned in small clusters throughout the organ, providing widespread efferocytic coverage through the tissue (Dzhagalov et al., 2013; H.-J. Kim et al., 2010; Tacke et al., 2015). The CD169+ macrophages, the predominant efferocytes in the bone marrow, are located within dense cellular regions adjacent to the sinuses (Bianconi et al., 2013; Morrison and Scadden, 2014). These macrophages appear optimally located to multi-task in the engulfment of apoptotic B cells, aged neutrophils and erythrocytes.

In some non-lymphoid tissues where moderate to high rates of apoptosis are normal, “specialized” phagocytes appear positioned to maximize the opportunity for encountering apoptotic cells. In this context, “specialized” refers to a population of tissue-resident phagocytes (of either hematopoietic or non-hematopoietic origin) that have evolved highly unique gene expression and functional characteristics that support the function of one type of tissue. For example, in the seminiferous tubules of the testes, approximately 75% of developing germ cells will undergo apoptosis before ever becoming mature sperm (Bailey et al., 2002; Braun, 1998; De Paepe, 2004; Elliott et al., 2009; Lee et al., 2016; Sunaga et al., 2013; Surh and Sprent, 1994). Sertoli cells are specialized phagocytes attached to the basal lamina of the seminiferous tubules that extend their processes toward the lumen, forming a network of membrane structures that intertwine with spermatagonia and can recognize and rapidly engulf specifically those germ cells undergoing apoptosis (Elliott et al., 2010; Griswold, 1998; Liu and Keller, 2016; Nakanishi and Shiratsuchi, 2004; Park et al., 2011; Pittet and Weissleder, 2011; Shiratsuchi et al., 1997). Similarly, in the brain, ongoing neurogenesis in the hippocampus features moderate rates of apoptosis, involving hundreds of developing neurons. Microglia, a specialized brain phagocyte, line the dentate gyrus and mediate engulfment of developing neurons that undergo apoptosis (Desch et al., 2011; Fourgeaud et al., 2016; Fujimori et al., 2015; Juncadella et al., 2012; Larson et al., 2016; Lee et al., 2016; Lu et al., 2011; Mattocks and Tropepe, 2010; Sierra et al., 2010; Yang et al., 2015). In contrast, in the pleural cavities, sentinel, tissue-resident macrophages can move freely or are loosely adhered to the membrane surfaces, enabling rapid response to pathogens outside the lung and visceral organs (Arandjelovic and Ravichandran, 2015; Green et al., 2016; Jenkins et al., 2011; Okabe and Medzhitov, 2014; Rosas et al., 2014). Within the lung and airways, specialized tissue-resident macrophages and dendritic cells are integrated into the epithelial layer that lines the airway. A subset of migratory lung DC (CD103+/CD11c+/MHCII^{hi}) appear to be the dominant DC subtype that captures apoptotic cells in the lung and can traffic to the draining lymph node for antigen presentation to naïve T cells (Davies et al., 2013; Desch et al., 2011; Dzhagalov et al., 2013; H.-J. Kim et al., 2010; Okabe and Medzhitov, 2015; Perdiguero and Geissmann, 2015). Recently, Bhattacharya and colleagues used intravital microscopy to demonstrate that a subset of alveolar macrophages forms gap junction channels with epithelial cells, enabling calcium-dependent signaling between macrophages and across many cell widths (Arandjelovic and Ravichandran, 2015; Casanova-Acebes et al., 2013; Furze and Rankin, 2008; Suratt et al., 2004; Westphalen et al., 2014). This is important, as unlike many tissue-resident macrophages, alveolar macrophages are mostly sessile. Considering the critical role of airway epithelial cells in the clearance of apoptotic cells during inflammation and allergic

responses, it is possible that both professional and non-professional phagocytes operate in concert to mediate efficient cell clearance and immune responses (Garrod et al., 2012; Juncadella et al., 2012; Klein et al., 2014; LeBien and Tedder, 2008). Although emerging evidence strongly suggests that tissue-resident phagocytes receive interstitial localization and positioning cues, the source and identity of these cues remains poorly understood. In light of the recent flood of information regarding the ontogeny and tissue-specific development of macrophages and dendritic cells (Gautier et al., 2012; Gray and Cyster, 2012; Hanayama et al., 2004; Okabe and Medzhitov, 2015; Perdiguero and Geissmann, 2015; Vinuesa et al., 2009), it seems likely that we are poised to apply new, sophisticated genetic models and gene expression analyses to understand how the positioning of tissue-resident interstitial phagocytes occurs and how the environmental, phenotypic, and functional heterogeneity are interconnected (Guilliams and van de Laar, 2015; van de Laar et al., 2016).

In contrast to homeostatic cell clearance, disease-associated tissue damage can lead to multiple waves of cell death, and the importance of cell clearance in these tissues is increasingly being understood as a key event in resolution, tissue repair, and the restoration of homeostatic tissue function (Dzhagalov et al., 2013; Headland and Norling, 2015; H.-J. Kim et al., 2010; N. D. Kim and Luster, 2015; Muñoz et al., 2015; Newson et al., 2014; Serhan, 2014; Tacke et al., 2015). In addition, tissue damage/infection also attracts circulating myeloid-derived phagocytes into the tissue to clear dying cells. Once in the tissue, monocytes can differentiate into a wide array of phagocytic phenotypes and may subsequently contribute to clearance of apoptotic cells (such as M1- and M2-like) (Basil and Levy, 2015; Headland and Norling, 2015; Larson et al., 2016; Newson et al., 2014; Serhan, 2014; Stanford et al., 2014). In these cases, localization of recruited phagocytes to apoptotic corpses is due to many different types of factors, including find-me signals, danger signals, and chemokines (Junger, 2011; Kolaczkowska and Kubes, 2013; Serhan, 2014). While it appears that both tissue-resident and recruited phagocytes contribute to the clearance of apoptotic cells in a damaged/infected tissues (Gautier et al., 2013; Uderhardt et al., 2012), the relative importance of each phagocyte type on the overall removal of dead cells in the tissue is still being worked out.

Communication Signals

The mobility of immune cells is a fundamental property of metazoan immunity that enables rapid and site-specific defense and wound-healing responses throughout the body. The movement of immune cells to and within tissues is controlled by attraction and repulsion factors produced by cells within local tissue environments, including chemokines, cytokines and soluble metabolites (e.g. lipids, nucleic acids). Like other immune cells, phagocytes rely on a range of different soluble cues emanating from apoptotic cells to sense and properly locate apoptotic cells. Activation of executioner caspases during apoptosis leads to the release of these soluble chemoattractants, called find-me signals (Lauber et al., 2004; Ravichandran and Lorenz, 2007). Find-me signals have been shown to enhance the localization of phagocytes to dying cells, and to date, at least four distinct find-me signal-receptor pathways have been identified primarily through in vitro studies (**Figure 1**) (Elliott et al., 2009; Gude et al., 2008; Lauber et al., 2003; Luo et al., 2016; Truman et al., 2008).

Importantly, three of these have been reported to contribute to efferocytosis in vivo: extracellular nucleotides ATP and UTP, CX_3CL1, and sphingosine-1-phosphate (S1P) (Elliott et al., 2009; Luo et al., 2016; Truman et al., 2008). However, due to the distinct experimental systems used to identify each of these factors as well as differences in the timing of release during cell death (**Figure 1**), it remains a challenge to understand the specific tissue settings where one or more of these factors would be operational, and how multiple find-me signals, when present concurrently, might be integrated to affect cell clearance.

As depicted in **Figure 1**, established find-me signals can be categorized based on when they accumulate at physiologically relevant levels following the induction of apoptosis (i.e. early, mid, and late apoptosis). Initiation of apoptosis occurs upon receipt of a death stimulus such as death receptor ligation (e.g. CD95-CD95L), nuclear DNA damage or mitochondrial stress. Cleavage and activation of caspases 3 and 7 triggers an irreversible cascade of enzymatic changes that result in the morphological and biochemical features of apoptosis. Of the find-me signals identified to date, the release of nucleotides (i.e. ATP and UTP) from the cytoplasm of apoptotic cells coincides most closely with the activation of caspases, occurring in nearly parallel kinetics with the accumulation of activated executioner caspases (Chekeni et al., 2010; Elliott et al., 2009; Poon et al., 2014a; Qu et al., 2011). Subsequent mechanistic studies confirmed that caspase-3/7 act on pannexin-1 channels: cleavage of the C-terminal tail of pannexin-1 monomers led to the opening of the hexameric channels, allowing for the extracellular release of cytosolic nucleotides (Chekeni et al., 2010; Poon et al., 2014a; Qu et al., 2011). Apoptotic cell-derived extracellular nucleotides are detected by macrophages and other immune phagocytes via the P2Y2 G-protein coupled receptor (GPCR) (Elliott et al., 2009). P2Y2 is expressed at relatively high levels on numerous myeloid cell populations in mice and humans, including tissue-resident macrophages in the lung and peritoneum of mice (Chen et al., 2006; Gautier et al., 2012; Kaufmann et al., 2005; Kronlage et al., 2010). With high sensitivity to both ATP and UTP (maximal responses in ~0.1μM range) (Junger, 2011), P2Y2 has been shown to promote vascular adhesion, motility, and apoptotic cell clearance in a number of different studies of acute and long term inflammation and adaptive immune responses (Elliott et al., 2009; Idzko et al., 2007; Jin et al., 2014; Klämbt et al., 2015; Ma et al., 2013b; McDonald et al., 2010; Shah et al., 2014; Wang and Kubes, 2016). In the context of cell clearance, depletion of extracellular nucleotides or deletion of P2Y2 in mice was shown to cause a delay in the clearance of apoptotic cells by macrophages in the thymus (Elliott et al., 2009). Similarly, UDP released by dying hippocampal neurons stimulates phagocytic clearance by microglia in vivo (Koizumi et al., 2007). Another find-me cue released in the early stages of apoptosis is CX_3CL1/fractalkine. Identified by Gregory and colleagues, CX_3CL1 was released by apoptotic Burkitt lymphoma cells in a manner that coincides temporally with early cellular hallmarks of apoptosis (**Figure 1**)(Truman et al., 2008). Recognition of CX_3CL1 from apoptotic cells by the GPCR CX_3CR1 was critical for recruitment of macrophages to the splenic germinal centers of mice in vivo (Truman et al., 2008). Thus, nucleotides and CX_3CL1 are released early by apoptotic lymphocytes and can play a role in the rapid and efficient clearance of apoptotic cell in tissues.

Two other prominent find-me signals, lysophosphatidylcholine (LysoPC) and S1P, accumulate to significant levels later in the apoptotic process (4-12 hours after induction) (**Figure 1**). LysoPC is a potent chemoattractant for monocytes and macrophages in vitro and in vivo (Hoffman et al., 1982; Ousman and David, 2000), and Lauber et al. reported that a number of transformed cell lines release LysoPC during apoptosis via caspase-mediated activation of calcium-independent phospholipase A_2 ($iPLA_2$) (Lauber et al., 2003). More recently, another chemotactic lipid, S1P, was shown to accumulate extracellularly following the induction of apoptosis, and S1P is also a potent macrophage chemoattractant in vitro (Gude et al., 2008; Luo et al., 2016; Weigert et al., 2010; 2006). Although the mechanisms of S1P release during cell death are not fully understood, it is interesting to note that Weigert et al. found that S1P release by apoptotic cells was associated with cleavage and activation of the inflammatory caspase, caspase-1, and that this release could be partially inhibited using the putative caspase-1 inhibitor YVAD (Weigert et al., 2010). Considering the timing of S1P accumulation following apoptosis induction (> 6 hours) and the well-described role of casp-1 in the execution of regulated necrosis (i.e. pyroptosis) it is possible that S1P may function as a find-me signal for the clearance of secondarily necrotic cells. Finally, additional find-me signals were recently reported by Cullen et al, who found that HeLa cells induced to apoptose by CD95 stimulation released chemokines MCP-1 and IL-8, both known myeloid/phagocyte chemoattractants (Cullen et al., 2013). However, these find-me cues appear more relevant for necrotic cell clearance, as the authors showed that chemokine release was due to caspase-independent necrosis. Along these lines, another necrotic cell find-me signal, cleaved annexin A1, was reported by Blume et al. to accumulate in the supernatants of late apoptotic or secondarily necrotic cells and could stimulate migration of myeloid cells in vitro (Blume et al., 2011).

The best in vivo evidence to date for the importance of find-me signaling in phagocyte recruitment and efficient cell clearance comes from studies where find-me signal receptors, all of which are GPCRs, have been ablated genetically or pharmacologically in mice. Inhibition or ablation of the ATP/UTP receptor P2Y2 in mice results in a significant inhibition in the recruitment of myeloid phagocytes in vivo as well as a delay in the clearance of apoptotic thymocytes (Elliott et al., 2009). In a separate study, deletion of P2Y2 significantly reduced the accumulation of macrophages infiltrating fibrosarcoma tumor tissues following apoptosis-inducing anthracycline chemotherapy (Ma et al., 2013a). Similarly, deletion of the fractalkine receptor CX_3CR1 reduces the accumulation of tingible body macrophages in the splenic follicles, although an increase in uncleared apoptotic cells was not observed at the time points used in this study (Truman et al., 2008). Receptors for S1P ($S1PR_{1-5}$) and lysoPC (G2A) have been implicated in autoimmunity, but their role in the clearance of apoptotic cells in vivo remains to be determined (reviewed in (Green et al., 2016; Medina and Ravichandran, 2016)).

Apoptotic cell motility

While the movement of phagocytes toward apoptotic cells has been studied extensively, there is now evidence to support the idea that the motile behavior of apoptotic cells themselves might contribute to cell clearance. Apoptosis is an active rather than a passive dismantling of the cell, and cells undergoing apoptosis often display rapid and dynamic

alterations of the cytoskeleton that lead to the membrane blebbing and motility patterns characteristic of apoptosis (the so-called ‘dance of death’) (**Figure 1**). Many of these morphological and motile behaviors are driven by the caspase-mediated activation of rho-associated coiled-coil-containing protein kinase 1 (ROCK1). Caspase-3 cleavage of the autoinhibitory domain of ROCK1 leads to constitutive kinase activity, myosin light chain (MLC) phosphorylation, actomyosin contraction, and blebbing (Atkin-Smith et al., 2015; Coleman et al., 2001; Sebbagh et al., 2001). More recently, intravital imaging studies in vertebrates have confirmed this ‘dance of death’ behavior in vivo. Using a transgenic mouse line expressing a FRET-based caspase-3 activation reporter, Yamaguchi et al. observed that intact embryonic cells undergoing apoptosis, as detected by FRET, carried out a rapid and somewhat erratic motility pattern along the neural crest for up to three hours (Yamaguchi et al., 2011). A subsequent report similarly showed that apoptotic neural cells in the developing zebra fish brain were very motile for up to three hours and frequently moved several cell diameters interstitially after becoming positive for surface phosphatidylserine (PtdSer) (van Ham et al., 2012). Similarly, intravital imaging of mouse thymic explants during negative selection revealed that the thymic macrophages were relatively sessile and that the apoptotic thymocytes themselves moved rapidly (apparently non-directionally) until encountering a macrophage and being engulfed (Dzhagalov et al., 2013). Also, during skeletal myoblast fusion, the emerging apoptotic myoblasts appear to move around in close apposition to the growing myotube without being engulfed (Hochreiter-Hufford et al., 2013). Conceptually, it seems possible apoptotic cell movement could enhance the ability of phagocytes to locate them, or alternatively might allow for aggregation of multiple apoptotic cells into a common area in the tissue, thereby facilitating clearance in tissues where professional phagocytes are sparse. In support of this, previous studies have shown that unengulfed apoptotic cells are often seen in clusters at specific sites within the tissue context (Elliott et al., 2009; Surh and Sprent, 1994; Wood et al., 2000). The molecular basis of this clustering or its relevance to cell clearance is currently unknown. Together these studies provide new ways to consider how apoptotic cells might be cleared in a tissue.

Possible integration of find-me signals during interstitial cell clearance

Considering the diversity of find-me signals identified to date, it will be important to understand distinct roles for each of these factors in efferocytosis as well as determine how these signals are integrated into the overall process of cell clearance in the tissue. Find-me signals released early, such as nucleotides and CX_3CL1, which do not appear to require active metabolic synthesis or gene expression, may provide key ‘alert’ information to neighboring phagocytes indicating apoptotic cell stress nearby. Factors that appear later and persist longer, such as LysoPC and S1P, might provide more stable cues that can stimulate recruitment of more distant phagocytes in settings where there are moderate to high levels of apoptosis. Another purpose for multiple and varied find-me signals may be that the condition the phagocytes or local tissue environments may need to be primed or altered in ways that enhance phagocyte mediated clearance. One recent example of this was the finding that S1P release by apoptotic cells triggers phagocytes to produce and release erythropoietin (EPO), which in an autocrine/paracrine manner activated PPARγ-dependent transcription of engulfment genes such as *Mfge8* and *Mertk* (Luo et al., 2016).

Dynamics of Selective Uptake

In a steady state tissue context, the phagocyte must specifically recognize and ingest the few dying cells among the sea of living cells. Accordingly, the physical interaction of phagocytes and target cells is regulated by a series of dynamic and temporally restricted checkpoints that function to ensure rapid clearance of appropriate targets. Dying cells undergo a continuum of changes on their surface and intracellularly that convey their state of viability. A canonical feature of apoptosis is the rapid, caspase-dependent exposure of PtdSer to the outer leaflet of the apoptotic cell plasma membrane. Exposed PtdSer is subsequently recognized by one or more of a cadre of heterogeneous surface receptors expressed on phagocytes (reviewed in (Green et al., 2016; Gregory and Pound, 2010; Hochreiter-Hufford and Ravichandran, 2013; Penberthy and Ravichandran, 2015). However, PtdSer recognition alone is likely not always sufficient to stimulate engulfment, as the duration of PtdSer exposure as well as additional eat-me ligands exposed can work in concert to help the phagocyte properly recognize and engage an apoptotic cell. It has become clear that both the dying cell and the phagocyte provide key spatiotemporal information that ultimately conveys the tissue and cell-specific nature of the dying cell.

The clearance code

Upon initiation of apoptosis, activated effector caspases cleave numerous substrates that result in the biochemical and morphological changes characteristic of apoptotic cell death (**Figure 1**). Among the most ubiquitous and best studied of these is the exposure of PtdSer on the exofacial leaflet of the plasma membrane (Fadeel and Xue, 2009; Fadok et al., 1992; Segawa and S. Nagata, 2015). In viable cells, PtdSer (and the other major aminophospholipids, such as phosphatidylethanolamine) are maintained in the cytoplasmic side of the membrane due to the enzymatic activity of flippases that shuttle PtdSer from the outer to the inner leaflet (Arandjelovic and Ravichandran, 2015; G. C. Brown and Neher, 2012; Segawa and S. Nagata, 2015). A member of the P4-type ATPse, ATP11C, was recently identified in a haploid screen for regulators of PtdSer exposure (Segawa et al., 2014). Cleavage of ATP11C by caspase-3/7 leads to irreversible loss of flippase activity, which is required for exposure of PtdSer during apoptosis. By contrast, scramblases, including the recently identified Xkr8, are a class of enzymes that drive PtdSer from the inner to outer leaflet (Suzuki et al., 2013; 2014). In the case of Xkr8, caspase-3/7 cleaves the C-terminal intracellular tail, leading to constitutive scramblase activity and PtdSer exposure on the cell surface.

It is important to note that the kinetics of caspase-3/7 activation in cells following a death stimulus are highly variable and depend greatly on the cell type and death stimulus. However, the measurement of caspase-3/7 activation in cells in real-time has been studied using sensitive fluorescent reporters (e.g. Casp3-FRET, FLICA). Results from these experiments indicate that although the time from delivery of a death stimulus to the first signs of effector caspase activation can vary widely, once caspase-3/7 are activated, maximal caspase activation is achieved rapidly (~1-4 hours) and that many of the characteristic features of apoptosis that are relevant to cell clearance are visible, including membrane blebbing, PtdSer exposure, and Pannexin-1-dependent nucleotide release (Chekeni et al.,

2010; Croker et al., 2011; Poon et al., 2014a). Thus, irreversible apoptosis is characterized by a rapid, intense and temporally linked series of biochemical and cellular events that provide cues to local phagocytes of the certain demise of the cell and thus the need for rapid clearance.

Even within the short time between minimal and maximal effector caspase activation, the exposure of eat-me signals on the dying cell occurs on a continuum of dynamic, distinct changes. In the case of PtdSer exposure, studies in lower organisms and mammalian cells have shown PtdSer on the outer leaflet of apoptotic cells can occur in aggregates or patches on the surface (Croker et al., 2011; Fairn et al., 2011; Gardai et al., 2005; van Ham et al., 2012). Consequently, localization of soluble PtdSer-binding proteins, like annexin-V, calreticulin, Gas6, and MFGE8, can also appear as patches on the surface of apoptotic cells. Interestingly, the earliest PtdSer molecules observed on apoptotic cells are present in one or two patches on the cell, with PtdSer becoming more uniform across the cell membrane as apoptosis progresses. Although the mechanisms responsible for the appearance or localization of PtdSer patches on apoptotic cells have not been precisely defined, it seems likely that the punctate localization of flippases and scramblases within the membrane are involved (Segawa et al., 2014; Suzuki et al., 2013). Functionally, the appearance of PtdSer-dense aggregates on early stage apoptotic cells, in combination with the blebbing activity, may contribute to the efficiency of clearance by enhancing the recognition and binding of PtdSer receptors on the phagocyte (discussed in more detail below). In addition to PtdSer, other changes on the surface of apoptotic cells contribute to recognition and likely convey important spatiotemporal information about the status of the target cell to phagocytes. These include ICAM-3, C1q, oxidized lipids and alterations in the glycocalyx/glycosylation patterns (Bilyy et al., 2011; Devitt et al., 1998; 2003; Fadok et al., 1998; Galvan et al., 2012; Torr et al., 2011; Ucker et al., 2012).

In contrast to the eat-me signals, some surface proteins can act as indicators of viability and act to suppress phagocytosis. These so-called “don't eat-me” signals include adhesion-related proteins CD47 and CD31. Originally identified as a viability marker on erythrocytes, CD47 has since been shown to be expressed on many different types of viable nucleated cells where it can interact with the inhibitory receptor SIRP1α on phagocytes to restrain the engagement of the phagocytic machinery (Willingham et al., 2012). While CD47 levels decrease as RBCs age, its downregulation during apoptosis has been observed, but is not a universal feature of apoptosis, and in many cases is not sufficient to override PtdSer-driven engulfment of apoptotic cells. In recent years, CD47 has become a potential therapeutic candidate for tumor cell eradication, as many different types of transformed cells express high levels of CD47, and antibodies targeting CD47 have shown promise in promoting clearance of tumor cells (Chao et al., 2010; 2011). Similarly, CD31 on viable cells can interact with CD31 on phagocytes to provide a spatially confined repulsion signal to prevent engulfment (S. Brown et al., 2002). Although CD31 expression varies widely on different cell types, certain leukocytes require the downregulation of CD31 for efficient efferocytosis to proceed (S. Brown et al., 2002).

Apoptosis leads to a wide array of changes to the cell surface. Understanding the specific molecular characteristics of the apoptotic cell surface at different times throughout apoptosis

will likely provide important contextual information to aid in our understanding of distinct mechanisms and consequences of apoptotic cells clearance.

Quality control checkpoints during engulfment

Despite being recognized as the canonical eat-me signal in efferocytosis, surface PtdSer is also seen on many different 'viable' cell types in a number of biological settings involving neither apoptosis nor cell clearance. Perhaps the most widely accepted model of engulfment, called 'tether-and-tickle', proposes that successful engulfment of apoptotic cells occurs in two molecularly distinct steps. Tethering receptors such as Tim4 can bind PtdSer with a relatively high affinity (K_d~2nM) (Miyanishi et al., 2007); however, Tim4 is unable to signal on its own to the engulfment machinery (Park et al., 2009), and additional engulfment receptors are required to 'tickle' the engulfment signaling pathways to mediate corpse internalization. In fact, a recent study in zebra fish showed that homologues of Tim4 and Bai1 worked concurrently to facilitate engulfment (Mazaheri et al., 2014). Interestingly, the affinity of other PtdSer-bridging proteins such as Gas6 (which link to the Mer tyrosine kinase receptor (Mer-TK)) are considerably lower that than of Tim4 (K_d 20-50nM) (Baroni et al., 2010; K. Nagata et al., 1996). Consistent with this notion, it has been shown in peritoneal macrophages that both Tim4 and Mer-TK worked concurrently to facilitate corpse uptake ex vivo (Nishi et al., 2014; Toda et al., 2012). However, a recent study by Dransfield et al. demonstrated that bone marrow-derived macrophages, which normally express Mer-TK but not Tim4, use Mer-TK for both tethering and tickling (Dransfield et al., 2015). These findings support the broader notion that the mechanisms that underlie the dynamics of engulfment are highly dependent on the subset of phagocytes being investigated. Further, in cells with high phospholipid scramblase activity (via calcium-induced activation of the lipid scramblase TMEM16F), this is not sufficient to induce engulfment of these PtdSer exposed cells; this is now thought to be due to PtdSer exposure being too transient with the exposed PtdSer being quickly flipped back to the inner leaflet by the aminophospholipid translocases (Segawa and S. Nagata, 2015; Segawa et al., 2011). This raises the dynamics of PtdSer exposure on apoptotic cells as another variable, with the possibility that tethering receptors like Tim4 could play a vital role in determining the viability of the target cell, i.e. PtdSer recognition by phagocytes only leads to internalization under conditions where PtdSer aggregation induces stronger and more persistent interactions between the target and the phagocyte.

Shifting Gears: How Motile Phagocytes Toggle between Motility and Engulfment

Efficient engulfment of apoptotic corpses by motile phagocytes requires the execution of two distinct but related cellular functions – migration toward the corpse and subsequent phagocytosis (**Figure 2**). Both of these energy-intensive processes involve large-scale reorganization of the phagocyte cytoskeleton and overlapping signaling pathways that regulate these changes. However, it is unclear how these events are integrated and act in synergistic and/or antagonistic manners. In addition, the complexity of soluble and cell-associated signals in a tissue context must be appropriately interpreted by the phagocyte to

ensure efficient clearance of non-viable targets. A number of recent studies have provided important insights that are beginning to define these relationships.

Surface proteins that function in motility and phagocytosis

Once the find-me signals have provided the cues necessary for phagocytes to move near a dying target, the phagocyte must arrest its migration to physically engage the target cells. Several lines of recent evidence suggest that a migration/arrest cycle is very likely operative in phagocytes to mediate efferocytosis. Indeed, a number of molecular pathways that control adhesion and migration also function in many instances as key mediators of efferocytosis. In fact, the first apoptotic cell recognition receptor identified on phagocytes was the vitronectin-binding integrin $\alpha_v\beta_{3/5}$, which is well linked to cell migration and also doubles as an apoptotic cell receptor via binding to MFGE8, a PtdSer binding protein (Hanayama et al., 2004; 2002; Hynes, 2002; Overstreet et al., 2013; Savill et al., 1990). In addition, another PtdSer receptor, BAI1, was originally identified as an adhesion-type GPCR expressed in brain tissues (Nishimori et al., 1997; Park et al., 2007). Additional surface proteins that function in both motility and efferocytosis in mammals include, ICAM-3, LRP1, CD14 and CD36 (Devitt et al., 2003; Gardai et al., 2005; Ogden et al., 2001; Savill et al., 1992; Torr et al., 2011). It is important to note that this dual functionality is an evolutionarily conserved feature of efferocytosis, as numerous studies have identified similar functional overlap of surface proteins in *C. elegans* (integrin homologues Ina1 and Ina2) and in *Drosophila* (MEGF-10 homologue Draper, and CD36 homologue Croquemort) (Franc et al., 1996; Hsu and Wu, 2010; MacDonald et al., 2006).

Intracellular signaling networks that function in motility and phagocytosis

The canonical Rho GTPases Rac1, Cdc42 and RhoA are well known to coordinate the actin dynamics that drive cell motility and phagocytosis (Heasman and Ridley, 2008; Ravichandran and Lorenz, 2007; Rossman et al., 2005) (**Figure 2**). Activation of Rac1 (i.e. Rac1-GTP) is an essential, evolutionarily conserved mediator of the actin polymerization required to form the phagocytic cup and F-actin-rich membrane protrusions that extend around and envelop the target cell (Kinchen and Ravichandran, 2010; 2007; Nakaya et al., 2008; 2006). Activation of Rac downstream of apoptotic cell recognition is mediated by the recruitment and activation of guanine nucleotide exchange factors (Rac-GEFs). One of the most important and evolutionarily conserved Rac-GEFs in efferocytosis is the bipartite Dock1-Elmo complex (Gumienny et al., 2001). In fact Elmo (which stands for ‘engulfment and cell motility’) was identified to regulate gonadal distal tip cell migration and engulfment of somatic and germ cell corpses in *C. elegans* (Gumienny et al., 2001). Similarly, mammalian Elmo1 and Dock are crucial for mammalian apoptotic cell engulfment and cell migration in vivo and in vitro (Brugnera et al., 2002; Côté and Vuori, 2007; Elliott et al., 2010; Grimsley et al., 2004; Park et al., 2007; Stevenson et al., 2014). Importantly, signaling through multiple chemotaxis and efferocytosis receptors converge at the Dock-Elmo-Rac module, indicating the essential role of this dual-function signaling complex in cell migration and phagocytosis.

Although the control of migration and phagocytosis by common signaling networks is known, it is not clear how these pathways are modulated to allow for the migration/arrest/

engulfment cycle of motile efferocytes. However, the likely key to toggling between migration and phagocytosis occurs at the level of Rac and RhoA antagonism and crosstalk (**Figure 2**). Many studies have shown that RhoA-GTP inhibits phagocytosis of apoptotic cells (Nakaya et al., 2006; Tosello-Trampont, 2003). In the context of cell migration, RhoA-GTP activates FilGAP, a filamin A-binding Rac1-GAP, leading to actomyosin contraction and inhibition of Rac-driven actin polymerization (Ohta et al., 2006). Although the role of FilGAP in apoptotic cell engulfment is presently unknown, its function as a toggling mechanism for the transition from Rac-dependent mesenchymal to RhoA-dependent amoeboid movement raises the possibility that a similar mechanism could underlie the transition from the polarized and directed migration of phagocytes induced by find-me signals to a partial arrest and probing behavior necessary for phagocytes to engage with apoptotic cells. Studies using FRET-based Rho GTPase activation reporter probes have produced a wealth of new information on the spatiotemporal dynamics of Rho and Rac activation during cell motility and efferocytosis that have altered the paradigm for understanding the nature of interplay and crosstalk between these signaling molecules (Machacek et al., 2009; Nakaya et al., 2008; Pertz et al., 2006). In the case of efferocytosis, a study by Nagata and colleagues using Rho and Rac FRET reporters revealed that active Rac rapidly accumulates at the site of phagocytic cup formation (Nakaya et al., 2008). Interestingly, it was observed (using wild-type and constitutively active Rac) that regions of the phagocyte membrane enriched for Rac-GTP acted as ‘portals’ for the engulfment of successive targets. However, since closure of the phagocytic cup and internalization of the target are hampered by constitutively active Rac (as has been elegantly shown for larger versus smaller particle uptake), the mechanical act of corpse internalization requires the spatially-defined termination of Rac signaling (Schlam et al., 2015). In contrast to Rac, the role of RhoA in efferocytosis is more nuanced. It is clear that globally activating RhoA (i.e. RhoA-GTP) in cells inhibits the phagocytosis of apoptotic cells; as a corollary, global inhibition of RhoA has an overall net positive effect on efferocytosis (Nakaya et al., 2006; Tosello-Trampont, 2003). However, the spatially restricted activation of RhoA appears to be important for controlling the closure of the phagocytic cup and internalization of the corpse within the phagolysosome. Thus, crosstalk between Rac and RhoA is a dynamic and essential element of efficient engulfment. The molecular regulation of this crosstalk during efferocytosis remains an important, but poorly understood area of apoptotic cell clearance.

Interstitial Cell Clearance

Given our current understanding of find-me and eat-me signals in the courting that occurs between dying cells and phagocytes, how might phagocytes ‘sense’ the need to arrest migration and engage the engulfment machinery in a tissue context? Even though it is common to consider find-me and eat-me signals as distinct, it is very likely that in vivo these events likely occur within a continuum. Efferocytosis in real-time likely involves a seamless integration of receptors that mediate the sensing of find-me to eat-me signals. Based on analogous biological systems, the physical localization of these surface proteins might be akin to a ‘phagocytic synapse.’ Such a synapse may be important for coordinating the signaling molecules on the phagocyte and to ensure maximum efficiency of target identification and uptake. Indeed, intercellular signaling synapses are observed in numerous

biological contexts, including the well-detailed examples in the nervous and immune systems. These synapses are known to be effective means for cells to focus signaling information between cells to specific sites within a cell, and subsequently translate the various go/no-go signals from different receptors to a specific cellular response. Considering the complexity of signals that control phagocyte morphology during efferocytosis, it is an exciting possibility that such a phagocytic synapse containing find-me receptors and eat-me receptors would function in the dynamic movement of the phagocytes, selective sensing of apoptotic cells (and avoiding viable cells) within tissues, and the subsequent efficient corpse removal (**Figure 2**). Signaling through the find-me receptors P2Y2, S1PR and G2A has been shown to stimulate RhoA activation (Donati and Bruni, 2006; Kabarowski et al., 2000; Liao et al., 2007). Thus, RhoA-dependent migration in response to a find-me cue will need to be overcome/turned off so that the essential Rac-dependent engulfment processes can occur. Whether these surface receptors use the engulfment synapse and the Rho/Rac antagonism to transition between migration, arrest, and subsequent target uptake, i.e. a hand-off in integrating find-me and eat-me cues, remains to be determined, and promises to be a fascinating area of future investigation (**Figure 2**).

Future challenges in defining apoptotic cell clearance in tissues

Our first insights into the complex intercellular interactions that underlie cell death and clearance in the tissue came from the pioneering work of Elie Metchnikoff 100 years ago. By light microscopy, Metchnikoff observed the role of phagocytes (motile and sessile) in the removal of damaged and unnecessary cells within inflamed tissues and during development in a number of different metazoan species (Metschnikoff, 1891; Tauber, 2003). The notion of regulated cell death as a component of cellular turnover was cemented in 1972 by the landmark study of Kerr *et al.* in which the term “apoptosis” was coined (Kerr et al., 1972). Using electron microscopy of human and rodent tissues, this group solidified the notion of interstitial cell death as an integral part of cellular turnover (Kerr et al., 1972). Further, the authors observed the engulfment of these apoptotic cells by tissue macrophages (‘histiocytes’). While we have made tremendous progress since these early studies in deciphering the key steps in apoptotic cell clearance, the scientific problem remains a beautiful one, with broad implications for basic physiology and many forms of diseases. A key challenge moving forward is the wide variety of tissue environments and the many different molecular mechanisms for recognition of dying cells that make it difficult to develop and apply broadly useful strategies for tracking and targeting efferocytosis in vivo (Arandjelovic and Ravichandran, 2015; Green et al., 2016; Hochreiter-Hufford and Ravichandran, 2013). The heterogeneity of cell clearance mechanisms in mammalian tissues was appreciated early on, and over the past 15 years investigators have generally focused on identifying the clearance mechanisms relevant for specific tissues and biological or disease states (reviewed in (Elliott and Ravichandran, 2010; Green et al., 2016; Poon et al., 2014b). For example, using intravital imaging a recent report by Lemke and colleagues suggested that microglial process extension in response to vascular injury was impaired in the absence of the efferocytosis receptors Axl and MerTK (Fourgeaud et al., 2016). Similarly, intravital imaging of peritoneal macrophages revealed that they remain relatively sessile but execute dead cell clearance via extension of pseudopodia that gradually disassemble and engulf dead

cell material (Wang and Kubes, 2016). Similarly, Casanova-Acebes used multiphoton imaging of calvarial bone marrow to track aged neutrophils as they return from circulation to the bone marrow, and were found to be engulfed by CD169+ bone marrow macrophages rapidly (Casanova-Acebes et al., 2013). Thus, although few in number, these studies highlight the possibilities for illuminating the true nature of phagocyte-apoptotic cell interactions that are important for interstitial cell clearance (**Figure 3**). Nevertheless, the reality is that there is tremendous heterogeneity at the dying cell and phagocyte levels. At the target level, there are different forms of apoptosis, different cell types undergo death at different rates, and different targets likely use different sets of find-me and eat-me cues. Further, the cells undergoing developmental apoptosis versus damage-induced apoptosis are certain to have additional differences in the markers they expose. From the phagocyte end, there are professional, non-professional, and specialized phagocytes, and they likely utilize distinct as well as overlapping sets of phagocytic receptors. Moreover, in many tissues, professional and non-professional phagocytes often reside nearby and how they may communicate with each other to divvy up their engulfment roles is a fascinating problem that remains to be addressed. Nevertheless, the recent progress in understanding the dynamics of cell clearance is already providing insights that were not apparent a few years ago, and the pace of learning about cell clearance in vivo is only going to increase, as the techniques and the power of our investigations continue to expand rapidly.

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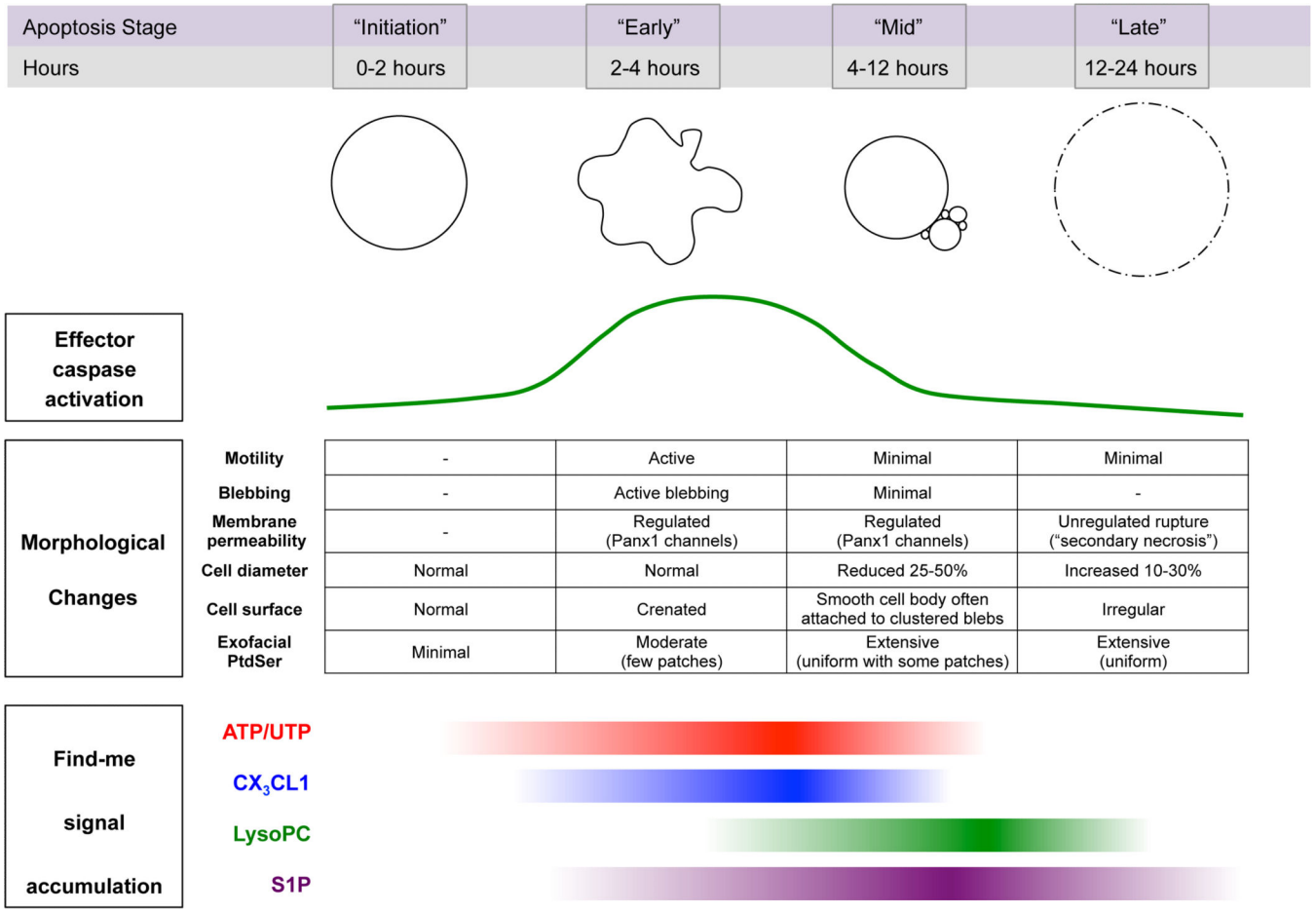

Figure 1. Spatiotemporal characteristics of apoptotic cells

Generalized depiction of the temporal relationships among key features of apoptotic cells at different stages after induction of apoptosis. Effector caspase activation (i.e. caspases 3 and 7) and prominent morphological changes in apoptotic cells are shown in relationship to the accumulation of the four main find-me signals identified to date: ATP/UTP, CX_3CL1, lysophosphatidycholine (LysoPC), and sphingosine-1-phosphate.

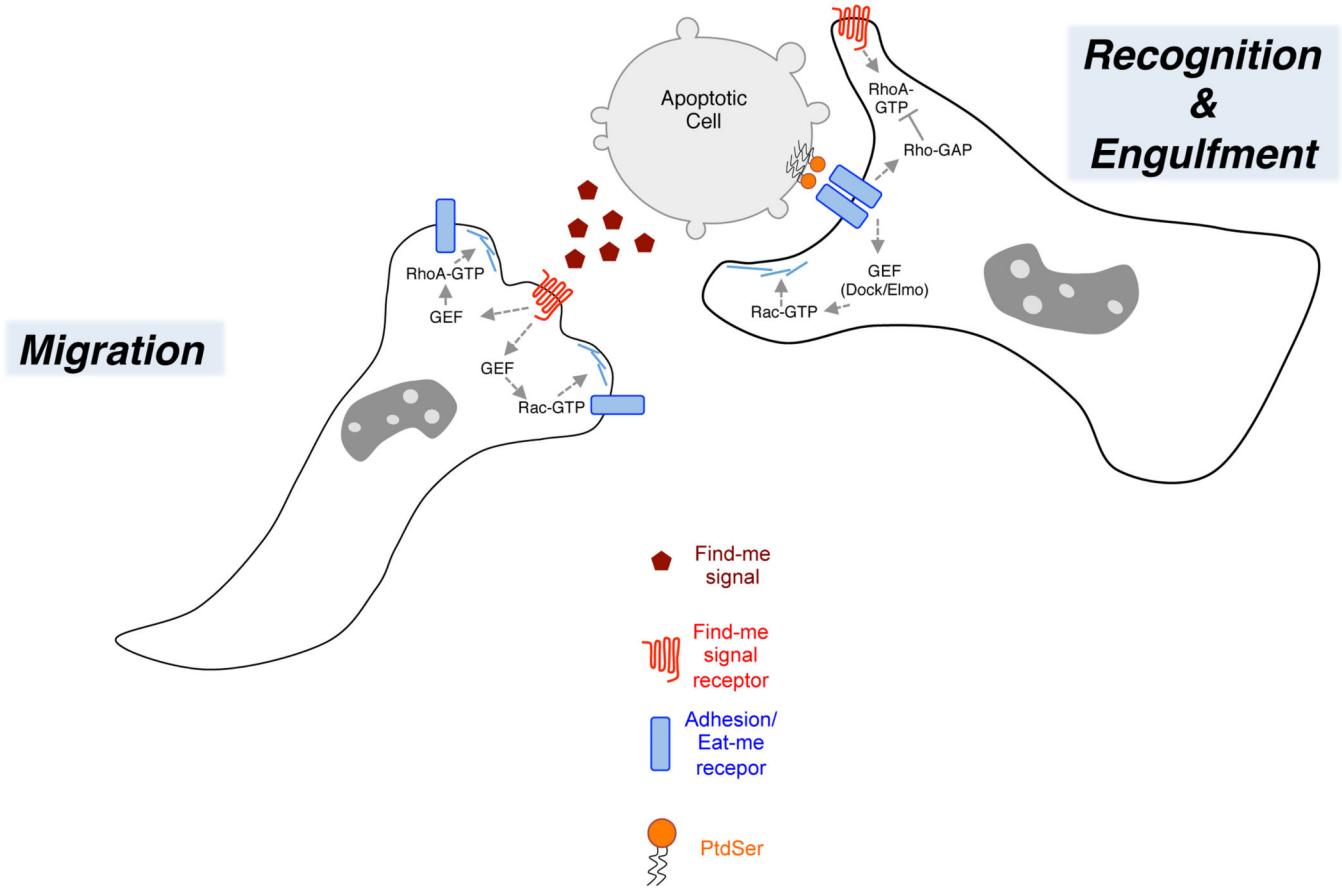

Figure 2. Model for hand-off of Rho signaling during transition from find-me to eat-me stages of efferocytosis

Detection of find-me signals by GPCR receptors on phagocytes stimulates migration and/or motility behavior in a manner dependent on RhoA and possibly Rac1 activation. During the find-me stage, some eat-me receptors, for example $\alpha v\beta_{3/5}$ integrins, might function in adhesion and cell motility. Upon encountering the apoptotic target, adhesion receptors can function to recognize PtdSer on the surface of the apoptotic cells, either directly or indirectly via bridging proteins like MFG-E8 or Gas6, and reorient in a putative phagocytic synapse at the site of intercellular contact between the apoptotic cell and phagocyte. RhoA-GTP is likely inhibited or spatially confined during this stage to prevent antagonism with Rac that would impair Rac-GTP-dependent actin polymerization and phagocytic cup formation.

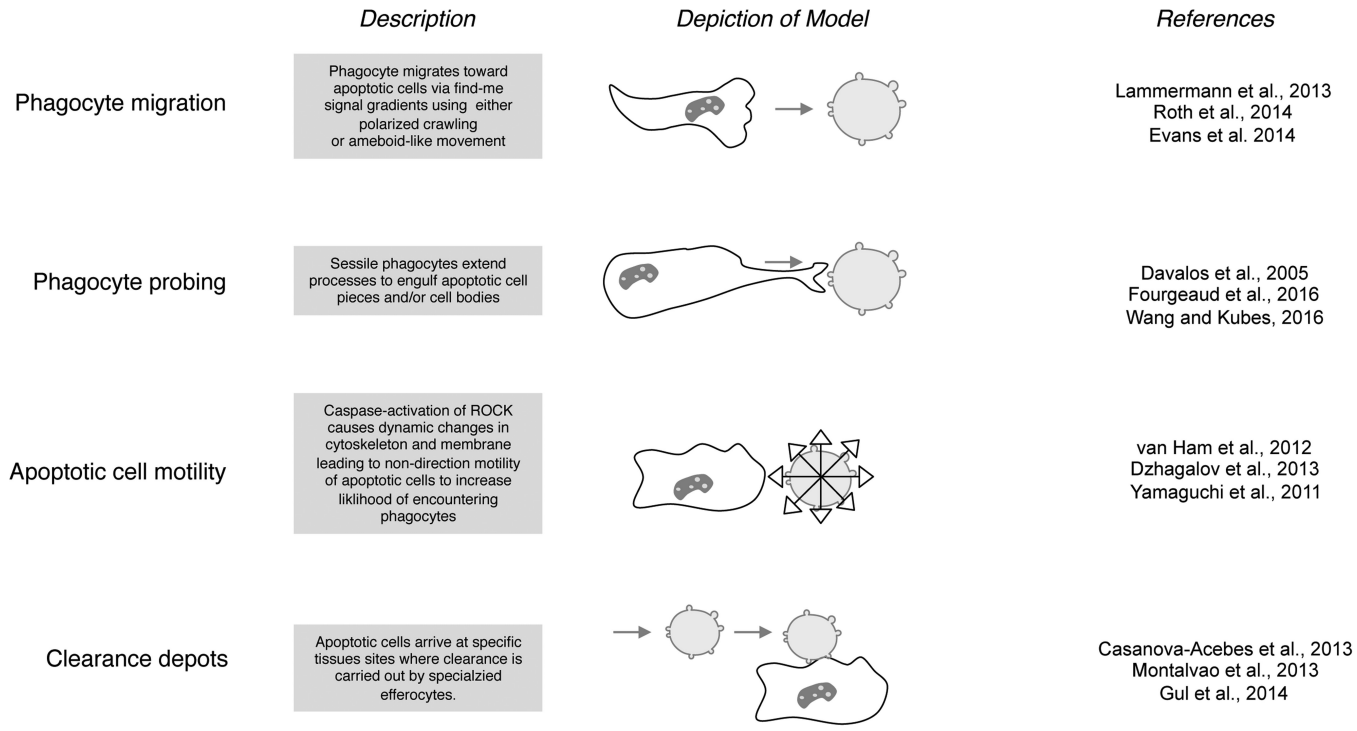

Figure 3. Different modes of phagocyte and apoptotic cell movement that can promote intercellular interaction and efferocytosis in a tissue

The four modes of efferocytosis related cell movements described are based on data from the indicated studies using intravital microscopy.