Clearance of apoptotic cells: implications in health and disease

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Recent advances in defining the molecular signaling pathways that regulate the phagocytosis of apoptotic cells have improved our understanding of this complex and evolutionarily conserved process. Studies in mice and humans suggest that the prompt removal of dying cells is crucial for immune tolerance and tissue homeostasis. Failed or defective clearance has emerged as an important contributing factor to a range of disease processes. This review addresses how specific molecular alterations of engulfment pathways are linked to pathogenic states. A better understanding of the apoptotic cell clearance process in healthy and diseased states could offer new therapeutic strategies.

Introduction

Apoptosis plays an essential role in the development and maintenance of all mammalian tissues. The apoptotic program ensures that damaged, aged, or excess cells are deleted in a regulated manner that is not harmful to the host. Beyond the cell intrinsic apoptotic program initiated after a variety of insults, an integral second step in apoptosis is the removal of the cell corpse (Kerr et al., 1972). Indeed, the physical removal and subsequent degradation of the corpse via phagocytosis represents the final act necessary for the successful removal of a cell fated to die. Recent advances in our understanding of apoptotic cell clearance have led to the identification of molecules and signaling pathways that orchestrate this process (Lauber et al., 2004; Ravichandran and Lorenz, 2007; Erwig and Henson, 2008).

The efficiency of the phagocytic clearance of apoptotic cells appears enormous when one considers that despite the loss of $>10^9$ cells per day, the incidence of histologically detectable apoptotic cells is rare in normal tissues (Mochizuki et al., 1996; Scott et al., 2001; Schrijvers et al., 2005; Yang et al., 2006; Elliott et al., 2009). The engulfment of apoptotic cells is performed by both professional phagocytes (such as macrophages and dendritic cells) and by nonprofessional "neighboring" phagocytes (such as epithelial cells, endothelial cells, and fibroblasts). Current evidence suggests that the steps involved in the phagocytic clearance of apoptotic cells are similar between professional and nonprofessional phagocytes (Fig. 1), although the kinetics may differ, with professional phagocytes exhibiting higher rates and capacity for phagocytosis (Parnaik et al., 2000).

Based on work from many laboratories over the past decade, several broadly defined steps have been identified in the recognition and removal of apoptotic cells by phagocytes. Each step appears to be tightly regulated by signaling events to ensure swift and efficient clearance (Fig. 1). At the early stage of apoptosis, the dying cells release "find-me" signals that are sensed by motile phagocytes, which help attract these phagocytes to the proximity of the dying cell. Several soluble chemoattractant find-me signals released during apoptosis have been recently defined, including triphosphate nucleotides (ATP/UTP), lysophosphatidylchloline (lysoPC), and the chemokine CX_3CL1 (Lauber et al., 2003; Truman et al., 2008; Elliott et al., 2009; Muñoz et al., 2010). Once in the proximity of the dying cell, the physical contact between the apoptotic cell and the phagocyte is mediated via ligands on apoptotic cells (referred to as "eat-me" signals) and engulfment receptors on phagocytes that can recognize these eat-me markers. Among the array of identified eat-me molecules (Ravichandran and Lorenz, 2007), the exposure of phosphatidylserine (PtdSer) on the outer leaflet of the apoptotic cell plasma membrane appears to be a key eat-me marker (Fadok et al., 1992; Vandivier et al., 2006). Phagocyte recognition of PtdSer is mediated directly via one or more PtdSer recognition receptors, including Bai1, Tim-4, and Stabilin-2 (Kobayashi et al., 2007; Park et al., 2007, 2008, 2009; Miyanishi et al., 2007; Nakayama et al., 2009), or by soluble bridging molecules that bind PtdSer on the apoptotic cell and a receptor on the phagocyte (MFG-E8/$\alpha v\beta_{3/5}$, Gas6/MER; Savill et al., 1990; Scott et al., 2001; Hanayama et al., 2004). Engagement of the PtdSer receptors initiates signaling events within the phagocytes that lead to activation of the small GTPase Rac, and subsequent cytoskeletal reorganization of the phagocyte membrane to allow corpse internalization (Albert et al., 2000; Gumienny et al., 2001). From studies in *Caenorhabditis elegans* and *Drosophila*

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Abbreviations used in this paper: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; COPD, chronic obstructive pulmonary disease; LDL, low-density lipoprotein; PtdSer, phosphatidylserine.

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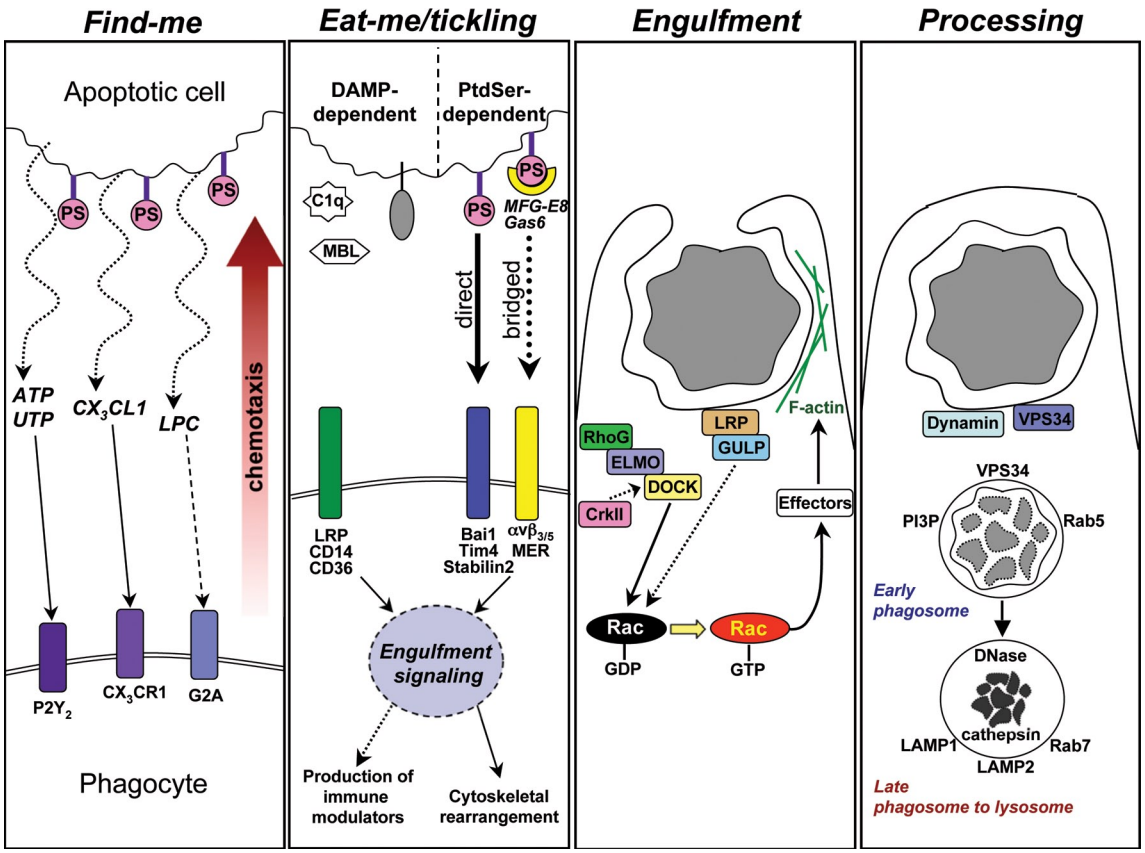

Figure 1. **Stages of apoptotic cell engulfment and associated cell signaling events that regulate each stage.** The four stages of apoptotic cell clearance are shown, with some of the specific key signaling players identified. The "find-me" step occurs when apoptotic cells release soluble chemoattractants that promote chemotaxis of phagocytes via corresponding receptors on the phagocyte. The broken line from LPC to G2A indicates uncertainty of direct ligand–receptor interaction. The "eat-me" stage is characterized by the appearance of ligands on the surface of the dying cell that mark it as a target to be engulfed by phagocytes bearing appropriate DAMP or PtdSer recognition receptors. The "engulfment" stage occurs when signaling downstream of the apoptotic cell recognition receptors stimulates Rac-dependent cytoskeletal rearrangement and formation of the phagocytic cup around the target and subsequent internalization. Once fully internalized, the cell corpse undergoes "processing" through the phagolysosomal pathway that results in the degradation and reprocessing of the dead cell material. DAMP, damage-associated molecular patterns; LPC, lysophosphatidylcholine; MBL, mannose-binding lectin; PS, phosphatidylserine.

melanogaster, and in vitro mammalian cell experiments, two key evolutionarily conserved Rac-dependent apoptotic cell engulfment pathways have been identified (Fig. 1; Reddien and Horvitz, 2004; Kinchen, 2010). In addition to receptors that can directly signal after engaging eat-me signals, there are also contributions from other "tethering" receptors (e.g., CD14 and CD31) that help the binding/specific recognition between the apoptotic cell and the phagocyte (Brown et al., 2002; Devitt et al., 2003). Once inside the phagosome, the ingested apoptotic cargo is processed via a phagolysosomal pathway that shares both overlapping and unique features with the endocytic machinery (Erwig et al., 2006; Kinchen et al., 2008; Yu et al., 2008; Kinchen and Ravichandran, 2010; Bohdanowicz and Grinstein, 2010). Because of this overlap, it is difficult to distinguish disease states related specifically to aberrant signaling in the phagosomal pathways from those involving endocytosis dysfunction. Indeed, a role for endocytosis in human disease has been well established (Mosesson et al., 2008; Ballabio and Gieselmann, 2009). Thus, we will focus on diseases related to engulfment signaling upstream of corpse degradation.

Although the clearance of apoptotic cells occurs throughout the body, the specific molecular pathways can vary by tissue. For example the intracellular engulfment signaling molecules Rac, ELMO, and Dock180 appear to be widely expressed (Hasegawa et al., 1996; Gumienny et al., 2001), whereas the expression of many of the surface molecules responsible for recognition of apoptotic cells varies widely among different tissues and cell types (Ferrero et al., 1990; Graham et al., 1994; Falkowski et al., 2003; Miyanishi et al., 2007; Park et al., 2007). Thus, because of the redundancy in the engulfment machinery among cell types, it is critical to know the expression pattern of identified phagocytic receptors when considering apoptotic cell clearance in a specific tissue or by a particular cell type. Interestingly, many of the disease states linked to failed clearance have been associated with aberrations in the recognition or eat-me step of clearance (Table I). This observation might reflect an investigator-induced bias toward phagocyte–corpse interactions, or it may be the result of selective expression of phagocytic receptors that reduces the redundancy of uptake mechanisms, and thus is more likely to reveal failures in clearance.

Regardless of the specific molecules mediating uptake, the ability to efficiently clear apoptotic cells is strongly linked to the homeostatic maintenance of healthy tissues in mammals. This is thought to be the result of two key features of the clearance process. The first is the obvious function of phagocytes

Table I. **A survey of disease states associated with defects in engulfment-related genes**

Gene	Disease relationship	Human/mouse	References
Find me			
G2A	AI	M	Le et al., 2001
CX_3CR1	Neuropathy	M	Cardona et al., 2006
CX_3CL1	Atherosclerosis	M	Combadière et al., 2003
Eat-me/tickling			
MER	AI, cancer, neuropathy, atherosclerosis	H/M	Gal et al., 2000; Scott et al., 2001; Cohen et al., 2002; Keating et al., 2006; Nandrot et al., 2007; Ait-Oufella et al., 2008; Thorp et al., 2008
MFG-E8	AI, atherosclerosis, neuropathy	M	Hanayama et al., 2004; Ait-Oufella et al., 2007; Nandrot et al., 2007
C1q	AI, atherosclerosis, neuropathy	M	Botto et al., 1998; Fonseca et al., 2004; Bhatia et al., 2007
$\alpha v\beta_{3/5}$	AI, atherosclerosis	M	Weng et al., 2003; Lacy-Hulbert et al., 2007
TIM-4	AI	M	Rodriguez-Manzanet et al., 2010
Gas6	Atherosclerosis	M	Lutgens et al., 2008
Engulfment			
ELMO1	Diabetic nephropathy[a]	H	Shimazaki et al., 2005; Leak et al., 2009; Pezzolesi et al., 2009a
GULP1	Arthritis[a], schizophrenia[a]	H	Qingchun et al., 2008; Chen et al., 2009
MEGF10	Schizophrenia[a]	H	Chen et al., 2009
Post-engulfment			
LXRα/β	AI	M	A-Gonzalez et al., 2009
PPARδ	AI	M	Mukundan et al., 2009
DNase II	AI	M	Kawane et al., 2003

Genes are grouped by known roles in engulfment (find-me, eat-me, engulfment, and post-engulfment). AI, autoimmune phenotype; H, human; M, mouse.
[a]There is evidence of genetic linkage but no direct causal relationship was established.

as "garbage collectors," mediating the physical removal of the dying cells. Such clearance sequesters the dying cell and prevents the release of potentially toxic or immunogenic intracellular contents from the dying cell into the local environment. This is a key distinction from necrotic cell death, where the unregulated release of dead cell material can cause very strong inflammatory responses (such as ischemic injury). The second homeostatic function of the clearance process is the production of anti-inflammatory mediators by phagocytes that suppress inflammation and facilitate the "immunologically silent" clearance of apoptotic cells.

The purpose of this review is to examine the current body of knowledge linking apoptotic cell clearance to disease pathogenesis. We will discuss several families of disease states that appear to have as a contributing factor some level of impaired cell clearance. We will also attempt to highlight how components of the engulfment signaling pathways may function in myriad disease processes.

Failed clearance, altered immune tolerance, and autoimmunity

Autoimmune disorders represent the best-characterized relationship between apoptotic cell clearance and disease pathogenesis (Table I; Savill et al., 2002; Gaipl et al., 2004; Erwig and Henson, 2007; Nagata et al., 2010). The self-contained, regulated nature of apoptotic cell death preserves membrane integrity and prevents the release of potentially inflammatory and immunogenic intracellular contents. However, if the apoptotic cells are not promptly cleared, the membrane integrity is lost over time, and apoptotic cells can progress to secondary necrosis. The release of intracellular contents from necrotic cells is thought to provoke an inflammatory response, particularly toward intracellular antigens and DNA released from the dying cells. This may provide the immunogenic impetus for the onset of some autoimmune disorders in humans, including systemic lupus erythematosus and rheumatoid arthritis (Gaipl et al., 2004). Early experiments in mice showed that the administration of excess syngeneic apoptotic cells or the masking of PtdSer on apoptotic cells via annexin V (to block PtdSer-mediated uptake) produces hallmarks of autoimmunity, such as autoantibody production and IgG deposition in the glomeruli (Mevorach et al., 1998; Asano et al., 2004). More recently, several genetic mouse models bearing defects in PtdSer-mediated recognition have further confirmed that the failure to efficiently clear apoptotic cells can result in autoimmunity (Botto et al., 1998; Scott et al., 2001; Cohen et al., 2002; Hanayama et al., 2004; Lacy-Hulbert et al., 2007; Rodriguez-Manzanet et al., 2010). Nuclear antigens, particularly DNA and DNA–protein complexes (e.g., high mobility group box 1–containing nucleosomes), appear especially crucial in human systemic lupus erythematosus and rheumatoid arthritis (Taniguchi et al., 2003). Studies in knockout mice demonstrated that to maintain self-tolerance, DNase-mediated degradation of apoptotic cell-derived DNA in the phagosome is necessary (Napirei et al., 2000; Krieser et al., 2002; Kawane et al., 2003). There is now a solid link between the inefficient engulfment of apoptotic cells and autoimmunity in humans (Ren et al., 2003; Gaipl et al., 2004).

An additional means for controlling the immune response to apoptotic cells is through the active production of anti-inflammatory mediators by phagocytes. The PtdSer-dependent

recognition of apoptotic cells by a phagocyte elicits the release of anti-inflammatory mediators such as IL-10, TGFβ, and prostaglandins in vitro (Voll et al., 1997; Fadok et al., 1998; McDonald et al., 1999; Ogden et al., 2005). Moreover, this recognition actively suppresses inflammatory cytokine release in vitro, particularly those elicited via Toll-like receptors (TLRs; Voll et al., 1997; Fadok et al., 1998). This immunosuppressive response extends in vivo, as studies in mice have shown that the systemic administration of apoptotic cells induces a tolerizing effect on the immune response in rodent allograft models (Sun et al., 2004; Wang et al., 2009). Recently, key insights into the signaling events that regulate the release of these immune modulators have been gained. PtdSer-dependent engagement of apoptotic cells induces in phagocytes the p38 MAPK-dependent transcriptional regulation of *IL-10*, as well as translational control of TGFβ in the phagocyte (Chung et al., 2007; Xiao et al., 2008). The ability of apoptotic cells to suppress TLR-dependent release of IL-6, IL-8, and TNF has also been shown to be regulated at the transcript level (Cvetanovic and Ucker, 2004). Thus, in addition to the physical removal of dying cells, the "tickling" of phagocytic receptors generates signals that lead to regulation of anti-inflammatory mediators and in turn, the elicitation of an immunosuppressive environment during removal of apoptotic cells. Even under normal healthy conditions, there is a turnover of >200 billion cells per day in many tissues throughout our body, and therefore interruptions to the finely tuned clearance system can lead to inflammation, tissue destruction, and the onset of disease.

Respiratory diseases and impaired cell clearance

Intriguingly, increased levels of apoptotic cells are seen in the sputum and lung tissue of several serious respiratory diseases, including chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and asthma (Henson and Tuder, 2008). Because aberrant lung inflammation is a common feature of these diseases, one possibility is that uncleared apoptotic cells progressing to secondary necrosis may contribute to lung inflammation. But a common underlying question is whether or not these "uncleared" apoptotic cells represent increased rates of apoptosis or defects in apoptotic cell clearance. In the past few years, several studies have established considerable links between respiratory disease and inefficient apoptotic cell clearance in the lung (Vandivier et al., 2002; Hodge et al., 2003; Huynh et al., 2005). Although the focus of these studies has primarily been on the phagocytic activity of lung resident macrophages (alveolar macrophages), it will be interesting to determine the relative contribution of healthy lung epithelial cells in the clearance of neighboring apoptotic cells.

The environment of the diseased lung contributes to poor apoptotic cell clearance. Cigarette smoking, the leading cause of COPD, is correlated with increased apoptotic cell debris in the lung (Hodge et al., 2005), and cigarette smoke impairs the uptake of apoptotic cells by alveolar macrophages in vitro (Kirkham et al., 2004; Hodge et al., 2007). Sputum from CF patients, when added to normal alveolar macrophages, inhibits their ability to engulf apoptotic targets in vitro (Vandivier et al., 2002). At least two factors in CF sputum have been shown to disrupt apoptotic cell engulfment, including elevated levels of neutrophil-derived elastase, which may cleave eat-me signals (Vandivier et al., 2002), and pyocyanin, a toxic by-product of *Pseudomonas aeruginosa*, a common infectious pathogen found in the lungs of about half of all CF patients (Bianchi et al., 2008). Finally, the inflammation associated with lung disease appears to create a cytokine milieu (notably increased TNF) that may suppress apoptotic cell engulfment (Borges et al., 2009), perhaps by hindering the differentiation of monocytes to macrophages, thus exacerbating these clearance defects.

Intrinsic defects in macrophages in the context of the diseased lung also appear to contribute to the reduced clearance seen in these respiratory diseases. Alveolar macrophages from COPD, CF, and asthma patients show a decreased ability to engulf apoptotic cells in vitro (Hodge et al., 2003, 2007; Huynh et al., 2005; Vandivier et al., 2009). To date, there are no reported links to specific engulfment pathways that are defective in these lung diseases, although decreased expression of at least two collectins (mannose-binding lectin and surfactant protein-D) in COPD patients suggests a possible role for decreased pattern recognition receptor (PRR)/C1q receptor–mediated uptake (Hodge et al., 2008). Intriguingly, Vandivier et al. (2009) recently found that cystic fibrosis transmembrane conductance regulator (CFTR)-deficient epithelial cells are defective in the phagocytosis of apoptotic cells, whereas CFTR-deficient alveolar macrophages show no engulfment defect. These findings suggest that a persistent disease state in the lung (i.e., COPD) and/or genetic anomalies may drive engulfment defects, and thus point to a prominent role for engulfment in the establishment and progression of disease. Moreover, the relative contributions of macrophages and the epithelial cells for apoptotic cell clearance, as well as the anti-inflammatory cytokines generated (or lack thereof), need to be determined in the context of lung inflammation. Future genetic studies that target engulfment genes in particular phagocyte populations may reveal some important information on the onset and progression of lung inflammation.

An interesting feature of defective apoptotic cell clearance in the diseased lung is the potential role of the small GTPase RhoA. During engulfment, activation of the small GTPase Rac in the phagocyte is crucial for actin rearrangement during corpse internalization (Fig. 1). In contrast, RhoA antagonizes Rac in this process, and increased levels of RhoA-GTP potently impair engulfment (Leverrier and Ridley, 2001; Tosello-Trampont et al., 2003; Nakaya et al., 2006). Independent studies have shown that CFTR deficiency in lung epithelial cells results in higher basal levels of activated RhoA (Kreiselmeier et al., 2003; Vandivier et al., 2009). Studies using in vitro treated lung epithelial cells similarly show increased basal levels of RhoA-GTP in response to cigarette smoke (Richens et al., 2009). Pharmacological inhibitors of RhoA activity, particularly statins, enhance apoptotic cell engulfment in vitro and in vivo, and thus suggest that elevated RhoA-GTP levels may play a significant role in the impaired clearance observed in diseased lungs (Morimoto et al., 2006). Although the molecular events leading to increased levels of RhoA-GTP levels are poorly understood, cigarette smoke exerts a similar effect (activation of RhoA) and

may in part explain the defective engulfment seen in COPD (Richens et al., 2009). There is currently no definite linkage between lung disease and specific engulfment receptors, and the high rate of cell death in the lung due to inhaled toxins could provide valuable insights into clearance mechanisms through the use of genetically modified mice.

Atherosclerosis and engulfment-related consequences

Macrophages play a prominent role in the development of atherosclerotic plaques, and their function in clearing apoptotic cells appears to be a key to the pathogenesis of this widespread and life-threatening disease. At the onset of plaque formation, monocytes in the blood adhere to intimal smooth muscle cells and differentiate almost exclusively to macrophages. These macrophages then take up low-density lipoprotein (LDL) via scavenger receptors and, once they are cholesterol-laden, are known as "foam cells." These foam cells eventually undergo apoptosis, yet early atherosclerotic lesions display few uncleared apoptotic cells, which suggests efficient clearance (Tabas, 2005). As leukocytes continue to infiltrate the lesion and release inflammatory mediators, cell death increases (Schrijvers et al., 2005). Indeed, late plaques feature much higher levels of free, uncleared apoptotic cells, and eventually a necrotic core forms and becomes unstable, leading to possible lesions that can cause thrombosis (Tabas, 2005).

In recent years, the role of apoptotic cell clearance has begun to be appreciated in atherogenesis. Through the use of atherosclerosis mouse models—*ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$—genetic studies of engulfment molecules have demonstrated the role of cell clearance in atherosclerosis (Table I). Mice deficient in the apoptotic cell-bridging molecules MFG-E8 (Ait-Oufella et al., 2007) and C1q (Bhatia et al., 2007) develop accelerated atherogenesis and display increased plaque-bound apoptotic cells on *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ genetic backgrounds, respectively. Likewise, mice deficient in transglutamase 2 (TG2), a cross-linking enzyme that promotes engulfment via $\alpha v\beta_{3/5}$ (Lorand and Graham, 2003; Szondy et al., 2003), also enhances atherosclerotic plaque formation in *Ldlr*$^{-/-}$-deficient mice (Boisvert et al., 2006), but not in ApoE-deficient mice (Williams et al., 2010). In addition, the receptor tyrosine kinase MER, which recognizes apoptotic cells via the PtdSer-binding Gas6 bridging molecule, functions in vivo to inhibit plaque formation and can promote apoptotic cell clearance in atherosclerosis models (Ait-Oufella et al., 2008; Thorp et al., 2008). Paradoxically, Gas6 deficiency on the *ApoE*$^{-/-}$ background leads to the formation of more stable plaques with smaller necrotic cores, fewer macrophages, and increased TGFβ levels (Lutgens et al., 2008), which suggests possible additional nonengulfment related antiatherogenic roles for MER. These studies suggest divergent roles for the receptor–ligand interactions in atherogenesis, which may be due to nonengulfment functions of both proteins or the lack of our full understanding of cell death/cell clearance in an atherosclerotic plaque.

Lipid handling by macrophages plays an important role in atherosclerosis, and so it is interesting that there is considerable overlap in the cellular mechanisms that regulate lipid metabolism and apoptotic cell engulfment. We and others have found that macrophages engulfing apoptotic cells up-regulate the key lipid transporter ABCA1, and this leads to enhanced cholesterol efflux from the phagocytes (Gerbod-Giannone et al., 2006; Kiss et al., 2006a). This cholesterol efflux requires PtdSer-dependent recognition and signaling within the phagocytes (Kiss et al., 2006a). These findings reveal that a phagocyte taking up an apoptotic cell has the ability to regulate and normalize the level of cellular material. Another intracellular engulfment signaling protein, GULP1, has been shown to promote cholesterol efflux, and GULP1 functions downstream of the LDL-receptor related protein 1 (LRP1), which is also linked to engulfment of apoptotic cells (Su et al., 2002; Gardai et al., 2005; Kiss et al., 2006b). Nuclear receptors, a family of transcriptional regulators that control the response to cellular lipids (Hong and Tontonoz, 2008), have been implicated in this response, as antagonists blocked this efflux (Gerbod-Giannone et al., 2006; Kiss et al., 2006a). As further evidence of the interplay between engulfment and lipid metabolism, mice deficient in the LXRα/β or PPARδ nuclear receptors showed decreased expression of engulfment genes, with impaired engulfment of apoptotic cells by macrophages in vitro and in vivo (A-Gonzalez et al., 2009; Mukundan et al., 2009). These mice also showed aberrant expression of inflammatory mediators and eventually develop hallmarks of autoimmunity. Because uncleared dead cells are a fundamental issue in atherogenesis, it would seem that the ability to modulate apoptotic cell clearance in this environment could serve as a useful and novel tool to prevent or treat disease.

Cell clearance defects in neurological diseases

Over a decade ago, several studies identified excess apoptotic cells associated with chronic neurodegenerative diseases, including in patients with Parkinson's, Alzheimer's, and Huntington's disease, and in aging brains (Su et al., 1994; Thomas et al., 1995; Zhang et al., 1995; Mochizuki et al., 1996). Microglia are one of the primary phagocytes for apoptotic cells and debris in the brain (Witting et al., 2000; Magnus et al., 2002; Stolzing and Grune, 2004; Garden and Möller, 2006). Considered to be of myeloid lineage, these highly motile cells provide necessary surveillance to respond to cell death associated with acute injury and stroke (Davalos et al., 2005; Garden and Möller, 2006). Upon the initiation of neuronal cell death, microglia migrate to the site of injury and mediate the inflammatory response (Davalos et al., 2005; Koizumi et al., 2007). Recently, engulfment signaling pathways have been implicated in glial function during chronic neurological diseases. Although the discussion in the following paragraph focuses on microglial cells, it is important to keep in mind that other cell types in the brain such as astrocytes can also engulf apoptotic cells (Chang et al., 2000; Magnus et al., 2002; Park et al., 2007) and thus may play a role in clearance and disease in the brain.

To date, MFG-E8 is the engulfment-related molecule best linked to clearance of apoptotic cells in the brain. Cultured astrocytes and microglia produce MFG-E8, and MFG-E8 can promote the phagocytosis of apoptotic neurons by microglia in vitro (Boddaert et al., 2007; Fuller and Van Eldik, 2008).

There is also a correlative relationship between MFG-E8 and Alzheimer's disease, as suppressed levels of MFG-E8 are associated with the disease in humans and mice (Boddaert et al., 2007; Fuller and Van Eldik, 2008). Additional evidence of engulfment signaling in the brain comes from studies of microglial chemoattractants. Dying neurons release find-me cues, namely extracellular nucleotides as well as CX_3CL1 (fractalkine or neurotactin) that promote chemotaxis of microglia via the P2Y and CX_3CR1 receptors, respectively (Harrison et al., 1998; Koizumi et al., 2007). Interestingly, both fractalkine and UDP appear to enhance glial cell engulfment: fractalkine by enhancing microglial secretion of MFG-E8, and UDP through an as yet unknown mechanism (Koizumi et al., 2007; Fuller and Van Eldik, 2008). The role of fractalkine signaling has been studied in the context of amyotrophic lateral sclerosis and Parkinson's disease using CX_3CR1-deficient mice. In these disease models, loss of fractalkine signaling resulted in increased numbers of dying neurons, which suggests a potential role for fractalkine as an important find-me signal in the maintenance of brain homeostasis (Cardona et al., 2006). A key unexplored area of clearance in the central nervous system is the immune response generated by microglial cells or astrocytes during engulfment (i.e., the release of anti-inflammatory mediators) and how that impacts homeostasis and disease. Finally, in the developed brain, cell turnover is thought to be quite low with the exception of restricted regions where adult neurogenesis takes place (Kempermann et al., 2004; Zhao et al., 2008; Taupin, 2009). Defining how apoptotic cell clearance impacts other developmental processes in the brain related to cell turnover, including adult neurogenesis, will require additional studies with appropriate neurological models.

Tumorigenesis and cell clearance

Because apoptotic cell clearance typically generates an immunosuppressive environment, its role in the development and progression of cancer is enigmatic. As has been reviewed elsewhere (Coussens and Werb, 2002; Condeelis and Pollard, 2006; Solinas et al., 2009), chronic inflammation is a key factor in tumorigenesis. Thus, the efficient clearance of dying cells, and the associated production of anti-inflammatory mediators, would be predicted to be beneficial in limiting tumorigenesis. However, within a tumor environment where rapid cell proliferation and apoptosis are ongoing, phagocyte-mediated clearance can exert an unwanted immunosuppressive effect. This is particularly the case upon the administration of antitumor chemotherapeutics, most of which act by inducing apoptosis of tumor cells. In this setting, efficient engulfment and the characteristic release of anti-inflammatory mediators, particularly TGFβ, upon encounter with eat-me signals during this process appear to suppress the antitumor immune response. Indeed, in several rodent tumor models, treatment with monoclonal antibodies to block PtdSer-mediated uptake retards the growth of tumors (Huang et al., 2005; Ran et al., 2005; He et al., 2009). Similarly, vaccination of mice with UV-irradiated lymphoma cells coated with annexin V to mask PtdSer provides significant tumor protection against subsequent challenge with living tumor cells, presumably by initiating an antitumor inflammatory response (Bondanza et al., 2004). Antibody depletion of MFG-E8 in mouse models of solid tumors also enhances antitumor activity (Jinushi et al., 2008; Jinushi et al., 2009). These findings suggest that interfering with PtdSer uptake promotes dendritic cell-mediated antitumor activity by favoring inflammatory uptake mechanisms. Still, despite what appears to be a plausible scenario wherein apoptotic cell clearance could have a profound impact on carcinogenesis, there is only limited genetic evidence to implicate specific engulfment signaling pathways in this process. Indeed, the expression of several key engulfment players, including MER (Linger et al., 2008) and $\alpha v\beta_5$ (Burvenich et al., 2008), is up-regulated in neoplastic cells, but the importance of this observation is unclear.

With the recent discovery of several "find-me" factors released by apoptotic cells that act to promote recruitment of phagocytes to apoptotic cells, new insights have been gained in our understanding of connections between cell clearance and tumorigenesis. Several insightful studies from the laboratory of C.D. Gregory (Ogden et al., 2005; Truman et al., 2008) have focused on how macrophages sense and subsequently engulf apoptotic Burkitt lymphoma cells and how these signaling events may impact disease progression. These neoplastic B cells express high levels of fractalkine on their surface that is cleaved during apoptosis and subsequently functions as a potent chemoattractant for macrophages (Truman et al., 2008). Recruitment of macrophages to splenic follicles is impaired in fractalkine receptor-deficient mice, an observation consistent with a role for fractalkine as a key mediator of macrophage recruitment to germinal centers (Truman et al., 2008). Within the germinal center environment, high levels of IL-10 (likely produced by the engulfing macrophages) appear to suppress tumor immunity, whereas the release of B cell survival factors by engulfing macrophages is thought to promote tumor growth (Ogden et al., 2005).

Additionally, we have recently found that apoptotic cells release nucleotide triphosphates (ATP/UTP) early during the apoptotic process (within 2–4 h), and that these nucleotides act as chemoattractants for monocytes and macrophages in vitro and in vivo (Elliott et al., 2009). The amount of ATP released by apoptotic cells under these conditions, which promotes silent clearance, represents a very small percentage of the total intracellular pool of nucleotides (<2%; Elliott et al., 2009). In contrast, a few other recent studies have demonstrated that ATP is released by tumor cells undergoing apoptosis in response to chemotherapeutics, with considerably higher amounts of ATP release (10–100 fold greater) seen at later times after induction (12–24 h; Ghiringhelli et al., 2009; Martins et al., 2009; Aymeric et al., 2010). This apoptotic cell-derived ATP stimulates activation of the NLRP3 inflammasome in dendritic cells via the P2X7 receptor (Ghiringhelli et al., 2009). This heightened activation state appears necessary to drive IL-1β secretion and subsequent priming of CD8+ T cells for IFNγ production and antitumor responses. These studies highlight an emerging role for factors released by apoptotic cells in shaping the immune response in normal and tumor environments. This has led to the concept of "immunogenic" versus "non-immunogenic" cell death, and the idea that immunogenic cell death may be beneficial in antitumor therapies (Green et al., 2009; Locher et al., 2009). Thus, whether apoptotic cell clearance has a beneficial or detrimental effect in the context of tumor progression

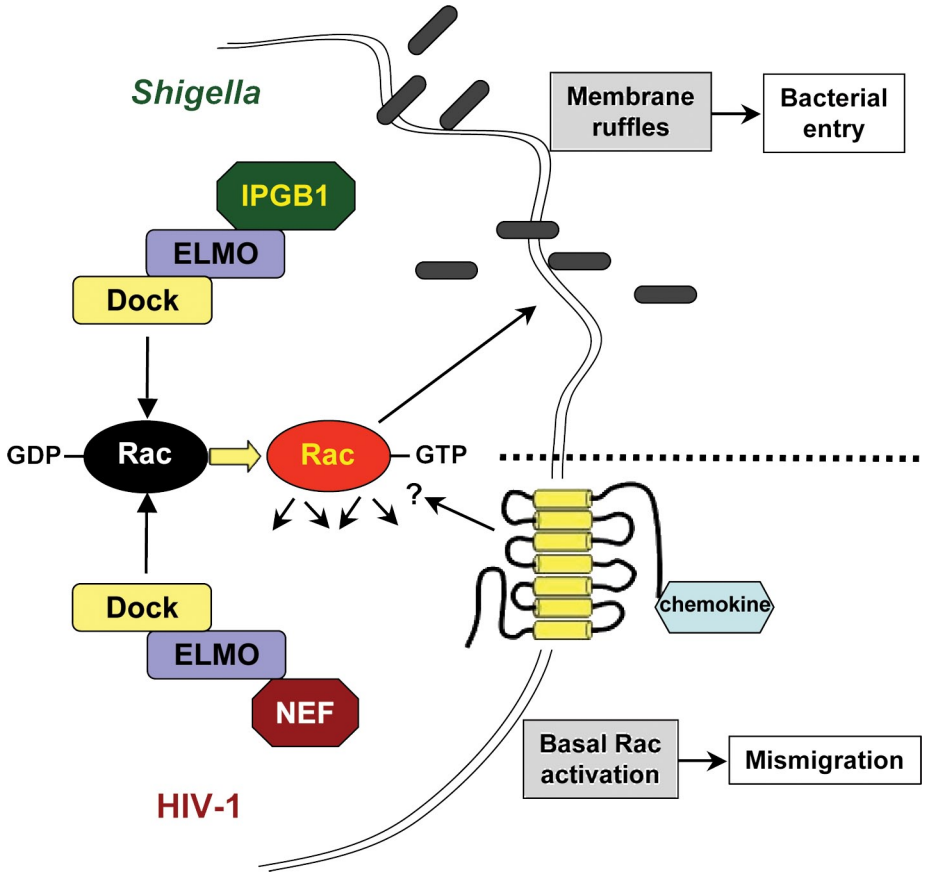

Figure 2. **Pathogens usurp the ELMO–Dock–Rac engulfment module.** Examples of mechanisms whereby microbial pathogens use the ELMO–Dock–Rac module to alter the host cellular response. The area above the broken line shows mechanism of enhanced *S. flexneri* invasion via IPGB1 interaction with ELMO, leading to enhanced Rac activation and membrane ruffles that serve as entry points for the bacteria. The area below the broken line shows that HIV-1 uses Nef interaction with the ELMO–Dock2 complex to disrupt CXCR4-dependent chemotaxis in CD4+ T cells.

or anticancer therapies will depend on gaining a better understanding of the role of factors released by apoptotic tumor cells.

Engulfment molecules in microbial pathogenesis

An emerging facet of engulfment signaling is how these pathways can be usurped by microbial pathogens. It has been known for some time that bacteria can hijack or mimic host signaling pathways to aid in pathogenic steps, including cell entry and immune evasion (Stebbins and Galán, 2001). This is achieved by delivery of bacterial effector proteins into the host cell that mimic a range of cellular activities. As key regulators of the cytoskeleton and numerous other cellular processes, small G proteins, particularly the Rho family (e.g., RhoA, Rac, and Cdc42), are frequent targets for these clever effector mechanisms (Mattoo et al., 2007). The signaling machinery that controls phagocyte morphology during apoptotic cell engulfment relies on these GTPases as well, and thus it is not surprising that several bacteria target these pathways. In particular, the RhoG–ELMO–Dock–Rac pathway has been found to be such a target (Fig. 2). The invasive pathogen *Shigella flexneri* utilizes a type III secretion system to inject effectors to promote entry into epithelial cells, including IPGB1 (Handa et al., 2007). IPGB1 promotes membrane ruffling via Rac activation in a mechanism that requires binding to ELMO1. The small GTPase RhoG acts upstream of ELMO1, and active RhoG-GTP interacts with ELMO1, and thereby recruits the ELMO–Dock180 complex to the membrane to promote Rac activation, membrane ruffling, and engulfment (Katoh and Negishi, 2003; deBakker et al., 2004). IPGB1 mimics the activity of RhoG-GTP, and the Rac-generated ruffles serve as a site of entry for *S. flexneri* (Handa et al., 2007). Similarly, *Yersinia enterocolitica* virulence factors Invasin and YopE also modulate Rac1 activity at the level of RhoG, and appear to do so in an ELMO–Dock180-dependent manner in cultured cells (Roppenser et al., 2009). However, neither of these *Y. enterocolitica* virulence factors have been reported to directly interact with ELMO–Dock180, and the role of this module was inferred by expression of a dominant-negative mutant of ELMO1 that did not further alter Rac activation in the presence of YopE (Roppenser et al., 2009).

Usurping the engulfment machinery is not exclusive to bacteria, and in fact can be used by viruses to promote pathogenesis. Janardhan et al. (2004) found that the Nef gene product of HIV-1 is able to complex with the ELMO2–Dock2 module in T cells to promote Rac activation. Further, we have found that Nef interacts with Dock2 in Jurkat T cells and promotes the activation of a key cytoskeletal Rac effector, p21-activated kinase (PAK; unpublished data). The outcome of this interaction appears to be dysregulated Rac activation, which is

associated with enhanced activation through the T cell receptor and improper CXCR4-dependent chemotaxis. However, the hijacking of the engulfment signaling machinery has only been shown using cultured cells, and it will be important to determine if in vivo pathogenesis is dependent on these activities as well.

Engulfment genes and other types of disease associations

Several recent studies have discovered associations with human disease and genetic mutations of components of the engulfment signaling machinery. For example, several point mutations in the intronic regions of *Elmo1* have been linked to diabetic nephropathy and diabetes (Shimazaki et al., 2005; Pezzolesi et al., 2009a,b). ELMO has also been shown to promote the invasive phenotype of glioblastoma cells in concert with Dock180 and Rac (Jarzynka et al., 2007). Although the role of MFG-E8 in cell clearance and self-tolerance in mice is well-established, there is now evidence that improper splicing of this gene in humans, which results in the production of a PtdSer-binding mutant protein, can be seen in some systemic lupus erythematosus patients (Yamaguchi et al., 2010). Finally, several mutations in engulfment-related genes have been seen in human diseases, including Alzheimer's disease, schizophrenia, and multiple types of cancer (Table I). It will be important to determine the contribution of these genes in these diseases and understand how they relate to engulfment- and nonengulfment-related cell signaling. As such, these studies point to the importance of the signaling molecules relevant for apoptotic cell engulfment, or the respective signaling pathways in disease, and may help unravel a few of the complex disease pathologies.

Conclusions

The past decade has seen an impressive expansion of our knowledge regarding the fundamentals of apoptotic cell clearance. Despite the complexity and what appears to be redundancy of this process, several key themes emerge, with relevance for disease onset and progression. First, the presence of excess apoptotic cells, particularly in a disease state, is not simply a sign of disease but is likely to have a role in pathogenesis. With the tools currently available, it is difficult to distinguish whether excess apoptotic cells observed in vivo are the result of normal cell death with failed clearance, or an increase in the rate of cell death. However, most tissues appear to have mechanisms in place to support very efficient clearance of apoptotic cells, and uncleared apoptotic cells thus likely represent, at least to some extent, a failure of clearance. A key question related to this idea is the relative contribution of the actual physical removal of the dying cell versus the anti-inflammatory signaling generated by this event in homeostasis and disease. This question will require a more thorough understanding of the signaling events that regulate these two closely related, but experimentally distinguishable, steps of engulfment. Finally, it is also important to carefully consider the impact of apoptotic cell clearance on progression of particular disease states, as in most cases apoptotic cell clearance is a beneficial event. Yet, as in the case of tumorigenesis, it may be that certain disease conditions are exacerbated by the clearance of apoptotic cells. The widespread role of apoptotic cell clearance in many tissues and the recent flood of information on this topic (using in vivo models) portend potentially therapeutic benefits by targeting the components of the engulfment machinery.

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