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The phagosome as the organelle linking innate and adaptive immunity

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Abstract

The means by which phagocytosis and antimicrobial defense mechanisms are linked have expanded greatly in recent years. It is now clear that the process of phagocytosis does more than just degrade internalized microbes, but also helps coordinate the actions of the innate and adaptive immune system. This review will discuss the means by which Toll-like Receptor signaling pathways are coordinated around the processes of phagocytosis, phagosome trafficking and autophagy and how these signaling pathways influence T-cell mediated immunity. In this regard, we propose that at the subcellular level, phagosomes represent the smallest definable unit that links innate and adaptive immunity.

Introduction

The process of phagocytosis, first discovered by Elie Metchnikoff over a century ago, represents one of the most ancient forms of defense used by multicellular organisms to kill microbes they encounter. This process involves the engulfment of the microbe by protrusions of the plasma membrane of a phagocyte, which results in the formation of a membrane-bound cytosolic compartment called a phagosome (1). Through highly regulated membrane fusion events, newly formed phagosomes undergo sequential interactions with early endosomes, late endosomes and lysosomes (2). These phagosome-endosome interactions are viewed classically as being essential to deliver degradative enzymes to break down the luminal cargo into peptides, nucleotides and metabolites. Recently, phagosomes have also been implicated as important sites of signal transduction by microbial sensors called Pattern Receptor Receptors (PRRs). PRRs recognize conserved molecules that are uniquely (or selectively) produced by microbes, such as lipopolysaccharide (LPS), double stranded RNA, flagellin and various lipoproteins (3). There are several families of PRRs, but the ones that detect extracellular microbes are the most likely to have their functions integrated with the general phagocytic machinery. These include the Toll-like Receptors (TLRs) and the C-type Lectin Receptors of the Dectin family of proteins (3).

This review will focus on the emerging idea that the functions of TLRs are linked intimately to the functions of phagocytosis and phagosome trafficking. We discuss evidence that microbial detection by PRRs can lead to phagocytosis and the recruitment of anti-microbial

activities to phagosomes. In addition, we will explore the idea that TLR signaling can occur from phagosomes, and how distinct endosomal or phagosomal compartments may lead to distinct signaling pathways being activated. We hope to convey our view of where the nascent field of “innate immune cell biology” is moving, with a focus on achieving an understanding of how the membrane trafficking events and TLR signaling events are interconnected.

PRRs that activate both phagocytosis and inflammation

Phagocytosis and TLR-induced signaling provide key protective mechanisms in mammals against bacterial infection. Evidence in support of this claim comes from human patients that are deficient for key components in the TLR signaling pathways such as IRAK4 (4), and those that lack neutrophils (5), which are the most abundant phagocytes in inflamed tissues. Under both instances, these patients are highly susceptible to colonization by bacteria that are classically defined as non-pathogenic. At the level of an infected tissue, the link between TLR signaling and phagocytosis is well-established, because TLR signaling leads to the expression of inflammatory cytokines and chemokines (6). These factors promote the recruitment of monocytes, neutrophils and other phagocytes to the infected tissue, where they are more likely to encounter microbes and engulf them. Thus, TLRs can promote phagocytosis by promoting their recruitment to infected tissues, but whether TLRs can enhance the antimicrobial actions of individual phagocytes remained unclear. However, in recent years, it has become clear that LPS can upregulate macropinocytosis in dendritic cells and macrophages, which is the actin-dependent engulfment of large volumes of extracellular media (7, 8). This upregulation of macropinocytosis is dependent on the LPS sensor, TLR4 (8) and the downstream ribosomal s6 kinases (RSK) that this receptor activates (9). LPS-induced macropinocytosis may also enhance the phagocytosis of bacteria. In addition, the protein MD-2, which is the LPS-binding moiety of the TLR4 signaling complex (10), can opsonize gram-negative bacteria and promote phagocytosis (11). Perhaps the most direct link between microbial detection and phagocytosis comes from the C-type lectin receptor Dectin-1. Dectin-1 was first described as a phagocytic receptor for fungal pathogens (12), but is now appreciated to also function as an inflammation-inducing PRR (13). Thus, TLRs and Dectin family members can function as PRRs that induce both phagocytosis and inflammatory cytokine expression. In this regard, both of these receptors function to promote the killing of microorganisms in a cell-intrinsic way.

It is noteworthy that not all phagocytic PRRs are intrinsically capable of inducing inflammatory responses. For example, the Mannose Receptor, Mannose Binding Lectin (MBL), C-reactive protein, Serum Amyloid protein, and the complement system all bind directly to microbial products and are therefore PRRs (6). These proteins have well characterized activities in promoting the phagocytosis and killing of microbes they recognize, yet they have no intrinsic functions in promoting inflammation. Despite the lack of intrinsic ability to promote inflammation, at least one of these proteins (*e.g.* MBL) can extract microbial products from intact bacteria so that they may be more easily accessed by TLRs (14). In this regard, even a classically defined opsonin such as MBL can integrate their actions with other PRRs that induce inflammation leading to clearance of bacterial pathogens.

TLR enhancement of phagosome maturation

TLR signaling not only induces global activation of DCs through the upregulation of costimulatory molecules and secretion of cytokines (15), but also acts locally in a phagosome-autonomous manner to enhance phagocytosis, degradation of pathogens, processing and presentation of antigens (Figure 1). Since the original report demonstrating

the TLR-dependent maturation of the phagosomes containing bacteria, but not apoptotic cells, within the same cell (16), a number of studies have begun to reveal downstream signaling events leading to TLR-induced maturation of the phagosomes (17). Individual TLRs promote phagocytosis to varying degrees with TLR9 being the strongest and TLR3 being the weakest (18). This increase in phagocytosis is the result of both a larger percent of phagocytes taking up bacteria, and the number of bacteria phagocytosed by individual cells (16, 18, 19). TLR-induced phagocytosis of bacteria depends on MyD88 signaling through IRAK4 and p38 leading to the up-regulation of scavenger receptors (16, 18). The downstream signaling pathways that enhance phagosomal maturation upon TLR engagement involve members of the MAPK family, as inhibitors of MAPK p38 block TLR-enhanced phagosomal maturation (16). A stark illustration of the importance of this pathway comes from pathogens that utilize TLR-mediated phagosome acidification to benefit their replication. In TLR-deficient cells, *Salmonella enterica* serovar typhimurium fail to upregulate *Salmonella* pathogenicity island 2 (SPI-2) genes and cannot form a replicative compartment. TLR signaling enhances the rate of acidification of the *Salmonella*-containing phagosome, which is utilized by *S. typhimurium* to regulate virulence genes necessary for intracellular survival and growth (20).

TLR enhancement of antigen presentation to T cells

Following phagocytosis of pathogens, DCs process the peptides for presentation on MHC class II. TLR engagement in DCs induces translocation of MHC class II molecules from the endosomes to the plasma membrane, where peptides are presented on MHC class II to naïve CD4 T cells (21). Presentation of antigen on MHC class II requires engagement of TLR within the same phagosome that has incorporated the antigen (22). This study predicts that antigens that are physically linked to TLR ligands should be highly immunogenic. Indeed, this seems to be the case, both with protein antigens that have been physically cross-linked to TLR agonist (23), and for naturally occurring protein antigens, profilin of *Toxoplasma gondii*, that acts as a ligand for TLR11 (24), and flagellin of bacteria, a ligand for TLR5 (25). Physical link between antigen and TLR ligand is also required for MHC class II presentation of dying tumor cells, as DCs require signaling through TLR4 and MyD88 for efficient processing and presentation of antigen from dying tumor cells in the setting of anticancer chemotherapy and radiotherapy (26). Signaling pathways leading to enhancement of MHC class II antigen presentation by phagosomal TLR remains unclear. A recent study indicated that AP-3 mediates phagosomal TLR trafficking and signaling for efficient MHC class II presentation of cargo antigens in DCs (Mantegazza et al. in press). In addition, in the absence of AP-3, peptide+MHC class II export to the cell surface is impaired. AP-3 promotes MHC class II presentation of TLR-conjugated antigens internalized by phagocytosis but not receptor-mediated endocytosis, indicating that TLR trafficking via AP-3 is restricted to phagosomes.

The importance of phagosomes in controlling antigen presentation also extends to the cross-presentation pathway, whereby internalized cargo can be loaded onto MHC class I molecules for presentation to CD8+ T-cells (27). The means by which proteins from the extracellular media gain access to the MHC class I machinery in the endoplasmic reticulum (ER) has long been mysterious. Recent work indicates that phagosomes can fuse with ER-derived vesicles by a process dependent on the SNARE Sec22b (28). Depletion of Sec22b results in a strong defect in cross presentation of phagocytosed *T. gondii* and *E. Coli*, but no defect in antigen presentation of MHC class II. The fact that TLRs regulate several aspects of MHC class II presentation suggests that innate immune signals may also control the Sec22b-mediated recruitment of ER-derived vesicles to phagosomes to promote cross presentation (28).

TLR signaling from the plasma membrane and phagosomes

In the sections above, we highlighted the means by which TLR signaling can enhance several important aspects of phagocytosis and phagosome transport. How are the TLR signaling pathways coordinated around the remarkably dynamic events at the plasma membrane and on phagosomes? We examine this question by focusing on recent efforts to define the subcellular sites of TLR signal transduction. Since all TLRs encounter their microbial ligands in the extracellular space or in endosomal vesicles (29), these compartments have been the focus of work in this area (Figure 2). In the case of TLRs that are uniquely localized to endosomal compartments, it is likely that they signal from these locations. This is the case for a subset of receptors whose biosynthetic trafficking is regulated by the protein Unc93B (30, 31). Unc93B binds to TLRs 3, 7, 9 and 13 in the ER and somehow delivers these receptors to endosomes (30–32), rather than the plasma membrane. Each of these receptors detects features of nucleic acids that are often found in microbial genomes such as double stranded RNA (TLR3), single stranded RNA (TLR7), unmethylated CpG containing DNA (TLR9) (33). The ligand detected by TLR13 is unknown. The positioning of these receptors in endolysosomal compartments likely facilitates ligand binding after a microbe has been degraded (34). Since these receptors are detected in endosomes and can be recruited to phagosomes (*e.g.* TLR9) (35), it is very clear that signaling occurs from these compartments. Interestingly, as will be described below, recent work indicates that upon ligand binding, TLR9 can move between endosomal compartments (36). While the precise site of TLR signaling within endosomes or phagosomes remains unclear, there is general agreement that in the case of nucleic acid binding TLRs, endosomal/phagosomal signaling does occur.

While it is a reasonable suggestion that TLRs uniquely found in endosomes induce signaling from endosomes, it is more challenging to determine where plasma membrane-localized TLRs signal from. Our best understanding of this process comes from studies of TLR4. TLR4 is found at the cell surface at steady state, where it encounters gram-negative bacteria (or soluble LPS) (37). LPS recognition by TLR4 is facilitated by interactions with the LPS-binding proteins LBP, CD14 and MD-2 (38). Upon detection of LPS, TLR4 is thought to oligomerize and creates a signaling platform on its cytosolic TIR domain to recruit the TIR domain containing adaptors TIRAP and MyD88 (39–41). These adaptors serve as a molecular link between the active receptor and the downstream kinases of the IRAK family that lead to the activation of inflammatory transcription factors such as NF- κ B, AP-1 and various Interferon Regulatory Factors (IRFs) (3). Evidence supporting the idea that TLR4 signaling through the TIRAP-MyD88 adaptors occurs at the cell surface is ample. For example, the TIRAP adaptor is a phosphatidylinositol 4,5-bisphosphate (PIP2) binding protein (42), which is enriched at the cell surface at steady state (1). Since TIRAP is required for TLR4 signaling through MyD88 (43, 44), its enrichment at the cell surface prior to microbial encounters suggests that this is the initial site of signal transduction. Moreover, TIRAP has the ability to recruit its downstream partner MyD88 to the plasma membrane (42), further suggesting that signaling occurs from the cell surface. Complementary work revealed that blocking endocytosis does not disrupt, but rather enhances, TLR4 signaling through TIRAP-MyD88 (45, 46).

More recently, it has become clear that TLR4 also induces signaling from endosomes or phagosomes (Figure 2). This was first shown by studies using dynamin inhibitors, which block TLR4 endocytosis and the activation of a second TLR4-mediated signaling pathway that does not require TIRAP or MyD88 (47). This second pathway is mediated by a distinct set of TIR domain containing adaptors called TRAM and TRIF (48, 49). Like the TIRAP-MyD88 pair, TRAM and TRIF are recruited to the TIR domain of TLR4 upon LPS treatment (50). Their recruitment leads to the activation of the transcription factor IRF3,

which regulates Type I Interferon (IFN) expression (51). Since IFN expression is not induced by MyD88 downstream of TLR4 (52), their expression can be used as a specific indicator of TRAM-TRIF signal transduction. The evidence that blocking endocytosis blocks IFN expression indicates that unlike the TIRAP-MyD88 signaling pathway, TRAM-TRIF signaling is not initiated until after endocytosis has occurred (47, 53). Blocking TLR4 endocytosis also prevents the activation of TRAF3, an E3 ubiquitin ligase that acts downstream of TRIF (50). Recently, the idea that TRAM-TRIF signaling occurs uniquely from endosomes was extended to phagosomes. Espevik and colleagues found that enhancing bacterial phagocytosis by using opsonizing antibodies strongly enhanced signaling through the TRAM-TRIF pathway, but signaling through the TIRAP-MyD88 pathway (which occurs at the cell surface) was largely unaffected (54). The authors also demonstrate that TLR4 continues to accumulate on phagosomes even after they have been formed. Based on this data, it was suggested that an intracellular pool of TLR4 is recruited from recycling endosomes to phagosomes to propagate TRAM-TRIF dependent signaling. In addition, the LPS-binding protein CD14 has recently been identified as the first factor that regulates the microbe-induced endocytosis of TLR4 (8). CD14-deficient macrophages are defective for TLR4 endocytosis in response to soluble LPS, LPS-coated beads or *E.coli*. Under these conditions, TLR4 signaling through the TIRAP-MyD88 pathway is intact, but TRAM-TRIF signaling is completely abolished.

Regulators of microbe-induced TLR trafficking

One of the central tenets of TLR signaling is that all cellular responses to TLR ligands are mediated by either the MyD88- or TRIF-dependent signaling pathways (55). Thus, cells deficient in both MyD88 and TRIF are often used to eliminate all signaling pathways induced by TLR ligands. Studying the process that CD14 regulates TLR4 endocytosis in response to LPS revealed a third signal transduction pathway that is activated by this TLR4 ligand (8). This third CD14-dependent signaling pathway does not require TLR4 signaling, nor does it require the TIR domain containing adaptors. Rather, CD14-dependent endocytosis of TLR4 is mediated by the tyrosine kinase Syk and its downstream effector PLC γ 2 (8, 56). Syk activation by CD14 is facilitated by the Immunoreceptor Tyrosine Activation Motif (ITAM)-containing transmembrane adaptors DAP12 and Fc ϵ R γ . Since TLR4 signaling through the TRIF pathway occurs from endosomes (47, 50, 53), this CD14-dependent endocytosis pathway is also required for TRAM-TRIF dependent signal transduction (8). Notably, Syk and PLC γ 2 are regulators of phagocytosis, in particular downstream of the Dectin family of PRRs that mediate the internalization of fungal pathogens, and Fc-Receptors (12, 57–59). The commonalities between the cytosolic responses induced by CD14, Dectin-1 and Fc-receptors raises the possibility that an inflammatory phagocytic pathway exists, which is induced by PRRs and antibody receptors, the function of which would be to link the internalization of potential pathogens with the induction of antimicrobial responses.

The inactivation of TLR4 signaling appears to be linked to its ability to be internalized into late endosomal multivesicular bodies (MVBs) (Figure 2). The biochemical aspects of TLR4 internalization into MVBs are unclear, but this process may involve interactions between TLR4 and regulators of MVB formation, such as Hrs (60). (46). Whether other TLRs are also internalized into MVBs is unknown.

Plasma membrane localized TLRs are not the only members of this receptor family whose transport is stimulated by microbial detection. Endosomal TLRs, TLR3, 7, and 9, are expressed in the ER at steady state. UNC93B transports these TLRs from the ER to the endosome by interacting physically via the TLR transmembrane domain (30, 32). Once inside the acidified endosome, these TLRs undergo sequential proteolytic processing by

cathepsins and asparagine endopeptidases to generate an active form of the receptor (35, 61, 62). Upon binding stimulatory DNA, TLR9 undergoes a conformational change that transduces an activating signal across the endosomal membrane to the TIR domain, which is transmitted by MyD88 to downstream signaling molecules (63). MyD88 subsequently interacts with the death domain of several IRAK proteins to induce two bifurcating pathways (Figure 2). The two distinct pathways emanating from endosomal TLRs are best studied in plasmacytoid dendritic cells (pDCs), in part because these cells produce copious amounts of cytokines and IFNs upon viral infection. The first pathway leads to the transcriptional activation of pro-inflammatory cytokines, and requires NF- κ B (64). The second pathway leads to the activation of type I IFN genes through phosphorylation of IRF7 (65). Although both pathways depend on MyD88 (41) and UNC93B (30), the latter pathway requires additional molecules (64, 66–73). TLR9 and TLR7 induction of type I IFN genes requires adaptor protein 3 (AP-3) (36, 74). AP-3 is a multi-protein complex of adaptors that sorts integral membrane cargoes primarily from early endosomes towards lysosomes and lysosome-related organelles (LROs) (75). In the absence of AP-3, CpG DNA-induced TLR9 trafficking to LRO-like compartment in pDCs is disrupted, and TLR9 remains stuck in LAMP2-negative endosomes that contain PI(3,5)P₂. These LAMP-2 negative endosomes only support NF- κ B-dependent cytokine induction, but not type I IFNs (36). Such selective deficiency in IFN synthesis was also found in pDCs lacking components of the biogenesis of lysosome-related organelles complex 1 (BLOC1) (unpublished observations), indicating that specialized LROs in pDCs are required for TLR-induction of IFN activation. Thus, TLR9 and TLR7 signal from distinct endosomal compartments to engage selective sets of adaptors and transcription factors that enable the same receptors to induce differential gene expression.

Do all TLRs signal from phagosomes?

The above sections explained how several TLRs (TLR4, 7 and 9) can induce signal transduction from endosomes or phagosomes, and that microbe-induced trafficking of these receptors dictates the type of signaling pathway activated by these receptors. Is the ability to induce signal transduction from endosomal locations a common feature of all TLRs? The signaling functions of TLR2 and its signaling partners TLR1 and 6 have been examined in this regard. In addition to other ligands, TLR2 detects bacterial lipoproteins that are enriched on the cell walls of gram-positive bacteria (33). Like TLR4, TLR2 is found at the plasma membrane and can induce signal transduction via the TIRAP-MyD88 adaptors (43, 44). However, this receptor does not induce TRAM-TRIF dependent IFN expression (52). Classic studies from Aderem and colleagues showed that TLR1, TLR2 and TLR6 can be found on phagosomes containing zymosan particles (76, 77), and other studies have shown TLR2 on phagosomes containing *Chlamydia* and *Borrelia* species (78, 79). However, it should be noted that microscopic examination of a TLR on a phagosome is not indicative of signal transduction. For example, while TLR1, 2, and 6 can be found on phagosomes containing bacteria they detect, these receptors can also be found on phagosomes containing no microbial ligands, such as those containing sheep red blood cells (76, 77). Moreover, even in the case of TLR9 (which certainly signals from phagosomes), its localization to these compartments is not indicative of signal transduction. For example, TLR9 can be detected in latex-bead containing phagosomes that contain no microbial products (35). Thus, it appears that most TLRs are recruited to phagosomes regardless of their cargo, probably to “sample” the compartment for the presence of microbial products (76). For these reasons, microscopic examination of TLR recruitment to phagosomes cannot be used as a marker of inflammatory signal transduction. Currently, no reliable marker has been identified to definitively demonstrate if TLR2 signaling occurs from phagosomes, or if signaling from phagosomes is distinct from signaling that occurs from the plasma membrane. Perhaps the best functional evidence to indicate that TLR2 signaling can occur from phagosomes comes

from two recent studies using infectious microbes as TLR2 ligands. Studies with *Staphylococcus aureus* revealed that MBL opsonization promotes bacterial phagocytosis, and this process leads to the enhancement of TIRAP-MyD88 dependent cytokine expression (14). Blocking internalization of *S. aureus* diminished cytokine expression, suggesting that delivery to phagosomes is both necessary and rate limiting for the ability to induce TLR2 signaling. Secondly, studies of Vaccinia Virus infection of inflammatory monocytes revealed that these cells have the unusual ability to induce the expression of Type I IFNs via TLR2 signaling (80). Inhibition of Vaccinia internalization prevented TLR2-mediated IFN expression, but not TLR2-mediated inflammatory cytokine expression. These latter results are remarkably similar to the behavior of TLR4, in that cytokine production is induced from the cell surface while IFN expression is induced from phagosomes or endosomes. While these experiments suggest that TLR2 signaling can occur in phagosomes, the fact that *S. aureus* and Vaccinia Virus contain ligands that activate PRRs other than TLR2 mandates further work to address this important issue.

In the case that TLR2 can signal from phagosomes or endosomes, the question then becomes how? TIRAP is required for TLR2 signaling, and the localization of this adaptor is mediated by interactions with PIP2 (42–44). Since PIP2 must be removed from the phagocytic cup in order for phagocytosis to be completed (57, 81), all phagosomes should lack PIP2, and hence all phagosomes should lack TIRAP. In principle, this idea would preclude TLR2 from having the ability to signal from phagosomes. Recent studies on the localized formation of PIP2 have provided a means by which TLR2 may indeed be able to signal from phagosomes. Grinstein and colleagues have shown that unlike Fc-mediated phagocytic events, phagosomes created by complement-mediated phagocytosis experience a second “wave” of PIP2 formation (82). This second wave of PIP2 formation is linked directly to the ability of these phagosomes to retain the Type I PI(4)P 5-kinase (PIP5K), which is responsible for creating PIP2 (82). The formation of the TIRAP receptor (PIP2) on some phagosomes raises the possibility that TIRAP could be recruited to phagosomes after they are formed. Since TLR2 is found on all phagosomes, it is therefore possible that TIRAP would be in a position to recruit MyD88 to phagosomes containing TLR2 ligands. Future work on this topic is required to test this hypothesis, but the genetic and cell biological tools appear to be in place to determine formally if TLR2 signaling can indeed occur from phagosomes.

Unusual partners in TLR regulation of antimicrobial actions in phagosomes

Engagement of TLRs in the phagosome results in enhanced maturation and degradation of its cargo (16). Recent evidence indicates that this process is at least in part mediated by the recruitment of proteins normally associated with autophagy. Autophagy is a highly conserved, tightly regulated cellular mechanism of lysosomal degradation of cytosolic constituents (83). Damaged organelles, long-lived proteins and even intracellular pathogens are engulfed by autophagosome, a double membrane vesicle that sequesters and fuses with lysosome for degradation (84). One of the hallmarks of autophagy is the incorporation of LC3 (human homologue of yeast Atg8) into autophagosomes, a process dependent on multiple Atg genes. A seminal work by Douglas Green and colleagues showed that in macrophages, particles that engage TLRs trigger the autophagosome marker LC3 to be rapidly recruited to the phagosome (85) (Figure 1). This process is preceded by the recruitment of beclin 1 and phosphoinositide-3-OH kinase activity, in a manner dependent on Atg5 and Atg7. However, translocation of beclin 1 and LC3 to the phagosome was not associated with double-membrane structures characteristic of conventional autophagosomes. Instead this was associated with enhanced phagosome fusion with lysosomes, leading to rapid acidification and enhanced killing of the phagocytosed yeast. TLR signaling from the phagosomal membrane was required to recruit LC3, as only the bead-coupled Pam3Cys, but

not soluble Pam3Cys, induced LC3 recruitment to the phagosomal membrane. Thus, this study showed for the first time that phagosomal maturation utilizes molecules involved in the autophagy pathway without invoking canonical autophagy.

The role of recruitment of Atg proteins to TLR-containing phagosomes extends beyond microbial degradation. This process is also utilized by DCs, professional antigen presenting cells, for more efficient processing of phagocytosed antigens for presentation by MHC class II (86). Atg proteins are recruited to the phagosomal membrane containing microbial antigens and facilitate fusion with lysosomes, thereby enhancing processing of antigens for presentation to MHC class II in dendritic cells. In vivo, conditional deficiency in Atg5 in DCs resulted in minimal priming of CD4 T cell Th1 response, resulting in increased mortality following intravaginal herpes simplex virus 2 (HSV-2) infection. DCs that lack Atg5 have an impaired ability to degrade extracellular antigens containing viral PAMPs, leading to reduced levels of MHC II+ peptide complex presented on their cell surface. As in macrophages (85), Atg-dependent enhancement of antigen processing only occurred with cargo containing TLR ligands, indicating that TLR signaling provides the necessary signal for Atg engagement in phagosome-specific manner. Similar requirement for Atg proteins is found for HIV-1 antigen processing for MHC class II in human DCs (87).

Phagosomes containing TLR ligands not only recruit molecules but also organelles to enhance microbial killing. Engagement of a subset of TLRs (TLR1, TLR2 and TLR4) results in the recruitment of mitochondria to phagosomes (Figure 1). This results in enhanced mitochondrial reactive oxygen species (ROS) production in macrophages. A TLR signaling adaptor, TRAF6, is translocated to the mitochondria, where it engages the ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) molecule. Interaction with TRAF6 leads to ubiquitination of ECSIT, resulting in increased mitochondrial and cellular ROS generation, and improved killing of phagocytosed bacteria (88). Thus, TLRs engage autophagic machinery and even mitochondria to enhance microbial killing within phagosomes. In DCs, TLR signaling couples Atg and AP-3 molecules for processing and presentation of phagocytosed antigen on MHC class II (Figure 1).

Concluding remarks

Over the last decade, research into the mechanisms of TLR signaling pathways has mainly taken a genetic approach to identify novel regulators that execute the signaling responses induced by microbial products. Today, there are dozens of known regulators of these pathways, but their precise functions and their mechanisms of action remain a very large “black box” that needs to be filled (89). This review highlights how cell biological analysis can be coupled to genetic studies to provide a more comprehensive view of how TLR signaling pathways are integrated into the general cellular infrastructure within which they operate. One of the most intriguing ideas that emerge from this work can be derived from Janeway’s original proposal that PRRs would serve as the link between innate and adaptive immunity (90). Work championed by Steinman and colleagues established professional antigen presenting cells as the cellular unit that links innate and adaptive immunity (91). We now propose that at the subcellular level, the phagosome’s ability to coordinate TLR signaling with antigen presentation makes this organelle the smallest definable unit that bridges innate and adaptive immunity.

As most microbes that enter mammalian cells must do so by a phagocytic route, this idea extends beyond any specific host-microbe encounter, and may apply to other families of microbial sensors that have yet to be studied in this regard. Many unanswered questions remain regarding cell biological aspects of TLR signal transduction. We still do not know the precise subcellular sites of TLR signaling, nor do we understand how

phagosome trafficking pathways are mechanistically linked to the activity of TLRs. Additionally, it remains to be determined how microbial pathogens manipulate TLR signaling pathways, and if their mechanisms of immune evasion are linked to alterations in phagosome trafficking. Finally, the question of how much of our knowledge of TLR signaling in mice translates into similar insight in humans remains to be determined.

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Figure 1. TLRs promote phagocytosis, microbial degradation, processing and presentation of antigens

Activation of surface TLRs by microbes enhance endocytic uptake of pathogens. Once taken up into phagosomes, endosomal TLRs can stimulate phagosomal fusion with lysosomes and induce degradation of pathogens. Phagosomal TLRs induce MAPK p38-dependent signals that lead to enhanced phagosomal maturation and antigen presentation for MHC class II in phagosome autonomous manner. TLR-induced signals also recruit components of autophagy machinery to the phagosomal membrane, resulting in incorporation of LC3-II and efficient fusion with the lysosome. Consequently, Atg5-deficient phagocytes are impaired in microbial degradation and antigen processing for MHC class II. Moreover, phagosomal TLR signaling recruits mitochondria, leading to ROS generation and enhanced bacterial killing.

Figure 2. Signaling by TLRs at several stages of phagosome formation and trafficking
 TLR-mediated activation of AP-1 and NF- κ B can occur at the site of endocytosis and phagocytosis, in PI(4,5)P2-rich regions of the plasma membrane. Newly formed phagosomes permit TLR4 to induce Type I IFN expression, which is mediated by IRF3. As phagosomes mature, TLR4 is internalized into multivesicular bodies (MVBs) and inactivated. Microbial cargo is also degraded as the phagosomes proceed, by an AP-3 dependent process, to late endosomal compartments and lysosomal related organelles (LRO). Microbial nucleic acids may then engage TLRs 7 and 9 to induce additional inflammatory responses. Depending on the nature of the late endosomal compartment, nucleic acid sensing TLRs can induce NF- κ B dependent cytokine expression or IRF7-mediated Type I IFN expression.