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Review

Phagocytosis of apoptotic cells: a matter of balance

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Abstract. Efficient clearance of apoptotic cells is required to control homeostasis in normal and pathological circumstances, and inappropriate clearance of cell corpses may lead to autoimmune diseases and inflammation. The multiplicity of phagocytotic mechanisms points to the relevance of removing apoptotic cells. A variety of surface molecules present in either the apoptotic bodies or phagocytes help in attachment and initiation of engulfment. Nonetheless, uncontrolled phagocytosis of apoptotic cells and other particles may lead to tissue injury; therefore, negative signals are important in balancing phagocytotic activity. This review aims at a systematic examination of positive and negative signals that modulate the uptake of apoptotic bodies and the signaling mechanisms involved in the clearance of apoptotic cells.

Key words. Apoptosis; programmed cell death; phagocytosis; macrophage; prion.

Introduction: clearance of apoptotic cells

Programmed cell death occurs constantly during both development and homeostasis and can generate large numbers of apoptotic bodies, i.e. the fragmented pieces of apoptotic cells, in degenerative diseases. These apoptotic bodies must be cleared to avoid tissue damage and inflammatory responses. The relevance of this process is highlighted by the large proportion of genes involved in the clearance of degenerating cells, relative to the total number of genes affecting programmed cell death (PCD), in the nematode *Caenorhabditis elegans*. Of 14 genes identified as being related to PCD (*ced* genes), at least 7 code for products involved in the clearance of dead cells (*ced-1*, *ced-2*, *ced-5, ced-6*, *ced-7, ced-10* and *ced-12*) [1–3]. Interaction with apoptotic cells usually produces an active anti-inflammatory response by macrophages, which is important in preserving homeostasis [4–6].

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Phagocytes constantly monitor cells, which can display either ‘eat me’ or ‘don’t eat me’ signals. Normal self cells present molecules that promote detachment from phagocytes or that inhibit phagocytic activity. Upon the induction of apoptosis, cells stop presenting these signals or disable them and begin to express surface markers that are recognized by phagocytes as positive signals for engulfment. In turn, phagocytes employ a variety of receptors and other surface molecules to recognize, attach and initiate phagocytosis of apoptotic cells.

A large variety of molecules involved in apoptotic cell phagocytosis have been identified both in cell culture systems and in vivo. Cultures containing isolated phagocytes that are allowed to interact with apoptotic cells are a major preparation used to identify these molecules. Recognition or internalization can be modulated by the addition of competitors or adjuvants of putative recognition systems, as well as inhibitors of intracellular signal transduction pathways [7–10]. An additional source of information are organisms (usually mice or *C. elegans*)

lacking proteins implicated in the clearance of apoptotic cells, some of which show persistence of apoptotic cells that are associated with impaired development or certain diseases [11–13].

The mechanisms of phagocytosis depend on the apoptotic cell type, the means of induction of cell death, the stage of apoptosis, the type and state of differentiation of the phagocyte, and the surrounding microenvironment [7, 14–16]. The present review examines the signals for phagocytosis of apoptotic cells described in a variety of experimental models and the major components of their signaling pathways identified to date. Both the positive signals driving the process of phagocytosis, which have been widely discussed in the literature, and the counteracting negative signals, which have been shown to be critical for this process, will be discussed.

'Eat me' signals on the surface of apoptotic cells

Apoptosis triggers changes in dying cells that are required for the recruitment of phagocytes, for tethering of the cells to the phagocytes and, finally, for engulfment. The surface of apoptotic cells exhibits a wide range of molecules that are absent in normal self cells, as well as modified versions of 'normal' molecules (table 1). The best-characterized signal of apoptotic cells is the exposure of the anionic phospholipid phosphatidylserine (PS) [17]. Usually PS is restricted to the inner layer of the plasma membrane, and this asymmetry is maintained by two mechanisms: (i) the action of a translocase of aminophospholipids, i.e. a Mg^{2+}-ATPase inhibited by Ca^{2+}, which transfers PS from the external layer to the inner layer, and (ii) the binding of PS to proteins of the membrane-associated cytoskeleton, e.g. fodrin. Artificial externalization of PS via treatment with calcium ionophores generates other ligands in addition to PS because inhibitors of integrin- or lectin-recognition systems impair the uptake of the treated cells. Thus, PS externalization and loss of membrane asymmetry may involve the exposure of other signals for phagocytosis apart from PS itself [18].

Exposure of PS is considered an early sign of apoptosis, but necrotic cells display PS even before the disintegration of their membranes [19, 20], and normal cells, such as activated T cells, activated platelets, differentiating myoblasts, myocardioblasts, megakaryocytes and macro-

Table 1. Molecules involved in promoting recognition or engulfment of apoptotic cells

Phagocyte receptor	Apoptotic cell	Extracellular signal	References
Lectins	modified sugar chains		[37–41, 85, 86]
PS receptor	PS	(annexin I)	[34, 35]
Scavenger receptor A	PS?		[46]
Scavenger receptor BI	PS?		[47, 76]
LOX-1	PS?		[48]
CD68	PS?, oxidized sites		[42–45]
CD14	ICAM-3		[36, 78, 83, 84]
CD36	PS?		[77]
Annexin I, annexin II	PS?		[80]
CD36 and $\alpha_v\beta_3$ integrin		thrombospondin, MGF-8 or Del-1	[7, 95–98]
CD36 and $\alpha_v\beta_5$ integrin	PS?		[102, 104]
β_1 integrin	CD29		[88]
β_2 integrins	CR3 and CR4	C3, C4, iC3b	[51–56]
		C1q	[57–59, 64, 66]
		MBL + C1q	[58, 64]
CD91 bound to calreticulin		MBL/SP-A/SP-D + C1q	[57, 66]
		collectins MBL, SP-A, SP-D	[57, 58, 64–66]
	PS	protein S	[70]
FcR	PS	β_2GPI	[61–63]
FcγRs		pentraxins CRP, SAP	[67–69]
	lysophosphatidylcholine	IgM	[60]
Receptor tyrosine kinases Axl/Sky/Mer	PS	Gas-6	[12, 81, 82]
Integrins?		vitronectin, fibronectin, collagen VI	[91]
CD44			[92]
	CD44, α_4 integrin		[93]
SHPS-1	CD47		[145]

phages, also exhibit PS on their external cell surface [18, 21]. However, these cells are not phagocytosed, indicating that PS, per se, is not sufficient for promoting engulfment. Possible explanations for this are: (i) the absence of some other factor relevant to recognition, (ii) the masking of PS or (iii) the localization of these cells in sites devoid of competent macrophages [22]. Nonetheless, certain pathogenic protozoa take advantage of their normal surface exposure of PS for invading and deactivating macrophages and increasing susceptibility to their own growth [23].

Interestingly, macrophages must also express PS on their surfaces for the phagocytosis of apoptotic cells to occur [18]. ABC1, a member of the ATPase membrane transporter family that is also involved in engulfment of apoptotic cells [24], and Ced-7, the ABC1 homologue of *C. elegans*, were likewise shown to be required in phagocytes and target cells [25, 26]. Inhibition of ABC prevents both exposure of PS and phagocytosis of cells expressing PS, but not phagocytosis of yeasts or opsonized particles, which depends on other mechanisms [25]. The mechanism of action for ABC1 may involve lipid translocation, such as that of other ABC transporters, and facilitate the recruitment of other signals for engulfment as well as phagocytic receptors involved in apoptotic cell clearance [27, 28]. Ced-7, together with Ced-6 and Ced-1, is involved in a pathway that is important for the engulfment of cell corpses [2, 26, 29, 30] and which is distinct from another pathway composed of Ced-2, Ced-5, Ced-10 and Ced-12 [2, 31, 32]. Ced-6 and its mammalian homologue GULP are adaptor proteins that are able to interact with the NPXY motifs in the intracellular domains of the receptors Ced-1 and CD91/LRP, which are both implicated in the engulfment of cell corpses [33].

Recently, annexin I was found to colocalize with PS in apoptotic cells and was associated with the efficient tethering and internalization of these cells [34]. Annexin I is recruited from the cytosol to the cell surface by mechanisms dependent on calcium release and activation of caspases. A specific PS receptor (PSR) was identified in phagocytic cells [35] (see below) and reported to participate in the recognition of apoptotic cells via annexin I. Indeed, the homologue gene of annexin I in *C. elegans* was silenced by small interfering RNA (siRNA), and these mutated worms revealed a defect in the clearance of apoptotic cells [34].

Parallel to PS-mediated signals, intercellular adhesion molecule 3 (ICAM-3), a highly glycosylated protein constitutively expressed in leukocytes [36], is somehow modified during apoptosis such that it switches its affinity for its counter receptor, leukocyte function-associated antigen 1 (LFA-1), to an alternative receptor on the surface of other cells. Antibodies against ICAM-3 and macrophage CD14 inhibit phagocytosis of apoptotic cells in a nonadditive way, suggesting that both molecules may share the same system of recognition [36].

The pattern of glycosylation of the cell surface is also modified during apoptosis. This may result from either desialization, leading to the exposure of sugar residues that were masked before apoptosis, or fusion of the plasma membrane with membranes from the endoplasmic reticulum and Golgi apparatus that contain incompletely synthesized carbohydrates [37, 38]. Sialic acids are ubiquitously expressed at the nonreducing ends of oligosaccharide chains and are important in various biological events. During apoptosis they can be enzymatically removed to expose asialoglycoconjugates, which are relevant in inducing phagocytosis [39–41].

Oxidation of proteins and lipids at the apoptotic cell surface is also important for engulfment [42–44]. Various apoptotic stimuli induce oxidative stress, leading to peroxidation of membrane lipids. Oxidized low-density lipoprotein (OxLDL) and apoptotic cells share many receptors, such as SRA, CD36, CD68, SR-BI and LOX-1, and compete in binding to macrophages [42, 45–48]. Antibodies against oxidized phospholipids label apoptotic cells and inhibit their engulfment by macrophages [44]. Moreover, it was shown that oxidation of PS is critical for efficient phagocytosis [49, 50].

Opsonization of apoptotic bodies is usually, though not always, a positive signal for phagocytosis. In addition to their recognized role in facilitating phagocytosis of invading pathogens, molecules of the complement system were implicated in the uptake of apoptotic cells [11, 51–59]. Both the classic pathway and the alternative pathway participate in recognition of apoptotic cells. Deletion of C1q and C4 in mice leads to an accumulation of apoptotic cells in glomeruli, high titers of autoantibodies and a disease similar to systemic lupus erythematosus [11, 59]. Immunoglobulin M (IgM) binds to lysophosphatidylcholine in late apoptotic cells and accounts for the removal of these cells through complement activation [60].

Other opsonins present in serum that have a role in the phagocytosis of apoptotic cells include β_2-glycoprotein I (β_2-GPI) [61–63]; collectins, such as mannose-binding lectin (MBL) [57, 58, 64] and surfactant proteins SP-A and SP-D [65, 66]; pentraxins, such as C-reactive protein (CRP) [67, 68]; serum amyloid P component (SAP) [68, 69] and serum-derived protein S [70]. Opsonins are strongly related to the innate immune response against pathogens, which leads to inflammation. However, the removal of apoptotic cells is usually characterized by suppressed production of inflammatory mediators, which seems to depend on exposure of PS [71].

Apoptotic cell-recognition molecules in phagocytes

Phagocytes, in turn, express a large repertoire of surface molecules for the recognition of apoptotic cells (table 1). Some of these molecules are considered tethering recep-

tors (e.g. CD14), while others are believed to transduce intracellular signals for the engulfment of apoptotic bodies (e.g. PSR) [72].

A cell surface glycoprotein that has at least one potential site of tyrosine phosphorylation was identified as a specific PSR in various cells, such as macrophages, fibroblasts, epithelial cells and endothelial cells [35, 73]. A homologue of PSR, PSR-1, which is also implicated in apoptotic cell clearance, was recently reported in *C. elegans* [32]. However, PSR-deficient mice showed contradictory results regarding the requirement of PSR for apoptotic cell engulfment in both in vivo and in vitro assays [73–75]. Besides PSR, various molecules that have affinity for anionic phospholipids are candidate receptors for PS (CD68, LOX-1, SRA, SRBI and CD36) [45–48, 76, 77]. CD14, initially included in this list [78], was recently discarded as a possible PS receptor because PS showed no specificity for soluble or membrane-anchored CD14, it was less active than phosphatidylcholine and phosphatidylethanolamine in binding to monocyte-derived macrophages, and PS-containing liposomes did not inhibit CD14-dependent phagocytosis of apoptotic cells [79]. Like some apoptotic cells, macrophages display both annexin I and annexin II, which have affinity for PS, on their surface. However, annexin I and annexin II cannot be removed from the macrophage surface by chelation of calcium, as they can in apoptotic Jurkat cells. It is not clear how these molecules are present in phagocytes, but they stimulate phagocytosis of apoptotic cells [80].

Mer is a member of the Axl/Mer/Tyro3 receptor tyrosine kinase family. Mice expressing a Mer protein that had a cytoplasmic truncation showed deficient clearance of apoptotic cells and increased numbers of nuclear autoantibodies [12]. This effect was specific for apoptotic cells, as opposed to other targets such as bacteria, latex beads and opsonized particles. Binding of apoptotic cells to phagocytes was, however, preserved in these mutant mice, suggesting that only engulfment is dependent on the cytoplasmic portion of Mer. Mer and apoptotic cells may possibly be bridged by Gas6, a ligand of Mer that has affinity for PS [81]. In fact, Gas6 enhances the engulfment of apoptotic bodies by phagocytes [82].

CD14 is a glycoprotein that is bound to the cell surface by a glycosylphosphatidylinositol (GPI) anchor. It binds various molecules, such as lipids, carbohydrates and proteins, and is broadly known as a receptor for lipopolysaccharide (LPS). CD14 plays a role in the recognition of apoptotic bodies by either resident or elicited macrophages [78, 83, 84] and possibly participates in a recognition complex, together with a modified form of ICAM-3, in apoptotic cells [36].

Several lectins and receptors are also implicated in mechanisms of recognition of exposed carbohydrates that vary according to the type of both apoptotic and phagocytic cells [37–39, 41, 85, 86]. Table 2 summarizes the results of assays using a variety of cell types and sugar inhibitors, which support the role of surface sugars in the control of phagocytosis.

CD93, also known as C1qRp, is expressed on macrophages and seems to have a positive role in apoptotic cell phagocytosis, because CD93-deficient mice have an impairment in clearance of these cells. However, the impor-

Table 2. Carbohydrates of apoptotic cells involved in recognition of phagocytes

Apoptotic cell type	Inhibitory sugars	Non-inhibitory sugars	Implied lectin	Phagocytes	References
Liver cells of neonatal rats	GalNAc, Gal, Man, ASF, Lac-BSA		ASGP-R MR	liver phagocytes of neonatal rats	[37]
Lymphocytes	Glu, Fuc, GalNAc, methyl-manno-pyranoside, Lac-BSA, ASF	BSA 20%, fetuin	MR galactose receptor	Kupffer cells	[38]
HT-29 (adenocarcinoma cell line)	Gal	Man, Fuc		THP-1 human monocytic cell line	[39]
HeLa	sialic acid derivatives	Neu5Ac, GlcNAc, Gal, Man	receptors for asialoglyco moieties	elicited peritoneal macrophages	[41]
Thymocytes	GlcNAc and Chito, GalNAc, Gal to a lesser extent	Fuc, Man, NANA		peritoneal macrophages	[85]
Neutrophils	Man, Fuc, Glsa, Glc	Glu, Gal, GlcNAc, GlsaNAc	mannose/ fucose lectin	fibroblasts	[86]
Leukocytes	fucoidin, mannan	Glu, GlcNAc, Gal, Fuc, Man, mannan	fucoidin receptor	endothelial cells	[93]

Gal, D-galactose; Glu, D-glucose; Fuc, D-fucose; Man, D-mannose; Glsa, galactosamine; GlsaNAc, N-acetylgalactosamine; Glc, glucosamine; GlcNAc, N-acetylglucosamine; ASF, asialofetuin; Lac-BSA, lactosylated bovine serum albumin; NANA, N-acetylneuraminic acid; Chito, N-N'-diacetylchitobiose; ASGP-R, asialoglycoprotein receptor; MR, mannose receptor.

tance of this molecule was not confirmed by in vitro assays using macrophages of wild-type and CD93-deficient mice [87].

The β_1 integrin (CD29) was associated with recognition and binding of apoptotic cells to leukocytes, but its ligand is still unknown [88]. Most integrins recognize various components of the extracellular matrix (ECM), and distinct integrins can interact in modulating phagocytic responses [89, 90]. β_1 integrins are also implicated in improving the engulfment of apoptotic neutrophils following adhesion of macrophages to fibronectin, vitronectin, collagen VI (but not collagen I), bovine serum albumin (BSA) or naked plastic [91].

β_2 integrins, such as CR3 and CR4, are receptors for iC3b, which can opsonize apoptotic cells. Antibodies against iC3b and its receptors inhibit the clearance of apoptotic cells [53, 55]. FcγRs, usually associated with phagocytosis of IgG-opsonized particles, can also function as a receptor for cyclic AMP receptor for C-reactive protein (CRP), which, together with SAP, binds to both apoptotic and necrotic cells and enhances the uptake of these cells [68].

The surface molecule of phagocytes, CD91, can bind calreticulin, a secreted protein, and together with the opsonins C1q and MBL initiate macropinocytosis and uptake of apoptotic cells tethered to phagocytes [57]. The collectins SP-A and SP-D also bind apoptotic cells, promoting their uptake in a mechanism dependent on calreticulin and CD91 [66].

CD44, a receptor for hyaluronan, enhances the phagocytosis of apoptotic cells when ligated by specific antibodies [92]. CD44, as well as the α_4 integrin, is also involved in engulfment of apoptotic leukocytes by endothelial cells, but only when present in the target apoptotic cell, not in the phagocyte [93]. Additional evidence of a role for this receptor stems from CD44-deficient mice, which are unable to resolve bleomycin-induced inflammation in the lung and which show impaired clearance of apoptotic neutrophils, accumulation of hyaluronan fragments, and impairment of transforming growth factor β activation [94].

Redundant and complex recognition systems

The preceding sections showed that apoptotic cells display various signals to be recognized by phagocytes and that phagocytes can display multiple recognition systems. Recognition and phagocytosis of apoptotic cells are complex processes, and the molecules employed depend on the activation state of macrophages [7], the phagocytic cell type, and the stage of apoptosis, as will be illustrated in the following section. Furthermore, these systems can act cooperatively and sequentially.

Nonactivated macrophages can recognize apoptotic bodies by a complex formed by CD36, the integrin $\alpha_v\beta_3$ and secreted thrombospondin. CD36 is a scavenger receptor that recognizes PS, but the molecules recognized by this complex in apoptotic cells are still unknown.

Similar to nonactivated macrophages, which secrete thrombospondin that bridges apoptotic cells and macrophages, activated macrophages secrete the glycoprotein milk fat globule-EGF factor 8 (MGF-E8). This protein bridges apoptotic cells by recognizing aminophospholipids (such as PS) and phagocytes, probably by binding to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ via its arginine-glycine-aspartic acid (RGD) motif [95]. This mechanism of recognition may be particularly relevant during involution of mammary glands, when suckling and milking ceases and a large number of epithelial cells degenerate by apoptosis. The secretion of these proteins by macrophages may explain how the related integrins participate in recognition of apoptotic cells without binding directly to PS. Del-1, a molecule that binds $\alpha_v\beta_3$ and that is structurally and functionally homologous to MGF-E8 also participates in engulfment of apoptotic cells [96]. Interestingly, distinct subsets of macrophages seem to express only one or the other molecule [96–98]. Moreover, it was recently shown that MGF-E8 and $\alpha_v\beta_5$ participate in phagocytosis of apoptotic cells by dendritic cells [99] and that $\alpha_v\beta_5$/MGF-E8 and MER/Gas-6 act synergistically in the engulfment of apoptotic cells, thus amplifying downstream intracellular events [100] (see next section for discussion).

Other systems related to phagocytosis by activated macrophages involve uncomplexed CD36 as well as other receptors, such as lectins and CD14 [14]. These data suggest that a combination of PS and other molecules may have a central role in generating complex signals recognized by various phagocyte receptors [14, 84, 101].

Like dendritic cells, retinal pigment epithelium (RPE) also makes use of $\alpha_v\beta_5$ integrin rather than $\alpha_v\beta_3$ in the recognition of apoptotic cells [102] and the outer segments of photoreceptors [103–105], respectively. CD36 also plays an important role in the phagocytic activities of both dendritic cells and RPE [102, 106, 107]. Vitronectin stimulates phagocytosis of the outer segments of photoreceptors by RPE, which seems to be mediated by $\alpha_v\beta_5$ integrin [108]. The mannose receptor is also involved in this system [109, 110].

It should be noted that apoptosis is a dynamic, active process, and the cell surface changes continuously. Distinct signals for recognition by phagocytes are exposed at different moments of the apoptotic process. Phagocytes, in turn, display various molecules that are engaged in complexes of recognition in a time-dependent manner, according to the degeneration stage of the target cell. Jurkat cells, classified in two distinct stages of apoptosis according to TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) staining, are recognized in two sequential mechanisms. An early stage depends on carbohydrate moieties, and a later stage depends on exposure of PS [111]. Anoxia-induced apop-

totic neuronal cells expose PS at their surface only in late stages of apoptosis, when cells have completely lost their adhesion properties. These cells are efficiently phagocytosed by microglial cells, unlike early apoptotic neuronal cells, which are loosely attached to the tissue culture dish [112]. On the other hand, apoptotic HT-29 cells were classified in three different stages of apoptosis, related to exposition of apoptotic molecules and cell viability, but were efficiently phagocytosed regardless of their apoptotic stage. PS externalization and galactose exposed on sugar chains are involved in recognition of apoptotic cells in all stages, while the vitronectin receptor is involved only in the latest stage. The authors suggested that the distinct complexes of recognition work successively in an efficient mechanism of cell clearance [39].

Despite efforts to understand the time course of apoptotic cell recognition, the wealth of molecules reviewed above were uncovered through the use of a large variety of experimental systems, and too little information is available from any single system to allow a comprehensive summary of the time sequence of marker appearance. A microarray technique was recently used to demonstrate the sequential patterns of gene expression in macrophages exposed to mycobacteria for different periods of time [113]. This approach may be useful in comprehending the dynamic nature of apoptotic cell recognition by phagocytes.

Intracellular signaling for phagocytosis of apoptotic cells

One of the requirements for proper phagocytosis is the polymerization and reorganization of the actin cytoskeleton underneath bound particles. Binding of apoptotic cells induces actin polymerization and protein phosphorylation at nascent phagocytic cups [10]. This is crucial for formation of membrane extensions around, and engulfment of, the target particle, irrespective of its nature. However, distinct particles mobilize different receptors, which recruit specific sets of intracellular molecules. Thus, it is likely that specific intracellular mechanisms mediate phagocytosis of apoptotic cells as compared with other particles, such as IgG-opsonized cells, complement-opsonized cells or latex beads.

Galectin-3, a member of the family of β-galactoside-binding lectins, can be found at the cell surface or inside cells, or in a secreted form, and was shown to be involved in actin polymerization and to interfere with phagocytosis. Galectin-3-null cells are less efficient than wild-type in clearing IgG-opsonized cells in vitro and apoptotic cells both in vitro and in vivo. The secreted form of galectin-3 is irrelevant for phagocytosis, as was demonstrated in co-culture experiments of wild-type and galectin-3-deficient macrophages. In resting macrophages, galectin-3 seems to be diffusely distributed inside the cells; it colocalizes with F-actin soon after the beginning of phagocytosis and is found in phagocytic cups and in both early and late phagosomes [114].

Uptake of apoptotic cells by dendritic cells is mediated by $\alpha_v\beta_5$ integrin and involves early phosphorylation of tyrosine residues in several proteins, such as the adaptor proteins p130Cas and CrkII, and the guanine nucleotide exchange factor for Rac, Dock180. CrkII binds p130Cas through its SH2 domain, and Dock180 through its SH3 domain. Disruption of these interactions abrogates phagocytosis of apoptotic cells. CrkII is recruited to the limiting membrane of phagosomes and seems to direct Dock180 to the plasma membrane, influencing the polarity of the cytoskeleton projections in response to stimuli for migration or signals from apoptotic cells. Dock180 activates the small guanosinetriphosphatase (GTPase) Rac1 [115], a pathway that was also demonstrated in *C. elegans* [31, 116, 117]. It was recently described that the formation of a $p130^{Cas}$/CrkII/Dock180 complex and subsequent activation of Rac1 is stimulated by Mer/Gas6. Activated Mer induces FAK^{Tyr861} phosphorylation, which is recruited to $\alpha_v\beta_5$ integrin. Both receptors act synergistically on this intracellular pathway [100]. The CED-2/CED-5/CED-10/CED-12 proteins are homologues of the mammalian proteins CrkII, Dock180, Rac1 and ELMO 1. In *C. elegans*, the engulfment pathway involving CED-5/CED-10/CED-12 can be activated by PSR-1 [32]. Recently, it was described that ELMO interacts with the small GTPase RhoG through its conserved Armadillo repeats. TRIO, the exchange factor for RhoG, was also implicated in this pathway. This event occurs upstream of the Dock-mediated activation of Rac. The *C. elegans* homologues of RhoG and TRIO, MIG-2 and UNC-73, respectively, participate in corpse clearance in nematodes as well, upstream of CED-12 [118].

Small GTPases of the Rho family have a central role in the reorganization of the cortical actin cytoskeleton during phagocytosis. Engulfment of apoptotic bodies in mammals requires Rac [10, 115, 119] and Cdc42 [10]. Activated (GTP-bound) Rac 1, Cdc42 and RhoA accumulate in EMC cells after activation of Mer by Gas6 stimulation. This results in tyrosine phosphorylation of the guanine exchange nucleotide factor Vav1 and its release from Mer, leading to replacement of guanosine diphosphate (GDP) by GTP in Rho GTPase members [120].

Phagocytosis of apoptotic cells is also stimulated by glucocorticoid [121, 122], which promotes cytoskeleton alterations associated with enhancement of Rac levels. Polymerization of actin filaments by Rho GTPases occurs through activation of Wiskott-Aldrich syndrome proteins (WASPs). These proteins recruit the actin-related proteins Arp2/3 complex to preexisting actin filaments and allow the nucleation of new filaments [123, 124]. Clearance of apopotic cells by WASP-deficient macrophages is delayed both in vitro and in vivo [125].

Also implicated in actin polymerization during phagocytosis of apoptotic cells is 12/15 lipoxygenase (12/15-LO). In peritoneal macrophages, this enzyme translocates from the cytosol to the plasma membrane, close to regions of contact with apoptotic cells. It colocalizes with emerging filopodia, and inhibition of its activity diminishes actin polymerization. The activity of this enzyme seems to correlate with the differential phagocytic ability of macrophages among a heterologous population. Only phagocytes expressing this enzyme are able to bind and phagocytose apoptotic cells [126].

Activation of phosphoinositide 3-kinase (PI3K) is also required for the phagocytosis of apoptotic cells, as well as other particles [10, 127]. Inhibition of PI3K does not inhibit binding of apoptotic cells, actin cup formation, accumulation of phosphotyrosine in actin cups [10] or translocation of 12/15-LO to the plasma membrane [126]. However, activation of PI3K seems to be relevant downstream of the activation of Rho GTPases [10]. Examination of distinct PI3K isoforms revealed that class I PI3K p110 is the major isoform required for phagocytosis of apoptotic cells, but a distinct class of PI3K may also be involved [128].

Protein kinase C (PKC) is implicated in phagocytosis of diverse particles, and also apoptotic cells [25, 127, 129]. Specifically, tissue macrophages were shown to require PKC II in order to engulf apoptotic thymocytes. It was suggested that the expression level of this isoform is related to the differential efficiency of phagocytosis observed between alveolar and peritoneal macrophages: the former present reduced levels of PKC II compared with the latter [129]. PKC activation also enables binding and subsequent engulfment of apoptotic cells by specific receptors, such as $\alpha_v\beta_5$ (but not $\alpha_v\beta_3$), and is involved in the association of $\alpha_v\beta_5$ with the actin cytoskeleton [103].

Negative regulation of phagocytosis

The large number of molecules involved in promoting phagocytosis and the redundancy of systems testify to the importance of removal of apoptotic corpses. Nevertheless, either too little or too much phagocytosis can produce undesirable results for the organism. Intense phagocytic response is associated with the production of reactive oxygen species and tissue injury [130]. Circulating monocytes must have a limited phagocytic capacity that is enhanced only following migration to inflamed sites [131]. Alveolar macrophages as well as monocytes recruited to the lung show limited phagocytosis of apoptotic cells compared with peritoneal macrophages, which possibly helps preserve lung tissue from inflammatory destruction [132, 133]. In addition, phagocytosis of apoptotic lymphocytes has been shown to be beneficial in the growth of pathogenic parasites within macrophages [134, 135].

Once triggered, phagocytosis must be restricted to sites where it is required, controlled and eventually finished. Although little is known concerning the negative regulators of phagocytosis, recent studies have contributed in clarifying how phagocytosis can be limited or terminated.

‘Don’t eat me’ signals and intracellular brakes for phagocytosis

Normal cells display molecules for signaling that they should not be eliminated. Macrophages establish interactions with leukocytes that will determine the fate of the latter, i.e. whether they will be phagocytosed or not. One major intracellular mechanism for inhibiting cell responses occurs through receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ITIMs are present in the cytoplasmic portion of inhibitory receptors that contain a consensus sequence of six amino acids (Ile/Val/Leu/Ser-X-Tyr-X-X-Leu/Val). These receptors attenuate activation signals initiated by other receptors [136]. Activation of ITIM receptors results in phosphorylation of tyrosines, generating recruitment sites for phosphatases containing SH2 domains, such as SHP-1, SHP-2 and SHIP, which then transduce inhibitory signals [137–139]. Some systems of recognition of normal cells by phagocytes use this general mechanism.

CD31 (platelet-endothelial cell adhesion molecule-1, PECAM-1) is a membrane glycoprotein that has ITIMs and that is expressed by leukocytes and macrophages. Homophilic ligation of CD31 from normal cells and macrophages promotes their detachment by an active and temperature-dependent mechanism. Apoptotic cells also express CD31, but signaling mediated by this molecule is disrupted. In contrast to CD31 from normal cells, CD31 from apoptotic cells shows reduced constitutive association with the cytoplasmic signaling molecule SHP-1, reduced pervanadate-mediated recruitment of SHP-2 and reduced CD31 tyrosine phosphorylation. Binding of antibodies fails to induce calcium transients. Thus, homophilic interaction of CD31 serves to avoid inadequate engulfment of healthy cells, and apoptosis disables this mechanism of repulsion [140].

Sialization of glycoproteins is another strategy used to mark viable cells. Sialized molecules are recognized by receptors known as Siglecs (sialic acid-binding Ig-like lectins), but their desialated forms are not recognized. Siglecs are transmembrane proteins, and their cytoplasmic portion contains ITIMs. These receptors seem to inhibit engulfment of normal cells, but not apoptotic cells that have lost sialic acid, as well as microbes that do not synthesize sialic acid [141].

CD47 is a surface marker recognized by signal regulatory protein (SIRP) that also displays an ITIM in its cytoplasmic domain. Interaction of these molecules impairs phagocytosis. Expression of CD47 is downregulated during

senescence of erythrocytes and platelets, which enables clearance of these senescent cells [142]. Phagocytosis of opsonized erythrocytes and platelets is negatively modulated by SIRP [143, 144], and CD47 has a recognized role in the negative modulation of phagocytosis of red blood cells. In sharp contrast, a requirement of CD47 was reported for efficient phagocytosis of lymphocytes [145]. The authors suggested that ligation of CD47 and SHPS-1 acts in the tethering of apoptotic cells to macrophages and that discrepancies with other studies may be related to either cell specificity or distinct conformations adopted by CD47 at the plasma membrane of the cell types studied [145]. Importantly, CD47, also known as IAP (integrin-associated protein), can interact with integrins and modulate phagocytosis negatively [90].

Integrins were also associated with negative modulation of the uptake of apoptotic neutrophils following previous ingestion of apoptotic neutrophils. This effect is mimicked by ligation of $\alpha_v\beta_3$, $\alpha_6\beta_1$ and $\alpha_1\beta_2$ integrins and does not seem to be due to the overload of macrophages. Integrins β_1 and β_2 may negatively modulate the function of $\alpha_v\beta_3$ [146]. It was suggested that after ligation with apoptotic neutrophils, integrin $\alpha_v\beta_3$ negatively regulates adhesion of macrophages and facilitates migration of these cells to lymphatic organs.

CD200 and its counter receptor, CD200R, are implicated in macrophage and myeloid cell regulation. CD200 is widely expressed in various cell types, whereas CD200R is restricted to myeloid cells. Macrophage lineage cells of CD200-deficient mice are more numerous and show an activated phenotype compared with their counterparts in wild-type mice. In in vivo models of nerve injury, $CD200^{0/0}$ macrophages and microglia are more readily activated. This indicates that CD200R transmits a negative signal that regulates macrophage responses [147–149].

The Rho GTPase RhoA is implicated in the negative modulation of apoptotic cell phagocytosis, opposite the promoting role of other Rho family enzymes, such as Rac and Cdc42 [150]. GTP-bound RhoA binds to and activates Rho-kinase (ROK), which is probably a primary effector in mediating this regulatory effect. ROK most likely acts upon contractility at the center of the cell, since its inhibition destabilizes the formation of stress fibers but does not alter the formation of protrusions at the cell's periphery [150].

Increased levels of cAMP inhibit phagocytosis of apoptotic lymphocytes, an effect that seems to be related to changes in macrophage adhesion. cAMP leads to altered distribution of cytoskeleton elements in contact with the substrate, such as actin, talin, vinculin and paxillin, and macrophages assume a round morphology with reduced numbers of membrane projections [151]. Also, the permeant cAMP analogue 8-Br-cAMP inhibits, whereas the PKA inhibitor Rp-cAMP mimics, the enhancing effect of lipoxin upon apoptotic cell phagocytosis [152].

Regarding negative signals from the extracellular milieu, it was shown that the long pentraxin PTX3 inhibits the internalization of late apoptotic neutrophils by dendritic cells [153]. The inhibitory effect of PTX3 was also attributed to inhibition of binding of late apoptotic neutrophils to the surface of monocyte-derived macrophages [154]. PTX3 is produced in response to inflammatory signals, such as LPS, IL-1 or tumor necrosis factor α [153]. PTX3 binds to C1q, which has a role in the positive regulation of apoptotic cell phagocytosis. Protein S, a blood glycoprotein that has a positive role in engulfment of apoptotic cells [70], can act negatively when complexed with C4b-binding protein (C4BP) [155]. These findings underscore the complexity of phagocytosis, with its multiple levels and possibilities of regulation.

PrP^c, a novel negative modulator of phagocytosis

The cellular prion protein (PrP^c) has an unexpected negative effect upon phagocytosis. Recently, we showed that phagocytes derived from mice devoid of PrP^c are more active than phagocytes from wild-type mice [156]. The negative regulation exerted by PrP^c upon phagocytosis was observed in various models combining distinct phagocytes (peritoneal macrophages and Müller glial cells) and targets (neuronal cells, thymocytes and neutrophils, as well as zymosan). Such an effect in several distinct experimental models indicates that PrP^c may have a conserved role in phagocytosis. The negative modulation of phagocytosis by PrP^c was observed when apoptotic cells were offered in low proportion relative to macrophages, suggesting a role in circumstances that are not characterized by massive cell death [156].

PrP^c is a GPI-anchored surface molecule. Similar to many engulfment receptors, such as CD36, CD44, SR-BI, CD14 and FcγRIII, PrP^c is present in lipid rafts [157], where these molecules may form multireceptor complexes. Interactions of GPI-anchored proteins with their ligands may modify the threshold for cellular activation or, alternatively, relocate receptors to lipid rafts. Such a mechanism is present in the nonopsonic phagocytosis of *Mycobacterium kansasii*, a phenomenon mediated by CR3 associated with GPI-anchored proteins [158].

Inflammatory responses in $PrP^{0/0}$ mice were also altered. These mice recruited more monocytes and fewer neutrophils than wild-type mice after injection of zymosan [156]. In the central nervous system (CNS), inflammation is limited [159], and activation of microglia must be well regulated because these cells produce both cytotoxic and inflammatory mediators. The ability to downregulate phagocytosis, and possibly other activities of activated microglia, would be an advantage in a tissue rich in PrP^c, such as that of the CNS. In vitro cultures of wild-type

microglia seem to be more activated than those of $PrP^{0/0}$ microglia because of augmented production of superoxide after stimulation with LPS or concanavalin A [160] and $PrP^{106–126}$ [161], and also of nitrite after stimulation with the PrP peptide $PrP^{106–126}$ [161]. Phagocytosis is negatively modulated by superoxide [162] and the superoxide derivative H_2O_2 [163–166] (but see [167]), as well as nitric oxide [168–170] and its derivative, nitrite [171]. These data are consistent with the observation of higher phagocytic activity in $PrP^{0/0}$ macrophages [156].

Neuronal cells can produce negative signals to avoid inadequate phagocytosis and to promote a macrophage response that allows tissue repair [172]. Preliminary results indicate that apoptotic neutrophils derived from wild-type or PrP^{c} null mice are phagocytosed with similar efficiency. Nevertheless, PrP^{c}-null macrophages are more effective in phagocytosing either type of neutrophil, as well as other types of apoptotic cells [C. J. G. de Almeida and R. Linden, unpublished results]. A similar control mechanism for phagocytosis may also be active within the nervous system. Interestingly, it was recently shown that the fibrillar prion peptide $PrP^{106–126}$ and the scrapie form of the prion protein (PrP^{Sc}) also have negative effects on phagocytosis by microglial cells [173]. In summary, the

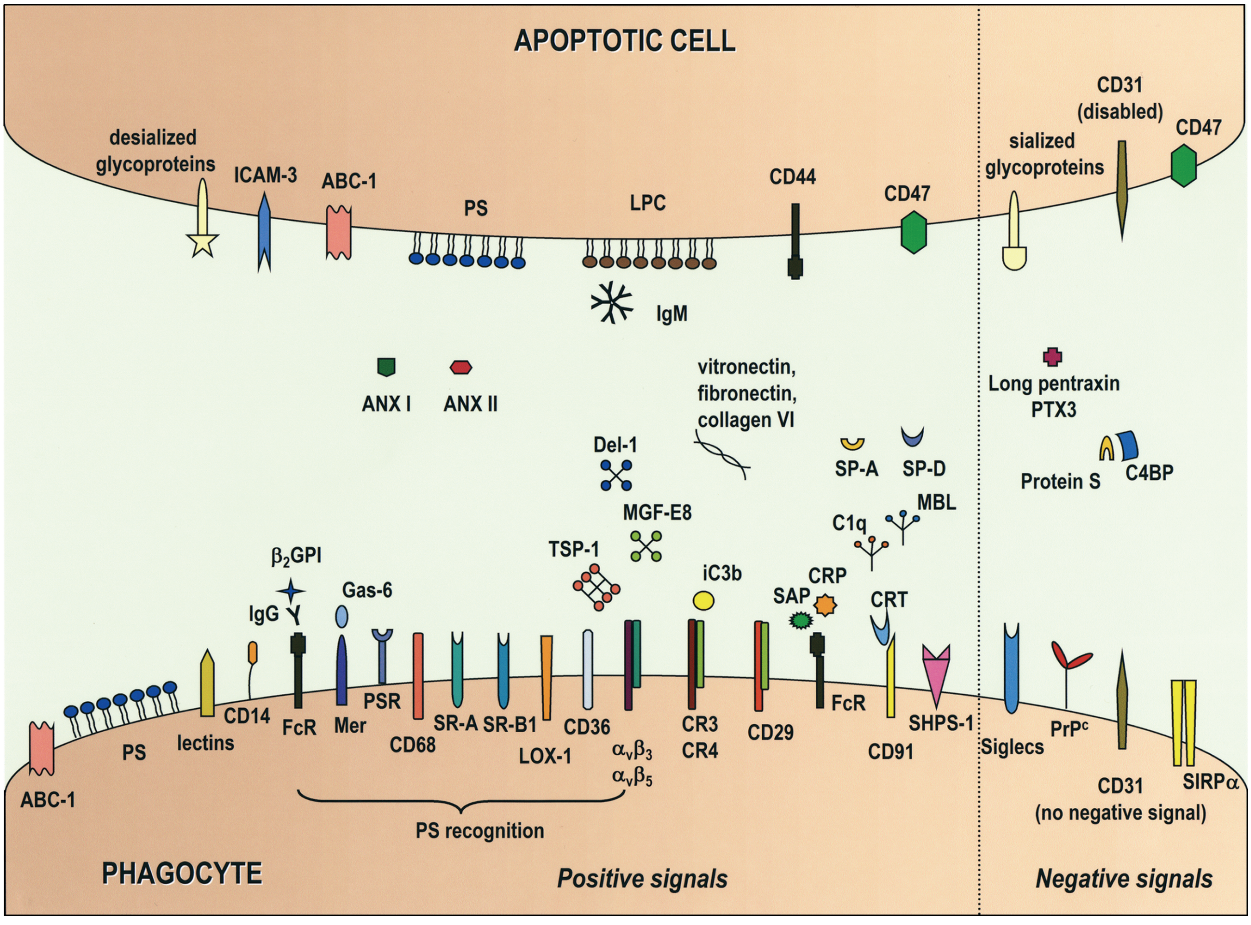

Figure 1. Positive and negative signals that regulate the recognition and engulfment of apoptotic cells. Phagocytosis of apoptotic cells is stimulated by various molecules displayed on the surface of both cell types. Some molecules present in viable cells avoid recognition by phagocytes and are modified during apoptosis, allowing clearance of degenerating cells. Besides the many molecules involved in the stimulation of phagocytosis, phagocytes also display molecules that counteract positive signals for engulfment and are relevant in controlling this process. ICAM-3, intercellular adhesion molecule 3; ABC-1, ATP-binding cassette transporter 1; PS, phosphatidylserine; LPC, lysophosphatidylcholine; CD44, receptor for hyaluronic acid; CD29, integrin β_1; CD47, integrin-associated protein (IAP); CD31, platelet/endothelial cell-adhesion molecule (PECAM1); ANX I, annexin I; ANX II, annexin II; IgM, immunoglobulin M; Del-1, developmental endothelial locus-1; MGF-E8, milk fat globule-EGF factor 8; TSP-1, thrombospondin 1; iC3b, complement protein iC3b; SAP, serum amyloid P component; CRP, C-reactive protein; C1q, complement protein C1q; SP-A, surfactant protein A; SP-D, surfactant protein D; MBL, mannose-binding lectin; PTX3, long pentraxin 3; C4BP, C4b-binding protein; CD14, lipopolysaccharide receptor; FcR, Fc fragment of immunoglobulin G receptor; Mer, a receptor tyrosine kinase; PSR, phosphatidylserine receptor; CD68, macrosialin; SR-A; scavenger receptor class A; SR-B1, scavenger receptor class B1; LOX-1, lectin-like oxidized low-density lipoprotein (LDL) receptor-1; CD36, thrombospondin receptor; $\alpha_v\beta_3$ and $\alpha_v\beta_5$, integrins that bind vitronectin; CR3 and CR4, complement receptors 3 and 4; CD91, α_2 macroglobulin receptor; SHPS-1, SHP protein-tyrosine phosphatases substrate-1; Siglec, sialic acid-binding immunoglobulin-like lectin; PrP^{c}, cellular prion protein; SIRPα, signal regulatory protein α.

Table 3. Negative modulators of apoptotic cell phagocytosis

Macrophage	Apoptotic cell	Extracellular	References
CD31	CD31		[140]
Siglecs	sialized glycoproteins		[141]
SIRP	CD47		[142]
Integrins			[146]
RhoA			[150]
cAMP			[151, 152]
		long pentraxin PTX3	[153, 154]
		protein S/C4BP	[155]
PrP^c			[156]

cellular prion protein adds to a growing list of negative modulators, which may be essential in balancing the threshold of phagocytic responses (fig. 1).

Conclusion: balancing the signals for and against phagocytosis of apoptotic cells

Studies of apoptotic cell phagocytosis began more recently than those of the uptake of IgG- and complement-opsonized particles. The search for understanding the mechanisms and consequences of apoptotic cell clearance has attracted considerable attention, and information on this issue is continuously growing. Nonetheless, many unanswered questions remain, such as what the links between the silent removal of apoptotic cells are and the roles of some of the molecules involved in inflammatory responses. Further studies are also needed to identify undisclosed molecules involved in the recognition of apoptotic cells by macrophages, as well as the intracellular mechanisms initiated by the interaction of phagocytes with apoptotic cells.

In particular, evidence that, despite the benefits of removing apoptotic bodies, uncontrolled phagocytosis may lead to tissue injury and inflammation has prompted the search for attenuation mechanisms of phagocytosis (table 3). Studies of the balance between positive and negative modulators of apoptotic cell phagocytosis should help in both understanding and managing inflammatory and autoimmune, as well as degenerative, diseases.

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