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Information processing during phagocytosis

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Abstract

Phagocytosis, the process by which macrophages, dendritic cells and other myeloid phagocytes internalize diverse particulate targets, is a key mechanism of innate immunity. The molecular and cellular events underlying the binding and engulfment of targets in phagosomes have been extensively studied. More recent data suggest that the process of phagocytosis itself provides information to myeloid phagocytes about the nature of the targets they are eating and that this helps tailor inflammatory responses. In this Review, we discuss how such information is acquired during phagocytosis, and how it is processed to coordinate an immune response.

Myeloid phagocytes including macrophages, dendritic cells (DCs), and neutrophils play numerous roles in immunity, inflammation and tissue repair. In addition to being key players in the innate immune response to microbes and in initiation of adaptive immune responses, they are critical for clearance of dead cells during normal tissue homeostasis and remodeling, as well as in response to damage. Phagocytosis is the process by which cells ‘eat’ a wide variety of particulate targets, including microbes, dead cells and environmental debris (Box 1).

BOX 1

Different Mechanisms of Internalizing

Endocytosis

Endocytosis is the actin-independent process by which all cells internalize small molecules. There are a variety of mechanisms of endocytosis including internalization into clathrin-coated pits which are roughly 100 nm in diameter and concentrate receptors and other surface molecules into early endosomes. Similarly, caveolae are cholesterol-rich invaginations of the plasma membrane that are generally a little smaller. Distinct endocytic processes requiring flotillins, RhoA, and Cdc42 have also been reported (62).

Macropinocytosis

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This process of “cell drinking” involves the eruption of membrane ruffles from the cell surface which collapse back and fuse with the plasma membrane to engulf surrounding liquid (see figure). Newly formed macropinosomes, typically 0.5–5 mm in diameter, contract and fuse with compartments of the normal endocytic pathway (63). This process requires extensive actin mobilization. All cells engage in macropinocytosis.

Phagocytosis

This is the actin-dependent process of specifically internalizing particulate targets typically >0.5 mm in diameter via diverse mechanisms (see figure). Targets may include microbes, dead or dying cells, or environmental debris. The ability to engage in phagocytosis is restricted to specialized cells.

Autophagy

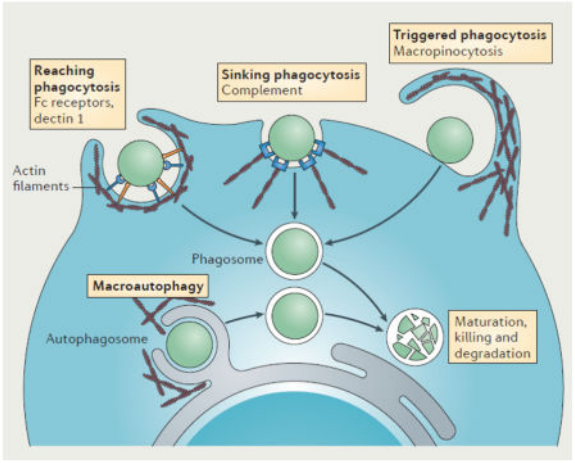
This is the process by which cells orchestrate engulfment of cytosolic material including soluble factors, organelles, and microbes (see figure). Newly formed “autophagosomes” fuse with lysosomes to degrade engulfed material. All cell types are capable of autophagy.

Cell Cannibalism

Sometimes cells actively eat other healthy live cells (e.g. tumor cells eating lymphocytes). The target cell is typically killed and degraded (64).

Entosis

This curious process, observed in a variety of cell types, involves one cell “burrowing” into a neighboring cell. The burrowing cell may either die within the target cell or re-emerge (64).



The response of a phagocyte to a target can be multi-layered. For instance, for effective clearance of pathogenic microbes, phagocytes must first detect the microbes using surface receptors, and then eat and kill them. In addition, some phagocytes process internalized targets to derive antigen for presentation to T cells, and most produce inflammatory cytokines and chemokines that orchestrate local and systemic inflammatory responses and

direct the development of adaptive immunity. Some phagocyte responses are dependent on internalization of the target, while others are not. For example, internalization is frequently required for microbial killing and essential for antigen processing and presentation. In contrast, inflammatory responses (e.g. cytokine production) can often be induced without the need for internalization; indeed, soluble components shed from microbial cell walls or fragments of degraded microbes can drive these responses by simply engaging receptors at the phagocyte surface.

Traditionally, we have thought of phagocytosis and the activation of intracellular signaling for inflammation as somewhat separate. ‘Phagocytic receptors’, such as dectin-1 and Fc receptors, trigger phagocytosis following direct or indirect recognition (via opsonization) of particulate targets (see excellent recent reviews defining the signaling processes involved in activating phagocytosis (1–3)). In contrast, innate immune receptors, such as Toll-like receptors (TLRs), trigger inflammatory cytokine and chemokine production (see excellent recent reviews detailing how these types of receptors regulate gene induction (4,5)). However, the lines between these processes have always been a little blurry. For instance, it has long been appreciated that Fc receptors can activate production of inflammatory mediators, and certain TLRs have been implicated in promoting phagocytosis. More recently it has become evident that the process of phagocytosis itself plays an integral role in shaping the response triggered during engagement of specific targets.

In this Review, we focus on the concept that the process of phagocytosis itself informs myeloid cells about the nature of the targets they are engulfing including the target’s physical form, whether it’s dead or alive, and how much of a threat it presents.

Properties of phagocytosis

The mechanisms by which internalization of particulate targets occurs are varied and depend on the target and its location (Box 1). Specific receptors on the cell surface instruct distinct types of phagocytosis: for example, ‘reaching’ phagocytosis (also known as ‘zippering’ phagocytosis) is initiated by Fc receptors and dectin-1 (discussed below), while ‘sinking’ phagocytosis is initiated by complement receptors. In addition, the process of macropinocytosis or “cell drinking” can be triggered by various microbial and self stimuli, resulting in internalization of particles bound to the cell surface. Phagosomes also form inside cells; during autophagy, intracellular membranes engulf organelles and cytoplasmic debris, and this process can be utilized to engulf intracellular microbes into a phagosome. In each instance, internalized particles are degraded as the compartment acidifies and fills with proteolytic enzymes. In the case of neutrophils, this maturation is enhanced by fusion with pre-packaged storage granules containing densely packed antimicrobial peptides and enzymes. Additionally, components of the NADPH phagocyte oxidase may assemble on the phagosome and generate antimicrobial reactive oxygen species (ROS) to facilitate killing. Thus phagocytosis is a dynamic and diverse process with many potential sites for regulation.

Receptors involved in phagocytosis

Although a number of different types of receptors have been demonstrated to play roles in phagocytic uptake of target particles (Table 1), and many more such receptors likely remain to be identified, the definition of a true phagocytic receptor can be tricky. Only a handful of receptors have been reproducibly shown to be sufficient to mediate phagocytosis when expressed alone in otherwise non-phagocytic cells. Such receptors include Fc receptors for antibody-opsonized particles and dectin-1 for fungal β-1,3-glucans (6,7). However, a receptor that is necessary for efficient binding of a particle participates in phagocytosis, even if it is not the receptor that actually instructs particle internalization. A ‘tethering and tickling’ model has been proposed to distinguish between receptors that bind a target to ‘tether’ it to the cell surface, and receptors that ‘tickle’ the cell to provoke it to internalize the target (8). For example, of the receptors that have been implicated in phagocytosis of apoptotic cells, most have been shown to be sufficient to tether targets to the cell surface but not to trigger their uptake (9). However, recent evidence suggests that αvβ3 integrin can provide the tickle signal, but only in collaboration with the secreted protein MFG-E8 (10).

Furthermore, since most target particles, such as bacterial cell walls or dead cells, engage many different receptors on the surface of a phagocyte, it is often difficult to determine which ones actually trigger internalization. In addition to demonstrating that overexpression of a receptor confers phagocytic activity on otherwise non-phagocytic cells, it is important to determine a receptor’s role in cells in which it is normally expressed. For example, TREM2, a DAP12-associated IgG domain-containing receptor, can bind bacteria and trigger phagocytosis via an ITAM mechanism when expressed in CHO cells (11), but cells lacking DAP12 or TREM2 showed only minor defects in internalizing bacteria. It may be more important in regulating inflammatory responses through interacting with an as-yet-undefined self ligand on the surface of myeloid cells (12,13). Perhaps if it were possible to examine particles (or bacteria) for which the as-yet-undefined bacterial TREM-2 specific ligand represented greater proportion of the signal for phagocytosis, the effects would be more profound. As it is, the bacteria studied are likely recognized by multiple receptors, many of which may provide internalization signals.

Receptors may also trigger internalization in general non-specific ways. Macrophages activated with soluble LPS, a ligand for TLR4, were shown decades ago to exhibit increases in membrane ruffling, adhesion, motility, and macropinocytosis, all processes that likely contribute broadly to binding and uptake of microbes. Macropinocytosis, in particular, has been shown to non-specifically lead to internalization of bacteria bound to the cell surface (3,14). These phenomena likely contribute to reports that TLRs are also phagocytic receptors.

Information gathered during phagocytosis

The idea that innate immune receptors convey information about the nature of the microbe being recognized has become a central principle of innate immunity. For example, TLR specificity identifies certain organisms: TLR4 recognizes LPS from Gram-negative bacteria, TLR5 recognizes flagellin from motile bacteria, and TLR3 recognizes viral nucleic acids.

Thus, differences in the signals propagated by individual TLRs contribute to tailoring of the immune response to be effective against the particular class of threat detected. Phagocytic receptors can often trigger responses that are different from other innate immune receptors. For example, dectin-1 triggers activation of the transcription factor NFAT (15–17). Combinatorial signaling by multiple receptors also shapes the response; crosstalk between receptors at the cell surface can lead to qualitatively different inflammatory responses than they might activate on their own. For example, when co-activated, dectin-1 and TLR2 trigger a collaborative response that is greater than the response either receptor induces on its own and results in enhanced production of several inflammatory cytokines, including TNF and MIP2 (18,19).

These examples relate to simple receptor—ligand interactions at the cell surface. However, additional information about a particulate target can be gathered while it is being eaten by phagocytosis, and once it is inside the cell. This can be important for shaping the immune response, which must be tailored to the specific target being eaten. The remainder of this review will focus on this emerging theme. We will break the process of phagocytosis down into four stages that we call ‘tasting’, ‘feeling’, ‘swallowing’, and ‘digesting’ (Fig. 1), and explore the idea that at these different steps phagocytes receive additional information about the targets they engage and the nature of the threat posed. By tasting and feeling a target, phagocytes collect information about its chemical and physical properties. During the process of swallowing, when a target is engulfed and internalized, phagocytes further assess its form and chemical composition, which can determine its fate within the cell. And digesting the targets releases additional ligands that further inform the cell about the target and determine the cell’s response to it. We will present key examples of information gathered at each of these steps in the progression of phagocytosis, and discuss how they collectively provide more information than a simple receptor activation model can convey, instruct how the target is internalized, and influence the orchestration of the immune response mounted against it.

Tasting, feeling, swallowing and digesting

Permission to play with your food

The initial contact of the target with the surface of a phagocytic cell triggers two events: chemical sampling of the target by surface receptor engagement (tasting) and physical exploration of the target by the plasma membrane (feeling). Recruitment and oligomerization of receptors that recognize specific chemical constituents of the target tethers the target to the phagocyte surface and initiates some intracellular signaling (for example, MyD88-dependent NF-κB activation by TLRs), which may be sufficient to induce cytokine production. Membrane ruffling, the extension of pseudopodia, and the engagement of specific phagocytic receptors, gather information about the physical properties of the target and determine whether and how internalization will proceed. These two processes may be linked: binding by non-phagocytic surface receptors can help the phagocyte get a better grip of the particle, and the collaborative signaling by phagocytic receptors can influence responses initiated by non-phagocytic receptors.

Simply assessing the size of the particle bound at the cell surface can have important consequences for the type of inflammatory immune responses activated. For example, determining whether to internalize a target through phagocytosis or endocytosis affects antigen handling decisions. It was reported that ovalbumin delivered to macrophages in 560 nm liposomes (which are phagocytosed) is more efficiently presented than ovalbumin delivered in 155 nm liposomes (which are endocytosed) despite similar total levels of ovalbumin uptake (20). Whereas vesicles containing smaller liposomes proceeded rapidly to fuse with lysosomes, larger liposomes remained for longer periods of time in early phagosomal compartments that efficiently recruited antigen-processing machinery to enhance ovalbumin presentation. Thus phagocytosis provides cells with a mechanism to more efficiently direct antigen to initiate an adaptive immune response.

The size of a phagocytosed particle can also influence the types of inflammatory cytokines that are produced. In one study, the authors evaluated cytokine production by human peripheral blood mononuclear cells (PBMCs) stimulated with ssRNA–protamine particles of various sizes (21). They observed interferon-α (IFNα) production in response to 220 nm and 500 nm particles, but not 1200 nm particles. By contrast, 220 nm particles failed to induce tumour necrosis factor α (TNF), but 500 nm and 1200 nm particles both induced robust TNF production. Other cytokines, such as IL-1β and IL-6, were induced equivalently regardless of particle size. Thus a phagocytic cell may be able to tailor its response to produce cytokines and chemokines that attract and activate the most appropriate cells to tackle an infection characterized by a particular microbial size range. For example, this may allow the immune system to distinguish between viruses (typically smaller) and bacteria (typically larger).

Phagocytes can also sense other physical properties of a target, such as its geometry and topography. A study of the binding and uptake of IgG-coated polystyrene beads of various shapes and sizes showed that macrophages engage larger spheres more efficiently than smaller spheres, but smaller rods more efficiently than larger rods (22). Interestingly, the optimal binding dimension of rod-shaped beads correlates with the average size of most rod-shaped bacteria (2–3 μm). The initial contact that a macrophage makes with a target also appears to influence its ability to respond. Macrophages engaging elliptical IgG-coated polystyrene particles at their pointed ends internalize them in just a few minutes, while binding of the flat regions of identical particles delays internalization for hours due to inefficient actin polymerization at the contact site (23).

It is not currently clear to what degree other phagocyte functions (for example, cytokine production) are influenced by information received about a target's geometry and topography. However, the strength and duration of target binding, combined with the speed at which the target is then internalized, no doubt impact the ability of different receptors to engage their ligands and propagate downstream signals. Signaling by dectin-1, for instance, appears to be terminated upon target internalization since blockade of actin polymerization prevents phagocytosis of β-glucan particles and results in elevated cytokine production (24). Furthermore, macrophages undergoing 'frustrated phagocytosis' upon engagement of β-glucan particles that are too large to be internalized produce elevated levels of inflammatory cytokines. Thus it appears that the longer the receptor engages β-glucan particles at the cell

surface, the stronger the response. Variation in the size and shape of different morphological forms of a fungus may therefore, in combination with changes in the composition of the fungal cell wall, impact receptor engagement and signaling, and ultimately determine the type of immune response that ensues.

Target rigidity may also influence the phagocyte response. For example, the ability of macrophages to phagocytose IgG-opsonized polyacrylamide beads is reportedly dependent on the acrylamide content – ‘stiff’ beads (0.2% bis-acrylamide) are several times more likely to be internalized than ‘soft’ beads (0.05% bis-acrylamide) which fail to induce actin polymerization at the contact site (25). Mechanosensing may provide a phagocyte with information about the nature of a target and the best way to deal with it. For example, since microbial cell walls are more rigid than the surfaces of mammalian cells, this information may influence the phagocyte’s decision to identify them as targets for further investigation.

Consistent with this, our recent studies have identified a molecular mechanism whereby dectin-1 can sense the physical properties of a target to distinguish binding to soluble versus particulate ligands. Dectin-1 can bind both β-glucan-containing particles (such as yeast cells) and soluble β-glucan polymers, but productive dectin-1 signaling is only activated when the receptor engages particulate β-glucans (26). At the molecular level, we showed that dectin-1 responses are instructed at a particle contact site by the formation of a ‘phagocytic synapse’ (Fig. 2). This structure is analogous to the ‘immunological synapse’ that forms between an antigen-presenting cell and a T cell. The T cell receptor (TCR) signals via immunoreceptor tyrosine-based activation motifs (ITAMs) in its associated CD3 and ζ chains; dectin-1 signals via a related ITAM-like motif termed a hemITAM. The tyrosine kinase cascades initiated upon activation of both receptors are regulated by membrane-intrinsic tyrosine phosphatases (CD45 and CD148), which are initially required to activate Src family kinases, but must subsequently be rapidly isolated from the clustered receptors to permit productive downstream signaling. The phagocytic synapse enables dectin-1 to distinguish between intact yeast, which it should eat and kill, and harmless pieces of fungal debris. Recent studies have indicated that the TCR complex may be a mechanotransducer (reviewed in (27)). Inside-out signaling and cytoskeletal contraction are proposed to control TCR activation. A similar mechanism could explain how Dectin-1 is capable of physically detecting β-glucans immobilized in fungal cell walls.

Mechanosensing by DCs has recently been reported in two studies, which showed that in addition to proteins, DCs can use membrane lipids to sense particulate targets (28,29). Using atomic force microscopy, one study reported extremely high contact forces (in the nanoNewton range) between uric acid crystals and DCs (29). Depletion of cholesterol from dendritic cell membranes abolished uric acid crystal binding and reduced uric acid crystal-induced upregulation of CD86 expression by these cells, whereas treatment to remove DC surface proteins had no effect. The precise mechanism of this receptor-independent binding and signaling is unknown, but it appears to involve lipid sorting at the binding site, actin polymerization and Src–Syk signaling. The forces involved, which are thought to be electrostatic, are much stronger than protein-based interactions (these are in the picoNewton range), but are similar in magnitude to the cumulative forces generated during receptor-dependent phagocytosis. Shi and colleagues subsequently showed that alum also interacts

directly with lipids in dendritic cell membranes and uses a similar, although not identical, mechanism to activate DC responses (28). Together the studies begin to suggest a mechanism by which the particulate nature of these targets is sensed and is crucial for activating cellular responses.

Now swallow

The process of internalizing material in a discrete intracellular compartment is in itself sufficient to alter inflammatory signaling mechanisms. Although receptors can be recruited non-specifically (for example, TLR2 is recruited into phagosomes even if the target does not have TLR2 agonist activity (30)) receptors with specificity for a target are concentrated in newly forming phagosomes, resulting in enhancement of their activation.

MyD88-dependent signaling by TLR4 (which triggers TNF-α production) was shown to occur from the cell surface, whereas TRIF-dependent signaling by TLR4 (which triggers IFNβ) occurs only after internalization of the receptor into endosomes (31). This concept was subsequently extended to include the idea that TRIF signaling from phagosomal membranes requires TLR4 delivery to phagosomes from an endocytic recycling compartment through a process that is RAB11a-dependent and requires the presence of TLR4 ligands (32). Consistent with this idea, it was shown that TRIF signaling by TLR4 in endosomes requires CD14-mediated internalization of TLR4 from the cell surface, whereas TRIF signaling from *E. coli*-containing phagosomes is CD14-independent (33). Thus different mechanisms control the delivery of TLR4 to endosomes and phagosomes, and TRIF-dependent signaling from these distinct intracellular compartments (Fig. 3). Together these findings support the idea that formation of the phagosome is associated with dynamic alterations in the receptors available to detect the contents and the mechanisms by which these receptors signal (32).

Signaling from a phagocytic compartment informs the cell about the threat posed by its contents. In addition to their antimicrobial activity, macrophages play an important role in the clearance of dead cells and cellular debris. Under such circumstances it is important for the phagocyte to determine how the cell died. Cells that die by necrosis caused by injury or infection alert phagocytes to the presence of danger that requires immune activation to deal with the threat (34). On the other hand, if cells die by apoptosis and there is no sign of infection or other danger, then they just need to be removed and recycled, without inducing cytokine and chemokine production. For example, immature DCs efficiently phagocytose both necrotic and apoptotic tumor cells, but only necrotic cells trigger DC maturation and activation of $CD4^+$ and $CD8^+$ T cells (35). Soluble factors produced by the necrotic cells appear to provide the danger signal in this example, but we can also see that the process of phagocytosis itself is an integral part of the response to apoptotic cells; phagosomes formed during internalization of apoptotic cells are instructed differently compared with phagosomes formed in other ways.

Indeed, it was noted that the rate of maturation (measured by acidification and lysosomal marker acquisition) of macrophage phagosomes containing unopsonized apoptotic cells was considerably faster than phagosomes containing IgG-opsonized target cells (36). The accelerated maturation of phagosomes containing unopsonized apoptotic cells was

dependent on enhanced activation of Rho and ERM (ezrin-radixin-moesin) family proteins. In DCs, however, the investigators observed no difference in Rho/ERM activation and phagosome maturation rates following the uptake of opsonized or unopsonized targets. This is not to say that DCs handle apoptotic cell phagosomes in the same way as other phagosomes. Indeed, it was reported that DCs segregate microbes and apoptotic cells into distinct phagosomes (37). Upon phagocytosis of apoptotic cells, MHC class II molecules localized to the phagocytic compartment but antigens were not processed and presented efficiently. However, adding TLR ligands to the phagosome allowed for efficient processing and presentation of apoptotic cell antigens. The authors suggest that TLR signaling within the phagosome is important to activate MHC class II processing and peptide loading pathways. Further, phagocytosis of apoptotic cells infected with *E. coli*, or loaded with LPS, triggers IL-6 and TGFβ production by DCs, through detection of pathogen-associated molecular patterns (PAMPs) and exposed phosphatidylserine respectively (38). This combination of cytokines drives T_H17 cell polarization. In contrast, uninfected apoptotic cells trigger only TGFβ release, which induces a regulatory T cell response. Taken together, the data demonstrate that phagocytosis provides information about the level of danger and is an integral part of the decision-making process directing differences in the inflammatory responses to apoptotic, necrotic, and infected apoptotic or necrotic cells.

TLR signaling by a newly formed phagosome can also influence the response within the compartment. It was recently reported that TLR signaling from phagosomes stimulates recruitment of mitochondria to the vicinity of the phagosomes, and that production of ROS by the mitochondria enhances bacterial killing by stimulating phagosomal ROS production (39). ROS production by phagosomes has also been linked to recruitment to the phagosomal membrane of microtubule-associated protein 1 light chain 3 (LC3), a factor involved in autophagy (40), and deficiency in LC3 recruitment to phagosomes results in reduced killing (41).

Some microbes are capable of escaping from phagosomes into the cytosol, where they may trigger activation of cytosolic innate immune sensors and be ‘recaptured’ into autophagosomes. Bacteria escaping into the cytosol of phagocytic cells can be recognized by a variety of systems including NOD receptors, and ubiquitin pathways (42). When *Listeria* escape from the macrophage phagosome they are detected by NOD2 (together with TLR2) and targeted for autophagy (43), although this process is actively antagonized by the expression of ActA and other factors by the bacteria, and is ultimately inefficient at restricting bacterial growth (44,45). Similarly, DCs challenged with *Salmonella* or *E. coli* were shown to sequester the bacteria into lysosomal compartments in a NOD2-dependent manner and NOD2 was shown to be important for the presentation of antigens on MHC class II molecules (46). NOD2 has also been implicated in triggering autophagy in response to *Shigella flexneri* in non-phagocytic cells (47), and a recent study showed that *S. flexneri* targeted for autophagy are trapped in ‘septin cages’ (filaments of polymerized septin that form around the bacteria) (48).

Break it down

Finally, the phagosome matures by fusion with lysosomes and the target is digested by hydrolytic enzymes in the low pH of the phagolysosome. Degradation of the target releases additional ligands that were previously obscured but can now be detected inside the cell, either by receptors on the phagolysosomal membrane or by sensors in the cytosol.

Although TLR2 detects lipopeptides found in the cell walls of Gram-positive bacteria and can recognize intact bacteria, optimal activation of TLR2 by *Staphylococcus aureus* in macrophages requires phagocytosis (49). Part of this enhanced activity associated with phagocytosis might come from recruitment of TLR2 to the phagosomes and the resulting enhanced sensitivity (50,51), but the investigators also observed that phagosome maturation, acidification and digestion by lysosomal hydrolases are necessary. The data suggest that while some TLR2 ligands are readily accessible to the receptor at the cell surface, a larger pool of ligands is buried in the cell wall and becomes accessible only after digestion of the cell wall.

In a similar study we observed that in addition to releasing TLR2 stimuli, bacterial degradation in phagosomes releases DNA that activates TLR9 to increase and extend inflammatory responses (52). Live *S. aureus* are relatively resistant to killing and degradation in macrophages due to chemical modification of peptidoglycan (O-acetylation) that makes it resistant to degradation by lysozyme. Mutant bacteria with lysozyme-sensitive peptidoglycan triggered greater inflammatory responses through TLR2 and TLR9. This enhanced signaling corresponded in time with when the bacterial cell wall became compromised within the phagolysosomes. TLR9 is an intracellular TLR that localizes to lysosomes, and even its recognition of soluble CpG-containing DNA molecules (that is, ligands which are not concealed intracellularly) requires internalization and delivery of the DNA to lysosomes. As such, recognition of DNA released during bacterial degradation by TLR9 in phagolysosomes makes sense. However, TLR2 is a cell surface receptor, and neither of these studies has yet explained where degradation-enhanced TLR2 activation occurs in the cell. Is there a pool of TLR2 receptors that can be recruited to mature intracellular compartments for recognition of newly-released TLR2 ligands, or do the released ligands need to be released from the cell to further stimulate surface receptors?

Enhanced degradation does not affect inflammatory signaling solely through TLR activation. It was demonstrated that degradation of *S. aureus* peptidoglycan in phagosomes is essential for activation of the NLRP3 inflammasome and optimal IL-1β and IL-18 secretion by macrophages following exposure to purified peptidoglycan or live *S. aureus* (53) (Box 2). Compared with *S. aureus* that have lysozyme-resistant peptidoglycan, *S. aureus* with lysozyme-sensitive peptidoglycan triggered greater IL-1β and IL-18 release from macrophages *in vitro* and augmented IL-1β– and IL-18-dependent inflammatory responses *in vivo*. The precise mechanism by which peptidoglycan degradation leads to NLRP3 inflammasome activation has not yet been elucidated. Curiously, a lysozyme-sensitive mutant of *Listeria monocytogenes* (that is also modified to prevent its escape from the phagosome) stimulated greater TLR-mediated cytokine production than a lysozyme-resistant strain (consistent with the discussion above) but did not significantly activate the Nlrp3 inflammasome (54). This finding might suggest either that differences in peptidoglycan

structure affect NLRP3 inflammasome activation during phagosome maturation, or that other differences in phagosome maturation between *S. aureus* and *L. monocytogenes* influence signaling. *Listeria*, of course, usually lyses the phagosome and escapes into the cytosol, and it was noted that the lysozyme-sensitive bacteria escaping into the cytosol activated the AIM2 inflammasome (54).

BOX 2

Inflammasomes

While most cytokines and chemokines are co-translationally inserted into the endoplasmic reticulum and secreted through the traditional constitutive secretory pathway, IL-1b and IL-18 are different. These cytokines are translated in the cytosol as proIL-1b and proIL-18 and must be processed into their mature forms by active caspase-1 in order to be secreted. Thus activation of caspase-1 is a second level of control of release of these cytokines. Caspase-1 is activated in multiprotein complexes called "inflammasomes" that link specific inflammatory signals (e.g. detection of bacteria or viruses) to caspase-1 (65). In certain cases, inflammasome activation can also lead to an inflammatory cell death process called "pyroptosis". Several key types of inflammasomes have been described and are defined by their associated nucleotide binding and oligomerization domain-like receptors (NLRs) or HIN-200 family proteins which are linked to specific types of microbial danger detection:

Nlrp3

The Nlrp3 inflammasome has been implicated in detection of the broadest array of stimuli including toxins (nigericin, maitotoxin), endogenous alarmins (ATP, uric acid crystals), viruses (influenza virus), Gram-positive bacteria (*S. aureus*, *S. pneumonia*), fungi (*C. albicans*), and environmental debris (silica, asbestos). The mechanism by which Nlrp3 might mediate detection of such a broad array of stimuli is not yet clear, but likely involves perturbations of mitochondria as a common factor.

Nlrc4

The Nlrc4 inflammasome detects bacterial flagellin and the structurally related basal body rod component of bacterial types III and IV secretion systems from diverse bacteria including *Salmonella* and *Pseudomonas*.

Nlrp1b

The Nlrp1b inflammasome is responsible for responding to *Bacillis anthracis* lethal toxin in the cytosol.

Aim2

Aim2 binds directly to microbial double-stranded DNA in the cytosol and contributes to caspase-1 activation in response to a variety of microbes including bacteria (*Francisella tularensis*, *Listeria*, and viruses (CMV, vaccinia virus).

Lysosomal rupture has been proposed as a mechanism by which inflammasomes in the cytosol are activated following phagocytic uptake of diverse particulate targets. Two

different bits of information could be delivered to cytosolic sensors by lysosomal rupture: release of components of the digested target could be recognized as ‘danger’ signals (as discussed above), and disruption of the integrity of the intracellular compartment itself could be recognized as ‘damage’. Rupture of the compartment releases phagolysosomal contents including endogenous components into the cytosol where they do not belong, and it has been proposed that this damage is detected as an endogenous danger signal for activation of the NLRP3 inflammasome (55). Using, among other approaches, a lysosomal destabilizing peptide Leu-Leu-OMe, it was shown that disruption of lysosomes can trigger NLRP3 activation. Various measurements of lysosomal integrity similarly revealed disruption of the organelles after ingestion of silica particles, alum crystals, or amyloid-beta aggregates (55,56). Lysosomal destabilization is not a general property of phagocytosis though, as lysosomes remain intact after macrophages ingest IgG-opsonized sheep red blood cells, polystyrene beads, or zymosan (53,57). The most direct explanation for the data is that the particles physically damage the lysosomes and it is this damage that causes inflammasome activation. However, an alternative explanation is that a signal generated by internalization of the particles leads to a more generalized instability of lysosomes throughout the cell. In support of this interpretation, much of the data generated to put forth the lysosomal damage hypothesis shows global disruption of lysosomes throughout the cell, and some bacteria that escape into the cytosol through phagosomal rupture (such as *F. tularensis*) fail to activate the NLRP3 inflammasome in mouse cells (58).

A recent study has also shown that release of bacterial components into the cytosol allows phagocytes to determine whether ingested microbes are dead or alive (59). This is an important consideration that enables them to carefully assess the level of threat that microbes pose, in order to balance the effective eradication of the organism with protection of the host from the toxic effects of an inflammatory response. If the microbes are dead then the activity of phagocytic cells alone may be sufficient to clear the organisms, but if they’re alive then back up, including activation of an adaptive immune response, may be required to ensure successful eradication of the pathogen. Consistent with this, vaccines containing live organisms usually induce more vigorous immune responses and more effective protection than vaccines containing inactivated or killed organisms. Recent observations have suggested that this is not simply due to the ability of live organisms to continue to grow and replicate within the immunized host, but that the process of phagocytosis is part of a mechanism for sensing microbial viability.

A recent study found that a variety of live Gram-negative bacteria trigger inflammatory responses in macrophages that are not triggered (or are triggered significantly less well) by heat-killed bacteria – these responses include IFNβ and IL-1β production, and pyroptosis (59). The authors hypothesized that macrophages might detect viability-associated PAMPs or ‘vita-PAMPs’ that are specifically associated with viable but not dead microbes. In agreement with this idea, they found that unlike LPS and genomic DNA, which can persist for long periods after bacterial death, RNA is rapidly degraded when *E. coli* are killed with heat, antibiotics, UV irradiation and ethanol, and that addition of bacterial (but not eukaryotic) mRNA was sufficient to trigger IFN-β, IL-1β and pyroptosis during phagocytosis of dead bacteria. The investigators reported that internalization of live *E. coli* (and to a lesser extent, dead *E. coli*) causes phagosomes to become ‘leaky’, which they

suggest would allow dsRNA released by degradation of live bacteria to enter the cytosol and induce TRIF-dependent IFN-β production and NLRP3-dependent IL-1β processing (Fig. 4). It is not clear whether this leakiness is due to membrane disruption or the opening of large pores, or how this phenomenon could be reconciled with the other functions of phagosomes such as killing, degradation and containment, which would seem to require maintenance of a sealed, impermeable compartment. Also, we don't yet know what it is about *E. coli* that triggers 'leakiness' while other phagocytosed particles (as discussed above) do not (57).

Perhaps consistent with the above idea that the phagosome can distinguish live from dead organisms, heat-killed *Candida albicans*, in contrast to live *C. albicans*, fails to activate the NLRP3 inflammasome (60,61). However, *C. albicans* is a dimorphic fungus that switches between growth in a budding yeast form and a filamentous form, an ability that is important for pathogenicity of the organism. Curiously, live mutants incapable of switching from the yeast to the filamentous form also fail to activate the NLRP3 inflammasome, suggesting that the phagocyte does not detect whether the yeast is alive, but instead that it has activated its filamentous growth pathway. The morphological switch to filamentous growth is crucial for escape of the fungus from the phagosome to permit its continued growth and virulence, and thus it may induce inflammasome activation through rupture of phagosomal membranes and release of lysosomal components into the cytosol. Release of IL-1β and other related factors (such as IL-18) may represent a reserve mechanism that can be employed if initial mechanisms to control the internalized yeast fail and more radical action is required to overcome a spreading infection.

Conclusion

Phagocytosis is widely understood as a process of clearing debris and killing microbes. As discussed here, the mechanism of phagocytosis is itself a central regulator of how a cell responds to a particle. Phagocytosis plays a key role in gleaning information about the particle, deciding what sort of threat it poses, and eliciting a set of responses that are appropriate to the threat. Developing a better understanding how phagocytosis regulates immune responses will lead to development of better antigen delivery tools for vaccines, new approaches to developing drugs to regulate inflammation, and better understanding of how pathological inflammation develops during microbial infection and autoimmune diseases.

Glossary terms

Toll-like receptor

A type of pattern-recognition receptor that recognizes unique structures that are associated with infection or tissue damage. Signalling through TLRs promotes inflammatory immune responses, cytokine production and cell activation.

NADPH phagocyte oxidase

An enzyme system that consists of multiple cytosolic and membrane-bound subunits. The complex is assembled in activated myeloid cells, mainly on the phagolysosomal membrane. NADPH oxidase uses electrons from NADPH to reduce molecular oxygen to form

superoxide anions. Superoxide anions are enzymatically converted to hydrogen peroxide, which is converted by myeloperoxidase to hypochloric acid, a highly toxic and microbicidal agent.

Pseudopodia
Transient cell membrane protrusions that extend around a target particle to engulf it. Pseudopodia also occur during cell migration.

Frustrated phagocytosis
Occurs when a phagocyte is unable to engulf its target because it is physcially too large to encompass. This can lead to the release of potentially toxic pro-inflammatory mediators into the surrounding environment.

Immunological synapse
A stable region of contact between a T cell and an antigen-presenting cell that forms through cell–cell interaction of adhesion molecules. The mature immunological synapse contains two distinct, stable membrane domains: a central cluster of TCRs, called the central supramolecular activation cluster (cSMAC) and a surrounding ring of adhesion molecules called the peripheral supramolecular activation cluster (pSMAC).

Mechanotransducer
A system for converting mechanical energy into chemical signals

Pathogen-associated molecular patterns
(PAMPs). Molecular patterns that are found in microbes but not in mammalian cells. Examples include terminally mannosylated and polymannosylated compounds (which bind the mannose receptor) and various microbial components, such as bacterial lipopolysaccharide, hypomethylated DNA, flagellin and double-stranded RNA (all of which bind Toll-like receptors).

Inflammasome
A large multiprotein complex, which contains certain NOD-like receptors, retinoic acid RIG-I-like receptors and IFI200 proteins, the adaptor protein ASC and pro-caspase-1. Assembly of the inflammasome leads to the activation of caspase-1, which cleaves pro-interleukin-Iβ (pro-IL-1β) and pro-IL-18 to generate the active forms of these pro-inflammatory cytokines.

Pyroptosis
An inflammatory form of cell death that is associated with antimicrobial responses and is dependent on inflammasome activation.

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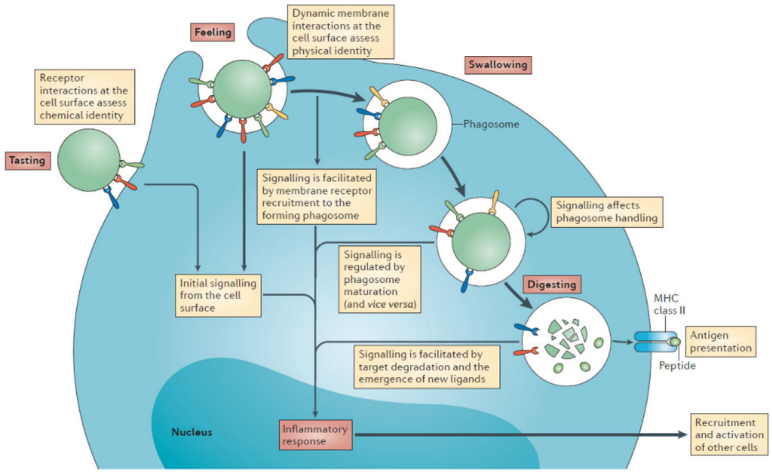

Figure 1. Information processing at different stages of phagocytosis

As phagocytosis proceeds from the initial binding of a target to actin-dependent internalization and ultimately to degradation of the target in the phagolysosome, myeloid cells acquire information about the target by a variety of mechanisms. At the surface, receptors sample the chemical constituents of the particle and membrane dynamics facilitate assessment of its physical properties. Additional information is gathered as the phagosome pinches off from the plasma membrane and as it matures through interactions with other intracellular compartments. Finally, degradation of the target exposes ligands that weren't previously accessible, and releases ligands into the cytosol for detection by intracellular receptors. The information gathered by all of these processes is integrated to shape the ensuing immune response.

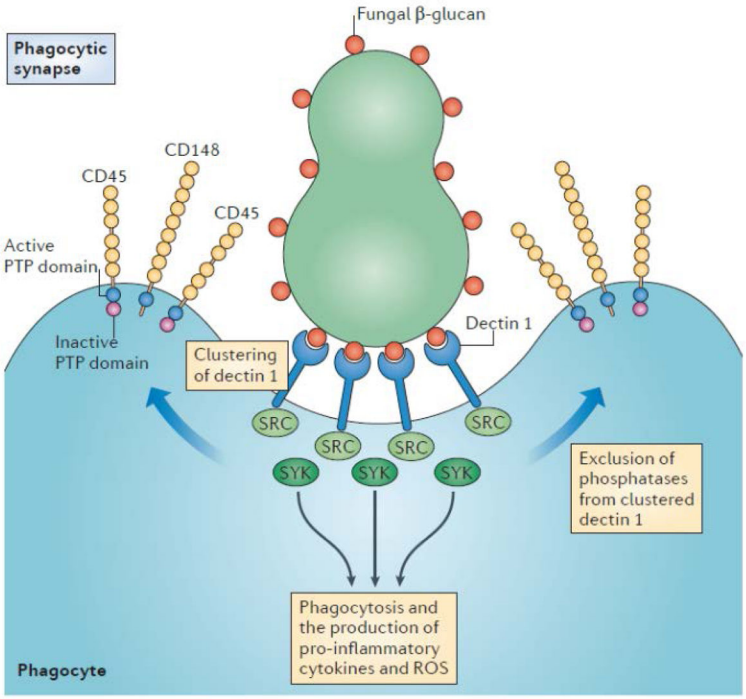

Figure 2. Phagocytic synapse formation permits Dectin-1 to distinguish between soluble and particulate β-glucans

Dectin-1 engages both particulate β-glucans and soluble β-glucan polymers, but only particulate β-glucans activate phagocytosis and inflammatory responses. CD45 and CD148 phosphatases regulate signal transduction by the Dectin-1 hemITAM, and must be isolated from the clustered receptors by the formation of a synapse in order to permit productive Dectin-1 signaling. Soluble β-glucans bind with high affinity to Dectin-1 but do not form synapses and therefore fail to trigger Dectin-1-mediated responses.

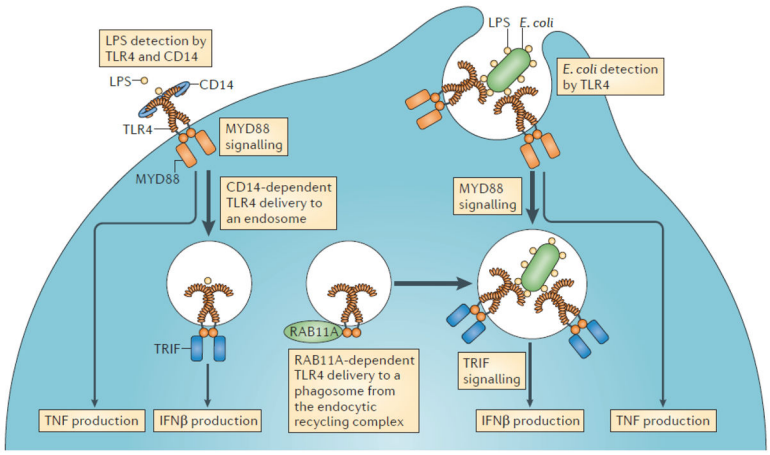

Figure 3. TLR4 signal transduction from the cell surface, endosomes and phagosomes

TLR4 signals via the MyD88 adaptor at the cell surface and via TRIF from intracellular compartments. Recruitment of TLR4 for TRIF-mediated signaling is differentially regulated when cells undergo endocytosis of soluble LPS and phagocytosis of LPS-containing bacteria.

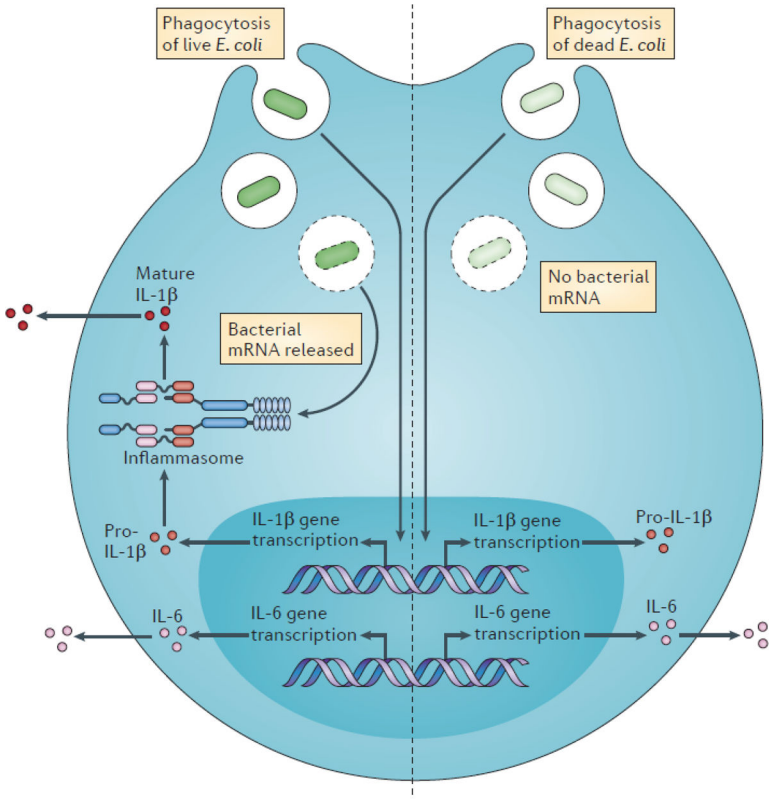

Figure 4. Macrophages can differentiate between live and dead *E. coli* by the detection of 'vita-PAMPs'

During phagocytosis, macrophages assess whether *E. coli* are alive or dead. While some responses are triggered by engagement of both live and dead bacteria at the cell surface (e.g. IL-6 production), other responses are only initiated upon internalization of live bacteria (e.g. inflammasome activation, which is required for processing of IL-1β). When live bacteria are degraded they release RNA, which is thought to leak out of the phagolysosome and be detected by cytosolic receptors. However, RNA is rapidly degraded when bacteria die, so no RNA is released when dead bacteria are phagocytosed.

Table 1

Selected receptors involved in phagocytosis

RECEPTORS	LIGANDS	Refs.
Opsonic Phagocytosis		
Fc Receptor family (FcγRI, FcγRIIA FcγRIIa)	Antibody-opsonized targets	(66)
Complement Receptors (CR1, CR3, CR4)	Complement-opsonized targets	(67)
$\alpha_5\beta_1$ (integrin)	Fibronectin	(68,69)
Non-Opsonic Phagocytosis		
Dectin-1	β-glucan	(70)
MARCO	Bacteria (undefined specific ligand)	(71)
Scavenger Receptor A	Bacteria (diverse charged molecules)	(72)
$\alpha_V\beta_5$ (integrin)	Apoptotic cells	(68)
Triggered (Non-Specific) Phagocytosis		
Toll-Like Receptors	LPS, Lipopeptides, etc.	(73)