Phagocytosis and innate immunity

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Phagocytosis is an evolutionarily conserved process utilized by many cells to ingest microbial pathogens, and apoptotic and necrotic corpses. Recent investigation has revealed a fundamental requirement for two co-ordinated cellular processes – cytoskeletal alterations and membrane trafficking – in the phagocytic event. Some elements of this machinery are co-opted by certain pathogens to gain entry into host cells.

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Abbreviations

DAG	diacylglycerol
DC	dendritic cells
FcR	Fc receptor
ITAM	immunoreceptor tyrosine-based activating motif
LPS	lipopolysaccharide
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PS	phosphatidylserine
PSR	PS receptor
TLR	Toll-like receptor

Introduction

Phagocytosis is the process by which leukocytes and other cells ingest particulate ligands whose size exceeds about 1 μm. This phylogenetically conserved process is critical for innate immunity. By ingesting microbial pathogens, phagocytic leukocytes accomplish two essential immune functions. Firstly, they initiate a microbial death pathway, in part by routing ingested pathogens to lysosomes, which are rich in hydrolytic enzymes, and also by targeting the phagocyte oxidase complex to the phagolysosome. Secondly, phagocytic leukocytes, particularly dendritic cells (DC), utilize phagocytosis to direct antigens to both MHC I and II compartments [1]. Thus, phagocytosis serves a dual role: as an innate immune effector as well as a bridge between the innate and acquired immune responses.

Here we focus on recent progress in the cell biology of phagocytosis and discuss the importance of these findings to innate immunity.

Signaling events during phagocytosis

Clustering of phagocytic receptors by ligation to multiple vicinal ligands on the surface of the target particle triggers signals that initiate engulfment. Many receptors are competent to engage the phagocytic machinery (Table 1). For Fc receptors (FcRs), the initial intracellular event appears to be phosphorylation of the receptors themselves, or associated immunoreceptor tyrosine-based activating motif (ITAM)-containing subunits, by members of the Src family [2•,3]. Lipid rafts may play a role in coupling the kinases to the receptors, but rafts are more likely to be important for phagocytosis triggered by unopsonized targets [4–6] or facilitated by extracellular-matrix proteins [7]. The phosphorylated receptor/subunit ITAMs then serve as docking sites for Syk. This tyrosine kinase is absolutely required for the internalization of IgG-opsonized particles, but not for particles taken up by other receptors [8,9], suggesting that other kinases must be involved in the latter cases.

The precise sequence of events thereafter is less clear, but adaptor proteins such as LAT [10], SLP-76, BLNK [11], Crkl [12], Nck [13] and possibly Fyb/SLAP (MG Coppolino *et al.*, unpublished data) are engaged by the activated receptor complex. A wave of lipid remodelling ensues. Phosphatidylinositol 3-kinase (PI3K) is activated, generating 3′-phosphoinositides at the phagosomal cup. This accumulation is sharply restricted in both space and time, consistent with a role in transducing some of the early signals that prompt pseudopodial extension. The abrupt dissipation of the 3′-phosphoinositide gradient is due, at least in part, to the recruitment of the lipid phosphatase SHIP to the phagocytic cup [14•,15•].

The synthesis of phosphatidylinositol-4,5-bisphosphate (PIP_2) is also accelerated during phagocytosis [16••]. This lipid is not only a substrate of PI3K, but is also the target of phospholipase C (PLC), which generates diacylglycerol (DAG) during phagocytosis (Figure 1). The latter mediator can activate both classical and novel isoforms of protein kinase C (PKC), which are recruited to the phagosome and have been implicated, by pharmacological evidence, in particle uptake [17]. Activation of both PLC and PKC requires prior activation of PI3K [18]. Other kinases implicated in phagocytosis include MEK1 and/or ERK — which may be selectively involved in FcγR-mediated phagocytosis in human neutrophils [19] but not in macrophages [20] — and PKA [21]. Lastly, PLA_2 and PLD are also activated and believed to participate in the phagocytic process [22,23]. The former may participate in vesicle trafficking during phagocytosis (see below) as well as contributing to the production of leukotrienes that amplify the phagocytic signal [24].

Cytoskeletal alterations during phagocytosis

Among the most striking features of phagocytosis is the rapid, focal accumulation of F-actin and associated proteins in the periphagosomal region (Figure 2). Members of the Rho family of GTPases signal actin assembly during phagocytosis. For FcR-mediated phagocytosis, Rac1

Table 1

Examples of phagocytosis-promoting receptors in mammalian cells that participate in innate immunity.

Cell type	Receptor	Target	Ligand	References
Leukocytes	FcγRs	Pentraxin-oposonized zymosan (yeast)	Serum amyoloid P, C-reactive protein	[86,87]
PMN, Mo, MΦ	CR1 (CD35)	Complement-opsonized bacteria and fungi	C3b, C4b, mannan-binding lectin	[82]
PMN, Mo, MΦ	CR3 (CD11b–CD18; αMβ2; Mac1)	Complement-opsonized bacteria and fungi	C3bi, C3d	[126]
		Gram-negative bacteria	LPS	
		Bordetella pertussis	Filamentous hemagglutinin	
		Yeast	β-glucan	
MΦ, DC	CR4 (CD11c–CD18)	*M. tuberculosis*	?	[127]
MΦ	CD43 (leukosialin/sialophorin)	*M. tuberculosis*	?	[128]
Mast cells	CD48	Enterobacteria	FimH	[129]
MΦ	Mannose receptor	*Pneumocystis carinii, Candida albicans*	Mannosyl/fucosyl residues	[130]
MΦ	Scavenger receptor AI/II	Apoptotic lymphocytes	?PS	[131–133]
		Gram-positive cocci	Leipoteichoic acid	
Sertoli cells, thymic Epi	Scavenger receptor BI	Apoptotic cells	PS	[134,135]
MΦ	MARCO	*Escherichia coli, S. aureus*	?	[136]
MΦ	MER	Apoptotic thymocytes	?Gas6/PS	[119•]
Many	PSR	Apoptotic cells	PS	[113••]
MΦ	CD36	Apoptotic PMN	PS/thrombospondin	[85,137]
MΦ	CD14	*Pseudomonas aeruginosa*	?LPS	[138,139]
		Apoptotic cells	?	
Many	β1 integrins	*Yersinia*	Invasin	[140]
MΦ	αvβ3	Apoptotic cells	?Thrombospondin	[84,85]
DC, Epi	αvβ5	Apoptotic cells	?	[141,142]
Epi	E-cadherin	*Listeria*	InlA	[72,143]
Epi	Met	*Listeria*	InlB	[73••]

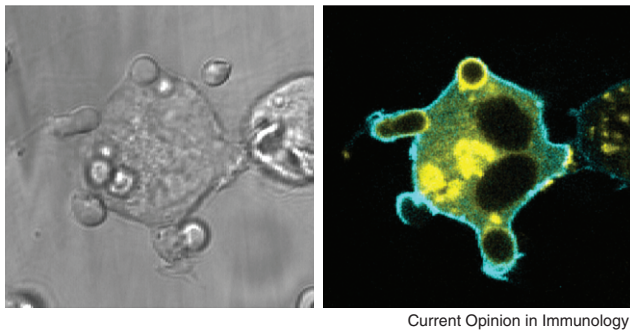
Specific inhibition of binding by these receptors correlates with inhibition of phagocytosis. However, with some notable exceptions (e.g. FcγRIIA and the macrophage mannose receptor), it is possible that the indicated receptor serves to enhance ligand binding, rather than to participate directly in the ingestion process. Epi, epithelial cells; Leuk, leukocytes; Mo, monocytes; MΦ, macrophages; PMN, polymorphonuclear leukocytes.

and Cdc42 (which are members of the Rac family) play prominent roles [25–28]; in contrast, Rho [27] has been implicated in phagocytosis mediated by complement receptor 3, a leukocyte integrin, although Rho may have additional roles in FcγR-mediated phagocytosis [29]. Work on *Caenhorabditis elegans* suggests that the requirement for Rac in phagocytosis, although not absolute, is evolutionarily conserved and critical for diverse forms of phagocytosis [30•]. Although no single Rac or Cdc42 effector has been unequivocally demonstrated to be essential for phagocytosis, members of the WASP family are likely to play key roles [31,32]. These proteins act as molecular scaffolds by associating with plasma-membrane-associated PIP_2 (in the case of WASP and N-WASP), with adaptor proteins and with the Arp2/3-based actin-nucleating machinery [33,34,35•]. Cdc42 accelerates the actin-nucleating activity of the Arp2/3 complex [33,34], thus providing a nidus for the generation of actin polymer at the base of the phagosome [35•].

ARF6, a member of the ARF family of GTPases, contributes to Rac-initiated cytoskeletal events [36,37]. ARF6 functions both upstream of Rac, inducing its activation [38] and plasma membrane redistribution [39], as well as downstream following plasma membrane targeting of Rac [37]. ARF6 also serves as a co-factor for the generation of PIP_2 at the plasma membrane [36].

PIP_2 is likely to play multiple roles in modifying the cytoskeleton during phagocytosis. It may signal the dissociation of actin-capping proteins, such as gelsolin [40] and CapG [41], from the barbed ends of actin filaments,

Figure 1



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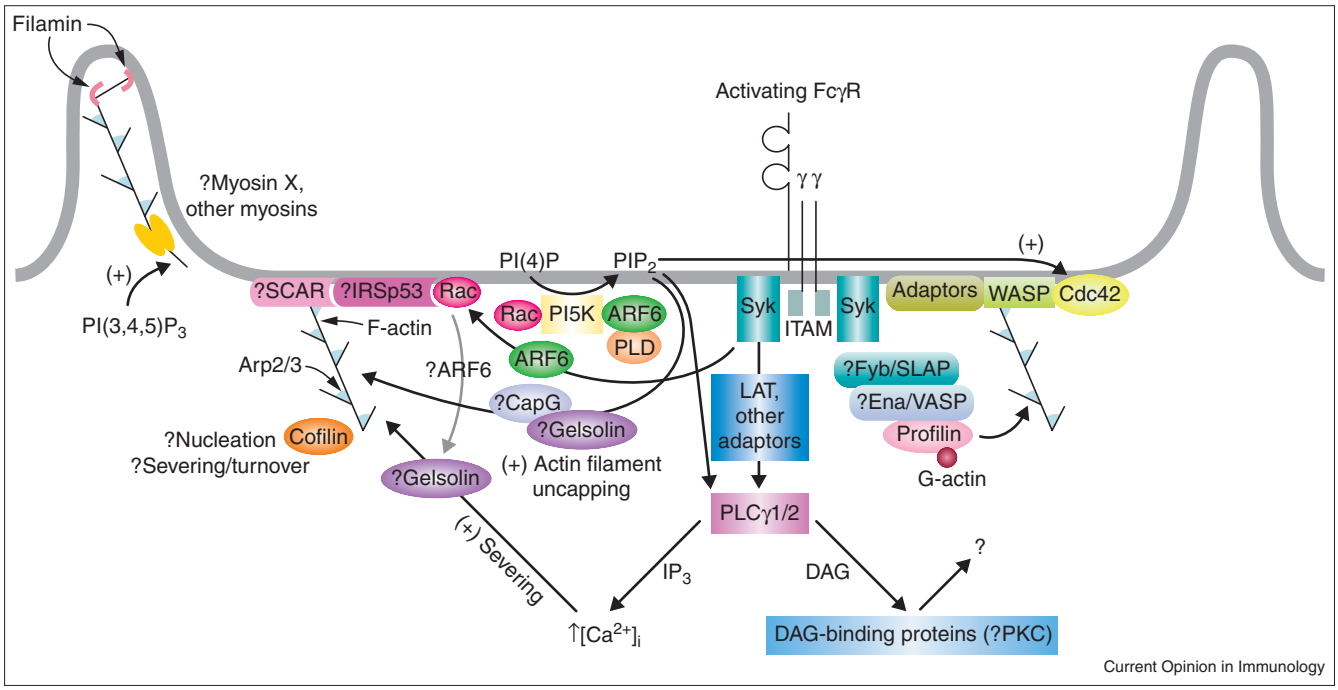
Lipid remodelling during phagocytosis. The figure illustrates localized changes in PIP_2 and DAG in macrophages engulfing opsonized particles (IgG-coated red blood cells). The left panel shows a differential interference contrast image; the right panel shows a dual-color confocal fluorescence image. The localization of PIP_2 was detected using a cyan fluorescent-protein-labelled PH domain from PLCδ (hence PIP_2 is blue), whereas DAG was detected with a yellow fluorescent-protein-tagged C1 domain of PKCδ. For details, see reference [16••].

Figure 2



Cytoskeletal alterations during FcγR-mediated phagocytosis. Actin polymerization ensues following receptor clustering by ligand (e.g. IgG) bound to the pathogen surface. Polymerization occurs following phosphorylation of receptor-associated ITAMs and recruitment of Syk. Actin polymerization requires multiple enzymatic activities, including PI 5-kinase (PI5-K) and an array of GTPases, notably Rac, Cdc42 and ARF6. Other GTPases, such as Rho [27] and Rap1 [144] may play a more prominent role in complement-receptor 3 (CR3)-mediated phagocytosis. Actin nucleation occurs principally through recruitment of the Arp2/3 complex. Additional roles for uncapping proteins (e.g. CapG) and severing/uncapping proteins (e.g. gelsolin and cofilin), probably accelerated by either the local enhanced production of PIP_2 or dephosphorylation (of cofilin), are likely. According to this model, the lipid product of PI3K, PI(3,4,5) trisphosphate, plays no direct role in promoting actin assembly, but rather participates in pseudopod extension (see text) and recruitment of unconventional myosins (e.g. Myosin X). Myosins assist in transducing mechanical energy necessary for pseudopodial dynamics. Question marks refer to hypothetical components of the model.

thus contributing to filament growth. Gelsolin also severs actin filaments; the coordination of severing and uncapping ensures the generation of short actin filaments that become incorporated into a force-generating network.

Cofilin is an actin-depolymerizing protein that, when dephosphorylated, contributes to actin remodeling by enhancing actin filament turnover [42,43] and creating new barbed ends [44]. The identity of specific phosphatase(s) that dephosphorylate phospho-cofilin *in vivo* is unknown. The major kinases that phosphorylate cofilin are LIM kinases 1 and 2 [45,46], but other cofilin kinases have been identified [47,48]. Interestingly, LIM kinase 1 is activated by effectors of Rac [45,46] and LIM kinase 2 is activated by the Rho effector, ROCK [49]. A Cdc42-activated kinase phosphorylates and activates both LIM kinase isoforms [50]. Cofilin dephosphorylation accompanies phagocytosis, and microinjection of anti-cofilin antibodies into macrophages inhibits phagocytosis of yeast [51].

Work with *Dictyostelium* has established a role for several unconventional myosins during phagocytosis [52,53]. These proteins may contribute to the generation of membrane tension and/or particle adhesion [54] as well as pseudopod extension, in the case of Myosin X (D Cox, JS Berg, J Chinegwundoh, BM Dale, RE Cheney, S Greenberg, unpublished data) and phagosome closure [55]. Given the diversity of the myosin superfamily and the recruitment of multiple members of this family to phagocytic cups, additional roles for myosins in phagocytosis are likely.

Membrane dynamics during phagocytosis

Professional phagocytes have an insatiable appetite: they can engulf multiple, often large particles. Such extensive phagocytosis requires the internalization of a vast area of the surface membrane. It has been estimated that, in extreme cases, macrophages can internalize the equivalent of >100% of their surface area within 30 minutes [56••]. Remarkably, this occurs without apparent reduction in exposed membrane surface. In fact, direct electrophysiological estimates revealed that the surface area of macrophages *increases* during the early stages of phagocytosis [57] and similar conclusions were reached by spectroscopic methods [58,59]. These results imply that the loss of membrane taken up into

phagosomes must be compensated by exocytic delivery of endomembranes to the surface.

At least part of the exocytosis triggered by phagocytosis occurs very near the site and at the time of particle engulfment. Direct visualization of endomembrane traffic in live cells undergoing phagocytosis reveals that cytoplasmic vesicles are delivered focally to the vicinity of the nascent phagosome. Moreover, the exposure to the surface of epitopes found in the lumen of such vesicles implies that fusion of endomembranes precedes sealing of the phagosomal membrane [60•]. The highly localized and rapid nature of the exocytosis suggests that, rather than being merely a compensatory reaction, membrane traffic is an essential requirement for efficient phagocytosis. Three observations support this notion. Firstly, phagocytosis is inhibited by blockers of PI3K [59,61], an enzyme that is involved in multiple membrane traffic events. Secondly, phagocytosis is reduced by cleavage of SNAREs using tetanus or botulinum toxins [58]. SNAREs are ubiquitous proteins thought to promote the docking and coalescence of lipid bilayers during membrane fusion events. Thirdly, a dominant-negative form of NSF similarly decreased phagocytic efficiency [62]. NSF is an ATPase that ensures the availability of active SNAREs for the fusion process.

Recent data indicate that early endosomes represent an important source of the membrane that is delivered to the nascent phagosome. The bacterial toxins that were found to depress phagocytosis are known to cleave and thereby inactivate VAMP3, a SNARE found in recycling endosomes. Accordingly, direct visualization of VAMP3-containing vesicles using a chimeric construct tagged with a fluorescent protein documents the occurrence of focal exocytosis at the phagosomal cup [60•]. Other proteins associated with endocytic vesicles that play a role in promoting phagocytosis/pseudopod-extension include amphiphysin IIm and dynamin 2, although their exact roles in these processes are uncertain [63,64•]. In addition, other sources of membrane may subserve similar and novel functions during phagocytosis. These include the endoplasmic reticulum [65] and lysosomes [66], the latter of which are required for invasion of *Trypanosoma cruzi*.

The tethering of secretory vesicles with their target membranes is thought to be mediated by small GTPases of the Rab family. One such protein, Rab11, promotes trafficking of sorting/recycling endosomes, the compartment that expresses VAMP3, to the plasma membrane. Ectopic expression of an active form of Rab11 potentiated phagocytosis and, conversely, an inactive Rab11 mutant decreased phagocytic efficiency [56••]. These observations further suggest a contribution of endosome exocytosis to the formation and closure of phagosomes. ARF6 has also been postulated to target recycling vesicles to the plasma membrane [67,68]. It is therefore noteworthy that expression of mutant forms of ARF6 also depressed phagocytic efficiency [69,70]. This may reflect the contribution of ARF6 to cytoskeletal rearrangements (see above), but a possible role in vesicle fusion cannot be discounted.

The cumulative evidence suggests that the complex cytoskeletal rearrangements that drive phagocytosis are accompanied by local membrane remodelling that may contribute to pseudopod extension and/or sealing.

Co-opting of the host-cell phagocytic machinery by invasive pathogens

The initial host response to most bacterial and fungal pathogens is phagocytosis. The particular route of entry is a function of both the specific host cell mediating ingestion and the pathogen itself [71]. For example, internalization of *Listeria* is mediated by the adhesins InlA, which binds to E-cadherin on host epithelia, and InlB, which binds to the Met tyrosine kinase and to gC1q-R on host cells [72,73••,74]. E-cadherin-mediated entry requires participation of catenins [75•] and Met-dependent signaling induces activation of PI3K [73••]. For *Yersinia*, recognition of invasin on the bacterial surface is mediated by β1 integrins on a variety of cells; bacterial uptake requires the participation of Src-family tyrosine kinases and focal adhesion kinase [76]. Many bacterial pathogens utilize multiple, possibly redundant mechanisms of entry into host cells. This is likely to be the case for *Neisseria*. Its adhesins include epitopes on the pili, which bind to the host cell protein CD46, and outer membrane proteins (Opa variants), which bind to members of the CD66 (CEACAM) family on phagocytes. CD46 ligation leads to calcium fluxes [77] whereas CD66 ligation results in activation of Src-family kinases and Rac [78]. Interestingly, activation of the Src-family members by *Neisseria gonorrhoeae* appears to require activation of an acid sphingomyelinase [79,80].

Opsonization of either invasive or non-invasive pathogens is an important mechanism used by the host to enhance the efficiency of phagocytosis. Complement fixation by the alternative or lectin pathways is one such example; however, the list of known non-immunoglobulin opsonins is growing and includes lung surfactant proteins and other collectins [81–83], extracellular matrix proteins [7,84,85], and pentraxins [86,87]. Some of these proteins may stimulate phagocytosis indirectly, by binding to phagocyte receptors that activate phagocytosis in general [7,88]. In some cases, the same proteins that act as opsonins for microbial pathogens also promote phagocytosis of apoptotic cells [89,90] (see below).

Several pathogens, such as *Salmonella* or *Shigella*, stimulate a 'trigger' mechanism of invasion, inducing a localized 'splash' of F-actin-rich membrane protrusions in the phagocyte that resemble forming macropinosomes. Work from Galan's group [91–93] has established a molecular basis for this form of phagocytosis: using a Type III secretion system, *Salmonella* injects SopE — a protein that serves as a guanine nucleotide exchange factor for Cdc42 and Rac — into host cells [91]. Yet another *Salmonella*

Table 2

***C. elegans* gene products involved in phagocytosis of apoptotic corpses.**

C. elegans gene product	Function	Mammalian equivalent	References
CED-1	Receptor	SREC	[116••]
CED-2	Adaptor	CrkII	[145••]
CED-5	Adaptor	DOCK 180	[146]
CED-6	Adaptor	hCED-6	[147,148]
CED-7	Transporter	ABC1	[111]
CED-10	GTPase	Rac1	[145••]

SREC, scavenger receptor expressed by endothelial cells.

protein, SipA, decreases actin depolymerization [92] and enhances actin-bundling activities of other actin-binding proteins [93,94]. In contrast, *Shigella*-induced ruffling and internalization are stimulated by secretion of IpaC, which activates Rac and Cdc42 indirectly [95], and IpaA, which binds to vinculin [96]. Rac and Cdc42 appear to be responsible for *Shigella*-stimulated actin nucleation, whereas Rho and IpaA-mediated alterations appear to modify the shape of the ruffles, rendering them competent to promote bacterial internalization [97,98].

Some pathogens synthesize anti-phagocytic factors [99]. For example, *Yersinia* secretes YopH, a tyrosine phosphatase that dephosphorylates the focal adhesion protein, Cas [100]. Another secreted product of *Yersinia*, YopE, is a RhoGAP [101]. Many pathogens evade killing by influencing post-phagocytic events, such as phagosome maturation [71,102–104]. This can result in evasion of biologically active lysosomal enzymes [71,102] or components of the NADPH-oxidase-containing vesicles [105,106]. Among the survival strategies employed by *Mycobacterium tuberculosis*, for example, is the recruitment of coronin 1 (TACO) [107] and suppression of calcium signaling [108], both of which are suggested to contribute to evasion of lysosome fusion by the *Mycobacterium*-containing phagosome.

Phagocytosis of apoptotic cells

Senescent cells generally undergo apoptosis. Extensive apoptosis also occurs during the course of organogenesis. Effective removal of such apoptotic cells is required for appropriate tissue renewal and remodelling. To a large extent, this occurs by phagocytosis, which facilitates both clearance of apoptotic bodies and completion of the cell death pathway [109••,110••]. Clearance of apoptotic corpses is mediated by macrophages as well as non-professional phagocytes, including epithelia.

Phosphatidylserine (PS) appears to be a major ligand on the surface of apoptotic cells that triggers phagocytosis. In normal cells, PS is largely confined to the inner leaflet of the plasma membrane. During apoptosis, PS becomes exposed at the outer leaflet of the membrane. This results from transmembrane lipid scrambling that is not counteracted by the flippases that maintain lipid asymmetry in normal cells. The ATP-binding cassette transporter 1 (ABC1), a structural ortholog of *C. elegans ced-7* [111], is required for efficient transbilayer redistribution of PS on phagocytic targets [112]; interestingly, its expression in macrophages is also required for maximal phagocytosis of apoptotic targets [112]. Various molecules have been postulated to function as possible PS receptors (PSRs) on the surface of mammalian phagocytes, including CD14, CD36, CD68 and LOX-1. Most of these, however, do not discriminate between PS and other phospholipids, whereas phagocytosis of apoptotic cells is particularly dependent on PS. Such selectivity is best explained by the recent identification of a distinct PSR with exquisite specificity for PS [113••].

The multiplicity of receptors implicated in phagocytosis of apoptotic cells, together with the dependence of the process on PS, prompted the notion that the process involves a 'tether and tickle' sequence. According to this model, introduced by Henson and colleagues [114••], one of a variety of receptors with comparatively high affinity would be involved in the initial attachment of apoptotic cells to the phagocyte. Such initial tethering is required for the PSR, which has comparatively lower affinity for its ligand. The low affinity of the PSR ensures that non-apoptotic cells, which expose only small amounts of exofacial PS, are not subjected to phagocytosis. This tandem mechanism is not unprecedented, since CD14 is thought to mediate the inflammatory response to lipopolysaccharides (LPSs) by initially attracting and then handing over the ligand to Toll-like receptors (TLRs).

The importance of phagocytosis of apoptotic cells is underlined by the evolutionary conservation of the process, which has been described in detail in *C. elegans* and in *Drosophila*. In fact, comparative studies to date suggest that many aspects of the phagocytic process, ranging from the extracellular receptors — for example, croquemort in *Drosophila* and CED-1 in *C. elegans*, which are analogous to mammalian scavenger receptors [115,116••] — to intracellular intermediates and effectors, are highly conserved across species (Table 2).

Little is known to date regarding the transduction of signals that lead to phagocytosis of apoptotic cells in mammalian cells. Initial indications suggest the usage of pathways similar to those for microbial engulfment, including PI3K and Rho-family GTPases [117,118]. However, conservation in signal transduction pathways between phagocytosis of apoptotic cells and other targets is not absolute; for example, ingestion of apoptotic thymocytes, but not IgG-coated targets, utilizes the receptor tyrosine kinase, MER. This kinase recognizes apoptotic cells indirectly by binding PS-recognizing opsonins, such as Gas6 [119•,120].

Phagocytosis and inflammation

Phagocytosis and inflammation co-exist. This naturally raises the question of whether phagocytes utilize common signaling intermediates to effect phagocytosis and gene

expression. Examples of distinct signaling paradigms exist, such as a requirement for Rac in the phagocytosis of *Pseudomonas aeruginosa*, but not in NF-κB-dependent gene expression induced by the same bacterium [121]. However, other pathogens, such as *Staphylococcus aureus*, trigger *trans*-activation of NF-κB via a multiprotein complex that includes Rac1 and TLR2 and in an apparently Rac-dependent manner [122]. The relationship between recruitment of various TLRs to phagosomes [123] and signaling phagocytosis remains to be clarified. It is noteworthy that, unlike the uptake of IgG-opsonized particles, phagocytosis of apoptotic cells by the PSR is not accompanied by the release of inflammatory mediators and is associated with the release of the anti-inflammatory growth factor, TGF-β [113••]. This ensures the ongoing clearance of apoptotic cells without concomitant inflammation.

Anti-inflammatory phagocytosis is also facilitated by serum proteins like C-reactive protein and the C3 component of complement, which coat apoptotic cells and mediate their uptake via non-inflammatory receptors [124••]. It is tempting to speculate that the striking association of complement-component deficiencies and systemic lupus erythematosus results from an inability to clear apoptotic corpses in a non-inflammatory manner, resulting in the generation of autoantibodies [124••]. Similarly, in the absence of functional MER, the inability to clear apoptotic cells efficiently results in the generation of anti-DNA antibodies [119•]. In contrast, necrotic cells may release pro-inflammatory substances, such as heat shock proteins [125••], or may be ingested by pro-inflammatory receptors that counteract the immunosuppressive effects of the PSR [113••].

Conclusions

Phagocytosis is a fundamental cellular process that serves multiple functions in immunity. The multiplicity of phagocytosis-promoting receptors contrasts with the convergence of signaling strategies designed to promote target engulfment. On the other hand, differences in the nature of specific phagocytic pathways determine the relative extent of the inflammatory response. The study of phagocytosis is rapidly evolving and complex, reflecting recent advances in a range of disciplines. Future insights into the mechanisms of phagocytosis may suggest novel strategies to modulate the immune response, holding promise for the treatment of an array of human diseases.

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