Xenophagy of invasive bacteria is differentially activated and modulated via a TLR-TRAF6-Beclin1 axis in echinoderms

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In marine environments, organisms are confronted with numerous microbial challenges, although the differential regulation of xenophagy in response to different pathogenic bacterial species remains relatively unknown. Here, we addressed this issue using A. japonicus as a model. We identified 39 conserved autophagy-related genes by genome-wide screening, which provided a molecular basis for autophagy regulation in sea cucumbers. Furthermore, xenophagy of two Gram-negative bacteria, Vibrio splendidus and Escherichia coli, but not a Gram-positive bacteria, Micrococcus luteus, was observed in different autophagy assays. Surprisingly, a significantly higher autophagy capacity was found in the E. coli–challenged group than in the V. splendidus–challenged group. To confirm these findings, two different lipopolysaccharides, LPS V. splendidus and LPS E. coli, were isolated; we found that these LPS species differentially activated coelomocyte xenophagy. To explore the molecular mechanism mediating differential levels of xenophagy, we used an siRNA knockdown assay and confirmed that LPS V. splendidus–mediated xenophagy was dependent on an A/JTLR3-mediated pathway, whereas LPS E. coli–mediated xenophagy was dependent on A/JToll. Moreover, the activation of different A/TLRs resulted in A/JTRAF6 ubiquitination and subsequent activation of K63-linked ubiquitination of A/Beclin1. Inversely, the LPS V. splendidus–induced A/JTLR3 pathway simultaneously activated the expression of A/JA20, which reduced the extent of K63-linked ubiquitination of A/Beclin1 and impaired the induction of autophagy; however, this finding was not evident with LPS E. coli. Our present results provide the first evidence showing that xenophagy could be differentially induced by different bacterial species to yield differential autophagy levels in echinoderms.

Autophagy is a highly conserved and mostly selective intracellular degradation pathway in eukaryotes that is mainly involved in the regulation of essential physiological processes (1–3). For autophagy activation, a portion of the cytoplasmic component is first surrounded by a cup-shaped structure called the phagophore (4, 5), and this phagophore then extends, closes, and forms a double-membrane autophagosome (6). Subsequently, autophagosomes fuse with lysosomes (in metazoan cells) or vacuoles (in yeast and plant cells) to degrade cytoplasm-derived materials by lysosomal/vacuolar hydrolases. Various autophagy-related genes and multiple signaling pathways are involved in these processes (7). Among these core sets of proteins, LC3/Atg8 are widely adopted as molecular markers to determine the occurrence of autophagy (8). Recent studies have shown that autophagy can selectively degrade specific cargoes via processes referred to as “selective autophagy,” which aim to maintain cellular homeostasis, regulate inflammatory responses, and eliminate invasive pathogens (9, 10). Special terms have been coined to describe selective autophagy according to the types of targeted cargoes, and these terms include mitophagy (mitochondria), pexophagy (peroxisomes), lipophagy (lipid droplets), aggrephagy (protein aggregates), and xenophagy (invading microorganisms) (10).

Xenophagy, a unique type of selective autophagy, has received extensive attention and plays a central role in innate immunity by targeting foreign entities, such as viruses, bacteria, and parasites and protecting host cells from fatal damage (11). In mammals, different types of pathogenic bacteria, including Salmonella enterica (12), Listeria monocytogenes (13), Shigella flexneri (14), and Helicobacter flexneri (15), distinctly lose the “battle” in different models and reportedly induce xenophagy. Interestingly, nearly all invading microbe-induced xenophagy is dependent on an interaction between the bacterial cell wall components and pattern recognition receptors (PRRs) in host cells, which provides the missing link between pathogen recognition and the initiation of autophagy (16–18). For instance, Khan et al. (19) demonstrated that two types of scavenger receptors, macrophage receptor with collagenous structure (MARCO) and scavenger receptor-B1 (SR-B1), in human mesenchymal stem cells (MSCs) could mediate xenophagy and enhance the intracellular killing of the pathogen by binding to lipoglycans from Mycobacterium tuberculosis. Travassos et al. (20) found that nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 of the family of NOD-like receptors (NLRs) in human epithelial HeLa cells activate xenophagy after specifically binding to peptidoglycan (PGN) from S. flexneri and L. monocytogenes. Toll/Toll-

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like receptors (TLRs) are considered the most important PRRs in mediating xenophagy. Delgado et al. (18) screened a pathogen-associated molecular pattern (PAMP) ligand library for effects on xenophagy in murine macrophage cell lines and found that different TLRs could identify different ligands from bacteria or viruses (i.e., TLR3 can identify polyinosinic-polycytidylic acid [poly(I:C)], TLR2 can identify zymosan, and TLR4 can identify LPS) to active xenophagy. However, lipopeptides such as Pam3CSK4 (TLR1/2 ligands), flagellin (TLR5 ligand), or CpG oligonucleotides (TLR9 ligand) cannot induce xenophagy. Moreover, TLR ligands induce xenophagy in a mainly cell-type-dependent manner, and the degradation mechanism of the cargoes might also differ among various species. For instance, single-stranded RNA (ssRNA) serves as a ligand for murine TLR7 and human TLR8 but is not recognized by human TLR7 (21) or murine TLR8 (22) in macrophages; thus, ssRNA cannot induce autophagy. In contrast, the same ligands from different bacteria might bind to different receptors to mediate xenophagy. In Drosophila primary hemocytes and S2 cells, two types of PGNs derived from different types of cells, DAP-type (from Lactobacillus plantarum) and lysine-type (from Staphylococcus epidermidis), can induce autophagy, but the receptor of peptidoglycan-recognition protein-LE (PGRP-LE) was responsible only for the induction of autophagy stimulated by DAP-type PGN, which suggests that cytoplasmic sensors other than PGRP-LE detect invading bacteria with cell walls containing lysine-type PGN (23, 24). Although the fundamental mechanism linking one pathogen with the autophagy machinery has been well studied, the mechanism through which host cells use distinct sensors to induce xenophagy in response to two or more microbes is largely unknown.

After the specific binding between PAMPs from microbes and PRRs from the host, the ubiquitination of some key autophagy-related proteins and cargoes is an important step for the formation of autophagosomes and subsequent autophagic degradation via xenophagy (7). TRAF6, as an E3 ubiquitin ligase, is widely involved in autophagy induction due to its autoubiquitination and heteroubiquitination activities (25). The stimulation of TLR4 and TLR3 with LPS and poly(I:C), respectively, triggers autophagy in lung cancer cells, which enhances the production of various cytokines by promoting TRAF6 ubiquitination and thus facilitates immune defense against pathogens (26). Moreover, accumulating evidence indicates that Beclin1 is the principal substrate of the ubiquitin ligases that regulate the autophagy machinery (27). K63-ubiquitination of Beclin1 promotes autophagy induction (28) but K48-linked ubiquitin to induce a negative pathway for autophagy regulation (29). Upon TLR engagement, TRAF6 promotes the K63-linked ubiquitination of Beclin1, Beclin1 is a core component of the class III phosphatidylinositol 3-kinase complex (PI3KC3), and the K63-linked ubiquitination of Beclin1 modulates the lipid kinase activity of PI3KC3 and thereby induces autophagy (25, 30). More importantly, the engagement of TLRs also triggers a signaling pathway that leads to the translocation of NF-κB to the nucleus and promotes the expression of A2O. A2O contains an N-terminal ovarian tumor (OTU) deubiquitinase domain that removes K63-polyubiquitin chains from TRAF6 to turn off the activation of autophagy (25, 31, 32), which indicates that A2O negatively regulates TLR-induced autophagy in human cells.

Aquatic organisms are confronted with numerous microbial challenges in their living environments, including pathogenic, nonpathogenic, and beneficial challenges. Despite the importance of xenophagy in innate immunity, which directly captures pathogens or indirectly mediates immune responses to control foreign microbe infection (16, 33), the mechanism regulating xenophagy in response to different bacterial infections in the same species is largely unknown. To address this knowledge gap between xenophagy and host innate immune responses in aquatic organisms, we used Apostichopus japonicus, an economically important marine species, as a model. The sea cucumber A. japonicus is a marine invertebrate belonging to the Echinodermata and widely distributed in China, Korea, and other Asian countries. These species play important roles in ecology and aquaculture, they provide commodity or medicine valued by humans, including food resources (34). In China, the gross production of A. japonicus reached as high as 196,564 tons in 2020 and obtained remarkable economic. Moreover, they occupy a taxonomic position that is believed to be important for understanding the origin and evolution of deuterostomes. Considering the important role of xenophagy in resisting microorganisms, we decided to investigate the different bacteria in the regulation of sea cucumber autophagy. In this study, we confirmed that two Gram-negative bacteria, Vibrio splendidus and Escherichia coli, showed differential capacity to regulate xenophagy via different TLR cascades, whereas the Gram-positive bacterium Micrococcus luteus could not activate autophagy activity. The molecular basis for the different autophagy levels induced by the two Gram-negative bacteria was also elucidated. The findings obtained in this study will provide valuable insights into the autophagy mechanisms through which xenophagy and innate immunity pathways intersect and their contribution to cell survival in marine invertebrate species.

Results

Sea cucumbers have a complete autophagy machinery

The occurrence of autophagy in vertebrates involves four main successive steps, namely autophagy initiation, phagophore elongation, autophagosome formation, and lysosome fusion, and many key proteins and adaptors are involved in these processes (35). In our previous study, we found that the mRNA expression of several autophagy-related genes, such as AjULK, AjAtg13, AjBeclin1, and AjLC3, was significantly changed after V. splendidus challenge, which indicates that sea cucumbers might regulate autophagy-related genes and activate autophagy to resist pathogen invasion (36). However, whether the complete machinery needed for the autophagy process exists in A. japonicus is largely unknown. To understand whether sea cucumber has the molecular basis for the autophagy machinery, key proteins encoding genes involved in autophagy regulation were screened from sea cucumber
transcriptome (37) and genomes (38, 39), and the sequences were used for in silico analysis and sequence alignment. In our study, we ultimately identified 39 Atgs in A. japonicus that belong to the five complexes implicated in the four major steps, namely initiation, elongation, completion, and fusion of the autophagosome (Table S1). The results in Figure 1 show the key proteins involved in the core molecular mechanism of autophagy in A. japonicus, and their amino acid sequences were conserved compared with those of Homo sapiens, Mus musculus, Danio rerio, and Strongylocentrotus purpuratus. Among these autophagy-related proteins, LC3 plays an important role in the elongation of the autophagosome because its cleavage and its lipidation protein product (LC3-II) decorate autophagosomes. Animal LC3 proteins comprise two subfamilies: microtubule-associated protein 1 light chain 3 (LC3A/B/C), gamma-aminobutyric acid receptor–associated protein (GABARAP)/Golgi-associated ATPase enhancer of 16 kDa (GATE16) (8). Our sequence data for sea cucumber included four homologous genes, AjLC3, AjLC3C, AjGABARAP, and AjGABARAPL2 (Table S1), and we found that all four proteins contain an ATG8 domain and a highly conserved glycine residue in C-terminal, which suggests that the sea cucumber LC3 conjugation system might have a function similar to that of other conserved LC3 proteins in eukaryotes (Fig. 1 and Table S1). The sequence alignment revealed that AjLC3 exhibited 70% identity with H. sapiens LC3B, and AjLC3C exhibited 56.7% identity with H. sapiens LC3C. In addition, autophagy receptors initiate isolation membrane formation by bridging ubiquitinated substrates and LC3 via their ubiquitin-binding region (UBR) and ATG8/LC3-interacting region (LIR), respectively. In our case, the two primary receptors Ajp62/SQSTM1 and AjNBR1 contained a UBR domain with an LIR motif at the C terminus (Fig. 1). In general, these results indicated that sea cucumbers possess the complete autophagy machinery and could be able to respond to bacterial infection.

Autophagy is activated by challenge with two Gram-negative bacteria of V. splendidus and E. coli but not a Gram-positive bacteria of M. luteus

Selective autophagy is a homeostatic regulation system that can specifically recognize substrates and play an antibacterial role in the immune response of the host (40). However, the xenophagy mechanism in response to other specific microbes found in aquatic environments is relatively unknown. Therefore, sea cucumber coelomocyte was challenged with three types of bacteria, V. splendidus, E. coli, and M. luteus, and the autophagy levels were detected by transmission electron microscopy (TEM). At 24 h after introduction of three bacteria into sea cucumbers, we observed double-membraned autophagosomes enclosed V. splendidus and E. coli within coelomocyte under Bafilomycin A1 (Baf-A1) treatment, but not in M. luteus–challenged group (Fig. 2A). The percentage of

Figure 1. Complete autophagy machinery of A. japonicus according to in silico analysis. Sea cucumbers contain the main processes of autophagy, namely autophagy initiation, phagophore elongation, autophagosome formation, and lysosome fusion, and the presence of all processes guarantees the integrity of the autophagy machinery and the accomplishment of xenophagy. The domains of 13 key proteins were detected using the SMART program and compared with those of Homo sapiens, Mus musculus, Danio rerio, and Strongylocentrotus purpuratus.
Figure 2. Characterization of A. japonicus coelomocyte xenophagy after challenge with V. splendidus, E. coli, and M. luteus for 24 h. A, TEM detection after V. splendidus and E. coli injection following with 10 nM Baf-A1 treatment, the red arrows indicated the double-membraned autophagosomes surrounding bacteria; B, LC3 fluorescence intensity detection without CQ or Baf-A1 injection; C, LC3 fluorescence intensity detection with CQ injection; D, LC3-positive autophagosomes colocalized with lysosomes with CQ injection. E, LC3-positive autophagosomes colocalized with lysosomes with Baf-A1 injection. After challenge, the cells were fixed and stained with anti-LC3 or combined with anti-LAMP antibodies at the indicated time point of 24 h. After nuclear staining with DAPI, green and red signals that represent autophagosomes and lysosomes, respectively, were visualized under a confocal microscope and statistically analyzed; scale bar = 5 μm. The relative LC3 positivity in 1000 cells from each indicated sample was determined. The data are presented as the means ± SDs (n = 3) relative to the control (0 h), are shown in bar graphs (lower panel in D and E). Asterisks indicate significant differences compared with the control group: *p < 0.05 and **p < 0.01 (t test).
intracellular bacteria in autophagosomes reached 7.8 ± 1.8% in V. splendidus group and 10.2 ± 2.7% in E. coli group when compared with control (0 h) group. Furthermore, Western blotting analysis indicated that the AjLC3-II/I level was significantly increased at 12 (1.41-fold, p < 0.01) and 24 h (1.67-fold, p < 0.01 and 2.22-fold, p < 0.01) after challenge with V. splendidus and E. coli compared with the level in the 0 h (control) (Fig. 3, B and C), but M. luteus did not trigger an elevation in the lipidation of AjLC3 (Fig. 3A). An autophagy receptor protein of Ajp62 exhibited significant decreases at 24 h post E. coli (0.69-fold, p < 0.01) and V. splendidus (0.76-fold, p < 0.05) challenge compared with the level in the 0 h (Fig. 3, B and C), but this change was not detected in M. luteus–challenged sea cucumber coelomocyte (Fig. 3A). Moreover, we found that the AjLC3-II/I ratio in three bacteria challenged groups was significantly increases for Baf-A1 treatments, and the Ajp62 proteins were also measured and showed increased levels for Baf-A1 (Fig. 3). Besides, an immunofluorescence analysis indicated a significant increase in LC3 green fluorescence at 24 h after V. splendidus and E. coli challenge, but no significantly vivid spots were observed in M. luteus–challenged coelomocyte (Fig. 2). The LC3-positive signal was significantly increased by 14.6- (p < 0.01) and 36.5-fold (p < 0.01) in the V. splendidus and E. coli groups, respectively. What is more, we found that the fluorescence intensity was clearly increased by Chloroquine (CQ) (Fig. 2C) in bacteria-infected sea cucumber coelomocyte and in the control group compared with that obtained without the injection of CQ (Fig. 2B), which suggested that bacteria induce functional degradative autophagy in A. japonicus. Moreover, to further verify the occurrence of autophagic flux in coelomocyte of A. japonicus, the accumulate LC3-positive puncta (AjLC3) induced by CQ or Baf-A1 was detected by immunofluorescence observation. Double-staining analysis indicated that the red fluorescence representing lysosomal membranes and the green signal representing autophagosomal membranes were colocalized at 24 h post V. splendidus and E. coli challenge (Fig. 2, D and E). The quantitative results showed that

Figure 3. Characterization of A. japonicus coelomocyte xenophagy after challenge with M. luteus, V. splendidus, and E. coli by Western blotting analysis. Sea cucumbers were injected with or without 10 nM Baf-A1 following M. luteus (A), V. splendidus (B), and E. coli (C) challenge for 12 and 24 h, respectively. The protein band density was calculated using ImageJ software. The data, which are presented as the means ± SDs (n = 3) relative to the 0 h (control), are shown in bar graphs (right panel in D and E). Asterisks indicate significant differences compared with the control group: *p < 0.05 and **p < 0.01 (t test).
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the LC3-positive signal was markedly upregulated by 23.6-fold ($p < 0.01$) and 73.5-fold ($p < 0.01$) in the V. splendius and E. coli groups based on CQ treatments (Fig. 2D). And the LC3 green fluorescence was significantly increased by 35.2-fold ($p < 0.01$) in the V. splendius group and 47.0-fold ($p < 0.01$) in the E. coli group under Baf-A1 treatments compared with that in the control group (Fig. 2E). All these results supported the conclusion that two Gram-negative bacteria, V. splendius and E. coli, but not M. luteus, could induce xenophagy. Interestingly, the autophagy levels induced by E. coli were significantly higher than those induced by V. splendius according to the number of autophagosomes, LC3 punctum formation, LC3-II/I ratio, and protein levels of two autophagy receptors (Figs. 2 and 3).

LPS from V. splendius and E. coli differentially induce xenophagy

LPS has been widely considered an important selective autophagy induction factor that regulates specific downstream cargoes and is targeted to the degradation pathway (41). To address the differential autophagy levels induced by V. splendius and E. coli depending on their different LPS sources, we isolated the LPSs from V. splendius and E. coli. Equal final concentrations of LPS (10 μg ml$^{-1}$) were added to cultured coelomocyte followed by Baf-A1 treatments, and we found that both LPSs could promote autophagosome formation (Fig. 4A). Furthermore, the A/JLC3-II/I level was significantly increased at 6 and 12 h after LPS$^V_{E. coli}$ exposure, and the protein level of A/jp62 was markedly decreased under the same condition, respectively (Fig. 4B). Similarity, the A/JLC3-II/I level was also markedly increased after LPS$^{V. splendius}$ stimulation, following with decreased expression of A/jp62 (Fig. 4B). What is more, significant increases in A/JLC3-II/I and A/jp62 were both observed after Baf-A1 exposures, with the higher magnitudes in LPS$^{E. coli}$ group (Fig. 4B). To confirm whether autophagic flux occurred after LPS stimulation, the accumulated LC3 green signal was also measured by immunofluorescence following CQ or Baf-A1 treatment. Our results showed that the fluorescence intensity of A/JLC3 was significantly increased at 12 h after the exposure of the coelomocyte to LPS$^{E. coli}$ following CQ (23.5-fold, $p < 0.01$) (Fig. 4C) and Baf-A1 (29.7-fold, $p < 0.01$) treatments (Fig. 4D). Moreover, the LC3 fluorescence intensity in LPS$^{V. splendius}$ stimulation group was also observably increased by 3.66-fold ($p < 0.01$) with CQ (Fig. 4C) and 6.82-fold ($p < 0.01$) with Baf-A1 at 12 h (Fig. 4D) compared with those in the control group. Besides, we found that the LC3 green signal in the PGN$^{M. luteus}$ group showed a slight increase after Baf-A1 treatment (Fig. 4D). To prove that the observed LC3 green signal is indeed autophagosome, we silenced A/jULK by siRNA and analyzed the changes in autophagy marker expression and LC3 green fluorescence. To be mentioned, the A/jULK was successfully silenced in our previous work, and the autophagy flux in coelomocyte was markedly decreased after A/jULK knockdown in vivo (36). Here, the A/JLC3-II/I level (Fig. 5) and LC3 immunofluorescence (Fig. 6H) were both damaged after interference of A/jULK based on two different LPS stimulations, whereas the A/jp62 level was significantly increased under the same condition (Fig. 5), suggesting that the observed LC3 puncta are really autophagosomes but not LC3-associated phagocytosis. Based on the overall results, we concluded that the different LPSs from V. splendius and E. coli both induced coelomocyte xenophagy in A. japonicus and that LPS$^E_{E. coli}$ stimulation and E. coli challenge induced a higher autophagic flux (Figs. 2–4).

Differential LPS-induced autophagy is dependent on different TLR cascades

To understand whether differential LPS-induced autophagy was activated by different PRR-mediated signaling pathways, we silenced the four identified PRRs, A/jTLR3, A/jToll, A/jNLR4, and A/jSR-B, and analyzed the changes in autophagy marker expression (A/JLC3 lipidation and A/jp62 degradation) based on the two different LPS stimulations (Figs. 5 and S1). All of these PRRs were found to bind to LPS in previous work (42–44). Our results indicated that LPS$^{V. splendius}$ exposure did not change the A/JLC3-II/I and A/jp62 levels in the A/jTLR3-knockdown group (Fig. 5A). In contrast, we found that the A/JLC3-II/I and A/jp62 levels were significantly increased and decreased, respectively, in a time-dependent manner in the A/jToll-, A/jNLR4-, and A/jSR-B-silenced groups and in the control (0 h) group (Fig. 5A), which supported the finding that LPS$^{V. splendius}$ induced autophagy through A/jTLR3 signaling. With LPS$^{E. coli}$, no changes in A/JLC3 lipidation and A/jp62 degradation were detected in the A/jToll-silenced group but not in the A/jTLR3-, A/jNLR4-, and A/jSR-B-silenced groups or the control (0 h) group (Fig. 5B). Overall, the results indicated that LPS$^{V. splendius}$ and LPS$^{E. coli}$ induced xenophagy via the A/jTLR3 and A/jToll-mediated signal cascades, respectively.

TLRs mediate LPS-induced xenophagy via the TRAF6-Beclin1 axis

TRAF6, as an important adaptor in the TLR signaling pathway, plays broad roles during immune responses, such as inflammatory cytokine secretion and autophagy initiation (45, 46). In our study, the protein expression of A/jTRAF6 was significantly increased after 12 h of exposure to LPS$^{V. splendius}$ and LPS$^{E. coli}$ (Figs. 6, A and B and S2, A and B). Furthermore, we found that the K63-linked ubiquitination level of A/jTRAF6 in the LPS$^{V. splendius}$ group was markedly increased at 12 h (Figs. 6A and S2A), and greater upregulation of this level was found in LPS$^{E. coli}$ group (Figs. 6B and S2B). In order to determine whether ubiquitination of A/jTRAF6 induced by TLR signaling pathways, the protein and K63-linked ubiquitination levels of A/jTRAF6 were analyzed after A/jTLR3 or A/jToll knockdown following with two types of LPS challenges. We found that the A/jTRAF6 protein expression and K63-linked ubiquitination level were both damaged in A/jTLR3 (Figs. 6C and S2C) and A/jToll knockdown groups (Figs. 6D and S2D) following two different LPS challenges; moreover, the expression of A/jTRAF6 and its ubiquitination level were also abolished after the silencing of A/jTRAF6 under
LPS* V. splendidus (Figs. 6E and S2E) and LPS* E. coli * challenges (Figs. 6F and S2F), as well as decreased expression of AjLc3-II/I (Figs. 6, E and F and S2, E and F) and impaired LC3 green signal (Fig. 6G) under the same conditions. Our results suggested that Aj/TRA6 serves as a common adaptor for the two TLR signaling pathways (Figs. 6 and S2).

More importantly, Beclin1 is a critical component of the autophagy initiation machinery that is ubiquitinated by TRAF6.
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Figure 5. The AjLC3-I-to-AjLC3-II conversion and protein level of Ajp62 in coelomocyte were detected after 0, 6, and 12 h of exposure to LPS V. splendidus and LPS E. coli following the mRNA silencing of AjTLR3, AjToll, AjSR-B, AjNLRC-4, and AjULK. A, Western blotting analysis of LPS V. splendidus-challenged group following mRNA silencing of AjTLR3, AjToll, AjSR-B, AjNLRC-4, and AjULK; B, Western blotting analysis of LPS E. coli-challenged group following mRNA silencing of AjTLR3, AjToll, AjSR-B, AjNLRC-4, and AjULK. The NC group was transfected with nontargeted double-stranded siRNA and served as the control. The protein band density was calculated using ImageJ software. The data, which are presented as the means ± SDs (n = 3) relative to the 0 h (control) are shown in bar graphs (lower panel in A and B). The asterisks indicate significant differences compared with the control group: *p < 0.05 and **p < 0.01 (t test).
Figure 6. *L. splendidus* and *L. coli* mediate the K63-linked ubiquitination of AjTRAF6 based on AjTLR3 and AjToll cascades, which interacts with AjBeclin1 to regulate xenophagy in *A. japonicus* coelomocyte. Sea cucumber primary coelomocyte were stimulated with *L. splendidus* (A) or *L. coli* (B) for the indicated times (0 and 12 h) following AjTLR3 (C), AjToll (D), and AjTRAF6 (E and F) silencing for 24 h. G, LC3 fluorescence intensity detection after interference of AjTRAF6 followed by *L. splendidus* or *L. coli* exposure for 12 h. H, LC3 fluorescence intensity detection after interference of AjULK followed by *L. splendidus* or *L. coli* exposure for 12 h. Western blotting analysis of immunoprecipitated (IP) AjTRAF6 samples was performed to determine the presence of K63-linked ubiquitin (Ub K63). The membranes in (A) and (B) were stripped and analyzed for interaction with AjBeclin-1. Whole-cell lysates were analyzed by western blotting as indicated. The protein band density was calculated using ImageJ software. The heavy chain of the TRAF6 antibody was detected by the secondary antibody and was labeled Ig band. The data, which are presented as the means ± SDs (n = 3) relative to the 0 h (control) in Fig. S2. For LC3 positive signal, the cells were fixed and stained with anti-LC3 at the indicated time points (0 and 12 h). After nuclear staining with DAPI,
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(25, 47). Thus, we explored Aj/TRAf6-mediated xenophagy depending on Aj/Beclin1 ubiquitination in sea cucumber coelomocyte. First, we found that the K63-linked ubiquitination of Aj/Beclin1 was also markedly increased under the same conditions (Figs. 7, A and B and S3, A and B). Then, we determined the K63-linked ubiquitination of Aj/Beclin1 after AjTLR3 or AjToll silencing and found that AjTLR3 or AjToll knockdown by specific siRNA impaired the LPS-induced K63-linked ubiquitination of Aj/Beclin1 (Figs. 7, C and D and S3, C and D), which suggested that AjTLR3 and AjToll mediated LPSV. splendidus- and LPS E. coli-induced ubiquitination of Aj/Beclin1 in sea cucumber coelomocyte. To further determine whether the ubiquitination of Aj/Beclin1 was dependent on its interaction with Aj/TRAf6 in coelomocyte, we analyzed the interaction between Aj/TRAf6 and Aj/Beclin1 through an immunoprecipitation assay. The protein level of Aj/Beclin1 after LPSV. splendidus or LPS E. coli stimulation was detected by Western blotting after precipitation with an Aj/TRAf6-specific antibody (Figs. 6, A and B and S2, A and B). Notably, the expression of Aj/Beclin1 and its ubiquitination level were both abolished after Aj/TRAf6 silencing in the LPSV. splendidus group (Figs. 7E and S3E) as well as in LPS E. coli challenge group (Figs. 7F and S3F). Our findings indicated that Aj/TRAf6 could directly ubiquitinate Aj/Beclin1, which results in its ubiquitination, and promote autophagy during challenge with different types of LPSs.

AjTLR3 but not AjToll promotes the expression of the autophagy inhibitor AjA20

We have demonstrated that AjTLR3 and AjToll are consistently involved in the regulation of autophagy by the Aj/TRAf6-Aj/Beclin1 axis during exposure to LPSV. splendidus and LPS E. coli, whereas the mechanism underlying the differential autophagy capacity in response to these two Gram-negative bacteria remains unknown. A20, as a deubiquitinating enzyme, has been proven to hinder the K63-linked ubiquitination of Beclin1 and further inhibit autophagy (25). In this study, the protein expression level of AjA20 was significantly increased in coelomocyte after 12 h of exposure to LPSV. splendidus (Figs. 7A and S3A), and its level was also significantly increased in the Aj/TRAf6-silenced group upon LPSV. splendidus challenge (Figs. 7E and S3E); these observations indicated that AjA20 might play a negative role in the regulation of autophagy induction. Moreover, we found that the elevation in the protein level of AjA20 was abolished by the knockdown of AjTLR3 under the same conditions (Figs. 7C and S3C), which suggested that AjTLR3 signaling promotes AjA20 expression under LPSV. splendidus stimulation. However, the analysis of E. coli–mediated autophagy showed that the exposure of coelomocyte to LPS E. coli did not trigger changes in AjA20 expression (Figs. 7B and S3B), and no changes were obtained with AjToll (Figs. 7D and S3D) or Aj/TRAf6 (Figs. 7F and S3F) silencing under the same conditions.

Discussion

Xenophagy is a particularly important type of selective autophagy and plays a pivotal role in host innate immune defense. Unlike bulk degradation of the packaged cargo in nonselective autophagy, xenophagy imparts selectivity to the degradation process by tagging and targeting cargoes into lysosomes (17). In mammalian species, the role of xenophagy in selectively eliminating disease-related pathogens, such as mycobacteria (19), HIV (48), and toxoplasma (49), has been well studied. However, the differential regulation mechanisms of xenophagy in response to different microbe challenges in a specific species, particularly an aquatic animal, remain largely unknown. In this study, 39 conserved Atgs were obtained by screening the sea cucumber genome, and the results indicated that A. japonicus possesses the complete machinery necessary for the autophagy process. We further found that the two Gram-negative bacteria V. splendidus and E. coli induced differential TLR-dependent xenophagy via their different LPSs, but the Gram-positive bacteria M. luteus did not induce xenophagy. Both autophagy pathways were dependent on activation of the TLR-TRAf6-Beclin1 axis through the ubiquitination of Aj/Beclin1. Moreover, V. splendidus–induced autophagy also increased the expression of the deubiquitinase Aj/A20, which impaired the K63-linked ubiquitination of Aj/Beclin1, but this cascade was not detected in the LPS E. coli–induced AjToll signaling pathway. Overall, our current study provides novel insights into the xenophagic mechanism through which different TLR-TRAf6-Beclin1 axis initiates autophagy in marine invertebrates.

Because A. japonicus lacks an adaptive immune system, this species must employ its innate immune system to protect itself against infections. The release of the genome of A. japonicus (38, 39) allowed us to identify autophagy-related proteins of sea cucumbers. Therefore, using multiple sequence alignment and SMART tools, we observed that the core proteins involved in the autophagy pathway appear to be functional in A. japonicus and could be divided into four main steps: initiation, phagophore elongation, autophagosome formation, and lysosome fusion (Fig. 1 and Table S1). Among all Atg-encoded proteins, LC3/Atg8 is one of the most important ubiquitin-like proteins that is synthesized as a precursor with a key Gly residue at its C terminus (called LC3-I), which is cleaved by Atg4 and ultimately forms mature LC3-II via reactions mediated by Atg3 and Atg7 (8). In our study, we found four LC3 homologs from A. japonicus (named AjLC3, AjLC3C, AjGABARAP, and AjGABARAPL2), and all of these shared a highly conserved ATG8 domain with other species, such as S. purpuratus and H. sapiens. Although these four LC3 homologs also shared a conserved Gly site in their C terminus with other LC3 proteins, the position of this key site in A. japonicus (AjLC3 in Gly121, AjLC3C in Gly121, AjGABARAP in Gly168, and AjGABARAPL2 in Gly129) is slightly different from that in the referenced species (consistently Gly120). This
difference in the position of the residue appears to not affect its cleavage by Atg4 but might decide its functional differentiation in other types of autophagy (50). Moreover, Beclin1 is the mammalian homologue of yeast Atg6, a key component of PI3KC3 that initiates autophagy by helping localize other autophagy proteins to the preautophagosomal membrane (51). The K63-linked ubiquitination of Beclin1 at Lys117 facilitates the oligomerization of Beclin-1 and enhances the lipid kinase activity of PI3KC3 (25, 51). In our study, AjBeclin1 showed good conservation with Beclin1 from other species and contained an Lys residue at position 115, a key target for linking ubiquitin chains. Furthermore, the xenophagy-mediated elimination of cytoplasmic cargoes depends on recognition by specialized receptors known as xenophagy adaptors (52). Almost all adaptors in mammals contain one or more ubiquitin-binding domains (UBDs, including UBA) and an LIR (53). In A. japonicus, the two xenophagy adaptors Ajp62 and AjNBR1 include a UBD and an LIR motif, which is consistent with the results found for mammalian species proteins. To date, several structures of LC3 orthologs bound to LIR motifs, including a crystal structure of LC3B bound to the p62 or NBR1 LIR peptide, have been analyzed, and the noncanonical LIR (CLIR) motif in the autophagy receptor NDP52 binds to LC3C (53). In our study, we could not find the NDP52 gene in the sea cucumber genome. Moreover, we concluded that the AjLC3 sequence was closer to that of LC3B, which binds to p62 or NBR1 to induce autophagy through the LIR motif. Therefore, we prepared human LC3B antibody to analyze sea cucumber coelomocyte autophagy during bacterial infection. We observed the accumulation of AjLC3-II/I and the formation of double-layer autophagosomes, and these findings were further confirmed by the degradation of Ajp62 after V. splendidus challenge. These data strongly indicate the existence of autophagic flux in A. japonicus (Figs. 2 and 3), which indicates that sea cucumbers possess a complete autophagy machinery similar to other advanced species.
Xenophagy is differentially modulated by TLR-TRAF6-Beclin1

Although the existence of complete autophagy machinery provides a molecular basis for the occurrence of xenophagy in *A. japonicus*, whether autophagy could be activated by different types of microbes remains largely unknown. Therefore, we investigated the autophagic flux in *A. japonicus* exposed to three different bacteria. Our results clearly indicated that two types of Gram-negative bacteria could induce coelomocyte xenophagy, but the Gram-positive bacteria *M. luteus* failed to activate xenophagy in *A. japonicus* (Figs. 2 and 3). Gibson et al. (54) showed that the Gram-positive bacteria *Staphylococcus aureus* could induce xenophagy in zebrafish. Moreover, Yano et al. (23) showed that *Drosophila* lacking PGRP-LE or expressing mutant PGRP-LE112 was unable to undergo autophagy, which resulted in increased susceptibility to *L. monocytogenes* infection. Recently, we found only a short-type PGRP (named *Aj*PGRP-S) in the sea cucumber genome (55), and further analysis indicated that the key site of *Aj*PGRP-S is not conserved to counterpart in *Drosophila*. Therefore, we speculated that this failure of *M. luteus*–induced xenophagy could be explained by the fact that the bacterial ligand might not be recognized by *Aj*PGRP-S.

Nearly all inductions of autophagy by invading microbes are dependent on the interaction between bacterial cell wall components and PRRs of host cells (16–18). In our study, we found that the two Gram-negative bacteria differentially induced xenophagy, and a higher autophagy ratio was found in the *E. coli* group. In addition, the *Aj*LC3-II/I ratio, the p62 protein level, and LC3 green signal were all significantly increased by CQ and Baf-A1 injections. As well known, Baf-A1 and CQ are commonly used that inhibit autophagy acceleration induced degradative xenophagy in *A. japonicus*. To date, most studies have primarily focused on the xenophagic mechanism of the host in response to infection with a single pathogen (19, 56). Juárez et al. (57) showed that NOD2 enhances the autophagy of alveolar macrophages after *M. tuberculosis* infection in humans. Moreover, Liu et al. (58) indicated that *Salmonella typhimurium*–induced autophagy in a RAW264.7 murine macrophage cell line is mediated by the TLR4 signaling pathway. In TLR4-deficient macrophages, LPS (from *S. Typhimurium*) is unable to induce autophagy. More importantly, the abovementioned studies demonstrated that LPS, flagellin, and CpG oligodeoxynucleotides from Gram-negative bacteria other than *Salmonella* activate TLR4, TLR5, and TLR9, respectively, and induce different degrees of autophagy in RAW264.7 cells. In our study, we suspected that the significant differences in the induction of autophagy might depend on different PRR signaling pathways that are activated by PAMPs from different bacteria to increase the expression of autophagy-related molecules (25, 26). LPS is a complex glycolipid endotoxin derived from the cell wall of Gram-negative bacteria (59) and is a powerful inducer of autophagy in many mammalian cell lines, including macrophages (60), hepatocytes (41), and myoblasts (61). First, to confirm that the mediation of autophagy by the two Gram-negative bacteria was dependent on LPS, we performed an autophagy assay using cultured coelomocyte and found that LPS*E. coli* and LPS*V. splendidus* induced autophagy in cultured sea cucumber coelomocyte (Fig. 4), which is consistent with the effects of the two bacteria on xenophagy. Multiple studies have shown that LPS induces autophagy through TLR cascades (58, 62). Wang et al. (63) demonstrated that the activation of autophagy by LPS via TLR4 represents an innate defense mechanism for controlling intracellular *E. coli* replication in human peritoneal mesothelial cells. However, only two TLRs, namely *A/J*TolR3 and *A/Toll*, have been identified in sea cucumbers (42). Subsequently, to determine whether differential LPS-induced accumulation of autophagosomes occurs via PRRs in *A. japonicus*, we detected the autophagy levels after silencing four types of PRRs, including *Aj*TLR4 and *Aj*SR-B. Our results clearly indicated that *Aj*LC3 lipiddation and *Aj*p62 degradation were highly correlated with the level of *Aj*TLR3 after LPS*V. splendidus* exposure and the level of *Aj*Toll after LPS*E. coli* stimulation (Fig. 5), respectively, which indicated that LPS*V. splendidus* induced coelomocyte xenophagy through *A/J*TolR3 and that LPS*E. coli* interacts mainly with *A/Toll* to accelerate autophagy. Norris et al. (64) used different types of LPS (from several strains of *Burkholderia pseudomallei*) to challenge RAW264.7 cells and found that green LC3 signal was visible in all the groups but was markedly more abundant in 576a–, MSHR435–, and *Salmonella* LPS-treated cells. However, the differential autophagy levels in RAW264.7 cells mediated by different TLR cascades are largely unknown. In general, LPSs containing different components might induce differential activation of TLR2 and TLR4 to facilitate the elimination of invading bacteria through autophagy (65). LPS consists of three parts: lipid A, a core oligosaccharide, and an O side chain (66). Among these parts, the shape of the lipid A component determines the bioactivity of LPS (67). In our study, the differential autophagy levels induced by different bacteria indicated that the constituents of lipid A in these two types of LPSs might differ. Netea et al. (68) demonstrated that an LPS with a conical shape (e.g., the LPS of *E. coli*) induces cytokine production through TLR4; however, a cylindrical LPS (e.g., that of *Porphyromonas gingivalis*) induces cytokine expression through TLR2. In addition, Nahori et al. (69) showed that parental leptospiral LPS (e.g., the LPS of *Leptospira interrogans*) was the predominant ligand for TLR1/TLR2 in human cells, whereas TLR2 and TLR4 contribute to activation in murine cells. Our results showed that the LPS from *E. coli* was mainly linked to *A/Toll* in sea cucumbers, and a previous study demonstrated that *A/Toll* exhibits high conservation with human TLR4 (46), which indicates that *A/Toll* was similar to human TLR4 and likely induced by the conical LPS from *E. coli*. Usually, double-stranded RNA (dsRNA) or its synthetic analog poly(I:C) acts as a ligand of TLR3 and stimulates autophagy in various mammalian cells (18, 26, 60). Our analysis of the sequence characteristics of *A/J*TolR3 revealed that the pivotal amino acid residues for phosphorylation, which are necessary for the dsRNA-mediated signaling pathway, were not present in *A/J*TolR3 (70). In addition, we found that the protein sequence of *A/J*TolR3 was close to that of...
human AjTLR2 (42), which indicated that AjTLR3 might function similarly to human TLR2 and could recognize LPS of V. splendidus.

In most mammalian cells, autophagy induced by different PAMPs also requires the E3 ligase TRAF6 (25). Based on TLR stimulation, TRAF6 promotes the K63-linked ubiquitination of Beclin1 to induce TLR-mediated autophagy (26). Inomata et al. (31) showed that poly(I:C) stimulation induces autophagy via TRIF and TRAF6. In atrophying skeletal muscle, the inhibition of TRAF6 also blocks the expression of K63-linked mono/polyubiquitination and impacts autophagosome formation (71). In our study, we discovered that the protein level of AjBeclin1 decreases after poly(Lys) stimulation in RAW264.7 cells. However, the K63-linked ubiquitination of AjBeclin1 was both significantly increased after challenge with the two types of LPS, although higher levels were found in the LPSV group than in the LPSEcoli group, and the K63-linked ubiquitination of AjTRAF6 was abrogated after AjTLR3, AjToll, or AjTRAF6 knockdown in these two groups (Fig. 6). Zhan et al. (26) reported that TLR3 and TLR4 activation induces autophagy in lung cancer cells through the promotion of TRAF6 ubiquitination, which is similar to our results. Moreover, Shi et al. (47) showed that an increased abundance of TRAF6 could promote the K63-linked ubiquitination of Beclin1 in RAW264.7 cells. In our results, we found that the K63-linked ubiquitination of AjBeclin1 was both significantly increased in the LPSV group and LPSV group, and their levels were impaired after AjTLR3 or AjToll knockdown (Fig. 7), which suggested that the involvement of AjTRAF6 in the ubiquitination of AjBeclin1 is essential for the activation of AjBeclin1 ubiquitination. To determine whether AjTRAF6 interacts with AjBeclin1 in coelomocyte, an immunoprecipitation assay was performed. We found that AjBeclin1 can directly combine with AjTRAF6; moreover, our results clearly indicated that the AjTRAF6-deficient coelomocyte exhibited impaired AjBeclin1 ubiquitination (Fig. 7), which suggested that the K63-linked ubiquitination of AjBeclin1 is important for both types of LPS-induced autophagy. In mouse and human species, the ubiquitination of Lys117 of Beclin1 could promote the oligomerization of Beclin1 and affect the activity of PI3KC3, which further results in formation of the PI3KC3-Beclin1 protein complex and the induction of autophagy (25, 51). In our study, we identified seven central proteins related to the PI3KC3 complex, such as AjPI3KC3, AjAMBRA1, and related regulatory subunits, which indicates that the nucleation of autophagosomes is highly conserved in A. japonicus compared with vertebrates.

A question that remains unaddressed is how challenge with different LPS types causes discrepant autophagy levels. A20 is a known ubiquitin-editing enzyme that mainly functions as an endogenous regulator of inflammation through the termination of NF-kB activation (34) and plays negative roles in the activation of autophagy by limiting TRAF6 E3 ligase activity and directly deubiquitinating Beclin1 (25). First, we found that the protein level of AjA20 was only markedly induced after LPSV stimulation but not in LPSEcoli group under the same conditions (Fig. 7). Because A20 reduces the ubiquitination of Beclin1 and limits the induction of autophagy (25), we hypothesized that the reduction in the autophagy level observed in the LPSV group might be mediated by AjA20. To more directly test the mechanism responsible for the discrepant autophagy levels induced by LPSV and LPSEcoli challenge, we analyzed the AjBeclin1 ubiquitination and AjA20 expression levels in response to stimulation with two different LPSs after reducing the progression of TLR cascades using specific siRNA. We found that the increase in the AjA20 protein level was abolished by the silencing of AjTLR3 under the same conditions, which suggested that AjTLR3 signaling under LPSV stimulation induced the expression of the gene encoding AjA20. Moreover, the AjA20 protein level after LPSV stimulation was significantly increased by the silencing of AjTRAF6, and this finding indicated that this strong expression of AjA20 might lead to a reduction in the extent of ubiquitination of AjBeclin1, which acts to limit autophagy under LPSV challenge. Most studies have reported that A20 overexpression can inhibit autophagy by limiting the K63-linked ubiquitination of TRAF6 or by directly deubiquitinating Beclin1 (47, 72). Thus, the precise mechanism of AjA20 and deubiquitination should be verified in future studies.

In conclusion, this study shows that V. splendidus induces autophagy through the activation of AjTLR3 signaling, but E. coli promotes autophagy via activation of the AjToll pathway (Fig. 8). Through these signaling pathways, ubiquitinated AjTRAF6 directly ubiquitiates AjBeclin1, which results in its ubiquitination and subsequently the induction of autophagy. The difference indicates that AjA20 plays a negative regulatory role in the activation of autophagy after LPSV stimulation. Overall, our study reveals that LPS utilizes diverse mechanisms to induce xenophagy in invertebrate marine animals.

**Experimental procedures**

**Ethics statement**

The sea cucumbers used in this work were commercially cultured animals, and all experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by the Experimental Animal Ethics Committee of Ningbo University, China.

**Homology analysis of autophagy machinery**

The A. japonicus autophagy-related genes that regulate the autophagy machinery were screened from the sea cucumber genome (https://www.ncbi.nlm.nih.gov/assembly/GCA_002754855.1) and transcriptome database (accession number of SRA080354). We used the Protein BLAST program of NCBI (https://www.ncbi.nlm.nih.gov/) to search for other Atg homologs using the A. japonicus Atg protein sequences collected from the UniProt database. The conserved domains, signal peptides, and internal repeats of all the proteins were detected using the Simple Modular Architecture Research Tool (SMART) program.
bacteria in autophagosomes was determined within 1000 coelomocytes in each group.

Cell culture, LPS, or PGN isolation and exposure

Primary coelomocytes were prepared according to our previous work (73). Briefly, the harvested cells at a final concentration of 10^6 cells ml\(^{-1}\) were resuspended in L-15 cell culture medium (Invitrogen) containing penicillin (100 U ml\(^{-1}\)) and streptomycin sulfate (100 mg ml\(^{-1}\)). NaCl solution was utilized to adjust the osmotic pressure to a final concentration of 0.39 M. The cells were then dispensed into a 24-well culture microplate with 500 μl of L-15 medium in each well, and the cell viability was checked by trypan blue (Solarbio). For LPS stimulation experiments, LPS was isolated from the two bacteria (E. coli and V. splendidus) using an LPS extraction kit (Beibokit) following the manufacturer’s instructions. PGN was isolated from M. luteus (Sigma). The cultured cells were exposed to 10 μg ml\(^{-1}\) LPS from E. coli (LPS\(^E\) \(\text{coli}\)), 10 μg ml\(^{-1}\) LPS from V. splendidus (LPS\(^V\) splendidus), or 10 μg ml\(^{-1}\) PGN from M. luteus (PGN\(^M\) luteus) for 0, 6, and 12 h. Primary cells was collected at 0 h in each group served as a control. For CQ and Baf-A1 treatments, primary coelomocytes were treated with 10 μM CQ or 2 nM Baf-A1. After challenge, the cells were collected and used for subsequent detection of autophagosome formation, determination of the protein levels of autophagy-related genes, and immunofluorescence assays.

RNA interference

Specific siRNAs for AjTLR3, AjToll, AjNLRC4, AjSR-B, AjTRAF6, and AjULK (36) were synthesized by GenePharma (Table S2). Control siRNA (Negative control, NC) that did not target any of the genes from the sea cucumber transcriptome served as a control. The experimental and control siRNAs were dissolved in RNase-free H\(_2\)O to obtain 20 μM stock solutions. Approximately 1 μl of each stock solution of siRNA...
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RNA isolation and real-time quantitative PCR

Total RNA was isolated using RNAiso Plus (TaKaRa), and cDNA was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). The relative mRNA expression of each gene was measured using an Applied Biosystem 7500 Real-time Quantitative PCR System (Thermo Fisher Scientific). The specific primers are listed in Table S2. Actin was determined to be a suitable housekeeping gene for the normalization of target quantification by Zhao et al. (74). Each reaction was performed in a final volume of 20 μl, which contained 2 μl of cDNA, 1 μl of each primer (10 μM), 6 μl of RNase-free H2O, and 10 μl of SYBR Green PCR Master Mix (TaKaRa). The amplification procedure was as follows: denaturation at 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. After the cycling stage, melting curve analyses were performed. The 2−ΔΔCT method was used to analyze the expression level of each gene (75).

Transmission electron microscopy (TEM)

After the sea cucumbers were challenged with three types of bacteria in vivo and subjected to different LPS stimulations in vitro, the coelomocytes were harvested as previously described. First, the collected coelomocytes were washed twice with sterilized isotonic buffer (0.001 M EGTA, 0.53 M NaCl, and 0.01 M Tris-HCl, pH 7.6). The pellets were then fixed in 2.5% glutaraldehyde in PBS at 4 °C for 2 h and washed once with 0.1 M PBS. Subsequently, the pellets were fixed in 1% osmium tetroxide for 1.5 h, dehydrated through a series of ethanol concentrations, and embedded in Epon resin. The samples were sectioned with a microtome, and the sections were double stained with 3% uranyl acetate and lead citrate before examination under a transmission electron microscope.

Antibody preparation

Several recombinant proteins were prepared and used to generate mouse polyclonal antibodies (e.g., Ajp62, Aj/Beclin1, and AjA20) or rabbit polyclonal antibodies (e.g., Aj/LAMP) according to our previous work (76). Briefly, the partial cDNA sequences of the above genes were cloned with specific primers (Table S2), double digested with restriction enzymes, and ligated into the pET28a(+) vector. The recombinant plasmids were then transformed into E. coli Rosetta (DE3) cells (Invitrogen), generated by adding IPTG at a final concentration of 1 mM, and purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (QIAGEN). After dialysis, the soluble target proteins were injected into 4-week-old mice or rabbits to acquire polyclonal antibodies according to our previously described protocol (76). In addition, antibodies against Ub-K63 (T56579S), β-actin (M20011S), TRAF6 (T55175S), Beclin1 (T55092S) were purchased from Abcam. HRP-conjugated anti-mouse (D110087) and anti-rabbit IgG (D110058) secondary antibodies were purchased from Sangon. Antibody against LC3B (ab51520) was purchased from ABCAM.

Immunofluorescence analysis

The tested coelomocytes obtained as above were seeded at a density of approximately 10⁵ cells ml⁻¹ on glass chamber slides with lysine and treated for 20 min at 16 °C. Thereafter, the supernatant from two independent experiments was discarded, and the coelomocytes in each well were fixed with 4% paraformaldehyde (PFA) for 30 min and permeabilized with 0.1% Triton X-100 (Sigma) for 10 min. The cells were washed three times with PBS (containing 0.05% Tween-20) and blocked with 5% BSA in PBS at 25 °C for 1 h. The supernatant was then removed, and the cells were incubated overnight with anti-LC3B antibody (1:500 dilution) or the combination of anti-LC3B antibody and anti-LAMP antibody (1:500 dilution) as the primary antibodies at 4 °C. After three washes with PBS, the cells incubated with the anti-LC3B antibody alone were incubated with an FITC-conjugated goat anti-mouse secondary antibody (1:1000 dilution) at 37 °C for 1 h, and the cells incubated with two primary antibodies were further incubated with Cy3-conjugated goat anti-rabbit (1:1000 dilution) and FITC-conjugated goat anti-mouse secondary antibodies for 1 h at 37 °C. After three additional washes with PBS, DAPI (diluted to 10 μg ml⁻¹ in PBS; Beyotime Biotechnology) was added to the cells to stain the nuclei. After a final three cycles of washing, the cells were mounted in antifade fluorescence mounting medium for observation with a laser-scanning confocal microscope (ZEISS).

Western blotting analysis

Western blotting analysis was performed as described in our previous work (73). The proteins from coelomocytes were extracted using cell lysis buffer (Beyotime Biotechnology), and the concentration was measured with a BCA protein assay kit (Sangon). Approximately 50 μg of protein in each well was separated by SDS-PAGE with a gel thickness of 1 mm and then electrophoretically transferred to a 0.45-μm-pore-size nitrocellulose membrane. The membrane was blocked with 5% skim milk at 25 °C for 1 h and then incubated with specific polyclonal antibodies (usually diluted 1:1000 in 5% skim milk) at 4 °C overnight. Subsequently, the membranes were washed three times with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) and incubated with the corresponding secondary antibodies (usually diluted 1:3000 in 5% skim milk) at 25 °C for 1.5 h. The membranes were then subjected to three 10 min washes with TBST, incubated with Western Lightning-ECL substrate (PerkinElmer) and exposed to X-OMAT AR X-ray film (Eastman Kodak). The protein and β-actin bands were quantified using the BioRad Quantity One software.

(continued)
package, and the results were derived from a statistical analysis of three independent experiments.

**Immunoprecipitation and ubiquitination**

For communoprecipitation assays, LPS-exposed coelomocytes were lysed in a buffer that contained 20 mM HEPES pH 7.4, 50 mM β-glycerophosphate, 1 mM Na3VO4, 0.5% Triton X-100, 0.5% CHAPS, and 10% glycerol with a protease inhibitor cocktail (Beyotime Biotechnology). The equal lysates were incubated with equal anti-AjTRAF6 or anti-AjBeclin1 antibody overnight at 4 °C and then incubated with Protein A + G Agarose (Beyotime Biotechnology) for 4 h at 4 °C. The immunoprecipitates were collected, washed four times with lysis buffer, and analyzed by SDS-PAGE and immunoblotting. For the detection of AjTRAF6 or AjBeclin1 ubiquitination, the samples were first incubated with an antibody against K63-linked ubiquitination overnight at 4 °C. The membrane was subjected to three 10 min washes with TBST and then incubated with HRP-conjugated secondary antibody at 25 °C for 1.5 h. After three 10 min washes with TBST, the membrane was incubated with Western Lightning-ECL substrate and exposed to X-OMAT AR X-ray film. To determine whether AjBeclin1 directly interacts with AjTRAF6, the membrane was stripped and then incubated with an antibody against AjBeclin1. The subsequent steps were the same as those described above. Because the protein molecular weight of AjBeclin1 was similar with heavy chain of the antibody, we used Goat Anti-Rabbit IgG HRP (M21008S, Abmart), which could remove both heavy chain of the antibody and light chain of the antibody.

**Statistical analysis**

Both in vivo and in vitro experiments were executed with three biological replicates, and the data are expressed as the means ± standard deviations (SDs) (n = 3). One-way ANOVA was applied to determine the significance of the differences between the control and experimental groups. Differences were considered significant at *p* < 0.05 and **p** < 0.01.

**Data availability**

Requests for access to the data, statistical code, questionnaires, and technical processes may be made by contacting the corresponding author at lichenghua@nbu.edu.cn.

**Supporting information**—This article contains supporting information.

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