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Prix Fixe: Efferocytosis as a Four-Course Meal

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Abstract

During development, stress, infection, or normal homeostasis, billions of cells die on a daily basis, and the responsibility of clearing these cellular corpses lies with the phagocytes of innate immune system. This process, termed efferocytosis, is critical for the prevention of inflammation and autoimmunity, as well as modulation of the adaptive immune response. Defective clearance of dead cells is characteristic of many human autoimmune or autoinflammatory disorders, such as systemic lupus erythematosus (SLE), atherosclerosis, and diabetes. The mechanisms that phagocytes employ to sense, engulf, and process dead cells for an appropriate immune response have been an area of great interest. However, insight into novel mechanisms of programmed cell death, such as necroptosis, has shed light on the fact that while the diner (or phagocyte) is important, the meal itself (the type of dead cell) can play a crucial role in shaping the pursuant immune response.

1 Introduction

The phagocytic cells of our innate immune system act as surveyors of the environment, constantly patrolling the body for unwanted, unneeded, and unexpected components and ridding them in a timely and orderly fashion. The ancient, evolutionarily conserved pathway of phagocytosis (“the cellular process of eating”) has been at the vanguard of immunology, developmental biology, and cellular biology since its nineteenth-century discovery (and 1908 Nobel Prize in Physiology and Medicine) by Ilya Metchinkoff and Paul Ehlirch (Krysko and Vandenabeele 2010). While clearance of invading pathogens is indeed a necessary function of phagocytes, the sensing, recognition, and removal of cellular corpses are a critical role that phagocytes play during times of development, cellular homeostasis, and stress (Nagata et al. 2010).

The formation of a “wild-type,” functioning organism is, in actuality, a process wrought with waste. A multitude of extra cells are generated during development, only to unceremoniously undergo programmed cell death (described below) and be cleared by phagocytes (Green 2011). During the development of *Caenorhabditis elegans*, a total of 1090 cells are generated, and 131 of them are destined for death (Kinchen 2010). Indeed, this cell death is critical for the correct development of the organisms, as animals deficient for a variety of caspases, endoproteases that mediate apoptotic cell death, are grossly malformed

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and often embryonic lethal (McIlwain et al. 2015). While the generation and subsequent destruction of these cells are necessary for proper development, as well as normal cellular homeostasis, wound healing, and immune responses in the adult organism, the ruin left in its wake would be catastrophic if not for the efficient work of the phagocytic system (Savill et al. 2002; Peter et al. 2010).

Despite the constant turnover of cells through programmed cell death mechanisms (not to mention those induced to die via stress or infection), it is rare to observe apoptotic cells under normal physiological conditions. Considering the average one million adult human cells that undergo apoptosis every second, one must truly appreciate the magnitude of the job facing phagocytes (Ravichandran 2010). Moreover, as a reoccurring and normal event in the life span of an organism, this process of dead cell clearance must occur in a quiescent manner, so as to not inappropriately alert the immune system (Hart et al. 2008).

In this chapter, we will explore efferocytosis not only as a process of cleanup, but also a critical regulator of the immune response. While the manner and efficiency in which dead cell cargo is degraded and processed by the phagocyte are important, the type of dead cell cargo is engulfed can also play a role in how the phagocyte responds.

2 Prepping of the Meal: Types of Cell Death

Death is a part of life. The process of generating, maintaining, and protecting a multicellular organism throughout its lifetime requires the creation and destruction of billions of cells. While damage can certainly cause unwanted cellular death, most cellular death is genetically programmed, and perturbations in these genetic programs can promote cell accumulation, autoimmunity, oncogenesis, attrition, and/or degeneration. Programmed cell death, such as apoptosis, necroptosis, or pyroptosis, is an active mechanism designed to sculpt, control, and aid the body in its development and survival. Like death itself, the innate immune system has tolerant systems in place to manage these morbid, yet necessary events. Death comes in a variety of flavors, some more appetizing than others.

2.1 Apoptosis

The most widely studied form of cell death is apoptosis. Apoptosis (from the Greek meaning “falling off”) is genetically programmed cellular suicide and involves the coordinated dismantling of intracellular components designed to prevent inflammation and limit damage to the surrounding environment (Green 2011). During apoptosis, the plasma membrane forms “blebs,” allowing membraned fragments containing intracellular contents to separate from the larger, dying cells as “apoptotic bodies.” This blebbing is a morphological characteristic of apoptosis and also a key mechanism for confining danger-associated molecular patterns (or DAMPs) and avoid alerting the immune system (Green et al. 2009). Another hallmark of apoptosis is DNA fragmentation and chromatin condensation (Green 2011). These and other characteristics of apoptosis are largely controlled by a family of cysteine proteases with endopeptidase activity called caspases (Taylor et al. 2008).

Caspases exist only in the animal kingdom and are broadly grouped into initiator caspases (caspase-8 and caspase-9), executioner caspases (caspase-3, caspase-6, and caspase-7), and

inflammatory caspases (human caspase-1, human caspase-4, and human caspase-5; rodent caspase-1 and rodent caspase-11) (Taylor et al. 2008; Green 2011). The main function of caspases is to cleave proteins to ensure an efficient yet rapid cell death. How then does the cell tolerate such a lethal family of proteases? First of all, caspases only cleave at specific sequence residues that end in aspartate residues (hence the “asp” of caspase) (Kumar 2007). Secondly, caspases exist in inactive forms, requiring dimerization (initiator caspases) or cleavage (executioner caspases) to gain activity (Kumar 2007; Taylor et al. 2008). Thirdly, caspases are not the only proteins involved in regulation of apoptosis (discussed below).

Upstream of apoptotic signaling events, initiator caspases exist as inactive monomers that must be dimerized to become active. This process is known as an “induced proximity model” and results in autocatalytic cleavage and stabilization of the dimer (Muzio et al. 1998; Boatright et al. 2003). Once active, initiator caspases are capable of cleaving numerous targets, most notably the executioner caspases (Green 2011). Executioner caspases exist as inactive dimers and cleavage by proteases, mainly initiator caspases, occurs between the large and small subunits, resulting in a conformational change that brings the two active sites of the executioner caspase dimer together to create a functional mature protease. These active executioner caspases can now cleave and activate other executioner caspases, leading to a rapid feedback loop to facilitate apoptosis (Riedl and Shi 2004).

What triggers this cascade of lethal events? Depending on the adapters and initiator caspases involved, most apoptotic programs fall into either the intrinsic or the extrinsic category. The major mechanism of apoptosis in mammals is the intrinsic or mitochondrial pathway. This pathway is activated by a variety of stress-inducing stimuli, including growth factor deprivation, cytoskeletal disruption, DNA damage, accumulation of unfolded proteins, and hypoxia, as well as developmental signals that instruct cells to die, such as hormones (Brenner and Mak 2009; Green 2011). These signals converge on the mitochondria, where the balancing act between pro-apoptotic and anti-apoptotic members of the BCL2 family orchestrates mitochondrial outer membrane permeabilization (MOMP) to release cytochrome c and other deadly sequestered proteins from within the mitochondria (Tait and Green 2010). The intricacies of BCL2 family interactions are discussed in detail in a number of other sources (Tait and Green 2010; Green 2011; Llambi and Green 2011; Llambi et al. 2011).

The result of MOMP and the release of cytochrome c is the formation of the apoptosome, a multimeric complex comprised of cytochrome c, caspase-9, and the adaptor APAF-1. The formation of the apoptosome activates caspase-9, which leads to the activation of the downstream executioner caspases, such as caspase-3 and caspase-7 (Shiozaki et al. 2002). The importance of the intrinsic apoptotic pathway is highlighted by the developmental phenotypes of gene-targeted mice deficient for components of this pathway. Unsurprisingly, mice deficient for caspase-9, APAF-1, or caspase-3 suffer from large brain outgrowths, characterized by reduced neuronal cell apoptosis, and subsequent perinatal lethality (Colussi and Kumar 1999). Thus, the fate of proper vertebrate development lies in the ability of one organelle, the mitochondria, to know when to maintain its integrity and when to release the mediators of death.

The extrinsic pathway of apoptosis, however, is triggered by signals that engage extracellular death receptors (DR). Signals such as tumor necrosis factor (TNF), CD95-ligand (CD95-L or Fas-L), and TNF-related apoptosis-inducing ligand (TRAIL) bind the DRs TNF receptor-1 (TNFR1), CD95 (or Fas), and TRAIL-R1/2 (DR4/5), respectively (Green 2011). Engagement of a DR ligand with its cognate receptor results in the recruitment of pro-caspase-8 to the death-inducing signaling complex (DISC) formed at the cytoplasmic tail of the engaged DR that also includes the adaptor proteins FADD or TRADD. Recruitment of these caspase-8 monomers results in dimerization and activation, leading to activity of caspase-3 and caspase-7 (Ashkenazi and Dixit 1998; Boatright et al. 2003). Caspase-8 activity can also cleave and activate BCL2 family proteins to trigger the intrinsic apoptotic pathway (discussed above) to induce efficient cell death (Tait and Green 2010; Green 2011).

Perhaps the most important result of apoptosis is the careful organization and packaging of potentially immunogenic cellular component into discreet, tolerogenic pieces. While it is easy to appreciate the appropriate execution of apoptosis in terms of proper development and suicide of infected cells, the real victory is sustained cellular renewal in the absence of immune activation. As described below, not all forms of cell death are tolerated as well.

2.2 Necrosis

While apoptosis is considered a genetically controlled and immunologically silent mechanism of cell death, necrosis has been characterized as a passive type of cell death, resulting in organelle swelling and cellular explosion that uncontrollably releases inflammatory cellular contents (Nikoletopoulou et al. 2013). Mechanistically, classical necrosis is typically not associated with a genetic program and occurs independently of caspase activation (Leist and Jaattela 2001). Unlike apoptosis, which is critical for proper development, necrosis is thought to mediate cellular death in response to catastrophic damage or pathology, including infarction, mechanical trauma, ischemia, frostbite, and animal venom (Raffray and Cohen 1997). Likewise, apoptotic cells that are not efficiently cleared by phagocytes can undergo secondary necrosis, a process that occurs completely independently of any apoptotic machinery.

Various molecules in our cells are pro-inflammatory if they are released from cells. Collectively referred to as damage-associated molecular patterns (DAMPs) or alarmins, they can activate neighboring macrophages and dendritic cells through TLR signaling and other mechanisms (Kono and Rock 2008). Morphologically, necrotic cells are characterized by cellular swelling (oncosis), nuclear distension, and plasma membrane rupture (Green 2011; Nikoletopoulou et al. 2013). This explosion of cellular contents, including proposed DAMPs such as HMGB1, HDGF, nucleotides, metabolites, and uric acid, is typically associated with an inflammatory reaction (Nikoletopoulou et al. 2013). Importantly, DAMP release and recognition might help alert the immune system to a cell-death-inducing pathogen, but if triggered inappropriately, it can also have deleterious effects, including autoimmunity. Importantly, both the extent and type of cell death represent major means of regulating DAMP release. When one considers the trauma that triggers necrotic death, it is unsurprising that inflammation follows, whereas apoptosis, occurring in a structured setting during carefully timed points in development, proceeds without alarm. Our understanding of

necrotic cell death is evolving though, as a new programmed form of necrosis, termed necroptosis, has recently been described.

2.3 Necroptosis

Like its name suggests, necroptosis is the marriage between the programmed nature of apoptosis and the morphological features of necrosis. As mentioned previously, mice deficient for caspase-3 or caspase-9 die perinatally and are characterized by an excess of neuronal cells and large brain outgrowths (Colussi and Kumar 1999). Intriguingly, mice deficient for caspase-8, or its anti-apoptotic homologue, FLIP, die at embryonic day 10.5 and have embryonic vascular, cardiac, and hematopoietic defects (Varfolomeev et al. 1998). Additionally, pharmacological inhibition of caspase-8 or knockdown of caspase-8 or FLIP via short interfering RNA (siRNA) sensitizes fibroblasts to TNF-induced necrotic death (Vercammen et al. 1998). This surprising observation, wherein the absence of a pro-apoptotic gene resulted in a deficit of cells, led researchers to examine alternate roles for caspase-8.

In the absence of caspase-8, ligation of the death receptor pathway of apoptosis, such as the TNF-TNFR pathway, can result in necrotic cell death and requires the kinase activity of receptor-interacting protein kinase-1 (RIPK1) and RIPK3 (Oberst et al. 2011; Weinlich and Green 2014). Strikingly, the embryonic lethality of the caspase-8-deficient mouse is fully rescued by co-ablation of receptor-interacting protein kinase-3 (RIPK3), and caspase-8/RIPK3 double knockout mice are developmentally normal, but develop a severe lymphoaccumulative disorder resembling that of mice or humans lacking Fas or FasL (Bidere et al. 2006; Kaiser et al. 2011; Oberst et al. 2011).

How then does engagement of the extrinsic pathway of apoptosis result in necroptosis? RIPK1 plays a paradoxical role in the survival of the cell. Ligated death receptors, such as TNFR1 (or TLR engagement of TRIF (Feoktistova et al. 2011; Dillon et al. 2014)), promote the recruitment and deubiquitination of RIPK1 to the adaptor TRADD. Together with TRAF2 and the ubiquitin ligases cIAP1, cIAP2, and LUBAC, this complex, termed “Complex I,” can activate the NF-κB signaling pathway, which can induce the expression of FLIP (Zhang et al. 2000; Micheau et al. 2001; Newton 2015). Subsequent formation of a cytoplasmic “Complex II” or “Ripoptosome” containing RIPK1, FADD, and caspase-8 drives apoptotic signaling upon the ligation of death receptors. However, in the absence of extrinsic apoptotic machinery, RIPK1 (or more specifically, the kinase activity of RIPK1) can promote necroptosis via RIPK3 activation. Therefore, while it has been known that FLIP blocks caspase-8-mediated apoptosis, the catalytically active complex of FADD, caspase-8, and FLIP also blocks signaling for necroptosis (Oberst et al. 2011; Newton 2015). Furthermore, inactive RIPK1 can block RIPK3 activity, even when RIPK3 is activated independently of RIPK1 (Oberst et al. 2011).

The activation of RIPK1 and RIPK3 is not the final executioner of the necroptosis pathway, however. Necroptosis also depends on the RIPK3-mediated phosphorylation of the pseudokinase, mixed lineage kinase-like (MLKL) (Kaiser et al. 2013; Rodriguez et al. 2015). Phosphorylated MLKL induces a conformational change that allows for its oligomerization and interaction with the plasma membrane by binding to

phosphatidylinositol lipids to directly disrupt membrane integrity (Wang et al. 2014; Rodriguez et al. 2015).

The involvement of RIPK1 adds another layer of complexity. In addition to its role in necroptosis, the kinase activity of RIPK1 is required for a variety of innate immune signaling pathways, such as Toll-like receptors (TLRs), interferons, and the RIG-I-MAVS pathway (Dillon et al. 2014; Weinlich and Green 2014). Whereas ablation of RIPK3 rescued caspase-8-deficient animals, RIPK1/caspase-8 double knockout mice die perinatally at day 1, similar to the RIPK1−/− mice (Dillon et al. 2014). During embryonic development, RIPK1 can trigger TNFR-mediated necroptosis in animals lacking apoptotic machinery, such as FADD, FLIP, or caspase-8, and RIPK1-mediated lethality at later developmental stages that could be mediated by similar signals (termed “signal 1”). Postnatally, RIPK1 can serve two functions: (1) RIPK1 is required to prevent a TNFR1-induced apoptosis, possibly due to its role in NF-κB activation and subsequent FLIP upregulation; and (2) RIPK1 is also required to prevent RIPK3-dependent lethality promoted by other “signal 2,” such as TRIF and IFN (Dillon et al. 2014). The balance and contribution of RIPK1 to cell survival, cell death, and immunity is certainly multifaceted and is discussed in elegant detail in a recent review (Weinlich and Green 2014; Newton 2015).

In addition to the role of RIPK1 in directly regulating inflammatory pathways, necroptosis has been reported to itself be an immunogenic type of cell death, and its inhibition, either genetically or with small-molecule inhibitors, has been demonstrated to lessen disease severity in several mouse models (Weinlich and Green 2014; Newton 2015). Furthermore, necroptosis is very common in vivo, not only in physical traumas, but also mainly in diverse forms of neurodegeneration, and death inflicted by ischemia or infection. Similar to classical necrosis, necroptosis results in the release of intracellular danger signals into the extracellular milieu, and these components can stimulate the immune system. Indeed, animals deficient for the necroptotic pathway are protected from various models of inflammation, such as chemically induced pancreatitis, intestine and skin inflammation, or ischemic reperfusion injury (Linkermann et al. 2013; Weinlich et al. 2013; Wu et al. 2013).

2.4 Pyroptosis

Previously, we discussed the caspases as mediators of apoptosis, but some caspases, such as caspase-1 and caspase-11 (or caspase-5 in humans), have roles in non-apoptotic, biological processes. The best-described function for caspase-1 is its key role in the processing of inactive IL-β and IL-18 into mature inflammatory cytokines. Additionally, excessive caspase-1 activity can cause pyroptosis, a non-apoptotic type of programmed cell death (LaRock and Cookson 2013; Jorgensen and Miao 2015). Execution of pyroptosis differs from apoptosis both at the biochemical and at the morphological level. Although caspase-1 can trigger apoptosis, caspase-1-mediated pyroptosis does not result in the cleavage of substrates of typical caspases. Rather, activated caspase-1 activates caspase-7 (Bergsbaken et al. 2009). Unlike apoptosis, MOMP is typically not associated with pyroptosis. Pyroptosis is characterized by lysis of the plasma membrane and the release of pro-inflammatory intracellular contents. Interestingly, nuclear DNA undergoes extensive fragmentation, similar

to that observed in apoptosis, although the mechanism by which this occurs remains unknown (LaRock and Cookson 2013).

Regardless of its pro-inflammatory or pro-pyroptotic outcome, caspase-1 is activated by dimerization at complexes termed inflammasomes that form in the cytosol and detect a diverse repertoire of pathogenic molecules, including bacterial toxins and viral RNA (Henao-Mejia et al. 2012). However, how active caspase-1 kills a cell remains a complete mystery, in the sense that no key substrates have been identified that would account for this rapid deadly event. We can speculate, though, on the evolutionary roots of caspase-1's dual roles. Bacterial and viral pathogens can subvert caspase-1-mediated IL-1β and IL-18 processing, thus dampening the host inflammatory response and facilitating infection (Bergsbaken et al. 2009; Jorgensen and Miao 2015). Could pyroptosis that represents a strategy by the infected host redirect the activity of caspase-1 toward killing the cell? Alternatively, it is possible that pyroptosis is required for the release of the mature, inflammatory cytokines.

Similar to other types of lytic cell death discussed above, pyroptosis might indeed be pro-inflammatory, as it results in the release of intracellular danger signals. While caspase-1 activation is required for cell death in a variety of experimental settings, including in the immune system, the cardiovascular system, and the central nervous system, mice deficient for caspase-1 develop normally, implying that this protease is redundant in vivo during development (Green 2011; McIlwain et al. 2015). It was recently reported, however, that caspase-1-deficient mice generated from strain 129 embryonic stem cells also harbor a mutation in the caspase-11 locus, and so are in fact caspase-1/caspase-11 double knockout mice (Kayagaki et al. 2011). While it is clear that inflammation mediated by caspase-1 (or caspase-11) is a critical component of pathogen clearance and sepsis, what remains to be elucidated is the role of pyroptosis in vivo inflammatory pathologies, and whether a pyroptotic death is handled or recognized differently by the innate immune system.

3 The Dining Experience: The Mechanisms of Efferocytosis

The meal is prepared, the table is set, but how do the diners know when and where to go? Phagocytes are not typically within close proximity to the dying cells, so they must be recruited and enticed to their cellular meal (Peter et al. 2010). But recruitment is just the beginning. Efferocytosis is a carefully orchestrated process wherein phagocytes, both professional (macrophages and dendritic cells) and non-professional (epithelial cells), migrate toward areas of cell death, recognize and engage cellular corpses via surface molecules specific to dead cells, and internalize, usually in its entirety, a dead cell for degradation and processing (Parnaik et al. 2000; Poon et al. 2014). For apoptotic cells, this is an immunologically tolerable event, as phagocytes recognize cells that have undergone this cell death program as biologically inert and dispose of these corpses before they release their potentially immunogenic intracellular contents, such as DNA (Nagata et al. 2010).

We now recognize that dying cell clearance is an important factor in many different disease models, including SLE, atherosclerosis, and Alzheimer's disease (Camins et al. 2008; Ravichandran 2010). Thus, there has been a tremendous effort to understand the specific

steps by which dying cells are recognized and cleared, as well as how these processes shape the immune response. Efferocytosis can be generally categorized into 4 steps: (1) the recruitment of phagocytes by “find-me” signals; (2) the recognition and engagement of “eat-me” signals; (3) the engulfment of the cellular corpse (Fig. 1); and (4) the processing, degradation, and immune response to the engulfed corpse (Fig. 2).

3.1 Perusing the Menu: “Find-Me” Signals

Even in tissues with high rates of cellular turnover, such as the thymus or bone marrow, uncleared apoptotic cells are rarely observed. Our earliest evidence of the phagocytic program of clearing dead cells comes from studies done in *C. elegans*, wherein phagocytes were recruited to sites of cell death and cleared dying cells before apoptosis (and hence complete death) was even fully executed (Reddien and Horvitz 2000; Hoeppner et al. 2001). Given that phagocytes do not often regularly reside in the tissues they must patrol, apoptotic cells must “advertise” their presence to phagocytes, essentially expediting their own clearance (Elliott et al. 2009).

Apoptotic cells release several distinct molecules, termed “find-me” signals, to attract phagocytic cells via a chemotactic gradient. To date, several potential “find-me” signals have been reported, although their relevance has not always been validated in vivo (Hochreiter-Hufford and Ravichandran 2013). B cells in germinal centers undergo increased rates of apoptosis during affinity maturation, and these apoptotic B cells release the membrane-associated molecule CX3CL1 (or fractalkine) in small vesicles or microparticles. This classical chemokine is sensed by CX3CR1 and mediates the migration of macrophages to the dying cells. However, mice deficient for CX3CR1 do not display a defect in apoptotic cell clearance in their germinal centers, indicating that other factors function to recruit phagocytic cells (Truman et al. 2008).

Lysophosphatidylcholine (LPC) is generated and released by the caspase 3-dependent activation of phospholipase A and was the first “find-me” signal of lipid origin (Lauber et al. 2003). LPS is sensed by the G-protein-coupled receptor G2A, and this interaction can stimulate macrophage recruitment (Peter et al. 2008). Similarly, the lipid sphingosine-1-phosphate (S1P) is secreted by apoptotic cells and sensed by multiple G-protein-coupled receptors S1P-R1-5 to mediate phagocyte chemotaxis (Gude et al. 2008). These lipids, however, are present in the circulation at a concentration higher than that released by apoptotic cells, and their function in vivo has not been assessed. Therefore, their activity is likely to be merely local.

Perhaps the most promising candidates as “find-me” signals are nucleotides. ATP and UTP are released in a caspase-dependent manner via activation of pannexin 1 channels (Chekeni et al. 2010) and are detected by phagocytic cells via purinergic receptors, like P2Y2. Moreover, disruption of the nucleotide/P2Y2 interaction results in an accumulation of apoptotic thymocytes following glucocorticoid treatment in vivo (Elliott et al. 2009). It should be noted that the release of nucleotides from apoptotic cells, while an active process, is significantly smaller (less than 2 % of intracellular ATP levels) than the release that occurs during necrosis (Ravichandran 2010). Furthermore, these released nucleotides are easily

degraded by extracellular nucleotidases. Thus, nucleotides most likely act to recruit tissue-resident phagocytes in a short-range capacity.

A number of caveats exist for the theory of “find-me” signals. Many cell types of different origins and function express receptors for these “find-me” signals, yet the vast majority of cells recruited to sites of cell death are macrophages. Is recruitment based on a synergistic effect of these signals or do additional signals exist to regulate migration? Lactoferrin, a glycoprotein released by apoptotic cells, has been shown to act as a “keep-out” signal, excluding neutrophils and eosinophils from sites of cell death (Bournazou et al. 2009, 2010). However, the lactoferrin-deficient animal model has yet to be characterized, so its in vivo role remains unknown. Additionally, the relatively low concentration of these “find-me” signals begs the question—what is the range of chemotactic gradients and how are circulating phagocytes recruited to sites of cell death (Elliott et al. 2009)? While these signals are often caspase-dependent (and hence apoptosis-dependent), these molecules are also released during other forms of cell death, such as necrosis or necroptosis (Iyer et al. 2009), often in greater quantities than in apoptosis. What are the migratory and inflammatory consequences of these disparate levels of potential “find-me” signals? Indeed, some of these “find-me” signals, such as ATP, can also act as a danger-associated molecular pattern (DAMP), thereby alerting the innate immune system. Necrosis, for example, results in the release of uric acid, which acts as both a “find-me” signal and an activating factor of inflammasomes (Kono et al. 2010). Do low levels of “find-me” signals promote macrophage migration, while higher concentrations stimulate a pro-inflammatory response?

Finally, in addition to chemotaxis, what other roles do these “find-me” signals play? There is evidence that sensing of these “find-me” signals, such as ATP or S1P, activates and prime phagocytes, increasing their phagocytic capability (Hanayama et al. 2004). These and other questions remain to be answered as studies unravel the mechanisms behind the active migration of phagocytes to their meal.

3.2 It is All in the Presentation: “Eat-Me” Signals

The ability to distinguish self from nonself is the defining hallmark of our immune system. Likewise, the ability to distinguish living cells from dead cells is critical to development, immunity, and the prevention of unwanted inflammation. How then do phagocytes, actively recruited to sites of cell death by “find-me” signals, target dying cells, while leaving healthy cells unperturbed? It stands to reason that the process of dying transforms healthy cells into targets for engulfment, rendering them distinguishable from living cells.

Lipid bilayers comprise the core structure of the plasma and organelle membranes, and the unique makeup of these different membranes confers distinct protein folding and permeability properties (Leventis and Grinstein 2010). Lipid distribution differs not only among membranes but frequently also between the two leaflets of the bilayer. Notably, the composition of the plasma membrane is an asymmetrical distribution of lipids, wherein the lipid phosphatidylserine (PS) is actively confined to the inner leaflet in viable cells (Balasubramanian and Schroit 2003). However, during apoptosis, PS is rapidly externalized in a caspase-dependent manner. This exposure occurs not only in mammals, but also in *C. elegans* (Venegas and Zhou 2007) and Drosophila (van den Eijnde et al. 1998). The calcium-

mediated cation channel TMEM16F has been shown to mediate lipid scrambling (Suzuki et al. 2010), and recent studies have demonstrated that the scramblase Xkr8 is cleaved by caspase-3 and facilitates PS exposure during apoptosis (Suzuki et al. 2013). The flippase ATP11C normally transports aminophospholipids from the extracellular to the cytoplasmic side. During apoptosis, though, ATP11C is inactivated by caspase-3 cleavage, and PS remains externally exposed (Segawa et al. 2014).

Despite its relatively minor presence in most biological membranes, PS is a lipid of great physiological importance (Leventis and Grinstein 2010). Extracellularly exposed PS is the most well-characterized "eat-me" signal and an essential factor in the recognition and clearance of apoptotic cells (Balasubramanian and Schroit 2003). Phagocytes recognize exposed PS via membrane receptors, such as T cell immunoglobulin mucin receptor 4 (TIM4), brain-specific angiogenesis inhibitor 1 (BAI1), and stabilin-2 (Park et al. 2007, 2008a; Rodriguez-Manzanet et al. 2010). Additionally, there exist bridging molecules, such as milk fat globule-EGF factor 8 (MFG-E8) and Gas6, capable of recognizing PS and being recognized by phagocytic cell surface receptors such as integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, or Tryo3-Axl-Mer (or TAM) receptors (Ishimoto et al. 2000; Hanayama et al. 2002; Zizzo et al. 2012). Engagement of these receptors can result in cytoskeletal rearrangements that facilitate the engulfment of the cellular corpse (discussed below).

While a hallmark of cell death, PS is found extracellularly in low levels on living or activated cells, yet these cells are not engulfed (van den Eijnde et al. 2001). Even forced extracellular levels of PS on viable cells, via constitutively active TMEM16F, do not result in engulfment (Segawa et al. 2011). How then does a phagocyte distinguish a PS-positive dead cell, primed for clearance, from a PS-positive cell that should live to see another day? One answer may lie in the presence of "don't eat-me" signals, such as CD31, CD47, and CD61. Engagement of these molecules, expressed on viable cells, can negatively regulate phagocytosis, thus signaling to the phagocyte that this cell, while PS-positive, is not intended for clearance (Oldenborg et al. 2000; Elward et al. 2005; Poon et al. 2014).

Further, PS is not the only "eat-me" signal identified. ICAM3, oxidized LDL-like molecules, glycosylated surface proteins, and C1q bound serum proteins have all been described to act as "eat-me" signals (Ravichandran 2010; Poon et al. 2014). The translocation of calreticulin (CRT) from the endoplasmic reticulum to the plasma membrane can also serve as an "eat-me" signal and stimulate engulfment by phagocytes (Gardai et al. 2005). While efferocytosis may be regulated by the balance of "eat-me" and "don't eat-me" signals or the synergistic effect of multiple "eat-me" signals, it is clear that dead cells actively promote their own clearance to phagocytes that have evolved to recognize and remove such cells from circulation.

3.3 Savoring the Meal: Phagocytosis of Cellular Corpses

Efferocytosis is an intricately choreographed process requiring action by both the dying cells and the phagocyte. While the dying cell actively recruits phagocytes to sites of cell death via "find-me" signals and advertises its desire to be cleared via "eat-me" signals, the phagocyte facilitates the actual engulfment via engagement of receptors that specifically recognize these signals. As PS is the most characterized "eat-me" signal, PS receptors are the most

characterized mechanism for dead cell recognition. While initial thinking hypothesized that a single PS receptor existed to mediate this recognition and phagocytosis, we now know that multiple PS receptors exist (Bratton and Henson 2008; Nagata et al. 2010; Poon et al. 2014). PS can be recognized via bona fide membrane receptors, such as Stablin-2 (Park et al. 2008a), BAI-1 (Park et al. 2007), RAGE (He et al. 2011), and TIM4 (as well as family members TIM1 and TIM3) (Miyanishi et al. 2007; Freeman et al. 2010; Rodriguez-Manzanet et al. 2010). Additionally, bridging molecules, such as MFG-E8 (Hanayama et al. 2004; Hu et al. 2009), protein S, and Gas6 (Rothlin et al. 2007; Lemke and Rothlin 2008), have been demonstrated to simultaneously recognize PS on dead cells and promote engulfment via engagement of their cognate membrane receptors. In order to facilitate phagocytosis, MFG-E8 associates with the integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$, while the Tyro3-Axl-Mer (TAM) family of receptors recognize protein S and Gas6 (Scott et al. 2001; Lemke and Rothlin 2008).

Just as other eat-me signals exist, so do their corresponding recognition receptors. For example, lectins can recognize modified glycoproteins and lipids (Ezekowitz et al. 1990); CD36, with $\alpha_v\beta_3$, can bind thrombospondin (Fadok et al. 1998b); scavenger receptors like SR-A can bind oxidized LDL-like moieties (Gordon 1999); CD14 can bind ICAM3 (Gregory et al. 1998); and CD91 (or LRP1) binds C1q via calreticulin (Gardai et al. 2005). Here, we will focus on the PS recognition system by phagocytes as a model for dead cell recognition and clearance.

Despite a common goal, PS receptors differ in their expression patterns, mode of PS recognition, and downstream signaling. In addition to professional phagocytes, these receptors are expressed in a variety of tissues, including bone marrow (BAI-1), spleen (BAI-1), brain (BAI-1), lungs (RAGE), kidney cells (TIM-1), and sinusoidal endothelial cells (stabilin-2) (Hochreiter-Hufford and Ravichandran 2013). The tissue specificity of these receptors may help to explain why multiple PS receptors are required for efficient efferocytosis, as different tissues may require specialized PS receptor mechanisms (Camins et al. 2008; Nagata et al. 2010; Poon et al. 2014). For example, defects in BAI-1, highly expressed in glial and neuronal cells, are associated with neurodegenerative disorders (Sokolowski and Mandell 2011), while stabilin-2 expression is highly expressed in endothelial cells within atherosclerotic plaques (Lee et al. 2011).

Likewise, recognition of PS by these molecules occurs via different domains. The TIM family of receptors utilizes an IgV domain (Santiago et al. 2007). BAI-1 binds PS via thrombospondin type 1 repeats (Park et al. 2007), while stabilin-2 contains EGF-like domains that mediate PS recognition (Park et al. 2008a). MFG-E8 binds PS via C1 and C2 domains (Hanayama et al. 2002). Why different receptors or bridging molecules have evolved different PS-binding motifs is unknown and adds an additional layer of complexity to the study of efferocytosis.

Engagement of these PS receptors (or surface receptors engaged by bridging molecules) results in cytoskeletal reorganization to facilitate phagocytosis. Studies to delineate the molecules involved in the engulfment of dead cells in *C. elegans* and subsequent identification of mammalian homologues have begun to clarify the intracellular signaling

events that occur (Reddien and Horvitz 2004). The uptake of dead cells is mediated by the Rho family of small GTPases, including members RhoA, Rac, Rab5, and Cdc42 (Nakaya et al. 2006), which cycle between the resting, inactive GDP-bound state and the active GTP-bound state, mediated by specific guanine nucleotide exchange factors (GEFs).

The physical engulfment of apoptotic cells is morphologically distinct from other types of phagocytosis. Whereas particles engulfed via complement-receptor-mediated phagocytosis seem to be absorbed into the phagocyte, dead cell engulfment involves active membrane ruffling by a process similar to macropinocytosis (Olazabal et al. 2002; Riento and Ridley 2003). Specific Rho family GTPases are conversely activated or inactivated during phagocytosis. Unlike its role in complement-receptor-mediated phagocytosis, it has been demonstrated that RhoA negatively regulates engulfment, as inhibition of this GTPase enhances engulfment. Conversely, overexpression of RhoA inhibits engulfment, and this is dependent on signaling by the RhoA-binding protein, Rho-associated coiled-coil-containing protein kinase (ROCK) (Erwig et al. 2006). An increase in the kinase activity of ROCK affects the status of myosin light chain (MLC) phosphorylation, to promote actomyosin assembly and cell contractility (Riento and Ridley 2003). This decrease in RhoA activation, and in turn the decreased signaling via ROCK, decreases stress fiber formation and probably facilitates cell shape changes during engulfment (Erwig et al. 2006).

Signaling during apoptotic cell engulfment converges on evolutionarily conserved pathway that leads to Rac1 activation, though the molecules used by specific receptors to activate the Rac1 pathway can differ. Unlike RhoA, Rac1 activation translates into membrane ruffles that are necessary to promote phagocytosis (Nakaya et al. 2006). Similarly, CDC42 has been linked to the engulfment of apoptotic cells, although its precise role is unclear (Leverrier et al. 2001)

Engagement of integrins, such as $\alpha_v\beta_3$ or $\alpha_v\beta_5$, or Mer by bridging molecules recruits the cytoplasmic protein, CrkII, which then associates with the adaptor proteins ELMO1 and DOCK180 to the phagocytic cup (Albert et al. 2000; Wu et al. 2005). Together, DOCK180 and ELMO1 form a bipartite GEF, which activates Rac1 (Brugnera et al. 2002). BAI1 also requires the activity of the DOCK180/ELMO1 complex for Rac1 activation, but BAI1 is able to recruit and bind ELMO1 independently (Park et al. 2007). The importance of DOCK180 and ELMO1 is highlighted by experiments in *C. elegans* wherein loss of the ELMO1 homologue (CED-12) results in failure to clear apoptotic cells during development (Wu et al. 2001). Moreover, overexpression of DOCK180 and ELMO1 results in greatly increased Rac1 activity and phagocytic capacity (Brugnera et al. 2002).

Not all “eat-me” signal receptors require the DOCK180/ELMO1 complex for Rac1 activation, though. Stabilin-2 requires the activity of the adaptor protein, GULP, to activate the Rac1 pathway (Park et al. 2008b, 2010b). Likewise, CD91/LRP, which binds calreticulin, interacts with GULP (Su et al. 2002). TIM4, however, contains a very short cytoplasmic region, which is not necessary for Rac1 activation, and currently, the signaling components downstream of TIM4 are unknown (Park et al. 2009).

Regardless of the path, Rac1 activation is a critical point in the engulfment process. GTP-bound Rac acts at sites of apoptotic cell recognition to promote Arp2/3 activation/actin polymerization/cytoskeletal rearrangement via the Scar/WAVE complex (Miki et al. 1998; Castellano et al. 2000). Once engulfment is complete, however, the job of phagocyte is not over. It has merely just begun.

3.4 Aperitifs and Digestifs: Digestion and Immune Response

Once encased inside of the phagocyte, the dead cell is now cargo destined for digestion and degradation. Late-stage RhoA activation is thought to promote apoptotic cell digestion by regulating the acidification of phagosomes (Erwig and Henson 2008). Similarly, GDP-bound small GTPase, Rab5, is recruited to the phagosome and activated by yet-unidentified GEFs. This GTP-bound, active Rab5 promotes activity of the Class III PI3 K, VPS34, which generates PI(3)P on the phagosomal membrane (Kinchen 2010). Recent studies connect Rab5 activation to Rab7 recruitment to the phagosome (Nordmann et al. 2010). Subsequently, the HOPS complex is recruited to the phagosome and activates Rab7, resulting in fusion of the phagosome with the lysosomal network (Kinchen et al. 2008).

Once the phagosome becomes mature via lysosomal fusion, acidic proteases and nucleases get activated and the apoptotic cell targets are degraded into their basic cellular components including fats, sterols, peptides, and nucleotides. DNAse II is a lysosomal enzyme required for the degradation of DNA, and DNAse II deficiency results in an accumulation of undigested DNA fragments within phagocytic cells, as well as polyarthritis and inflammation in joint tissues (Kawane et al. 2001, 2006).

But like any meal, it can have a profound effect on the eater. An area of growing interest is how a phagocyte handles the metabolic stress of ingesting a cellular corpse and essentially doubling its content of cellular components. One such component, cholesterol, can have a profound effect on the phagocyte's response to engulfed dead cells. In order to maintain their homeostasis in the face of increased cholesterol, phagocytes increase their basal cholesterol efflux activity from the cell (Noelia et al. 2009). Engagement of PS receptors leads to the activation of peroxisome proliferator-activated receptor γ/δ (PPARγ/δ) and liver x receptor (LXR) families, both important regulators of cellular lipid homeostasis and the clearance of apoptotic cells (Mukundan et al. 2009; Roszer et al. 2011). This induction results in the upregulation of phagocytic receptors, such as members of the TAM family, and basal cholesterol efflux machinery, such as 12-transmembrane protein ABCA1 (ATP-binding cassette subfamily A, member 1), to accommodate the increase in cholesterol associated with engulfment (Han and Ravichandran 2011). Moreover, $PPAR\gamma^{-/-}$ and $PPAR\delta^{-/-}$ macrophages show a defect in apoptotic cell uptake. The dual functions of PPARs and LXRs in both lipid apoptotic cell clearance and lipid homeostasis suggest the interconnectedness between efferocytosis and metabolism.

Apoptotic cell death occurs in healthy organisms as part of normal tissue turnover and thus needs to be immunologically silent with regard to its resolution (Henson and Hume 2006). One of the hallmarks of apoptotic cell clearance is its non-inflammatory and non-immunogenic nature, and cholesterol homeostasis plays a critical role in this tolerant pathway (Hochreiter-Hufford and Ravichandran 2013; Poon et al. 2014). Phagocytes that

have engulfed apoptotic cells have been shown to secrete anti-inflammatory cytokines, such as TGFβ and interleukin-10 (IL-10) (Fadok et al. 1998a, 2001). Moreover, the uptake of apoptotic cells actively suppressed pro-inflammatory cytokines, such as tumor necrosis factor (TNF), IL-1 and IL-12 (Kim et al. 2004). Intriguingly, PPARγ and PPARδ are central players in the polarization of M2 macrophages, the phenotype of which is anti-inflammatory. Agonists for both PPARγ and LXR have been shown to inhibit inflammatory responses (Mukundan et al. 2009; Noelia et al. 2009; Poon et al. 2014).

How then do apoptotic cells program their engulfers to tolerate their presence? Engulfment of necrotic cells or opsonization of corpse debris via FcR-mediated phagocytosis does not induce enhanced cholesterol efflux in the phagocytes, despite providing excess cholesterol for the engulfing cells (Kiss et al. 2006). These data suggest it is not the burden of extra cholesterol, but the engagement of ligands on apoptotic cells that induce a “prophylactic” cholesterol efflux from phagocytes. One key ligand seems to be the exposed PS on apoptotic cells. Coculture with mere apoptotic membranes or PS liposomes can induce the cholesterol efflux, anti-inflammatory cytokine production, and suppression of pro-inflammatory genes (Huynh et al. 2002; Kim et al. 2004). Collectively, these data suggest that recognition of apoptotic cells via engagement of “eat-me” signals, specifically PS, contributes to the silent clearance of these cells. Although PS recognition seems to be relevant for such immune tolerance, the roles of other “eat-me” signals and cognate receptor(s), as well as other signaling pathways involved, are unknown.

Despite engaging PS receptors, necrotic, necroptotic, or pyroptotic cells do not elicit an anti-inflammatory response (Martinez et al. 2011, 2013). Moreover, lytic cell death, such as necrosis, necroptosis, and pyroptosis, often results in an increased release of “find-me” signals (Ravichandran 2010). In the absence of efficient efferocytosis, apoptotic cells will undergo secondary necrosis and lyse, causing an inflammatory response (Juncadella et al. 2013). Of note, it is hypothesized that merely caspase activation results in dampening of the immunogenicity of DAMPS, and therefore, apoptotic cells that have transitioned through secondary necrosis are not inflammatory (Kazama et al. 2008; Luthi et al. 2009). However, recent data have shown that apoptotic cells can indeed be inflammatory if they are not phagocytosed in a timely manner (Obeid et al. 2007; Michaud et al. 2011).

How the phagocyte handles the ingested corpse in terms of its processing, degradation, and subsequent influence on the pursuant immune response is an area of growing interest. From the perspective of a single-celled organism, the two ancient systems of phagocytosis and autophagy represent two modes of nutrient acquisition—phagocytosis when extracellular fuel is abundant and autophagy when nutrients are scarce. However, these scenarios become decidedly more complex when one considers the engulfment of pathogens or dead cells (Martinez et al. 2013). The discovery of LC3-associated phagocytosis (LAP) has shed some light on this issue. LAP is a process that marries the evolutionarily conserved pathways of phagocytosis and autophagy into a fundamentally new concept, allowing us to reimagine the impact of the autophagy machinery on innate host defense mechanisms. LAP is triggered wherein an extracellular particle, such as a pathogen, immune complex, or dead cell, is sensed and phagocytosed, and this engulfment recruits some, but not all, members of the autophagy machinery to the cargo-containing, single-membraned vesicle (Sanjuan et al.

2007; Martinez et al. 2011). Engagement of multiple types of receptors, including TLR1/2, TLR2/6, TLR4, FcR, and TIM4 has been shown to induce translocation of autophagy machinery and ultimately LC3 to the cargo-containing phagosome, termed the LAPosome (Florey et al. 2011; Martinez et al. 2011; Henault et al. 2012; Martinez 2015). It is the activity of these autophagic players that facilitates the rapid destruction of the cargo via fusion with the lysosomal pathway. This is not macroautophagy, per se, but a distinct process, and it is triggered upon phagocytosis of particles containing ligands that engage a receptor-mediated signaling pathway.

Molecularly, LAP differs from canonical autophagy in multiple ways. LAP proceeds independently of the preinitiation complex, composed of ULK1/2, FIP200, and ATG13, whereas autophagy requires its activity (Florey et al. 2011; Martinez et al. 2011). Both LAP and autophagy utilize the Class III PI3 K complex and its core components Beclin-1 and VPS34, but LAP exclusively utilizes the UVRAG-containing Class III PI3 K complex (Martinez et al. 2015). In addition to the Class III PI3 K complex, LAP also requires Rubicon (RUN domain protein as Beclin-1 interacting and cysteine-rich containing), which negatively regulates autophagy, via its inhibition of VPS34 (Matsunaga et al. 2009; Zhong et al. 2009) or by blocking GTPase Rab7 activation (Sun et al. 2010). Rubicon associates constitutively with the UVRAG-containing Class III PI3 K complex (Matsunaga et al. 2009), as well as the NOX2 complex, also required for LAP (Martinez et al. 2015). Rubicon is crucial for both the interaction of the Class III PI3 K complex with the LAPosome and subsequent PI(3)P production as well as promoting the production of ROS via the NOX2 complex (Yang et al. 2012; Martinez et al. 2015).

Importantly, LAP can have a profound effect on the immune response to the engulfed material. LAP deficiency results in a failure to efficiently degrade intraphagosomal yeast in vitro (Sanjuan et al. 2007) or clear *Aspergillus fumigatus* in vitro or in vivo (Martinez et al. 2015). Furthermore, lungs and serum from animals with LAP deficiency display increased levels of pro-inflammatory cytokines when challenged intranasally with *Aspergillus fumigatus* (Martinez et al. 2015). Additionally, LAP is a critical regulator of the type I interferon response to immune complexes (IC) by plasmacytoid dendritic cells (pDC). Engagement of the FcγR by the IC induces LAP, resulting in LC3 translocation to the IC-containing phagosome in an ATG5- and ATG7-dependent, but ULK1-, FIP200-, and ATG13-independent manner. This failure to translocate LC3 results in a failure to acquire a late-endolysosomal phenotype, and subsequently a failure to form the specialized IRF7-signaling compartment required for TLR9-mediated activation of interferon regulatory factor 7 (IRF7). IFN-α production was completely ablated in ATG7$^{-/-}$, but not ULK1$^{-/-}$, pDC in response to DNA-IC, suggesting that LAP could affect the functional immune response elicited by this autoantigen (Henault et al. 2012).

LAP can act as a critical defense mechanism against autoimmune responses. Whereas much has been explored in terms the link between the uptake of dead cells with autoimmunity, how the phagocyte degrades the engulfed corpse is also a critical component to preventing unwanted immune responses. Billions of cells die daily as a result of stress, infection, or normal homeostasis, and it is the responsibility of the phagocytes of the immune system, such as macrophages, to rid the body of these cellular corpses, thus preventing inflammation

and autoimmunity (Han and Ravichandran 2011; Martinez et al. 2011). Importantly, LAP has been demonstrated to play a critical role in the efficient clearance of dying cells, as well as promoting the anti-inflammatory response to apoptotic cells. Engagement of the PS receptor, TIM4, results in recruitment of the autophagic machinery to the dead-cell-containing, single-membrane phagosome. Macrophages deficient for ATG7, but not ULK1, fail to recruit LC3 to the phagosome, which results in failures in phagosomal acidification and subsequent corpse degradation. Whereas the phagocytosis of apoptotic cells is generally considered an "immunologically silent" event, ATG7-deficient macrophages produce dramatically increased levels of IL-1β and IL-6 when fed apoptotic cells. Moreover, these ATG7-deficient macrophages produce significantly less anti-inflammatory cytokines, such as IL-10, upon such engulfment (Martinez et al. 2011). How the LAP pathway modulates the immune response to apoptotic cells remains to be elucidated. The process of phagosome maturation and its effect on the immune response are actively being studied, in order to further understand the role of dead cell clearance in the prevention of autoimmunity and other pathologies (discussed below).

4 Food Poisoning: Pathologies Associated with Aberrant Efferocytosis

The lack of detectable apoptotic cells under physiological conditions speaks to the sheer efficiency of efferocytosis. Conversely, but not surprisingly, many different diseases are characterized by the presence of uncleared dead cells or a defect in properly handling engulfed cellular corpses. Furthermore, the unwanted inflammatory response to uncleared dead cells can exacerbate some conditions (Hochreiter-Hufford and Ravichandran 2013; Poon et al. 2014). Non-resolving inflammation contributes to tissue damage and organ dysfunction in a wide array of pathologies. Accumulating evidence indicates that efferocytosis is impaired in many autoimmune and inflammatory disorders.

4.1 Systemic Lupus Erythematosus (SLE)

Perhaps the most common pathological disorder associated with aberrant dead cell clearance is systemic lupus erythematosus (SLE). SLE is a chronic systemic autoimmune disorder affecting the skin, lungs, kidneys, and central nervous system. Patients with SLE display persistence of apoptotic cells within lymph nodes, blood, and skin (Baumann et al. 2002). As discussed earlier, apoptotic cells, while regarded immunologically silent initially, can undergo secondary necrosis if cleared in efficiently. This results in rupture of the protective plasma membrane and the release of intracellular autoantigens, such as DNA, ATP, and HMGB1, normally compartmentalized within an apoptotic cell (Raffray and Cohen 1997; Peter et al. 2010; Nikoletopoulou et al. 2013). SLE patients show a strong correlation between disease progression and the failed clearance of apoptotic cells, as well as increased inflammation (Liu and Davidson 2012). SLE patients also contain elevated levels of DNA or nucleosomes in the circulation, as well as autoantibodies that are specific for nuclear or other "self" components (Rumore and Steinman 1990). These autoantibodies can bind to circulating autoantigens, forming immune complexes that accumulate or deposit in the glomerular and vessel walls of the kidney, causing lupus nephritis (Berden 1997; van Bruggen et al. 1997).

It has been demonstrated that mice with deficiencies for engulfment (and hence clearance) of apoptotic cells, such as MFGE8-, BAI1-, TIM4-, or MerTK-deficient animals, accumulate apoptotic corpses within lymph nodes and develop an SLE-like disease that involves autoantibody formation, splenomegaly, and glomerulonephritis (Cohen et al. 2002; Hanayama et al. 2004; Park et al. 2007; Rodriguez-Manzanet et al. 2010). Furthermore, genetic polymorphisms and aberrant splicing of MFGE8 have been reported in a small subset of patients with SLE, indicating that this pathway of apoptotic cell recognition and clearance could be deregulated in some patients (Hu et al. 2009). Similarly, deficiencies in components of the complement pathway, particularly C1q which plays a key role in apoptotic cell clearance, have been also associated with SLE (Botto et al. 1998).

Not only is phagocytic capacity critical for the prevention of SLE, but the efficient degradation of engulfed dead cells is also an important factor. Mice deficient for DNAse I, critical for the degradation of chromatin, are prone to SLE-like glomerulonephritis (Napirei et al. 2000). Intriguingly, genome-wide association studies have identified polymorphisms in *atg5* (Zhou et al. 2011) and possibly *atg7* (Clarke et al. 2014), genes involved in both canonical autophagy and LAP (Mizushima 2007; Florey et al. 2011; Martinez et al. 2011, 2015; Henault et al. 2012; Kim et al. 2013), as predisposition markers for SLE.

4.2 Rheumatoid Arthritis

Rheumatoid arthritis is a chronic autoimmune disease associated with progressive joint destruction. It is a systemic inflammatory disorder characterized by increased circulating autoantibodies against citrullinated peptides or the complement protein, C3 (Luban and Li 2010; Kenyon et al. 2011). While there exists little direct evidence that rheumatoid arthritis is caused by defects in efferocytosis, site of inflammation often contain DAMPS, such as HMGB1 or histones H3 and H4, characteristic of uncleared apoptotic cells (Friggeri et al. 2012). These components can bind to phagocytes and inhibit efferocytosis, therefore perpetuating progression to secondary necrosis and the release of immunostimulatory materials (Hurst et al. 1983; Friggeri et al. 2010). Mice deficient for DNAse II, and thus defective for the degradation of engulfed cellular cargo, develop polyarthritis and anemia associated with significant increased inflammatory markers, reminiscent of human rheumatoid arthritis (Kawane et al. 2006) While direct genetic links to efferocytosis have not been described for human rheumatoid arthritis, studies have demonstrated that increasing the levels of bridging molecules for TAM receptor or activating the LXR/PPARγ can have therapeutic benefits in mouse models of inflammatory arthritis (Park et al. 2010a).

4.3 Type 1 Diabetes

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease that results from destruction of the insulin-producing β-cells in the islets of Langerhans in the pancreas. The clearance of apoptotic cells (a source of self-antigens) by phagocytes, predominantly dendritic cells, induces a tolerogenic response. However, defective clearance of cellular corpses, however, can result in increased rate of β-cells apoptosis, inflammation, and loss of tolerance, as inefficiently cleared apoptotic cells undergo necrosis, releasing danger signals and autoantigens into the extracellular milieu (Vives-Pi et al. 2015).

One of the key factors in T1D is the loss of T cell tolerance to self-antigens. While the mechanisms by which efferocytosis induces selective immunosuppression are not fully understood, recent evidence indicates that impaired clearance of dying cells contributes to immunogenic, not tolerogenic, DC maturation and chronic inflammation. Previous work has demonstrated that macrophages from non-obese diabetic (NOD) mice, which spontaneously develop type 1 diabetes mellitus, have a profound defect in the phagocytosis of apoptotic cells in vitro. NOD mice also display impaired efferocytosis in vivo when challenged with apoptotic stimuli. This defect in apoptotic cell clearance by NOD mice also translated into increased production of antinuclear autoantibodies (ANA) (O'Brien et al. 2006). Another study demonstrated that the tolerogenic behavior of dendritic cells after islet cells efferocytosis is central to silencing autoreactive T cells in type I diabetes (Pujol-Autonell et al. 2013).

Impaired wound healing is a common complication encountered by patients with both type 1 and type 2 diabetes mellitus. A large number of neutrophils are recruited to the wound site but must be cleared adequately by macrophages to initiate the next stage of wound healing. Macrophages isolated from diabetic wounds display dysfunctional efferocytosis, resulting in increased dead cell burden at the wound site, increased inflammation, and delayed wound healing (Maruyama et al. 2007; Khanna et al. 2010). While it has been demonstrated that diabetes is associated with compromised efferocytosis and high levels of pro-inflammatory cytokines, the mechanisms underlying these defects have not been characterized.

4.4 Atherosclerosis

Atherosclerosis is associated with chronic inflammation of the vascular wall, predominantly caused by the recruitment and accumulation of monocytes, macrophages, and dendritic cells (DCs). These phagocytic cells engulf oxidized lipids, causing the lipid-laden cells to undergo apoptosis, wherein they themselves become engulfed by neighboring phagocytes (Tabas 2005; Ley et al. 2011). While efficient efferocytosis can compensate during the initial onset of atherosclerosis, mature atherosclerotic lesions (known as plaques) are characterized by the presence of foam cells, macrophages that have taken up necrotic cells at the core of plaques and failed to stimulate cholesterol efflux (Vucic et al. 2012; Poon et al. 2014). Pathologically, this results in further reduced clearance of apoptotic cells, secondary necrosis, expansion of plaque lesions, and eventual plaque rupture, leading directly to acute coronary syndromes and stroke in humans (Schrijvers et al. 2005; Tabas 2005).

Mouse models with defects in the efferocytosis machinery, such as MerTK-, MFG-E8-, or C1q-deficient models, display an accumulation of apoptotic cellular debris within plaques and exacerbated atherosclerosis (Tabas 2005; Bhatia et al. 2007; Thorp et al. 2008). Apoptotic cell uptake via interaction with ICAM3 can be bound and inhibited by oxidized lipids present in plaques (Miller et al. 2003).

While the correlation between decreased efferocytosis and atherosclerotic lesions is not incompletely understood, several clinical observations linked to possible mechanisms have been described. HMG-CoA reductase inhibitors (also known as statins) are commonly used pharmacological agents for the treatment of atherosclerosis and vascular disease. In addition to lowering cholesterol and inflammation, an additional mechanism of action of statins is the

inhibition of RhoA, a negative regulator of engulfment that is highly expressed in atherosclerotic lesions (Loirand et al. 2006).

Downstream of engulfment, activation of the nuclear receptors liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs) are critical to promoting tolerance, as well as by upregulating MerTK expression. Moreover, synthetic agonists to LXR or PPARs have been demonstrated to be beneficial to the treatment of atherosclerosis (Noelia et al. 2009). Intricately linked to the pathological progression of atherosclerosis is cholesterol efflux. Uptake of apoptotic cells by phagocytes stimulates cholesterol efflux, primarily though upregulation of ABCA1, a critical molecule that transports free cholesterol from within the cells to lipid-poor apoA1 that is then modified in the plasma for transport to the liver and excretion (Oram and Heinecke 2005; Cuchel and Rader 2006). Indeed, decreased ABCA1 activity in mouse models promotes inflammation and atherosclerotic lesions. Conversely, overexpression of ABCA1 can dampen the inflammatory response and reverses the disease (Tang et al. 2009; Zhu et al. 2010). Finally, mice with deficiencies in the autophagic machinery, shared by the LAP pathway, such as LysM-Cre Atg5flox/flox mice, display more atherosclerotic lesions, suggesting the possibility that LAP-mediated processing is required for the prevention of inflammation (Liao et al. 2012; Razani et al. 2012).

4.5 Lung Inflammation

Upon the induction of lung inflammation, neutrophils, the most abundant cell type involved in the innate immune response, are quickly recruited airway to airway. Neutrophils, however, as a short-lived population, undergo apoptosis in order to prevent the release of histotoxic contents and subsequent damage to surrounding tissues. Clearance of these apoptotic neutrophils by phagocytes is central to the successful resolution of the inflammatory response (Fox et al. 2010). Both mouse and human models of chronic obstructive pulmonary disease and cystic fibrosis have demonstrated that impaired efferocytosis of dying neutrophils during inflammation can lead to a prolonged inflammatory response. Chronic obstructive pulmonary disease (COPD) is a common, yet complex disease, highly associated with cigarette smoke. Chronic inflammation, extracellular matrix destruction, and increased airway epithelial cell and neutrophil apoptosis are reported in COPD models, and alveolar macrophages from COPD patients demonstrate a decreased phagocytic capacity for apoptotic cells (Hodge et al. 2003). Cystic fibrosis (CF) lung disease is characterized by early, protracted inflammation associated with a massive influx of neutrophils and other inflammatory cells, and the efficient clearance of these inflammatory cells (now apoptotic) by phagocytes is critical to the resolution of inflammation. Similar to COPD patients, alveolar macrophages from CF patients display defective efferocytosis, possibly attributable to necrotic neutrophil-derived proteases capable of cleaving PS receptors on the surface of phagocytes (Vandivier et al. 2002a, b).

Human allergic asthma is a chronic inflammatory disorder of the airways and is characterized by airway inflammation, persistent airway hyperresponsiveness (AHR), and intermittent, reversible airway obstruction. “Airway remodeling,” including airway fibrosis, goblet cell hyperplasia, and other structural changes, is thought to be the result of chronic

inflammation and serves to exacerbate the asthma symptoms (Nials and Uddin 2008). Alveolar macrophages from patients with sever asthma or poorly controlled asthma are defective in clearing apoptotic cells (Huynh et al. 2005; Fitzpatrick et al. 2008). Similarly, patients with non-eosinophilic asthma have increased numbers of neutrophils in the airways, and alveolar macrophages from these patients show an impaired ability to phagocytose apoptotic cells (Simpson et al. 2013).

While the exact mechanisms underlying defective phagocytosis in patients with severe asthma are not yet understood, the use of corticosteroids, the most common treatment in asthma, induces eosinophil apoptosis as well as eosinophil engulfment by macrophages in vitro, via the binding of protein S to apoptotic eosinophils and the upregulation of MerTK on the surface of macrophages (Liu et al. 1999; McColl et al. 2009). Unsurprisingly, this treatment is significantly less effective in non-eosinophilic asthma and other neutrophil-dominated lung inflammatory disorders (Vago et al. 2012; Poon et al. 2014).

4.6 Neurodegenerative Disorders

In the peripheral immune system, phagocytes tasked with efferocytosis are mainly comprised of macrophages, monocytes, and dendritic cells. In the brain, however, microglia act as resident macrophages to accomplish this function. Microglia have phenotypical similarities to peripheral macrophages, in that they express and utilize PS receptors for the recognition and uptake of dead cells (Witting et al. 2000). Multiple neurodegenerative disorders have been associated with defective efferocytosis, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Mattson 2000). The most direct link of efferocytosis to neurodegenerative diseases is MFG-E8. Microglia express MFG-E8, and treatment of microglia cultures with fractalkine (CX3CL1) increases MFG-E8 mRNA levels (Leonardi-Essmann et al. 2005; Fuller and Van Eldik 2008). Inhibition of MFG-E8 in microglia cells resulted in decreased engulfment of apoptotic neurons (Fuller and Van Eldik 2008). Of note, it has also been reported that MFG-E8 also mediates the phagocytosis of viable, LPS-activated neurons, resulting in death of the engulfed neuron (Fuller and Van Eldik 2008; Fricker et al. 2012). Finally, MFG-E8 levels in the brains of the Tg2576 mouse model of Alzheimer's disease were severely decreased with age compared to wild-type controls (Fuller and Van Eldik 2008). Taken together, MFG-E8 seems to play a central role in the clearance of dead cells in the brain, and deficiencies in MFG-E8 can contribute to the onset and severity of Alzheimer's disease.

Other proteins that have tangential roles in efferocytosis have been implicated in neurodegenerative disorders. Apolipoprotein E (apoE) is a cholesterol transport protein expressed in liver, central nervous system, vascular smooth muscle cells, adrenals, macrophages, and adipocytes. The E4 isoform of apoE (apoE4) is a major genetic risk factor for multiple inflammatory metabolic diseases, including atherosclerosis and Alzheimer's disease. Peritoneal macrophages isolated from *APOE4* mice demonstrate defects in efferocytosis and increased inflammatory response, presumably through ER stress (Cash et al. 2012). How the cells of the brain mediate the clearance of potentially damaging dead cells, as well as other debris, is currently an area of great interest.

5 Conclusions

The controlled cell death program of apoptosis is an integral part of maintaining development and cell turnover, yet like a large meal, too much of a good thing can be detrimental. The sheer magnitude of the task undertaken by professional phagocytes to keep an organism free of potentially dangerous dead cells, and thus unwanted autoimmunity and inflammation, is a daunting one, but one performed with exquisite accuracy under normal conditions. Indeed, many autoimmune conditions have been clearly linked to defects in efferocytosis, in terms of recognition, engulfment, and digestion of its cellular corpse cargo. While the field of efferocytosis is relatively young, these new insights into the underlying mechanisms of dead cell clearance will provide invaluable opportunities to attack autoimmune and autoinflammatory diseases at its core. Bon appetit!

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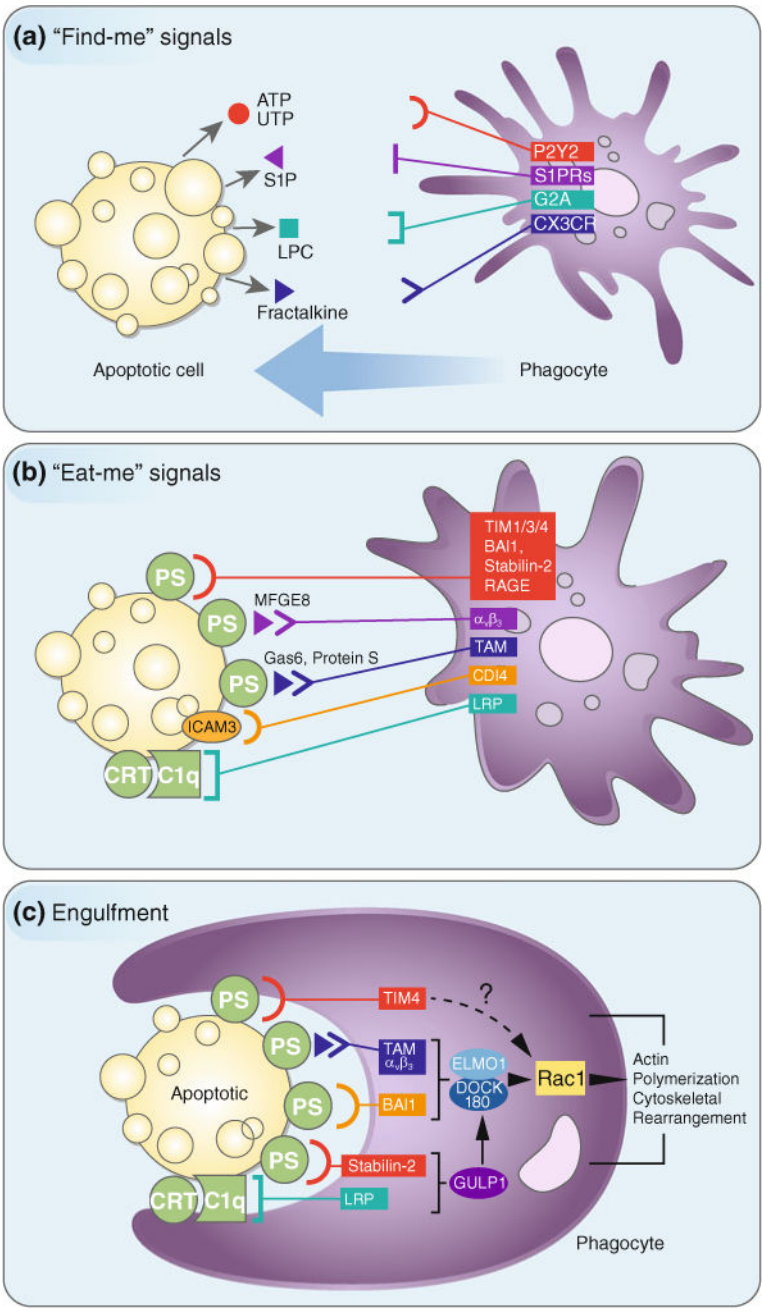

Fig. 1.

The recruitment of phagocytes to sites of cell death and recognition and engulfment of apoptotic cells by phagocytes. **a** Apoptotic cells (or other dying cells) release "find-me" signals, such as ATP, UTP, S1P, LPC, or fractalkine, that act to recruit phagocytes to sites of cell death. Phagocytes sense these "find-me" signals via cognate receptors (P2Y2, S1PRs, G2A, and CXCR3, respectively). **b** Phagocytes employ a system of receptors and bridging molecules to recognize and engage apoptotic cells (or other dying cells) via "eat-me" signals exposed on apoptotic cell surfaces. The most common "eat-me" signal is phosphatidylserine (or PS), which engages the PS-specific receptors, TIM1, TIM3, TIM4, BAI1, stabilin-2, and

RAGE, as well as the PS-specific bridging molecules MFG-E8, Gas6, and protein S. These bridging molecules engage other surface engulfment receptors ($\alpha_v\beta_3$ or TAM) to facilitate uptake. Other “eat-me” signals, such as calreticulin (CRT) and ICAM3, exist and mediate recognition and engulfment via the receptors LRP (via C1q) and CD14, respectively. **c** Once the engulfment receptors are engaged, actin polymerization and cytoskeletal rearrangement are initiated via activation of the Rac1 pathway. While some engulfment receptors utilize the ELMO1/DOCK180 complex ($\alpha_v\beta_3$, TAM, stabilin-2, LRP), the mechanism by which TIM4 activates the Rac1 pathway is unknown. Perturbations at any step of this process can result in inflammation and autoimmunity

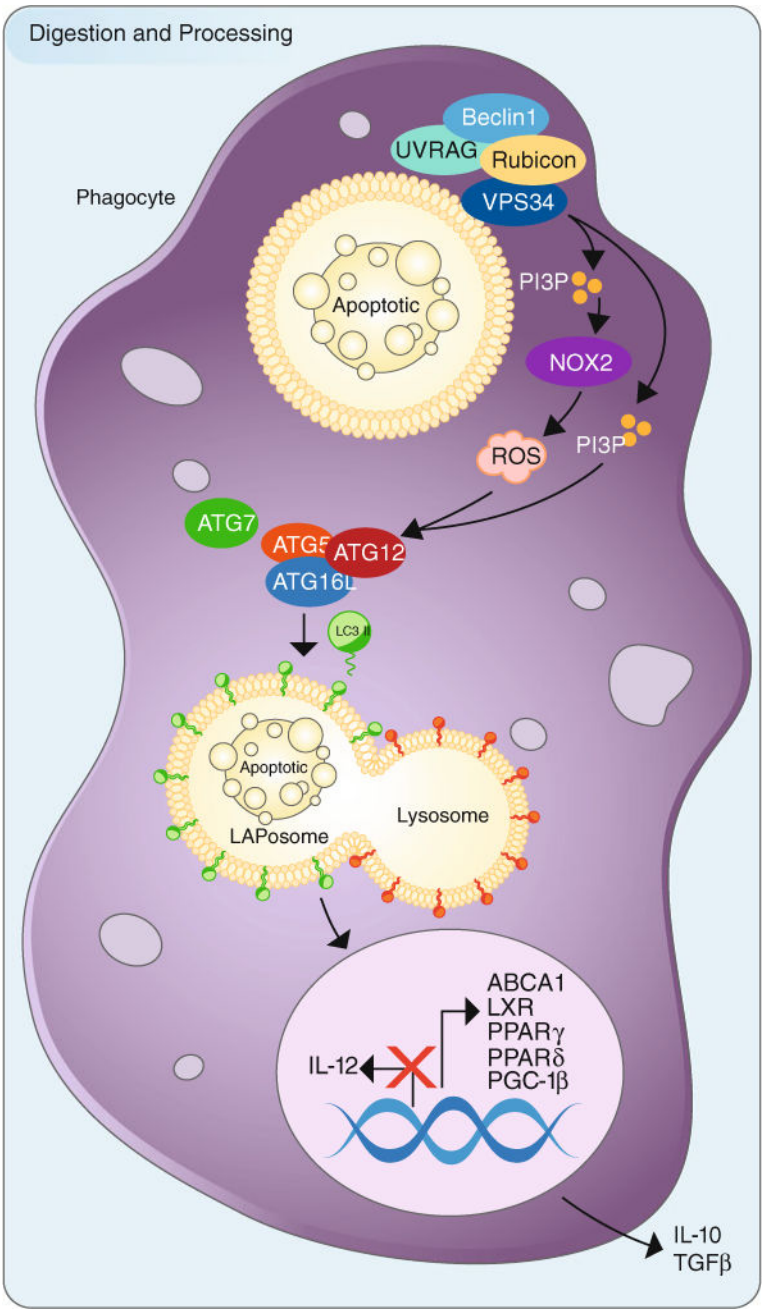

Fig. 2.

The processing and digestion of engulfed apoptotic cells utilizes LC3-associated phagocytosis and promotes an anti-inflammatory response. Upon engulfment of apoptotic cells (or other dying cells), components of the LC3-associated phagocytosis (LAP) pathway are recruitment to dead cell-containing phagosome (or LAPosome). The Class III PI3 K complex, composed of Beclin-1, VPS34, UVRAG, and Rubicon, is critical to the sustained and localized production of PI(3)P at the LAPosome. PI(3)P serves two roles—the recruitment of the downstream autophagic/LAP machinery (such as ATG5, ATG12, ATG16L, ATG7) and stabilization of the NOX2 complex for the production of ROS. Of

note, Rubicon is also required for the stabilization of the NOX2 complex. Both ROS and PI(3)P are required for lipidation and translocation of LC3-II to the single membrane of the LAPosome, and LC3-II is required for fusion to the lysosome and maturation of LAPosome. The anti-inflammatory effects of efferocytosis are mediated by the activity of lipid and cholesterol sensors, such as ABCA1, LXR, PPARγ, PPARδ, and PGC-1β, leading to the production of anti-inflammatory mediators, IL-10 and TGFβ. Pro-inflammatory mediators, such as IL-12, are actively repressed. Perturbations in this process can result in inflammation and autoimmunity