Chapter 4
Autophagy Regulation of Bacterial Pathogen Invasion

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Abstract Autophagy pathway is highly conserved in all eukaryotic species and responsible for targeting of cytosol components, such as protein aggregates, damaged or unnecessary organelles, and intracellular bacterial pathogens for lysosome-dependent degradation. Besides severing as a catabolic process, autophagy pathway furthermore has been discovered to function pivotally in both innate and adaptive immune responses. At present, it has been well demonstrated that certain types of bacteria could be targeted by autophagy upon their invasion. However, several bacterial pathogens have developed strategies to evade this degradation and clearance. Here, we review the role and mechanism of autophagy in the regulation of bacteria invasion, which may facilitate the designing of clinical drugs for efficient and safe cure of infection diseases caused by toxic bacteria.

Keywords Autophagy · Bacteria · Invasion · Degradation · Exnophagy

4.1 Introduction

The autophagy process is highly conserved among eukaryotes from yeast to humans [40] and plays fundamental roles in a variety of both physiological and pathological conditions [13, 31]. In the cell, autophagy pathway selectively targets intracellular pathogens, removes damaged or excessive organelles, and eliminates potentially toxic protein aggregates. Autophagy is functional for the clearance of proteins and other macromolecules for nutrients under starvation conditions. Moreover, autophagy plays fundamental roles in a variety of both physiological and pathological conditions, such as survival during starvation, aging, metabolic diseases, cancer, and neurodegeneration [31]. Furthermore, numerous studies have linked autophagy with

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the innate immune responses including function in regulation of invasion, clearance, tolerance, and inflammation [8]. Autophagy could be effectively activated by immuno-receptors such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) that respond to bacterial pathogens and toxic damage-associated molecular pattern molecules (DAMPs) [6, 66]. Autophagy also functions in adaptive immune reaction by production of antigen peptides that could be presented to T cells through MHC I and MHC II molecules [16]. Furthermore, autophagy is functional for polarization of Th1/Th2 cells and activation of macrophages [67].

Autophagy is initiated at a special region associated with the endoplasmic reticulum (ER), Golgi apparatus, and ER-Golgi intermediate compartment (ERGIC) [21]. The autophagosomes fuse with the lysosomes (in mammalian cells) or the vacuoles (in yeast and plants) for final degradation [37, 64]. The autophagy process involves a group of factors called ATG (autophagy related) proteins [37]. Growth and expansion of the phagophore require class III phosphatidylinositol 3-kinase (PI3 K) complex I that comprises lipid kinase Vps34, regulatory kinase Vps15, Beclin-1/Atg6, and Atg14 [5, 30]. The PI3 K complex generates phosphatidylinositol-3-phosphate (PI3P) from phosphatidylinositol to change the lipid composition of the phagophore [44, 62]. As the only transmembrane protein essential in autophagy, Atg9 traffics and transports membrane components for the growing of phagophore [28]. Upon closing, completed formation of the phagophore, the mature autophagosome moves to, docks on and subsequently fuses with lysosome/vacuole, in a process that is mediated by the SNARE proteins syntaxin-17, SNAP29 and VAMP8 as well as the HOPS complex [68].

Upon first observation, autophagy was thought to be a nonselective degradation process. However, it has been revealed clearly that this process can selectively target protein aggregates (aggrephagy); cellular organelles such as mitochondria (mitophagy), peroxisomes (pexophagy), endoplasmic reticulum (ER-phagy), ribosomes (ribophagy), lipid droplets (lipophagy), and bacterial and virus pathogens (xenophagy) [48, 65]. In host cells, autophagy is an efficient pathway for selective engulfment and degradation of bacterial pathogens. Here, we will review generally and briefly the function of autophagy in bacterial invasion.

4.2 Autophagosome Scaffold LC3 as a Platform for Receptor-Bacteria Recruitment

Autophagy is a very evolutionarily conserved degradation pathway for lysosomal degradation of long-lived proteins, big aggregates, and whole organelles. Autophagy in yeast is usually kept at a very low level in rich medium culture condition and is dramatically induced under starvation or rapamycin treatment. Whereas in mammalian cells, autophagy level is different depending on cell background, although in most cases it is constitutively activated [7, 11, 19].

Autophagy process begins from the formation of a double-membrane vesicle called autophagosome, where LC3 is conjugated to engulf cytosolic cargoes [40].

Autophagosome grows from a single spot called phagophore assembly site (PAS), where the following steps of initiation, nucleation, elongation, and finally the closure of the cup-shaped double-layer membrane, phagophore, happen successively [50]. Genetic screens led to the identification of over 40 ATG genes function in different steps in autophagosome formation [58]. Initiation of autophagy is regulated by the Atg1/Atg13/Atg17 kinase complex, which is inhibited by target of rapamycin (TOR) kinase at rich medium culture condition. The next step, nucleation of phagophore at PAS is controlled by a lipid kinase complex containing Vps34, regulatory subunits Atg14, Atg6/Vps30, and Vps15. The following elongation step is controlled by Atg9, the only transmembrane protein in autophagy pathway that provides lipid membrane for the expanding phagophore through shuttling between various vesicle compartments and PAS depending on Atg1 and Vps34. Besides Atg9, two highly conserved ubiquitin-like protein (Atg12 and Atg8) conjugation systems, Atg12–Atg5 and Atg8-phosphatidylethanolamine (PE), also contribute to this process [46]. Ubiquitin is a small protein containing 76 amino acids and is highly conserved from yeast to human. It is tightly folded to form a globular structure composed of five-stranded beta-sheet wrapped surrounding a central helix. Ubiquitin is synthesized first as a precursor protein. Subsequent proteolytic cleavage exposes its active C-terminal glycine amino acid, which allows ubiquitin to be conjugated to a lysine (or N-terminal methionine) in the substrate protein or in the first ubiquitin moiety. A cascade of catalytic enzymes involving activating (E1), conjugating (E2) and ligating (E3) enzymes, generate ubiquitin conjugates containing either multiple mono-ubiquitin or poly-ubiquitin chains (mostly Lys48, Lys63, or linear). Diverse types of ubiquitin modification confer diverse functions, such as regulation of cytoplasm membrane receptor endocytosis, targeting proteins for degradation or functioning in signalling complex assembly [20]. Proteins containing ubiquitin-binding domains (UBDs) can act as ubiquitin receptors through interaction with ubiquitin non-covalently. The ubiquitin conjugates can be reversed by a large class of de-ubiquitinating proteases (DUBs) that cleave the ubiquitin moieties from their substrates. Atg12 was the first ubiquitin-like protein (UBL) to be identified in autophagy pathway [54]. Different from ubiquitin, it is synthesized as a C-terminal glycine-exposed form. Autophagy core protein Atg7 (E1) directly transfers Atg12 onto Atg10 (E2) in a cascade manner. Atg12 is finally conjugated to Atg5. The Atg12–Atg5 conjugate then recruits factors important for phagophore elongation and closure. The Atg12–Atg5 conjugate works together with dimerized coiled-coil protein Atg16, which promotes the association of this conjugate with PAS and phagophore elongation. The main role of Atg12–Atg5–Atg16 complex functions as E3 for the LC3-PE conjugation. LC3, another UBL functions in autophagy, similar to ubiquitin, is synthesized as a precursor and cleaved by cysteine protease Atg4. Matured form of LC3 with exposed C-terminal glycine is activated by and conjugated to Atg7 (E1), transferred to Atg3 (E2) and then finally conjugated to PE lipid incorporated in phagophore with the help of Atg12–Atg5 conjugate. Conjugation of LC3 to phagophore is essential for expansion and also important to function as a platform for recruitment of cargoes, which is mediated by autophagy receptors [2].

4.3 Autophagy Receptors for Degradation of Bacterial Pathogens

Autophagy was first considered to be a bulk degradation pathway, but it is now accepted that many autophagy receptors exist for recognition of distinct cargo substrates. Based on the category of cargoes, several types of selective autophagy have been found: aggrephagy (clearance of protein aggregates, mitophagy (clearance of damaged mitochondria), ribophagy (clearance of excessive ribosomes), xenophagy (clearance of invading pathogens), pexophagy (clearance of peroxisomes), ER-phagy (clearance of endoplasmic reticulum), nucleophagy (clearance of nuclear envelope), lipophagy (clearance of liposomes) [1]. Selective autophagy mediated by different receptors plays an important role in maintaining intracellular homeostasis.

How is it achieved for selectively targeting different cargos (such as pathogenic bacteria) by autophagy? One way is to tag the invading bacteria by ubiquitin chains through different ubiquitin ligases. After the cargo bacteria get ubiquitinated, they are recognized by several autophagy receptors. These receptor proteins serve as a bridge between the cargo bacteria and LC3 on the membranes of the nascent autophagosomes. Thus, these receptor proteins share three common feature domains: LC3 interacting region (LIR) domain, oligomerization domain and ubiquitin moiety binding domain [4, 41]. At present, there are at least four key autophagy receptors including p62, NDP52, OPTN, and TAX1BP1. p62, also called SQSTM1, is the first molecule identified to function as a mammalian autophagy receptor. Initially, p62 is implied for the function in selective autophagy degradation of ubiquitinated protein aggregates. p62 interacts with several ubiquitin ligases such as TRIM50, TRAF6, and MURF2 that ubiquitinate substrates of p62 [52]. Notably, p62 also functions in pexophagy and mitophagy. The role of p62 in antibacterial autophagy was first explored in eliminating the invading *Salmonella Typhimurium* [69]. Soon later, several other types of bacterial pathogens including *Shigella flexneri* and *Mycobacterium tuberculosis* were also found to be subject to selective autophagy mediated by p62 [18]. It has been found that p62 co-localizes with *M. tuberculosis* in host cells after its invasion and controls its survival and replication in macrophages. Consistently, knockdown of p62 upregulates the invasion and survival of infected *M. tuberculosis* in macrophages. Besides, p62 also exerts a role in anti-inflammation through suppressing inflammatory responses induced by globular adiponectin [60]. In addition to p62 itself, its modulating proteins are also found to be involved in xenophagy. For insistence, TBK1 kinase can stimulate p62 function in bacterial autophagy through phosphorylation of Serine 403 at the UBA domain of p62. Such phosphorylation effectively increases the function of p62 for clearance of *M. tuberculosis*. p62 is also shown to be activated through TAK1-mediated phosphorylation, which promotes the binding of p62 with Keap-1 and finally inhibits inflammatory reactions induced by TLR, NLR, or IL-1 cytokines [24].

NDP52 is an important autophagy receptor protein initially found in mitophagy to maintain cell health by clearance of damaged mitochondria [26]. It was also found to function in the regulation of bacterial invasion. NDP52 could transfer different

types of bacterial pathogens such as *Streptococcus pyogenes*, *Salmonella enterica*, and *S. flexneri* by autophagy for their selective degradation [36].

Because of the redundancy of receptors, more than one receptor can target the same bacteria to autophagosomes. It has been found that p62 and NDP52 together target *Shigella* to autophagosomes, while p62 and NDP52 are recruited separately to *Listeria* [24]. NDP52 could interact with all the human LC3 orthologs with a preference for LC3C by its noncanonical LIR (CLIR) domain in antibacterial autophagy function. Ubiquitin ligase Parkin and TBK1 modify the function of NDP52 in bacterial autophagy [26]. GTPase protein Rab35 has been found to control Group A Streptococcus (GAS) degradation by autophagy through binding with NDP52 [36]. Besides, NDP52 also functions in the downregulation of inflammation by inhibiting the NF-κB signalling pathway. Optineurin (OPTN) is a 67 kDa size protein functioning in various tissues and has several domains including C-terminal zinc-finger, leucine zipper domain, an LIR domain, ubiquitin-binding UBAN domain, and coiled-coil motifs that mediate its oligomerization [57]. OPTN is found to function as autophagy receptor in mitophagy, aggrephagy, and xenophagy. Studies have found that OPTN can restrict the growth of *S. enterica* upon invasion [32]. Similarly, TBK1 phosphorylates OPTN within its LIR domain at Ser-177 and regulates its activity in selective autophagy. Besides, OPTN can inhibit inflammation by negatively regulating NF-κB signalling pathway [57]. In addition, OPTN also reduces ER-Stress in intestinal cell by targeting IRE1-α for degradation, which inhibits the ER based inflammation response [36]. On the other hand, OPTN mediates IRF3 activation, which results in type I IFN production for bacterial clearance [56]. TAX1BP1, also called CALCOCO3, is a closely related paralog of NDP52. Its function in xenophagy was first demonstrated by its involvement in the autophagic clearance of *S. typhimurium* [61]. The removal of the bacteria relied on the binding of TAX1BP1 to myosin motor VI that functions in the fusion of autophagosomes with lysosomes [47]. TAX1BP1 overexpression in the heart alleviates inflammatory reaction, oxidative stress, and cell apoptosis in Streptozotocin (STZ)-infection mouse models. It has been shown that TAX1BP1 can interact with MAVS virus, which induces the recruitment of ubiquitin ligase Itch to MAVS for its ubiquitination and degradation leading to restricted cell apoptosis [9].

4.4 Bacterial Pathogens that are Regulated by Autophagy upon Invasion

Although the exact mechanism of bacterial recognition by autophagy has not been discovered clearly, it is known that these recognition processes depend on the ubiquitination of the substrates [45]. Autophagy receptors including p62, NBR1, NDP52, OPTN, and TAX1BP1 mentioned above are a subset of pattern recognition receptors (PRRs). These receptors target ubiquitinated substrates to autophagosomes through binding LC3 [12]. At present, it has become clear that autophagy has a crucial role in the elimination of many types of pathogens [23].

The capacity to degrade bacteria by autophagy was first demonstrated in GAS. In 2004, it was found that in GAS-infected HeLa cells, almost all of the bacteria were recruited into autophagosomes. When autophagy was blocked in ATG5$^{-/-}$ cells, the bacteria survived within the cells [38]. Interestingly, upon invasion, the CD46 receptor could induce autophagy and GAS elimination by activating BECN1 and PI3 K complex [55]. Upon invasion, bacteria are first targeted by endosomes, several Rab GTPase family members that are found both in endocytosis and autophagy are found to be involved in bacterial autophagy, such as Rab7, Rab23, and Rab9A [42]. Several studies have shown that *S. typhimurium* is also a substrate of the autophagy pathway. *S. typhimurium* is an intracellular bacterium that usually resides in a *Salmonella*-containing vacuole (SCV) after invasion. In the cytosol, *S. typhimurium* is coated with ubiquitinated proteins detected and bound by p62, which co-localizes with LC3 and LAMP1 [59]. OPTN also plays an important role in the elimination of *S. typhimurium*. The kinase TBK1, which functions in activation of the transcription of type I interferons, could phosphorylate Serine177 of OPTN, thus enhances the affinity of LC3 binding with OPTN. *M. tuberculosis* usually infects and survives in human alveolar macrophages. Upon invasion, *M. tuberculosis* could arrest phagosome maturation and phagolysosomal fusion, which inhibits the processing and presentation of bacterial antigens. In 2004, studies showed that autophagy could efficiently inhibit the replication of *M. tuberculosis* in macrophages through elimination [23]. Interestingly, it has been found that vitamin D, or 1, 25-dihydroxy vitamin D (1,25D3), induces autophagy in human monocytes via the transcription of *BECN1*. Recently, it was found that upon invasion, *M. tuberculosis* containing phagosomes were highly labeled by LC3 and ATG12 [63]. Other than regulation of bacteria by autophagy, certain bacteria could also inhibit autophagy. For example, *Legionella pneumophila* could enhance the secretion of autophagosomes. Upon invasion, *Legionella* is internalized into a phagosome, then autophagy proteins such as ATG7 and LC3 are recruited and eventually facilitate the degradation of bacteria in lysosomes. *L. pneumophila* could evade autophagy by the secreted effector protein RavZ. RavZ is an ATG4-like cysteine protease that could hydrolyze the amide bond at the C-terminal of LC3 that is conjugated to phosphatidylethanolamine (PE) [10]. Beside of *L. pneumophila*, *S. flexneri* is also found to interfere with autophagy pathway. As a gram-negative pathogen, *S. flexneri* can escape from endosome upon invasion and transfer into cellular cytosol. *S. flexneri* then secrets toxin factors such as IcsB and IcsA (VirG) that can reduce binding of autophagy components like ATG5, which eventually inhibits the recognition of bacteria by the autophagy [43]. *Listeria monocytogenes* is an example of bacterial pathogen that can evade recognition by autophagy upon invasion. At the first phase of infection by *Listeria*, autophagy functions as host immune defense. In ATG5-deficient host cells, comparing to wild-type cells, *L. monocytogenes* rapidly replicates in mouse embryonic fibroblasts, which suggests a pivotal role for autophagy [49]. *Listeria* uses its surface-expressed ActA and InIK proteins to prevent its ubiquitination and recruitment of autophagy receptors such as p62 and NDP52 [15]. There are also certain bacteria that could exploit

autophagic vacuole for multiplication, such as *Coxiella burnetiid* and *Porphyromonas gingivalis*, two types of bacterial pathogens that are associated with cardiovascular diseases [14, 51].

4.5 LC3-Associated Phagocytosis

Recently, a pathway named as LC3-associated phagocytosis (LAP) has been found for the function of autophagy induced by bacterial invasion (Fig. 4.1). The detailed mechanism of the LAP has not been completely understood yet. What is known at present is that LAP uses several common components of the autophagy machinery especially the LC3 conjugation system. For instance, the ubiquitin-like reaction systems for conjugation of LC3 to the membrane lipid PE, ATG12 conjugation system consists of ATG7 (E1-like) and ATG10 (E2-like), the LC3 conjugation system of ATG7 (E1-like), ATG3 (E2-like) and a complex of ATG16L1, ATG5, and ATG12 (E3-like as a complex), have been found to be similarly needed for LAP and canonical autophagy [34]. However, there are also some differences in the molecular machinery between LAP and canonical autophagy. For example, canonical autophagy is induced by the upstream kinases mTORC1 and AMPK by activating the initiation complex composed of ULKs, FIP200, ATG13, and ATG101 [40]. By contrast, LAP is induced with no necessity of this initiation complex [34]. Instead, LAP is induced by cellular surface receptors such as TLRs, Dectin-1, Dectin-2, and Mac-1/CR3/integrin αmβ2

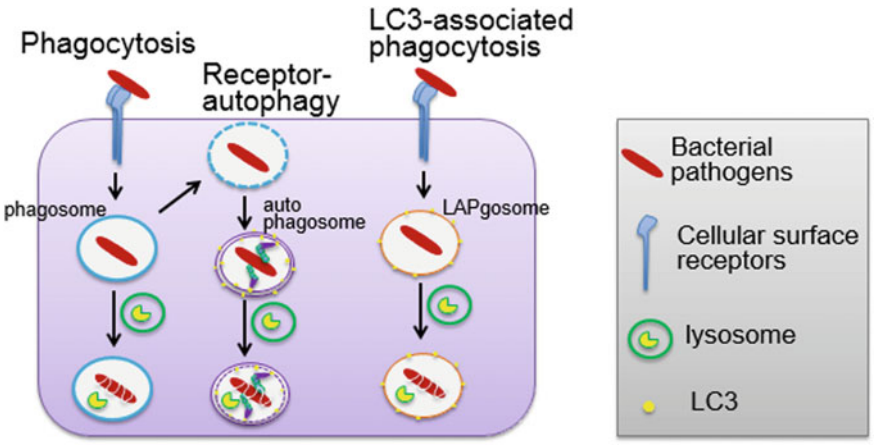

Fig. 4.1 Schematic overview of phagocytosis, canonical autophagy and LC3-associated phagocytosis upon the invasion of bacterial pathogens. Left, upon invasion, extracellular bacteria are recognized by specific surface receptors for phagocytosis. Middle, when pathogens evade from phagocytosis, they are modified by ubiquitination and recognized by autophagy receptors for degradation. Right, a noncanonical autophagy pathway called LC3-associated phagocytosis referred as LAPosomes, during which LC3 can be recruited to phagosomes directly

[25]. Both initiation complex and surface receptor activation (during LAP) can induce generation of the membrane phosphor-lipid PI3P at the target sites, which is mediated by class III PI(3)K complexes (PI3 KC3) [3]. PI3 KC3 contains the core components VPS34, VPS15, Beclin-1, and different Beclin-1 binding proteins. The LAP associated PI3KC3 contains UVRAG and Rubicon which is essential for PI3P formation during LAP but negatively regulates canonical autophagy [29]. In the process of canonical autophagy, a complex containing WIPI and ATG2 bind to the PI3P generated in the target site of membranes. The WIPI-ATG2 complex then binds to PI3P and ATG16L1, which helps recruit the LC3 conjugation systems, resulting in catalytic linkage LC3 to the target membrane. For LAP pathway, ATG16L1 recruitment to the PI3P-containing target membrane sites is also essential. The second big difference in LAP and autophagy induction is that phagocyte oxidase Nox2 generated reactive oxygen species (ROS) is specifically needed for LAP but not for autophagy [17]. To activate the production of ROS, Nox2 forms a catalytic complex with the cytosolic subunits p67, p47, p40, and Rac1/2. Rubicon upregulates Nox2 activities [34]. At the moment, how Nox2-derived ROS stimulates LAP is not clear at all.

The main function of the LAP pathway is to directly promote the fusion of phagosomes with lysosomes for quick degradation of the cargo. For example, fusion of phagosomes containing microbes like *Aspergillus fumigatus*, *Legionella dumoffii*, and *L. monocytogenes* were efficiently targeted to lysosomes by LAP pathway [22]. Additionally, LAP also enhances the fusion with lysosomes of dead cells containing phagosomal vesicles [33]. Moreover, LAP can also delay phagosome maturation, which then results in enhanced antigen presentation by MHC II [53]. The molecular mechanisms how LAP enhances its fusion with lysosomes are not well understood. The fusion of vesicles with lysosomes relies on a number of factors such as membrane lipid composition factors, combining machinery components Rab7, RILP, PLEKHM1, and the HOPS-SNARE complex [39]. At present, the specific composition of membrane lipid of LAPosome is totally unknown. For fusion of LAPosomes with lysosomes, LC3 proteins like the GABARAP family have been found to directly bind and recruit PLEKHM1 which is an adapter protein needed for Rab7 and HOPS complex recruitment to autophagosomes [35]. Another mechanism is that LAP can promote phagosome fusion with lysosomes through enhancement of specific SNARE complex. The SNARE complex mediating fusion of phagosomes with lysosomes is composed of STX17, SNAP29, VTI1b, and lysosomal membrane anchored VAMP8 [27]. The detail and clear verification of function LAP need further molecular and genetic studies.

4.6 Conclusion

Upon invasion of bacterial pathogens, autophagy, both canonical and noncanonical autophagy pathways are stimulated react for defense. The pathogens are eventually eliminated in lysosomes transferred by autophagy process. Sensing the bacterial pathogens upon invasion induces autophagy, especially noncanonical autophagy

LAP, which is a quick and direct way from invasion to lysosomal degradation. The escaped pathogens are again selectively targeted by autophagy through different bridging receptors. Autophagy is also activated by pathogen invasion, to sequester, degrade, and present antigens to host cells, leading to activated immune response against invading bacteria. However, there are also harmful effects conferred by autophagy on host cells. Through specific virulence factors, invading pathogens can evade or inhibit autophagy, or even take advantage of autophagosome for proliferation. Another thing is that in certain situations, over-enhanced autophagy activity causes excess immune response and inflammation. Thus, autophagy plays an important role in invaded host cells for the appropriate immune response to bacterial pathogens, and clinical drugs are then expected based on autophagy for treatments of various infectious diseases caused by bacterial pathogens.

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