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Clearing the Dead: Apoptotic Cell Sensing, Recognition, Engulfment, and Digestion

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Clearance of apoptotic cells is the final stage of programmed cell death. Uncleared corpses can become secondarily necrotic, promoting inflammation and autoimmunity. Remarkably, even in tissues with high cellular turnover, apoptotic cells are rarely seen because of efficient clearance mechanisms in healthy individuals. Recently, significant progress has been made in understanding the steps involved in prompt cell clearance in vivo. These include the sensing of corpses via "find me" signals, the recognition of corpses via "eat me" signals and their cognate receptors, the signaling pathways that regulate cytoskeletal rearrangement necessary for engulfment, and the responses of the phagocyte that keep cell clearance events "immunologically silent." This study focuses on our understanding of these steps.

Multicellular organisms execute the majority of unwanted cell populations in a regulated fashion via the process of apoptosis (Henson and Hume 2006; Nagata et al. 2010). Examples of unwanted cells include excess cells generated during development, cells infected with intracellular bacteria or viruses, transformed or malignant cells capable of tumorigenesis, and cells irreparably damaged by cytotoxic agents. Swift removal of these cells is necessary for maintenance of overall health and homeostasis and prevention of autoimmunity, pathogen burden, or cancer. Quick removal of dying cells is a key final step, if not the ultimate goal of the apoptotic program.

The term "phagocytosis" refers to an internalization process by which larger particles, such as bacteria and dead/dying cells, are engulfed and processed within a membrane-bound vesicle called the phagosome (Ravichandran and Lorenz 2007). A phagocyte is any cell that is capable of engulfment, including "professional" phagocytes such as macrophages, immature dendritic cells, and neutrophils. Metazoa have multiple mechanisms for clearing apoptotic cells, often depending on the tissue and apoptotic cell type (Gregory 2009). Macrophages and immature dendritic cells readily engulf dead or dying cells in tissues such as bone marrow (where a large number of new hematopoietic cells are generated), spleen (during or after an immune response), and the thymus (in young animals during T-lymphocyte development). In other tissues, neighboring "nonprofessional" phagocytes can also mediate the clearance of apoptotic targets. For example, in the mammary

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epithelium, viable mammary epithelial cells engulf apoptotic mammary epithelial cells after cessation of lactation (Monks et al. 2005, 2008). What distinguishes the phagocytosis of apoptotic cells from the phagocytosis of most bacteria or necrotic cells is the lack of a pro-inflammatory immune response (Henson 2005). This article discusses apoptotic cell engulfment, specifically the recruitment of phagocytes, through "find me" signals, the recognition of apoptotic cells by phagocytes via "eat me" signals, the internalization process and signaling pathways used for cytoskeletal rearrangement, and finally the digestion of apoptotic cells and phagocytic response to this process (Fig. 1).

RECRUITMENT OF PHAGOCYTES TO THEIR APOPTOTIC MEAL

Remarkably, even in tissues with high cellular turnover, apoptotic cells are rarely seen in situ, which is thought to be due to efficient clearance mechanisms. Early studies in the nematode *Caenorhabditis elegans* suggested that apoptotic cells are recognized and cleared before they are "fully dead" (Hoeppner et al. 2001; Reddien et al. 2001). This work led to the idea that apoptotic cells advertise their status to local and distant phagocytes at their earliest stages of death, perhaps via the release of "find me" signals (Ravichandran 2003).

"Find Me" Signals: Establishing a Chemotactic Gradient to Direct Phagocyte Migration

The role of "find me" signals is to establish a chemotactic gradient stimulating the migration of phagocytes to the apoptotic cell. To date, several proposed "find me" signals released by dying cells have been reported (Fig. 2). These include fractalkine, lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), and

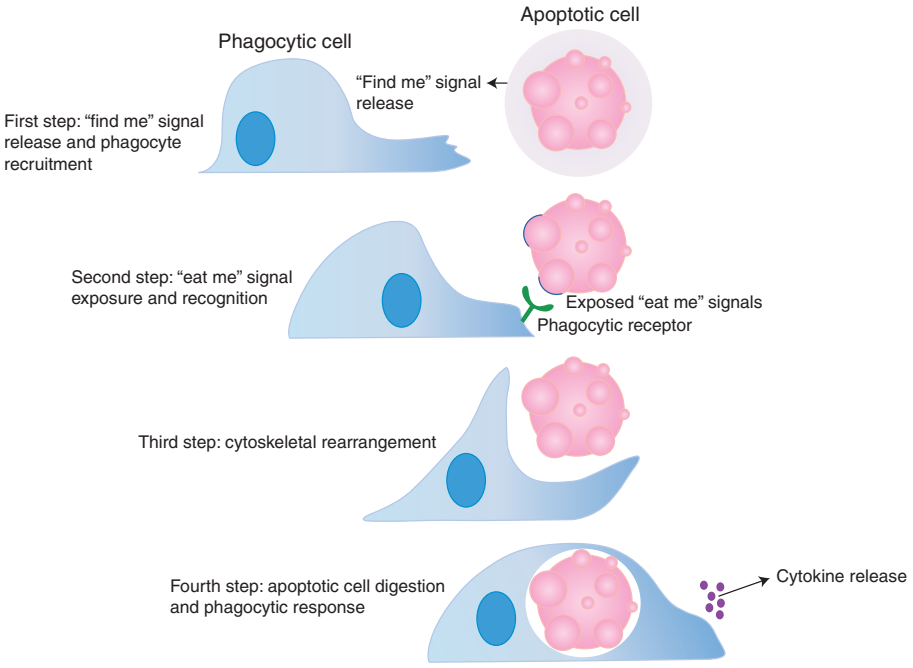

Figure 1. The steps of efficient apoptotic cell clearance. First, "find me" signals released by apoptotic cells are recognized via their cognate receptors on the surface of phagocytes. This is the sensing stage and stimulates phagocyte migration to the location of apoptotic cells. Second, phagocytes recognize exposed "eat me" signals on the surface of apoptotic cells via their phagocytic receptors, which leads to downstream signaling events culminating in Rac activation. Finally, further signaling events within the phagocyte regulate the digestion and processing of the apoptotic cell meal and the secretion of anti-inflammatory cytokines.

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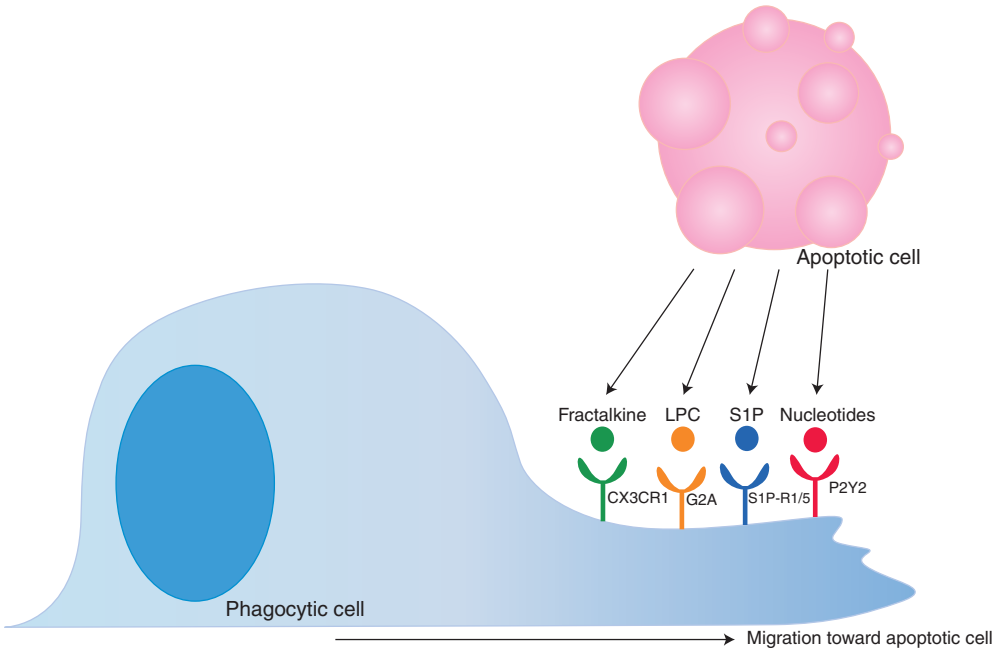

Figure 2. "Find me" signals and their receptors. Apoptotic cells release "find me" signals including fractalkine, LPC, S1P, and nucleotides. These molecules bind their cognate receptors (CX3CR1, G2A, S1P-R1/5, and P2Y2, respectively) present on the phagocyte surface. "Find me" signal recognition by the phagocyte stimulates migration toward the dying target.

the nucleotides ATP and UTP (Lauber et al. 2003; Gude et al. 2008; Truman et al. 2008; Elliott et al. 2009).

Fractalkine (i.e., CXC3CL1) is currently the only classical chemokine "find me" signal identified (Peter et al. 2010). It is a membrane-associated protein that is released from apoptotic B cells and neurons by a yet unknown protease; the released fractalkine is sensed via CX3CR1, which, in turn, directs macrophages to the dying targets (Truman et al. 2008). During affinity maturation of an antibody response, B cells in germinal centers undergo a high rate of apoptosis, and in an experiment measuring the clearance of these apoptotic B cells, fractalkine/CX3CR1 attraction was shown to influence macrophage recruitment to germinal centers in vivo (Truman et al. 2008). Interestingly, no increase in the presence of apoptotic or secondarily necrotic cells was observed in the germinal centers of CX3CR1 knockout mice, suggesting that fractalkine-mediated macrophage attraction is not required for clearance. Expression of fractalkine is limited to only a few cell types, so other mechanisms of phagocyte attraction must also exist.

LPC was the first discovered lipid "find me" signal, and it is released from apoptotic cells by the caspase-3-dependent activation of phospholipase A2, leading to the conversion of phosphatidylcholine to LPC (Lauber et al. 2003). The recognition of LPC is thought to occur via the G-protein-coupled receptor G2A and thereby stimulates macrophage chemotaxis toward apoptotic cells (Peter et al. 2008). However, the concentration of LPC reported to be required for macrophage chemotaxis appears to be quite high. In addition, the amount of LPC present in circulation is higher than LPC levels released by apoptotic cells, making LPC an unlikely candidate for a chemotactic mediator (Nagata et al. 2010). S1P, another lipid reported to function as a "find me" signal for apoptotic cells, is produced by sphingosine kinase 1 (SphK1) and secreted by apoptotic cells (Gude et al. 2008).

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S1P stimulates chemotaxis by binding to the G-protein-coupled receptors S1P-R1 through S1P-R5. At this time, no one specific S1P-R has been primarily associated with phagocyte chemotaxis, and macrophages are known to express all five (Rosen and Goetzl 2005).

Recently, nucleotides ATP and UTP have been implicated as a new class of "find me" signals. Small amounts of intracellular ATP and UTP are released in a regulated manner early during apoptosis to establish a gradient for monocyte attraction in vitro and in vivo (Elliott et al. 2009); this small amount of nucleotide release (<0.1% of the cellular content) should be distinguished from the release of significantly higher levels of nucleotides after other types of cell death such as necrosis. Subsequent studies showed that the release of ATP and UTP is mediated via the pannexin channels, which are opened during apoptosis by caspase-dependent cleavage of their carboxy-terminal tail (Chekeni et al. 2010). How are the nucleotides sensed? Current evidence suggests that the P2Y2 receptor on monocytes binds the released nucleotides and stimulates phagocyte chemotaxis; consistent with a role for P2Y2 in vitro, disruption of the nucleotide/P2Y2 "find me" network resulted in uncleared apoptotic thymocytes after glucocorticoid injection in vivo (Elliott et al. 2009). Because nucleotides are readily degraded by extracellular nucleotidases, they are unlikely to serve as long-range "find me" signals to phagocytes in circulation; rather, they attract tissue resident macrophages (Ravichandran 2010).

The nature of these identified "find me" signals is varied both in composition and recognition. It is currently unclear whether they function together in an additive or synergistic manner during the phagocyte attraction to apoptotic cells.

Additional Roles for "Find Me" Signals: Whetting the Appetite and Keeping the Meal Quiet

Nucleotides have obvious cellular functions independent of their role as "find me" signals (i.e., DNA building blocks and as energy currency). It is not surprising that the other "find me" signals also serve additional roles, even in the context of apoptotic cell clearance. For example, fractalkine was shown to induce milk fat globule EGF factor 8 (MFG-E8) expression in peritoneal macrophages (Miksa et al. 2007). MFG-E8 (described below) is a bridging molecule that facilitates recognition of apoptotic cells by phagocytes. This priming of fractalkine-dependent MFG-E8 expression enhanced the clearance of apoptotic thymocytes in vitro. LPC was shown to induce the expression of MCP-1 and RANTES in human vascular endothelial cells, thereby potentiating the recruitment of monocytes toward apoptotic cells (Murugesan et al. 2003; Peter et al. 2010). In addition, LPC has been described as a surface-bound target for IgM and may serve an additional role during the removal of apoptotic cells as an "eat me" signal (Kim et al. 2002). "Find me" signals may also influence a non-immunogenic response to apoptotic cells as seen with S1P-enhanced secretion of IL-10 and PGE_2 by tumor macrophages (Weigert et al. 2007; Johann et al. 2008; Ravichandran 2010). Why are there additional functions? Perhaps not every phagocyte requires "find me" signal-mediated chemotaxis to their target; there are many instances in which targets and phagocytes are in close proximity, and there is no need for the phagocyte to actually find the dying cell. In these cases, "find me" signals may have alternative functions such as enhancing the phagocytic machinery within the phagocyte or the ability to digest the ingested cargo. These possible functions remain to be addressed more carefully using different targets and different phagocytes.

"Stay Away" Signals

Phagocytosis of apoptotic cells is generally recognized as a non-immunogenic, non-inflammatory process with minimal, if any, neutrophil recruitment (Savill 1997; Savill et al. 2002). Of the known "find me" signals, however, LPC, S1P, and nucleotides are all known to stimulate neutrophil chemotaxis as well (Chen et al. 2006; Florey and Haskard 2009). Then how might the system avoid recruitment of neutrophils under

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these circumstances? One possibility is via the iron-binding glycoprotein lactoferrin (LTF), which is also released by apoptotic cells and appears to specifically inhibit neutrophil migration (Bournazou et al. 2009). This "stay away" signal is the first of its kind discovered, and currently mechanisms of LTF release and the identity of LTF receptor(s) on neutrophils remain unknown.

RECOGNITION OF CORPSES BY PHAGOCYTIC CELLS

Once in the proximity of their targets, phagocytes must be able to distinguish live from dead cells. Apoptotic cells display "eat me" signals on their surface, which phagocytes detect via receptors on their cell surface. Detection is, however, not always direct. Although certain phagocytic receptors can directly engage ligands on the dying cells, other receptors use soluble bridging molecules as intermediaries to recognize the "eat me" signals on apoptotic cells. Additionally, living cells have mechanisms in place to prevent their unwanted removal. The section below details the modes of specific recognition of apoptotic cells and the mechanisms used by live cells to avoid engulfment by phagocytes.

"Eat Me" Signals: Distinguishing the Live from the Dead

Numerous "eat me" signals have been identified to date including changes in glycosylation of surface proteins or changes in surface charge (Kinchen and Ravichandran 2007), binding of serum proteins such as thrombospondin and complement C1q to the apoptotic cell (Ravichandran and Lorenz 2007), expression of intercellular adhesion molecule 3 (ICAM3) and oxidized low-density lipoprotein (LDL)-like moiety (Devitt et al. 1998; Fadok et al. 1998b; Aderem and Underhill 1999; Schlegel et al. 1999), and the exposure of certain intracellular proteins such as calreticulin and annexin I (Arur et al. 2003; Gardai et al. 2005; Obeid et al. 2007a,b). However, the most widely studied and universally detected "eat me" signal is the exposure of the phospholipid phosphatidylserine (PtdSer) (Fig. 3) (Fadok et al. 1992, 1998a, 2001).

In viable cells, PtdSer is kept exclusively on the inner leaflet of the lipid bilayer via ATP-dependent translocases (Balasubramanian and Schroit 2003). In apoptotic cells, the concentration of PtdSer on the outer leaflet of the lipid bilayer is estimated to increase by more than 280-fold within only a few hours after induction of apoptosis (Borisenko et al. 2003; Ravichandran 2010). The exact mechanism by which PtdSer exposure occurs is just beginning to be better defined, but it appears to be caspase dependent (Nagata et al. 2010). Recently, a member of the TMEM16 family of Ca^{2+}-activated Cl^- channels, TMEM16F, was shown to mediate Ca^{2+}-dependent phospholipid scrambling (Suzuki et al. 2010). Overexpression and knockdown experiments determined that TMEM16F regulates the exposure of PtdSer and phosphatidylethanolamine (PtdEtn) to the outer leaflet of the plasma membrane in response to apoptotic stimuli, and a mutation of this channel was linked to a patient with Scott syndrome, a rare bleeding disorder, which results from defective phospholipid scrambling (Suzuki et al. 2010). Currently it is also unknown whether PtdSer recognition alone is sufficient to trigger phagocytosis; several studies have suggested that PtdSer recognition is both necessary and sufficient for clearance of apoptotic cells (Fadok et al. 1998a); however, some evidence suggests otherwise. For example, PtdSer exposure can occur on viable cells, yet these cells are not engulfed (van den Eijnde et al. 2001; Huppertz et al. 2006; Helming and Gordon 2009). Furthermore, viable cells forced to expose PtdSer (in equivalent amount to that detected on apoptotic cells) via expression of a constitutively active form of TMEM16F were not engulfed (Segawa et al. 2011). In addition, some phagocytes have a preference for certain targets over others, although both targets expose PtdSer similarly, as measured by annexin V staining (Ravichandran 2010). Perhaps apoptotic cells expose multiple "eat me" signals like those described above, and the combination of these with PtdSer may enhance engulfment. Calreticulin and annexin I have both been shown to colocalize with PtdSer on the surface

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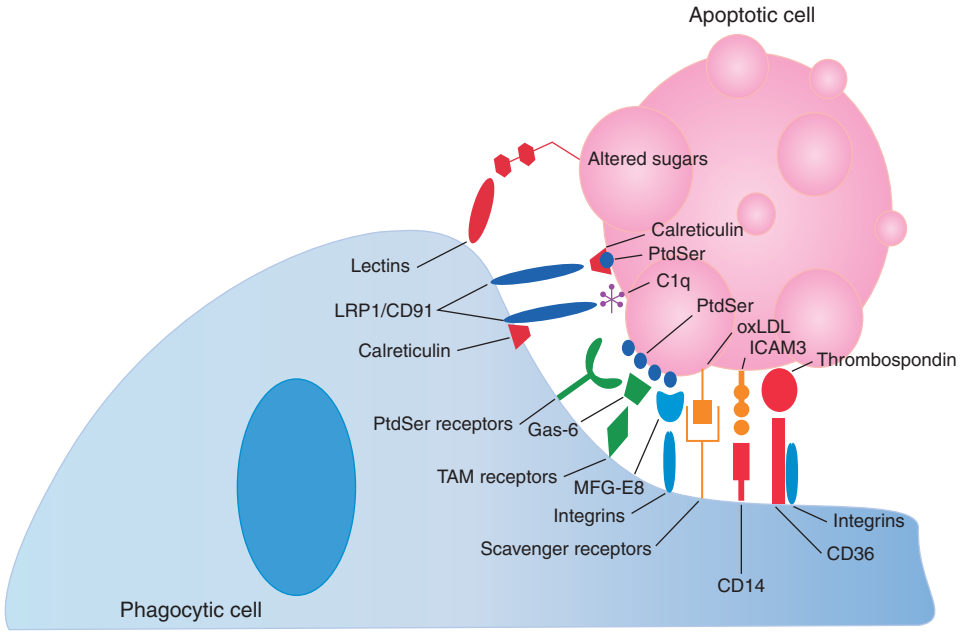

Figure 3. Apoptotic cell "eat me" signals and phagocytic receptors. As apoptotic cells undergo programmed cell death, they begin to expose "eat me" signals on their surfaces. Phosphatidylserine (PtdSer) is the best studied "eat me" signal; however, several others are also pictured here. "Eat me" signals are recognized by phagocytic engulfment receptors either directly (as with PtdSer receptors including TIM-4, BAI1, and Stabilin-2) or indirectly via bridging molecules or accessory receptors (as with Gas-6/TAM receptors, MFG-E8/$\alpha_v\beta_{3/5}$, and $\alpha_v\beta_{3/5}$ in conjunction with CD36 in the recognition of thrombospondin).

of apoptotic cells and enhance the uptake of targets by phagocytes (Arur et al. 2003; Gardai et al. 2005). Furthermore, some reports have detected oxidized PtdSer on the surface of apoptotic cells (Kagan et al. 2002), so perhaps PtdSer modification is another method by which dead cells identify themselves.

Apoptotic Cell Engulfment Receptors

A plethora of receptors are expressed on the surface of phagocytic cells that recognize "eat me" signals displayed on dying cells (Fig. 3). These include the lectins that bind altered sugars on apoptotic cells (Ezekowitz et al. 1990), CD36 (in conjunction with integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$) that binds thrombospondin (Savill et al. 1990), LRP1/CD91 (in conjunction with calreticulin) that binds complement C1q (Ogden et al. 2001), CD14 that binds ICAM3 (Gregory et al. 1998), and the scavenger receptors that bind oxidized LDL (Gordon 1999). Furthermore, PtdSer is recognized by an assortment of bridging proteins and direct recognition receptors, adding complexity to the process of dying cell recognition. For example, LRP1/CD91 has also been shown to bind calreticulin colocalized with PtdSer (Gardai et al. 2005). In addition, secreted proteins such as MFG-E8, growth-arrest-specific 6 (Gas6), and protein S have also been shown to bind PtdSer on the surface of apoptotic cells and promote engulfment via their cognate receptors on phagocytes. MFG-E8 (originally discovered in milk fat globules in the mammary gland) is expressed and secreted by professional phagocytes, associates with the $\alpha_v\beta_{3/5}$ integrins on phagocytes via its RGD motif, and binds PtdSer on apoptotic cells via its C1 and C2 domains (Hanayama et al. 2002; Nagata et al. 2010). Gas6 and protein S bridge PtdSer on

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apoptotic cells with the Tyro-3-Axl-Mer family of receptors (TAM receptors) on phagocytes (Nakano et al. 1997; Nagata et al. 2010).

Recently, receptors that directly recognize PtdSer on apoptotic cells and promote phagocytosis have been discovered, each with a unique expression pattern. Brain-specific angiogenesis inhibitor 1 (BAI1) is a transmembrane protein belonging to the adhesion-type G-protein-coupled receptor family that binds PtdSer via thrombospondin type 1 repeats (Park et al. 2007). BAI1 is expressed in macrophage cell lines and immunological tissues such as the bone marrow and spleen; however, BAI1 is expressed at a much higher degree in the brain glia and neuronal cells (Mori et al. 2002). Another set of recently described PtdSer receptors, T-cell immunoglobulin and mucin-domain-containing molecule 4 (TIM-4) and TIM-1, are small transmembrane proteins that bind PtdSer via their IgV domain (Kobayashi et al. 2007; Miyanishi et al. 2007; Santiago et al. 2007). TIM-4 is highly expressed by professional phagocytes; however, TIM-1 is primarily expressed in kidney cells. In contrast to the other identified PtdSer receptors, TIM-4 (and perhaps TIM-1) does not mediate direct signaling for engulfment and therefore likely functions as a tethering receptor (Park et al. 2009). Stabilin-2 (also known as hyaluronic acid receptor for endocytosis, or HARE) is a large type I membrane protein that stereospecifically recognizes PtdSer on the surface of apoptotic cells via its epidermal growth factor-like domains (Park et al. 2008a,c). It is primarily expressed on sinusoidal endothelial cells; however, Stabilin-2 is also expressed on human monocyte-derived macrophages (Park et al. 2008a). The most recent PtdSer receptor described to date is the receptor for advanced glycation end products (RAGE), which binds PtdSer in both its membrane-bound and soluble forms (He et al. 2011). The soluble form of RAGE appears to act as a decoy receptor, blocking PtdSer recognition and engulfment mediated by the membrane-bound form and other PtdSer receptors. RAGE is primarily expressed in lung tissue, including alveolar macrophages; however, its expression is induced by inflammation, perhaps suggesting a role for RAGE in the resolution of inflammation (Armstrong and Ravichandran 2011). Finally, the protein referred to as simply the "phosphatidylserine receptor" (PSR), was originally described as capable of binding PtdSer and mediating engulfment; however, the identity of PSR has turned out to be that of a Jumonji domain-containing nuclear protein, which does not directly mediate phagocytosis of apoptotic cells (Bose et al. 2004; Bratton and Henson 2008). The signaling mechanisms activated by the aforementioned receptors are discussed in the following section.

"Don't Eat Me" Signals

In addition to detecting the "eat me" signals on the surface of apoptotic cells, phagocytes can further distinguish between live and dead targets by the presence of "don't eat me" signals on the surface of living cells. CD47 (also known as integrin-associated protein) is a membrane protein expressed on the surface of healthy cells, recognized by its cognate receptor, SIRPα, which inhibits engulfment by phagocytes even in the presence of PtdSer (Gardai et al. 2005; Tsai and Discher 2008). CD47 expression is suppressed or down-modulated during apoptosis, thereby permitting clearance. Recently, mouse hematopoietic stem cells were shown to transiently increase CD47 expression during inflammation-mediated migration in order to avoid clearance; however, constitutive CD47 expression was detected in both mouse and human myeloid leukemias (Jaiswal et al. 2009). This poses a clever mechanism by which cancer cells may evade immune recognition and clearance, and new clinical therapies for the treatment of acute lymphoblastic leukemia are currently being developed that use blocking antibodies for CD47 (Chao et al. 2011). Research has shown that CD47 blocking antibodies increases engulfment in vitro and inhibits tumor engraftment in vivo; however, one large caveat to such treatment is that CD47 expression is important for the survival of many cell types including erythrocytes (Oldenborg et al. 2000), suggesting that blocking its recognition, even temporarily, may cause severe side effects, including anemia. CD31 is another example of a

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"don't eat me" signal (Brown et al. 2002); however, currently the CD47 literature is more advanced. Finally, the inhibitory receptor CD300a, broadly expressed on many hematopoietic cell types, has been recently shown to bind both PtdSer and PtdEtn on the surface of apoptotic cells and block their engulfment by macrophages (Simhadri et al. 2012). Interestingly, this form of regulation is intrinsic to the macrophage and independent of the apoptotic target.

ENGULFMENT SIGNALING PATHWAYS FOR CYTOSKELETON REARRANGEMENT

Once the apoptotic target is captured, the phagocyte undergoes cytoskeletal rearrangements necessary for corpse internalization. Genetic studies in *Caenorhabditis elegans* provided the initial insight into our understanding of the engulfment signaling pathways leading to the rearrangement of the phagocyte cytoskeleton necessary for corpse internalization (Kinchen 2010). The ongoing identification of the fly and mammalian homologs, as well as identification of new proteins exclusive to the mammalian contexts, are further defining signaling pathways in higher organisms (Table 1).

Rho-Family GTPases

GTPases are the molecular "switches" that turn signaling pathways on or off depending on the state of their bound guanine nucleotide. Active GTPase is the GTP-bound form, which is formed by the action of specific guanine-nucleotide-exchange factors (GEFs) that catalyze the GDP-to-GTP exchange. Conversely, GTPase-activating proteins (GAPs) stimulate the irreversible hydrolysis of GTP to GDP, effectively turning off the GTPase switch. The Rho family GTPases are members of the Ras superfamily of small signaling proteins with known functions in regulation of cellular movement (Ridley 2001). Members of this family include RhoA, Cdc42, and Rac. RhoA loss or suppression is associated with increased engulfment of apoptotic cells, and, conversely, forced activation is associated with inhibited engulfment (Tosello-Trampont et al. 2003; Nakaya et al. 2006). Regulation of clearance by RhoA is executed via the Rho-associated coiled-coil-containing protein kinase (ROCK). Active, or GTP-bound, Rho increases the kinase activity of ROCK, which, in turn, mediates phosphorylation of the myosin light chain (MLC) and promotes cell contraction (Riento and Ridley 2003). Contractility

Table 1. Evolutionarily conserved apoptotic cell engulfment signaling proteins

C. elegans	*Drosophila*	Mammal	Function
CED-1	DRPR	LRP1 (CD91) /MEGF10	Surface receptors on phagocytes that recognize ligands on the surface of apoptotic cells
CED-2	DCrk	CrkII	Adaptor protein that is proposed to localize the CED-5/CED-12 complex to the membrane; the precise role in apoptotic cell engulfment signaling is currently unclear
CED-5	Myoblast City	Dock180	RAC GEF containing a Docker (DHR2) domain that functions as a DH domain
CED-6	Dced-6	GULP	Adaptor proteins that bind CED-1; the precise role in apoptotic cell engulfment signaling is currently unclear
CED-7	Unknown	ABCA1/ABCA7	ABC transporter involved in cholesterol efflux downstream from apoptotic cell engulfment; the precise role is unknown, but its function is required in both the phagocyte and the apoptotic cell
CED-10	DRac	Rac	Rho family GTPase; regulates Arp2/3 activation, actin polymerization, and cytoskeletal rearrangement via the Scar/WAVE complex
CED-12	Dced-12	ELMO	Adaptor protein containing a PH domain; binds to CED-5 and enhances its RAC GEF activity

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likely inhibits the extension of pseudopods and phagocytic cup formation, necessary for the early stages of engulfment. RhoA activation at subsequent stages of engulfment, however, is thought to promote apoptotic cell digestion by regulating the acidification of phagosomes (Erwig and Henson 2008). In contrast to RhoA, the current function of Cdc42 in the clearance of apoptotic cells is unclear; however, some reports suggest that it may serve a beneficial role in this process (Leverrier and Ridley 2001). Finally, GTP-bound Rac has an evolutionarily conserved positive effect on engulfment, and Rac activation at sites of apoptotic cell recognition subsequently leads to Arp2/3 activation/actin polymerization/cytoskeletal rearrangement via the Scar/WAVE complex (Miki et al. 1998; Castellano et al. 2000). Presently, at least three distinct signaling pathways governing apoptotic cell clearance have been described, which ultimately lead to downstream activation of Rac.

Rac Activation via the CrkII–Dock180–ELMO Pathway

A combination of studies in *C. elegans*, *Drosophila melanogaster*, and mammalian models led to the identification of a signaling pathway for Rac activation composed of the proteins Dock180 (homologs include CED-5 in *C. elegans* and Myoblast City in the fly), ELMO (*C. elegans* CED-12 and fly DCed-12), and CrkII (*C. elegans* CED-2 and fly DCrk) (Wu and Horvitz 1998b; Reddien and Horvitz 2000; Gumienny et al. 2001; Zhou et al. 2001a). This group of proteins is involved in several cytoskeletal rearrangement processes including cell migration, neurite growth, muscle fusion, and phagocytosis of apoptotic cells (Nagata et al. 2010). Dock180 was identified as a Rac GEF that lacks the traditional Dbl-homology and pleckstrin-homology (DH-PH) domains required among GEFs to mediate GTP exchange (Brugnera et al. 2002). Although Dock180 contains a Docker (DHR2) domain that functions as a DH domain, monomeric Dock180 exists in a "closed" confirmation, thereby preventing the interaction of the DHR2 domain with Rac (Lu et al. 2005). ELMO is an adaptor protein that binds directly to Dock180, creating an unconventional bipartite Rac GEF (Brugnera et al. 2002). There are at least three possible mechanisms by which ELMO enhances Dock180 activity. First, binding of ELMO to the carboxy-terminal region of Dock180 relieves a steric inhibition that blocks the Dock180 DHR2 domain interaction with Rac (Lu et al. 2005). Second, the PH domain of ELMO then stabilizes the bond between Rac and Dock180 (Lu et al. 2004). Finally, ELMO targets Dock180 to the phagocytic membrane via direct binding to the carboxyl terminus of the transmembrane receptor BAI1 (Park et al. 2007).

Our understanding of Dock180–ELMO regulation is currently evolving. Original reports suggested that CrkII, an adaptor protein, recruits Dock180 and ELMO to the cell membrane after cell surface receptor/ligand engagement (Gumienny et al. 2001). Further experiments showed that CrkII can be coimmunoprecipitated with the Dock180–ELMO complex and is indeed required for efficient engulfment; however, direct interaction between CrkII and Dock180 is not essential for the removal of dead cells (Tosello-Trampont et al. 2007). Moreover, it was shown that ELMO also possesses a membrane-targeting signal and that activation of RhoG by the GEF Trio leads to membrane recruitment of the Dock180–ELMO complex via binding of ELMO Armadillo (ARM) repeats to active RHOG (deBakker et al. 2004). Finally, other studies have determined that Dock180, too, has a membrane-targeting signal, raising further doubt regarding the requirement of CrkII for this purpose (Cote et al. 2005). Therefore, the exact role of CrkII in this pathway is presently unknown.

Several phagocytic receptors introduced in the previous section have been shown to use the CrkII–Dock180–ELMO pathway for clearance of apoptotic cargo. These include integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which recognize PtdSer via bridging molecules (Albert et al. 2000; Akakura et al. 2004; Hanayama et al. 2004), and the TAM receptor Mer, which recognizes PtdSer via Gas6 (Wu et al. 2005). Likewise, the PtdSer receptor BAI1 also binds ELMO (via its cytoplasmic tail)

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and activates this signaling pathway during cell clearance (Park et al. 2007).

Rac Activation via the LRP1/MEGF10–GULP–ABCA1/ABCA7 Pathway

The second evolutionarily conserved signaling pathway for the removal of apoptotic cells includes LRP1 (CD91)/MEGF10 (*C. elegans* homolog CED-1 and fly Draper), GULP (*C. elegans* CED-6 and fly dCed-6), and ABCA1/ABCA7 (*C. elegans* CED-7) (Liu and Hengartner 1998; Wu and Horvitz 1998a; Zhou et al. 2001b; Su et al. 2002; Manaka et al. 2004). Although this pathway leads to downstream Rac activation (Kinchen et al. 2005), the intermediate steps are unclear. LRP1/CD91 is a surface receptor expressed on many cells, including phagocytes that recognize "eat me" signals on the surface of the apoptotic cells. Ligands for LRP1 (including calreticulin and thrombospondin) and the fly homolog, Draper (including Pretaporter), are known (Kuraishi et al. 2009); however, the ligand(s) for the worm homolog, CED-1, remains unidentified. After apoptotic cell recognition, LRP1 directly interacts with the adaptor protein, GULP, and may lead to cytoskeletal rearrangements (Su et al. 2002). Interestingly, the PtdSer receptor Stabilin-2 was also shown to interact with GULP via its NPXY motif, and knocking down endogenous GULP significantly reduced Stabilin-2-mediated engulfment of apoptotic cells, suggesting that it uses this pathway for clearance (Park et al. 2008b). Presently, the role of ABCA1/ABCA7 in this pathway is less well characterized. ABCA1/ABCA7 are members of the ATP-binding cassette transporter (ABC-transporter) protein family. How these proteins function in this engulfment signaling pathway is currently unclear, although their function is required in both the target and phagocyte (Wu and Horvitz 1998a). Interestingly, these proteins function in the transport of cholesterol and maintenance of lipid subdomains on the plasma membrane and may thus play a role in cholesterol homeostasis in the phagocyte during apoptotic cell engulfment (Kiss et al. 2006a,b; Landry et al. 2006).

A Third Parallel Engulfment Signaling Pathway?

In addition to the two described parallel engulfment signaling pathways, the existence of a third pathway has long been proposed because of the remaining (albeit inefficient) engulfment that occurs in the *C. elegans* CED-1, CED-5, or CED-10 mutants (Kinchen 2010). In fact, a recently described pathway was proposed to participate in engulfment signaling, consisting of the tyrosine kinase Abl (*C. elegans* ABL-1) and the Abl-interacting protein Abi (*C. elegans* ABI-1) (Hurwitz et al. 2009). Using *C. elegans*, this group reported that ABI-1 is required for engulfment of apoptotic cells and proper distal tip cell migration. ABI-1 was proposed to act in parallel to the two known engulfment signaling pathways, and ABL-1 likely opposes apoptotic cell engulfment by inhibiting ABI-1 function. The molecular pathways functioning upstream and downstream of these proteins are not yet defined, and it is still unclear whether ABI-1 signals through Rac (CED-10) similar to the other signaling pathways described above.

APOPTOTIC CELL DIGESTION AND PHAGOCYTE RESPONSE

After recognition and engulfment, the process of apoptotic cell clearance is not complete. Recent studies suggest that events downstream from internalization (collectively termed phagosome maturation) influence the phagocytic capacity of a cell to internalize additional targets (Wu et al. 2000; Krieser et al. 2002; Schrijvers et al. 2005; Park et al. 2011). Proper recognition, clearance, and degradation of apoptotic cell material are vital to maintaining an environment that protects the host against unchecked inflammation and eventual autoimmunity. Unengulfed apoptotic cells have the propensity to leak their cellular contents over time (secondary necrosis), resulting in inflammation, exposure of self-antigens, and a break in tolerance (Franz et al. 2006). On the other hand, recognition of apoptotic cells can trigger the secretion of anti-inflammatory cytokines (such as TGF-β and IL-10) from the phagocyte, thereby dampening or resolving

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inflammation (Henson 2005; Elliott and Ravichandran 2010). Furthermore, the proper degradation and processing of apoptotic cell material by professional phagocytes, particularly dendritic cells, are imperative for self-antigen presentation necessary to establish and maintain tolerance (Delamarre et al. 2005; Erwig and Henson 2008). The steps involved in apoptotic cell digestion and the effects this event has on the phagocyte are discussed further below.

Phagosome Maturation: Preparing the Meal for Digestion

Presently, the process of phagosome maturation is actively studied, and our knowledge is evolving. Following internalization, phagosomes (membrane-bound compartments containing the phagocytosed target) become increasingly acidic, ultimately fusing with lysosomes, which contain the digestive enzymes required for degradation (Kinchen and Ravichandran 2008). This process begins with recruitment of the large GTPase, Dynamin (*C. elegans* DYN-1), to the apoptotic cell/phagocyte interface (Fig. 4) (Yu et al. 2006). Dynamin interaction with Vps34 [a phosphatidylinositol(3)-kinase] on the forming phagosome leads to recruitment of the GDP-bound small GTPase, Rab5, to the phagosome surface (Kinchen et al. 2008). Rab5 is subsequently activated by an unidentified protein (the currently known Rab5 GEFs are not required in this process). In mammalian cells, however, depletion of the GEF, Gapex-5, was shown to inhibit Rab5 activation at sites of engulfment (Kitano et al. 2008). GTP-bound, active Rab5 promotes Vps34 activation, leading to generation of PtdIns(3)P on the phagosome surface, which is subsequently removed by the lipid phosphatase MTM-1 (Zou et al. 2009; Kinchen 2010). Recent studies describe that Mon1a (*C. elegans* SAND-1) and its binding partner, Ccz1, tie Rab5 activation to Rab7 recruitment to the phagosome (Kinchen and Ravichandran 2010) and its subsequent activation (Nordmann et al. 2010). At the Rab7-positive stage, the HOPS complex is recruited to the phagosome, leading to Rab7 activation and, ultimately, fusion of the phagosome with the lysosomal network (Kinchen et al. 2008). It is at this stage that acidic proteases and nucleases get activated and the apoptotic cell targets are degraded (Lennon-Dumenil et al. 2002). Rab5 must also be inactivated, and PtdIns(3)P must be removed from

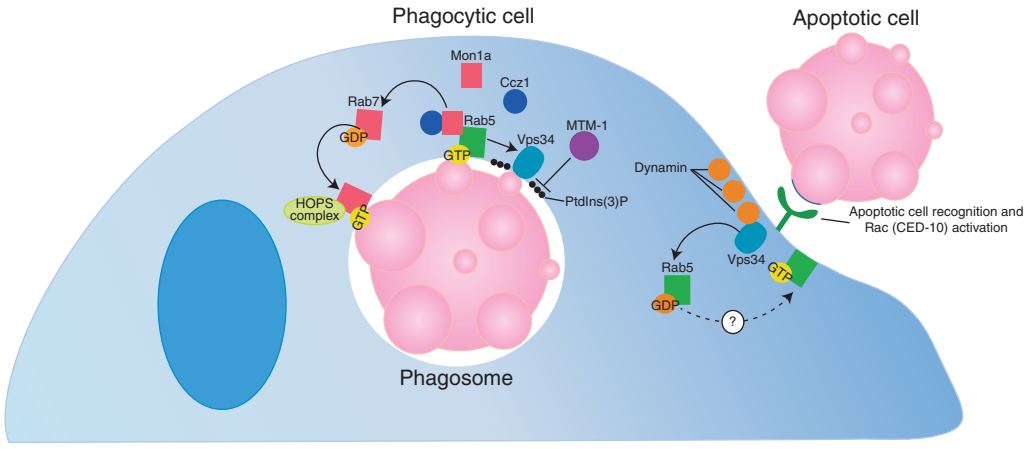

Figure 4. Phagosome maturation: The post-engulfment processing of apoptotic cells. In the first step of phagosome maturation, Dynamin is recruited to the apoptotic cell/phagocyte interface. Dynamin interacts with Vps34, leading to Rab5 recruitment and activation. Activated Rab5 further promotes Vps34 activation, leading to generation of PtdIns(3)P on the phagosome surface (subsequently removed by MTM-1). Mon1a and its binding partner, Ccz1, tie Rab5 activation and Rab7 recruitment to the phagosome. At this stage, the HOPS complex is recruited, leading to Rab7 activation and eventual fusion of the phagosome with the lysosome.

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the phagosome surface for maturation to proceed (Li et al. 2009; Zou et al. 2009).

Degradation and Processing of Apoptotic Cells

After engulfment, apoptotic cells are digested into their basic cellular building blocks including nucleotides, fats, sterols, and peptides/amino acids. Phagocytes often consume multiple corpses at one time; therefore, they must efficiently process the apoptotic cell constituents to maintain homeostasis. Several mechanisms have been identified that help a phagocyte maintain homeostasis. The mitochondrial membrane protein UCP2, which uncouples oxidative phosphorylation from ATP synthesis and reduces mitochondrial membrane potential in cells, has been shown to positively regulate the engulfment capacity of phagocytes (Park et al. 2011). Overexpression of UCP2 effectively enhanced phagocytosis of apoptotic cells in vitro, and loss of UCP2 expression reduced phagocytosis both in vitro and in vivo, suggesting that mitochondrial membrane potential modulates engulfment. UCP2 may thus provide a mechanism by which the energy balance within phagocytes is maintained during the process of engulfment. DNase II is a lysosomal enzyme responsible for degrading DNA, and lack of this enzyme leads to accumulation of DNA fragments within phagocytes (Kawane et al. 2001, 2003). Furthermore, conditional deletion of DNase II in the mouse led to the development of polyarthritis and an increase of inflammatory cytokines in the joint tissues (Kawane et al. 2006). This inflammation is thought to result from the buildup of target cell DNA in macrophages, which, in turn, were shown to produce the inflammatory cytokine TNFα (Kawane et al. 2006). Cholesterol also serves several important functions within a cell (regulates plasma membrane fluidity, plays a role in cell signaling and transport, etc.) and is therefore strictly regulated. Intriguingly, studies show that apoptotic cell-derived cholesterol becomes part of the cholesterol pool within the phagocyte, and phagocytes handle the additional sterol load by increasing their basal efflux mechanism via ABCA1 (Gerbod-Giannone et al. 2006; Kiss et al. 2006a). Conversely, increased cholesterol efflux is not seen when macrophages are fed necrotic cells or when macrophages are forced to internalize apoptotic cells via the FcR, suggesting an apoptotic-cell-specific trigger. PtdSer on the surface of apoptotic cells appears to be one such trigger; blocking PtdSer recognition was shown to inhibit enhanced cholesterol efflux, whereas providing purified PtdSer liposomes to macrophages augments it (Kiss et al. 2006a). Finally, processing of apoptotic cell proteins is linked to establishment and maintenance of self-tolerance via cross-presentation of engulfed cell peptides by the major histocompatibility complex (MHC) class I molecules (Bellone et al. 1997; Albert et al. 1998; Huang et al. 2000). Presentation of apoptotic-cell-derived peptides appears to be excluded from MHC class II presentation, perhaps reflecting the endogenous source of antigen (Blander and Medzhitov 2006).

Consequences of Apoptotic Cell Recognition and Internalization

The hallmark feature of apoptotic cell clearance is the non-inflammatory nature of this process. Many human diseases are caused or worsened by inflammation; however, the inflammatory process can also be of benefit to host survival as in the case of infection or cancer (Henson 2005). It is currently understood that a balance occurs between immunogenic and tolerogenic cell death/clearance depending on considerations such as the phagocytic cell type that clears the debris and the factors produced by the engulfing cells in response to apoptotic cell recognition.

Apoptotic cell death occurs in healthy organisms as part of normal tissue turnover (Henson and Hume 2006). The location of cell death and the means by which the cells are cleared will affect the response (or lack thereof) of the immune system. For example, organs such as the spleen and liver are considered tolerogenic, and apoptotic cells localized to these tissues will not generally elicit an immune response (Green et al. 2009). In addition, phagocytes are "programmed" to elicit specific responses after

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recognition of targets, dependent on their cell type. For instance, engulfment of uninfected apoptotic cells by tissue resident macrophages is usually immunologically silent; however, engulfment of apoptotic cells via infiltrating macrophages and/or dendritic cells in an inflamed environment has the potential to drive an antigen-specific immune response and break tolerance (Geissmann et al. 2010; Hochreiter-Hufford and Ravichandran 2012). Signals on the surface of dying cells can direct specific phagocytic cell types (i.e., dendritic cells or macrophages) toward them for clearance; however, in a recent study, lipid oxidation by one type of phagocyte was shown to inhibit phagocytosis of apoptotic cells via another phagocytic cell type. Briefly, expression of 12/15-lipoxygenase by resident peritoneal macrophages led to exposure of oxidized lipid on their cell surface; this oxidized lipid sequestered the MFG-E8 necessary for inflammatory monocyte recognition and engulfment of apoptotic cells (Uderhardt et al. 2012). This is the first example of a phagocyte-intrinsic mechanism for regulating apoptotic cell sorting to specific phagocytes. Annexin V is a naturally occurring PtdSer binding protein that blocks phagocytosis of apoptotic cells by macrophages but not dendritic cells (Krahling et al. 1999; Munoz et al. 2007; Frey et al. 2009). Necrotic cell immunization of Annexin V-deficient mice did not lead to elicitation of an immune response, unlike wild-type mice; macrophages isolated from Annexin V-deficient mice were shown to secrete significantly higher levels of IL-10 in response to incubation with LPS and necrotic cells than macrophages from wild-type mice (Frey et al. 2009). The investigators suggest that Annexin V blocks PtdSer-mediated anti-inflammatory cytokine release as well as phagocytosis of necrotic cells by macrophages, thereby allowing engulfment by dendritic cells and the initiation of an immune response. Further studies will elucidate whether this mechanism functions in an in vivo setting.

As mentioned above, one method by which apoptotic cells induce tolerance is by stimulating phagocyte secretion of anti-inflammatory and immunosuppressive cytokines such as TGF-β and IL-10. These cytokines, in turn, induce differentiation of regulatory T-cells and T helper 2 cells important in prevention of an inflammatory response (Green et al. 2009). Likewise, apoptotic cells have been shown to repress inflammatory cytokine secretion by monocytes in vitro (Voll et al. 1997; Kim et al. 2004). Interestingly, internalization of apoptotic cells is not required for stimulated cytokine secretion. In fact, in in vitro studies, apoptotic cell membranes were sufficient to inhibit IL-12 secretion by LPS-induced macrophages, and PtdSer vesicles, alone, stimulated TGF-β secretion by macrophages (Huynh et al. 2002; Kim et al. 2004). Work is currently underway to piece together the receptors and signaling molecules that connect PtdSer recognition with cytokine secretion.

Although apoptotic cell clearance is typically considered immunologically silent, there are exceptions in which apoptotic cells can elicit an immune response. The clearance of apoptotic tumor cells is an example in which immunogenic cell death is beneficial to the host so that residual tumor cells and metastases are maintained under control. Engulfment of oncogenic cells can give rise to the presentation of altered self-peptides, which may be presented to the immune system (Segal et al. 2008); however, the tolerogenic nature of cell clearance can dampen a subsequent immune response. Certain forms of chemotherapy and radiotherapy have been linked to the exposure or release of factors by dying cells that can initiate a robust immune response. For example, treatment of tumor cells with antracyclins led to cell-surface exposure of calreticulin early during the death program, which was essential for phagocytosis of dying tumor cells by dendritic cells and the initiation of an immune response (Obeid et al. 2007b). Additionally, calreticulin exposure was linked to immunogenic cell death caused by irradiation and UVC-light treatment (Obeid et al. 2007a). Uncleared, late apoptotic or necrotic cells with "leaky" plasma membranes can release damage-associated molecular-pattern molecules (DAMPs), such as heat shock proteins, high mobility group box 1 (HMGB-1) protein, and nucleic acids. These released intracellular molecules activate Toll-like receptors (TLRs) on phagocytes, facilitating antigen presentation

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and the initiation of an immune response (Peter et al. 2010; Kepp et al. 2011).

CONCLUDING REMARKS

Apoptotic cell death is an integral part of cell turnover in many tissues, and proper corpse clearance is vital to maintaining tissue homeostasis in all multicellular organisms. All living tissues have some mechanism(s) in place to handle corpse clearance, and most cell types (not just professional phagocytes) possess the ability to phagocytose apoptotic cells, underlining the relevance of this process in metazoan health.

Defects in clearance are believed to play a role in a wide variety of human pathologies including autoimmune diseases (systemic lupus erythematosus [SLE] and rheumatoid arthritis); pulmonary diseases (chronic obstructive pulmonary disease [COPD] and asthma); cardiovascular diseases (atherosclerosis); neurological diseases (Alzheimer's disease); infectious diseases (bacterial invasion by certain *Shigella*, *Yersinia*, and *Salmonella* species); and cancer (Elliott and Ravichandran 2010). Furthermore, the tolerogenic nature of apoptotic cell clearance has led to the emergence of potential new therapies using apoptotic cells for the prevention of transplant rejection and modulating autoimmune conditions such as type 1 diabetes (Castro et al. 2010; Morelli and Larregina 2010).

The field of apoptotic cell clearance is relatively young, and we are just beginning to better define the individual steps of this process and the consequences to the phagocyte, the tissue where it resides, and the organism. A better understanding of the sensing, recognition, engulfment, and processing of apoptotic cells will likely present new targets for therapy in the treatment of multiple immunological and metabolic diseases.

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Clearing the Dead: Apoptotic Cell Sensing, Recognition, Engulfment, and Digestion

Amelia Hochreiter-Hufford and Kodi S. Ravichandran

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