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Review

Molecular mechanisms of phagocytic uptake in mammalian cells

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Abstract. Phagocytosis is a highly conserved, complex process that has evolved to counter the constant threat posed by pathogens, effete cells and debris. Classically defined as a mechanism for internalising and destroying particles greater than 0.5 μm in size, it is a receptor-mediated, actin-driven process. The best-studied phagocytic receptors are the opsono-receptors, FcγR and CR3. Phagocytic uptake involves actin dynamics including polymerisation, bundling, contraction, severing and depolymerisation of actin filaments. Recent evidence points to the importance of membrane remodelling during phagocytosis, both in terms of changes in lipid composition and delivery of new membrane to the sites of particle binding. Here we review the molecular mechanisms of phagocytic uptake and some of the strategies developed by microbial pathogens to manipulate this process.

Keywords. Phagocytosis, signalling, receptors, actin, G proteins, membrane, pathogens.

Introduction

Eukaryotes are constantly exposed to microbial pathogens. Furthermore, multicellular organisms need to deal with "self" cells when they become dangerous, because of apoptosis, necrosis or transformation, and get rid of debris generated during development, tissue turnover and repair. With the exception of yeasts, animal eukaryotic cells have evolved a mechanism for engulfing and destroying particulate material: phagocytosis. In its classical sense, phagocytosis is receptor-mediated, actin-driven and proceeds in a zipper-like manner [1]. However, particle internalisation and bacterial invasion can also occur through macropinocytosis [2].

Phagocytosis is a multi-step process that is initiated by particle recognition. This is mediated by a wide variety of cell surface receptors that bind directly or indirectly, through opsonins (*e.g.* complement fragments, IgG), to the particle. Presumably due to evolutionary pressure, ligands for phagocytic receptors tend to be common to classes of pathogens. For example, β-glucans, which are expressed at discrete sites on the surface of yeasts, such as *Candida albicans*, are directly recognised by the phagocytic receptor Dectin-1, primarily expressed on antigen-presenting cells [3]. However, an essential part of *Candida* pathogenesis is the ability to switch to a filamentous form that is not recognised by Dectin-1, thereby preventing efficient binding by phagocytic cells [4]. As yet, it is unclear how much non-opsonic receptors, such as scavenger receptors (SR), really contribute to phagocytosis, although it was shown recently that bone marrow-derived dendritic cells from scavenger receptor A (SR-A)-knockout mice are less able to internalise the Gram-negative pathogen *Escherichia coli* than control animals [5]. Perhaps further work on non-opsonic receptors will reveal more that are capable of medi-

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ating phagocytosis, and to what extent they really contribute to phagocytosis *in vivo*.

In parallel to the direct recognition of ligands on the surface of phagocytic objects (microorganisms or cells), opsonisation by complement fragments or IgG provides common host-derived ligands for the abundantly expressed receptors that recognise them: complement receptors and Fc γ receptors, respectively. Although these receptors are thought to account for most of the recognition of phagocytic material *in vivo*, new opsono-receptors are still being discovered. For example, CD44 was shown to internalise hyaluronan-coated particles in murine macrophages in a process that also involves complement receptor 3 (CR3) [6, 7]. The reality of particle recognition is, therefore, complex and likely to involve a combination of surface receptors cooperating with each other. Because of this complexity, studies of phagocytic signalling in mammalian cells have so far concentrated on individual receptor types.

In this review we address the recent advances in our understanding of phagocytic uptake, focussing on the early signalling events that control actin and membrane dynamics in mammalian macrophages. Where pertinent, analogies with non-mammalian systems and alternative phagocytic cells are developed.

Actin dynamics

Once a particle has bound to surface receptors, transient, localised actin polymerisation is a necessary requirement for phagocytosis, as highlighted by the effects of cytochalasins. These drugs inhibit actin filament assembly and block particle uptake in both mammalian phagocytes and model organisms, such as the single-celled amoeba, *Dictyostelium discoideum* [8–10]. Actin dynamics are regulated by signalling pathways activated downstream of ligated phagocytic receptors. In mammalian macrophages, the best-understood pathways governing actin dynamics during phagocytosis originate from the FcγR and CR3 receptors, two opsono-receptors that recognise IgG- and C3bi-coated particles, respectively.

Receptor activation and signalling

The earliest signalling event detected following ligation of the FcγR with complexed IgG is the rapid phosphorylation of tyrosine residues within an immunoreceptor tyrosine-based activation motif (ITAM) domain, located either in the cytoplasmic tail of the receptor (human FcγRIIA) or in the associated homodimeric γ-subunit (other FcγR) [11]. This is mediated by Src-family tyrosine kinases and is essential for local actin polymerisation and phagocytosis [12]. An interesting recent development is the question of whether ligand-induced receptor clustering and Src kinase activity are coupled. Sobota et al. [13] showed that a mutant FcγRIIA that is unable to be phosphorylated does not properly cluster underneath bound IgG-beads, and proposed that there is a positive feedback loop, where phosphorylation by the Src kinase Lyn facilitates clustering, which further increases the efficiency of particle binding. This model also seems to apply to non-opsonic tyrosine kinase-dependent phagocytic receptors. For example, soluble β-glucan is able to bind the ITAM-containing receptor Dectin-1 but cannot activate signalling, whereas discrete regions of clustered β-glucan, found on zymosan or yeast bud-scars, can activate signalling, implying that cross-linking of receptors is critical [4]. As the resolution of imaging techniques increases, it will be possible to further dissect what is occurring at the molecular level during these early events.

Once phosphorylated, the ITAM-domain tyrosine residues function as docking sites for Src homology 2 (SH2) domain-containing proteins. Of these, the tyrosine kinase Syk has a particular importance to ITAM-dependent phagocytosis, as macrophages treated with pharmacological inhibitors of Syk kinase activity or derived from Syk knockout mice are unable to internalise IgG-opsonised particles [12, 14–16]. When activated, Syk triggers the activation and tyrosine phosphorylation of a number of downstream proteins, which impact on actin dynamics (see below).

By contrast to FcγR-dependent uptake, CR3-dependent binding and phagocytosis of C3bi-opsonised particles is not constitutively active and requires preactivation of phagocytes with phorbol esters, such as phorbol myristate acetate (PMA) or extracellular agonists [17, 18]. Importantly, CR3 is a member of the β_2 integrin family. Small GTPases of the Ras subfamily regulate integrin binding activity by inside-out signalling in a number of cell types including macrophages. Accordingly, the small G protein Rap1 is sufficient to activate CR3 ($\alpha_M\beta_2$), promoting binding and phagocytosis of C3bi-opsonised targets; furthermore, Rap1 is activated in response to inflammatory mediators and its activity required for agonist-induced activation of CR3 [19]. Interestingly, unlike the FcγR, CR3 does not rely on tyrosine phosphorylation to induce phagocytosis in macrophages, providing evidence for receptor-specific pathways in the early phases of uptake.

A central role for Rho-family G proteins

Signalling elements critical to the control of actin reorganisation during a variety of phagocytic signalling processes are the Rho GTP-binding proteins

[20–22]. This family of molecules act as molecular switches downstream of a variety of cell surface receptors. When activated and bound to GTP, they interact with downstream effectors, for example to mediate Arp2/3 recruitment and actin polymerisation during phagocytosis [23]. Inactive GDP-bound, Rho-family G proteins are thought to be cytosolic and bound to a guanine dissociation inhibitor (GDI) [24]. This association is very tight (nanomolar range) and renders the bound G protein unable to bind its effectors or other regulators [24]. The latter includes guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP). GEF activity is required to activate Rho proteins by catalysing GDP exchange to GTP. GAPs increase the intrinsically low catalytic activity of G proteins causing GTP hydrolysis and rapid inactivation.

Work using dominant negative alleles of Rac1 and Cdc42 has suggested that these Rho-family proteins are essential for actin assembly at nascent phagosomes during FcγR-mediated internalisation. Phagocytic uptake but not initial particle attachment is inhibited by dominant negative constructs, suggesting that Rac and Cdc42 activities are required downstream of FcγR ligation [20]. Importantly, blocking the activity of both Rac and Cdc42 abolishes the formation of actin cups, while abrogating the activity of only one of them still results in some local actin polymerisation. This indicates that Rac and Cdc42 have complementary functions, possibly because they act at different spatial and temporal phases of the phagocytic process [16, 25]. A recent exquisite FRET study has revealed that accumulation of active Cdc42 to forming phagosomes occurs early and is preferentially restricted to the tips of extending pseudopodia [25]. In contrast, activation/localisation of Rac1 occurs throughout the phagocytic cup and during closure [25]. A regulatory role for a third member of the Rho-family, Rac2, at the base of FcγR phagosomes has also been proposed [26]. Local Rac2 activity would precede Rac1 activation, thought to coincide with particle contraction and phagosome closure [25, 26].

By contrast, in macrophages and transfected fibroblast cell lines, actin remodelling during CR3-mediated phagocytosis depends exclusively on the activity of another Rho-family protein, RhoA [20]. Both dominant negative Rho and the specific Rho inhibitor, C3 transferase, completely abolish CR3-dependent internalisation while having no effect on FcγR-mediated uptake [20]. Actin polymerisation downstream of Rho is regulated by two distinct regions of the β_2 cytoplasmic tail. Whereas a 16-amino acid, membrane proximal α-helical region controls the activation of Rho, a TTT (threonines 758–760) motif at the C-terminal end of β_2 cytoplasmic domain is responsible for the recruitment of Rho to forming CR3 phagosomes [27]. During complement-mediated internalisation, F-actin assembly itself also differs dramatically from the continuous F-actin cups induced by the FcγR, in that the phagocytic cup consists of discrete foci of F-actin and other cytoskeletal-associated proteins [20, 28].

The interesting dichotomy between the proposed Type I (FcγR, Cdc42/Rac-dependent) and Type II (CR3, Rho-dependent) modes of phagocytosis received additional credence when it was found that Vav, a RacGEF activated in response to tyrosine kinase signalling was accumulating at Fc but not CR3 cups in J774.A1 macrophages. Moreover, a dominant negative approach suggested that Vav activity was necessary for FcγR but not CR3-mediated uptake [29]. However, several reports have challenged these findings. One study suggested that Rho activity is essential to the enrichment of F-actin at phagocytic cups and calcium signalling during FcγR-mediated uptake in RAW264.7 macrophages [30]. Another study performed in bone marrow-derived macrophages from wild-type and Vav-knockout mice suggested that Rac and Rho activities were necessary for both CR3- and FcγR-mediated phagocytosis [31]. These discrepancies may arise simply from the disparity of the models and assays used to study phagocytosis and are likely to be resolved over the next few years, as more information becomes available on the receptor pathways and model systems. Nevertheless, it is worth mentioning that the notion of two modes of uptake, Type I (Cdc42/Rac-dependent) and Type II (Rho-dependent) is also receiving support from the elucidation of the downstream pathways triggered by phagocytic receptors (see below) and from studies on other receptors, cell types and organisms. Indeed, Lee et al. [32] have recently established a role for the GEF DOCK-180 and adapter molecule CrkII in recruiting and activating Rac downstream of FcγR.

As indicated above, the best studied of the Rho-family G proteins are RhoA, Rac1 and Cdc42. One or more of these proteins control uptake of inert particles, bacteria and cells through a variety of receptors and in all model systems so far [33, 34]. There is, however, increasing evidence that other GTPases might be important for phagocytosis and bacterial invasion. As well as an emerging role for Rac2 in mammalian and insect phagocytosis [25, 34], there may be other G proteins involved. The *Salmonella* Type III secretion (T3S) effector SopB activates SGEF, an exchange factor for RhoG, which contributes to the actin remodelling responsible for *Salmonella* internalisation by non-professional phagocytes [35]. RhoG function has also been implicated in apoptotic cell

uptake by mammalian phagocytes [36]. Investigating more systematically the role of all Rho-family members in phagocytosis is an interesting avenue for further research.

The general importance of Rho-family small G proteins during internalisation is clearly demonstrated by the extent to which they are targeted by pathogenic microorganisms (see Table 1 for bacterial effectors and references). Enteric pathogens such as *Salmonella*, *Listeria*, *Shigella* and enteropathogenic *Yersinia* species are examples of bacteria that hijack host responses to their own end. Such bacteria can be classified either into those like *Salmonella* and *Listeria* that promote their own internalisation by phagocytes and other cell types (*e.g.* epithelial cells) and replicate intracellularly, or bacteria from *Clostridium* and *Yersinia* species that are unequipped to survive within macrophages and alter phagocyte signalling to remain extracellular. In both cases, however, there is the physical barrier of the plasma membrane, which separates the bacterium from the cytosol, where critical signalling molecules are located. *Listeria monocytogenes* activates actin dynamics by binding to and clustering cell surface receptors, mimicking a phagocytic event. By contrast, for *Shigella*, *Salmonella* and *Yersinia,* highly specialised organelles, T3S apparatus, mediate the injection of bacterial effectors into the host cytosol to stimulate (*Shigella*, *Salmonella*) or block (*Yersinia*) uptake. For other pathogens, interference with host cell signalling pathways involves entry of the virulence factor (*e.g.* C3 transferase from *Clostridium botulinum*) through an associated secreted factor that forms a pore in the host membrane.

Of the bacteria that promote their internalisation two general models are described: the trigger and zipper mechanisms. Examples of 'triggering' bacteria are *Salmonella* and *Shigella*, which manipulate host signalling through injection of effectors (such as RhoGEF) as described above. In the case of "zippering" bacteria, the receptors on the host cell surface initiate signalling that results in circumferential binding of the prey by phagocytic receptors and internalisation of the bacterium. The term 'zippering' likens this process to classical FcγR internalisation, which is reliant on the engagement of receptors all around the particle being phagocytosed. An archetypal example of a "zippering" bacterium is *L. monocytogenes.* This bacterium interacts with host epithelial cells *via* its adhesins InlA and InlB, which bind the host cell receptors E-cadherin and Met, respectively [37]. Once intracellular, bacteria escape the phagocytic vacuole and are free to move inside and between cells of the epithelial monolayer, resulting in dissemination and widespread disruption of the gut barrier.

Pathogenic *Yersinia* provide a model organism for studying the mechanism of T3S and, as such, their effectors are intensely studied [38]. Following breach of the intestinal barrier, the synergistic activity of six *Yersinia* outer membrane proteins (Yop) leads to a block in phagocytosis, as well as preventing cytokine secretion and inducing apoptosis in phagocytic cells. Interestingly, three Yop effectors YopE, YopT and YopO, target Rho-family GTPases, albeit by different mechanisms. YopE is a RhoGAP, YopT is a cysteine protease, cleaving the C-terminal region of the Rho proteins, releasing them from membranes, and YopO has a GDI-like activity [39, 40]. It is not yet understood how these effectors work in concert to disrupt Rho protein signalling during phagocytosis, as, on first glance, it seems counterproductive to have so many proteins targeting the same group of molecules. Current research is pointing towards the idea that there are exquisite and non-redundant roles for each of these Yop proteins in *Yersinia* virulence [41]. Indeed, the idea that bacterial effectors are acting specifically, locally and temporally is generally becoming more prominent.

Altogether, internalisation of material by zipper or trigger, in primitive organisms, non-professional and professional phagocytes alike, all have in common the local activation of selective Rho-family G proteins. Accordingly, bacterial pathogens that induce or block their uptake do so by virulence factors or toxins that stimulate or antagonise the local activation of one or more Rho proteins (Fig. 1; see also Table 1).

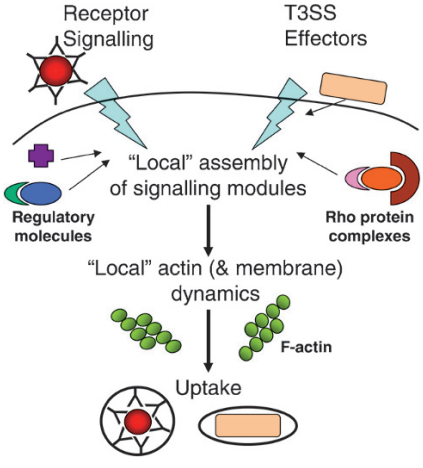

Figure 1. The convergent effects of receptor-mediated and pathogen-induced signalling. Whether occurring through a "zipper" or "trigger" mechanism, internalisation of bacteria, cells, and inert particles follows a similar sequence. The 'local' recruitment and activation of signalling complexes leading in particular to the activation of Rho-family G proteins, is an early step in the pathway. Locally assembled complexes trigger localised actin dynamics, which, together with membrane remodelling, leads to engulfment of the particle. T3SS, Type 3 secretion system.

Table 1. Bacterial toxins that modulate Rho-family G proteins and the actin cytoskeleton.

Species	Effector/toxin	Target	Effect	Mode of action	Reference
Aeromonas salmonicida	AexT	Rac1, Cdc42, RhoA	Inactivating	GAP activity	[166]
Burkholderia pseudomallei	BopE	Rac, Cdc42	Activating	GEF activity	[167]
Clostridium, Bacillus, S. aureus	C3 exotoxin	RhoA/B/C	Inactivating	ADP-ribosylation – interferes with RhoGEF stimulation	[168]
C. difficile	Toxin A and toxin B	Rho family (GDP form)	Inactivating	Mono-glucosyltransferase – inhibits GEF activation, blocks hydrolase activity, inhibits interaction with effectors	Ibid
C. botulinum, Bacillus cereus	C2, Iota toxin, VIP toxin	Actin – Arg177	ADP-ribosylation	Dissolution of actin cytoskeleton	Ibid
E. coli, Bordetella spp.	Cytotoxic-necrotizing factor (CNF1, DNT)	RhoA	Activating	Deamidation at Gln63 results in constitutively active Rho	Ibid
E. coli	EspG	RhoA (indirect)	Activating	Activates GEF-H1 by microtubule disruption	[169]
Helicobacter pylori	CagA	Rac1	Activating	As yet unknown	[170]
Listeria	ActA	VASP	Causes unipolar intracellular movement	Actin nucleation through activation of Arp2/3 complex	[171]
Pseudomonas aeruginosa	ExoS	Rac, Cdc42, Rho	Inactivating	RhoGAP, and ADP-ribosylating activity for Ras and Rabs	[172]
	ExoT	Rac, Cdc42, Rho	Inactivating	RhoGAP	[173]
Rickettsia	RickA		Causes unipolar intracellular movement	Actin nucleation through activation of Arp2/3 complex	[174]
Salmonella typhimurium	SopE/SopE2	Rac1, RhoG, Cdc42	Activating	GEF activity	[35]
	SopB	Cdc42, RhoG (indirectly)	Activating	Activates endogenous GEF, SGEF, how is unknown but requires PIs	Ibid
	SptP	Rac1, Cdc42	Inhibitory	GAP, undoes what SopB and SopE do	[175]
	SpvB	Actin – Arg177	ADP-ribosylation	Dissolution of cytoskeleton	[176]
	SipA	Actin	Actin polymerisation promoting uptake	Inhibits ADF/cofilin-directed depolymerisation both by preventing binding of ADF/ cofilin and by displacing them from F-actin	[80, 81]
	SipC	Actin	Causes actin nucleation and bundling	Bundling is independent of host components	[177]
Shigella flexneri	IpaC	Cdc42, Rac (through Cdc42)	Activating	Unknown	[178]
	VirA	Cdc42, Rac (indirect)	Activating	Possibly activates GEFs and destabilises microtubules	[179]
	IcsA	N-WASP	Causes unipolar intracellular movement	Actin nucleation through activation of Arp2/3 complex	[180]
Staph aureus	EDIN, EDIN-like factors	RhoA	Inactivating	ADP-ribosylation – leads to GDI binding and sequestration in cytosol	[181]
Yersinia species	YopE	Rac, Rho	Inactivating	GAP activity	[182]
	YopO	Rac, Rho	Inactivating	GDI-like	[182]
	YopT	RhoA	Inactivating	cysteine protease	[182]

Downstream regulators of uptake

The Arp2/3 complex. The local re-organisation of actin that occurs at phagocytic cups is mainly mediated by the recruitment and activation of actin-nucleating proteins, in particular the Arp2/3 complex [23]. WASP (Wiskott-Aldrich Syndrome Protein) and Scar/WAVE (Suppressor of cAMP receptor/WASP family Verprolin-homologous) proteins, often referred to as nucleation-promoting factors, trigger actin nucleation through direct binding of their C-terminal VCA (verprolin homology, cofilin homology and acidic region) domain to the Arp2/3 complex, stimulating its activation [42]. The Arp2/3 complex is composed of seven proteins including the actin-related proteins Arp2 and Arp3, and functions by promoting *de novo* actin polymerisation through stimulating the branching of new filaments on existing actin filaments [43]. The Arp2/3 complex is functionally important for both FcγR- and CR3-mediated engulfment as it co-localises with actin filaments beneath bound particles in receptor-expressing COS cells and J774.A1 macrophages and since expression of ScarWA, the conserved C-terminal region of the Arp2/3-binding protein Scar1, which disperses the Arp2/3 complex, prevents its localisation to the phagosome and local actin polymerisation [23].

Rho-family GTPases exert their effects on the actin cytoskeleton during phagocytosis by locally recruiting and interacting with a number of downstream effectors in an active, GTP-dependent manner. In turn, it is these regulators that are then able to bind and activate the nucleating capacity of the Arp2/3 complex and, hence, afford the signalling cascade a link between upstream signals and actin assembly (Fig. 1). The requirement for Arp2/3 complex activity was shown for classical FcγR- and CR3-mediated uptake, *Dictyostelium* phagocytosis and bacterial invasion [23, 44, 45].

Actin nucleators. As discussed above, two key effectors that stimulate actin filament assembly downstream of Cdc42 and Rac are WASP/N-WASP and the related Scar/WAVE-family proteins, respectively [46]. The central importance of WASP in FcγR-mediated phagocytosis is demonstrated by the impairment in actin cup formation and uptake observed in WAS patient-derived macrophages, which lack functional WASP protein [47]. Moreover, both overexpressed N-WASP [23] and endogenous WASP [47] localise at phagocytic cups in FcγR-transfected COS cells and macrophages. Interestingly, GFP-tagged N-WASP also accumulates at CR3-mediated phagosomes in receptor-transfected COS-7 cells [23]. In line with this, $WAS^{-/-}$ neutrophils exhibit defects in several β2 integrin-dependent functions, such as adhesion, migratory ability and activation of degranulation and respiratory burst. These defects are thought to stem from the dramatically reduced clustering of β2 integrins in these knockout cells [48]. However, the precise role of WASP/N-WASP during CR3-dependent uptake is currently elusive, as Cdc42 activity is not required in Type II phagocytosis [20].

WASP exists in the cell in an autoinhibitory conformation [49]. Several *in vitro* studies have established that this structural constraint can be relieved through binding to active, GTP-bound Cdc42 and phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] to produce an active form of WASP that can consequently interact with and activate the Arp2/3 complex [50–52]. It therefore seems likely that the coinciding events of local recruitment/activation of Cdc42 and the transient enrichment of $PI(4,5)P_2$ at the nascent phagosome represent an activation signal for WASP [53]. Additionally, WASP is found to be phosphorylated on tyrosine 291 during FcγR-dependent phagocytosis, which, together with formation of a complex between WASP and WIP (WASP-interacting protein), is critical to the assembly of a phagocytic cup around IgG-opsonised particles [54]. Two other activators of WASP have recently come to light, namely Felic/CIP4b and Toca-1. Felic/CIP4b is a scaffolding protein capable of binding both GTP-bound Cdc42 and WASP, which localises to sites of phagocytosis in RAW 264.7 macrophages [55], while Toca-1, a PCH (Pombe Cdc15 homology)-family protein, has been identified in a reconstituted biochemical system to interact with activated Cdc42 and N-WASP/WIP complex to bring about N-WASP activation and, subsequently, actin nucleation *via* the Arp2/3 complex [56]. Whether Toca-1 is physiologically important in the context of phagocytosis is yet to be elucidated.

Recently, other Arp2/3-regulating proteins have come to the forefront as potential key players in the remodelling of cytoskeletal actin at phagocytic cups. Ena/vasodilator-stimulated phosphoprotein (VASP) proteins, which are related to and interact with WASP-family members, can regulate actin dynamics at the plasma membrane [57]. Ena/VASP proteins accumulate with F-actin at nascent FcγR-induced phagosomes in both primary and immortalised macrophages, probably as part of a multimolecular complex with WASP and the adaptor proteins SLP-76 [(SH2)-domain-containing leukocyte protein of 76 kDa], Fyb/SLAP (Fyn-binding/SLP-76-associated protein) and Nck [58]. Another indication that these proteins could be important regulators of normal phagocytosis comes from the well-studied model system of actin-based motility in *L. monocytogenes*. *Listeria* ActA is able to recruit and promote the actin-nucleating capacity of Arp2/3 through VASP [59]. The observa-

tion that phagocytosis and actin cup formation are impaired in RAW 264.7 macrophages expressing ActA repeats, which effectively act to titrate Ena/VASP proteins from their targets, suggests a fundamental contribution for Ena/VASP proteins in actin remodelling downstream of FcγR ligation [58].

Other regulators. The signalling events linking activation of RhoA, downstream of CR3 ligation, to the assembly of actin structures are still poorly understood. However, some work has addressed candidate regulatory molecules. Two recognised downstream targets of Rho, Rho-kinase (ROK) and mammalian diaphanous-related formin (mDia), and a downstream target of ROK, myosin II, have roles in phagocytosis of C3bi-opsonised particles [60, 61]. Use of the ROK inhibitor, Y-27632 showed that enrichment in Arp2/3 complex and F-actin was diminished at CR3-induced phagosomes, correlating with the selective deficiency in CR3- and not FcγR-dependent uptake [60]. The same phenotype was observed with inhibitors of myosin II [ML7 and 2,3-butanedione monoxime (BDM)] [60]. Yet, the contractile protein myosin II is also required in FcγR-mediated phagocytosis, at least for particle internalisation, if not for generation of the actin cup [60]. More recently formins, specifically mDia, have also been identified as being involved in actin dynamics during CR3-mediated phagocytosis [61]. In mouse macrophages, the RhoA effector mDia1 localises with polymerised actin at CR3 phagosomes and small interfering RNA (siRNA) directed against mDia1 inhibit F-actin accumulation and CR3-mediated uptake without affecting particle attachment, highlighting the participation of mDia1 in the early stages of CR3-mediated phagocytosis [61]. It should be noted that the effect on CR3-dependent phagocytosis of either ROK or mDia inhibition is only partial, possibly suggesting cooperation/compensation by other molecules. Alternatively, there might be another component yet to be placed in the signalling pathway. Despite the latest findings, the exact mechanisms of action of ROK and mDia still remain to be seen.

Of late, another protein capable of enhancing actin polymerisation has been shown to shape actin dynamics in phagocytosis. Coronins can bind F-actin and localise at phagocytic cups in *Dictyostelium* [10], mouse [62, 63] and human [64] cells. In particular, coronin-1B controls the formation of filament networks through inhibition of Arp2/3-induced actin nucleation [65, 66]. When expressed in RAW264.7 macrophages, a dominant negative fragment of coronin-1 blocks accumulation of Arp2/3 at cups and the completion of phagocytosis, while having no effect on initial binding and signalling from the FcγR. This indicates that coronin-1 is required downstream of earlier signalling events, for example activation of tyrosine kinases and Rho-family G proteins [67]. Whether coronin is involved in phagocytic events mediated by other receptors in mammalian phagocytes is unclear at this stage. Finally, several actin bundling and cross-linking proteins (reviewed in [68]) have been shown to be involved in phagocytosis, although they are poorly studied at a functional level in this context.

Actin severing and depolymerisation

During phagocytosis, as in many actin-dependent cellular processes, actin dynamics are not restricted to nucleation and polymerisation but are also regulated by capping/uncapping, depolymerisation and severing [69]. Disassembly of actin filaments occurs both during internalisation, albeit at very low levels, and after completion of uptake. In any case, every stage of actin disassembly requires a precise spatial and temporal coordination of an array of actin-depolymerising proteins. Yet, it appears that it is not just the presence of specific functional proteins but also the absence and/or depletion of signalling molecules previously involved in earlier stages of phagocytosis, that contribute to the control of actin disassembly. Prompt disappearance of F-actin from phagosomes has been observed in *Dictyostelium* and in mammalian phagocytes, following closure of the phagocytic cup [70, 71]. A recent study suggests that, at least in human neutrophils, inactivation of Cdc42 is required for depolymerisation of the F-actin network, phagosomal maturation and phagolysosome fusion [72]. In contrast to these findings, Rac1 and Cdc42 were found to remain active at young phagosomes despite actin depolymerisation in RAW264.7 macrophages [73]. These experiments suggest that it is the hydrolysis of $PI(4,5)P_2$ from the phagosomal membrane that coincides with the dissociation of the actin network [73]. This is consistent with the idea that optimal WASP/N-WASP-induced, Arp2/3-dependent actin polymerisation depends on two inputs on WASP: active Cdc42 and $PI(4,5)P_2$ [74]. Cdc42 activity need not be terminated; if WASP is no longer localised or activated by $PI(4,5)P_2$, it will not be able to bind and activate the Arp2/3 complex, halting actin nucleation and polymerisation.

The role of gelsolin. Gelsolin is a calcium- and $PI(4,5)P_2$-regulated molecule that has pleiotropic effects on F-actin. In effect, severing, uncapping and binding of actin filaments by this protein increase filament number and provide many free polymerising ends. Gelsolin has been found enriched at phagosomes [75]. Additionally, Gelsolin-null ($Gsn^{-/-}$) mur-

ine neutrophils have a specific deficit in FcγR-mediated phagocytosis, although both attachment and ingestion of IgG-opsonised particles were inhibited [76]. Actin formation around the phagocytic cup was still occurring in these cells, as were other functions associated to neutrophil phagocytosis, such as granule translocation and NADPH oxidase activation [76]. Similarly, binding and phagocytosis of collagen beads, which depend on the integrin $\alpha_2\beta_1$ are also defective in $Gsn^{-/-}$ fibroblasts [77]. Nevertheless, CR3-mediated uptake was only slightly delayed in gelsolin-deficient neutrophils [76]. This suggests a selective role for gelsolin in early stages of phagocytosis, downstream of specific receptors. Gelsolin might dissolve the cortical actin cytoskeleton and allow receptor clustering and new localised actin polymerisation. Interestingly, some evidence supports the notion that gelsolin regulates actin remodelling through Rac activation, which correlates with the ability of Rac to trigger the dissociation of gelsolin from actin filaments and the receptor preference indicated above [78, 79]. It is also worth noting that a *Salmonella* invasion protein, SipA is capable of impeding actin turnover partly by blocking the severing function of gelsolin [80, 81]. By contrast, uptake of both complement- and IgG-opsonised particles is impaired in CapG-null macrophages [82]. CapG belongs to the same family of proteins as gelsolin but lacks severing activity. The data therefore suggest that CapG and gelsolin perform non-overlapping functions during phagocytosis, with CapG likely to be the more predominant capping protein in macrophages.

A conserved role for cofilin? As hinted above, the majority of the information available on actin disassembly comes from work focussing on *Dictyostelium*. One particular group of actin regulatory proteins possibly dictating disassembly are the ADF/cofilin family. Cofilin, a member of this family, functions both by severing and depolymerising ADP-actin filaments. It was originally identified as enriched at *Dictyostelium* phagocytic cups, along with the presumed cofilin-regulatory protein, DAip1 [83, 84]. DAip1-null *Dictyostelium* cells exhibit a defect in phagocytosis [84]. Interestingly, microinjection of anti-cofilin antibodies into differentiated macrophage-like U937 cells leads to a partial reduction in phagocytosis of serum-opsonised zymosan [85]. Cofilin is enriched at nascent phagosomes in human neutrophils phagocytosing opsonised *C. albicans* [86]. Cofilin is predicted to be involved in a range of phagocyte functions, as when treated with an antisense oligonucleotide to cofilin and challenged with opsonised zymosan, J774.1 macrophages show an approximate doubling of the overall amount of intracellular F-actin, a threefold increase in superoxide production and a mild augmentation of phagocytosis relative to control cells [87]. This finding suggests that cofilin indeed regulates actin dynamics during phagocytic uptake but that perhaps other proteins/mechanisms contribute more to the actin disassembly responsible for the termination of phagocytosis.

Cofilin works in concert with LIM kinase (LIMK) to control actin filament turnover. LIMK can block the actin binding and depolymerising activity of cofilin by phosphorylating it on a serine residue at position 3. In fact, a dominant negative mutant (D460A) of LIMK1 can substantially abrogate the total amount of F-actin in cells, indicating that LIMK is responsible for the tight regulation of the activity of cofilin and, hence, the balance between actin assembly and disassembly [88]. Interestingly, the different isoforms of LIMK can be activated through phosphorylation by downstream effectors of the Rho GTPases, namely the Ser/Thr p21-activated kinase 1 (PAK1), an effector for Rac1 and Cdc42, and ROK, the RhoA effector [89–91]. This in turn suggests that the Rho GTPases could control actin depolymerisation downstream of both the FcγR and CR3 receptors during phagocytosis. Confirmation of the regulation of actin turnover during zipper-like uptake has come from pathogenic bacteria. InlB-mediated entry of *L. monocytogenes* is thought to involve a cofilin phosphocycle influenced by the activity of LIMK and Rac [92].

Phagosome closure does not necessarily denote the end of all actin polymerisation. Indeed, it is likely that actin assembly recommences during phagosome maturation, long after internalisation, as suggested in *Dictyostelium* using GFP-tagged LimEΔ, a truncated Lim domain protein that exclusively labels F-actin [70]. This new actin filament assembly, after all or most of the original F-actin meshwork has disappeared from around the young phagosome, has been proposed to be instrumental in driving the movement of phagosomes through the cytoplasm [70].

Regulation of myosin-based contractility

Contractile activities generated by the myosin superfamily of motor proteins represent another driving force for phagosome formation. A large number of myosin isoforms have been implicated in uptake, in particular during FcγR-mediated phagocytosis. This is consistent with the idea that different steps of phagocytosis are controlled by distinct subsets of myosins. Indeed, immunofluorescence analysis of myosin distribution in macrophages undergoing FcγR-mediated phagocytosis has revealed that myosins II and IXb were concentrated at early phagocytic cup structures, likely modulating pseudopod exten-

sion, while myosin IC was associated with the forming phagosome at later time points, consistent with a role in phagosome closure [93]. By contrast, myosin V recruitment was found to increase continuously during formation of the nascent phagosome and was prominently recruited after phagosome closure [94, 95].

The influence on uptake of the contractile activity associated with phagocytosis was observed in time-lapse video microscopy studies where two macrophages were attempting to ingest a single IgG-opsonised erythrocyte; pseudopodia were extended by both macrophages around the soft target that became constricted. Despite the formation of two nascent phagocytic cups, a connection was maintained between the macrophages through a linking stretch of erythrocyte membrane. Upon treatment with BDM, a myosin inhibitor, phagocytosis was inhibited and constriction of the shared erythrocyte no longer took place, although pseudopodia extension and actin polymerisation still occurred [94].

Even though a range of myosin isoforms were described as recruited and accumulating at phagosomes, their exact roles during phagocytosis have remained largely vague. Several studies are beginning to define individual contributions. Myosin II has been implicated in phagocytic cup squeezing; treatment of macrophages with ML-7, a myosin light chain kinase (MLCK) inhibitor that selectively targets myosin II function, does not affect pseudopod extension but prevents closure of phagocytic cups [96]. Myosin II is also involved in CR3-dependent uptake, however with a preponderant role in actin assembly [60]. Myosin IC is the only myosin isoform localised to the linking strings of erythrocyte membrane shared by competing phagocytes, supporting a role for this particular myosin in the so-called 'purse-string-like' contraction accompanying phagosome closure [94]. Myosin X is also recruited to phagocytic cups in bovine pulmonary alveolar macrophages in a PI3-kinase (PI3K)-dependent manner. Its function within the phagocytic cup appears related to maximal pseudopod extension, as phagocytosis of large (6 μm) but not small (2 μm) IgG-coated particles is inhibited in cells expressing a truncated myosin X mutant [97]. This size dependency was also exhibited by cells treated with PI3K inhibitors [98], strongly suggesting that myosin X is a downstream effector of PI3K, controlling pseudopod extension.

Myosin V appears only after phagosome closure and has been proposed to regulate phagosomal movement inside cells. Myosin Va-null macrophages displayed a surprising increased rate of phagosomes accumulating in the perinuclear region. This suggests a role for myosin Va in short-range movements at the cell periphery, in the absence of which microtubules are likely to drive long-range translocation of phagosomes to the cell centre [99]. It has further been proposed that the saltatory phagosome movement observed in normal macrophages is a direct result of the myosin Va-F-actin interactions counteracting microtubule-dynein-based transport, possibly to allow phagosome fusion with endosomes in the cell cortex [99].

Membrane trafficking and remodelling

During internalisation, be it by a 'trigger' or a 'zipper' mechanism, the plasma membrane of phagocytes extends around the particle until a new distinct compartment, the phagosome, has been released into the cytosol. Measurement of cell surface area by flow cytometry and analysis of cell capacitance during phagocytosis have shown that, contrary to expectation, the cell surface area actually increases during internalisation of multiple particles [100–102]. This phenomenon is due to an active process of membrane remodelling, involving changes in membrane composition, membrane depletion and membrane delivery.

Phospholipids

Local, signalling-induced changes in the lipid composition of the plasma membrane are deeply involved in the process of membrane remodelling during phagosome formation. Among all the lipids possibly involved in this process, phosphoinositides (PIs) are the best characterised. They all derive from phosphatidylinositol, which can be phosphorylated by specific kinases at positions 3, 4 and 5 of the inositol ring, to generate progressively phosphatidylinositol mono-, bis- or tris-phosphate. Their role is related to their ability to interact with specific protein domains, including the pleckstrin-homology (PH) domain, which is the most widespread PI-binding protein module in higher eukaryotes interacting with $PI(3,4,5)P_3$. PH domains are found, for example, in Arf GTP-binding proteins, PDK1 and Akt/PKB. FYVE (conserved in Fab1, YOTB, Vac1, EEA1) domains bind PI(3)P and with less affinity PI(5)P, and Phox-homology (PX) domains preferentially bind PI(3)P [103–105]. These domains have been used as probes in several fluorescent and electron microscopy studies to document changes in PI composition during phagocytosis.

Specifically, this type of approach has revealed that $PI(4,5)P_2$ localises at the inner leaflet of the plasma membrane during the early stages of phagocytic cup formation. PI(4)P-5kinase (PIPKI), which generates $PI(4,5)P_2$, is activated by small G proteins, such as Rac1, and is itself recruited in response to FcγR

ligation. Also localised there is phosphatidic acid (PA), the product of phospholipase D (PLD) 1 and 2 activity. Inactivation or silencing of these two PLD isoforms partially inhibits particle engulfment [106]. $PI(4,5)P_2$ plays multiple roles in actin assembly and remodelling, namely: nucleation [51, 52], uncapping the barbed ends of existing filaments [107, 108], severing and cross-linking filaments [109]. These functions can explain the localisation of this particular PI at the edges of pseudopods, thought to need actin polymerisation to extend around the particle [73]. Also interesting is the function suggested for the accumulation of PA, a cone-shaped lipid, which can induce concave curvature on membranes. PA could promote wrapping of advancing pseudopodia around the particle and phagosome closure. Within 2–3 min from the onset of phagosome formation, before the sealing of the phagosome, depolymerisation of the actin cytoskeleton starts at the base of the cup and the local density of $PI(4,5)P_2$ decreases in parallel [73, 110]. Both phospholipase C (PLC, producing IP_3 and DAG), and PI3K (forming $PI(3,4,5)P_3$), are involved in $PI(4,5)P_2$ disappearance, at a time where small G proteins (such as Rac1 and Cdc42) are still active, possibly suggesting that these Rho-family proteins do not contribute to the process of actin depolymerisation observed at this stage. In line with this, inhibition of PLC and PI3K blocks actin depolymerisation from the forming phagosome [73]. Dissociation of the actin meshwork at the forming phagosome could avoid an excessive membrane rigidity, which would impede on one hand the curving necessary for particle enclosure and on the other hand the access of incoming endomembranes targeted to the nascent phagosome. In agreement with this model, stimulation of actin synthesis by overexpression of PIPKI, which converts PI(4)P into $PI(4,5)P_2$ at the plasma membrane, aborts phagosome closure and engulfment [73].

$PI(3,4,5)P_3$ starts to be formed soon after FcγR ligation and is observable until 1 min after phagosome sealing [110, 111]. Its accumulation at the phagocytic cup requires the function of class I PI3K, which is recruited to the receptor complex through an interaction of the p85 SH2 domain with phosphotyrosines on the tyrosine kinase Syk [111, 112]. Noticeably, inhibition of PI3K activity by wortmannin or LY294002 has no effect on actin polymerisation during phagocytosis or on phagocytosis of small beads; PI3K inhibitors abrogate uptake of large (>3 μm in diameter) particles [98, 113]. This suggests a role for class I PI3K in large scale membrane remodelling, possibly because additional activities are required for internalising larger particles, for example myosin X function [97, 114]. Alternatively, or in parallel, PI3K activity could also allow membrane delivery from intracellular compartments to the site of phagocytosis and help build up larger pseudopods. $PI(3,4,5)P_3$ is also emerging as a general regulator of small GTPases, with data available on Arf6 [115] and, possibly, Rac1 (reviewed in [110]). It is conceivable that larger particles would require a different regulation of these signalling molecules. Disappearance of $PI(3,4,5)P_3$ also seems important for the timely completion of particle engulfment. It can be regulated by the activity of SH2 domain-containing inositol 5'-phosphatases (SHIP), which can associate with both the ITAM and immunoreceptor tyrosine-based inhibition motif (ITIM) regions of Fcγ receptors. Overexpression of these phosphatases causes inhibition of FcγR- and CR3-mediated phagocytosis in macrophages, which is not particularly surprising if inhibition of PI3K activity also blocks uptake [116]. A novel $PI(3,4,5)P_3$-5-phosphatase, 72-kDa-5-phosphatase, has recently been reported to inhibit FcγR-mediated phagocytosis, affecting pseudopod extension and phagosome closure [117].

As suggested above, the localisation of specific lipids at the site of phagosome formation may also explain the focal character of the membrane delivery. No lipid diffusion from the site of phagosome formation to the neighbouring unengaged membrane has been observed. Different hypotheses have been proposed to explain these localised enrichments: the presence of degradative enzymes at the rim of the cup and in the unengaged membrane; the association of lipids with components of the receptor signalling complex, which is supported by the reduced diffusion of lipid-anchored proteins at the phagocytic cup, especially in the inner leaflet of the membrane [118]. Another hypothesis is that lipids are trapped at the edges of advancing pseudopods by a diffusional barrier, possibly formed by integral membrane components interacting with the underlying cytoskeleton, in structures resembling the tight junctions of polarised cells [110].

Another important feature that makes lipids crucial for the process of targeting signalling proteins involved in phagosome formation and closure is the creation of a negative surface charge at the inner leaflet of the plasma membrane. Electrostatic changes at the membrane may affect the specific recruitment and localisation of signalling molecules (*e.g.* GTP-binding proteins) and transmembrane proteins with cationic motifs [119–121]. During phagocytosis, ongoing membrane and lipid remodelling events dramatically alter the surface charge on the phagosome membrane, influencing localisation and also activation of signalling molecules. As has been shown for Rho-family GTP-binding proteins, the net charge at the phagosomal membrane can lead to the displacement of the G proteins from the cytosolic GDI,

towards the inner face of the plasma membrane, where they can be activated by GEF proteins. A change in membrane charge can also promote the deactivation of small G proteins by GAP [24, 122, 123].

Membrane sources

The process of endomembrane delivery to the site of phagosome formation through secretory vesicles that fuse with the plasma membrane has been called 'focal exocytosis' [124]. It explains the increase in total cell surface area that is observed during particle uptake. Although the plasma membrane is the main quantitative contributor of membrane during phagocytosis, intracellular membranes contribute to phagosome formation. Membrane sources identified in mammalian phagocytes are recycling endosomes, late endosomes, and endoplasmic reticulum (ER) (Fig. 2). In eukaryotes, SNARE (soluble *N*-ethylmaleimide-sensitive-factor accessory protein receptor) proteins have been implicated in every step of membrane fusion in intracellular trafficking pathways. The specific pairing of resident SNARE proteins on donor and target membranes constrains trafficking and fusion of the corresponding compartments (reviewed in [125]). Accordingly, detection of specific SNARE and associated markers at the phagocytic cup has been used to prove the contribution of intracellular organelles to phagosome formation. Cellubrevin/vesicle-associated membrane protein 3 (VAMP3) from recycling endosomes was shown to interact at the phagocytic cup with the cognate SNARE complex syntaxin 4 (STX4)-SNAP23 during ingestion of yeast and IgG-opsonised targets. Similarly, a role for tetanus-insensitive (TI)-VAMP/VAMP7 from late endosomes, possibly interacting with STX2, STX3 and/or STX4 has been documented during FcγR- and CR3-mediated phagocytosis ([125] and reviewed in [126]).

There is general agreement regarding the absence of membrane contribution from the Golgi apparatus, since phagocytosis can proceed in the presence of the fungal metabolite Brefeldin A (BFA) [127, 128]. By contrast, ER involvement is still a subject of controversy. Desjardins and colleagues [129] initially proposed that ER membranes were fusing with the plasma membrane. This finding raised the appealing possibility for antigens derived from internalised material to be mounted on MHC class I molecules, produced in the ER (cross-presentation). In agreement with this idea, ER markers (such as calnexin and calreticulin) have been detected at nascent phagosomes in J774.A1 macrophages internalising latex beads, opsonised particles, bacteria and parasites [129]. ER markers have also been observed by others

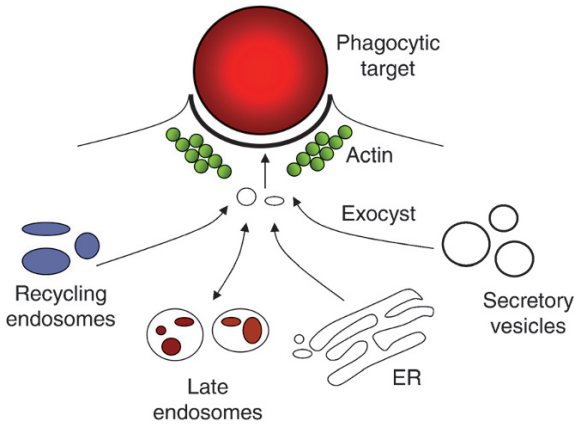

Figure 2. Sources of membrane contributing to particle internalisation. The exact contribution of the various endomembrane compartments is controversial and the subject of continued research. Focal exocytosis is the mechanism by which membrane is delivered to the forming phagosome. It is thought both recycling and late endosomes can provide membrane to the phagocytic cup. Endoplasmic reticulum markers have been observed at the phagosomal membrane, and recently a new role for the *Drosophila* exocyst complex in membrane delivery during phagocytosis has been revealed. See text for more details.

in other cell lines, at least with some phagocytic particles [130]. Accordingly, Becker et al. [128] have shown that interfering with the ER-specific SNARE protein ERS24/Sec22b, leads to an impairment of phagocytosis of IgG-opsonised 3 μm beads by J774.A1 macrophages, although the uptake of 0.8 μm particles was not affected. The same SNARE protein, ERS24/Sec22b, was previously shown to interact with the plasmalemmal Sso1/Sec9c in yeast studies [131]. Another ER-localised SNARE protein, Syntaxin18 (STX18), has been suggested to be recruited at the phagosomal membrane during the early stages of phagocytosis, and both the expression of dominant negative mutants and siRNA silencing of STX18 led to a significant reduction in the uptake of IgG-opsonised beads by FcγRIIA-transfected 293T cells [132]. However, the contribution of ER to phagocytosis has been dismissed as not quantitatively relevant, regardless of the particle size [133, 134]. The Grinstein group convincingly showed, by biophysical and biochemical assessments, that physical continuity between ER and the phagocytic cup never occurred [133]. These data obviously do not exclude the possibility of a later communication between the phagosome and the ER, which could be involved during the process of phagosome maturation and/or antigen cross-presentation. Alternatively, a recent hypothesis explains cross-presentation by a retrograde traffic of internalised antigens through the Golgi into the ER, based on the repositioning of the microtubule organising centre and the Golgi during phagocytosis [135, 136].

The delivery of endomembranes to the base of the phagocytic cup is a primary mechanism of membrane remodelling at the nascent phagosome, affecting the lateral displacement of plasmalemma markers from the cup [134]. Several molecules, representative of the outer and the inner leaflets of the plasma membrane, have been used to analyse the change in membrane composition, after labelling with electron-dense or fluorescent probes: GPI (glycosylphosphatidylinositol), GM1 gangliosides and a plasma membrane inner leaflet marker (a diacylated probe containing the N-terminal domain of Lyn) were all cleared from the nascent phagosome [134]. Inhibition of exocytosis attenuates phagocytosis in a size-dependent manner, with uptake of 8 μm IgG-opsonised beads blocked but internalisation of 3 μm beads unaffected. Different systems previously used to block exocytosis (colchicine treatment, PI3K inhibition and *N*-ethylmaleimide-sensitive factor blocking) were used to show that in these conditions, phagocytic cups form but there is no clearance of the membrane markers [134]. Interestingly, however, this process of clearance does not seem to involve the receptors engaged in particle binding: ligand-bound FcγRIIA persists at the phagocytic cup, with no new receptors coming from intracellular stores.

Additional processes that may be involved in remodelling the cell membrane at the site of phagosome formation are endocytosis and lateral segregation of lipid rafts [134, 137]. However, these two mechanisms, which would remove membrane from the phagocytic cup, do not interfere extensively with membrane composition and do not counterbalance the outwards traffic of endomembranes, so that the net balance is still an increased cell surface area [134]. The role of endocytosis can be envisaged in recycling of components to sustain the process of exocytosis or as part of the mechanism of antigen presentation.

The function of membrane dynamics, besides the physical ability to surround and internalise particles, is likely to be the recruitment of molecules involved in the following steps of phagocytosis (phagosome maturation). At the same time it might represent a strategy to regulate the signalling pathway, by displacement of factors that have to be switched on or off to let the whole process continue, as has been proposed to explain Lyn exclusion from the phagocytic cup during FcγR-mediated phagocytosis [134].

Role of the microtubule cytoskeleton in membrane delivery

The major candidate for the delivery of endomembrane to the site of phagosome formation is the microtubule-based transport system. Although their specific contribution is not clearly understood, microtubules have been reported to extend deep into the site of phagosome formation [112, 138]. Microtubules are thought to be required for podosome formation in primary human macrophages and pseudopod extension around large particles [112, 139]. They do not seem to be involved in the actual fusion process between the forming phagosome and other membrane compartments but are most likely required for the correct respective positioning of the organelles, and in trafficking the necessary vesicles and molecules to the site of phagocytosis. For instance, microtubules regulate the recruitment and activity of PI3K at the sites of podosome formation [112] and there are some examples of direct or indirect binding of PI3K regulatory subunits to tubulin [140, 141].

Arf and other regulators of membrane traffic

Members of the ADP-ribosylation factor subfamily of small G proteins, specifically Arf1 and Arf6, have been implicated in membrane delivery and dynamics during phagocytosis. Previous studies assumed that Arf1 was not participating in phagocytosis. Arf1 was indeed thought to be regulated by BFA-sensitive GEF, and phagocytosis could still occur in the presence of BFA [127]. Later analysis revealed that both Arf1 and Arf6 are activated during phagocytosis [115]. Moreover, other ArfGEFs, not Golgi-associated, such as the cytohesin/ARNO family-members, can regulate, at least *in vitro*, the activation of Arf1 and Arf6 [115, 142, 143]. In the context of phagocytosis, Arf6 has been found to mediate the focal delivery of vesicles to the forming phagosome [144]. Arf6 is activated immediately after binding of opsonised particles to macrophages and localised at the advancing edges of pseudopodia; by contrast, Arf1 activation occurs during pseudopod extension and active Arf1 is generally distributed over most of the engulfed particle. Also, the two Arf proteins are co-ordinately regulated, with Arf6 activation being PI3K-independent, whereas Arf6 deactivation and Arf1 activation are PI3K-dependent. Expression of Arf1 and Arf6 mutants revealed that the closure of FcγR-mediated phagosomes requires the normal cycling of both Arf1 and Arf6. In fact, macrophages expressing Arf mutants have been shown to initiate phagocytosis but are unable to progress past pseudopod extension [115]. Initially, it was suggested that Arf6 may control the early events of focal exocytosis of VAMP3-carrying vesicles from recycling endosomes to the nascent phagosome [144]; it may also contribute to actin polymerisation [127, 144]. It has now been proposed that Arf6 is required for recruitment of the Rho-family GEF Kalirin and subsequent Rac activation and membrane ruffling [145]. This contradicts earlier findings suggesting that Rac activation preceded Arf6

activation [144]. Arf6 can also activate the lipid-modifying enzyme PIPKI, thereby potentially amplifying the PI3K signalling cascade through PIPKI activity and PI(4,5)P_2 formation. Finally, Arf6 can activate PLD, known to mediate several activities at the plasma membrane, such as membrane ruffling and cell motility [146, 147], in addition to its role in phagosome formation during CR3- and FcγR-mediated phagocytosis [106, 148], (reviewed in [110]).

Arf1, whose activity is downstream of PI3K signalling, has been suggested to be involved in the oxidative burst coupled to FcγR-mediated phagocytosis, owing to its ability to bind Arfaptin (which could make Rac, also bound by Arfaptin, free to incorporate into functional oxidase complexes) [115]. In addition, a recent study from the Niedergang group has shown that Arf1 regulates the early recruitment of the endosome-associated clathrin adaptor complex AP-1 to the site of attachment of IgG-opsonised particles to macrophages, an activity that is again BFA-insensitive [142]. This suggests an involvement of Arf1 in the control of endosomal dynamics during phagocytosis, with the role proposed for AP-1 being to retain cargo proteins, such as TNFα, at the level of endosomes, rather than participate in formation of clathrin buds [142].

Interestingly, clathrin has not been found to be recruited with the AP complexes [142] and this observation is in line with previous studies inferring that clathrin function is not required for FcγR-mediated phagocytosis, although clathrin has been repeatedly observed at phagocytic cups. Accordingly, internalisation of IgG-coated beads is not compromised in cells expressing antisense mRNA to clathrin [149]. Whether clathrin localisation indicates frustrated endocytosis, triggered following FcγR clustering but unable to proceed because of the size of the bound particle, or is related to clathrin involvement in subsequent fission of vesicles from the phagosome during maturation is still unknown [149].

Another important GTPase involved in the process of membrane remodelling around the target particle during phagocytosis is dynamin II. Dynamin II belongs to a family of proteins that have been involved in fission of vesicles at various membrane compartments, including endocytosis at the plasma membrane, vesicle formation at the trans-Golgi network and possibly at recycling endosomes (reviewed in [150]). Amphiphysin IIm, with its C-terminal SH3 domain, can bind and recruit dynamin II at the phagocytic cup [151]. Expression of a dominant negative form of dynamin II blocks particle internalisation before phagosome closure, by preventing membrane extension [152]. Inhibition of Amphiphysin IIm function, by deleting the dynamin binding domain, prevents phagocytosis of zymosan, C3bi- and IgG-opsonised particles in RAW264.7 macrophages, meaning that it has a general role in particle internalisation [151]. Interestingly, antagonising dynamin II function abolished the capacitance rise normally observed at the macrophage cell surface after challenge with opsonised particles, suggesting that dynamin II has a role in the early stages of phagocytosis, possibly linked to the process of focal exocytosis of vesicles that contribute to pseudopod extension around the target particle. Since dynamin II has been localised by electron microscopy to tubular recycling endosomes [135, 153] and GFP-dynamin II has been detected all around particles in phagocytosing macrophages challenged with IgG-beads, it can be speculated that it controls vesicle budding from the endosomal compartment to the extending pseudopods. Indeed, dynamin II enrichment shows the highest intensity at the edges of the phagocytic cup and is rapidly lost after phagosome closure [102]. Other studies revealed, by total internal reflection microscopy, that there is a close association between vesicle fission by dynamin at the plasma membrane and assembly of actin, reinforcing the possibility of a role of dynamin in a force-generating system that powers movement of vesicles in the cytoplasm and coordinates vesicle production and actin assembly [154]. Dynamin, in fact, has been shown to associate to actin at the plasma membrane during phagocytosis and cell migration, and actin-binding proteins such as syndapin II and cortactin, have been identified as dynamin targets [150, 155].

A very exciting recent development in the elucidation of how new membrane is delivered at forming phagosomes comes from *Drosophila*. Several components of the exocyst complex, a multimolecular protein complex that tethers secretory vesicles to the plasma membrane, were identified by mass spectrometry of the membrane on latex bead phagosomes [34]. Silencing by RNA interference of exocyst subunits decreased phagocytosis of bacteria by *Drosophila* S2 cells [34]. How this new mechanism ties in with receptor signalling and membrane dynamics will surely be the focus of intense investigation in future years.

Role of membrane dynamics in bacterial uptake

As the importance of lipid dynamics during phagocytosis becomes apparent, so bacterial effectors are discovered that manipulate it. One important *Salmonella* T3S effector that contributes to invasion of intestinal cells is SopB. This P5-phosphatidylinositol phosphatase is responsible for activating Cdc42 and RhoG, both of which are required for *Salmonella* invasion [35, 156]. IpgD is the SopB homologue in

Shigella flexneri; it also modifies the host cell cytoskeleton to induce internalisation of the bacterium. Although an IpgD deletion mutant does not totally abrogate *Shigella* invasion, it cannot induce the formation of fully structured entry sites [157]. As shown for SopB, IpgD converts PI(4,5)P_2 to PI(5)P, which activates the PI3K/Akt pathway during infection [158, 159]. It was recently shown that VAMP8 is recruited to *Salmonella*-induced F-actin ruffles in a SopB-dependent manner. SopB is also the source of PI(3)P that recruits VAMP8 [160], a SNARE present on early and late endosomes and on the trans-Golgi network, where it is responsible for homotypic fusion. As yet though, there is no described role for VAMP8 during normal phagocytosis. However, these studies may point to the existence of additional regulators of membrane dynamics during phagocytosis.

Pizarro-Cerda et al. [161] recently found a role for PI4 kinase II α and β in *Listeria* internalisation. Specifically, siRNA-induced knockdown of PI4 kinase II α and β or of the PI(4)P ligand AP-1 (a clathrin adapter) inhibited bacterial entry. Importantly, this is the first time a role for PI(4)P during internalisation has been demonstrated and could suggest a role for PI(4)P during macrophage phagocytosis.

These examples further illustrate the similarity in principles and general mechanisms between classical macrophage phagocytosis and bacterial invasion. They also emphasize how much the study of bacterial invasion can inform on host cell signalling pathways.

Concluding remarks

Through the years, phagocytosis has progressed from a specific function displayed by amoebae and a subset of mammalian immune cells to a conserved cellular process displayed by a variety of cell types in all animal organisms, except yeasts. Phagocytosis is truly the general cell function through which particulate material (cell debris, certain viruses, microorganisms, apoptotic cells) is captured into an intracellular organelle and normally degraded. Phagocytic uptake can be performed equally well by professional phagocytes (*e.g.* neutrophils and macrophages) and non-phagocytic cell types (*e.g.* epithelial, fibroblast and endothelial cells). In all cases, similar molecular mechanisms – and to a large extent also signalling pathways – are induced by particle binding, namely localised remodelling of the actin cytoskeleton, phospholipid signalling and membrane delivery. This conservation is clearly demonstrated by the ability to transfer high phagocytic potential to epithelial or fibroblast cells by transfecting phagocytic receptors normally expressed on professional macrophages. Nevertheless, two ultrastructural modes of phagocytic internalisation need to be distinguished. The zipper mechanism, involving circumferential engagement of host cell receptors with ligands on the particle surface, is at play during most phagocytic events and during uptake of *L. monocytogenes* and *Yersinia* species by their host cells. By contrast, the trigger mechanism, illustrated by the entry process of *Salmonella typhimurium* and *S. flexneri* is less dependent on intimate contact between particle and host cells and more akin to macropinocytosis [162].

Over the past 30 years, developments in cell biology, pharmacology, molecular cell biology and signalling have led to the appreciation of the large number of receptors that could perform phagocytosis, and of the even greater number of molecules involved in phagocytic uptake. Whereas such abundance can be overwhelming, it merely reflects the fact that different receptors use specific signalling pathways to link receptor ligation to the build-up and activation of conserved signalling nodules and effectors, at so-called "constriction points" [163]. These critical steps are the accumulation of active members of the Rho-family proteins underneath bound particles, to regulate actin polymerisation, and the regulation of membrane dynamics. A lot remains to be learned as to how these two fundamental aspects of phagocytic uptake are coordinated and spatiotemporally regulated.

Further progress in our understanding of the molecular mechanisms of phagocytic uptake is likely to come from three major types of approach. Firstly, comprehensive, high-throughput approaches (proteomics analyses, genetic screens in genetically tractable organisms, and functional genomics analyses) are already yielding a wealth of proteins and pathways regulating phagocytosis, as recently illustrated in mammalian or *Drosophila* phagocytes and in *Caenorhabditis elegans* [34, 164, 165]. Secondly, elucidation of the mechanisms and pathways used by bacterial virulence factors to mimic or block phagocytosis will continue to provide interesting candidate regulators of uptake. Finally, the continued development of high-resolution live imaging [25] will help establish the spatiotemporal patterns of signalling complex assembly and signal transduction during phagocytosis. Altogether, these complementary strategies will help resolve most of the currently burning questions in the field of phagocytosis. What are the critical receptors and pathways regulating uptake of the various types of particles *in vivo?* How significant is the cooperation of receptors in this context? Trying to set aside the existing controversies, how many modes of uptake really coexist and when/why did they evolve during evolution? Do particle type, size and stiffness matter,

when considering the signalling pathways of phagocytosis? What are the biophysical constraints to phagocytosis and can a minimal phagocytic machine be modelled? Finally, in a broader context, to what extent are phagocytic pathways similar to other cellular processes also dependent on actin polymerisation and membrane dynamics such as cell adhesion and motility? Exciting times are ahead of us!

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