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Phagosome maturation during the removal of apoptotic cells: receptors lead the way

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Abstract

In metazoan organisms, cells undergoing apoptosis are rapidly engulfed and degraded by phagocytes. Defects in apoptotic-cell clearance result in inflammatory and auto-immune responses. However, little is known about how apoptotic-cell degradation is initiated and regulated and how different phagocytic targets induce different immune responses from their phagocytes. Recent studies in mammalian systems and invertebrate model organisms have led to major progress in identifying new factors involved in the maturation of phagosomes containing apoptotic cells. These studies have delineated signaling pathways that promote the sequential incorporation of intracellular organelles to phagosomes and have also discovered that phagocytic receptors produce the signals that initiate phagosome maturation. Here, we discuss these exciting new findings, focusing on the mechanisms that regulate the interactions between intracellular organelles and phagosomes.

Introduction: apoptotic cells are engulfed and degraded within phagocytes

Phagocytosis is an actin-based cell-internalization process for particles $>0.5 \mu\text{m}$ [1]. In metazoans, invading microorganisms, parasites and host cells undergoing apoptosis or necrosis are the major sources of phagocytic targets [1]. During phagocytosis, phagocytes recognize their targets via specific cell-surface receptors and extend pseudopods along the surface of phagocytic targets to form ‘phagocytic cups’ via the reorganization of the actin cytoskeleton [1] (Figure 1a). The progressive extension and the eventual fusion of pseudopods leads to the formation of ‘phagosomes’, which are intracellular vacuoles containing cargos [1] (Figure 1a). Immediately after phagosome formation, the actin scaffold disassembles from the phagosome surface. The cargo is then degraded through ‘phagosome maturation’, a process that involves extensive remodeling of phagosomal membranes and contents [2] (Figure 1). During the life of an animal, a large number of unwanted cells undergo apoptosis, a genetically controlled cell suicide [3,4]. In mammals, macrophages and dendritic cells function as ‘professional’ phagocytes that engulf and degrade most apoptotic cells [5]. Several other cell types can also function as ‘non-professional’ phagocytes, which have important roles in removing apoptotic cells or cell fragments, in particular in tissues that macrophages cannot infiltrate [5,6]. In simpler organisms such as the nematode *Caenorhabditis elegans*, several types of neighboring cells engulf and degrade apoptotic cells [7].

Efficient removal of apoptotic cells helps sculpt organs, maintain homeostasis and eliminate abnormal, non-functional or harmful cells [4,5]. Moreover, removing apoptotic cells prevents harmful inflammatory and auto-immune responses owing to release of potentially harmful contents. Furthermore, macrophages that engulf apoptotic cells elicit anti-inflammatory responses that facilitate the resolution of regional inflammation [3]. Inefficient engulfment of apoptotic cells, or degradation of apoptotic-cell contents, results in chronic inflammatory and auto-immune diseases [3,8].

Compared with the recognition and engulfment of apoptotic cells, which have been extensively studied, little is known about how apoptotic cells are degraded inside phagosomes. Unlike apoptotic cells, ingested microorganisms induce macrophages to elicit pro-inflammatory responses [9]. This raises some important questions. How do macrophages elicit different responses to different phagocytic targets? Does the phagosome-maturation process have any role in these differential responses? Recent progress focusing on the degradation of apoptotic cells in mammals, *C. elegans* and *Drosophila melanogaster*, which provides clues to some of these questions, is discussed here. For related topics not covered here, such as apoptotic-cell-DNA degradation, antigen cross-presentation and the detailed mechanisms of phagosome maturation, see Refs [2,8,10,11].

The events and mechanisms involved in phagosome maturation

Until recently, knowledge of phagosome maturation was almost entirely derived from studies of opsonized particles (including red blood cells and inert latex beads) or microbes engulfed by mammalian cells. Macrophage phagocytic receptors that recognize specific surface features of microbes, or opsonins [such as immunoglobulin G (IgG)] coated on particle surfaces, mediate actin-dependent phagocytosis [12,13]. After the disassembly of the actin cytoskeleton surrounding the cytoplasmic side of the phagosome, nascent phagosomes fuse sequentially with early endosomes, late endosomes, which are the differentiated derivative of early endosomes and, ultimately, lysosomes, which are the terminal degradative compartment [14–17] (Figure 1b). These fusion events change the composition of the phagosomal membrane and lumen, load digestive enzymes to phagosomes and promote phagosome acidification (Figure 1b).

The functions of phosphatidylinositol 3-phosphate (PtdIns3P) and Rab GTPases in the maturation of phagosomes containing opsonized cells or microbes have been well studied [2]. Based on cell-culture studies, Rab GTPases are thought to target and tether specific organelles to phagosomes [2,18,19]. Rab5, which is localized to the surface of early endosomes and nascent phagosomes, is essential for tethering these two organelles, and for recruiting the class-III phosphatidylinositol 3 (PI3)-kinase Vps34, which produces PtdIns3P [20–22]. PtdIns3P accumulates on endosomal and phagosomal surfaces and assists Rab5 in recruiting early endosomes [21,23–25]. In addition, PtdIns3P attracts additional molecules, which possess PtdIns3P-binding moieties such as the Phox (PX) domain or Fab1–YOTB–Vac1–EEA1 (FYVE) domain, to phagosomal surfaces [25]. For example, components of the nicotinamide adenine dinucleotide phosphate (NADPH)–oxidase complex, which mediates the killing of internalized microbes, are recruited to phagosomes through their PX domains [26–28]. Rab7 is specifically localized to late endosomes, lysosomes and phagosomes and is implicated in tethering and fusion between them [14,29,30]. However, until recently, the functions of endogenous Rab5, Rab7 and Vps34 in phagosome maturation in whole animals, which would help understand the true physiological and developmental roles of these factors, has not been studied.

The phagosomal lumen becomes increasingly acidic during maturation, owing to the activity of vacuolar (V)-type ATPase [2]. V-type ATPases are present on plasma and intracellular

membranes [31] and are presumably recruited to phagosome membranes from these sources. The final maturation stage of the phagosome, known as the phagolysosome, is characterized by the abundance of resident acid hydrolases, and an acidic luminal pH that activates these enzymes [2] (Figure 1b).

Although there are many data on the processing of opsonized particles, until recently, the cellular events leading to the maturation of phagosomes containing apoptotic cells had not been systematically studied. Lysosomes and the acidification of phagosomes were implicated in apoptotic-cell degradation in macrophages [32,33]. The fusion of endosomes and lysosomes with phagosomes containing apoptotic cells has also been observed [33]. However, whether such fusion events require the key molecules identified above, such as Rab5, Rab7 and PtdIns3P, was unknown. It was also unclear whether any unique factors existed that specifically controlled the degradation of apoptotic cells, as opposed to other phagocytic cargos. In addition, in several reports, apoptotic cells were observed to be engulfed through a process that resembled macro-pinocytosis, in which case phagosomes display a different morphology from those containing opsonized cells or microbes – they are spacious and contain a substantial amount of fluid [34–36]. In general, it remains to be determined whether the maturation of phagosomes containing apoptotic cells or other phagocytic targets uses the same or different mechanisms.

Studies of apoptotic-cell degradation in mammals

Differential regulation of the maturation of phagosomes carrying different cargos

In a study by Erwig *et al.* [37], the maturation rates of phagosomes containing IgG-coated cells (which are engulfed through the Fc receptor that binds the Fc fragment of immunoglobulins) or apoptotic cells were compared between phagocytes of different identities, focusing on parameters such as lumen acidification and the appearance of lysosomal landmarks on phagosomal surfaces and inside lumen. In several types of macrophages and fibroblast cells ('non-professional' phagocytes), phagosomes containing apoptotic cells always mature faster than those containing opsonized cells [37]. These observations indicate that phagocytic targets can differentially affect the maturation rate, perhaps through their phagocytic receptors, or other host-cell factors.

Erwig *et al.* [37] further identified the specific contribution of a Rho GTPase and its downstream targets, the ERM (ezrin–radixin–moesin) proteins, in accelerating the acidification of phagosomes containing apoptotic cells. Rho was activated at early time points only when apoptotic cells, but not opsonized cells, were ingested [37]. It remains unknown, however, how the Rho-mediated signal pathway is activated. Phagocytic receptors and guanine-nucleotide-exchange factors (GEFs) for Rho might be involved.

Dendritic cells actively inhibit phagosome maturation to preserve antigens for cross-presentation

Dendritic cells are important antigen-presenting cells; among other substances, they engulf viral-infected apoptotic cells or apoptotic tumor cells and cross-present viral or tumor antigens, in complex with major histocompatibility complex (MHC) I molecules, to cytotoxic T cells, which subsequently initiate full-scale immune responses [38–40]. Dendritic cells seemingly use common mechanisms to recognize and engulf apoptotic cells; however, unlike other phagocytes, they have developed unique strategies to enhance the efficiency of the cross-presentation of antigens, one of which is to reduce the degradation rate of ingested cells [40–47]. In dendritic cells, phagosome acidification occurs at a slower rate than in macrophages; consistently, the Rho-signaling pathway, which accelerates phagosome maturation in macrophages and fibro-blasts, does not seem to have such a role [37]. In addition, the fusion of a subset of lysosome-related organelles, which carry the

NADPH-oxidase complex NOX2, to phagosomes limits phagosome acidification and, thus, preserves antigens from complete degradation [46]. The NOX2 complex alkalinizes the phagosomal lumen by consuming protons generated by the V-type ATPases [46]. Recently, it has been reported that the fusion of the NOX2-complex-carrying, lysosomal-like organelles to phagosomes was mediated by the small GTPase Rab27 in dendritic cells [47]. These findings strongly indicate that different types of phagocytes employ different mechanisms to modulate phagosome maturation. Although NOX2 is also assembled on phagosomal surfaces in macrophages and neutrophils, it does not seem to affect phagosome acidification in these cells, indicating that the effects induced by NOX2 vary between different types of phagocytes [48].

Dynamic regulation of Rab5 during phagosome maturation

In addition to its well-known function in the maturation of phagosomes containing opsonized particles, Rab5 was recently found to be essential for the maturation of phagosomes containing apoptotic cells [49]. Using a fluorescence resonance energy transfer (FRET)-based time-lapse assay, Kitano *et al.* [49] observed that Rab5 accumulated to, and was activated on, the phagosome surface immediately after the disassembly of the actin coat from nascent phagosomes containing apoptotic cells. Among the GEFs known for Rab5, only Gapex-5 is essential for Rab5 activation during the maturation of phagosomes carrying apoptotic cells [49]. Furthermore, Rab5 activation is dependent on end-binding protein 1 (EB1), a microtubule-tip-associating protein that also interacts with Gapex-5 [49]. It has been proposed that the recruitment of Gapex-5 to phagosomes through the microtubule network leads to the subsequent recruitment and activation of Rab5 [49]. Previously, not much was known about the mechanisms promoting the accumulation and activation of Rab5 on phagosomal surfaces, except that the PtdIns3P signal did not seem to be involved [29]. Thus, the identification of Gapex-5 and EB1 as essential factors provides a molecular mechanism that involves the novel and crucial role of microtubules for these events.

Kinchen *et al.* [50] propose a different model, in which Vps34 activates Rab5 by mediating the interaction between Rab5 and dynamin 2, based on protein-protein interaction studies in mammalian cells and genetic studies in *C. elegans*. This study also indicates that receptor-mediated endocytosis (RME)-6, the *C. elegans* ortholog of Gapex-5, is not involved in the regulation of RAB-5 regarding phagosome maturation [50]. Whether these protein-protein interactions occur on the phagosome-limiting membrane has not been tested. In the endocytic pathway, Vps34 has been shown to function as a downstream effector of Rab5 [51–53]. Studies of phagosomes containing opsonized beads also indicate that the recruitment of Rab5 to phagosomal surfaces is independent of Vps34, as discussed earlier [29]. Whether this model can be reconciled with previous observations, and the model proposed by Kitano *et al.* [49], requires further investigation.

C. elegans as a new model system for studying the degradation of apoptotic cells

Two partially redundant pathways control the engulfment of apoptotic cells

The nematode *C. elegans*, a small, free-living round worm, is an excellent model organism for studying apoptosis owing to its simple anatomy, known cell lineage, well-established genetics and easily distinguishable apoptotic-cell morphology [54,55]. Apoptotic cells, which are referred to as ‘cell corpses’, can be recognized within living animals under the Nomarski differential interference contrast (DIC) microscope [56–58]. Cell corpses are rapidly engulfed and degraded by multiple types of neighboring cells [57–59].

The study of cell-corpse removal has traditionally focused on the engulfment process. Seven *C. elegans* genes, *ced-1* (cell death abnormal), *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10* and *ced-12*, were identified from genetic screens for mutants that are viable but contain persistent cell corpses [7]. These genes function to control engulfment in two partially redundant pathways [60,61] (Figure 2a), which are reviewed here because, recently, both pathways were found to have additional functions in the subsequent degradation of apoptotic cells [62]. In one pathway, CED-2, CED-5 and CED-12 work together to regulate CED-10, a Rac GTPase, which promotes cytoskeletal reorganization during engulfment [54] (Figure 2b). In the other pathway, composed of CED-1, CED-6 and CED-7, CED-1 is an engulfing cell-specific, single-pass transmembrane protein that functions as a phagocytic receptor [59] (Figure 2b). CED-1 recognizes cell corpses, clusters in the extending pseudopods and initiates pseudopod extension in response to extracellular 'eat me' signals, one of which is phosphatidylserine [59,63,64]. CED-7, a homolog of mammalian ABC1 transporters [65], is implicated in presenting the 'eat me' signals [59,63]. CED-6 functions downstream of CED-1, possibly as the adaptor of CED-1 [59,66,67]. An additional activity of CED-1 and CED-6 in actin reorganization mediated by CED-10 has also been suggested [68].

Recently, Yu *et al.* [69] isolated 14 loss-of-function alleles of *dyn-1*, which encodes the *C. elegans* large GTPase dynamin (DYN-1), from a genetic screen for a new class of mutants that are embryonic lethal and accumulate cell corpses. Dynamins are evolutionarily conserved factors mediating multiple aspects of intracellular-vesicle trafficking [70]. Dynamin 2 was reported to promote phagocytosis of opsonized cells in mammalian cells, but its role in apoptotic-cell uptake was not previously characterized [71]. It has been discovered that inactivation of DYN-1 in *C. elegans* results in defects in pseudopod extension around cell corpses, thereby indicating that *dyn-1*, like its mammalian counterpart, is required for phagocytosis [69]. Furthermore, DYN-1 is also essential for the degradation of cell corpses, a novel function not reported previously [69]. DYN-1 was subsequently identified as a new member of the CED-1 pathway [69] (Figure 2b). Later, we discuss the molecular mechanisms that support the important roles of DYN-1 in both the engulfment of apoptotic cells and phagosome maturation.

The dynamics of multiple cellular events during the removal of apoptotic cells

Previously, in *C. elegans*, transmission electron microscopy provided the only available method to determine whether a cell corpse was engulfed [58–60,72]. In wild-type animals, the few apoptotic cells present in the body are usually engulfed, whereas, in the *ced* mutants most, if not all, of the persisting cell corpses remain outside the engulfing cells, leading to the conclusion that *ced* genes are only required for the engulfment of apoptotic cells [60]. Despite its high resolution, electron microscopy only produces static snapshots of a dynamic process; therefore, it is not sufficient to detect changes in engulfment or phagosome maturation kinetics [60,72]. Recently developed techniques have revealed new functions in phagosome maturation for multiple factors previously thought to function only in engulfment, as discussed later [62].

A time-lapse fluorescent-microscopic-recording protocol has recently been developed that enables monitoring of the engulfment and phagosome-maturation processes, and the dynamic changes of the subcellular localization of GFP or monomeric red fluorescent protein (mRFP) tagged proteins, in living *C. elegans* embryos [62,69,73] (Figure 3a,b). All *C. elegans* embryos develop following the same cell-division lineage, making it possible to analyze the same cell corpses in wild-type and mutant backgrounds [57]. For instance, the three cells we often monitor always undergo apoptosis at mid-embryonic stage (~330 min post first cleavage) and can be reliably identified by their distinct positions [69] (Figure 3a). Thus, any defects observed in a mutant can be unambiguously attributed to the genetic difference instead of individual variability of different apoptotic cells or engulfing cells [74].

Time-lapse recording also enables accurate quantification of phenotypic defects, such as the delay of the engulfment, degradation, or protein translocation [62,69,73]. By a combination of time-lapse recordings and quantitative surveys of the engulfment and degradation status of all cell corpses at particular embryonic stages, detailed and unbiased evaluation of gene functions in cell-corpse removal can be obtained [62,69,73].

In developing embryos, the dynamics of multiple events essential for phagocytosis and phagosome maturation have been characterized, including the rate of pseudopod extension and phagosome degradation, the recruitment and incorporation of endosomes to extending pseudopods, and of endosomes and lysosomes to maturing phagosomes, and the sequential recruitment of signaling molecules CED-1, DYN-1, PtdIns3P, RAB-5, RAB-2 and RAB-7 to the extending pseudopods and phagosomes [62,69,73]. It is observed that, in wild-type embryos, the engulfment process, from the initial extension of pseudopods to the full closure of a phagocytic cup, lasts only 4–6 min, whereas, it takes 50–70 min for a nascent phagosome to be degraded [62,69] (Figure 3b,c). The phagosomal localization of each molecule displays a distinct spatial distribution and temporal kinetics (Figure 3c). These observations indicate that, despite quantitative differences, a largely similar series of cellular events occur during the maturation of phagosomes containing apoptotic cells in *C. elegans* and phagosomes containing opsonized cells in mammalian systems [2,62,69,73].

The signaling pathways that drive phagosome maturation

Both standard genetic screens and a genome-wide RNA interference (RNAi) screen have identified multiple *C. elegans* factors important for the degradation of apoptotic cells, with many not previously implicated in phagosome maturation. Live-cell-imaging studies have further determined their dynamic phagosomal localization patterns and the particular defects in phagosome maturation caused by their inactivation. As a result, the functions of these factors have been ordered in pathways that control phagosome maturation in *C. elegans*.

At first, there are dynamin and PtdIns3P

Using both fluorescence time-lapse recording and transmission electron microscopy, it has been discovered that loss of dyn-1 activity in *C. elegans* results in reduced speed of pseudopod extension around cell corpses [62,69]. In addition, *dyn-1* mutations cause major delays or blockages of phagosome maturation, leading to severe defects in the degradation of cell corpses [62,69]. The cell-corpse degradation activity of dynamin was recently confirmed by Kinchen *et al.* [50], who identified *dyn-1* in a genome-wide RNAi screen. However, by analyzing a weak and temperature-sensitive allele [69,76], Kinchen *et al.* [50] did not reveal the engulfment activity of *dyn-1*.

How does *dyn-1* control both engulfment and phagosome maturation? The extension of pseudopods around phagocytic targets requires a continuous supply of membrane material. ‘Focal exocytosis’, the accumulation and fusion of endosomes and other intracellular organelles to the plasma membrane in the phagocytic-cup region, is considered a primary mechanism by which to accomplish this goal [75]. The recruitment and fusion of endosomes to pseudopods are both defective in *dyn-1* mutants [69]. In addition, mutations in *dyn-1* severely delay or block the recruitment and fusion of both endosomes and lysosomes, organelles that provide phagosome-maturation factors, including digestive enzymes, to phagosomes [62]. These defects indicate that DYN-1 controls both engulfment and phagosome maturation by promoting the delivery of essential organelles to pseudopods and phagosomes.

How, then, does DYN-1 promote targeted vesicle delivery? DYN-1 is enriched on phagocytic cups and (albeit transiently) on phagosomal surfaces, a localization pattern

crucial for its cell-corpse removal activities [50,62,69] (Figure 3c). Two molecular mechanisms, which are not necessarily mutually exclusive, are consistent with crucial functions of DYN-1 on phagosomal surfaces. First, dynamins and dynamin-related proteins are known to polymerize over lipid surfaces and promote the fission and fusion of membranes [70,77]. Electron microscopy analyses have revealed that *dyn-1* (RNAi) results in the accumulation of abnormal vesicles inside engulfing cells [69]. DYN-1 could function to maintain an intracellular-vesicle pool and promote the fusion of vesicles with phagosomal membrane [69]. Second, the function of DYN-1 is likely to be related to its proposed signaling activity [50,62,69]. DYN-1 is needed for the enrichment of PtdIns3P, RAB-5 and RAB-7 on phagosomal surfaces (Table 1), which promote the fusion of endosomes and lysosomes with phagosomes [50,62]. DYN-1 can, thus, be the key organizer of multiple signaling cascades that initiate different cellular events such as the recruitment and fusion of intracellular organelles with phagosomes (Figure 4). Furthermore, dynamins are known to regulate the actin cytoskeleton and, thus, might also contribute this activity to phagosome maturation [70,78].

With the aid of 2xFYVE–GFP, a fluorescence reporter that binds PtdIns3P, rapid synthesis of PtdIns3P on nascent phagosomes and its persistent presence throughout phagosome maturation were observed in *C. elegans* [62,73]. Consistent with this, RNAi-mediated inactivation of *C. elegans vps-34* has been reported to cause a mild increase in the number of germ-cell corpses [50]. DYN-1 function is essential for PtdIns3P synthesis on phagosomes [62], but not vice versa [50] (Table 1), indicating that *dyn-1* functions upstream of *vps-34*. The milder defect observed in *vps-34* (RNAi) animals compared with *dyn-1* mutants indicates that DYN-1 might also regulate additional downstream events [50,69] (Figure 4b).

Along the way come the Rab GTPases

Three *C. elegans* Rab GTPases, RAB-5, RAB-2, and RAB-7, have specific roles during the degradation of cell corpses. In *rab-7* deletion or RNAi animals, apoptotic cells are engulfed with normal kinetics but remain un-degraded [50,62]. RNAi knockdown of *rab-5* results in the accumulation of germ-cell corpses [50]. Genetic screens have discovered that mutations in a less well-studied Rab, RAB-2, also lead to phagosome-maturation defects in *C. elegans* [73,79]. What are the molecular functions of these Rabs and how are they related functionally?

C. elegans RAB-7 is recruited to the surface of phagosomes containing cell corpses after PtdIns3P synthesis (Figure 3b) and drives phagosome maturation in a GTP-dependent manner, as observed during the uptake of opsonized particles by mammalian cells [62]. RAB-7 is specifically required for the incorporation of lysosomes to phagosomes [62] (Table 1). RAB-7 mediates the extension of lipid tubules from phagosomes to recruit lysosomes, similarly to what has been reported for mammalian Rab7, and promotes the fusion between these two compartments after docking occurs [30,62]. These findings indicate that RAB-7 is capable of mediating not only homotypic vesicle fusion, like its yeast counterpart, but also the fusion of different types of organelles [62,80]. RNAi knockdown of *vps-11*, *vps-16*, *vps-18*, *vps-33*, *vps-39* and *vps-41* homologs of yeast HOPs complex (homotypic fusion and vacuole-protein-sorting complex) components and known activators and effectors of yeast Rab7, resulted in the accumulation of RAB-7-positive phagosomes [50]. Thus, the HOPs complex could function as an effector of RAB-7 in phagosome maturation [50]. Whether the above *vps* genes are involved in RAB-7-dependent lysosome-phagosome fusion remains to be investigated.

Inactivation of *rab-5* impairs PtdIns3P presentation on phagosomes, indicating that it functions at an early phagosome maturation stage [50], consistent with the reported function

of mammalian Rab5 in phagosome maturation and PI3 kinase recruitment to endosomes [20–22,29,49,51].

RAB-2 is required for the recruitment and fusion of lysosomes to phagosomes and the acidification of phagosomal lumen, but not for the recruitment of early endosomes or the presentation of PtdIns3P on phagosomal surfaces [73] (Table 1). It remains to be elucidated whether mammalian Rab2 has a similar role in the maturation of phagosomes. Consistent with this notion, proteomic studies in *Drosophila* and mammals have identified Rab2 as a component of phagosomes [81,82].

The function of DYN-1 is essential for the phagosomal attachment and function of both RAB-5 and RAB-7 (see earlier) (Table 1). The dynamic phagosomal localization of RAB-5, RAB-2 and RAB-7 display a strikingly sequential pattern [62,73] (X. Y. and Z. Z., unpublished) (Figure 4c). Do these three Rabs function in a sequential manner? In *rab-7* (*RNAi*) worms, phagosomes are labeled with GFP–RAB-5 [50], indicating that RAB-5 functions before RAB-7 in phagosome maturation. However, whether the function of RAB-5 is necessary for the phagosome recruitment and function of RAB-7 has not been determined. How RAB-2 is functionally related to RAB-5 or RAB-7 is also currently unknown. In *rab-2* mutant embryos, the recruitment of RAB-7 to phagosomes is normal [73]. Interestingly, RAB-2 is essential for acidification of phagosomes, whereas RAB-7 is not [62,73]. These results indicate that RAB-2 and RAB-7 could control lysosome and phagosome fusions in parallel. In addition, they could each contribute to a different subset of events related to phagosome maturation (Figure 4).

The phagocytic receptor CED-1 leads the way

What, then, regulates DYN-1? Multiple lines of evidence indicate that the signaling pathway led by CED-1 performs this job [62,69]. An epistasis analysis places *dyn-1* in the pathway composed of *ced-1*, *ced-6* and *ced-7* [69]. In addition, the continuing enrichment of DYN-1 on extending pseudopods during engulfment, and on phagosomes after engulfment, was monitored separately over time in all known engulfment mutants. Localization of DYN-1 to both regions was dependent on *ced-1*, *ced-6* and *ced-7*, but not on *ced-5*, *ced-10*, or *ced-12* [69]. Furthermore, two of the important signaling events promoted by DYN-1, the phagosomal synthesis of PtdIns3P and the recruitment of RAB-7 to phagosomes, also require the functions of CED-1 and CED-6, but not the CED-5 pathway members [62,69]. These results indicate that vesicle trafficking is a novel event regulated by the CED-1 pathway; they further imply that CED-1, by controlling DYN-1 activity, also regulates phagosome maturation. An alternative model indicates that engulfment *per se* is required for DYN-1 recruitment to phagosomes [50]. The study that leads to this model did not monitor the dynamic localization of DYN-1 on phagosomes in the engulfment *ced* mutants over time, neither did it verify that those cell corpses examined were engulfed [50].

Does the CED-1 pathway directly regulate phagosome maturation? A previously overlooked fact is that mutations in *ced-1* or other engulfment genes only block the engulfment of some, but not all, apoptotic cells; the fates of the engulfed cell corpses in these mutants have not been monitored previously [60,62]. By monitoring these engulfed cell corpses, it has been discovered that, like *dyn-1* mutations, *ced-1* mutations not only reduce engulfment efficiency but also delay the degradation of engulfed apoptotic cells [62].

How does CED-1 regulate phagosome maturation? Signaling events that require CED-1 activity, including the synthesis of PtdIns3P and the recruitment of DYN-1 and RAB-7 to phagosomes also require CED-6 – a postulated adaptor for CED-1 [62]. As a consequence, *ced-1* and *ced-6* mutants are both defective in the recruitment and fusion of early endosomes and lysosomes to phagosomes [62]. Although CED-1 is only transiently localized to

phagosomal surfaces, it co-exists with DYN-1 and other phagosomal markers, including PtdIns3P, RAB-5, RAB-2 and RAB-7, for a period of time [62,69] (Figure 3c). Thus, through CED-6, CED-1 could recruit DYN-1 to phagosomes and further activate DYN-1 to promote a downstream signaling cascade that ultimately leads to apoptotic-cell degradation (Figure 4).

Unlike dynamin and Rabs, the activities of phagocytic receptors are intrinsically specific and are likely to be specified by their phagocytic targets. The findings discussed here thus indicate that different phagocytic receptors are able to promote different phagosome maturation modes and, subsequently, induce phagocytes to elicit different responses. It remains to be elucidated whether mammalian homologs of CED-1, such as human multiple-EGF-like-domain protein 10 (mEGF10), function as the initiation signal for phagosome maturation [83,84]. Toll-like receptors (TLRs), which recognize the common cell-surface pattern of many microorganisms, are enriched on the surface of phagosomes containing bacteria and function there to induce the proinflammatory responses of the host cell [85,86]. TLRs are not detected on phagosomes containing apoptotic cells [87,88]. Whether molecules equivalent to TLRs exist on phagosomes containing apoptotic cells to promote anti-inflammatory responses remains unknown. The transient phagosome localization of phagocytic receptors for apoptotic cells not only enables them to initiate phagosome maturation, but might also enable them to promote additional host-cell responses, including anti-inflammatory responses.

The removal of apoptotic cells in *Drosophila*

In *Drosophila*, macrophages, glial cells and epithelial cells all clear apoptotic cells [11]. In recent years, genetic studies have identified several *Drosophila* genes that encode important engulfment factors, which demonstrate the involvement of CED-1-like and class-B scavenger-receptor-like phagocytic receptors, a tyrosine-kinase-signaling pathway, and the ubiquitin-mediated protein-degradation mechanism in the engulfment of apoptotic cells [89–96] (Table 2). Draper, the *Drosophila* ortholog of CED-1, is implicated in the removal of not only apoptotic cells, but also injured axons and axon fragments generated during pruning, indicating that the mechanisms for the removal of apoptotic cells are also used by animals to remove cell debris [91,92]. Given that all of the known factors except Draper seem to function specifically in the step of phagocytosis, they are not described in detail here (Table 2). Recently, Draper was found to have a specific role in the maturation of phagosomes containing apoptotic cells, indicating that the phagosome maturation-initiating function of CED-1 is evolutionarily conserved [95].

Concluding remarks

Systematic characterization performed in both mammalian cultured cells and *C. elegans* have demonstrated that the sequential incorporation of endocytic organelles into phagosomes is essential for the degradation of apoptotic cells, as known previously for opsonized cells or invading pathogens (Figure 4a).

However, major differences exist in the rate of phagosome maturation when different types of particles are internalized [37]. A pathway mediated by a Rho GTPase has been implicated in accelerating the maturation of phagosomes containing apoptotic cells [37]. In addition, the kinetics of PtdIns3P phagosome localization and the requirement for Rab7 for lumen acidification are different depending upon the ingested particles [21,30,62,73]. These differences might be closely related to the different immunological responses induced by different phagocytic targets. Studies focusing on the degradation of apoptotic cells thus provide a new platform for investigating the mechanisms underlying the differential processing of different phagocytic targets.

Different types of phagocytes also display distinct features in processing the same type of prey. Dendritic cells deliberately reduce the extent of cargo degradation to better suit the antigen-presentation process [37,46,47]. In addition, engulfing cells in the *C. elegans* soma and those in the gonad display different dynamic features in degrading apoptotic cells [50,62,69]. The distinct behavior of different phagocytes is likely to have profound physiological significance, as demonstrated by dendritic cells. The mechanisms behind these differences are just starting to be revealed.

Extensive studies in the past two decades in mammalian cells have uncovered numerous factors that mediate phagosome maturation. Studies performed in the nematode *C. elegans* have further elucidated the activities of some of these factors in the context of an intact animal. Factors known to be required in mammals for phagosome maturation show similar functions in *C. elegans*, indicating the evolutionary conservation of these mechanisms. Powerful genetic screens performed in *C. elegans* have since expanded the known repertoire of phagosome-maturation factors [69,73,79]. The proteomic studies of phagosomes in both mammals and *Drosophila* and the genome-wide RNAi screen performed in *C. elegans* will lead to the characterization of additional novel factors [50,81,82]. Recently, live imaging in zebrafish has also revealed a crucial role for the V0-ATPase $\alpha 1$ subunit in lysosome-phagosome fusion [97]. These factors provide an excellent starting point for identifying equivalent mammalian homologs functioning in phagosome maturation. In addition, genetic studies in *C. elegans* have established the order in which phagocytic-maturation factors function, which further extends an understanding of the function and regulation of their mammalian counterparts (Figure 4b).

Further study of these factors is required to answer many remaining questions regarding the regulation of phagosome maturation during the removal of apoptotic cells. The detailed molecular mechanisms behind each step in the genetic pathway (Figure 4) need to be revealed, in particular, it will be of great interest to determine how DYN-1 serves as a key mediator of multiple downstream events, what the effectors of PtdIns3P are and how the three Rab GTPases work together to complete the process. In addition, the identity of the factors catalyzing the fusion of each type of intracellular organelle with the phagosome remains unknown. Furthermore, in addition to vesicle trafficking, other cellular events are likely involved in phagosome maturation. In fact, *C. elegans* CED-5 also regulates lysosome-phagosome fusions [62]. Given that CED-5, CED-2 and CED-12 form a protein complex to activate CED-10, it is likely that CED-2, CED-12 and CED-10 also contribute to phagosome maturation, perhaps by rearranging the cytoskeleton (Figure 4b).

Traditionally, phagocytic receptors were thought to specifically promote phagocytosis. Thus, discoveries that *C. elegans* CED-1 and its *Drosophila* homolog, Draper, have important roles in phagosome maturation have established a precedent that phagocytic receptors could also have key roles in regulating phagosome maturation [62,95]. The essential role of TLRs in inducing pro-inflammatory responses to bacteria is well known. What we have learned here might provide clues from the viewpoint of apoptotic cells for understanding how macrophages elicit different immune responses against apoptotic cells versus foreign pathogens. Further studies along this line will provide insights for developing treatments for bacteria infections and for coping with the immunological consequences caused by massive numbers of apoptotic cells, generated either by defects in their removal or by chemo- and radiation- cancer therapies.

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Figure 1.

Apoptotic cells are engulfed by phagocytes and, subsequently, digested inside phagosomes in metazoans. **(a)** The process of apoptotic-cell engulfment and degradation. Cell death proceeds in four stages: (i) the specification of a death fate (not shown here); (ii) cell-death execution; (iii) recognition and engulfment by phagocytes; and (iv) degradation within phagosomes. During phagosome maturation, a variety of intracellular organelles are sequentially incorporated into phagosomes. **(b)** The sequential incorporation of early endosomes, late endosomes and lysosomes to phagosomes drives phagosome maturation. These vesicles (identities indicated by particular colors) are recruited to the phagosomal surfaces and then fuse with phagosomes, providing the phagosome with a variety of protein and lipid materials. Phagosome luminal PH starts to decrease after the completion of engulfment and reaches the lowest level when a phagosome evolves into a phagolysosome. A phagolysosome gradually decreases in size and eventually disappears.

Figure 2.

The *C. elegans* engulfment genes and their molecular functions. **(a)** Two parallel and partially redundant pathways that control the engulfment of apoptotic cells in *C. elegans*. Members of each pathway were identified from genetic screens for mutations that impair cell-corpse removal. Each gene was placed in one of the two pathways based on the results of epistasis grouping assays and phenotype characterizations. The order of genes in each pathway was determined by epistasis analyses (for details, see text and Ref. [54]). The identities of the corresponding mammalian homologs are indicated inside parentheses. **(b)** The molecular functions and protein–protein interactions of the eight genes listed in (a). Yellow spheres coating the cell corpse represent the 'eat me' signal presented by cell corpses to attract engulfing cells. Among the eight proteins, CED-7 is the only one that functions in both dying and engulfing cells. All other proteins function specifically in engulfing cells for cell-corpse engulfment. CED-2, CED-5 and CED-12 form a protein complex under the plasma membrane to regulate the CED-10 Rac GTPase, which promotes cytoskeletal reorganization and pseudopod extension during engulfment. CED-5 and CED-12 function as a bipartite guanine-nucleotide-exchange factor that activates CED-10. CED-10 associates with the plasma membrane through its prenylated C terminus. Two deep blue lines, which represent a transmembrane receptor that interacts with an 'eat me' signal molecule and a nearby question mark indicate that the transmembrane receptor(s) expected to interact with the SH2 domain of CED-2 with its intracellular domain and activate the CED-2, -5 and -12 complex has not been identified. In the other pathway, CED-7 mediates the presentation of an 'eat me' signal on the surface of apoptotic cells, and might possess a different engulfment-promoting activity on the surface of engulfing cells. CED-1 is a phagocytic receptor that uses its extracellular domain to recognize the 'eat me' signal, clusters around the cell corpse, and uses its intracellular domain for downstream signaling. CED-6 interacts with CED-1 and functions as the adaptor of CED-1. DYN-1 is localized to the surface of pseudopods and phagosomes in a manner dependent on the functions of CED-1, -6 and -7. DYN-1 mediates the signaling of CED-1 to promote the delivery and incorporation of intracellular vesicles to pseudopods through 'focal phagocytosis', a

mechanism that provides membrane material to support pseudopod extension. DYN-1 also mediates CED-1 signaling to promote phagosome maturation. An additional activity of CED-1 and CED-6 in actin reorganization, possibly mediated by CED-10, has also been suggested. For review, see main text and Refs [9,50,54,62,68,69]. Figure adapted, with permission, from Ref. [61].

Figure 3.

Time-lapse recording of the engulfment and degradation of apoptotic cells and the dynamic phagosome localization pattern of multiple essential factors in *C. elegans* embryos. **(a)** DIC image of an embryo at 3330 min post the first embryonic division. Anterior is to the top. Ventral faces readers. Arrowheads indicate the three ventral hypodermal cells that engulf apoptotic cells C1, C2 and C3 (arrows); the identity of each engulfing cell is labeled. **(b)** Real-time observation of the engulfment and degradation of cell-corpse C3. Time-lapse images of the co-expressed CED-1-GFP (1–7) and 2xFYVE-mRFP reporters (8–18) around C3 in a wild-type embryo. CED-1-GFP labels pseudopods, enabling the visualization of the pseudopod extension and fusion along C3. 2xFYVE-mRFP, which associates with PtdIns3P, labels phagosomes throughout their entire duration, and is used here to demonstrate the formation and the gradual shrinkage of a phagosome containing C3, a process that represents phagosome maturation and the degradation of cargo. 0 min: the time point that the engulfing cell starts to extend pseudopods around C3. White arrows indicate the edge of pseudopods. Scale bar: 3 μ m. **(c)** A summary of the temporal order of the phagosome localization of multiple essential factors for phagosome maturation and of the incorporation of endosomes and lysosomes, using GFP or mRFP-fusion reporters. Data represent mean values obtained from time-lapse recording monitoring the engulfment and degradation of multiple C1, C2, and C3 cell corpses. '0 min' represents the time point when budding pseudopods are first detected by CED-1-GFP. The pale pink colors used for endosomal and lysosomal incorporation into phagosomes indicate that a very weak signal was detected at an early stage and signal intensities gradually increase. For review see Refs [62,69,73].

Figure 4.

Intracellular-vesicle-transport factors that are involved in phagosome maturation and the two genetic pathways that control degradation of apoptotic cells in *C. elegans* **(a)** In *C. elegans*, phagocytic receptor CED-1, large GTPase Dynamin and small GTPases, Rab5, Rab2 and Rab7, sequentially localize to the phagocytic cup, nascent phagosomes and phagolysosomes to mediate phagosome maturation through different stages. At each stage, endosomes and lysosomes fuse with maturing phagosomes. Golgi apparatus might also take part in the regulation of phagosome maturation through the activity of Rab2. **(b)** The two parallel genetic pathways that were previously identified to regulate cell-corpse engulfment in *C. elegans* are now found to also control phagosome maturation. Proteins, the names of which are in blue, are those that have been found to be required for degradation of apoptotic cells using loss-of-function mutations. Proteins in black are in the engulfment pathways that have not been tested for their phagosome-maturation activity. The positions in the pathway of those protein and lipid molecules listed in the red box have not been fully determined (for details, see main text and Table 1). The two pathways would eventually converge on the formation of a phagolysosome, after which step the phagosome degrades its content irreversibly.

Table 1
The defects of multiple phagosome maturation events caused by gene inactivation in *C. elegans*

Gene inactivated	Incorporation of		Enrichment on phagosomes of				
	Endosomes	Lysosomes	PtdIns3P	RAB-5	RAB-2	RAB-7	DYN-1
<i>dyn-1^a</i>	Defective [69]	Defective [62]	Defective [62,50]	Defective [50]	ND	Defective [62,50]	NA
<i>vps-34^b</i>	ND	ND	Defective [50]	Defective [50]	ND	ND	Normal [50]
<i>rab-5^b</i>	ND	ND	Defective [50]	NA	ND	ND	ND
<i>rab-2^a</i>	Normal [73]	Defective [73]	Normal [73]	ND	NA	Normal [73]	ND
<i>rab-7^{a,b}</i>	Normal [62]	Defective [62]	Normal [62,50]	Normal [50]	ND	NA	ND

Abbreviations: NA, not applicable; ND, not determined.

^a Gene inactivation by genetic mutations.

^b Gene inactivation by RNAi knockdown.

Table 2*Drosophila* proteins known to function in the engulfment of apoptotic cells^a

Protein	Molecular identity	Phagocytosis-related functions	<i>C. elegans</i> and mammalian homologs	Refs
I. Phagocytic receptors				
Draper	Transmembrane receptor with multiple EGF-like repeats	Recognition and engulfment of apoptotic cells and degenerative axon fragments; Phagosome maturation.	CED-1, mEGF10, mEGF11, SREC	[83,91–94]
SIMU	Transmembrane receptor with multiple EGF-like repeats	Recognition and engulfment of apoptotic cells; acting upstream of Draper	CED-1, mEGF10, mEGF11, SREC	[83,95]
Croquemort	Transmembrane receptor	Recognition and engulfment of apoptotic cells and <i>Staphylococcus aureus</i>	CD36	[89,90,11]
II. Adaptors and protein kinases				
dCed-6	PTB-domain adaptor protein	Mediating the signaling of Draper in engulfing axon fragments	CED-6, GULP	[92]
Src42A	Src family tyrosine kinase	Candidate kinase that phosphorylates Draper cytoplasmic domain and enables its association with Shark	Src family kinases	[94]
Shark	non-receptor tyrosine kinase	Mediating Draper signaling in engulfing apoptotic cells and axon fragments	Syk and Zap-70	[94]
III. SCF E3 ubiquitin ligase complex components and a ubiquitin conjugating enzyme				
Pallbearer	F box protein, SCF complex component	Engulfment of apoptotic cells	F-box protein 28	[96]
SkpA	SCF complex component	Engulfment of apoptotic cells	Skp1	[96]
Lin19	SCF complex component	Engulfment of apoptotic cells	Cullins	[96]
Effete	E2 ubiquitin-conjugating enzyme	Engulfment of apoptotic cells	E2 ubiquitin-conjugating enzymes	[96]

^a Abbreviations: SCF complex, Skp–Cullin F box complex; SIMU, six microns under.