REVIEW

Engulfment signals and the phagocytic machinery for apoptotic cell clearance

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The clearance of apoptotic cells is an essential process for tissue homeostasis. To this end, cells undergoing apoptosis must display engulfment signals, such as 'find-me' and 'eat-me' signals. Engulfment signals are recognized by multiple types of phagocytic machinery in phagocytes, leading to prompt clearance of apoptotic cells. In addition, apoptotic cells and phagocytes release tolerogenic signals to reduce immune responses against apoptotic cell-derived self-antigens. Here we discuss recent advances in our knowledge of engulfment signals, the phagocytic machinery and the signal transduction pathways for apoptotic cell engulfment.

Several billion senescent or damaged cells in the body physiologically undergo apoptosis every day. Rapid removal of apoptotic cells from tissues is important for maintaining tissue homeostasis and preventing inappropriate inflammatory responses in multicellular organisms. During this process, apoptotic cells express engulfment signals such as 'find-me' and 'eat-me' signals that indicate they should be removed from tissues, and phagocytes engulf apoptotic cells using multiple types of phagocytic machinery. At this point, apoptotic cell phagocytosis is distinguished from other types of phagocytosis and is designated 'efferocytosis' ('effero' means 'to carry to the grave').[1] This review focuses on several recent advances in our understanding of engulfment signals, the phagocytic machinery and signal transduction during efferocytosis.

ENGULFMENT SIGNALS

'Find-me' signals

Cells undergoing apoptosis secrete molecules, so-called 'find-me' signals (also referred to as 'come-to-get-me' signals), to attract phagocytes toward them. To date, four representative 'find-me' signals have been identified, including lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), CX3C motif chemokine ligand 1 (CX3CL1, also referred to as fractalkine), and nucleotides (ATP and UTP; Figure 1). LPC is released from apoptotic cells and binds to the G-protein-coupled receptor G2A on macrophages, facilitating the migration of macrophages to apoptotic cells.[2] In apoptotic cells, caspase-3 activation induces cleavage and activation of calcium-independent phospholipase A2 (iPLA2; also referred to as PLA2G6), which in turn processes phosphatidylcholine into LPC.[3] Recently, ATP-binding cassette transporter A1 (ABCA1) was shown to be required for the release of LPC from apoptotic cells.[4] CX3CL1 is generated as a membrane-associated protein and then released from apoptotic cells by proteolytic processing.[5] The secreted CX3CL1 binds to CX3C motif chemokine receptor 1 (CX3CR1) on microglia and macrophages, resulting in the migration of phagocytes. However, the roles of LPC and CX3CL1 as 'find-me' signals have not been clarified in an in vivo animal model. S1P is generated from sphingosine by sphingosine kinase. It is secreted by dying cells in a caspase-3-dependent manner and binds to S1P receptors on macrophages, leading to the recruitment of macrophages to apoptotic cells.[6] Nucleotides, including ATP and UTP, are released from apoptotic cells in a caspase-3-dependent manner and are sensed by purinergic receptors on phagocytes, resulting in the recruitment of phagocytes to apoptotic cells.[7] The release of nucleotides from apoptotic cells is mediated by pannexin 1 channels, which are activated in apoptotic cells in a caspase-3-dependent manner.[8] Although these molecules are defined as 'find-me' signals, many unanswered questions remain to be elucidated, including their reaction range, functional mode (cooperativity or redundancy) and in vivo relevance.

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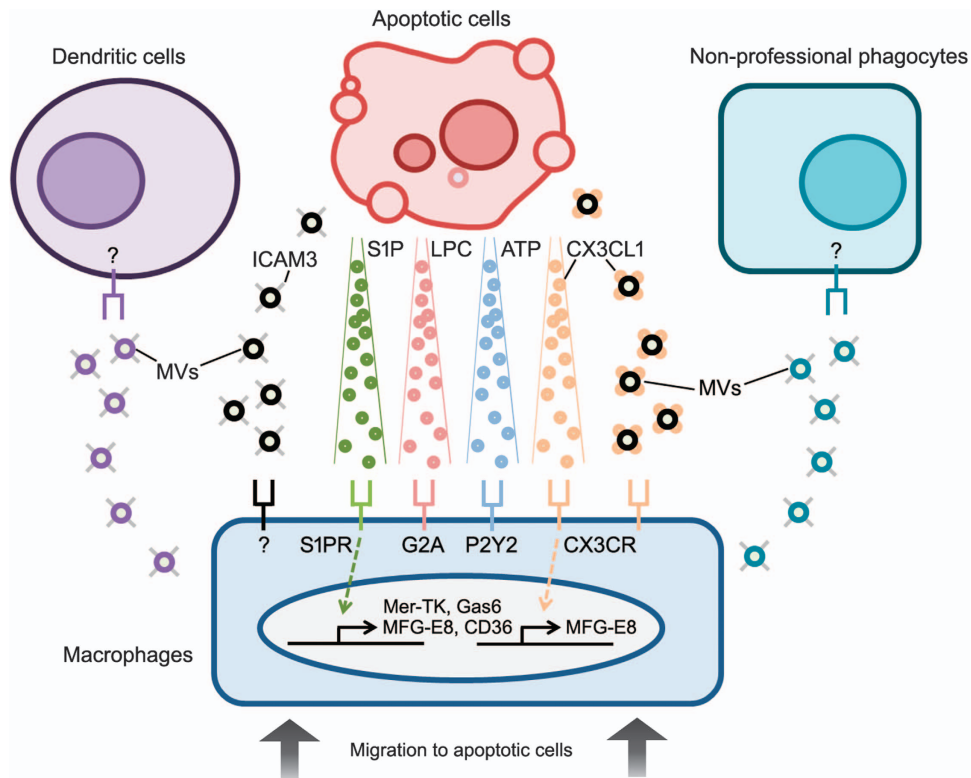Received 6 December 2016; accepted 15 December 2016

Figure 1 'Find-me' signals released by apoptotic cells and extracellular vesicles. Four representative 'find-me' signals released by apoptotic cells have been identified, including S1P (sphingosine-1-phosphate), LPC (lysophosphatidylcholine), nucleotides (ATP or UTP) and CX3CL1 (CX3C motif chemokine ligand 1; fractalkine). They bind to S1PR, G2A, P2Y2 and CX3CR, respectively, on the phagocyte surface, promoting phagocyte migration to apoptotic cells. Extracellular vesicles released by apoptotic cells and phagocytes appear to modulate functions of phagocytes during efferocytosis. Apoptotic cell-derived microparticles also attract macrophages to sites of cell death through CX3CL1 and ICAM3. Phagocyte-derived microvesicles and exosomes modulate phagocytic capacity in epithelial cells and the transfer of apoptotic cell-derived antigens to dendritic cells, respectively.

In addition, 'find-me' signals have multiple roles in efferocytosis. CX3CL1 appears to upregulate MFG-E8 expression in microglial cells and peritoneal macrophages.[9,10] S1P released by apoptotic cells acts as an anti-apoptotic mediator and attenuates macrophage apoptosis,[11] suggesting that apoptotic cells can prevent damage to neighboring cells to maintain tissue homeostasis. Recently, S1P has been shown to trigger the activation of erythropoietin (EPO)–EPO receptor (EPOR) signaling, which increases the expression of phagocytic receptors through peroxisome proliferator-activated receptor-γ.[12]

'Eat-me' signals

Dying cells also express 'eat-me' signals on the cell surface to indicate they should be engulfed by macrophages (Figure 2). Although a variety of potential 'eat-me' signals have been proposed, the best-characterized 'eat-me' signal is the expression of phosphatidylserine on the cell surface. Phosphatidylserine is a plasma membrane phospholipid that is localized on the inner membrane leaflet of the lipid bilayer in healthy cells and externalized on the cell surface in response to apoptotic stimuli.[13] The externalization of phosphatidylserine on the cell surface during apoptosis and its role in cell corpse clearance has also been identified in *Caenorhabditis elegans* and *Drosophila*.[14,15]

Recently, Xk-related protein 8 (Xkr8) has been shown to mediate surface expression of phosphatidylserine in apoptotic cells in a caspase-3-dependent manner.[16] This process is also mediated by the Xkr8 ortholog CED-8 in *Caenorhabditis elegans*, indicating a conserved mechanism for apoptotic phosphatidylserine exposure.[17] More recently, Xkr8 has been shown to associate with basigin or neuroplastin at the plasma membrane in response to apoptotic stimuli, and this complex is required for the proper scrambling activity of Xkr8.[18] In addition, the P-type ATPase ATP11C acts as a flippase to transport aminophospholipids from the outer leaflet to the inner leaflet of the lipid bilayer to maintain membrane asymmetry. In cells undergoing apoptosis, it is inactivated by caspase-3-mediated cleavage, permitting phosphatidylserine externalization.[19]

Calreticulin (CRT) is another potential 'eat-me' signal expressed on the apoptotic cell surface. In dying cells induced by endoplasmic reticulum (ER) stress, activated protein kinase RNA-like ER kinase phosphorylates eIF2α, which induces caspase-8 activation, Bap31 cleavage and Bax activation, resulting in the translocation of CRT from the ER to the Golgi and SNARE-mediated exocytosis.[20] CRT on the apoptotic cell surface is sensed by low-density lipoprotein receptor-related protein (also referred to as CD91) on phagocytes to promote
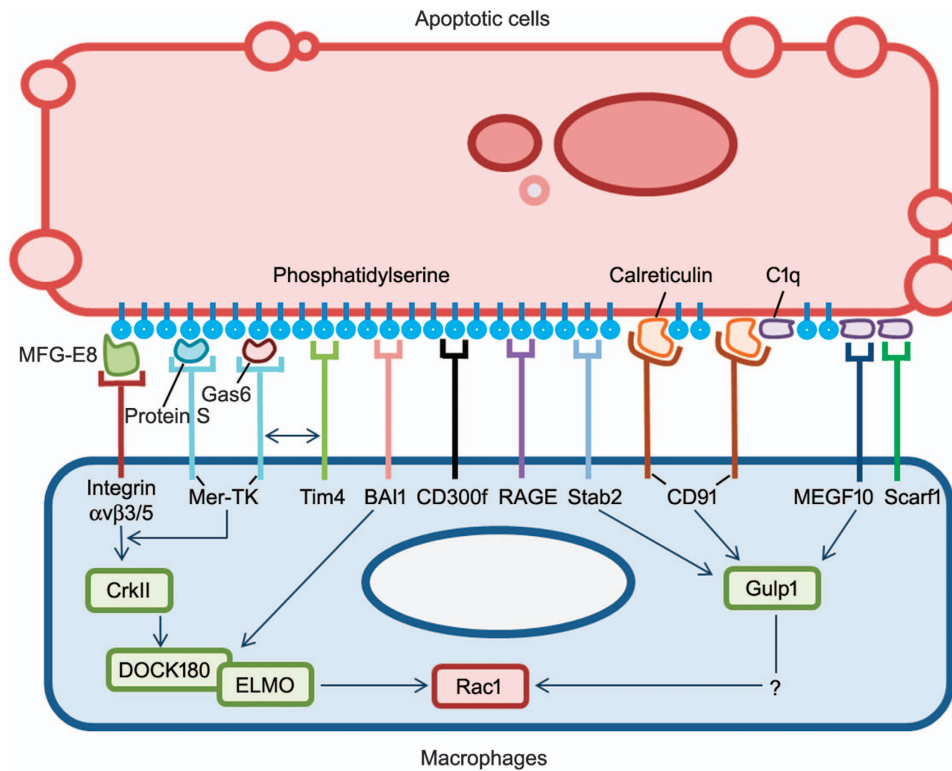
Figure 2 'Eat-me' signals, phagocytic machinery and signaling pathways. Apoptotic cells express 'eat-me' signals, such as phosphatidylserine and calreticulin, on the cell surface in response to apoptotic stimuli. Exposed phosphatidylserine on the apoptotic cell surface is recognized directly by phosphatidylserine receptors (Tim family proteins, BAI1, Stabilin-2, CD300f and RAGE) or indirectly by bridging molecules (MFG-E8, Gas6, protein S and C1q). MFG-E8 bound to phosphatidylserine is recognized by integrin αvβ3/5 on the phagocytes, and Gas6 or protein S bound to phosphatidylserine is sensed by Mer-TK. Bridging molecule C1q is recognized by MEGF10 or scarf1. Another 'eat-me' signal, calreticulin, is associated with phosphatidylserine or C1q on the apoptotic cell surface and recognized by CD91 (LRP1). Integrin αvβ3/5 and BAI transduce signals for cytoskeletal rearrangement through DOCK180/ELMO1, whereas Stabilin-2, MEGF10 and CD91 use adaptor protein Gulp1 as an engulfment signaling pathway.

engulfment.[21] Recently, CRT is shown to bind to phosphatidylserine via its C-terminal acidic region, leading to apoptotic cell phagocytosis.[22] Furthermore, phagocytosis of cells expressing CRT on the cell surface appears to induce immunogenic responses,[23] suggesting that recognition of CRT by specific phagocytes, especially dendritic cells, might trigger immunogenic signals rather than self-tolerance signals. However, it is unclear whether recognition of CRT is sufficient to trigger a signal to induce immunogenic responses in phagocytes (macrophages and dendritic cells). CRT is also expressed on the cell surface of macrophages through TLR and Btk signaling, stimulating cancer cell phagocytosis.[24] It is therefore possible that CRT is a PS-binding bridging molecule released from apoptotic cells and phagocytes rather than an 'eat-me' signal.

In addition to the role of phosphatidylserine as an 'eat-me' signal, the recognition of phosphatidylserine by phagocytes can enhance cholesterol efflux from cells to maintain cellular homeostasis[25] and trigger release of anti-inflammatory cytokines to induce immunogenic tolerance for apoptotic cell-derived antigens.[26] These findings suggest that phosphatidylserine exposure is not only an 'eat-me' flag to detect apoptotic cells but also a trigger of endogenous signaling for cellular homeostasis in phagocytes. However, the molecular

details of phosphatidylserine-mediated signaling remain to be clarified further.

'Don't eat-me' signals
Healthy cells display 'don't eat-me' signals, such as CD47 and CD31, on the cell surface to avoid efferocytosis. CD47 (also referred to as integrin-associated protein) is a membrane protein composed of an immunoglobulin (Ig) domain, five membrane-spanning regions and cytoplasmic region. Oldenborg et al.[27] found that CD47-deficient erythrocytes injected into mice are more rapidly removed by splenic macrophages than are CD47-positive erythrocytes. They suggested that CD47 functions as a signal for discrimination between self and non-self. In healthy cells, CD47 interacts with signal regulatory protein alpha (SIRPα; also referred to as SHPS-1 and CD172a) on macrophages. The CD47–SIRPα interaction induces tyrosine phosphorylation of the immunoreceptor tyrosine-based inhibitory motif in the SIRPα cytoplasmic tail and subsequent recruitment and activation of the inhibitory tyrosine phosphatases SHP-1 and SHP-2, resulting in the negative regulation of actin cytoskeletal rearrangement for phagocytosis. Senescent or damaged cells exhibit decreased CD47 expression or an altered pattern of CD47 distribution,

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thereby permitting efferocytosis.[21,28] Several cancer cell types, such as circulating leukemic stem cells and acute myeloid leukemia, were found to highly express CD47 on their surface to evade immune cells.[29,30] Recent studies showed that a neutralizing CD47 antibody or soluble SIRPα variants promote tumor cell engulfment by macrophages and suppress tumor growth in *in vivo* tumor models.[31–33]

Another candidate 'don't eat-me' signal is CD31 (also referred to as platelet and endothelial cell adhesion molecule 1). A CD31–CD31 homotypic interaction between viable neutrophils and phagocytes acts as a repulsive signal, thereby mediating detachment of viable cells from phagocytes. In contrast, apoptotic cells do not trigger this repulsive signal and are efficiently engulfed by phagocytes.[34] However, the intracellular signaling pathways for CD31-mediated repulsion remain to be clarified.

Extracellular vesicles

Almost all cells release membrane vesicles, which play an important role in intercellular communications.[35] Apoptotic cells can mediate the recruitment of phagocytes through the release of microparticles (Figure 1). ICAM-3 in apoptotic cell-derived microparticles induces the migration of macrophages towards apoptotic cells.[36] CX3CL1-positive microparticles are shown to induce the recruitment of macrophages to apoptotic cells.[37] Adipocyte-derived microparticles are released in a caspase-3 and Rho-kinase-dependent manner and facilitate microphage migration to obese adipose tissues.[38] Recently, microparticles released from apoptotic cells have been shown to induce immune responses to apoptotic cell-derived antigens in the presence of IFN-α.[39] Chromatin on the apoptotic cell surface appears to be a self-antigen that triggers immunogenic responses.[40] Thus, microparticles elicited from apoptotic cells might be removed to maintain tissue homeostasis and prevent aberrant inflammation. However, the clearance mechanism of apoptotic cell-derived microparticles remains to be investigated.

Phagocytes also appear to emit microparticles.[41] Recent studies showed that macrophages can communicate with other professional or nonprofessional phagocytes through the release of extracellular vesicles (Figure 1). Insulin-like growth factor-1 (IGF-1) released from macrophages promotes the engulfment of macrophage-derived microvesicles by epithelial cells, leading to reduced inflammatory responses in epithelial cells.[42] Macrophages are capable of transferring dead-cell-associated antigens to dendritic cells through the release of exosomes in a ceramide-dependent manner.[43] These observations suggest that microvesicles derived from apoptotic cells or phagocytes can modulate efferocytosis.

TOLEROGENIC SIGNALS

In the absence of infection or inflammation, apoptotic cell clearance is immunogenically silent. At this point, apoptotic cells and phagocytes might express signals to suppress the immune response to self-antigens. Apoptotic cells release signals to inhibit the recruitment of inflammatory cells, known as 'keep out' or 'stay away' signals. Lactoferrin is expressed in response to apoptotic stimuli and selectively inhibits the migration of granulocytes (neutrophils and eosinophils) but not monocytes and macrophages.[44,45] However, the role of lactoferrin in the negative regulation of the migration of inflammatory cells requires clarification in an *in vivo* animal model.

Annexin A1 was originally defined as an engulfment signal for the efficient clearance of apoptotic cells.[46] Annexin A1 on the apoptotic cell surface is known to inhibit dendritic activation, which in turn inhibits inflammatory cytokines and T-cell activation for apoptotic cell-derived antigens.[47] Annexins A5 and A13 also suppress dendritic cell activation for apoptotic cell-derived antigens, resulting in immunogenic tolerance.[48] However, deficiency in individual annexins did not show an obvious phenotype such as autoimmunity, suggesting that annexin proteins may have a redundant function. Thus, it remains to be defined whether annexin proteins are tolerogenic factors to suppress immune responses for apoptotic cell-derived antigens.

The 12/15-lipoxygenase in resident peritoneal macrophages causes the cell surface exposure of oxidized phosphatidylethanolamine, which sequesters the MFG-E8 required for the clearance of apoptotic cells in inflammatory monocytes, suggesting that oxidized phosphatidylethanolamine on resident macrophages may be a signal to reduce immune responses.[49] Recently, the chromatin on microparticles secreted from apoptotic cells was shown to be a self-antigen that induces immunogenic responses. In this context, DNase1L3 produced by macrophages and dendritic cells digests chromatin in apoptotic cell-derived microparticles,[40] suggesting that secreted DNase1L3 is a molecular mechanism for achieving immune tolerance for apoptotic cell-associated antigens.

PHAGOCYTIC MACHINERY

Phagocytes can recognize phosphatidylserine on the apoptotic cell surface through two types of phosphatidylserine recognition machinery: phosphatidylserine receptors and soluble bridging molecules. Phosphatidylserine receptors on the surface of phagocytes directly bind to phosphatidylserine on apoptotic cells, whereas soluble bridging molecules recognize phosphatidylserine on the apoptotic cell surface and function as a bridge between apoptotic cells and cell surface receptors on phagocytes (Figure 2).

Phosphatidylserine receptors

T-cell immunoglobulin and mucin domain-containing molecule (Tim) family proteins, Tim-1 (also referred to as kidney injury molecule 1 (Kim-1)), Tim-3 and Tim-4, act as phosphatidylserine receptors to clear apoptotic cells.[50–52] Tim-1 and Tim-4 bind to phosphatidylserine through a metal-ion-dependent ligand-binding site in their immunoglobulin V domain.[53] Tim-1 is highly expressed in damaged kidney epithelial cells and confers phagocytic capacity to them.[54] Tim-1-mediated efferocytosis is responsible for protecting the kidney after acute injury through PI3K-dependent

downregulation of NF-κB.[55] Tim-3 is expressed in peritoneal exudate cells and CD8-positive dendritic cells and contributes to the clearance of apoptotic cells and cross-presentation of apoptotic cell-associated antigens.[52] Tim-4 is expressed by professional phagocytes (macrophages and dendritic cells) and controls phosphatidylserine-dependent efferocytosis and adaptive immunity.[50,56] However, Tim-4 does not seem to transduce a signal for engulfment, which suggests that Tim-4 functions as a tethering receptor to recognize phosphatidylserine on the apoptotic cell surface and may be required for other proteins to trigger internalization of apoptotic cells.[57] Indeed, recent studies identified that Mer-TK and integrin β1 act as partners to transduce signals after Tim-4-mediated phosphatidylserine recognition.[58,59]

Brain-specific angiogenesis inhibitor 1 (BAI1) is a member of the G-protein-coupled receptor family; it has seven transmembrane regions and binds to phosphatidylserine through its thrombospondin type 1 repeats.[60] BAI1 interacts with the DOCK180/ELMO1 complex through an α-helical region in its cytoplasmic tail, thereby providing the signal for Rac1 activation. However, BAI1 is predominantly expressed in neuronal cells of the cerebral cortex,[61] suggesting that its role may be tissue-specific. Recently, BAI1 is known to contribute to phagosome formation and transport during the phagocytosis of apoptotic neurons by microglial cells.[62] In skeletal muscle, BAI1 and its homologous protein BAI3 bind to apoptotic myoblasts and transduce signals to fuse myoblasts.[63,64]

Stabilin-2 (also referred to as hyaluronic acid receptor for endocytosis (HARE) and FEEL-2) is a large membrane protein that is composed of seven FAS1 domains, eight atypical epidermal growth factor (EGF)-like domains, fifteen EGF-like domains, a Link domain, a transmembrane region and a cytoplasmic domain.[65] Stabilin-2 binds to phosphatidylserine via its EGF-like domain repeats, promoting apoptotic cell engulfment.[66] The histidine residue in the PS-binding loops is conserved in four EGF-like-domain repeats and plays an important role in pH-dependent phagocytic activity.[67] Stabilin-1 (also referred to as CLEVER-1 and FEEL-1), a homologous protein of stabilin-2, mediates apoptotic cell engulfment through phosphatidylserine recognition.[68] Stabilin-1 and -2 are expressed in sinusoidal endothelial cells and macrophages.[65,69–71] In hepatic endothelial cells, they act as tethering receptors for the capture of phosphatidylserine-exposed damaged erythrocytes through phosphatidylserine recognition.[72] However, the functions of stabilin-1 and -2 on efferocytosis require clarification in a knockout mouse model.

CD300 family proteins, including CD300b and CD300f, have recently been shown to act as phosphatidylserine recognition receptors to clear apoptotic cells.[73,74] CD300f regulates the engulfment of apoptotic cells via the PI3K pathway, leading to the activation of Rac1/Cdc42 GTPases to regulate F-actin.[75] CD300b is associated with DAP12 through its ITAM motif and activates the PI3K/Akt pathway.[73] In contrast, another CD300 family protein, CD300a, inhibits the uptake of apoptotic cells through binding to phosphatidylserine and phosphatidylethanolamine.[76] How the recognition of phosphatidylserine by CD300 family proteins induces stimulatory and inhibitory signals for apoptotic cell removal remains to be investigated.

Receptor for advanced glycation end products (RAGE) also binds to phosphatidylserine and has a role in the clearance of apoptotic cells.[77] RAGE is a type I membrane protein that belongs to the immunoglobulin protein family and specifically binds to phosphatidylserine. However, various soluble forms of RAGE also bind to phosphatidylserine on the apoptotic cell surface, thereby preventing apoptotic cell engulfment by phagocytic receptors. The physiological role of soluble RAGE proteins remains to be studied. In addition, several scavenger receptors have been proposed as receptors for apoptotic cell clearance, including CD36 and CD14. CD36 associates with integrin to engulf apoptotic cells in a thrombospondin-dependent manner and directly binds to oxidized phosphatidylserine.[78,79] CD14 has been proposed to be a receptor for apoptotic cell engulfment.[80] Studies of CD14-deficient mice showed that CD14 is a tethering receptor but not an engulfment receptor for apoptotic cells.[81]

Bridging molecules that recognize phosphatidylserine

Several soluble proteins have been identified as bridging molecules that recognize the 'eat-me' signals on the surface of apoptotic cells, including milk fat globule EGF factor 8 (MFG-E8, also referred to as lactadherin), growth arrest-specific 6 (Gas6), protein S and C1q. They bind to both phosphatidylserine on the apoptotic cell surface and phagocytic receptors on phagocytes, providing a link between apoptotic cells and phagocytes. MFG-E8 secreted by macrophages and immature dendritic cells binds to phosphatidylserine on apoptotic cells through its C1 and C2 domains and interacts with αvβ3 or αvβ5 integrin on phagocytes through the RGD (Arg–Gly–Asp) motif in its EGF domain, resulting in the promotion of apoptotic cell phagocytosis.[82,83] Gas6 and protein S share a similar domain structure and bind to phosphatidylserine on apoptotic cells to promote efferocytosis.[84,85] They are composed of a Gla domain at the N terminus, four EGF-like domains, and two laminin G-like domains at the C terminus. They bind to phosphatidylserine in a calcium-dependent manner via their Gla domain and associate with Tyro3–Axl–Mer (TAM) family tyrosine-kinase receptors on phagocytes through laminin G-like domains.[86,87]

Mer tyrosine kinase (Mer-TK) is the best-characterized TAM receptor and is known to transduce an important signal for apoptotic cell engulfment.[88] Mer-TK signaling is functionally associated with multiple engulfment systems for the efficient removal of apoptotic cells. Signaling from Mer-TK is induced by binding to Gas6 and functionally associated with αvβ5 integrin-mediated signaling.[89] Scavenger receptor A (SR-A) associates with Mer-TK to transduce signals during apoptotic cell engulfment.[90] Galectin-3 was found to be a new ligand of Mer-TK for apoptotic cell clearance.[91] Mer-TK mediates signal transduction from Tim-4-mediated efferocytosis.[58] Considering that Mer-TK plays a crucial role in inhibiting dendritic cell activation for apoptotic cell-associated antigens,[92] it is possible that Mer-TK has a common

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role in multiple engulfment systems to regulate immune responses. Recently, the TAM receptor tyrosine kinases Mer-TK and Axl were shown to function as phagocytic receptors under different environments. Mer-TK is primarily expressed at steady-state or under immune suppressive conditions and maintains immune tolerance, whereas Axl is expressed in response to proinflammatory stimuli and suppresses immune responses.[93]

Another potential bridging molecule is C1q, the first component of complement, which binds to phosphatidylserine on the apoptotic cell surface.[94] C1q binds to apoptotic cells likely via its globular head and interacts with calreticulin-CD91 on phagocytes to promote apoptotic cell engulfment.[95] SCARF1 (also referred to as scavenger receptor expressed by endothelial cell 1) acts as a receptor that recognizes C1q bound to apoptotic cells.[96] Recently, MEGF10 has also been shown to mediate apoptotic neuron clearance by astrocytes through bridging molecule C1q.[97] Furthermore, the activation of macrophages by C1q regulates Mer-TK and Gas6 expression.[98]

Possible link between many engulfment signals and the phagocytic machinery

Why are multiple phagocytic receptors necessary for efficient efferocytosis? Such multiple apoptotic cell recognition systems may be useful at several levels. First, discriminating dying cells from live cells during efferocytosis is important for proper cellular turnover. Although several 'eat-me' signals are present, there are many cases in which phosphatidylserine is substantially expressed on the cell surface of live cells, including cell-cell fusion, T-cell activation and platelet activation.[99] It is possible that specificity for apoptotic cell recognition can be improved if multiple phagocytic receptors bind to specific 'eat-me' flags on the apoptotic cell surface, or that sufficient mechanical force for efferocytosis can be provided by multiple phosphatidylserine recognition systems. Second, the eating of apoptotic cells by macrophages requires various cellular events, including the tethering of apoptotic cells on phagocytes, cytoskeletal rearrangement for internalization, suppression of immune responses, and disposal of metabolic burden. Thus, multiple phagocytic receptors may be required to perform various cellular processes during efferocytosis. Recently, several receptors have been proposed to act cooperatively for efferocytosis. Stabilin-2 associates with integrin αvβ5 through its FAS1 domain and functions cooperatively for apoptotic cell engulfment.[100] In peritoneal macrophages, Tim-4 functions as a tethering receptor for adhesion between apoptotic cells and macrophages, and Mer-TK acts as a tickling receptor to transduce signals for cytoskeletal rearrangement.[58] During the engulfment of apoptotic neurons, BAI1 is involved in the formation and transport of phagosomes, whereas Tim-4 contributes to phagosome stabilization.[62] Third, particular types of phagocytic machinery for phosphatidylserine recognition may be required for the efficient efferocytosis of specific phagocytes or under specific conditions. For example, Tim-4 is indispensable for tissue homeostasis in resident peritoneal macrophages, whereas MFG-E8 is essential for apoptotic cell

clearance in inflammatory macrophages.[101] Mer-TK is activated by Gas6 or protein S and acts as a receptor for the maintenance of self-tolerance in resting macrophages. In contrast, Axl is activated by only Gas6 under inflammatory conditions and acts as a receptor for immune suppression.[93]

SIGNALING FOR APOPTOTIC CELL ENGULFMENT
Signaling pathways for cytoskeletal rearrangement

Genetic analyses in *Caenorhabditis elegans* identified three signaling pathways that mediate apoptotic cell clearance: (1) the CED-1, 6 and 7 pathway; (2) the CED-2, 5, and 12 pathway; and (3) the ABI-1 and ABL-1 pathway.[102–105] In the first pathway, multiple EGF-like domains 10 (MEGF10) and Jedi (also referred to as MEGF12), mammalian homologs of CED-1, act as phagocytic receptors for apoptotic cell clearance.[106,107] MEGF10 indirectly recognizes phosphatidylserine on the apoptotic cell surface through the bridging molecule Clq,[97] whereas the molecular mechanism by which apoptotic cells are recognized by Jedi remains to be studied. MEGF10 and Jedi bind to Gulp1 (phosphotyrosine-binding domain-containing engulfment adaptor protein 1), a mammalian ortholog of CED-6, through an NPxY motif in their cytoplasmic region, leading to the transduction of a signal for cytoskeletal rearrangement.[108,109] ABCA1 and ABCA7, mammalian orthologs of CED-7, are members of the ATP-binding cassette containing transporter family that transport a variety of substances across the plasma membrane. They are involved in apoptotic cell clearance through unknown mechanisms.[110,111] ABCA1 also has multiple functions in apoptotic cells and phagocytes during efferocytosis, including the release of 'find-me' signals, protection from oxidative stress-induced apoptosis, and enhancement of cholesterol efflux.[4,25,108,112] The Gulp1 signaling pathway converges on Rac1 (an ortholog of *Caenorhabditis elegans* CED-10)[113] and is the downstream signaling pathway through which several phagocytic receptors, such as low-density lipoprotein receptor-related protein-1, Stabilin-1 and Stabilin-2, regulate apoptotic cell engulfment.[114–116] Stabilin-2 is known to coordinate the activities of the two phagocytic pathways (the Gulp1 pathway and ELMO1/DOCK180 pathway) through a direct interaction with integrin αvβ5.[100] However, the intermediates between Gulp1 and Rac1 in this pathway are largely unknown. In the second pathway, the mammalian homologs of CED-2, 5 and 12 are CrkII, DOCK180 and ELMO1, respectively. CrkII associates with DOCK180, a guanine-nucleotide exchange factor, which in turn triggers Rac1 activation.[117] ELMO1 associates with DOCK180 and acts as a positive regulator of Rac1 activation in *Caenorhabditis elegans* and mammalian cells.[118,119] In addition, TRIO/UNC-73 and RhoG/MIG-2 signaling also contribute to DOCK180-mediated Rac1 activation for proper phagocytosis.[120] This pathway is downstream of the PS-receptor BAI1 as well as integrin αvβ5.[60,117] In the third signaling pathway, ABI-1 (Abi) promotes apoptotic cell clearance through regulation of Rac-1 activity or an independent pathway. ABL-1 interacts with ABI-1 and negatively regulates engulfment by inhibiting ABI-1.[121] However, the role

of the mammalian counterparts of the genes involved in this pathway remains to be defined.

Other signaling pathway during apoptotic cell engulfment

Signaling of the tumor suppressor p53 is shown to regulate apoptotic cell engulfment. p53 controls phagocytosis of apoptotic cells by regulating the expression of death domain 1α.[122] Death domain 1α is an immunoglobulin superfamily receptor that mediates homophilic interactions between apoptotic cells and phagocytes, leading to the removal of apoptotic cells. The phosphatidylserine receptor BAI1 is also a specific target of p53 in the brain.[61] However, the molecular mechanism by which p53 signaling is activated in phagocytes remains to be defined. Several factors that can regulate cellular metabolic processes have been proposed as modulators for efferocytosis. Uncoupled protein 2, which reduces mitochondrial membrane potential in cells through uncoupling oxidative phosphorylation from ATP generation, has been shown to positively regulate the engulfment capacity of phagocytes.[123] Peroxisome proliferator-activated receptors and liver X receptors are activated by the engagement of apoptotic cell and regulate apoptotic cell engulfment, likely increasing the expression of phagocytic receptors or bridging molecules.[124–126] The nuclear receptor Nr4a1 contributes to anti-inflammatory effects during apoptotic cell phagocytosis.[127] These findings suggest that apoptotic cell clearance is associated with metabolic processes. However, it remains to be seen how the recognition of apoptotic cells activates nuclear receptors.

CONCLUSIONS

Apoptosis and efferocytosis are processes for homeostatic cell turnover in multicellular organisms, and proper corpse clearance is important to prevent inappropriate inflammatory responses such as autoimmunity. Over the past two decades, numerous studies have been performed to unveil the molecular mechanisms of apoptotic cell clearance, leading to a significant increase in our knowledge of this area. However, multiple unanswered questions concerning clearance mechanisms remain. Why are multiple phagocytic components necessary for efferocytosis? What is the signaling cascade mediated by receptors for engulfment signals? Can particular engulfment signals or phagocytic mechanisms determine immunogenic or tolerogenic clearance of apoptotic cells? To answer these questions, further understanding of engulfment signals and the phagocytic machinery is required. Furthermore, defective clearance of apoptotic cells in tissues is associated with the pathogenesis of various diseases, including autoimmune diseases, chronic obstructive pulmonary disease, atherosclerosis, Alzheimer's disease and cancer.[128] Thus, an understanding of the precise mechanism of apoptotic cell engulfment could be useful for the development of therapeutic strategies for controlling diseases associated with defective efferocytosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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