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Critical Review

Autophagy as a Macrophage Response to Bacterial Infection

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Summary

The macrophage is a key component of host defense mechanisms against pathogens. In addition to the phagocytosis of bacteria and secretion of proinflammatory mediators by macrophages, autophagy, a process involved in turnover of cellular material, is a recently identified component of the immune response to bacterial infection. Despite the bactericidal effect of autophagy, some species of intracellular bacteria are able to survive by using one or more strategies to avoid host autophagic attack. Here, we review the latest findings on the interactions between bacteria and autophagy in macrophages.
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Keywords autophagy; innate immunity; intracellular bacteria; macrophage; phagocytosis.

INTRODUCTION

Macrophages, together with other professional phagocytic cells including neutrophils and monocytes, play a crucial role in the host-defense system through recognition and elimination of invading pathogenic bacteria (*1*). Macrophages have the means to destroy pathogens directly or indirectly via innate and adaptive immune responses, respectively (*2*). The direct bactericidal features of these cells include the generation of reactive oxygen species (ROS) and phagocytosis, a process involving the engulfment of bacteria into phagosomes. The bacteria-containing phagosomes fuse with late endosomes or lysosomes in a process of "maturation" leading to the eventual degradation of the bacteria (*3*). The indirect macrophage immune response involves inflammation, a process characterized by the increased production of many inflammatory cytokines and chemokines, which together promote the recruitment of blood leukocytes to the site of infection and the activation of additional immune cells (*4*). In certain cases, the protective responses of the host are overcome by the invading pathogen triggering the death of activated macrophages. As a front-line component of host defense macrophages represent a useful model to study host-pathogen interactions.

Autophagy is a degradation and recycling system conserved amongst eukaryotes that eliminates unwanted and damaged cellular components including proteins and organelles. Autophagy plays an important role in many physiological and pathological pathways, including the cellular response to starvation, cell development, and tumor suppression (*5, 6*). More recently, autophagy has been recognized as a key component of host immune defense, and responsible for eliminating intracellular pathogens including bacteria, viruses, fungi, and parasitic protozoa (*7, 8*) in a process termed xenophagy (*9*).

Although three forms of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy (*6*), have been described in mammalian cells, only macroautophagy has to-date been associated with the elimination of intracellular bacteria (*10*). Macroautophagy (hereafter referred to as autophagy or canonical autophagy) involves the sequestration of a portion of the cell cytoplasm into a double-membrane vesicle or autophagosome. The process is regulated by a number of autophagy-related genes (ATGs) including those that encode proteins required for signaling (Beclin 1) and autophagosome formation/cargo recognition (LC3) [for details of the different mechanisms see reviews (*11, 12*)]. Autophagosomes fuse with lysosomes, forming single-membraned autolysosomes, in which the contents are degraded by lysosomal acid hydrolases (*6*).

To survive and proliferate in macrophages, some intracellular bacteria have developed different strategies to evade or withstand host defense systems. These strategies include: (i) escape from the phagosome into the cytosol to avoid lysosomal killing as exemplified by *Listeria monocytogenes*, *Shigella flexneri*,

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Burkholderia pseudomallei, and *Streptococcus pyogenes* (*13*); (ii) remodeling of the phagosomal compartment to block phagosomal maturation and fusion with lysosomes as exemplified by *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Salmonella typhimurium*, and *Brucella abortus* (*14*); and (iii) remodeling of the phagosomal compartment to allow survival and replication in acidic phagolysosomes as exemplified by *Coxiella burnetii*. Moreover, many bacterial pathogens have developed the ability to either evade autophagic attack or manipulate the autophagy pathway for their own benefit (*15*).

Here, we review the latest findings related to autophagy in bacteria-infected macrophages. In particular, we focus on how autophagy is triggered in response to invading bacteria, and how intracellular bacteria deal with autophagic attack (Table 1).

AUTOPHAGY INDUCTION IN INFECTED MACROPHAGES

As a housekeeping activity, autophagy plays a cytoprotective role to enable cellular homeostasis (*47*). In this process, autophagy is activated in response to many cellular stresses including starvation, endoplasmic reticulum stress, oxidative stress, and exposure to certain chemicals, radiation, and hypoxia (*48*). Similarly, bacterial infection and inflammation are stressors that trigger autophagy in macrophages and other immune cells (*49*). Autophagy when induced in infected macrophages promotes the clearance of pathogenic bacteria including *S. typhimurium* and *S. flexneri* (*50*).

Pathogenic bacteria induce autophagy in macrophages via virulence factors that fall into one of three categories: (i) pathogen associated molecular patterns (PAMPs), (ii) toxins, and (iii) secreted effector proteins (Fig. 1). Toll-like receptors (TLRs) represent host-surface PAMP recognition receptors (PRRs) that are activated by their cognate PAMPs (*51*). It has been reported that activation of TLR4 and TLR7 by bacterial lipopolysaccharide (LPS) and single-stranded DNA, respectively, induced autophagy in mouse macrophages (*52, 53*). In these cells, TLR4 activation enhances the interaction of the TLR adaptors MyD88 and Trif with Beclin 1. Consequently, the binding of Beclin 1 by Bcl-2 is reduced leading to an increase in autophagy (*54*). *Pseudomonas aeruginosa* LPS and pili also are able to induce autophagy in macrophages via TLRs (*22*). Other PRRs/PAMP interactions are able to induce autophagy (*55*). For instance, activation of both nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 by NOD-like receptors (NLRs) activates autophagy by recruiting Atg16L1 to the plasma membrane at the entry site of the invading *S. flexneri* and *L. monocytogenes* leading to their efficient sequestration in autophagosomes and subsequent killing (*56*). The bactericidal effect of autophagy in macrophages can be induced by other TLR-activated immune signals including cytokine interferon γ (IFN-γ) (*16*), p47 GTPase (mouse LRG-47 or human IRGM) (*17*), and ATP (via the $P2X_7$ receptor) (*57*). Interestingly, it has been reported that compared with macrophage cell lines the level of autophagy induced by T helper 1 (Th1) cytokine IFN-γ in primary macrophages is lower. This observation is likely due to the inhibition of IFN-γ-induced autophagy by Th2 cytokines interleukin 4 (IL-4) and IL-13 suggesting that Th1-Th2 polarization differentially affects the adaptive immune control of pathogens by fine-tuning autophagic activity (*58*). Taken together, these findings indicate that autophagy in macrophages can be stimulated directly via PRRs and indirectly by specific cytokines induced upon PRR activation.

The second category of virulence factors able to induce autophagy includes certain bacterial toxins. *Helicobacter pylori* vacuolating toxin VacA induces autophagy which in turn has been proposed to serve as a mechanism of protecting infected host cells from VacA toxin-induced cell death (*20*). Induction of autophagy is also observed as a consequence of expression of *Bacillus anthracis* anthrax lethal toxin (LT) (*44*), *Bacillus sphaericus* binary toxin (*59*), as well as some pore forming toxins including *Vibrio cholera* cytolysin (VCC) (*46*), *L. monocytogenes* listeriolysin O (LLO) (*25*), *S. aureus* α-toxin, streptolysin O, and *Escherichia coli* hemolysin (*60*). These pore forming toxins can activate AMP-activated protein kinase, and thereby downregulate target of rapamycin complex 1 (TORC1), a control node in the regulation of starvation-induced autophagy (*60*). It was recently reported that *B. anthracis* Edema toxin and *V. cholera* cholera toxin are able to inhibit antibacterial autophagy in macrophages by stimulating cAMP production (*45*). Because both *B. anthracis* and *V. cholera* each express at least one other toxin, which can induce autophagy, LT and VCC, respectively (*44, 46*), it is suggested that these bacteria use different toxins at different stages of infection to regulate host autophagy (*45*).

The third category of virulence factors able to induce autophagy in macrophages are some effector proteins secreted by the type III (T3SS), or type IV (T4SS) bacterial secretion systems. The *S. typhimurium* (SPI-1) T3SS effectors SipB and SopB are involved in autophagy induction (*30, 31*). The T3SS effector VopQ from *Vibrio parahaemolyticus* induces phosphatidylinositol-3-kinase (PI3K)-independent autophagy to limit phagocytosis (*41*). Increased autophagic activity has also been reported as a consequence of T3SS activity in *S. flexneri* infection (*23*) and of T4SS activity in *L. pneumophila* (*38*) and *C. burnetii* (*61*) infections. Although the mechanism by which *S. flexneri* T3SS function mediates autophagy induction is poorly understood, it is suggested to be linked to the escape of bacteria from the phagosome into the cytosol. An *S. flexneri* T3SS mutant unable to escape from phagosomes does not stimulate autophagy in macrophages (*23*).

RECOGNITION AND MODES OF AUTOPHAGIC ATTACK ON BACTERIA

Following autophagy induction, host recognition of invading bacteria as an autophagy target is another major step in antibacterial autophagy or xenophagy. Xenophagy is regarded as a selective form of autophagy, which relies on specific signals,

Table 1
Interaction between autophagy and bacteria

Bacteria	Host autophagy response	Bacterial response	Reference
Mycobacterium tuberculosis	Bacteria-containing phagosomes are targeted to autophagosomes.	Lipoprotein LpqH activates autophagy via TLR. Eis protein suppresses autophagy.	(*16–19*)
Helicobacter pylori	Infection-induced autophagy targets to bacteria-containing phagosomes.	Vacuolating toxin VacA is required for autophagy induction.	(*20, 21*)
Pseudomonas aeruginosa	Infection-induced autophagy targets to bacteria-containing phagosomes.	*P. aeruginosa* LPS and pili are involved in autophagy induction.	(*22*)
Shigella flexneri	Bacteria-induced autophagy prevents pyroptotic cell death. However, bacteria can block autophagy recognition.	TTSS effector IcsB masks recognition of VirG by Atg5 to avoid anti-microbial autophagy.	(*23, 24*)
Listeria monocytogenes	Cytosolic bacteria are targeted by autophagosomes; however, bacteria are able to avoid autophagic sequestration by recruiting host proteins on their surface.	Listeriolysin O (LLO), phospholipase C, actin polymerization protein ActA, and internalin InlK are involved in autophagy evasion.	(*25–29*)
Salmonella typhimurium	Damaged bacteria-containing phagosomes are targeted by autophagosomes. Ubiquitination, NDP52, and p62 are involved in autophagy recognition.	SPI-1 T3SS effectors SipB and SopB are involved in bacterial invasion, cytoskeletal rearrangement, autophagy induction, and cell death.	(*30–33*)
Burkholderia pseudomallei	Bacteria-containing phagosomes recruit LC3.	T3SS and its effector BopA are required for escape from phagosomes and LC3-associated phagocytosis.	(*34, 35*)
Francisella tularensis	Cytosolic bacteria are targeted to autophagosomes containing MHC class II.	Bacteria downregulate autophagy genes to avoid anti-microbial autophagy.	(*36*)
Yersinia enterocolitica	β1 integrin-dependent engulfment of bacteria is coupled to the activation of anti-microbial autophagy.	Adhesins invasin and YadA induce autophagy; however, bacteria globally suppress autophagy by T3SS effectors.	(*37*)
Legionella pneumophila	Bacteria modify the phagosomes by blocking their maturation via fusion with autophagosomes.	dot/icm T4SS effectors are involved in autophagy induction and inhibition of autophagosome maturation.	(*38*)
Brucella abortus	Bacteria develop autophagosomes without fusion with lysosomes to support their replication.	Two-component regulatory system BvrS-BvrR and T4SS effector VirB are involved in autophagy subversion.	(*39*)
Burkholderia cenocepacia	Bacteria linger in autophagosomes without fusion with the lysosomes.	*B. cenocepacia* downregulate autophagy genes, leading to the deterioration of autophagy.	(*40*)
Vibrio parahaemolyticus	Infection-induced autophagy prevents phagocytosis to benefit bacterial survival.	T3SS effector VopQ is involved in autophagy induction in infected cells.	(*41*)
Anaplasma phagocytophilum	Bacteria replicate in early autophagosomes with blocked maturation.	Unknown.	(*42*)
Yersinia pseudotuberculosis	Bacteria survival in modified autophagosomes without fusion with lysosomes.	Unknown.	(*43*)
Bacillus anthracis	Autophagy targets and degrades anthrax lethal toxin.	Lethal toxin induces autophagy, whereas Edema toxin inhibits it.	(*44, 45*)
Vibrio cholerae	Autophagy targets and degrades cholerae toxin.	Cytolysin induces autophagy, whereas cholerae toxin inhibits it.	(*46, 45*)

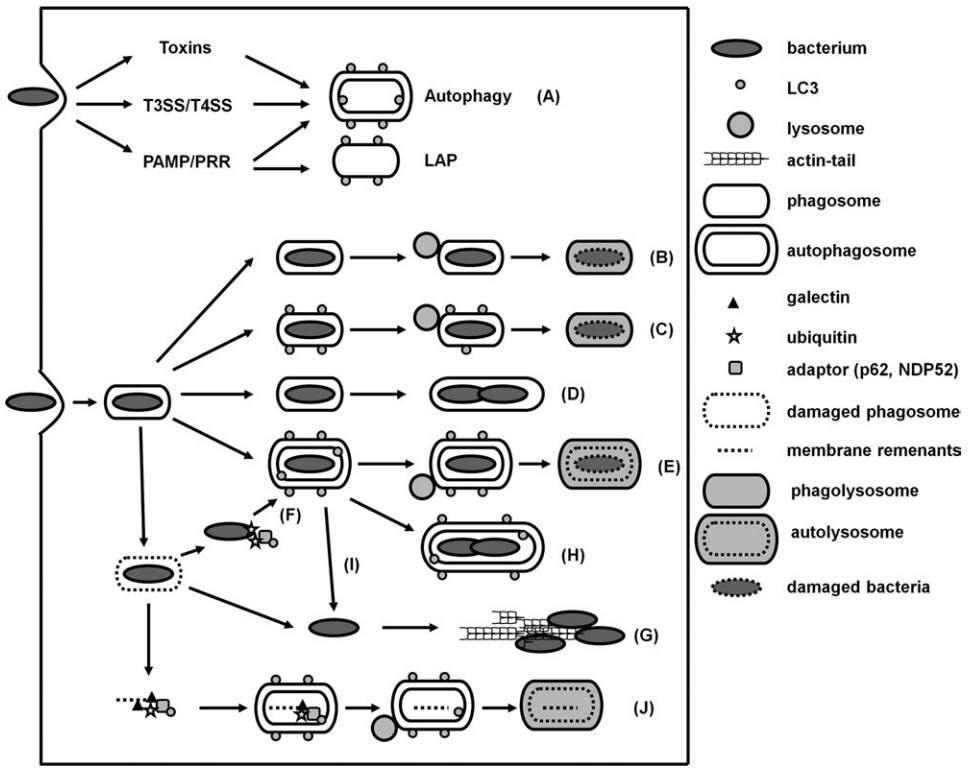

Figure 1. Interactions between intracellular bacteria and components of the autophagic machinery. Host autophagy and/or LC3-associated phagocytosis (LAP) are induced by three categories of bacterial virulence factors (A) including PAMPs (via PRRs such as TLRs), toxins, and T3SS/T4SS effectors. Following autophagy induction bacteria are targeted by a number of different cellular host cell strategies. Bacteria unable to escape phagosomes are degraded by phagocytosis (B), or subjected to LAP requiring recruitment of LC3 to phagosomal membranes (C). Some bacteria survive in phagosomes by blocking phagosome maturation (D). Some bacteria-containing phagosomes are targeted and degraded by canonical autophagy (E). For bacteria that have escaped from phagosomes, they may be recognized by canonical autophagy via ubiquitination and recruitment of adaptor proteins p62 and NDP52 (F), or reside in the cytosol and replicate there (G). Some bacteria entrapped in autophagosomes are able to survive there by blocking autophagosome maturation (H), or even escape into the cytosol (I). The membrane remnants of damaged phagosomes may also be targeted of autophagy via ubiquitination and recruitment of galectins, p62 and NDP52 (J). Examples of bacteria targeted by the strategies described in this figure are: *B. pseudomallei*, (C); *L. pneumopilia* (D); *M. tuberculosis*, (E); *S. typhimurium*, (F); *S. flexneri*, (G); *B. abortus*, (H); *L. monocytogenes*, (I), and *S. typhimurium*, (J).

adaptors, and receptors to deliver cargo into autophagosomes (*8, 9*). Host ubiquitination of targets is a known autophagic signal that together with the adaptor protein p62 facilitates selective autophagy of many substrates including protein aggregates, peroxisomes, mitochondria (*62*), and bactericidal peptides (*63*). Ubiquitination also serves as a signal for recognition of cytosolic bacteria such as *S. typhimurium* (*64*). Following escape from phagosomes to the cytosol bacterial components which remain poorly defined become associated with ubiquitin. Ubiquitin separately recruits the autophagy adaptors p62 and NDP52 (*32*), and in turn LC3 promoting sequestration by autophagosomes (*33, 65*). It was recently reported that membrane remnants of damaged phagosomes from which internalized bacteria including *S. typhimurium*, *L. monocytogenes*, and *S. flexneri* have escaped are also polyubiquitinated following the recruitment of p62 and NDP52 (*66, 67*). These ubiquitinated membrane remnants also recruit the galectins 3, 8, and 9. Galectins, a family of glycan-binding proteins, carry out a range of intracellular and extracellular functions through glycoconjugate-mediated recognition (*68*). Galectin-8 is required for recognition of bacteria by the autophagic machinery suggesting this galectin acts as a general danger sensor to facilitate the elimination of bacteria that escape from the phagosome (*67*).

Autophagy in macrophages targets invading bacteria using a number of different modes (Fig. 1). For bacteria unable to escape phagosomes after infection, such as *M. tuberculosis* and *H. pylori*, bacteria-containing phagosomes are sequestered by or fused with autophagosomes to form double-membrane or multimembrane compartments (Fig. 1, mode E) (*16, 21*). Bacteria such as *S. flexneri* and *L. monocytogenes* that have escaped the

phagosome to the cytosol can be engulfed by autophagosomes directly (Fig. 1, mode F; *23*, *26*). We have reported that *B. pseudomallei* is targeted by a process called LC3-associated phagocytosis (LAP), in which the autophagy-related protein LC3 is recruited directly to single-membrane phagosomes which stimulates phagosome maturation to kill the bacteria (Fig. 1, mode C; *34*). However, the majority of *B. pseudomallei* bacteria can escape to the cytosol from phagosomes, and intriguingly, are resistant to attack from canonical autophagy (Fig. 1, mode G; *34*). The mechanism by which *B. pseudomallei* evades LAP and canonical autophagy is presently under investigation in our laboratory.

LAP was first reported for experiments where macrophages infected with *Saccharomyces cerevisiae* or *E. coli* (*69*). In addition to inducing autophagy, many PAMP ligands such as LPS also induce LAP by triggering receptors such as TLR4, leading to the rapid translocation of Beclin 1 and LC3 to the pathogen-containing phagosomes (*70*). LAP in common with canonical autophagy requires a number of autophagy-related proteins including Beclin 1 and is inhibited by the PI3K inhibitor wortmannin, but lacks the formation of the characteristic double-membrane autophagosomes; accordingly, it is considered a non-canonical form of autophagy (*69, 71*). In another study, it was reported that ULK1, a protein kinase required for canonical autophagy, plays no role in LAP, suggesting that distinct differences exist in regulation of these two pathways (*72*). Although details of the molecular mechanism responsible for triggering LAP remains unclear, one report suggests that it is dependent on the generation of ROS by TLR-activated NOX2 NADPH oxidase (*73*). Proteomic analysis of isolated phagosomal membranes from naïve uninfected macrophages revealed the presence of LC3, the level of which increases in response to starvation-induced autophagy. This result suggests a direct relationship between phagocytosis and autophagy, and possibly a more general role for LAP in the physiology of macrophages (*74*). Indeed, LAP plays an important role in the autoimmune response by efficient phagocytic clearance of apoptotic cells (*72*). A process involving the formation of single-membrane vesicles and recruitment of both Beclin 1 and LC3 was reported to be important in macrophages for engulfment of extracellular fluid in macropinocytosis, and engulfment and clearance of living cells by epithelial cells in a process called entosis (*75*).

Apart from the sequestration of bacteria in autophagosomes via canonical autophagy or in phagosomes via LAP, autophagy plays additional roles in the innate immune system. Host autophagy uses the adaptor protein p62 to deliver ubiquitinated cytosolic proteins to autolysosomes, where they are converted into neo-bactericidal peptides that efficiently kill the entrapped *M. tuberculosis* (*63, 76*). Furthermore, autophagy facilitates adaptive immune responses such as MHC class II antigen presentation (*77*), although this process might be more important in dendritic cells than in macrophages. Taken together, autophagy in macrophages represents a series of defense mechanisms against pathogens in inflammatory process.

BACTERIAL EVASION OF AUTOPHAGY

Some host-adapted intracellular bacteria including *S. flexneri*, *L. monocytogenes*, *B. pseudomallei*, and *S. typhimurium* have developed one or more strategies to avoid entry into the host autophagic pathway (Table 1). These strategies include damage of sequestering (phagosome or autophagosome) membranes, masking to prevent recognition by the autophagy machinery, transcriptional control of ATGs, and formation of replicative niches.

Cytosolic *L. monocytogenes* use three PrfA-regulated virulence factors [listeriolysin O (LLO), phospholipase C (PLC), and actin polymerization protein ActA] to avoid entrapment in autophagosomes (*26*). In this process, LLO and PLC damage the membrane of phagosomes or autophagosomes. Expression of ActA on the bacterial surface recruits the host cell proteins Arp2/3 complex, VASP, and actin, which help prevent marking of the bacteria by ubiquitination and recognition by components of the autophagic pathway (*27, 28*). Other bacterial factors appear to be involved in disguising *L. monocytogenes* from autophagic recognition. Most recently it was reported that *L. monocytogenes* uses internalin InlK to recruit the host major vault protein to the bacterial surface, which prevents their ubiquitination and recognition by p62 (*29*).

The T3SS effector protein IcsB of cytosolic *S. flexneri* competes with Atg5 for binding to the actin polymerization protein IcsA (VirG), and in doing so masks recognition of bacteria by the autophagic machinery (*24*). Additionally, IcsB plays a role in preventing accumulation of ubiquitin proteins and the recruitment of p62 and NDP52 (*78*). Thus, only a small portion of *S. flexneri* in the cytosol are recognized by autophagy via polyubiquitination and p62/NDP52 recruitment in a process that requires IcsA-mediated actin polymerization and the assembly of bacteria-containing cages from septin, a component of the host cytoskeleton (*23, 78*). Other bacteria such as *B. pseudomallei* may be killed by LAP, but can escape into the cytosol, a process facilitated by the T3SS effector BopA (*34*). Once in the cytosol *B. pseudomallei* escape recognition by the autophagic machinery using a mechanism yet to be elucidated (*34, 35*).

Bacteria may also manipulate autophagy regulation at the level of gene transcription (Table 1). For example, cytosolic *Francisella tularensis* can downregulate the transcription of six autophagy related genes including Beclin 1, and as a result evade autophagic killing by the host macrophage (*36*). Similarly, *Burkholderia cenocepacia* are able to significantly reduce autophagic activity in murine macrophages by downregulating the transcription of four ATGs including the gene encoding LC3 (*40*). In *Yersinia enterocolitica*-infected macrophages, $\beta 1$ integrin-dependent engulfment of bacteria is coupled to the anti-microbial autophagy that requires bacterial adhesins invasin and YadA. However, wild-type bacteria are able to globally suppress host autophagy (as assessed by the accumulation of LC3-positive vesicles) via secretion of several T3SS effectors including YopE (*37*). The *M. tuberculosis* lipoprotein LpqH has been shown to be required for activating autophagy via TLR2/

1/CD14 (*18*), whereas another bacterial protein Eis inhibits autophagy through redox-dependent signaling and alterations in gene expression (*19*). The strategy of using toxins produced by a single species to differentially manipulate host autophagy at different stages of infection has also been noted for *B. anthracis* and *V. cholera* (*45*).

Autophagy has a role in the regulation of macrophage cell death (*79*). Accordingly, some bacteria are able to manipulate host cell death pathways through their regulation of autophagy thereby favoring bacterial survival (Table 1). For example, autophagy induction in *S. flexneri*-infected macrophages is negatively regulated by caspase-1 and the NLR Ipaf, and prevents pyroptotic cell death, providing a bacterial replication niche (*23*). Details of the mechanism are not known. Autophagy induced by *V. parahaemolyticus* TTSS effector VopQ has been proposed to inhibit phagocytosis in macrophages by sequestering the necessary membrane components required for phagocytosis, which favors bacterial survival by minimizing the phagocytic uptake of extracellular *V. parahaemolyticus* (*41*).

Unlike *M. tuberculosis* and *H. pylori* that are vulnerable to autolysosomal killing once entrapped in autophagosomes some bacteria enter the autophagic pathway but are able to stall further progress creating an intracellular niche compatible with survival of the pathogen (Table 1) (*80*). *L. pneumophila* (*38*), *B. abortus* (*39*), *C. burnetii* (*61*), *Anaplasma phagocytophilum* (*42*), and *Yersinia pseudotuberculosis* (*43*) are able to modify the autophagosome by preventing acidification and fusion with lysosomes, allowing bacterial replication. Although poorly understood, it has been suggested that this process requires bacterial virulence factors such as the T4SS effector VirB and the two-component regulatory system BvrS-BvrR in *B. abortus* (*39*), the dot/icm T4SS effectors in *L. pneumophila* (*38*), and the T4SS effectors in *C. burnetii* (*61*).

CONCLUDING REMARKS

Recent studies suggest that autophagy in macrophages plays an important role in host immune response against invading bacteria. Host autophagy is induced by various bacterial virulence factors including PAMPs, toxins, and the T3SS/T4SS effectors. Once stimulated, the autophagic machinery then recognizes and targets bacteria in different patterns. In addition to direct killing of bacteria in autophagosomes (xenophagy), autophagy also regulates many immune functions including inflammatory process, phagocytosis (LAP), antigen presentation, and the release of bactericidal factors (ROS, NO). The importance of autophagy in innate immunity makes it a potential drug target for anti-infection treatment. However, it is difficult to determine whether autophagy induction or inhibition favors infection due to the complexity of autophagy-mediated immune regulation and the versatility of bacterial strategies dealing with autophagy. Therefore, more comprehensive investigation, especially through *in vivo* studies, will complete our understanding of the interactions between macrophage autophagy and bacterial infection, and provide better therapeutic strategies against the problematic pathogens.

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