# THE ROLE OF AUTOPHAGY IN INFECTIOUS DISEASES

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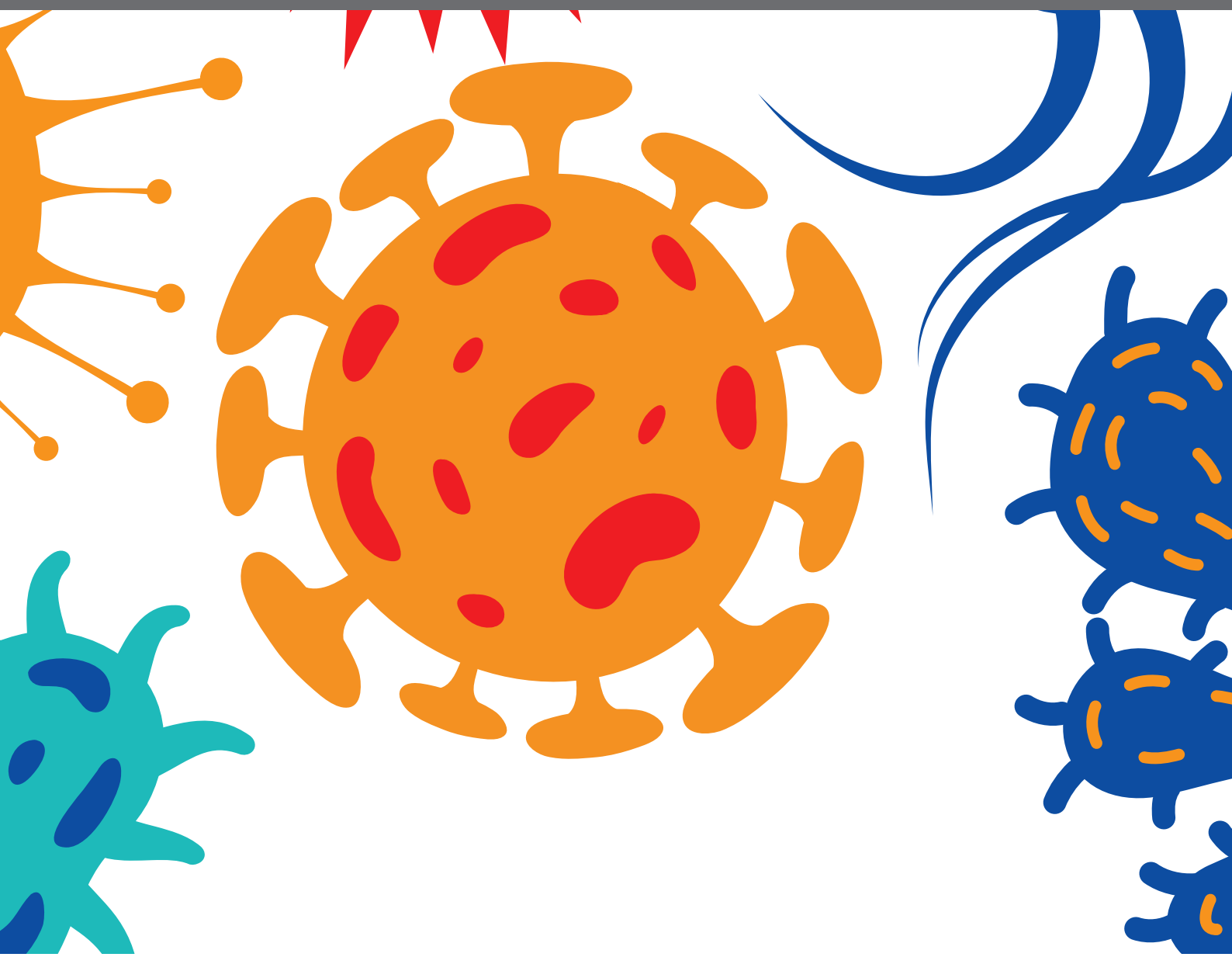

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# THE ROLE OF AUTOPHAGY IN INFECTIOUS DISEASES

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# Editorial: The role of autophagy in infectious diseases

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**autophagy, infectious diseases, pathogens, autophagic regulation, host defense**

**Editorial on the Research Topic**
The role of autophagy in infectious diseases

## Introduction

Autophagy is an intracellular catabolic process that sequesters and digests cytosolic components to maintain cellular homeostasis in health and diseases (Biasizzo and Kopitar-Jerala, 2020). Canonically, a series of evolutionarily conserved proteins identified as autophagy-related proteins participate in the autophagy process, forming double-membrane autophagosomes in the cytosol to digest the cytosolic components (Tsukada and Ohsumi, 1993). Autophagy has been reported to play critical roles in clearing intracellular pathogens and modulating the inflammatory response during host defense (Deretic et al., 2013). These make the autophagy process as an attractive target for developing therapeutic strategies for infections.

However, emerging studies indicate that the autophagy process is complicated and appears to be pathogen-specific. First, three forms of autophagy, macroautophagy, microautophagy and chaperone-mediated autophagy, are identified in mammalian cells according to diverse mechanisms of transporting cellular materials into lysosomes. Second, alternative mechanisms bypassing the canonical autophagy pathway have been recognized in the autophagy process in response to pathogen invasion. Furthermore, the autophagy process crosstalks with other cellular activities to modulate the host defense. Finally, some pathogens can hijack or exploit the autophagy process for their invasion. Therefore, further studies are urging to understand the pathogen-specific mechanisms of autophagic regulation during infection and the crosstalk between autophagy and other host defense mechanisms.

In this Research Topic issue, we have collected a series of research articles, reviews and perspective on recent advances in the mechanism of autophagic regulation in response to bacterial and viral infections, as well as autophagy in other aspects.

## Advances in autophagy during bacterial infection

Here we have collected two research articles, three reviews, and one perspective covering the role of canonical and noncanonical autophagy pathways in bacterial infection. The research article by Nikouee et al. showed that activating Beclin-1 in mice by forced expression of active mutant *Becn1F121A* or by treatment of Beclin-1–activating peptide enhanced autophagy and alleviated adverse outcomes of pneumonia-induced sepsis. This work shows the therapeutic potential in treating pneumonia-induced sepsis by activating Beclin-1. Pellegrini et al. reviewed studies on autophagy in host defense against an intracellular microorganism, *Mycobacterium tuberculosis*, in tuberculosis patients. Although Autophagy is known to mediate pathogen clearance and plays an important role in the control of inflammatory response during bacterial infections, some infectious agents, such as *Salmonella* Typhimurium, have developed mechanisms to escape or hijack autophagy for their benefit. One known mechanism is *via* direct interactions between the effector proteins secreted from the pathogen and autophagy proteins. Using a computational network analysis approach, Demeter et al. have identified and validated that the *Salmonella* Pathogenicity Island-1 effector protein, SpoE, directly interacts with the host SP1 transcription factor. Furthermore, SpoE negatively regulates the expression of the autophagy-related protein, MAP1LC3B and modulates the autophagy flux during *S.* Typhimurium infection in intestinal epithelial cells. Flores-Vega et al. summarized the principal strategies used by *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* to escape or hijack microbicidal mechanisms within the autophagic pathway in cystic fibrosis patients. Besides the canonical autophagy pathways, non-canonical autophagy pathways, such as LC3-associated phagocytosis (LAP) and Pore-Forming Toxin-Induced Non-Canonical Autophagy (PINCA), are implicated in host defense during bacterial infection. Grijmans et al. reviewed the recent advance in LAP against bacterial pathogens. Herb et al. discussed the molecular differences and similarities between LAP, PINCA and xenophagy, a selective form of macroautophagy, in macrophages during bacterial infections.

## Advances in autophagy during viral infection

Autophagy is implicated in the life cycle and resistance of virus infection. Here Mauthe et al. showed that HSBP1, a very small cytoplasmic coiled-coil protein, interacts with FIP200-ATG13-containing complexes to control the stability of ULK complex for autophagy induction and picornaviral replications in U2OS cell lines. Li et al. showed that Interferon alpha 2a (IFNα-2a), a treatment for chronic Hepatitis B virus (HBV) infection, interplays with the Akt/mTOR signaling and AMPK signaling to regulate the autophagy process and HBV replication under various glucose concentrations. These findings may help improve the therapeutic efficacy of IFNα-2a in treating HBV infection. Matsui et al. reviewed and highlighted the interaction between Hepatitis C virus (HCV) NS5A protein and hepatocyte-nuclear factor 1α (HNF-1α) together with the chaperone protein HSC70 to promote the lysosomal degradation of HNF-1α *via* chaperone-mediated autophagy (CMA), resulting in HCV-induced pathogenesis. These call for further investigations of HCV NS5A-interacting proteins containing CMA-targeting motifs to understand HCV-induced pathogenesis. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the pandemic in the past three years without effective treatment. Here Silva et al. summarized the interplay between coronaviruses and autophagy regarding virus life cycle, cell resistance, and inflammation and discussed the autophagy-targeted pharmaceuticals being tested in clinical trials with distinct mechanisms. Autophagy is not exclusive to animals or humans. It also happens in other organisms. Wu et al. summarized the mechanisms of how virus evades host immune responses by disrupting and manipulating host autophagy in plant and animals. Picot et al. reported that autophagy is activated in hemolymph and the mantle of pacific oysters in response to infection by the virus OsHV-1. This study may help find solutions for mortality outbreaks of young Pacific oysters, which have seriously affected the oyster-farming economy in several countries worldwide.

## Advances in autophagy in other aspects

The research article by Zhou et al. investigated the roles of Atg1 and Atg13 homologs in a nematode-trapping filamentous fungus, *Arthrobotrys oligospora*. The authors characterized the phenotypes in Atg1 mutant and Atg13 mutant strains. They found that compared to the wild type, these two mutants both are defective in autophagosome formation, highlighting the crucial roles of these Atg genes in the autophagy process in *A. oligospora*. Furthermore, they showed Atg1 contributes to other phenotypes, such as sporulation and nematode predation, indicating the additional roles of Atg1 in the growth and development of *A. oligospora*.

Autophagy and nitroxidative stress both promote the clearance of invading pathogens and intertwine with each other. TLR4 activates autophagy and causes nitroxidative stress through downstream signal pathways after engaging pathogen-associated molecular patterns (PAMPs). The review article by Zhang et al. summarized signaling pathways that connected TLR4, autophagy, and nitroxidative stress in infectious diseases, and discussed their triangular relationships that affect cellular homeostasis.

## Conclusion and perspective

This Research Topic provides updated knowledge into current understanding about the interaction between host autophagy and pathogens. Further studies are required to understand the pathogen-specific autophagy pathways and the crosstalk between autophagy and other signaling pathways in the host against pathogen infections.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

## Publisher's Note



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ORIGINAL RESEARCH
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# Beclin-1–Dependent Autophagy Improves Outcomes of Pneumonia-Induced Sepsis

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**Objective:** We previously demonstrated that promoting Beclin-1–dependent autophagy is cardiac protective during endotoxemia shock, suggesting that autophagy-based approaches may become a promising therapeutic strategy for sepsis. In this study, we applied both genetic and pharmacological approaches to evaluate whether Beclin-1 activation improves sepsis outcomes in a model of pneumonia-induced sepsis.

**Methods:** Sepsis was induced in mice by *Klebsiella pneumoniae* infection *via* intubation, and outcomes of clinical sickness scores, systemic infection, inflammation, survival, and pulmonary pathology were examined. Evaluation of Beclin-1 activation was achieved by comparing strains of C57BL/6J wild type and *Becn1F121A* that carries a transgenic expression of Beclin-1–active mutant F121A, and by comparing animal groups treated with Beclin-1–activating peptide, Tat-beclin-1 peptide (TB-peptide), or with vehicle control. The status of autophagy in the lung tissue was examined in autophagy reporter mice, CAG-RFP-EGFP-LC3, by fluorescence microscopy.

**Results:** Pulmonary infection by *K. pneumoniae* produced an insufficient, maladaptive autophagy in the lung. Activation of Beclin-1 by forced expression of active mutant *Becn1F121A* or by treatment with TB-peptide enhanced autophagy and significantly reduced sickness scores, systemic infection, and circulating and pulmonary cytokine production. Both approaches demonstrated notable benefits in limiting post-infection pathogenesis in the lung, such as decreases in alveolar congestion, hemorrhage, infiltration of inflammatory cells, and alveolar wall thickness.

**Conclusion:** Data suggest that targeted activation of Beclin-1 alleviates adverse outcomes of pneumonia-induced sepsis, and thus, possess a therapeutic potential.

**Keywords: autophagy, pneumonia, sepsis, infection, inflammation**

# INTRODUCTION

Sepsis is a life-threatening condition of organ dysfunction caused by a deregulated host response to infection (Singer et al., 2016). Despite improvements in antibiotic therapies and critical care techniques (Levy et al.), sepsis remains a leading cause of death in critical care units (Singer et al., 2016), and its reported incidence is still increasing (Iwashyna et al., 2010). Understanding of the pathological mechanisms and exploration of new therapeutic interventions for sepsis are in urgent need.

Research in our laboratory has been using the heart as a model to investigate the pathophysiologic mechanisms of sepsis-induced multi-organ failure. We previously demonstrated that sepsis triggers damage in mitochondria, resulting in an overproduction of mitochondria-derived danger-associated molecular patterns (DAMPs), such as mitochondrial reactive oxygen species (mtROS) and fragmented mitochondrial DNA (mtDNA) (Zang et al., 2007; Zang et al., 2012a; Zang et al., 2012b). These harmful molecules exacerbate myocardial inflammation and cardiac dysfunction during sepsis (Zang et al., 2012b; Yao et al., 2015). We recently examined the impact of autophagy, a survival lysosome-dependent process of removing damaged proteins and organelles (Mizushima and Levine, 2010), on cardiac performance during endotoxemia induced by lipopolysaccharide (LPS), a major pathogen-associated molecular patterns (PAMPs) from gram-negative bacteria (Sun et al., 2018). We discovered that enhancing autophagy *via* the specific activation of Beclin-1, a universally expressed autophagy initiation factor (Liang et al., 1998; Liang et al., 1999), protects mitochondria, reduces mitochondrial DAMPs, and alleviates inflammation in the heart during endotoxemia (Sun et al., 2018). More importantly, the specific activation of Beclin-1, either genetically or pharmacologically, significantly improves cardiac performance under the challenge of septic shock by LPS (Sun et al., 2018). This study leads us to postulate that the targeted activation of autophagy factors may become an effective approach to boost adaptive autophagic responses, and thus, improves outcomes in sepsis.

Clinically, pneumonia-induced sepsis is one of the most common sepsis etiologies and is associated with the highest rate of mortality (Esper et al., 2006; Mayr et al., 2014). To enhance the clinical relevance of our investigation, we established a preclinical mouse model of pneumonia-induced sepsis according to literature, in which sepsis is induced by an intratracheal injection of bacteria (Zang et al., 2012a; Zang et al., 2012b). In the studies summarized in this report, we evaluated the impact of Beclin-1–dependent autophagy on the outcomes after pneumonia-induced sepsis by both genetic and pharmacologic approaches. Our results suggest that autophagy-based approaches provide a promising therapeutic potential for sepsis.

# MATERIALS AND METHODS

## Experimental Animals

Wild type C57BL/6 mice were obtained from Charles River laboratories (Massachusetts, MA) and in-campus mouse breeding core facility at The University of Texas Southwestern Medical Center (UTSW). All animals were conditioned in-house for 5 to 6 days after arrival with commercial diet and tap water available at will. Mouse strains carrying autophagy reporter CAG-RFP-EGFP-LC3 (Li et al., 2014), a F121A mutation in *beclin-1* (*Becn1*$^{F121A/F121A}$) (Fernandez et al., 2018), and haploinsufficient for *beclin-1* (*Becn1*$^{+/-}$) (Qu et al., 2003) were previously developed. Animal work described in this study was reviewed and conducted under the oversight of UTSW Institutional Animal Care and Use Committee and conformed to the "Guide for the Care and Use of Laboratory Animals" when establishing animal research standards.

## Pneumonia-Related Sepsis Model

(1) Preparation of inoculum: Upon received, *Klebsiella pneumoniae* type 3 (ATCC, Rockville, MD, catalog number 43816) was inoculated into 2 ml nutrient broth medium (NBM) (Difco nutrient broth, BD Diagnostic, Burlington NC; supplier number 23400) and amplified on shaker incubator at 37°C for overnight. 0.5 ml of this bacterial suspension was further cultured in 50 ml NBM at 37°C for about 3.5 h, growing to log phase till the $OD_{600}$ density reached 0.8 when measured by spectrophotometer. The culture was then aliquoted and stored at 80°C until used. To prepare viable bacteria used for infection, the frozen bacteria stock was thawed, inoculated, and cultured to log phase as described above. This bacterial preparation was kept at 4°C and was ready to infect animals within the next three days. To determine the colony-formation units (CFU), a small amount of this bacterial suspension was diluted by folds of $10^7$ and $10^8$, and multiple 100-µl diluents were plated on 10- cm nutrient agar plates (made with Difco nutrient agar from BD Diagnostic, Burlington NC; supplier number 21300). After incubation at 37°C for overnight, bacterial colonies were counted and the number of CFU was calculated. (2) Induction of sepsis: Sepsis was induced by endotracheal delivery of *K. pneumoniae via* intubation by otoscope. The dose of bacteria inoculated into the animals was $3 \times 10^7$ CFU per 25 grams body weight. The prepared bacterial suspension was adjusted to concentration at $3 \times 10^7$ CFU per 50 µl using NBM. 10- to 12-week-old male mice were weighed individually to determine the exact amount of bacterial suspension injected into each animal. Mice were anesthetized with 90 mg/kg ketamine/10 mg/kg xylazine cocktail *via* i.p. prior to intubation. Bacterial suspension, $3 \times 10^7$ CFU per 50 µl per 25 g body weight, was used for infection and the uninfected group received 50 µl PBS as control. Tat-Beclin-1 peptide (TB-peptide) was synthesized according to published sequence (Pietrocola et al., 2016) by NonoPep (Shanghai, China). In the case that when animals receiving TB-peptide treatment, it was administered i.p. at a dose of 16 mg/kg in 100µl PBS post inoculation.

## Post-Infection Monitoring and Evaluation of Sickness Conditions

Following infection, animals were monitored three times daily for a period of 5-day post infection. For each animal, the progress

of clinical sickness was recorded according to a pre-designed scoring system that evaluates the parameters of overall physiological conditions, appearance, movements, behavior, respiration, and other abnormalities (**Table 1**). The assessment allowed the determination of humane endpoints in survival studies, in which a total score above 6 or a single category score above 3 indicates fatality. In addition, this rating system allowed the comparison of an overall progress in sickness between groups with or without a genetic trait or a treatment.

## Evaluation of Systemic Infection

When animals were sacrificed, blood was collected using Vacutainer rapid serum tubes (RST) (BD Diagnostics, Franklin Lakes, NJ; catalog number 368774). Organs were harvested and homogenized in PBS. The presence of bacterial infection was examined by culturing the blood or tissue lysates on nutrient agar plates for overnight at 37°C. Numbers of colony formation were normalized with the volume of blood or with the amount of protein in tissue lysates.

## Histology Analysis of Lung Injury

Fresh lung tissues were perfused in PBS, followed by fixation in 4% paraformaldehyde, and then left in the fixation buffer for 24 h at 4°C. For dehydration, fixed tissues were first transferred to 10% sucrose/PBS for 24 h, then to 18% sucrose/PBS for another 24 h, and both steps were performed at 4°C. Tissue samples were embedded in OCT, sectioned at 8 μm, air-dried, and stored at −80°C until used. Frozen slides were thawed, rehydrated, and subjected to histological staining. Lung injury were quantified by an investigator blinded to the treatment groups as described previously (Jiang et al., 2017). In brief, the following four pathological changes were measured and normalized by the total areas examined: alveolar congestion, hemorrhage, infiltration of inflammatory cells or aggregation of neutrophils in air space or the vessel wall, and alveolar wall thickness. Twenty random high-power fields were examined per animal.

## Detection of Autophagy by Fluorescence Microscopy

Autophagy in the lung tissue was evaluated using the mouse strain of CAG-RFP-EGFP-LC3 (Li et al., 2014). OCT-embedded tissue slides were sealed with DAPI/antifade mounting solution (Thermo Fisher Scientific, Rockford, IL; catalog number 36931) and examined under Zeiss Axiovert 200M inverted fluorescence microscope at 20× magnification.

## Preparation of Serum and Tissue Lysates and Cellular Fractions

Freshly collected blood was immediately centrifuged at 3,000*g* for 15 min at 4°C to isolate serum. The serum preparations were then allocated and stored at −80°C until analyzed. Tissues were harvested, washed in PBS, snap clamp frozen, and kept at −80°C. Tissue lysates were prepared using tissue protein extraction reagent (Thermo Fisher Scientific, Rockford, IL; catalog number 78510). Protein concentrations were quantified using detergent compatible Bradford assay kit (Thermo Fisher Scientific, Rockford, IL; catalog number 23246).

## Measurements of Cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine levels in serum or in total tissue lysates were measured using Bio-Plex Mouse Cytokine Panel A 6-Plex (Bio-Rad, Hercules, CA; catalog number M6000007NY) according to vendor's instructions. Results were normalized by volume of serum samples or by protein amount in tissue lysates.

## Statistical Analysis

Results were expressed as mean ± SEM using the indicated number of experiments or mice. Student t-tests were applied

**TABLE 1** | Criteria of Clinical Sickness Scores.

| | | Normal | Mild | Moderate | Severe |
|---|---|---|---|---|---|
| **Score** | | **0** | **1** | **2** | **3** |
| Body Weight | | 0-5% decrease | 6-10% decrease | 11-15% decrease | >20 % decrease |
| Body Condition Score | | good muscle mass & body fat | slight loss of muscle mass & body fat | moderate loss of muscle mass & body fat | pelvic bones, ribs, and/or spine visible |
| Appearance | Posture | Balanced & symmetrical | Slight asymmetry | Moderate difference from cohorts | hunched or asymmetrical |
| | Coat | well groomed with sheen | loss of sheen & slightly ruffled; mild dermatitis | dull, rough appearance; moderate dermatitis | ungroomed/unkempt, and thin; persistent, nonhealing dermatitis >20% body area |
| | Eyes | Bright, clear, no discharge | slightly closed / no discharge | slightly closed with discharge and/or swelling | closed with discharge or open with swelling |
| Movement | | moving well with no impediment | moving slowly or with slight difficulty / irregularity | moving slowly with difficulty or restlessness | not moving; staying away from cohorts; obsessive activity; not able to obtain food and/or water normally |
| Natural (Unprovoked) Behavior | | interacts with cohorts & environment | mild interest in environment & minimal interaction with cohorts | isolated from cohorts & disinterested in environment | vocalizing or unaware of surroundings, Immobile or hyper reactive; self-mutilation |
| Respiration | | normal (163-220 breaths/min.) | mild increase or decrease (difference <20 %) | moderate increase, or labored (+/- 25%) | rapid or severely labored (+/-50%) |
| Other Signs | | no other issues | mild local issue e.g. scratch or licking a part of its body frequently | moderate systemic issue (e.g. slightly swollen abdomen) | serious systemic issue (e.g. prolapsed organ; rectal prolapse; bleeding) |

for comparisons between groups. Nonparametric Kruskal-Wallis H test was applied to compare the mean rank of multiple pain score groups. Kaplan-Meier survival curves and relevant Log-rank statistical test were applied in the survival study. Differences were considered statistically significant when $p \leq 0.05$, and all samples were tested at least in triplicate.

# RESULTS

## Beclin-1 Activation Boosts Autophagy Response in the Lung Under Septic Infection

In the pneumonia-induced sepsis model, mice were infected with gram-negative *K. pneumoniae via* intubation. Infection dose at $3 \times 10^7$ CFU per mouse resulted in a mortality rate about 60% to 70% during the 5-day post-infection period, and bacteremia was confirmed 24 h post infection. Based on published results as well as observations in our laboratory, male and female mice showed significantly different susceptibility to respiratory and systemic symptoms in the pneumonia-induced sepsis model (Kadioglu et al., 2011). Thus, male but not female mice were chosen for the experiments presented in this report.

Autophagy reporter mice, CAG-RFP-EGFP-LC3, were chosen to examine autophagy flux in the lung tissue of the pneumonia-induced sepsis model. In these mice, the expression of a tandem red fluorescent protein (RFP)-EGFP-LC3 fusion protein was constructed under the CAG promoter (Li et al., 2014). Taking the advantage of differences in acid sensitivity between RFP and EGFP, both EGFP and RFP fluorescence are present during autophagosomal maturation (pH 5.9), indicating the early step of autophagic flux, whereases RFP signals in acid autolysosomes (pH 4.5) which formation occurs in a later step.

A mouse strain with transgenic expression of active mutant F121A in *beclin-1*, *Becn1*$^{F121A/F121A}$, was utilized as a genetic approach to up-regulate autophagy (Fernandez et al., 2018). We generated a new strain by crossing *Becn1*$^{F121A/F121A}$ with CAG-RFP-EGFP-LC3, and its autophagy response in the lung under *K. pneumoniae* infection was compared with that in CAG-RFP-EGFP-LC3 mice. As shown in **Figure 1A**, mice were infected with *K. pneumoniae* and PBS was used in the uninfected group. In the lung tissue samples harvested 48 h post infection, the status of autophagy in areas of alveoli and bronchioles were evaluated under fluorescence microscopy. Presence of autophagosomes was shown in yellow, due to overlying the colors of green from EGFP emission and red from RFP. Autolysosomes were shown in red, due to signals from RPF. DAPI for nucleic acid staining was used to visualize the location of cells. In CAG-RFP-EGFP-LC3 mice, an infection-associated reduction was evident in the autophagosome population, suggesting an inhibition in initiating autophagy flux. As expected, forced expression of active mutant of Beclin-1 resulted in a significantly enhanced signal of autophagosomes in CAG-RFP-EGFP-LC3 (X) *Becn1*$^{F121A/F121A}$ mice, in consistent with the function of Beclin-1 as an autophagy initiation factor.

Previous research from others and ours showed that a cell-permeable Beclin-1 activating peptide, Tat-beclin-1 (TB-peptide), is a pharmacological approach of promoting autophagy *in vitro* and *in vivo* (Shoji-Kawata et al., 2013; Pietrocola et al., 2016; Shirakabe et al., 2016; Sun et al., 2019; Sun et al., 2021). We evaluated its effects in the model of pneumonia-induced sepsis, choosing a dose that inducing sufficient autophagy without causing detectable toxicity (Sun et al., 2019; Sun et al., 2021). As shown in **Figure 1B**, CAG-RFP-EGFP-LC3 mice were given bacterial infection and received the treatment of TB-peptide (16 mg/kg, i.p.) 1 h post-infection. PBS was given in the vehicle control groups. Lung tissue samples harvested 24 and 48 h post infection were evaluated under fluorescence microscopy. In the vehicle-treated groups, the infection caused a visible decrease in the population of autophagosomes in the areas of both alveoli and bronchioles, suggesting an inhibitory effect on starting autophagy. However, the treatment of TB-peptide boosted autophagy, as demonstrated by the intensified signals of autophagosomes in the areas of alveoli and bronchioles. These results confirmed that TB-peptide promotes autophagy in the lung under the challenge of septic infection.

## Beclin-1–Dependent Autophagy Controls Local and Systemic Infection and Reduced Sickness Scores

To examine the impacts of Beclin-1–dependent autophagy on sepsis outcome after pneumonia infection, mice of wild type (WT) and *Becn1*$^{F121A/F121A}$ were given *K. pneumoniae* infection. In parallel experimental groups, WT mice received the treatment of TB-peptide. Degrees of local infection in the lung tissue and systemic infection in blood and in distant organs, such as heart and liver, were compared. As shown in **Figure 2A**, examined at 48 h post infection, a 10-fold or more decreases in colony formation were observed in mice with Beclin-1 activation, either *Becn1*$^{F121A/F121A}$ mice or TB peptide–treated mice, when compared with that in the WT counterparts.

Additional parallel groups of mice were monitored for a period of 5-day post infection, clinical sickness scores were evaluated by observing the body physiological conditions, appearance, movements, behavior, respiration, and other abnormalities, as described in the method section (**Table 1**). Statistical analysis was applied according to published methods (La Colla et al., 2009), and data revealed that activation of Beclin-1 either genetically (*Becn1*$^{F121A/F121A}$) or pharmacologically (TB-peptide) significantly reduced the overall sickness scores in response to pneumonia-induced sepsis (**Figure 2B**).

Lastly, survival rates of infected mice of WT, *Becn1*$^{F121A/F121A}$, and WT plus TB-peptide treatment were examined (**Figure 2C**). No statistical significance was detected when *Becn1*$^{F121A/F121A}$ mice or TB peptide–treated mice were compared with WT or vehicle-treated mice under the indicated experimental setting. However, a strain of haploinsufficient of Beclin-1 (*Becn1*$^{+/-}$) showed a significantly decreased survival rate when compared with the rest of the groups. The result suggests that Beclin-1 signal is at least essential for survival under the challenge of pneumonia-induced sepsis.

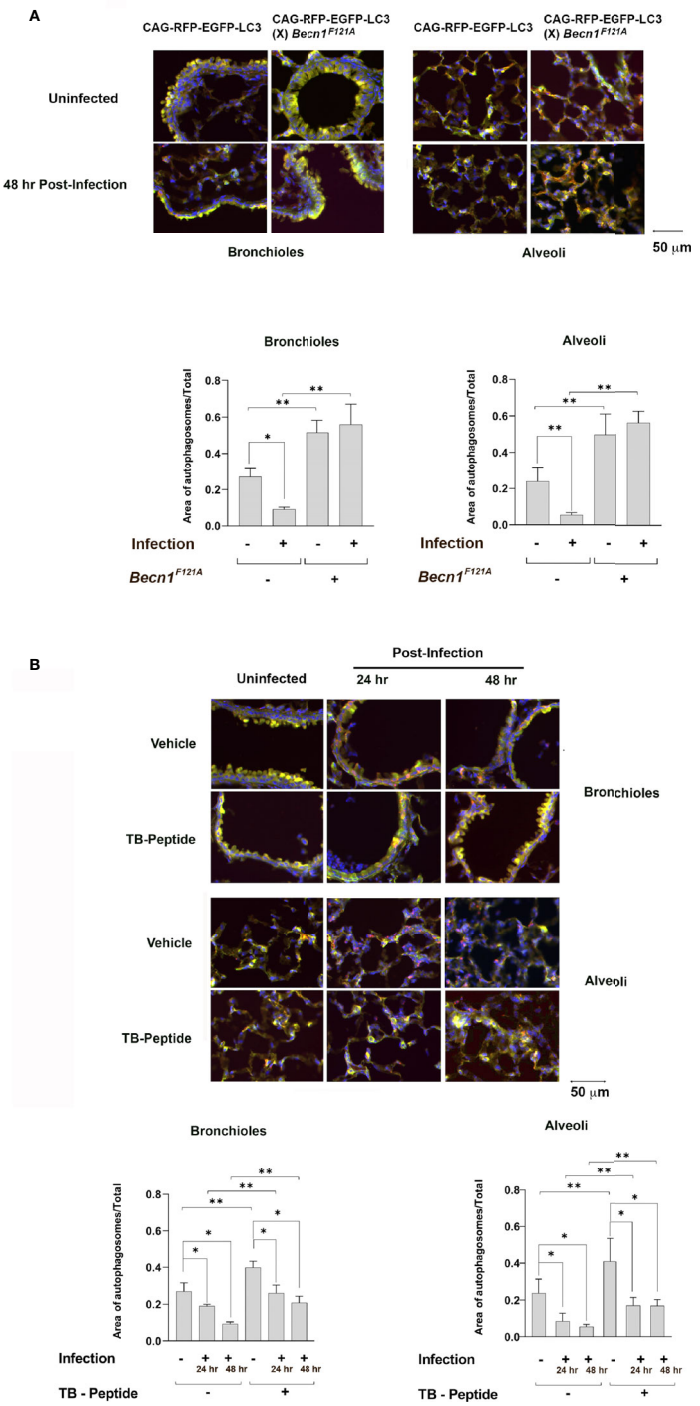


**FIGURE 1** | Lung autophagy in response to pneumonia-induced sepsis and the effects of Beclin-1 activation. **(A)** Autophagy reporter CAG-RFP-EGFP-LC3 mice or mice of CAG-RFP-EGFP-LC3 (X) *Becn1*$^{F121A/F121A}$ were given septic infection of *K.pneumoniae* (3X10$^{7}$ CFU/mouse) or PBS (uninfected) *via* intubation. Lung tissue samples were harvested 48 h post infection, and lung tissue slides were evaluated under fluorescence microscopy. Neutral autophagosomes, shown in yellow, allow emissions from both EGFP (green) and RFP (red). Acid autolysosomes, shown in red, are due to signals from RPF. DAPI in blue indicates nucleic acid staining. **(B)** CAG-RFP-EGFP-LC3 mice receiving *K. pneumoniae* infection or PBS (uninfected) were given TB-peptide (16.5 mg/kg, i.p.) or PBS control vehicle 1 h post-infection. Lung tissue samples were harvested 24 and 48 h post infection, and lung tissue slides were evaluated under fluorescence microscope as described in **(A)** In both **(A, B)** autophagosome areas were quantified as ratios to the total area examined. Images are representative of n ≥ 5 animals per group and ten random high-power fields were examined per animal. All values are means ± SEM. Significant differences are shown as * for uninfected *vs*. infected, ** for WT *vs*. *Becn1*$^{F121A/F121A}$ **(A)** or for TB-peptide treated *vs*. untreated **(B)** ($p < 0.05$, student t-test).

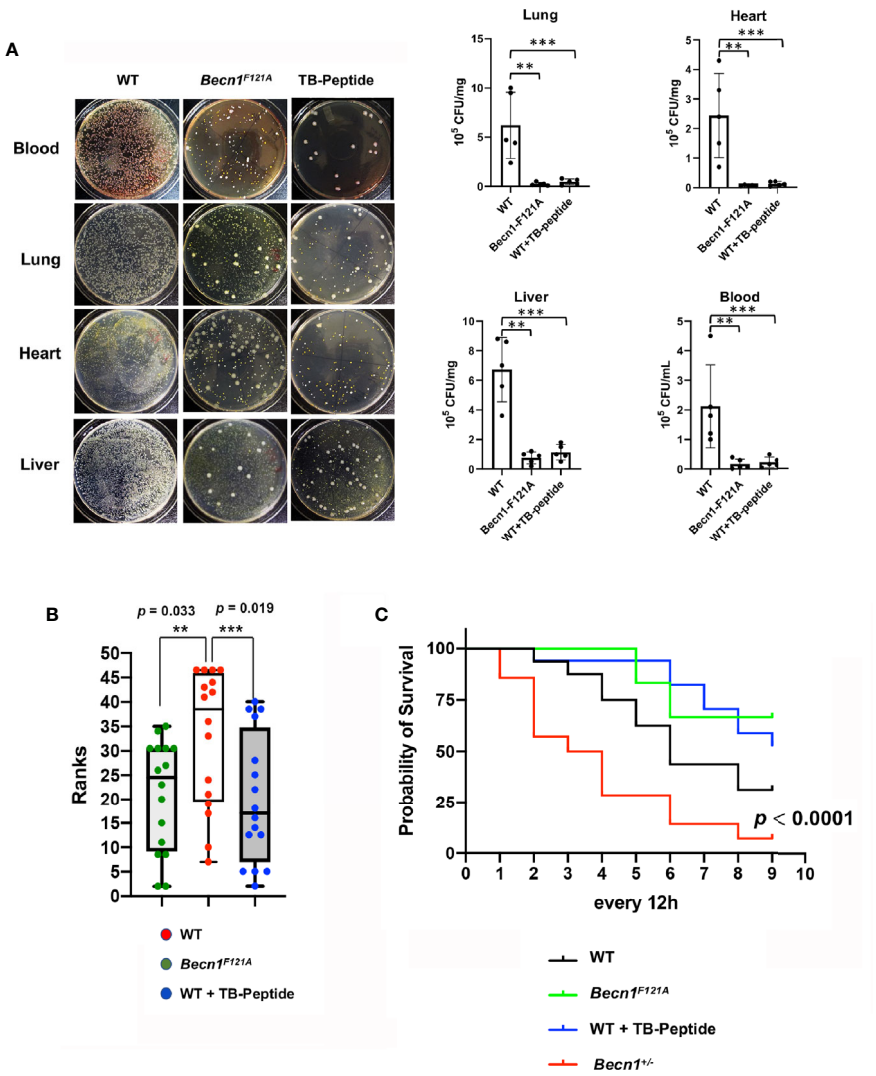


**FIGURE 2** | Effects of Beclin-1–dependent autophagy on local and systemic infection, clinical pain scores, and survival in pneumonia-induced sepsis. Mice of WT, *Becn1*$^{F121A/F121A}$, or *Becn1*$^{+/-}$ were given septic infection by *K. pneumoniae* (3X10$^{7}$ CFU/mouse, intubation). A parallel group of infected WT mice were treated with TB-peptide (16.5 mg/kg, i.p.) 1 h post-infection. **(A)** To evaluate infection, blood was collected, and organs harvested 48 h post infection. Blood serum and tissue lysates were plated on nutrient agar plates and incubated at 37°C for overnight. Bacterial colony formation was normalized with serum volume or lysate protein concentration. All values are means ± SEM. Significant differences are shown as ** for WT *vs*. *Becn1*$^{F121A/F121A}$, and *** for TB-peptide treated *vs*. untreated ($p < 0.05$, n = 5, student t-test). **(B)** Post-infection physiological conditions were monitored and scored according to **Table 1** at indicated time points. Kruskal –Willis H test for mean rank adjusted for multiple testing was applied, and differences were considered statistically significant when $p \leq 0.05$ (** for WT *vs*. *Becn1*$^{F121A/F121A}$ and *** for TB-peptide treated *vs*. untreated). **(C)** Survival curves were compared by a log-rank test, and differences were considered statistically significant when $p \leq 0.05$. In B and C: n = 16 for WT, n = 12 for *Becn1*$^{F121A/F121A}$, n = 17 for WT plus TB-peptide, and n = 14 for *Becn1*$^{+/-}$.

## Beclin-1–Dependent Autophagy Attenuates Pulmonary and Systemic Cytokine Productions in Pneumonia-Induced Sepsis

Whether increasing Beclin-1–dependent autophagy has an effect on the control of overwhelming inflammation induced by pneumonia-induced sepsis was also addressed. Mice of WT or *Becn1*$^{F121A/F121A}$ were given septic infection by *K. pneumoniae*. In parallel groups of WT mice, TB-peptide or control vehicle PBS was administered 1 h post infection. At 48 h post-infection, blood was collected and lung tissue harvest. Cytokines present in serum and in the lung tissue lysates were compared. As shown in **Figures 3A, B**, activation of Beclin-1 by forced expression of *Becn1*$^{F121A/F121A}$ or by TB-peptide provided similar levels of substantial reduction in circulating cytokines, as well as in pulmonary cytokines, indicating that Beclin-1–dependent autophagy provides anti-inflammatory effects during sepsis.

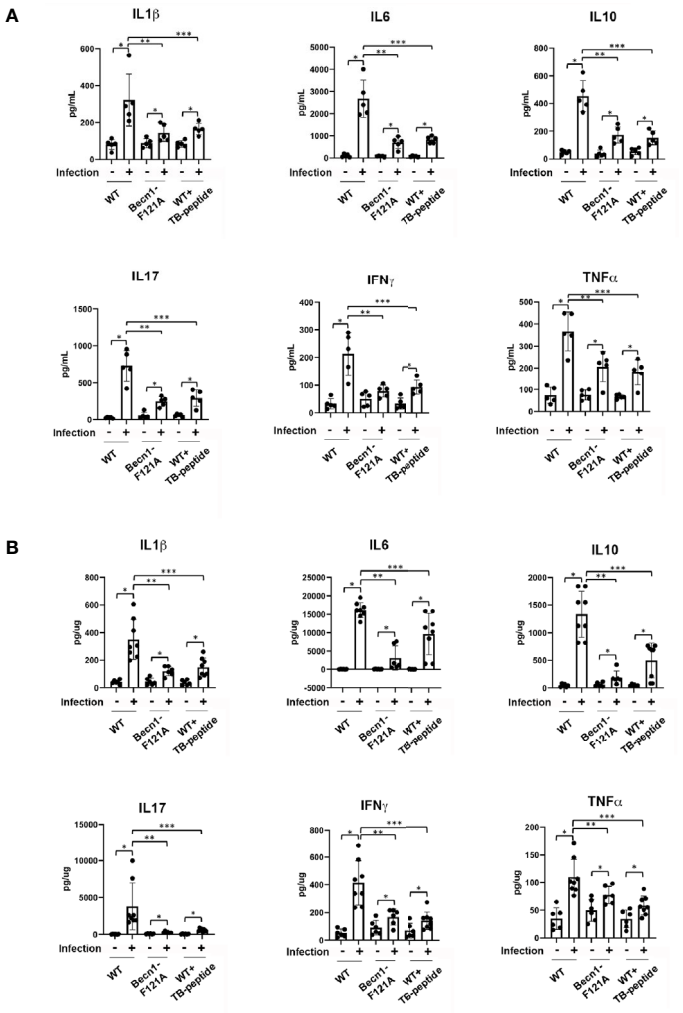


**FIGURE 3** | Beclin-1–dependent autophagy attenuates systemic cytokines and pulmonary cytokines in pneumonia-induced sepsis. Mice of WT and *Becn1*$^{F121A/F121A}$ were given septic infection by *K. pneumoniae* (3X10$^7$ CFU/mouse, intubation) or PBS (uninfected). In parallel groups, infected and uninfected mice were treated with TB-peptide (16.5 mg/kg) or PBS vehicle 1 h post-infection. Serum was collected and lung tissue harvested 48 h post-infection. Cytokines in serum **(A)** and in lung tissue lysates **(B)** were measured by ELISA assays. All values are means ± SEM. Significant differences are shown as * for uninfected *vs*. infected, ** for WT *vs*. *Becn1*$^{F121A/F121A}$, and *** for TB-peptide treated vs. untreated ($p < 0.05$, n ≥ 5, student t-test).

## Beclin-1–Dependent Autophagy Alleviates Pulmonary Pathology in Pneumonia-Induced Sepsis

Since infection was induced in the lung in this sepsis model, pulmonary pathology was examined according to criteria described in literature (Jiang et al., 2017). Degrees of alveolar congestion, hemorrhage, infiltration of inflammatory cells or aggregation of neutrophils in air space or the vessel wall, and alveolar wall thickness were compared between infected mice of WT, *Becn1*$^{F121A/F121A}$, and WT receiving TB-peptide treatment. As shown in **Figure 4A**, the lung tissue slides were subjected to Hematoxylin and Eosin (H&E) staining. At 48 h post infection, areas of bacterial infection, infiltration of immune cells, hemorrhage, alveolar congestion, and increases in alveolar wall thickness were dramatic in the lung of WT mice, in contrast to those in mice of *Becn1*$^{F121A/F121A}$ and WT treated with TB-peptide. Especially, presence of bacterial infection was barely detected in the latter two groups, suggesting a stronger bactericidal activity in response to Beclin-1 activation. Quantification of those pathological areas in percentage of the total areas examined showed that activation of Beclin-1, either by mutation at F121A or by treatment with TB-peptide, significantly reduced lung injury in response to the septic challenge (**Figure 4B**).

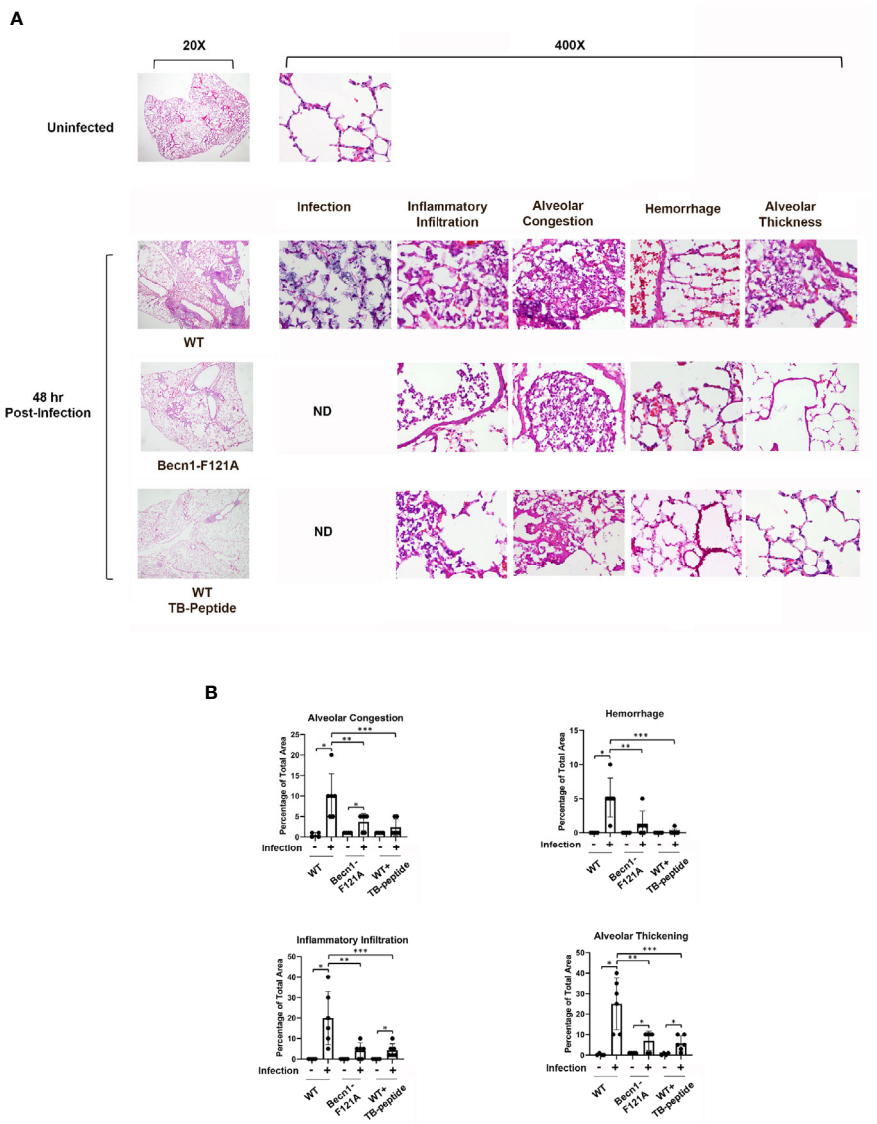


**FIGURE 4** | Beclin-1–dependent autophagy limits the progress of pulmonary pathology in pneumonia-induced sepsis. Mice of WT and *Becn1*$^{F121A/F121A}$ were given septic infection by *K. pneumoniae* (3X10$^{7}$ CFU/mouse, intubation) or PBS in the uninfected group. In parallel groups, infected and uninfected WT mice were treated with TB-peptide (16.5 mg/kg, i.p.) or PBS vehicle 1 h post-infection. Lung tissue was harvested 48 h post-infection, and tissue slides were stained with Hematoxylin and Eosin (H&E). **(A)** H & E images of lung histology were analyzed under Olympus BX43 microscope at magnifications of 20X and 400X. Images are representative of n=5 per group. **(B)** Areas of inflammatory infiltration, alveolar congestion, alveolar thickness, and hemorrhage were quantified as percentage of total area using Image J All values are means ± SEM. Significant differences are shown as * for uninfected vs. infected, ** for WT *vs*. *Becn1*$^{F121A/F121A}$, and *** for TB-peptide treated *vs*. untreated ($p < 0.05$, n = 5, student t-test).

## DISCUSSION

We previously showed that activation of autophagy initiator Beclin-1 provided a benefit of cardiac protection during endotoxemia (Sun et al., 2018). In this investigation, we designed a study to further address whether targeted activation of Beclin-1 possesses therapeutic potential for sepsis using a previously established model of pneumonia-induced sepsis, which has more clinical relevance (Zang et al., 2012a; Zang et al., 2012b). In this model, pulmonary infection of gram-negative *K. pneumoniae* was introduced *via* intubation, and sepsis occurrence was confirmed by systemic infection. The impact of Beclin-1 activation was addressed using both genetic and pharmacological approaches. In our experimental setting, we found that promoting Beclin-1–dependent autophagy significantly improved sepsis outcomes, including reductions in sickness scores, infection, and inflammation. Examination of lung pathology revealed that enhanced Beclin-1 signaling

alleviated lung injury, as shown by limiting the degrees of alveolar congestion, hemorrhage, infiltration of inflammatory cells, and alveolar wall thickness, and therefore, alleviated lung injury. Together, the data provide a strong support for the notion that Beclin-1–dependent autophagy presents therapeutic values to improve sepsis outcomes.

In this study, we used a strain of autophagy reporter mice CAG-RFP-EGFP-LC3 to examine the status of lung autophagy post septic infection. We found that infection hindered autophagy flux at the time points examined, suggesting an insufficient, maladaptive autophagy response (**Figure 1**). A previous study using a mouse model of polymicrobial sepsis, cecal ligation and puncture (CLP), showed elevated autophagy in the lung tissue (Lee et al., 2014). This discrepancy is likely due to the differences in the time points chosen for experiments and/or the septic responses in different models. Autophagy is triggered as a defense mechanism during the early stage of sepsis but attenuated with the progression in severity. For example, signals detected in the heart of a mouse model of endotoxemia and in the liver of CLP sepsis indicate that a decline in autophagy is tightly associated with the occurrence of organ failure (Chien et al., 2011; Takahashi et al., 2013; Sun et al., 2018). In the study reported here, the lung tissue was evaluated at 48 h post infection, at which time point signs of obstructed lung function such as rapid and labored respiration were observed. Nonetheless, we confirmed that activation of Beclin-1 either genetically by a forced expression of activation mutant *Becn1F121A* or pharmacologically by an activating peptide, TB-peptide, indeed dramatically enhanced autophagy signaling in the lung under the condition of infection.

Bacterial clearance by macrophages *via* phagocytosis is the first step of defense to remove invaded pathogens. Enhanced bactericidal activity of macrophages was previously observed in *Becn1F121A* mice, which carry an active mutant Beclin-1 (Fernandez et al., 2018). The anti-pathogenic function of autophagy in macrophage defense capacity was also suggested in models with reduced expression of autophagy factors. For example, atg7 deficiency led to impaired host defense in macrophages, and thus resulting in magnified infection, inflammation, and worsened injuries in the lung of animals infected by *K. pneumoniae* (Ye et al., 2014). Consistent with these studies, we observed that activate mutant Beclin-1 *Becn1F121A* or treatment with TB-peptide provided a significant reduction in infection, locally in the lung and systemically in blood and in distant organs, in response to infection by *K. pneumoniae* (**Figures 2** and **4**). In this report, infection was examined at the tissue level rather than in cell types such as in macrophages. In our ongoing investigations, we indeed observed that TB-peptide improved bactericidal activities in cultured macrophages (data not shown). However, roles of other cell types in this process of pathogen clearance may not be excluded since changes in autophagy alter metabolic and inflammatory responses in immune cells as well as non-immune cells of the host body (Painter et al., 2020). Crosstalk between different cell types plays an important role in maintaining the microenvironment that is critical for pathogen survival. Further investigations regarding how host autophagy may be utilized to eradicate evaded microbial pathogens will reveal new opportunities for developing effective therapies for sepsis.

We detected that promoting Beclin-1–dependent autophagy has an anti-inflammatory effect in this model of pneumonia-induced sepsis, as shown by quantification of cytokine production (**Figure 3**) and the evaluation of lung injury (**Figure 4**). The results are consistent with previous observations in models of endotoxemia (Sun et al., 2018) and CLP (Lee et al., 2014). Autophagy interacts with inflammation at multiple layers, and one of those pivotal component mediators are ROS. It is well known that sepsis and acute injuries trigger a surge in ROS production, leading to oxidative stress that contributes to the induction of overwhelming inflammation (Zang et al., 2007; Zang et al., 2012b). Though intracellular ROS are generated at multiple locations, ROS from mitochondria constitute a main portion. In mitochondria, mtROS are produced as by-products of respiratory chain reaction. Upon challenge under pathological conditions, functional deficient and/or structural disrupted mitochondria release various harmful molecules including mtROS that function as DAMPs to stimulate inflammation. Autophagy, on the other hand, can remove dysfunctional organelles, such as mitochondria, through its "self-eating" process, and thus, to control the production of mitochondria-derived DAMPs. In mice subjected to endotoxemia, mitochondrial damage induces inflammation *via* activation of NLRP3-dependent inflammasome in macrophages (Nakahira et al., 2011). In the same model, stimulating Beclin-1–dependent autophagy improves the quality control of mitochondria and reduces inflammation in the heart (Sun et al., 2018), suggesting a mechanism of using autophagy as an approach to mitigate inflammation. We expect similar events occurred in the lung during pneumonia-induced sepsis, and detailed molecular signals in various cell types of the lung tissue will be further interrogated in future studies. It is also noteworthy that the role of autophagy in pathogenesis varies according to different types of conditions, as deleterious side of autophagy was reported in chronic problems such as pulmonary hypertension (Teng et al., 2012) and cardiac hypertrophy (Zhu et al., 2007).

Developing strategies that harness autophagy as effective therapies has received substantial attention in recent years. However, most available autophagy-based approaches have often focused on reagents with broad-spectrum impacts but with limited specificity to autophagy factors (Laplante and Sabatini, 2012). Thus, autophagy-inducing approaches with target specificity and fewer toxic side effects are still limited. A milestone development in this area was the development of TB peptide, which specifically activates Beclin-1. This peptide has been shown to provide beneficial effects in several preclinical disease models (Shoji-Kawata et al., 2013; Pietrocola et al., 2016; Shirakabe et al., 2016). Our evaluation of TB-peptide previously in the endotoxemia model (Sun et al., 2018) and currently in the pneumonia-induced sepsis model have provided novel evidence supporting the notion that pharmacological approaches targeting Beclin-1 signaling possess important therapeutic

values for sepsis. In the studies described in this report, our data showed that TB-peptide provided evident benefits in controlling infection and inflammation (**Figures 2** and **4**). It remains unclear whether this treatment can improve survival since no statistical significance was detected (**Figure 2**). Possible reasons behind this observation could be that the sample size is not large enough to reach statistical significance, or the effect of this treatment may only slow down the progress of sepsis symptoms, and therefore, potential benefits in a longer post-infection time course remains to be determined. Nonetheless, the results suggest that TB-peptide, used by itself or in combination with other therapies, has a potential to improve outcomes after sepsis. Additional evaluation of this treatment in other sepsis models or acute injury models will help to fully validate its therapeutic effectiveness and to understand its mechanisms of action.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

This study has been reviewed and conducted under the oversight of UTSW Institutional Animal Care and Use Committee and conformed to the "Guide for the Care and Use of Laboratory Animals" when establishing animal research standards.

## AUTHOR CONTRIBUTIONS

QZ conceived the project, designed the study, and wrote the manuscript. AN, MK, XD, and YS conducted the experiments and contributed to the data analysis. All authors contributed to the article and approved the submitted version.

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frontiers in Cellular and Infection Microbiology

MINI REVIEW
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# Bacterial Subversion of Autophagy in Cystic Fibrosis

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Cystic fibrosis (CF) is a genetic disease affecting more than 70,000 people worldwide. It is caused by a mutation in the *cftr* gene, a chloride ion transporter localized in the plasma membrane of lung epithelial cells and other organs. The loss of CFTR function alters chloride, bicarbonate, and water transport through the plasma membrane, promoting the production of a thick and sticky mucus in which bacteria including *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* can produce chronic infections that eventually decrease the lung function and increase the risk of mortality. Autophagy is a well-conserved lysosomal degradation pathway that mediates pathogen clearance and plays an important role in the control of bacterial infections. In this mini-review, we describe the principal strategies used by *P. aeruginosa* and *B. cenocepacia* to survive and avoid microbicidal mechanisms within the autophagic pathway leading to the establishment of chronic inflammatory immune responses that gradually compromise the lung function and the life of CF patients.

**Keywords: cystic fibrosis, autophagy, subversion, *Burkholderia cenocepacia*, *Pseudomonas aeruginosa***

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## INTRODUCTION

Autophagy is a self-degradative process that plays a key housekeeping role in removing misfolded or aggregated proteins located in the cytosol. This cellular process contributes to the removal of damaged organelles including mitochondria, peroxisomes, and endoplasmic reticulum (Dikic and Elazar, 2018). Autophagy also plays an important role in the regulation of inflammasome activation, particularly in the removal of inflammasome-activating endogenous signals as well in the sequestration and remotion of inflammasome components (Harris et al., 2017). In innate immunity, autophagy plays a role in controlling the intracellular spread of cytosolic bacteria and restricting bacteria contained in vacuoles or phagosomes. During bacterial infection, infected cells form double-membrane compartments (known as autophagosomes) around free bacteria or associated to damaged vacuoles by intracellular pathogens that usually are delivered to lysosomes for their degradation (Huang and Brumell, 2014). As a cellular process, autophagy is highly efficient; nevertheless, some intracellular bacteria have evolved different strategies to avoid its degradation by the autophagic pathway.

## PATHOPHYSIOLOGY OF OPPORTUNISTIC INFECTIONS IN PATIENTS WITH CF

Cystic fibrosis (CF) is an autosomal recessive congenital disease (O'Sullivan and Freedman, 2009; Shteinberg et al., 2021) that principally affects lungs, pancreas, liver, kidneys, and intestine of at least 70,000 people worldwide (Jackson and Goss, 2018). The condition is due to a mutation in the *cftr* gene (Tsui et al., 1985), that codes a CF transmembrane conductance regulator (CFTR) involved in the transport of chloride and sodium ions, $HCO_3^-$, and water across the lung epithelia (Shteinberg et al., 2021). Defective CFTR function produces a thick and sticky mucus (Boyle, 2007) that rapidly clogs the lower airways in which diverse bacterial pathogens might produce infection and inflammation that gradually decrease the lung function (Blanchard and Waters, 2019), leading to the production of thick sticky mucus. Affected individuals develop shortness of breath, cough, and chronic infections that eventually decrease lung function, which increases the mortality risk. Several mutations are described in the *cftr* gene (Bareil and Bergougnoux, 2020). The most common mutation is the deletion of phenylalanine in the position 508 (F508del). This mutation is associated with inflammation and decreased autophagy (Luciani et al., 2010). The first bacterial pathogens associated with the lower airways of children with CF are non-typable *Haemophilus influenzae* and *Staphylococcus aureus* (Cox et al., 2010). This initial colonization is progressively replaced by *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* during adolescence and adulthood. Both of the latter opportunistic pathogens produce chronic infections that gradually reduce the lung function (Cox et al., 2010; Rossi et al., 2020; Rosales-Reyes et al., 2021). The inefficient bacterial clearance by affected individuals with CF is associated with a reduced bactericidal activity of macrophages, neutrophils, and respiratory epithelial cells (Yoshimura et al., 1991; Smith et al., 1996; Painter et al., 2006; Porto et al., 2011). In the mouse model, the phagocytic activity of alveolar macrophages with a deficiency in CFTR ($cftr^{-/-}$) is not affected; however, its lysosomes fail to acidify and kill internalized bacteria (Di et al., 2006). *B. cenocepacia* invades macrophages and resides in a vacuole (BcCV) that shows a delay in lysosomal fusion (Lamothe et al., 2007). The delay in the lysosomal fusion with the BcCV is more pronounced in macrophages defective in CFTR (Lamothe and Valvano, 2008). In addition, *P. aeruginosa* survives more efficiently in macrophages with defective CFTR function (Porto et al., 2011) due to a deficiency in its lysosomal acidification (Di et al., 2006). In this mini-review, we describe how the subversion of autophagy by two important bacterial pathogens, *B. cenocepacia* and *P. aeruginosa*, contributes to the establishment of chronic infections in individuals with CF.

## AUTOPHAGY IN CF

Autophagy is a cellular process that plays an important role in innate immunity, specifically by restricting the replication of bacterial pathogens contained in vacuoles or phagosomes. In CF, phagocytic cells increase the production of reactive oxygen species (ROS). The cells also increase the activation of the transglutaminase-2 (TGM2) that inactivates the Beclin1 (BECN1) complex resulting in an inefficient autophagy process (Luciani et al., 2010). Beclin1 is cross-linked by TGM2, and this new complex is sequestered in the cytosol to form aggresomes. The treatment of cells that express CFTR-F508del with Cysteamine corrects the autophagy deficiency by increasing the function of the BECN1 complex with a reduced level of sequestosome 1 (SQSTM1, also known as p62) (De Stefano et al., 2014; Ferrari et al., 2017). Cells with deficient autophagy in the airways of CF patients show accumulation of SQSTM1, a protein that works as an adaptor in the regulation of the formation and elimination of aggregates containing ubiquitinated proteins (Komatsu et al., 2007; Nezis et al., 2008). In addition, the accumulation of SQSTM1 at the endosomal level reduces the pool of the small GTPases Rab5 (Villella et al., 2013) and Rab7 (Gilardini Montani et al., 2019) that are essential for maturation to early and late endosomes, respectively. In addition, the dendrimer-based cysteamine analogue (PAMAM-$DEN^{CYS}$) partially rescues the function of cells carrying the F508del mutation. This analogue significantly reduces the aggresome bodies formation in IB3 cells (Brockman et al., 2017). Thus, the autophagy dysfunction could be exploited by intracellular pathogens such as *B. cenocepacia* or *P. aeruginosa* to survive and persist in eukaryotic cells (Porto et al., 2011; Assani et al., 2014).

### *Burkholderia cenocepacia*

*B. cenocepacia* is a nonfermenting, anaerobic Gram-negative bacterium that belongs to the *Burkholderia cepacia* complex (Bcc) (Mahenthiralingam et al., 2005). *B. cenocepacia* and *B. multivorans* are two opportunistic pathogens that cause infections in individuals with CF. *B. cenocepacia* produces a chronic infection that is characterized by the establishment of a strong inflammatory immune response and cell death (Kopp et al., 2012). This bacterial infection decreases lung function (Scoffone et al., 2017) and reduces the survival of colonized individuals (Isles et al., 1984; Tablan et al., 1985).

*B. cenocepacia* invades macrophages and epithelial cells in which it persists and replicates (Burns et al., 1996; Saini et al., 1999; Martin and Mohr, 2000). In CF epithelial cells, *B. cenocepacia* resides in autophagosomes that fail to fuse with lysosomes (Sajjan et al., 2006). In macrophages, *B. cenocepacia* survives in a membrane-bound vacuole (BcCV) (Burns et al., 1996; Martin and Mohr, 2000) in which the bacteria delays the lysosomal fusion with the BcCV (Lamothe et al., 2007). *B. cenocepacia* also modulates macrophage function through the translocation of bacterial effectors by their type VI secretion system (T6SS) to inactivate the small GTPase Rac1 and decrease the ROS production (Rosales-Reyes et al., 2012b). During this process, the overexpression of the T6SS damages the membrane of the BcCV allowing leakage of its content to activate the inflammasome NLRP3 (Rosales-Reyes et al., 2012a) (**Figure 1**). The damaged membrane of the BcCV could be a signal to induce

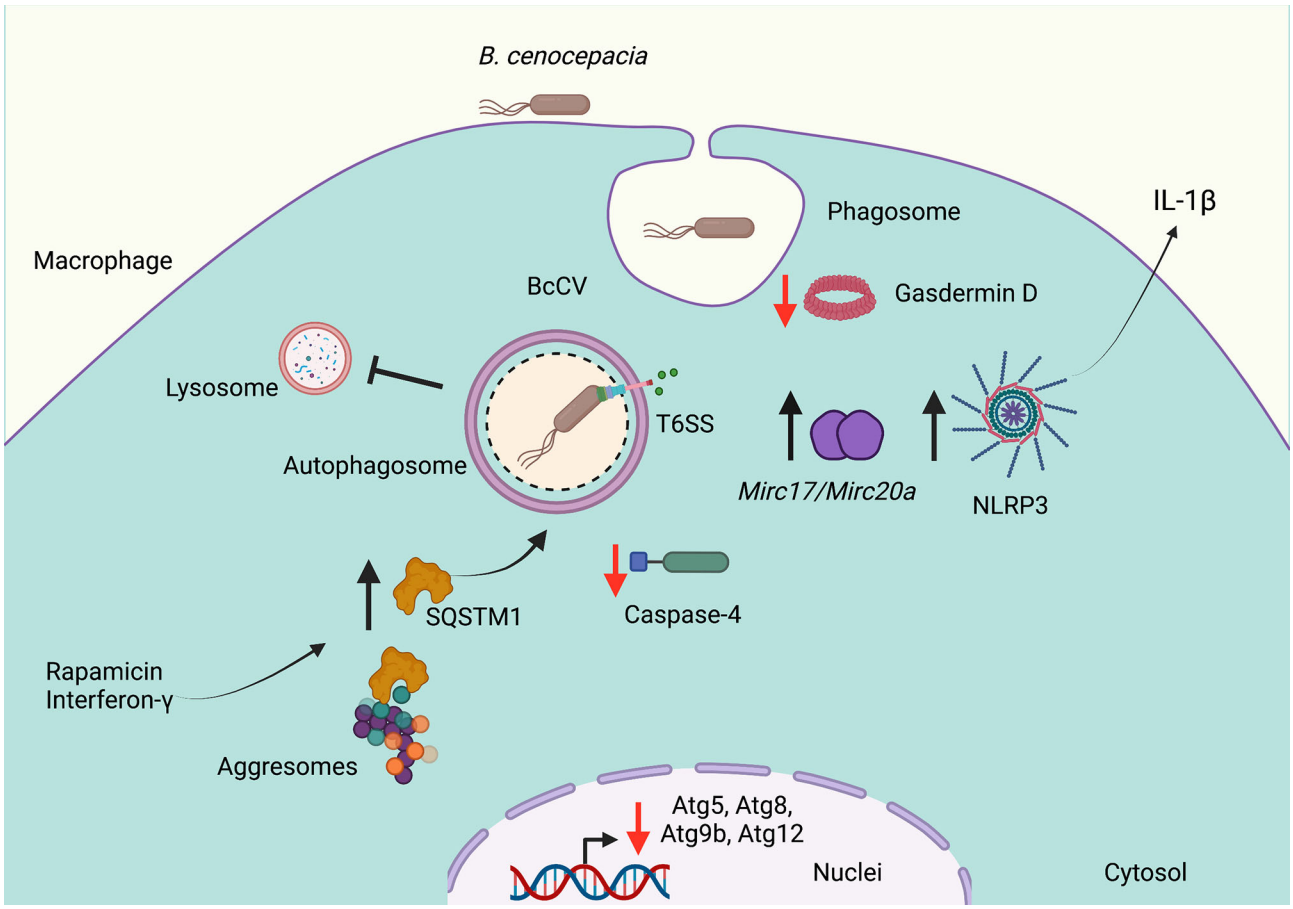


**FIGURE 1** | *Burkholderia cenocepacia* subverts autophagy in cystic fibrosis. *Burkholderia cenocepacia* invades phagocytic cells and resides in a vacuole (BcCV) that shows delayed fusion with lysosomes. The activity of the Type VI Secretion System (SST6) damages the membrane of the BcCV, allowing leakage of its content to activate the inflammasome NLRP3 and to release IL-1β. The damaged membrane of the BcCV might be surrounded by autophagosome membranes. Bacterial infections decrease the *Atg9b, Atg5, Atg12*, and *Atg8* transcription. The decrease of Gasdermin D and caspase-4 expression in *B. cenocepacia*-infected macrophages decreases the autophagosome formation. The downregulation of *Mir17* and *Mir20a* partially restored the autophagy deficiency. Rapamycin or IFN-γ stimulation induces the release of SQSTM1 from aggresomes to increase the mature autophagosome formation. Red arrows indicate decreased autophagy, and black arrows indicate increased autophagy. Created with BioRender.com.

autophagy; however, *B. cenocepacia* impairs the formation of mature autophagosomes. The deficiency of caspase-4 (CASP-4, a protein associated to non-canonical activation of inflammasome) increases bacterial replication, with reduced association of LC3 at the BcCV. These observations suggest that CASP-4 has an important role in the autophagosome formation to control intracellular *B. cenocepacia* (Krause et al., 2018). In this manner, macrophages carrying mutation F508del in CFTR also show a reduced association of LC3B with the BcCV (Abdulrahman et al., 2011). Importantly, intracellular *B. cenocepacia* decrease the transcription of Atg9b, Atg5, Atg12, and Atg8, suggesting that the downregulation of these autophagic components could be an additional strategy used by this bacterium to survive inside CF macrophages (**Figure 1**). In addition, in CF macrophages, the *Mirc1/Mir17-92* cluster works in a way similar to a negative regulator of autophagy. Thus, the downregulation of *Mir17* and *Mir20a* expression partially increases the clearance of *B. cenocepacia* by autophagy (Tazi et al., 2016). Therefore, the induction of autophagy with rapamycin on macrophages carrying mutation F508del in CFTR reduces the intracellular bacterial load and decrease the inflammation of the lungs of *B. cenocepacia*-infected mice F508del (Abdulrahman et al., 2011). Moreover, the autophagosome maturation in murine macrophages needs the expression of SQSTM1 (Komatsu et al., 2007). The depletion of SQSTM1 in F508del macrophages infected with *B. cenocepacia* results in the release of BECN1 from cytosolic CFTR aggregates with a consequent redistribution at BcCV in which LC3 is recruited to form functional autophagosomes (Abdulrahman et al., 2013). The pre-activation of macrophages either with IFN-γ or rapamycin increases the colocalization of SQSTM1 with BcCV to produce mature autophagosomes (Assani et al., 2014) (**Figure 1**). In addition, macrophages pre-activated with IFN-γ increase their ability to control intracellular *B. cenocepacia* to process and present bacterial antigens by class II MHC molecules to CD4 T-

cells (Rosales-Reyes et al., 2020). Surprisingly, *B. cenocepacia* survives more efficiently in macrophages deficient in Gasdermin D (*gsdmd*$^{-/-}$), an executioner of pyroptotic cell death. The deficiency of Gasdermin D is associated with a low rate of autophagosome formation (Estfanous et al., 2021).

In contrast, Al-khodor et al. report that *B. cenocepacia* strain J2315 in human monocyte-derived macrophages or mouse bone marrow-derived macrophages disrupt the membrane of the BcCV to escape into the cytosol, in which the bacterium is surrounded by actin, and recruits KDEL, ubiquitin, SQSTM1, and LC3B to form functional autophagosomes (Al-Khodor et al., 2014).

Altogether, the downregulation of autophagic pathway is a key strategy used by *B. cenocepacia* to survive and persist for long periods of time causing a severe inflammatory immune response that triggers lung deterioration in CF patients.

### *Pseudomonas aeruginosa*

*P. aeruginosa* is an environmentally ubiquitous Gram-negative bacterial pathogen that is associated with increased morbidity and mortality among CF patients (Govan and Harris, 1986). This bacterium colonizes the lower airways of CF-affected individuals. The ability of *P. aeruginosa* to survive in this microenvironment requires the efficient evasion of their recognition by the immune system. The downregulated expression of diverse virulence factors by constant acquisition of mutations in global regulator genes as the quorum sensing and the mismatch repair system are general mechanisms used by *P. aeruginosa* to mediate their adaptation and survival in this microenvironment (Rossi et al., 2020; Rosales-Reyes et al., 2021).

Although *P. aeruginosa* was considered to be an extracellular opportunistic pathogen, it has been shown that it has the ability to gain access to phagocytic cells (Speert and Simpson, 1999). This bacterium induces autophagy in both macrophages and mast cells (Yuan et al., 2012; Junkins et al., 2013). In mouse and human macrophages, intracellular *P. aeruginosa* promotes autophagy to decrease phagocytosis and their intracellular bacterial killing (Wu et al., 2016). In these studies, the knockdown of ATG7 or Beclin1 increases both macrophage phagocytic activity as well as intracellular killing. Nevertheless, the autophagy induction by rapamycin decreases the expression of phagocytic receptors for *P. aeruginosa* (Wu et al., 2016). Additionally, in macrophages, *P. aeruginosa* induces the assembly and activation of the NLRP3 inflammasome; thus, active NLRP3 inflammasomes reduce the efficiency of macrophages to kill *P. aeruginosa* by the decreased formation of autophagosomes (**Figure 2**) (Deng et al., 2016). In addition, the inflammasome activation by *P. aeruginosa* does not require the type III secretion system. The inflammasome activation leads to TRIF processing by caspase-1 and decreases the NLRP3 inflammasome activation. Thus, inhibition of TRIF cleavage by caspase-1 increases the bactericidal activity mediated by autophagy (Jabir et al., 2014).

The ability of *P. aeruginosa* to modulate the formation of mature autophagosomes is a key strategy to ensure its survival in phagocytic cells. For example, Annexin A2, a member of the annexin family, interacts with Fam13A to activate the Rho GTPase to regulate the autophagosome formation after *P. aeruginosa* invasion through the Akt1-mTOR-ULK1/2 pathway (Li et al., 2015). *P. aeruginosa* produces pyocyanin (PYO), an important virulence factor required for their full virulence. PYO is a redox-active released pigment that interferes with several cellular functions in host cells including electron transport, gene expression, energy metabolism, cellular respiration, and an innate immune response (Rada and Leto, 2013). Deletion of the *phzM* gene (required for pyocyanin biosynthesis) significantly decreases autophagy induction. In addition, pyocyanin induces autophagy through the EIF2AK4/GCN2 (eukaryotic translation initiation factor 2 a kinase 4)–EIF2S2/eIF2α (eukaryotic translation initiation factor 2 subunit α)–ATF4 (activating transcription factor 4) pathway (**Figure 2**). The reduced pyocyanin production during chronic infections has been associated with better bacterial adaptation into the host (Yang et al., 2016). ExoS, a T3SS effector with the activity of ADP ribosylation, inhibits the host autophagy by decreasing the Vps34 kinase activity (Rao et al., 2021). Thus, the survival of *P. aeruginosa* inside phagocytic and epithelial cells requires a coordinated mechanism that ends in decreased autophagosome formation, leading to *P. aeruginosa* survival and persist for long periods of time, increasing the inflammatory immune response that gradually decreases lung function of individuals affected with CF.

## AUTOPHAGY IN THE CONTROL OF INTRACELLULAR BACTERIA

*B. cenocepacia* and *P. aeruginosa* are two important opportunistic pathogens that produce chronic infection in CF lungs. Their ability to survive and persist into eukaryotic cells leads to the establishment of chronic inflammation and cell death. The bacterial survival in vacuoles suggests that the induction of autophagy could be an important strategy to destroy these pathogens that subvert autophagy. Rapamycin is a drug that induces autophagy, decreasing the intracellular load of *B. cenocepacia*. In the mouse model, Rapamycin also decreases lung inflammation induced by the *B. cenocepacia* infection (Abdulrahman et al., 2011). In addition, the treatment with gamma-interferon (IFNγ) also promotes the formation of autophagosomes, in which *B. cenocepacia* is destroyed (Assani et al., 2014). Thus, the intracellular processing of *B. cenocepacia* by pre-activated macrophages with IFNγ produces peptides that are presented by class II MHC molecules to CD4 T cells (Rosales-Reyes et al., 2020). Similar findings have been observed in macrophages infected with *P. aeruginosa* in which the treatment with rapamycin or IFNγ also induces autophagosomes in which the bacterium is destroyed (Yuan et al., 2012). These observations suggest that the induction of autophagy might decrease the intracellular survival of *B. cenocepacia* and *P. aeruginosa* to decrease the chronic colonization and inflammation of the CF lungs.

## CONCLUDING REMARKS

Eukaryotic cells can destroy intracellular microorganisms through the induction of autophagy. Autophagy is considered to be one of

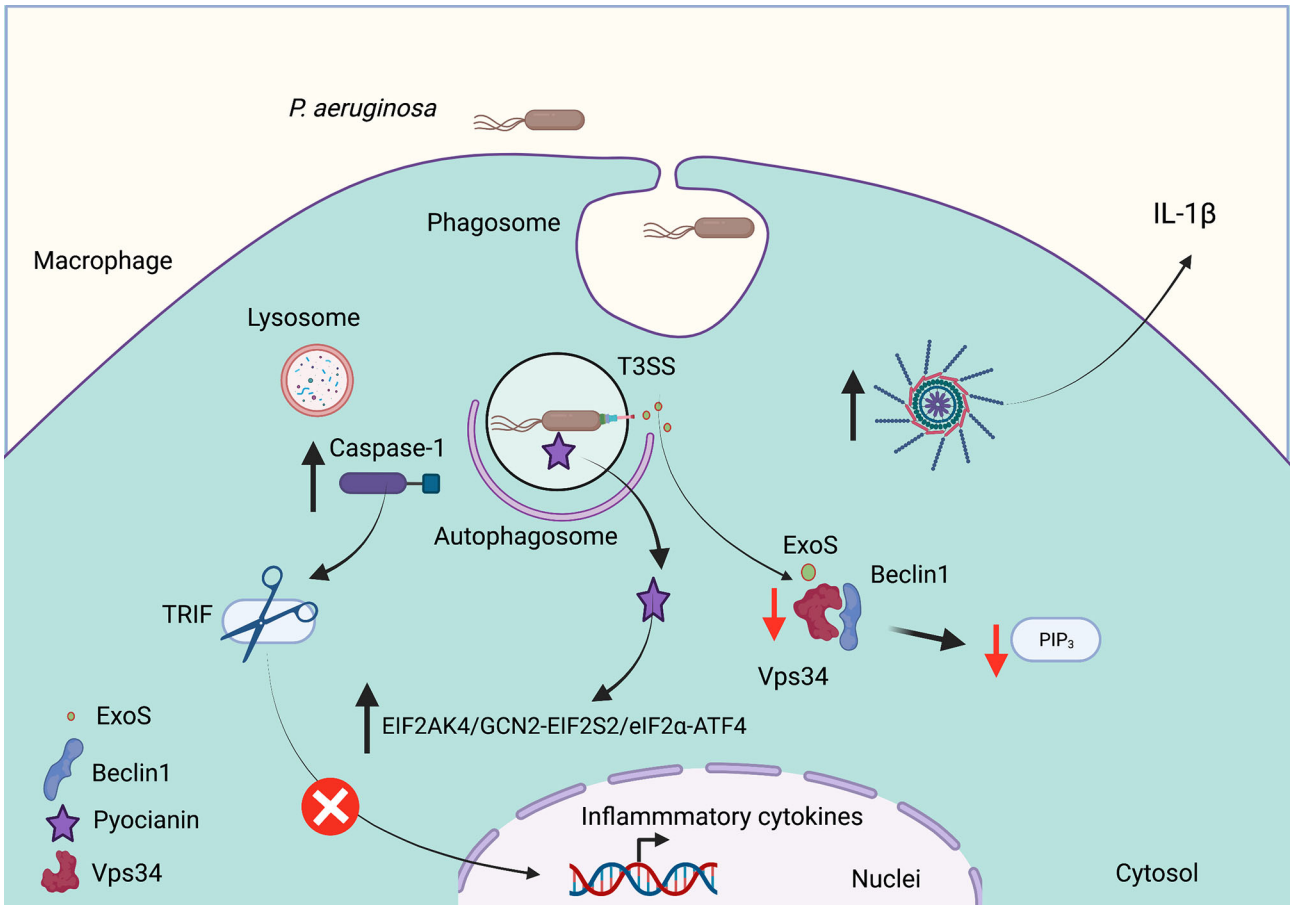


**FIGURE 2** | *Pseudomonas aeruginosa* subverts autophagy in cystic fibrosis. *P. aeruginosa* invades phagocytic and epithelial cells, which modulates the autophagic pathway to survive. Cell infection induces NLRP3 inflammasome activation to release IL-1b. Caspase-1 activation also mediates TRIF degradation to decrease the inflammatory response. The Type III Secretion System (T3SS) releases ExoS, a toxin with ADP ribosylation activity that decreases the Vsp34 activation to produce phosphatidylinositol (3,4,5) trisphosphate ($PIP_3$). The release of pyocyanin promotes autophagy through the EIF2AK4/GCN2 (eukaryotic translation initiation factor 2 a kinase 4)–EIF2S2/eIF2α (eukaryotic translation initiation factor 2 subunit α)–ATF4 (activating transcription factor 4) pathway. Red arrows indicate decreased autophagy, and black arrows indicate increased autophagy. Created with BioRender.com.

the first antimicrobial defense mechanisms used by several eukaryotic cells. This cellular process uses a distinct set of proteins that assemble a membrane around the vacuoles containing bacteria culminating in the destruction of the intracellular microorganisms. Bacterial antigens are processed in the autophagosome and the peptides generated are presented by class II MHC molecules to CD4 T cells to activate an adaptative immune response. Several microorganisms have evolved by developing strategies to evade autophagic degradation, allowing their survival and persistence. The bacterial persistence in the lungs of individuals affected with CF through the subversion of autophagy is a key factor that promotes chronic inflammation and decreases lung function, which ultimately compromises the life of affected individuals. The first bacterial pathogens associated with the colonization of the lower airways of newborn children with CF are non-typable *H. influenzae* and *S. aureus*. Progressively, these pathogens are replaced by *P. aeruginosa* and *B. cenocepacia* during adolescence and adulthood. The mechanisms described herein by which *B. cenocepacia* and *P. aeruginosa* subvert autophagy could help to establish better strategies to combat these intracellular pathogens that produce chronic infections in patients with CF.

## AUTHOR CONTRIBUTIONS

RR-R conceived and supervised the review topics. VF-V, SV-R, and RR-R wrote the first draft. VF-V, SV-R, JL-F, RL, JS-P, and RR-R edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Mechanisms of TLR4-Mediated Autophagy and Nitroxidative Stress

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Pathogenic infections have badly affected public health and the development of the breeding industry. Billions of dollars are spent every year fighting against these pathogens. The immune cells of a host produce reactive oxygen species and reactive nitrogen species which promote the clearance of these microbes. In addition, autophagy, which is considered an effective method to promote the destruction of pathogens, is involved in pathological processes. As research continues, the interplay between autophagy and nitroxidative stress has become apparent. Autophagy is always intertwined with nitroxidative stress. Autophagy regulates nitroxidative stress to maintain homeostasis within an appropriate range. Intracellular oxidation, in turn, is a strong inducer of autophagy. Toll-like receptor 4 (TLR4) is a pattern recognition receptor mainly involved in the regulation of inflammation during infectious diseases. Several studies have suggested that TLR4 is also a key regulator of autophagy and nitroxidative stress. In this review, we describe the role of TLR4 in autophagy and oxidation, and focus on its function in influencing autophagy-nitroxidative stress interactions.

**Keywords: TLR4, autophagy, nitroxidative stress, interaction, homeostasis**

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## INTRODUCTION

Autophagy is a physiological metabolic compensatory process of eukaryotic cells that maintains homeostasis. However, autophagy is also a conserved defense mechanism that evolved during cell evolution and which has an important role in the process of pathogenic infection. Generally, autophagy degrades or digests intracellular aging or damaged organelles, protein, metabolin, and even pathogenic microorganisms by autophagosome encapsulation and lysosomal binding (Nakatogawa et al., 2012). Autophagy of immune cells resists the invasion of pathogens by regulating cellular functions. For example, promoting autophagy helps to clear *Streptococcus*, *Helicobacter. pylori*, and *Pneumonia* (Wang et al., 2009; Ye et al., 2015; Nozawa et al., 2017). However, some microorganisms have evolved the ability to inhibit, escape, and even use autophagy, allowing them to continue to survive in the host body (Starr et al., 2012; Sharma et al., 2021). Moreover, autophagy is involved in the regulation of inflammation. Moderate autophagy maintains

homeostasis by negatively regulating inflammation (Liu et al., 2016b; Zhong et al., 2016). Incorrect autophagy can damage the body and even cause organ failure by triggering cytokine storms (Lu et al., 2019; Zhao et al., 2019). How to regulate and use autophagy to protect the body is a hot spot of current research.

Nitroxidative stress is a state of physiological imbalance mainly related to the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is well-known that nitroxidative stress is a general factor in many pathological conditions, including autoimmune diseases, infectious diseases, and tumor (Su et al., 2017; Smallwood et al., 2018). ROS and RNS also have dual-roles in the processes of removing pathogenic microorganisms and maintaining homeostasis. Low concentrations of ROS/RNS help maintain normal physiological metabolism and protect against infectious pathogens (Valko et al., 2007). However, the excessive ROS/RNS leads to an imbalance of redox states, metabolic disorders, and even damage of tissues and organs (Islam, 2017; Di Meo and Venditti, 2020). In addition, a serious pathogenic infection can induce severe nitroxidative stress followed by cytokine storm, resulting in the acute injury of tissues and organs (Mrityunjaya et al., 2020). Therefore, understanding how the body regulates nitroxidative stress levels is important.

The recognition of pathogenic microorganisms by pattern recognition receptors (PRRs) is the initial step in host innate immune responses. The Toll like receptor (TLR) family is an important class of PRRs that recognize a variety of bacteria and viruses, and induces the secretion of inflammatory cytokines and chemokines. Moreover, the activation of TLRs directly enhances the phagocytosis and killing capacity of innate immune cells that promotes the elimination of pathogens (Vasselon and Detmers, 2002). TLR4 is mainly expressed on the membranes of macrophages, dendritic cells and neutrophils. Activation of TLR4 is closely related to inflammation, autophagy, and nitroxidative stress during a pathogenic infection (Deng et al., 2020). In this review, we discuss recent studies on the activities of TLR4, autophagy, and nitroxidative stress during pathogenic microorganism infections, with a particular focus on the mechanisms of TLR4-mediated autophagy and nitroxidative stress.

## THE INITIATION OF AUTOPHAGY AND NITROXIDATIVE STRESS DURING BACTERIAL INFECTION

### Initiation of Autophagy

Autophagy is also known as type II programmed cell death. Moderate autophagy can degrade damaged organelles, denaturated macromolecules and intracellular pathogens, and then provide raw materials for cell metabolism (Levine and Kroemer, 2019; Zhu et al., 2019). Excessive autophagy and insufficient autophagy lead to disease (Lavandero et al., 2015). It was confirmed that autophagy is closely associated with proliferation of pathogenic microorganisms (Wang et al., 2020b). The stage and site of pathogenic infection, type of infected cell, and physiological state of the host body all affect the activity and outcome of autophagy. Therefore, identifying the initiation process of autophagy can help us understand its multiple functions.

Mammalian target of rapamycin (mTOR) is a key factor of autophagy initiation. It is widely known that mTOR is composed of two protein complexes mTORC1 and mTORC2, which have different structures and functions and are involved in many physiological processes. Unc-51-like kinase 1 (ULK1) is an indispensable component in autophagy vesicles, which can form the ULK1 complex with autophagy related 13 (Atg13), 200-kDa FAK family kinase interaction protein (FIP200), and Atg101 to induce autophagy (Chen et al., 2014; Hurley and Young, 2017). Under normal circumstances, mTORC1 suppresses autophagy by directly inhibiting the ULK1 complex activity. However, under stress conditions, mTORC1 is phosphorylated leading to the rapid disinhibition of ULK1 and Atg13, which triggers autophagy. In addition, mTORC1 also negatively regulates autophagy by phosphorylating autophagy/Beclin-1 regulator 1 (Ambra1) at Ser52 and 4',6-diamidino-2-phenylindole (DAP1) at Ser3 and Ser51 (Koren et al., 2010; Cianfanelli and Cecconi, 2015). Conversely, mTORC2 indirectly inhibits autophagy through activation of the AKT/mTORC1 signaling axis. The mechanism involves the AKT-dependent phosphorylation inhibition of tuberous sclerosis complex 1/2 (TSC1/2) which induces Rheb activity that promotes mTORC1 activation (Bernard et al., 2020). Another major mTOR related signaling pathway is the AMP-activated protein kinase (AMPK) -mTOR pathway. The regulation of AMPK is extremely complex. Activation of AMPK phosphorylates TSC2 at Ser792 of mTORC1. Furthermore, PIM2 directly phosphorylates TSC2 at Ser1798 to activate mTORC1 (Lee et al., 2010; Lu et al., 2013). Moreover, AMPK initiates autophagy by directly phosphorylating ULK1 at Ser317 and Ser777 in response to stress (Pagano et al., 2014). In addition, a novel signaling axis AMPK- E3 ligase S-phase kinase-associated protein 2 (SKP2)-co-activator-associated arginine methyltransferase 1 (CARM1), was found to regulate autophagy activation by nutrient starvation (Shin et al., 2016). A recent study indicated that PKCα phosphorylated ULK1 at Ser423, which prevented autosomal formation and inhibited autophagy (Wang et al., 2018a). Atg13, ULK1, and TFEB can be negatively regulated by mTORC1. The secretion of TFEB during stress helped regulate the expression of genes involved in lysosomal biogenesis and lipid catabolism (Settembre et al., 2013).

The processes of autophagy initiation induced by different pathogenic microorganisms are also different. It was shown that *Salmonella typhimurium* invasion breaks through vesicles and enters the cytoplasm to selectively activate mTOR and degrade AMPK to escape autophagy (Liu et al., 2018). Streptococcus pneumoniae PavA activates the AMPK signaling pathway to induce autophagy in alveolar epithelial cells by inhibiting the mTOR pathway (Kim et al., 2017). In *H. pylori* infected gastric epithelial cells, inactivated transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) inhibits AMPK phosphorylation, as well as autophagy and cell survival (Lv et al., 2014).

## Formation of Nitroxidative Stress

Nitroxidative stress is a state of physiological imbalance mainly caused by excessive ROS and RNS. Under normal conditions, low levels of ROS/RNS trigger the hosts protective immune response, which is of great significance for anti-infection, anti-inflammatory, and tumor inhibition. However, excessive ROS/RNS directly leads to membrane rupture, DNA peroxidation damage, and cell dysfunction, such as loss of energy metabolism, changes in cell signal transduction, and gene mutation. During some pathogenic microorganisms infection, the host's antioxidant system and metabolic balance of free radicals becomes disordered, resulting in nitroxidative stress.

ROS components include $O_2$, $H_2O_2$ and –OH, and RNS components includes NO, $NO_2$, and ONOO– (Stamler et al., 1992). ROS functions as a signaling molecule in a variety of intracellular processes, which lead to cell proliferation, apoptosis, and defense against microorganisms (Fratelli et al., 2005; Scherz-Shouval et al., 2007). Endogenous ROS is mainly induced by the mitochondrial respiratory chain (Zou et al., 2017). Specific enzymes of the NADPH oxidase (NOX) family and double oxidase family are involved in ROS production. These enzymes catalyze the conversion of intracellular $O_2$ to $O_2^-$, and then $O_2^-$ is converted to $H_2O_2$ in the presence of superoxide dismutase. Subsequently, $H_2O_2$ reacts with metal ions to form –OH (Si et al., 2015). For example, sodion, a second messenger, interacts with phospholipids and controls oxidase in mitochondria. Furthermore, sodion helps transfer electrons from the substrate to oxygen ions, and subsequently promotes ROS formation and causes oxidative stress (Hernansanz-Agustin et al., 2020). Studies reported that SoxRS and OxyR are the main redox response transcription factors involved in oxidative stress in bacteria. OxyR senses $H_2O_2$ and organic peroxide, and SoxRS regulates $O_2^-$-mediated oxidative stress. In addition, other stress factors including RpoS and PerR are also involved in regulating oxidative stress responses (Fei et al., 2020). ROS and RNS are produced through different processes including ultraviolet irradiation, metal-catalyzed reactions, electron transport reactions, and inflammation (Valko et al., 2006). Many studies have shown that a variety of pathogens induce nitroxidative stress (Li et al., 2019). The increase of ROS/RNS production was found during microbial infection, or infection with hepatitis C virus, Herpes simplex virus type 1, *E. coli*, and *Salmonella* (Molteni et al., 2014; Rhen, 2019). When pathogenic microorganisms infect a host, ROS and RNS can be beneficial and detrimental *via* anti-inflammatory, anti-pathogen, protective immunity, or cytotoxicity functions (Umezawa et al., 1997). Moderate insulin-like growth factor 1 promoted mitochondrial ROS synthesis and NO synthase gene expression which participated in the elimination of *P. falciparum* (Drexler et al., 2014). Activation of TLR4 helped clear invading pathogens through the production of NO which was induced by increasing the activity of iNOS *via* guanosine triphosphate cyclohydrolase (Deng et al., 2015). Inhibition of mitochondrial ROS production impaired the ability of immune cells to kill *Salmonella typhimurium* in mice (West et al., 2011). In addition, excessive ROS/RNS was closely associated with tissue and organ damage (Deng et al., 2012). For example, excessive ROS triggered apoptosis by inducing the expression of NLRP3, caspase-1, and ASC (Dai et al., 2019).

## Antioxidant Systems

Because persistent and excessive nitroxidative stress can lead to host tissue damage, organisms have an antioxidant system that maintains redox homeostasis. There are two types of antioxidant systems: antioxidant enzymes and non-enzymatic antioxidants (Halliwell, 1996). Antioxidants help to mediate peroxidation by catalyzing the formation of active oxygen intermediates which lead to remove free radicals of oxygen and nitrogen. For example, the massive accumulation of ROS promotes the production of superoxide dismutase (SOD), which catalyzes the conversion of superoxide into $O_2$ and $H_2O_2$ (Lipinski et al., 2010). Then, catalase scavenges peroxide by catalyzing the conversion of $H_2O_2$ into $O_2$ and $H_2O$ (He et al., 2017). In addition, the GPx family and Trx system, endogenous antioxidants, also participate in the elimination of nitroxidative stress (Papp et al., 2007; Liu et al., 2014a). Moreover, several signaling pathways, of which nuclear factor erythroid 2-related factor 2 (NRF2) signaling is the most important, are involved in regulating nitroxidative stress. ROS directly oxidizes the cysteine residues on Kelch-like ECH-associated protein 1 (KEAP1) freeing NRF2 from the KEAP1-NRF2 complex. After free NRF2 translocates into the nucleus, it promotes the expression of multiple antioxidant genes by binding to their regulatory regions (Schieber and Chandel, 2014). Furthermore, $NO^-$ triggers NRF2 transcription and the expression of ferroportin which activate the antioxidant system and nutrition immunity (Nairz et al., 2013). In turns, inhibiting NRF2 signaling increased ROS levels by suppressing the expression of HO-1 during *H. pylori* infection (Ko et al., 2016).

# OVERVIEW OF TLR4: AN IMPORTANT SIGNAL TRANSDUCTION PROTEIN

Studies over the past few decades have shown that the recognition of microbes is based on hosts genes encoding PRRs, which consist of TLRs, RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and Nod-like receptors (NLRs) (Janeway, 2013). TLRs are Type I transmembrane proteins that recognize various microbial pathogen-associated molecular patterns (PAMPs). More than a dozen TLRs have been identified to date. Different TLRs can recognize different PAMPs in different cell compartments, including the cell membrane, endosomes, cytoplasm, and endosome (Akira et al., 2006). The correct localization of TLRs is crucial for the regulation of signal transduction. Furthermore, cell type specific signaling downstream of TLRs determines specific innate immune responses (Kawasaki and Kawai, 2014). Generally, TLR signaling is divided into two types: MyD88-dependent and the MyD88-independent signaling pathways. Both pathways activate downstream signaling molecules to promote the production of pro-inflammatory cytokines, chemokines, and type I interferon (IFN) which help remove

pathogens (Yamashita et al., 2012). Here, we mainly focus on studies of TLR4 which is also involved in autophagy and nitroxidative stress.

## TLR4 Signal Transduction

TLR4 is known for recognizes a broad variety of substances including bacterial lipopolysaccharide (LPS), viral structural protein, fungal mannan, and parasitic glycoinositolphospholipids and has a key role in activating innate immunity (Kawai and Akira, 2011; Mu et al., 2011). For example, when Gram-negative bacteria infect a host, bacterial LPS is directly recognized by TLR4 in the presence of LPS binding protein, CD14, and myeloid differentiation factor 2 (MD-2). Subsequently, TLR4 undergoes oligomerization and recruits downstream adaptor proteins through its Toll-interleukin-1 receptor domains, which initiates signal transduction (Lu et al., 2008). Similar to other TLRs, TLR4 also has two types of signal transduction. After TLR4 is combined with MyD88, phosphorylated IRAK-4 activates the TRAF6-TAK1-NF-κB/MAPK signaling axis which promotes the release of inflammatory factors, such as IL-1β, IL-6, and TNF-α (Monlish et al., 2016; Dajon et al., 2017). Theses cytokines are responsible for inflammation, autophagy and nitroxidative stress. In the MyD88-independent pathways, TRAM is selectively recruited to TLR4 to link TLR4 and TRIF, which induces the production of type I IFN and other proinflammatory cytokines through the activation of IRF3, NF-κB, and MAPK signaling (Kawai and Akira, 2011).

## TLR4-Mediated Innate Immune Responses Have Dual Functions

However, when TLR4 is overactivated or its negative regulatory system is obstructed, TLR4 can induce endotoxic shock, autoimmune disease, and even cytokine storm where the immune system attacks the host. The SARS-CoV-2 virus has spread worldwide since 2020, infecting billions of people and killing millions of people (Harrison et al., 2020). Cytokine storm is thought to be an important cause of death in patients with severe and critical COVID-19, which is caused by the SARS-CoV-2 virus (Chen et al., 2020). Compared with a severe influenza group, PBMCs from COVID-19 patients showed a high inflammatory response, and a marked TNF/IL-1β driven inflammatory response. IFN, TNF-α, and IL-1β co-drive inflammatory responses in the monocytes of patients with severe COVID-19, but not in those of patients with mild COVID-19 (Lee et al., 2020). Further research showed that TLR4 is probably involved in recognizing the spike protein of SARS-CoV-2 and then inducing inflammatory responses (Bhattacharya et al., 2020; Choudhury and Mukherjee, 2020). After SARS-CoV-2 virus infects a host, the SARS-CoV-2 spike trimer directly binds to TLR4 and the trigger section of IL-1β. Inhibiting TLR4 completely blocks SARS-CoV-2-induced IL-1β. In contrast, ACE2-deficient or TMPRSS2-inhibition did not affect SARS-CoV-2-induced IL-1β (Zhao et al., 2021). This indicates that if TLR4 signals are not effectively controlled, they can seriously threaten health. Several tightly-regulated mechanisms regulate TLR4 signal transduction to avoid excessive immune response (**Table 1**) (Liu et al., 2016a).

# REGULATORY RELATIONSHIPS AMONG TLR4, AUTOPHAGY, AND NITROXIDATIVE STRESS

In previous studies, researchers mainly investigated the functions of TLR4 with regard to the activation of innate immune responses and production of proinflammatory cytokines during infection. Recently studies have suggested that TLR4 is involved in regulating autophagy and nitroxidative stress during various physiological states (Wang et al., 2016; Wang et al., 2018b; Wang et al., 2020a). Here, we summarize signaling pathways that connect TLR4, autophagy, and nitroxidative stress, and discuss their triangular relationships that affect cellular homeostasis.

## Key Signaling Nodes That Link TLR4, Autophagy and Nitroxidative Stress

### mTOR Signaling Pathway

Identifying the signaling pathways shared by TLR4, autophagy and nitroxidative stress help us better understand the process of host resistance to pathogens. mTOR is involved in many cellular

**TABLE 1** | The negative regulatory molecules and targets of TLR4 signaling.

| Regulated TLRs | Negative regulator molecule | Target | Refs |
|---|---|---|---|
| TLR4 | RP105 | Competing ligands for TLR4 | (Liu et al., 2003) |
| TLR4/9 | Rab7b | Promoting TLR4 degradation, inhibiting NF-κB/MAPK | (Wang et al., 2007) |
| TLR4 | SHIP1 | Inhibiting combination of TLR4 and MyD88 | (Cekic et al., 2011) |
| TLR4/7/9 | SOCS-1 | Inhibiting IRAK activity | (Mansell et al., 2006) |
| TLR3/4 | SARM/TRAF1/TRAF4 | Inhibiting TRIF | (O'Neill and Bowie, 2007; Ermolaeva et al., 2008) |
| TLR4 | Tollip/SHP1 | Inhibiting self-phosphorylation of IRAK1 | (O'Neill et al., 2003; Das et al., 2012) |
| TLR4 | ATF3/Notch | Inhibiting NF-κB | (Kim et al., 2010; Yao et al., 2013) |
| TLR3/4 | Rhbdd3/Ash1l | Inhibiting NEMO ubiquitination | (Xia et al., 2013; Liu et al., 2014b) |
| TLR4 | IRG1/USF-1 | Promoting A20 activity | (Shi et al., 2005; Tiruppathi et al., 2014) |
| TLR3/4/9 | Zc3h12a | Promoting degradation of IL-6 and IL-12p40 | (Chen et al., 2018) |
| TLR4 | Tet2/Daxx | Promoting histone deacetylation, inhibiting IL-6 | (Yao et al., 2014; Liang et al., 2020) |
| TLR2/4/5 | miR-146a | Inhibiting IRAK and TRAF6 activity | (Chassin et al., 2010) |
| TLR4 | miR-21 | Inhibiting PDCD4 | (Sheedy et al., 2010) |
| TLR2/3/4 | Setdb2 | InhibitingH3K9me3 levels in Cxcl1 promoter region | (Das et al., 2016) |

physiological processes, such as inflammation, energy metabolism, oxidation and autophagy (Zhou et al., 2018). The TLR4-MyD88-MAPK and TLR4/PI3K/Akt signaling pathways affect mTOR activity and influence autophagy (Huang et al., 2020). Generally, nitroxidative stress affects the phosphorylation of AMPK and PI3K signaling, which then influence autophagy (Hinchy et al., 2018; Mistry et al., 2019). Mechanistically, bacteria activate mTORC1 *via* the upstream TLR4-PI3K-Akt signaling cascade, which inhibits autophagy by suppressing autophagy initiating kinase ULK1 (Shariq et al., 2021). Down regulating TLR4 and MyD88 promoted autophagy by suppressing the phosphorylation of MAPK, mTOR and p65. However, activated TLR4-TRIF and TLR4-MAPK signaling pathways induced autophagy by promoting the dissociation of Beclin 1 and Bcl-2. The release of Beclin 1 from Bcl-2 biased cells toward autophagy (Shi and Kehrl, 2008). Then, mTOR-dependent autophagy precisely regulates downstream NF-κB activation, which leads to the production of nitroxidative stress (Zhou et al., 2018). ROS and RNS signaling were demonstrated to be related to TLR4-dependent NF-κB activation and inflammatory cytokines production (Weigert et al., 2018). The membrane-associated enzyme complex NADPH acts as a key intermediate in regulating the TLR4-mediated production of ROS. Furthermore, the inhibition of mTOR or mTORC1 down-regulated the TLR4-mediated production of ROS and RNS. mTOR-induced the expression of NOX2 and NOX4, which are essential for ROS formation (Sohrabi et al., 2018).

## TRAF6 Signaling Pathway

TLR4-MyD88 signaling activates of TRAF6, another important factor that links autophagy and oxidation. TRAF6 promotes mtROS production during LPS stimulation or bacterial infection through the direct ubiquitylation of domains of evolutionarily conserved signaling intermediates in Toll pathways (ECSIT), which is a key mitochondrial respiratory chain assembly factor (West et al., 2011; Wi et al., 2014). Moreover, the inhibition of TRAF6 ubiquitin-ligase activity suppresses autophagy (Min et al., 2018). Mechanistically, activated TRAF6 promoted the K63-linked polyubiquitination of Beclin-1, which is essential for the initiation of autophagy (Lee et al., 2018). Furthermore, activated TRAF6 also induced the stabilization and activation of ULK1, which is the most important factor for the biogenesis of autophagosomes by inducing its Lys63 ubiquitination (Han et al., 2019). In addition, under non-autophagic conditions, mTOR induces the phosphorylation of Ambra1. Then, activated Ambra1 interacts with TRAF6 to mediate the ubiquitination of ULK1 that further inhibits the initiation of autophagy (Nazio et al., 2013). Furthermore, TRAF6 is necessary for the translocation of mTORC1 to lysosomes. The p62/TRAF6 complex induces the activation of mTORC1 *via* the K63-linked polyubiquitination of mTOR (Linares et al., 2013). These intriguing studies indicate that mTOR and TRAF6 are factors joints that connect TLR4 signaling, autophagy, and nitroxidative stress (**Figure 1**).

## TLR4 and Nitroxidative Stress Induce Autophagy

The process of TLR4 activation is always accompanied by autophagy and nitroxidative stress in host immune cells (**Figure 2**). For example, *mycobacterium tuberculosis* infection, recognized by TLR4, induced SIRT3-mediated autophagy and nitroxidative stress in mouse BMDM (Kim et al., 2019). *E. coli* LPS stimulation also induced autophagy and nitroxidative stress *via* MAPK signaling in macrophages (Wang et al., 2020a). The host recognition of bacteria is the first step for activation of the immune system. Generally, TLR4 recognizes PAMPs and induces the secretion of inflammatory cytokines, including type I IFN, which induces the production of ROS/RNS. Moderate intracellular oxidation is an important method for the host to eliminate pathogenic microorganisms. Evidence suggests that TLR4 interacts directly with NADPH oxidase. Indeed, the TIR-domain of TLR4 interacts directly with the carboxy-terminal region of Nox2/4 under LPS stimulation (Park et al., 2004). Activated NADPH oxidase induces the

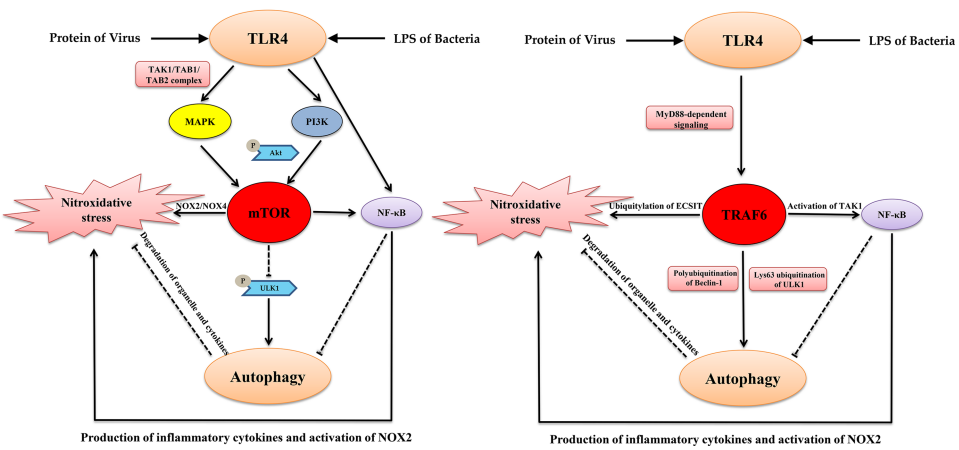


**FIGURE 1** | The key signaling nodes that link TLR4, autophagy and nitroxidative stress. TLR4, Autophagy, and Nitroxidative Stress: A Triangular Relationship That Affects Cellular Homeostasis.

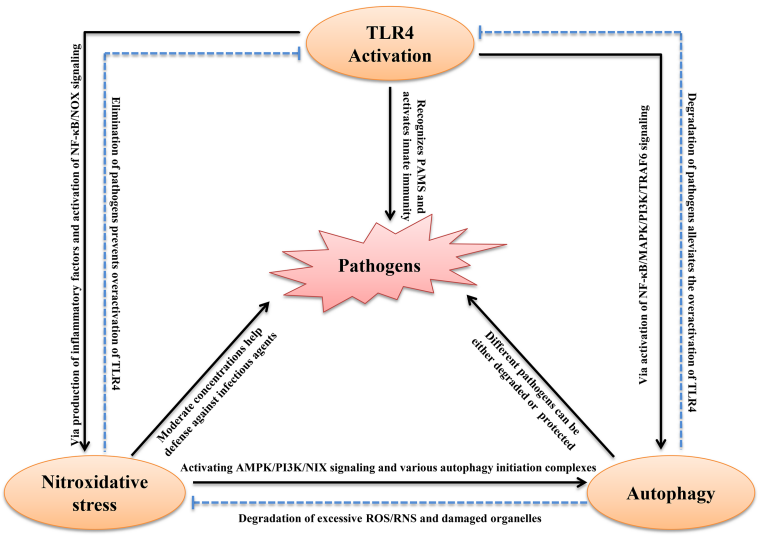


**FIGURE 2** | Schematic diagram of the interplay between TLR4, autophagy and nitroxidative stress in infectious Diseases. See text for explanation.

transmembrane transport of electrons and production of ROS. Furthermore, TLR4 promotes the expression of iNOS *via* NF-κB signaling. This promotes NO production that suppresses SOD activity and promotes MDA production, which aggravates nitroxidative stress (Li et al., 2019). In addition, TLR4-mediated pro-inflammatory cytokines, such as TNF-α and IL-1β, and also induces ROS (Sasaki et al., 2008). Moreover, inhibiting the expression of TLR4 significantly decreased the level of autophagy (Kandadi et al., 2012). The excessive accumulation of ROS/RNS is harmful to homeostasis and can cause body oxidative damage in the host. It is widely thought that ROS/RNS induces autophagy, which maintains oxidation intermediates at a low level. ROS directly phosphorylated the p65 subunit of NF-κB at Ser-536, which activated the autophagy receptor P62 (Song et al., 2017). In addition, many oxidation intermediates promote the transcription of *BNIP3* and *NIX*, which dissociate Beclin-1from Bcl2 to induce autophagy (Mahalingaiah and Singh, 2014; Xu et al., 2020). Moreover, $H_2O_2$ mediated the binding of AMPK and GSH which helped phosphorylate the ULK1 complex leading to autophagy (Filomeni et al., 2015). Furthermore, excessive RNS and ROS cause DNA damage (Wiseman and Halliwell, 1996). Upon DNA damage, Ataxia telangiectasia mutated promoted AMPK phosphorylation and the p53-dependent expression of autophagic genes, including ATG4, ULK1 and UVRAG (Hurley and Bunz, 2007; Fullgrabe et al., 2014). Nrf2 sensed cellular ROS or RNS, and then activated AMPK, which suppressed mTOR signaling leading to autophagy (Kapuy et al., 2018). In addition, the inhibition of ROS by *N*-acetyl-l-cysteine abolished autophagic flux in porcine trophectoderm cells and ROS production activated MAPK and PI3K/Akt pathways, which were involved in this process (Luo et al., 2019). As mentioned above, TLR4 regulated autophagy *via* the TLR4-MyD88-MAPK/NF-κB and TLR4/PI3K/Akt/mTOR signaling pathways. However, whether TLR4 activation has an active role in the induction of autophagy remains controversial. Most studies have shown that LPS or infection can induce autophagy *via* TLR4 signaling, and that the knock-down/knock-out of TLR4 down-regulates autophagy (Chen et al., 2015; Zhao et al., 2016; Wang et al., 2020a). However, a study reported that the activation of TLR4 by bacterial LPS inhibited autophagy through the MyD88-TAK1-MAPK-dependent phosphorylation of mTOR (Zhou et al., 2018). Another recent study showed that the intraperitoneal injection of LPS promoted neuroinflammation by activating TLR4, inhibiting autophagic markers, and inducing excessive oxidation intermediates (Jamali-Raeufy et al., 2021a). We hypothesize that this opposite result is related to the different concentrations and duration of stimulation or different types of cells used. However, there is no doubt that TLR4 is involved in regulating autophagy.

## Autophagy Regulates Reactive Intermediates Production and TLR4 Activity

A recent study reported autophagy also regulated nitroxidative stress. Autophagy often helps remove damaged organelles and excess metabolic intermediates to maintain redox homeostasis in all types of cells (Kroemer et al., 2010). Autophagic dysfunction results in mitochondrial damage and the accumulation of oxidative intermediates, which promote the production of proinflammatory cytokines that can lead to ROS-mediated cell death (Larabi et al., 2020). Deletions of autophagy-related genes, such as *ATG5* and *ATG16* promoted high cellular ROS levels (Asano et al., 2017; Saxena et al., 2018). Moreover, nitroxidation intermediates have dual roles in the regulation of autophagy. NO activates autophagy *via* mTOR activity. Furthermore, NO accumulation triggered nitrosative stress, which promoted ATM/LKB1/AMPK/TSC2 signaling cascades that initiated autophagy *via* the inhibition of mTORC1 (Tripathi et al., 2013). NOS, another nitroxidation

intermediate, interacted with PINK1 to activate selective autophagy by triggering the translocation of Parkin (Han et al., 2015). To date, no studies have shown that autophagy directly eliminates RNS. However, evidence has shown that autophagy removes substances that induce RNS production. For example, autophagy eliminated damaged mitochondria and inflammatory cytokines to reduce RNS accumulation (Shi et al., 2012; Kaminskyy and Zhivotovsky, 2014). Therefore, autophagy can be considered a non-canonical antioxidant system that helps to degrade excessive ROS/RNS. The intracellular redox state can affect the fate of cells, and the timely removal of oxidizing molecules is beneficial to the maintenance of homeostasis. Following the removal of pathogenic microorganisms by autophagy and nitroxidative stress, the activation of TLR4 is alleviated. During infection by pathogenic microorganisms, this physiological homeostasis helps to eliminate pathogens or helps pathogens survive. Conversely, if this homeostasis is disrupted, the host will be seriously challenged. The above studies have identified extensive crosstalk between TLR4, autophagy and nitroxidative stress (**Figure 2**).

## CONCLUDING REMARKS

As a pattern recognition receptor, TLR4 is involved in mediating pathogen-inducing inflammation. Moderate inflammatory responses help fight against pathogenic infections, but the excessive activation of inflammatory responses can trigger cytokine storm, which causes the immune system to attack the host leading to multiple organ failure and even death. Autophagy and nitroxidative stress are involved in the regulation of inflammation, and TLR4, autophagy, and nitroxidative stress have vital roles during pathogenic microorganism-induced host immune responses. Following the pathways of autophagy and nitroxidative stress and the signaling transduction of TLR4 has indicated a strong connection between them. Studying the detailed regulatory relationships between TLR4, autophagy and nitroxidative stress might help enhance our understanding of the interactions between pathogenic microorganisms and hosts. The activation of TLR4 always contributes to autophagy and the production of reactive intermediates including ROS/RNS. mTOR and TRAF6 are key factors that connect TLR4 signaling, autophagy and nitroxidative stress. Furthermore, promoting mTOR and TRAF6-dependent autophagy and nitroxidative stress *via* upstream TLR4-MyD88-NF-κB/MAPK and TLR4-TRAF6 signaling pathways, and downstream NOX, AMPK signaling and inflammatory cytokines regulates the elimination of pathogens and maintenance of body health. Furthermore, nitroxidative stress can induce autophagy through the activation of NF-κB, AMPK, and Nrf2 pathways. Then, autophagy helps to degrade excessive ROS/RNS to balance the intracellular redox state.

An important question is how to harness the links between TLR4, autophagy, and nitroxidative stress to control pathogenic microorganism infections. Many natural substances and small molecular compounds were reported to control autophagy and oxidation by regulating TLR4 activity (Jamali-Raeufy et al., 2021b). Of note, TLR4 activity, autophagy and oxidative stress all affect the survival of pathogenic microorganisms. According to the above ideas, we should identify targets from the signal pathways or key node proteins involved in TLR4/autophagy/nitroxidative stress simultaneously. Among them, mTOR, TRAF, NF-κB and their upstream and downstream molecules are worth investigating. In addition, we should choose different treatment strategies according to the types of pathogenic microorganisms and the characteristics of the infected organs. Finally, there are some outstanding questions: What are the differences between different TLR4 downstream signaling-mediated autophagy and nitroxidative stress pathways? Are TLR activation, autophagy and nitroxidative stress caused by infection and non-infectious diseases different? What are the specific molecular mechanisms and processes of the autophagic degradation of ROS/RNS? What are the detailed mechanisms of the activation of autophagy by endogenously produced RNS? Does excessive autophagy help to eliminate the ROS/RNS? If so, what is the relationship between excessive autophagy-mediated cell death and low ROS/RNS levels? These questions need to be answered in future studies.

## AUTHOR CONTRIBUTIONS

SW, KZ, and SD conceived the manuscript. SW, KZ, and QH wrote the manuscript. SW, KZ, QH and YY prepared the Figure. SW and QH revised the manuscript. Funding acquisition was by SW and JL. All authors contributed to the article and approved the submitted version.

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# HSBP1 Is a Novel Interactor of FIP200 and ATG13 That Promotes Autophagy Initiation and Picornavirus Replication

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ATG13 and FIP200 are two subunits of the ULK kinase complex, a key regulatory component of the autophagy machinery. We have previously found that the FIP200-ATG13 subcomplex controls picornavirus replication outside its role in the ULK kinase complex and autophagy. Here, we characterized HSBP1, a very small cytoplasmic coiled-coil protein, as a novel interactor of FIP200 and ATG13 that binds these two proteins *via* FIP200. HSBP1 is a novel pro-picornaviral host factor since its knockdown or knockout, inhibits the replication of various picornaviruses. The anti-picornaviral function of the FIP200-ATG13 subcomplex was abolished when HSBP1 was depleted, inferring that this subcomplex negatively regulates HSBP1's pro-picornaviral function during infections. HSBP1depletion also reduces the stability of ULK kinase complex subunits, resulting in an impairment in autophagy induction. Altogether, our data show that HSBP1 interaction with FIP200-ATG13-containing complexes is involved in the regulation of different cellular pathways.

**Keywords: autophagy, infection, ULK kinase complex, EMCV, CVB3, EV71**

## BRIEF RESEARCH REPORT

## INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is a cellular degradation pathway that is evolutionary conserved (Lahiri et al., 2019). This process is characterized by the selective or non-selective sequestration of cytoplasmic cargoes within double-membrane autophagosomes, which subsequently fuse with lysosomes to deliver their cargo into the hydrolytic interior of these organelles (Dikic and Elazar, 2018; Nakatogawa, 2020). Autophagy is active at basal levels in every eukaryotic cell and can be enhanced by stresses such as nutrient starvation and pathogen infection (Galluzzi et al., 2014; Deretic, 2021). Autophagosome biogenesis is orchestrated by the autophagy-related (ATG) proteins (Nakatogawa, 2020). Four of them, the kinase unc-51 like autophagy activating kinase (ULK) 1 (or ULK2), ATG13, RB1 inducible coiled-coil 1 (FIP200/RB1CC1) and ATG101, form the ULK kinase complex, which is a key regulator of autophagy

induction (Licheva et al., 2021). Activation of the ULK kinase complex initiates a signaling cascade that leads to the formation of autophagosomes (Licheva et al., 2021).

Numerous ATG proteins have other functions than the ones in autophagy (Bestebroer et al., 2013; Mauthe and Reggiori, 2016; Galluzzi and Green, 2019). In a previous study, we performed a siRNA-based screen to identify in an unbiased fashion the extent of the non-autophagic roles of the ATG proteins. In particular, we examined the impact of the depletion of each component of the ATG proteome on the replication of 6 viruses from 6 different virus families. With this approach, we also identified an anti-viral role of the subcomplex formed by ATG13 and FIP200, outside the context of the ULK kinase complex (Mauthe et al., 2016). This anti-viral role is specific for picornaviruses, a large virus family of non-enveloped, small (~30 nm in diameter) viruses with a positive-stranded RNA genome, which cause diseases in humans and animals (Mauthe et al., 2016). Picornaviruses are classified into different genera based on a complex set of rules (ICTV taxonomy) (Zell et al., 2017). Examples of genera are enteroviruses, which include coxsackievirus B3 (CVB3) and enterovirus 71 (EV71), and cardioviruses, a member of which is the encephalomyocarditis virus (EMCV) (Zell et al., 2017).

In this study, we have investigated heat shock factor binding protein 1 (HSBP1), a protein that we found binding to FIP200 and ATG13 (Mauthe et al., 2016), to gain additional insights into the anti-picornaviral function of the FIP200-ATG13 complex. HSBP1 is a small coiled-coil protein of 12 kDa that forms trimers (Liu et al., 2009). HSBP1 has been identified as a negative regulator of heat shock factor 1 (HSF1) (Satyal et al., 1998), the primary mediator of transcriptional responses to proteotoxic stresses (Vihervaara and Sistonen, 2014). Recently, it has also been shown that HSBP1 is crucial for the assembly of the Wiskott–Aldrich syndrome protein and SCAR homolog (WASH) complex (Visweshwaran et al., 2018), an actin-regulating complex that is recruited to endosomes by interaction with the retromer complex, that plays a role in endosomal protein sorting (Seaman et al., 2013). Here, we identified two new functions of HSBP1. First, we found that HSBP1 is important for the stability of ULK kinase complex subunits and therefore for autophagy initiation as well. Second, HSBP1 is a pro-picornaviral host factor that dissociates from the cytoplasmic FIP200-ATG13 subcomplex and translocates into the nucleus after picornavirus infection. We also provide evidence that the pro-picornaviral role of HSBP1 could be negatively controlled by the FIP200-ATG13 subcomplex, which thereby acts as an anti-picornaviral factor.

# MATERIAL AND METHODS

## Antibodies and Reagents

The following primary antibodies were used: rabbit anti-LC3 (Novus Biologicals, Littleton, CO), mouse anti-LC3 (Nanotools, Teningen, Germany), mouse anti-p62 (Abcam, Cambridge, UK), mouse anti-tubulin (Sigma-Aldrich, St. Louis, MO), rabbit anti-ATG13 (Sigma-Aldrich), mouse anti-HSBP1 (Sigma-Aldrich, clone 2C3), rabbit anti-ATG16L1 (MBL, Woburn, MA), mouse anti-actin (Merck, Darmstadt, Germany), mouse anti-GFP (Clontech, Shiga, Japan), rabbit anti-ULK1 (Santa Cruz, Dallas, TX), rabbit anti-FIP200 (Bethyl Laboratories, Montgomery, TX), mouse anti-enterovirus (DAKO, Glostrup, Denmark), mouse anti-dsRNA (English & Scientific Consulting Bt., Budapest, Hungary), rabbit anti-capsid (EMCV, a kind gift from Ann Palmenberg, University of Wisconsin, Madison, WI), mouse anti-VP1 (EMCV, a kind gift from Hanchun Yang, China Agricultural University, Beijing, China), mouse anti-4G2 (Dengue virus (DENV) and Zika virus (ZIKV), Merk Millipore, Billerica, MA) and rabbit anti-E1 (Chikungunya virus (CHIKV), from Jolanda Smit, University Medical Center Groningen, The Netherlands), mouse anti-NP (influenza A virus (IAV), BioRad, Hercules, CA). The secondary antibodies were from Thermo Fisher Scientific (Waltham, MA) and were AlexaFluor488-conjugated goat anti-mouse or chicken anti-rabbit; AlexaFluor568-conjugated goat anti-mouse or donkey anti-rabbit; AlexaFluor680-conjugated goat anti-mouse or goat anti-rabbit; and AlexaFluor800-conjugated goat anti-mouse secondary antibodies were used for the visualization of the primary antibodies, and they were all from Thermo Fisher Scientific (Waltham, MA). Hoechst33342 was from Sigma Aldrich while bafilomycin $A_1$ (BafA1) was from BioAustralis (Smithfield NSW, Australia).

To induce autophagy, cells were washed two times with Earle's balanced salt solution (EBSS, Sigma-Aldrich) and then incubated in the same medium for 2 h.

## Virus Stocks and Infection

Virus stocks of EMCV, EMCV-Zn, Rluc-EMCV, CVB3, RLuc-CVB3, EV71 (kind gifts from Frank van Kuppeveld, University of Utrecht, Netherlands), A/Puerto Rico/8/1934 (H1N1) IAV (kind gift from Anke Huckriede, University Medical Center Groningen, The Netherlands), DENV-2 strain 16681, ZIKV (clinical isolate from Surinam) and CHIKV (La Reunion OPY1) (all a kind gift from Jolanda Smit, University Medical Center Groningen, The Netherlands) were generated and propagated as described previously (Mauthe et al., 2016; Bhide et al., 2019; Diosa-Toro et al., 2019; Troost et al., 2020).

Virus infections for EMCV, EMCV-Zn wt, Rluc-EMCV, CVB3, RLuc-CVB3 were performed at a multiplicity of infection (moi) of 0.25 (or 1 for the co-immunoprecipitation experiments) and virus inoculums were left onto the cells for 6 h. For DENV, CHIKV, ZIKV and IAV, cells were infected at a moi of 0.8 for 26 h, at a moi of 10 for 10 h, at a moi of 0.8 for 26 h and at a moi of 0.5 for 6 h, respectively.

## Cloning

GFP-HSBP1 was generated by cloning HSBP1 cDNA (Source BioScience, Nottingham, UK, cat# IRQMp5018C039D) into the pEGFP-C1 vector as EcoRI/SacII fragment. Construct correctness was confirmed by DNA sequencing and protein expression by western blot (WB).

## Cell Lines and Cell Culture

U2OS (a kind gift from Ger Strous), HeLa (a kind gift from Peter van der Sluijs), U2OS cells stably expressing either GFP (GFP

U2OS) or GFP-HSBP1 (GFP-HSBP1 U2OS), *hsbp1*$^{-/-}$ U2OS (HSBP1KO), *atg7*$^{-/-}$ U2OS (ATG7KO) (Janssen et al., 2018), *atg13*$^{-/-}$ U2OS (ATG13KO) cells and HeLa RFP-GFP-LC3 cells (a kind gift of Tamotsu Yoshimori) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technology, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS), at 37°C in 5% $CO_2$ humidified atmosphere. The culture medium of HeLa RFP-GFP-LC3, GFP U2OS and GFP-HSBP1 U2OS was supplemented with 0.6 µg/ml G418 (Thermo Fisher Scientific). The HSBP1KO and ATG13KO cells were generated as previously described (Janssen et al., 2018). In brief, for the generation of HSBP1KO and ATG13KO cells using the CRISPR/Cas9 system, guides targeting exon 1 (CGTCCCTTACCACCGAGGTGAGG) and exon 2 (GGATATTTCTCCCAATGATCTGG) of HSBP1 and exon 3 (TTTGCTTCATGTGTAACCTCTGG and AGTCG GGAGGTCCATGTGTGTGG) of ATG13, respectively, were designed using optimized CRISPR design (http://crispr.mit.edu/). Guides were cloned into pX458 plasmid (Addgene #48138) allowing expression of guide RNAs and Cas9 along with GFP. U2OS cells were transfected for 48 h and subsequently clonally sorted based on GFP expression using a SH800S Cell Sorter (Sony biotechnology, San Jose, CA). Clones were then sequenced and protein expression was assessed by WB to verify the deletion of *HSBP1* and *ATG13*. Characterization of the ATG13KO cells can be found in **Figure S2C**.

To generate stable GFP or GFP-HSBP1 U2OS cells, U2OS cells were transfected with the pEGFP or the pEGFP-HSBP1 plasmid, respectively and then selected in a medium containing 0.6 µg/ml G418 for 10 days, resulting in a stable GFP U2OS or GFP-HSBP1 U2OS bulk population.

## Co-Immunoprecipitations

U2OS cells either stably or transiently expressing EGFP and EGFP-HSBP1 and grown in a 10 cm dish, were treated with EBSS for 2 h, EMCV infected for 6 h or left untreated before being subjected to lysis on ice in the following buffer: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM DTT, 0.5% Tween, Complete protease inhibitor (Roche, Basel, Switzerland), 1 mM PMSF. Co-immunoprecipitations were performed using the GFP-trap beads (Chromotek, Planegg, Germany). Beads were incubated with the lysates for 2 h at 4°C and washed with the washing buffer (20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 2 mM $MgCl_2$, 5 mM DTT, 0.5% Tween-20). Proteins were eluted by boiling the beats in Laemmli loading buffer (65.8 mM Tris-HCl, pH 6.8, 26.3% glycerol, 2.1% SDS, 0.01% bromophenol blue) (Laemmli, 1970) and then examined by WB.

## Western-Blot Analyses

Cells grown in 6-well or 24-well plates were washed with PBS and harvested in 100 µl of lysis buffer (20 mM Tris-HCl, pH 7.6, 130 mM NaCl, 1% Triton-X100, Complete protease inhibitor). The lysates were incubated on ice for 30 min, vortexed and centrifuged at 14,000 g for 10 min at 4°C. Supernatants were finally collected and mixed with the Laemmli loading buffer. Alternatively, cells were directly lysed in the Laemmli loading buffer and sonicated for 1 min. Equal protein amounts were separated by SDS-PAGE and after WB, proteins were detected on PDVF membranes (Merck) using specific antibodies and the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE). Densitometric values of the bands were quantified on WB images at non-saturating exposures using the ImageJ software (Schneider et al., 2012), and normalized against the loading control. For the detection of the small HSBP1 protein, we followed an optimized protocol previously described (Visweshwaran et al., 2018). In brief, after running, the gels were put into a renaturation buffer (20% glycerol, 50 mM Tris–HCl, pH 7.4) for 30 min at room temperature and after transfer, PVDF membranes were incubated in PBS containing 0.4% paraformaldehyde for 30 min to cross-link the proteins before proceeding with the detection.

## Immunofluorescence Microscopy

Cells were fixed with 3.7% paraformaldehyde or 100% methanol, washed and blocked with blocking buffer (PBS, 1% bovine serum albumin, 0.1% saponin). Primary and secondary antibodies were diluted in the blocking buffer and incubated for 1 h at room temperature. Nuclei were stained with Hoechst33342 during the incubation with the secondary antibody for automated image acquisition. Alternatively, nuclei were stained with DAPI (Thermo Fisher Scientific) for confocal microscopy. Fluorescent microscopy images were collected with a DeltaVision RT fluorescence microscope (Applied Precision, Issaquah, WA) equipped with a CoolSNAP HQ camera (Photometrix, Kew, Australia). Images were generated by collecting a stack of 6 to 16 images with focal planes 0.30 µm apart, and subsequently deconvolved using the SoftWoRx software (Applied Precision). Quantification of puncta number was performed using the Icy software (http://icy.bioimageanalysis.org) using spot detector plugin or the ImageJ software. For automatic acquisition, fluorescence images were automatically acquired using a TissueFAXS (TissueGnostics, Vienna, Austria), which is based on a high-end fully motorized Zeiss AxioObserver Z1 microscope with a Zeiss- LD "Plan-Neofluar" 20x/0,4 Corr Dry objective (Zeiss, Oberkochen, Germany). The following filters were used: DAPI for the imaging of the nuclei, GFP for the acquisition of the GFP-HSBP1 and LC3 signals, and TexasRed for the imaging of the p62, EMCV and CVB3 capsid signal. The GFP and TexasRed filter were used for the GFP-RFP-LC3 tandem analysis. The acquired images were analyzed using the TissueQuest fluorescence analysis software (TissueGnostics GmbH, Vienna, Austria) to determine the cell count (based on the nuclei staining), the percentage of infected cells (based on the signal intensity in the cells), the mean signal intensity of infected cells and the translocation of GFP-HSBP1 into the nucleus (based on the signal intensities of GFP in the cytosol versus the nucleus). LC3 and p62 puncta were automatically quantified using the icy bioimage analysis software (De Chaumont et al., 2012).

## siRNA and DNA Transfections

U2OS cells were transfected for 48 h with 20 nM of either control siRNA or siRNA targeting HSBP1 (SMARTpool from Dharmacon, Lafayette, CO) using 0.1 µl, 0.5 µl or 2 µl of Lipofectamine RNAiMAX (Thermo Fisher Scientific) for 96-, 24- or 6-wells plate cultures, respectively, according to the manufacturer's protocol. For the GFP and GFP-HSBP1 transfection, U2OS cells

were seeded in 10 cm dishes (for co-immunoprecipitation, Co-IP) or in 6-well plates (for making stable cell lines), followed by a transfection procedure with Fugene (Promega, Madison, WI), according to the manufacturer's protocol.

## Luciferase Assays

Cells grown in 96-well plates were washed with PBS and incubated with 50 µl of Lysis buffer (Thermo Fisher Scientific) at room temperature for 15 min, before storing the cell lysates at -20°C. 25 µl aliquots of thawed cell lysates were then used to measure renilla luciferase expression using the Renilla luciferase flash assay kit (Thermo Fisher Scientific). Alternatively, renilla luciferase activity was measured in the following reaction buffer: 45 mM EDTA, 30 mM sodium pyrophosphate, 1.425 M NaCl, 10 µM coelenterazine h (Promega) (Baker and Boyce, 2014).

Enzymatic activities were measured using a GloMax®-Multi Detection System (Promega) and the following program: 25 µl substrate; 2 s delay; 10 s measuring. Background luminescence was subtracted from each value and the results were normalized towards cells transfected with control siRNA.

## RNA Isolation and RT-qPCR and RNA Sequencing

The Power SYBR® Green Cells-to-CT™" kit (Thermo Fisher Scientific) was used according to manufacturer's protocol to isolate RNA, reverse transcribe it and synthesize cDNA. Quantitative PCR was performed in a CFX connect Thermocycler (Bio-Rad, Hercules, CA) using the following specific primers (Mauthe et al., 2016): HSBP1 (TATCGCGGACCTCATGACAC and TAGCAACCTTCAACTCTTTTGCG), ULK1 (TGGGCAA GTTCGAGTTCTCC and CTCCAAATCGTGCTTCTCGC), ULK2 (TGGAGACCTCGCAGATTATTTGC and ACACTCTGATCGTGTCTTCACT), ATG101 (TCCTCCA GCTTCCGAGTCCA and CCACGTAACCAGGGAGGAAC), FIP200 (CTCAAACCAGGTGAGGGTGCTTCA and TGTTTT GTGCCTTTTTGGCTTGACA), ATG13 (TCCAGACAGTT CGTGTTGGG and CTCAAATTGCCTGGTAGACATGA), GAPDH (GGGAACGCATTGACTGTTTT and CTCGGGCTTCTCAAAGTCAC).

The mRNA expression levels were first normalized against the expression of GAPDH, before comparing gene expression levels in HSPB1-depleted cells with those in the control cells.

## Statistical Analyses

Statistical significance was evaluated using two-tailed heteroscedastic *t*-testing before calculating the *p*-values. Individual data points from each independent experiment (the number of the independent experiments is indicated in each figure legend) were used to determine significances.

# RESULTS

## HSBP1 Interacts With the ULK Kinase Complex Through FIP200

We have found previously that FIP200 and ATG13 restrict picornavirus replication independently of their roles as components of the ULK kinase complex (Mauthe et al., 2016). We furthermore identified two potential binding partners that were shared by ATG13 and FIP200, i.e., HSBP1 and cell cycle progression 1 (CCPG1) (Mauthe et al., 2016). Since CCPG1 has recently been characterized as an ER-phagy receptor (Smith et al., 2018), we focused our attention to HSBP1 because no connection to autophagy or virus replication has been previously reported. To verify that HSBP1 is indeed interacting with FIP200 and ATG13, we performed Co-IP experiments in U2OS cells ectopically expressing GFP-HSBP1 (GFP-HSBP1 U2OS cells) using the GFP-Trap® resin (**Figure 1A** and **Figure S1A**). We could confirm that GFP-HSBP1 specifically interacts with all the subunits of the tested ULK kinase complex, i.e. FIP200, ATG13 and ULK1 (**Figure S1A**). Next, we explored how HSBP1 binds to the ULK kinase complex also with Co-IP experiments. When ATG13 was knocked down in GFP-HSBP1 U2OS cells, the interaction between HSBP1 and ULK1, but not with FIP200 was abolished (**Figure 1A**). ULK1 knockdown did not influence the interaction of both FIP200 and ATG13 with HSBP1. In contrast, FIP200 depletion eliminated the binding between HSBP1 and ULK1 or ATG13 (**Figure 1A**). Altogether, these Co-IP experiments revealed that HSBP1 binds to the ULK kinase complex *via* FIP200.

## HSBP1 Is Required for Full Autophagy Induction

The ULK kinase complex is essential for autophagy initiation (Licheva et al., 2021). Therefore we tested whether HSBP1 depletion alters the autophagic response to nutrient deprivation (**Figures 1**, **2** and **Figures S1**, **S2**). First, we performed a classical autophagic flux assay (Klionsky et al., 2021) in which we treated control and HSBP1-depleted cells (**Figures S1B**, **S4**) with EBSS for 2 h, to induce autophagy in the presence or absence of BafA1, a lysosomal inhibitor (**Figures 1B, C**). As a readout, we measured the conversion of non-lipidated microtubule associated protein 1 light chain 3 (LC3/MAP1LC3)-I into lipidated, autophagosomal membrane-associated LC3-II (**Figure 1B**) and the levels of sequestosome 1 (p62/SQSTM1) (**Figure 1C**) by WB, and examined the RFP-GFP-LC3 fluorescent reporter by fluorescence microscopy (**Figure S1C**), which are all assays that allow to assess autophagy induction and progression (Klionsky et al., 2021). The WB analyses revealed that BafA1 treatment increased the LC3-II and p62 levels in control and HSBP1-depleted cells to a similar extend (**Figures 1B, C**). Moreover, the number of formed autolysosmes was also equal in these cells (**Figure S1C**). Together, these data clearly show that autophagic progression is not blocked in the absence of HSBP1. Nonetheless, we detected a reduced autophagosome formation rate under starvation conditions, i.e., lower ratio of LC3-II/LC3-I, in HSBP1-depleted cells in comparison to the control (**Figure 1B**). To confirm the reduced autophagosome formation rate, we treated control and HSBP1-depleted cells with EBSS for 2 h and quantified endogenous ATG13 puncta by immunofluorescence microscopy (IF), which represent autophagosome formation sites (Karanasios et al., 2013). Knockdown of HSBP1 caused a significant reduction in the number of ATG13 puncta per cell (**Figure 1D**). We also generated a HSBP1 knockout cell line in U2OS cells, HSBP1KO (**Figures S2A**, **S4**), and

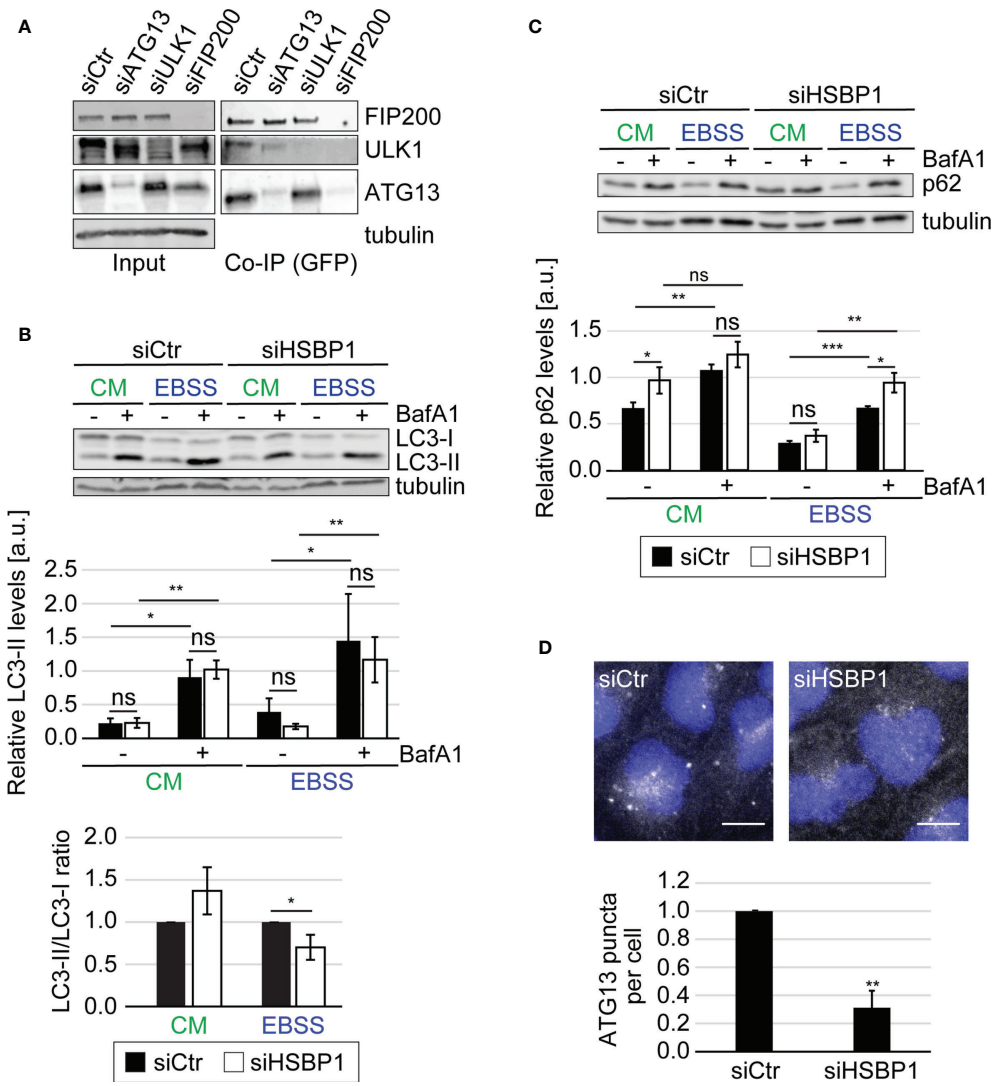


**FIGURE 1** | HSBP1 interacts with the ULK kinase complex. **(A)** GFP-HSBP1 U2OS cells were transfected with control siRNA (siCtr) or siRNAs against ATG13 (siATG13), ULK1 (siULK1) or FIP200 (siFIP200), lysed and subjected to co-immunoprecipitation using GFP-trap beads 48 h after siRNA transfection. Input lysates and Co-IP were examined by WB using antibodies against ATG13, ULK1, FIP200 and tubulin. Tubulin served as the loading control. One representative blot is shown (n = 3). **(B, C)** U2OS cells were transfected with either siCtr or siHSBP1 for 48 h and then maintained in the control medium (CM) or transferred into EBSS to induce autophagy, in the presence (+) or the absence (-) of 200 nM BafA1 for 2 h. Cells were then lysed and proteins examined by WB using anti-LC3 **(B)**, anti-p62 **(C)** and anti-tubulin antibodies. Tubulin served as the loading control. LC3-II and p62 signals were normalized to tubulin (a.u., arbitrary units) and LC3-II/LC3-I ratios determined, and values are presented in the depicted graphs. Error bars represent the standard deviations (SDs) of 3 independent experiments. **(D)** U2OS cells were transfected with either siCtr or siHSBP1 for 48 h and then transferred into EBSS medium for 2 h, before being processed for IF using anti-ATG13 antibodies. Representative images are shown and the number of ATG13-positive puncta per cells was quantified. Error bars represent SDs of 3 independent experiments. Scale bars: 10 μm. The statistical significances were calculated to the controls. The symbols *, ** and *** indicate significant differences of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively and ns indicate not significant.

repeated the autophagic flux assay to corroborate the effects that HSBP1 depletion had on autophagy (**Figures 2A, B** and **Figure S2B**). Analogously to the result observed in HSBP1-depleted cells, we detected an impairment in autophagosome formation reflected by a lower LC3-II/LC3-I ratio (**Figure 2A**). We furthermore observed a significantly reduced number of p62 puncta per cell in HSBP1KO under control and starvation conditions (**Figure 2B**), whereas LC3 puncta were reduced under some conditions, but not significantly (**Figure S2B**). p62 forms discrete puncta when autophagy is induced and therefore measuring the amount of those is widely used as a read-out for autophagy induction (Orhon and Reggiori, 2017). Altogether, these results show that depletion of HSBP1, a novel interactor of the ULK kinase complex, impairs nutrient starvation-induced early autophagy events.

Two scenario could explain the negative impact that HSBP1 knockdown has on the nutrient-induced autophagic response.

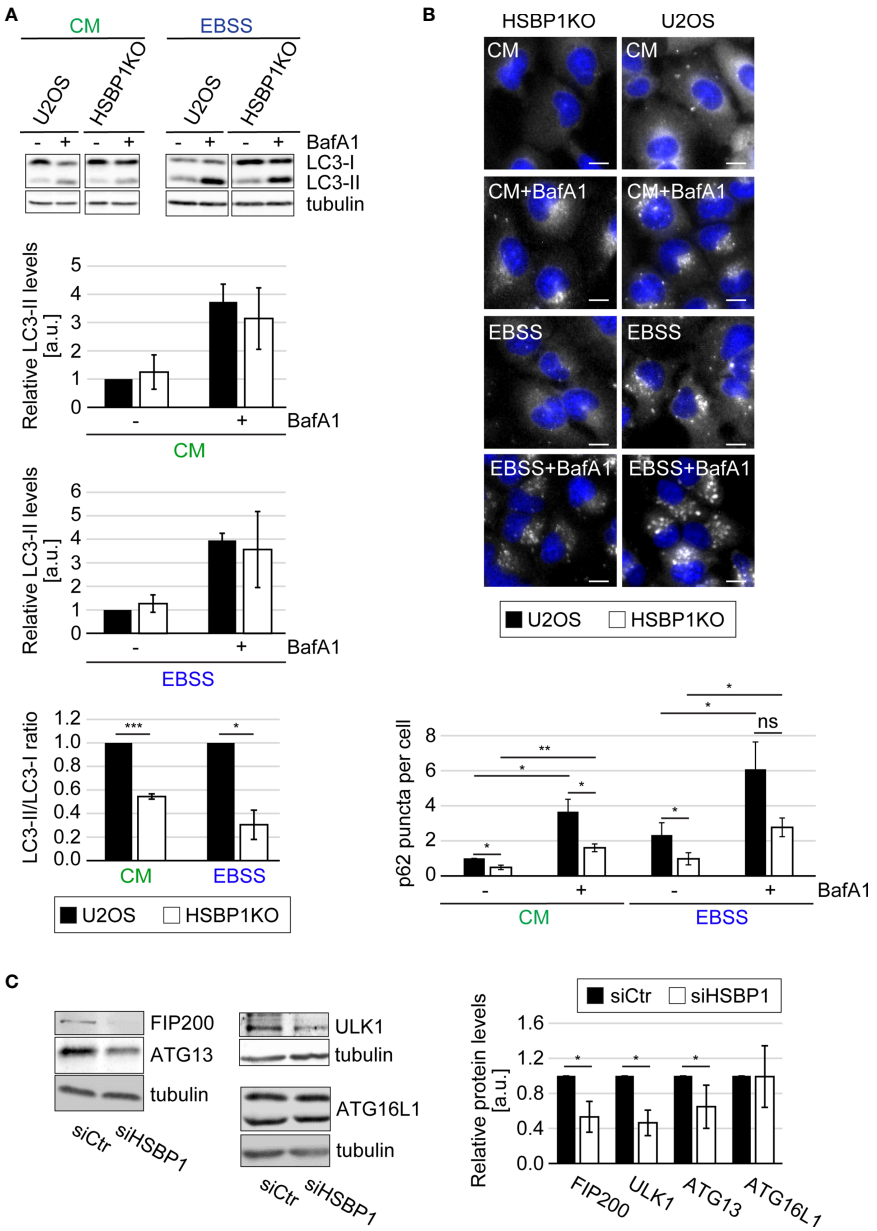


**FIGURE 2** | HSBP1 is required for full autophagy induction. **(A)** U2OS and HSBP1KO cells were maintained in CM or transferred into EBSS medium in the presence (+) or the absence (-) of 200 nM BafA1 for 2 h. Cells were subsequently lysed and WB performed using anti-LC3 and anti-tubulin antibodies. Tubulin served as the loading control. LC3-II WB signals were normalized to tubulin (a.u.) and LC3-II/LC3-I ratios determined. Error bars represent SDs of 3 independent experiments. **(B)** U2OS and HSBP1KO cells were kept in CM or transferred into EBSS medium in the presence (+) or the absence (-) of 200 nM BafA1 for 2 h. Cells were processed for IF using anti-p62 antibodies. Representative images are shown and the number of p62-positive puncta per cells was quantified. Scale bars: 10 µm. Error bars represent SDs of 3 independent experiments. **(C)** U2OS cells were transfected with either siCtr or siHSBP1 for 48 h before to be lysed and carrying WB analyses with antibodies against HSBP1, ATG13, FIP200, ULK1, ATG16L1 and tubulin. Tubulin was used as the loading control. Signal intensities were normalized to tubulin (a.u.). Error bars represent SDs of 3 independent experiments. The symbols *, ** and *** indicate significant differences of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively and ns indicate not significant.

The first is that HSBP1 is a positive regulator for the ULK kinase complex. The second is that HSBP1 could be important to stabilize this complex, a possibility evoked by the fact that HSBP1 has been shown to be crucial in the assembly and stabilization of the WASH complex (Visweshwaran et al., 2018). This second scenario was tested by knocking down HSBP1 and examine the levels of the ULK kinase complex subunits by WB. Indeed, HSBP1 depletion led to a significant decrease of ATG13, FIP200 and ULK1 levels (**Figure 2C**). This effect was specific to these proteins since the expression levels of

other ATG proteins, i.e. ATG16L1, was not influenced by HSBP1 knockdown. Since the decrease in protein expression was not due to a reduced mRNA expression (**Figure S2D**), this result shows that HSBP1 is important to stabilize the ULK kinase complex.

## HSBP1 Is a Novel Host Factor That Promotes EMCV and CVB3 Replication

Since HSBP1 binds to FIP200, we next examined whether HSBP1 also functions together with the ATG13-FIP200 subcomplex in controlling picornavirus infection. We repeated the Co-IP experiments using GFP-HSPB1 as a bait and measured the binding to ATG13 after either autophagy induction or EMCV infection (**Figure 3A**). EMCV is a member of the picornavirus family that we have previously employed to characterize the anti-picornaviral role of the FIP200-ATG13 subcomplex (Mauthe et al., 2016). The binding of HSBP1 to ATG13 remained unchanged upon autophagy induction (**Figure S3A**) showing that nutrient starvation and subsequent activation of the ULK kinase complex activity is not regulated through dynamic HSBP1 binding. In contrast, when the cells were exposed to EMCV, HSBP1 association with ATG13 was significantly reduced (**Figure 3A**). This result and the fact that HSBP1 binds the ULK complex *via* FIP200 (**Figure 1A**), suggests that HSBP1 is also involved and possibly regulates the ATG13-FIP200 subcomplex function in controlling the replication of EMCV and by extension, of other picornaviruses. To test whether HSBP1 influences EMCV replication, we infected control and HSBP1-depleted U2OS cells with a wild-type EMCV strain and quantified both viral capsid expression and the percentage of virus infected cells. In parallel, the same cells were also infected with a luciferase-expressing EMCV strain to assess virus replication by measuring luciferase activity (Mauthe et al., 2016). We found that HSBP1 knockdown reduced EMCV replication to 40-60% in comparison to control cells (**Figure 3B**). We also infected the HSBP1KO cells and observed that EMCV replication was significantly reduced, confirming the results obtained with the siRNA (**Figure 3C**). Conversely, GFP-HSBP1 overexpression led to an increase in EMCV replication, revealing that HSBP1 is a novel host factor that positively regulates EMCV propagation (**Figure 3C**). Finally, we also tested CVB3 replication in HSBP1-depleted cells to determine whether what we observed is EMCV specific or not. Measurement of luciferase expression and quantification of virus-positive cells in HeLa and U2OS cells upon HSBP1 knockdown (**Figure 3D**) and in HSBP1KO cells (**Figure S3B**) revealed HSBP1 depletion also reduced CVB3 replication. These data established HSBP1 as a novel host factor that is required for optimal EMCV and CVB3 replication.

## EMCV Infection Triggers GFP-HSBP1 Translocation From Cytoplasm Into the Nucleus

Picornaviruses cause a so-called nucleocytoplasmic traffic disorder by modulating the nuclear pore complexes and thereby disrupting the regulated transport of material, i.e., proteins and mRNA, in and out of the nucleus (Lidsky et al., 2006; Lizcano-Perret and Michiels, 2021). Interestingly, we also observed a relocalization of GFP-HSBP1 from the cytoplasm into the nucleus upon EMCV infection by fluorescence microscopy (**Figure 4A**). This redistribution was already seen at 3 h post-infection, in agreement with the notion that the nucleocytoplasmic traffic disorder is an early event during EMCV infection (Lidsky et al., 2006), while the capsid protein expression was only detected at 4-6 h post-infection when more than 90% of the infected cells showed nuclear GFP-HSBP1 (**Figures S3C, D**). GFP-HSBP1 relocalization was strongly reduced when we inhibited virus replication by either treating cells with cycloheximide 1 h after exposure to EMCV or inoculating cells with inactivated EMCV, showing that HSBP1 redistribution is induced by an active EMCV infection (data not shown). Previous studies have demonstrated that EMCV-induced nucleocytoplasmic traffic disorder is caused by the viral Leader (L) protein, which alters the phosphorylation status and thereby the function of the nuclear pore complexes (Lidsky et al., 2006). We also found that GFP-HSBP1 translocation into the nucleus depends on L since infection with EMCV-Zn, an EMCV strain lacking L (Hato et al., 2007), did not lead to the same change (**Figures S3C, D**). The mechanism underlying nucleocytoplasmic traffic disorders differs between members of the cardioviruses (*Picornaviridae*) like EMCV and members of the enteroviruses (*Picornaviridae*) such as CVB3 and EV71 (Lizcano-Perret and Michiels, 2021). Thus, we tested whether the relocalization of HSBP1 is specific to EMCV or also occurs upon infection with CVB3 and EV71. To this aim, we infected GFP-HSBP1 U2OS cells with EV71 and CVB3 before imaging them. Cell infection with these viruses also caused a relocalization of HSBP1 from the cytoplasm into the nucleus, i.e. more than 90% of infected cells displayed nuclear GFP-HSBP1 (**Figures S2**, **3C, D**), showing that HSBP1 redistribution is triggered by all the tested picornaviruses. Although it remains unclear whether the presence of HSBP1 in the nucleus is beneficial for the virus or simply a consequence of the nucleocytoplasmic traffic disorder, it is clearly specific for picornaviruses since viruses from other families (e.g., IAV, ZIKV, DENV or CHIKV) or treatments triggering autophagy *via* endoplasmic reticulum stress, did not cause HSBP1 translocation into the nucleus (**Figures S3E, F**, and data not shown).

## The Anti-Picornaviral Effect of the FIP200-ATG13 Subcomplex Involves HSBP1

Replication of EMCV and other picornaviruses is enhanced when ATG13 and FIP200 are depleted (Mauthe et al., 2016). Since HSBP1 depletion has an opposite effect, we examined the functional relationship between these three proteins. To this aim, we infected wild type, ATG7KO (Janssen et al., 2018) and ATG13KO (**Figure S2C**) cells after knocking down or not HSBP1, and then measured EMCV infection by IF (**Figure 4B**). In agreement with previous results (Mauthe et al., 2016), EMCV replication was more efficient in the ATG13KO than in wild type and ATG7KO cells. Interestingly, HSBP1 depletion reduced EMCV infection also in ATG13KO cells, when compared to control siRNA treated cells (**Figure 4B**). To confirm that this observation is not specific for EMCV infection, we repeated the same experiment using CVB3, obtaining an identical result (**Figure 4B**). Collectively, these data show that the effect of HSBP1 on picornavirus replication is not mediated through its function in autophagy since HSBP1 depletion decreased viral replication in ATG7KO cells. Moreover, these

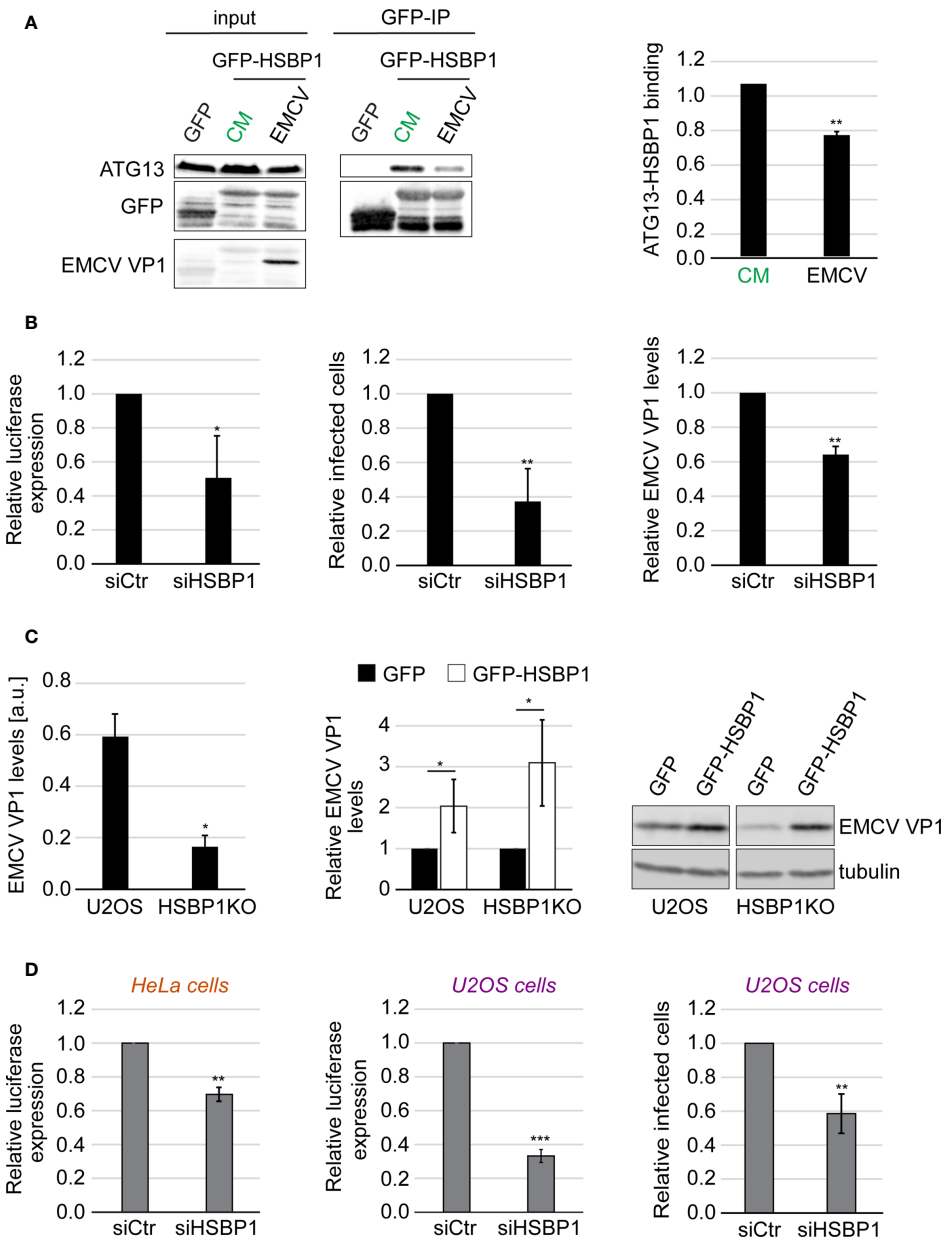


**FIGURE 3** | HSBP1 promotes EMCV and CVB3 replication. **(A)** U2OS cells were transfected with a plasmid carrying GFP or GFP-HSBP1 for 24 h and then kept in CM or were infected with EMCV for 6 h. Cells were then lysed and immunoprecipitated using GFP-trap beads. Input lysates and Co-IP were examined by WB using antibodies against ATG13, GFP and EMCV VP1. Signal intensities were quantified and the of ATG13/GFP-HSBP1 ratios in the Co-IP were determined before to be normalized to that of the CM samples. Error bars represent SDs of 4 independent experiments. **(B)** U2OS cells were transfected with either siCtr or siHSBP1 for 48 h before being infected with EMCV or a luciferase-expressing EMCV strain (left panel) for 6 h. EMCV replication was quantified by luciferase expression (left panel), EMCV VP1-positive cells by IF (middle panel) and EMCV VP1 levels by WB (right panel). Error bars represent SDs of 3 (WB, right panel) or 4 independent experiments (luciferase and IF, left and middle panels). **(C)** U2OS and HSBP1KO cells were transfected with a plasmid expressing either GFP-HSBP1 or GFP for 24 h before being infected with EMCV for 6 h. Cells were then lysed and WB membranes probed with anti-EMCV VP1 and anti-tubulin antibodies. Tubulin was used as the loading control. Signal intensities were quantified and normalized to tubulin. EMCV VP1 levels in U2OS and HSBP1KO cells expressing GFP are compared in the left panel. EMCV VP1 levels in U2OS or HSBP1KO cells carrying GFP-HSBP1 were expressed relative to those in the same cells but carrying GFP (right panel). Error bars represent SDs of 4 independent experiments. **(D)** U2OS or HeLa cells were transfected with either siCtr or siHSBP1 for 48 h, before being infected with CVB3 (right panel) or a luciferase-expressing CVB3 strain (left and middle panels) for 6 h. CVB3 replication in HeLa cells was measured by luciferase expression (left panel) and in U2OS cells by either assessing luciferase expression (middle panel) or the percentage of CVB3 VP1-positive cells (right panel). Error bars represent SDs of 3 (HeLa cells) or 4 (U2OS cells) independent experiments. The statistical significances were calculated to the controls. The symbols *, ** and *** indicate significant differences of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

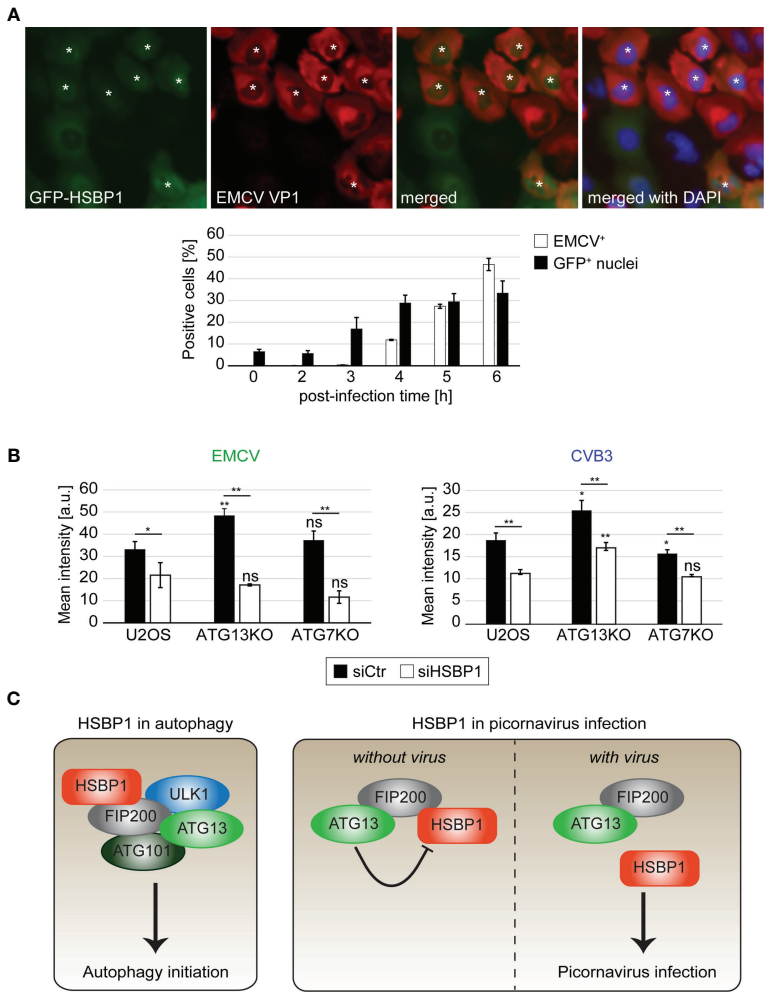


**FIGURE 4** | HSBP1 counteracts the anti-picornaviral function of ATG13 and translocates to the nucleus upon infection. **(A)** GFP-HSBP1 U2OS cells were infected with EMCV for the indicated times before being fixed and immunostained with anti-EMCV VP1 antibodies. IF images were automatically acquired and analyzed using the TissueFAXS microscope and software. EMCV VP1 positive cells (EMCV$^+$) and cells positive for GFP-HSBP1 signal in the nucleus (GFP$^+$ nuclei) were quantified. Error bars represent SDs of 3 independent experiments. White asterisks highlight GFP-HSBP1 U2OS cells with signal in the nucleus. **(B)** U2OS, ATG13KO and ATG7KO cells were transfected with either siCtr or siHSBP1 for 48 h, before being infected with EMCV (left panel) or CVB3 (right panel) for 6 h. Cells were immunostained using antibodies against EMCV and CVB3 VP1 to identify the infected cells and determine the degree of infection. The mean signal intensities were quantified and error bars represent SDs of 3 independent experiments. **(C)** Schematic model for the functions of HSBP1 in autophagy and picornavirus replication. The statistical significances were calculated to the controls. The symbols * and ** indicate significant differences of $p < 0.05$ and $p < 0.01$, respectively and ns indicate not significant. If not indicated otherwise, statistical differences were calculated to the control (U2OS cells).

results indicate that the anti-picornaviral function of the FIP200-ATG13 subcomplex is connected to HSBP1, since after the depletion of HSBP1 in ATG13KO cells (**Figure 4B**), the effect caused by the loss of ATG13 and FIP200 is lost.

## DISCUSSION

Autophagy is an important cellular survival mechanism that keeps cellular homeostasis under several stress conditions (Lahiri et al., 2019). The ULK kinase complex is crucial for autophagy initiation, and its function is modulated through the interaction of its subunits with various binding partners, including SMCR8-C9orf72 complex subunit (SMCR8) (Yang et al., 2016), autophagy and beclin 1 regulator 1 (AMBRA1) (Nazio et al., 2013) and acidic lipids (Karanasios et al., 2013). Here, we identified a novel ULK kinase complex binding partner, HSBP1, which interacts with this complex *via* FIP200 (**Figure 4C**). We have found that HSBP1 knockdown decreases the formation rate of autophagosomes but does not influence the overall autophagic flux. Since the depletion of HSBP1 caused a reduction in the levels of ULK kinase complex components, we suggest that HSBP1 could be involved in the assembly and/or stability of this complex in a similar manner as it has been shown for the WASH complex

(Visweshwaran et al., 2018). However, the autophagy defect caused by HSBP1 depletion is much less severe than when an ULK kinase complex component is ablated, e.g. ATG13, since this causes a severe autophagic flux impairment (**Figure S2C**). Thus, HSBP1 has more a regulatory role and it might be required to fine tune the activity of this complex under specific conditions. Alternatively, HSBP1 may act as a chaperone promoting the assembly of the ULK kinase complex.

We have also found that HSBP1 knockdown reduces picornavirus replication, establishing HSBP1 as a novel host cell regulator of picornavirus infection. It has been shown that autophagy induction promotes picornavirus replication under some circumstances (Klein and Jackson, 2011; Huang and Yue, 2020), which could explain why HSBP1 depletion has a negative impact on picornavirus propagation. However, we detected a negative effect of HSBP1 depletion on EMCV replication also in autophagy deficient cells (**Figure 4B**). This result shows that the proviral function of HSBP1 is autophagy independent. In fact, we found that HSBP1 suppresses the anti-viral function of the FIP200-ATG13 subcomplex that we have previously discovered (**Figure 4C**). Thus, our data favors a possible model in which through binding HSBP1, the FIP200-ATG13 subcomplex inhibits the pro-picornaviral function of HSBP1. Upon picornaviral infection, HSBP1 dissociates from this complex and is released from the inhibitory action of FIP200-ATG13 subcomplex, fulfilling its proviral function. This would mean that the FIP200-ATG13 subcomplex carries out its anti-picornaviral function by preventing the pro-picornaviral function of HSBP1. This notion is corroborated by the observation that both the anti-picornaviral role of FIP200-ATG13 subcomplex is abolished when HSBP1 is depleted (**Figure 4B**) and picornavirus replication is reduced under the same situation (**Figure 3**). This notion is further supported by the finding that HSBP1 overproduction enhances EMCV replication (**Figure 3C**), possibly because the levels of FIP200-ATG13 subcomplex are insufficient to inhibit excess HSBP1. In line with this consideration, FIP200 overexpression reduces picornavirus replication (Mauthe et al., 2016), probably because the higher FIP200 levels can more effectively inhibiting HSBP1. Although this model can explain our data, we cannot exclude that HSBP1 functions in a step of the picornaviral life cycle that is epistatic to the one controlled by the FIP200-ATG13 subcomplex. For example, the disruption of the WASH complex by HSBP1 depletion (Visweshwaran et al., 2018) could negatively affect picornaviral cell entry. Consequently, HSBP1 could have even a dual role in this scenario, i.e., promoting cell entry through the stabilization of the WASH complex (or other yet uncharacterized functions of HSBP1) and counteracting the virus by stabilizing the FIP200-ATG13 subcomplex.

The nuclear relocalization of HSBP1 observed in picornavirus-infected cells is not associated with a defect in the general protein shuttling between the cytoplasm and the nucleus because viruses such as DENV and ZIKV, which are known to target nuclear pore complexes (Wubben et al., 2020), did not trigger HSBP1 redistribution into the nucleus (**Figures S3E, F**). An early report identified HSBP1 as a negative regulator of HSF1 and showed that it inhibits HSF1 function in the nucleus (Satyal et al., 1998). However, we have no indications of a possible functional connection between enhanced HSF1 activity and HSBP1 relocalization, since HSBP1 did not move to the nucleus upon ER stress caused by tunicamycin (data not shown), which is known to induce HSF1 activity (Liu and Chang, 2008). Additionally, HSF1 knockdown does not interfere with HSBP1 subcellular distribution (data not shown). Since HSBP1 has no apparent nuclear localization signals, we cannot exclude that the translocation into the nucleus is facilitated by a yet unknown binding partner. It also still remains to be determined whether HSBP1 fulfills its pro-picornaviral function within the nucleus or not.

In conclusion, we identified two new functions of HSBP1, both mediated *via* its binding to FIP200. On the one hand, HSBP1 regulates the early steps of autophagy by stabilizing the ULK kinase complex, and on the other hand, it functions as a positive regulator for picornavirus replication independently of its function in autophagy. Future studies will be required to unveil how HSBP1 is exactly promoting in the picornavirus life cycle.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

MM and FR designed the study. MM, NB, PV, and ND performed experiments. MM and FR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.745640/full#supplementary-material

**Supplementary Figure 1 |** HSBP1 binds the ULK kinase complex components. **(A)** U2OS cells were transfected with plasmids expressing GFP-HSBP1 or GFP for 24 h. Cells were subsequently lysed and co-immunoprecipitated using GFP-trap beads. Input lysates and Co-IP were examined by WB using antibodies against GFP, ATG13, ULK1 and FIP200. **(B)** HSBP1 levels in U2OS cells treated with siHSBP1 for 48 h, were assessed by WB. Tubulin is used as the loading control. **(C)** RFP-GFP-LC3 HeLa cells were transfected with either siCtr or siHSBP1 for 48 h and kept in CM or transferred into EBSS medium in the presence (+) or the absence (-) of 200 nM BafA1 for 2 h. Cells were fixed and images were automatically acquired and analysed. Representative images are shown and the number of autophagosomes (GFP-positive LC3 puncta) and autolysosomes (RFP-only positive LC3 puncta) per cell was quantified. Scale bars: 10 µm. Error bars represent SDs of 4 independent experiments. The symbols * and ** indicate significant differences of $p < 0.05$ and $p < 0.01$, respectively.

**Supplementary Figure 2 |** HSBP1 depletion does not impair autophagy progression and ULK kinase complex component mRNA expression. **(A)** HSBP1 levels in HSBP1KO cells were assessed by WB. Tubulin is used as the loading control. **(B)** U2OS and HSBP1KO cells were kept in CM or transferred into EBSS medium in the presence (+) or the absence (-) of 200 nM BafA1 for 2 h. Cells were processed for IF using anti-LC3 antibodies. Representative images are shown and the number of LC3-positive puncta per cells was quantified. Scale bars: 10 µm. Error bars represent SDs of 3 independent experiments. **(C)** U2OS and ATG13KO cells were kept in CM or transferred into EBSS medium in the presence (+) or the absence (-) of 200 nM BafA1 for 2 h, before to be lysed and WB probed with antibodies recognizing ATG13, LC3 and actin. **(D)** U2OS cells were transfected with either siCtr or siHSBP1 for 48 h. Cells were subsequently lysed and mRNA levels of HSBP1, ATG13, FIP200 and ULK1 were measured using quantitative real-time PCR. Error bars represent SDs of 4 independent experiments. The symbol *** indicates a significant difference of $p<0.001$.

**Supplementary Figure 3 |** Nuclear translocation of HSBP1 is specifically triggered by picornavirus infection. **(A)** U2OS cells were transfected with a plasmid carrying GFP-HSBP1 for 24 h and then kept in CM or transferred into EBSS medium for 2 h. Cells were then lysed and immunoprecipitated using GFP-trap beads. Input lysates and Co-IP were examined by WB using antibodies against ATG13 and GFP. Signal intensities were quantified and the ATG13/GFP-HSBP1 ratios in the Co-IP were determined before to be normalized to that of the CM samples. Error bars represent SDs of 4 independent experiments. **(B)** CVB3 replication in HSBP1KO cells was measured by either assessing luciferase expression (left panel) or determining the percentage of CVB3 VP1-positive cells (right panel). Error bars represent SDs of 3 (left panel) or 6 (right panel) independent experiments. The statistical significances were calculated to the controls. **(C)** GFP-HSBP1 U2OS cells were infected with EMCV, EMCV-Zn, EV71 and CVB3 for 6 h before being fixed and immunostained with anti-EMCV VP1 (for EMCV and EMCV-Zn), anti-CVB3 VP1 (for CVB3) or anti-dsRNA (for EV71) antibodies. Images were automatically acquired and analyzed using the TissueFAXS microscope and software. The average percentage of virus positive cells +/- SD is indicated. **(D)** Virus positive cells with the GFP-HSBP1 signal in the nucleus (GFP+ nuclei) were quantified. Error bars represent SDs of 5 independent experiments. **(E)** GFP-HSBP1 U2OS cells were infected with EMCV (for 6 h), CHIKV (for 10 h), DENV (for 26 h), ZIKV (for 26 h) or IAV (for 6 h). Cells were then fixed and immunostained with virus specific antibodies before automatically acquire images and analyze them using the TissueFAXS microscope and software. The average percentage of virus positive cells +/- SD is indicated. **(F)** Virus positive cells with GFP-HSBP1 signal in the nucleus ($GFP^+$ nuclei) were quantified. Error bars represent SDs of 3 or 4 independent experiments. The symbols ** and *** indicate significant differences of $p < 0.01$ and $p < 0.001$, respectively.

**Supplementary Figure 4 |** Depletion of HSBP1 in U2OS cells. **(A)** Original, uncropped WB used for **Figure S1B**. HSBP1 and tubulin bands used for figure S1B are indicated in red squares. **(B)** Original, uncropped WB used for **Figure S2A**. HSBP1 and tubulin bands used for **Figure S2A** are indicated in red squares.

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REVIEW
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# The Role of Chaperone-Mediated Autophagy in Hepatitis C Virus-Induced Pathogenesis

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Lysosome incorporate and degrade proteins in a process known as autophagy. There are three types of autophagy; macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Although autophagy is considered a nonselective degradation process, CMA is known as a selective degradation pathway. All proteins internalized in the lysosome *via* CMA contain a pentapeptide KFERQ-motif, also known as a CMA-targeting motif, which is necessary for selectivity. CMA directly delivers a substrate protein into the lysosome lumen using the cytosolic chaperone HSC70 and the lysosomal receptor LAMP-2A for degradation. Hepatitis C virus (HCV) NS5A protein interacts with hepatocyte-nuclear factor 1α (HNF-1α) together with HSC70 and promotes the lysosomal degradation of HNF-1α *via* CMA, resulting in HCV-induced pathogenesis. HCV NS5A promotes recruitment of HSC70 to the substrate protein HNF-1α. HCV NS5A plays a crucial role in HCV-induced CMA. Further investigations of HCV NS5A-interacting proteins containing CMA-targeting motifs may help to elucidate HCV-induced pathogenesis.

**Keywords: hepatitis C virus, chaperone-mediated autophagy, CMA-targeting motif, LAMP-2A, HSC70, lysosome**

## INTRODUCTION

The molecular mechanisms of autophagy were discovered by Prof. Yoshinori Ohsumi and his team *via* the identification of the autophagic-related genes (ATGs) in yeast in the early 1990s (Ohsumi, 2014). ATG genes are well-conserved among eukaryotes. The field of autophagy has been developed rapidly on the basis of these great discoveries. Lysosomes, together with other proteolytic systems, are involved in the constant turnover of intracellular constituents. Using this mechanism, cells eliminate aggregate-prone proteins and organelles, bulk cytoplasm, and infectious pathogens. Moreover, there is growing evidence of autophagy's roles in cell death, differentiation, aging, growth control, antigen presentation, cell defense, and adaptation to hostile conditions (Cuervo, 2004; Mizushima, 2007).

Because of the numerous functions of autophagy in the cells, interference with this process could be associated with various human diseases. Many diseases, such as cancer, neurodegenerative

diseases, metabolic dysfunction, liver diseases, and cardiovascular diseases, have been linked to disruptions in autophagy. (Levine and Kroemer, 2008; Yang and Klionsky, 2020). The failure of autophagic clearance is linked to the intracytoplasmic accumulation of misfolded and aggregate-prone protein in most adult-onset neurodegenerative disorders (Nixon, 2013). Autophagy is also critical in the adaptive immune response, specifically in the processing and presentation of major histocompatibility complex (MHC) class II antigens, in addition to its role in innate immunity (Levine and Deretic, 2007). Consequently, numerous intracellular pathogens hijack this pathway by evading autophagic detection, changing the autophagic route, and manipulating the autophagosomal compartment to their benefit (Ogawa et al., 2011).

In mammalian cells, proteins are incorporated into lysosomes by (1) macroautophagy, (2) microautophagy, and (3) chaperone-mediated autophagy (CMA) (**Figure 1**). In macroautophagy, a protein with other cytosolic components and organelles is entrapped in a double-membrane-bound vesicle, called an autophagosome. The autophagosome fuses with the lysosome, followed by degradation of the sequestered components. In microautophagy, cytosolic components are directly ingested by lysosomes through invagination of the lysosomal membrane. The third form of autophagy, CMA, is distinct from the other types of autophagy in terms of identification of protein target by chaperone protein HSC70 and mechanism of delivery to the lysosomal lumen (Cuervo, 2004; Mizushima, 2007; Glick et al., 2010).

## THE MOLECULAR MECHANISM OF CHAPERONE-MEDIATED AUTOPHAGY

Although autophagy was commonly regarded in the past as a nonselective breakdown system, CMA turned out to be a type of selective autophagy. CMA selectively recognizes substrate proteins by the specific protein recognition and translocation into the lysosomal membrane in association with HSC70, a heat shock protein of around 70 kDa. All of the protein substrates degraded by CMA have a specific pentapeptide motif (KFERQ-motif; CMA-targeting motif) in their amino acid sequences (Kirchner et al., 2019; Kacal et al., 2021). A potential CMA-targeting motif can be found in 30-40% of soluble cytosolic proteins. However, additional motifs are made possible by posttranslational modifications, such as phosphorylation or acetylation, thus increasing the number of possible substrates.

A protein-containing CMA-targeting motif is recognized by the cytosolic chaperone HSC70 (Chiang et al., 1989; Cuervo, 2011; Kirchner et al., 2019). The next step of the CMA pathway is the binding of the protein complex, a target protein and HSC70, to the cytosolic tail of lysosome-associated membrane protein type 2A (LAMP-2A) at the lysosomal membrane (Cuervo and Dice, 1996). LAMP-2A is one of the three splice variants of LAMP-2 genes: LAMP-2A, LAMP-2B, and LAMP-2C. LAMP-2A is the crucial determinant of the CMA pathway. LAMP-2A protein is necessary for CMA, but not for other types of autophagy. The production, elimination, and sub-compartmentalization of LAMP-2A receptor modulate the

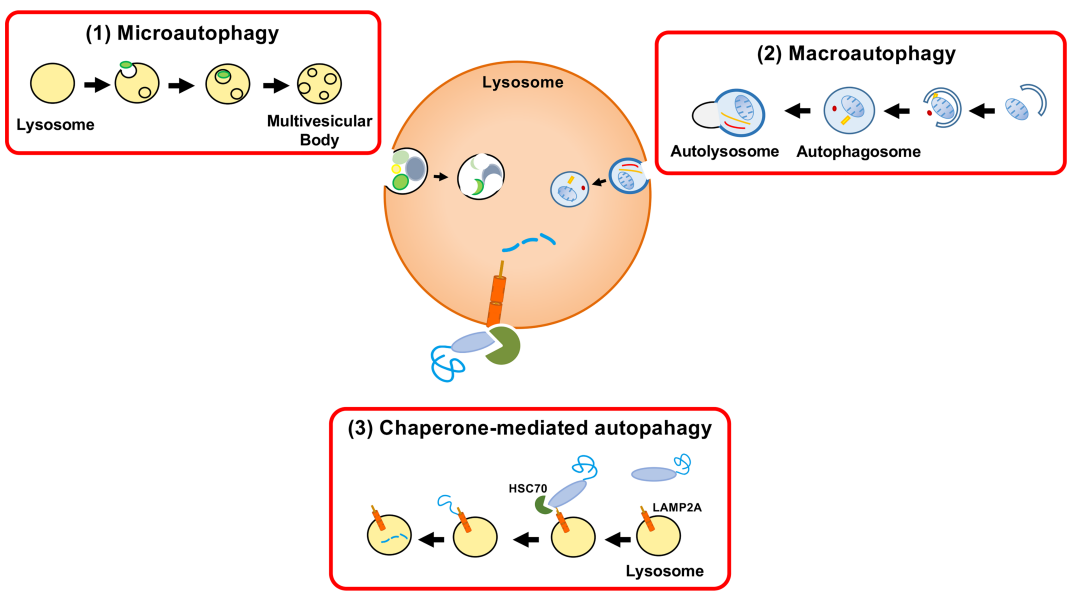


**FIGURE 1** | Three autophagy pathways. Proteins are incorporated into lysosomes by **(1)** macroautophagy, **(2)** microautophagy, or **(3)** chaperone-mediated autophagy (CMA). Autophagy was formerly considered a nonselective bulk degradation process. However, CMA results in the selective degradation of the cytosolic proteins. Macroautophagy involves the encapsulation of a protein with other cytosolic components and organelles in a double membrane-bound vesicle (autophagosome). The autophagosome fuses with the lysosome and the sequestered components are degraded. Microautophagy is a process in which lysosomes directly engulf cytosolic components through membrane invagination. CMA involves the selective destruction of CMA-targeting motif-containing proteins transported to lysosomes by the chaperone HSC70 and the internalization of proteins by LAMP-2A.

CMA activity in the cells (Kaushik and Cuervo, 2012; Kaushik and Cuervo, 2018; Arias and Cuervo, 2020). Multimerization of LAMP-2A is required for translocation of unfolded substrate protein into the lysosome lumen (Cuervo and Wong, 2014). Finally, the target protein is rapidly degraded in the lysosome, followed by the dissociation of the translocation complex and monomerization of LAMP-2A (**Figure 2**).

## CMA AND EMI

Endosomal microautophagy (eMI) is another type of selective protein degradation using HSC70 for recognition of a CMA-targeting motif. In contrast to CMA, the unfolding and LAMP-2A binding of cytosolic proteins is not required for eMI. A substrate protein for eMI is sequestered by the formation of the invagination in the surface of the endosomal membrane through the coordinated function of ESCRT I (TSG101) and three accessory proteins: VPS4A, VPS4B and Alix. After binding to a substrate protein, HSC70 interacts with phosphatidylserine of the endosomal membrane. HSC70 is internalized along with the substrate protein in microvesicles involved in the endosomal sorting complex required for transport (ESCRT). Substrate proteins in vesicles are degraded in the late endosome (**Figure 3**). However, it's still unclear whether the entire ESCRT machinery is necessary for the eMI pathway (Tekirdag and Cuervo, 2018; Sahu, et al., 2011). In the CMA pathway, HSC70 is released back to the cytosol after the substrate is transferred back to LAMP-2A. On the other hand, HSC70 is internalized and degraded with the target protein in the eMI pathway (Sahu, et al., 2011; Madrigal-Matute and Cuervo, 2016). Although both CMA and eMI use the CMA-targeting motif for substrate recognition, the substrates of CMA and eMI do not fully overlap. The CMA-targeting motif is necessary and sufficient for HSC70-induced degradation on CMA, whereas the CMA-targeting motif is not sufficient for the targeting degraded proteins in eMI (Tekirdag and Cuervo, 2018; Kichner et al., 2019). Microtubule-associated protein Tau, involved in axoplasmic transport in normal neurons, is known to be degraded by both eMI and CMA (Mukherjee et al., 2016). The intrinsic properties of the substrate protein may be accountable for the shifting between these two pathways. Because CMA and eMI require different receptors to transport the target protein to the appropriate location of degradation, the knock-down of each receptor will assist in the analysis of protein degradation; that is, the knock-down of LAMP2A membrane protein increases the amount of target protein in the CMA pathway. On the other hand, the substrate protein level increases in the eMI pathway after the knock-down of the VPS4A/B protein (Tekirdag and Cuervo, 2018).

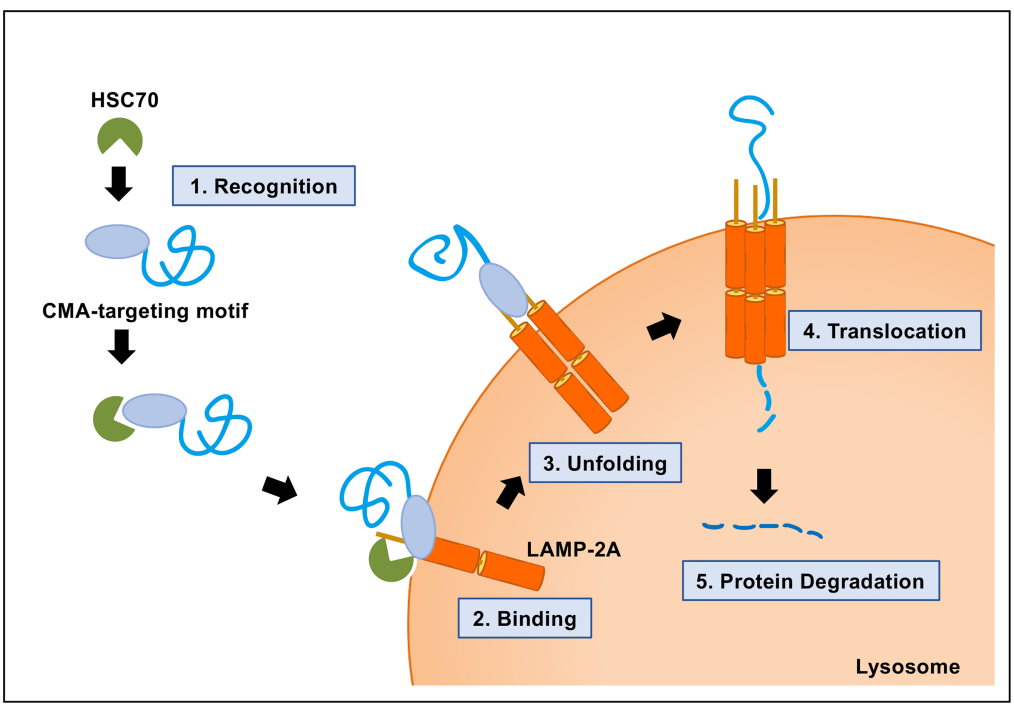


**FIGURE 2** | Molecular mechanism of chaperone-mediated autophagy (CMA). CMA is a five-step process. Recognition of the CMA-targeting motif in the substrate protein by HSC70 (step 1); binding of the substrate–chaperone complex to LAMP-2A (step 2); unfolding of the protein substrate (step 3); multimerization of LAMP-2A and translocation of the protein to the lysosomal lumen mediated by lysosomal HSC70 (step 4); protein degradation and disassembly of LAMP-2A multimer (step 5).

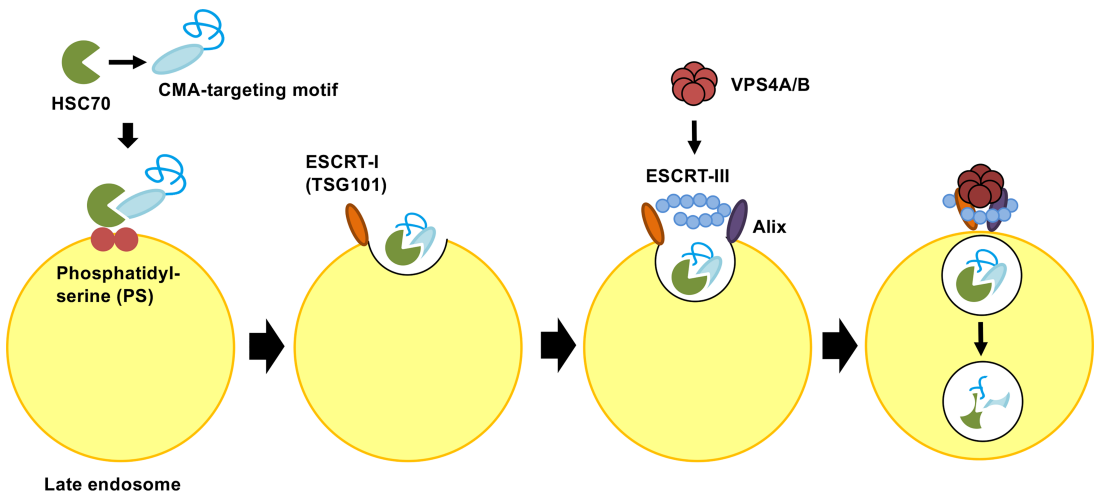


**FIGURE 3** | Molecular mechanism of endosomal microautophagy (eMI). Multifunctional chaperone HSC70 recognizes protein bearing CMA-targeting motifs. Upon cargo binding, HSC70 directly interacts with phosphatidylserine (PS) in the endosomal membrane. HSC70 is internalized along with protein into microvesicles *via* the coordinating functions of ESCRT I (TSG101), ESCRT III, VPS4A/B, and Alix. The degradation of microvesicles occurs in the endosomal lumen or lysosome *via* endosome–lysosome fusion. ESCRT, endosomal sorting complex required for transport.

## CMA AND HUMAN DISEASES

Many studies have discovered the association of impairment of the CMA process and human diseases. CMA is known to be involved in Parkinson's disease (Wong and Cuervo, 2010), Huntington's disease (Bauer et al., 2010; Koga et al., 2011; Qi et al., 2012), Alzheimer's disease (Liu et al., 2009; Wang et al., 2009), prostate cancer (Lv et al., 2011), and renal diseases (Sooparb et al., 2004). *Salmonella enterica*, an invasive intracellular bacterium, exploits LAMP-2A and HSC70 to promote proliferation (Singh et al., 2017). This bacterium activates the CMA pathway to degrade tripartite motif (TRIM) 21, an E3 ubiquitin ligase which is involved in regulating the IFN-I response, to escape the host immune system (Hos et al., 2020).

## CMA AND HEPATITIS C VIRUS INFECTION

HCV is an enveloped, positive single-stranded RNA virus that belongs to the *Flaviviridae* family, *Hepacivirus* genus (Ray et al., 2013). The HCV genome consists of a 9.6kb RNA encoding a polyprotein of 3,010 amino acids (aa). The polyprotein is cleaved into three structural proteins (core, envelope 1 [E1], and envelope 2 [E2]) and seven nonstructural proteins (p7, nonstructural protein 2 [NS2], NS3, NS4A, NS4B, NS5A, NS5B) proteins by viral proteases and host signal peptidase (Ray et al., 2013). The structural proteins are responsible for the formation of virions, whereas the nonstructural protein is involved in viral replication (Lohmann et al., 1999; Blight et al., 2000). Approximately 56 million people (0.8% of the global population) are chronically infected with HCV (World Health Organization, 2021). Within two or three decades after infection, around 20% of HCV carriers will develop cirrhosis and hepatocellular carcinoma, either of which requires liver transplantation (Roudot-Thoraval, 2021).

Several studies have associated both structural and nonstructural HCV proteins with macroautophagy (Guevin et al., 2010; Su et al., 2011; Wang et al., 2014; Lee et al., 2019). As macroautophagy serves various functions in the host cell, it also serves to sustain HCV life cycle. However, the molecular mechanism by which HCV induces macroautophagy is still unclear (Ke and Chen, 2014).

HCV infection causes not only intrahepatic diseases but also extrahepatic manifestations, such as metabolic disorders (Ramos-Casals et al., 2017; Koike, 2009). We reported that HCV infection suppresses GLUT2 gene expression *via* selective lysosomal degradation of transcription factor HNF-1α protein (Matsui et al., 2012). HCV infection induces lysosomal degradation of this protein *via* interaction with NS5A protein (Matsui et al., 2015). We then discovered the HCV-induced selective degradation of HNF-1α *via* CMA (Matsui et al., 2018).

Other groups reported that CMA targets IFNAR1 degradation in the lysosome in free fatty acids-treated HCV cell culture (Kurt et al., 2015; Dash et al., 2016). They also reported that CMA promotes Beclin1 degradation through Nrf2 signaling in persistently infected HCV cell cultures (Aydin et al., 2018; Dash et al., 2020).

It has been reported that ER stress induces CMA *via* activation of p38 MAPK, resulting in phosphorylation of LAMP-2A and accumulation of LAMP-2A on lysosomal membrane (Li et al., 2017). Dash et al. (Dash et al., 2019) have described that ER stress uses the p38 MAPK-CMA pathway to maintain cell survival under

stress. HCV was reported to induce ER stress (Wang et al., 2019). Therefore, it remains to be elucidated whether HCV infection induces ER stress to activate CMA pathway.

## MOLECULAR MECHANISM OF HCV-INDUCED CMA PATHWAY

To clarify the molecular mechanism underlying the HCV-induced CMA pathway, we searched for the CMA-targeting motif within HNF-1α. We identified the CMA-targeting motif of HNF-1α raging from aa 130 to 134, QREVV (**Figure 4**). HSC70 binds HNF-1α *via* its CMA-targeting motif, $^{130}$QREVV$^{134}$. Protein Complex NS5A/HSC70/HNF-1α is transported to the lysosome, resulting in the association of HNF-1α with LAMP-2A. HNF-1α crosses the membrane with the assistance of LAMP-2A. HNF-1α is degraded in the lysosome. We propose that HCV-induced HNF-1α degradation *via* CMA suppresses GLUT2 gene expression, leading to the downregulation of cell surface expression of GLUT2 and the disruption of glucose uptake into the cells (Matsui et al., 2012; Matsui et al., 2015; Matsui et al., 2018) (**Figure 4**).

## ANALYSIS OF HCV-INDUCED CMA PATHWAY

### Detection of the CMA-Targeting Motif in the Sequence of Substrate Protein

The CMA-targeting motif in the amino acid sequence of the substrate protein is essential for the interaction between HSC70 and a substrate protein. Cuervo's group proposed the basic requirements for the CMA-targeting motif (Kaushik and Cuervo, 2018; Kirchner et al., 2019). A CMA-targeting motif contains one or two of the positively charged residues: lysine (K) or arginine (R); one or two of the hydrophobic residues: phenylalanine (F), isoleucine (I), leucine (L), or valine (V); one of the negatively charged residues: aspartic acid (D) or glutamic acid (E); and one glutamine (Q) on either side of the pentapeptide (**Figure 4**). The removal of the pentapeptide amino acid in a target protein inhibited its lysosomal degradation, underscoring the importance of this motif in the CMA pathway (Dice et al., 1990; Wing et al., 1991; Kaushik and Cuervo, 2012). Free Web-based software, KFERQ finder V0.8 (https://rshine.einsteinmed.org/) was developed by Cuervo's group to facilitate rapid identification of this motif in any protein sequences (Kirchner et al., 2019).

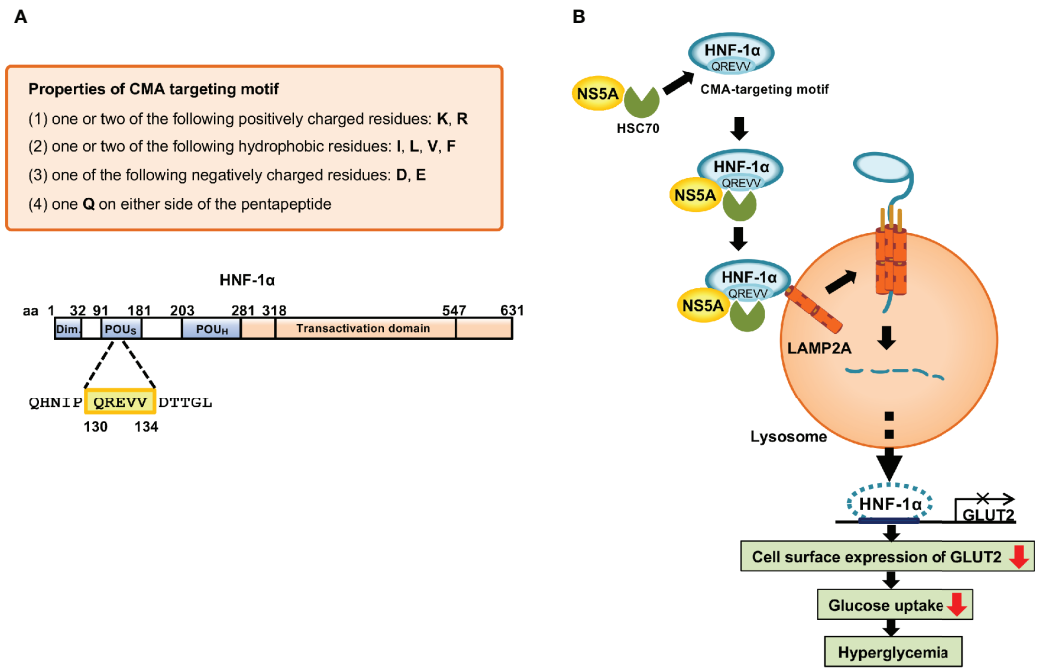


**FIGURE 4** | Mechanism of the HCV-induced degradation of HNF-1α *via* CMA. **(A)** The basic requirements of the CMA-targeting motif. One glutamine residue (Q) flanked on either side by one or two basic amino acids (K or R), an acidic amino acid (D or E), and one or two bulky hydrophobic amino acids (F, I, L or V). Following this rule, we identified the CMA-targeting motif ($^{130}$QREVV$^{134}$) in the POUs domain of HNF-1α. **(B)** HCV NS5A interacts with HSC70 and recruits HSC70 to HNF-1α protein. HSC70 binds to the CMA-targeting motif of HNF-1α. The protein complexes are delivered to the surface of the lysosomal membrane to bind to LAMP-2A. Once HNF-1α binds to LAMP2A, HNF-1α unfolds and crosses the lysosomal membrane with the assistance of LAMP-2A. Finally, HNF-1α is degraded by lysosomal proteases, resulting in the downregulation of the GLUT2 transcription. GLUT2 mRNA levels and GLUT2 expression decrease, resulting in decreased glucose uptake to the cell, which in turn leads to hyperglycemia.

### HCV NS5A Plays a Crucial Role in HCV Induced-CMA Pathway

HCV infection enhances the interaction between HSC70 and HNF-1α (Matsui et al., 2018). The selective lysosomal degradation of HNF-1α protein is induced by HCV NS5A. Domain I of NS5A plays a crucial role in the interaction with HNF-1α and the degradation of HNF-1α protein (Matsui et al., 2015).

HCV NS5A interacts with HSC70 to promote the recruitment of HSC70 to the substrate protein. NS5A binds both HSC70 and the substrate protein. HSC70 binds to its substrate protein *via* the CMA-targeting motif. Protein Complex NS5A/HSC70/substrate protein is transported to the lysosomal membrane, resulting in association with LAMP-2A. The substrate protein can then cross the membrane with the assistance of LAMP-2A.

### HSC70 and LAMP-2A Are Key Components of the CMA Machinery

The cytosolic chaperone HSC70 recognizes the host protein *via* the CMA-targeting motif (Bonam et al., 2019). The HCV-induced degradation of HNF-1α is restored by the siRNA knockdown of HSC70. To investigate whether LAMP-2A plays a role in the HCV-induced degradation of HNF-1α, we knocked down LAMP-2A mRNA by siRNA. The knockdown recovered the level of substrate protein in HCV-infected cells. This result suggests that HNF-1α is degraded through CMA, but not through eMI (Matsui et al., 2018).

### HCV NS5A Is Colocalized With a Substrate Protein in the Lysosome

In HCV-uninfected cells, HNF-1α is localized mainly in the nucleus. When cells are infected with HCV, HNF-1α is localized in both the nucleus and in the cytoplasm. Since HCV NS5A is localized in the cytoplasm, NS5A is colocalized with HNF-1α protein in the cytoplasm in HCV-infected cells. HCV NS5 protein binds to HNF-1α and retains it in the cytoplasm, which may facilitate the CMA-induced degradation of HNF-1α. We performed immunofluorescence staining to confirm the subcellular colocalization of NS5A and HNF-1α in the lysosome. When cells were treated with a lysosomal enzyme inhibitor, pepstatin A, the colocalization of HNF-1α protein with HCV NS5A was detected in the lysosome.

### Treatment of Lysosomal Inhibitor

$NH_4Cl$, an inhibitor of lysosomal proteolysis, is known to neutralize the acidic lysosomal pH. When HCV infection induces protein degradation of the substrate through the CMA pathway, treatment of the cells with 5mM $NH_4Cl$ restores the substrate protein levels.

## CMA AND OTHER VIRUSES

As an obligate intracellular pathogen, viral replication depends strongly on the host machinery. Viruses utilize the autophagy system of the host, including the CMA pathway, to maintain their life cycles. In addition, some viruses interact with HSC70 and its co-chaperones, resulting in either a positive or negative life cycle regulator (Kaushik and Cuervo, 2018; Wang et al., 2020). Recently, it was reported that the NS2A protein of Zika virus promoted degradation of karyopherin subunit alpha 2 (KPNA2) *via* CMA, resulting in increased Zika virus production. The KNPA2 protein level was restored in the LAMP-2A knockdown cells infected with the Zika virus, indicating the important role of the CMA pathway in this viral replication (He et al., 2020).

## CONCLUSIONS AND FUTURE PERSPECTIVES

We clarified the molecular mechanism underlying HCV-induced CMA. We demonstrated that HCV NS5A interacts with chaperone HSC70, and recruits it to the substrate protein for lysosomal degradation *via* CMA, thereby facilitating HCV pathogenesis. There are two crucial requirements of the substrates for HCV-induced CMA; NS5A binding and a CMA-targeting motif. We and other groups have been investigating NS5A-interacting proteins (Matsui et al., 2012; Sianipar et al., 2015; Ross-Thriepland and Harris, 2015; Chen et al., 2016; Minami et al., 2017; Abe et al., 2020). Using software, we can easily do a search to determine whether the NS5A-interacting proteins contain potential CMA-targeting motifs. We have already found that at least 40 NS5A-binding proteins that contain potential CMA-targeting motifs. Further identification of novel substrates for HCV-induced CMA pathways is required to clarify the physiological relevance of the CMA-dependent degradation of host proteins in HCV infection. We provided evidence suggesting that NS5A-HSC70 complex is important for HCV-induced CMA. Small molecules that can inhibit the NS5A-HSC70 interaction may contribute to the therapeutic strategy for HCV-induced pathogenesis.

## AUTHOR CONTRIBUTIONS

CM and PY outlined and wrote the first draft. IS edited and finalized the manuscript. CM, PY, LD, and TA conceived and produced the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Clearance or Hijack: Universal Interplay Mechanisms Between Viruses and Host Autophagy From Plants to Animals

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Viruses typically hijack the cellular machinery of their hosts for successful infection and replication, while the hosts protect themselves against viral invasion through a variety of defense responses, including autophagy, an evolutionarily ancient catabolic pathway conserved from plants to animals. Double-membrane vesicles called autophagosomes transport trapped viral cargo to lysosomes or vacuoles for degradation. However, during an ongoing evolutionary arms race, viruses have acquired a strong ability to disrupt or even exploit the autophagy machinery of their hosts for successful invasion. In this review, we analyze the universal role of autophagy in antiviral defenses in animals and plants and summarize how viruses evade host immune responses by disrupting and manipulating host autophagy. The review provides novel insights into the role of autophagy in virus–host interactions and offers potential targets for the prevention and control of viral infection in both plants and animals.

**Keywords: virus–host interaction, immune response, autophagy, virus manipulation, infection**

## INTRODUCTION

Plant and animal viruses are among the most difficult "foes" to deal with. Plant virus infections can lead to a substantial decrease in crop yield and represent a serious threat to food security. Global economic losses due to plant viruses are estimated to be as high as 30 billion dollars annually (Nicaise, 2014). The damage caused by mammalian viruses is even more widespread, as evidenced by the coronavirus disease 2019 (COVID-19) pandemic, with 200 million cases of infection and more than 4 million deaths being reported worldwide (Kumar et al., 2021). In addition, viruses such as human immunodeficiency virus (HIV), influenza virus, and hepatitis virus, among others, are endemic in humans and have long represented a threat to human health. Viruses are specialized parasites containing genomes with limited genome coding potential and, consequently, require the host's intracellular machinery to replicate, express viral proteins, and establish infection (Cesarman et al., 2019; He et al., 2020; V'Kovski et al., 2021). In turn, hosts have evolved a variety of defense mechanisms to limit viral replication and spread. Plants have developed innate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI),

effector-triggered immunity (ETI), RNA silencing, and a variety of other mechanisms to inhibit viral infection (Incarbone and Dunoyer, 2013; Mandadi and Scholthof, 2013). Similarly, animals control viral invasion by initiating innate and adaptive immune responses (Daugherty and Malik, 2012; Mukherjee, 2020). Interestingly, increasing evidence has shown that autophagic processes play an indispensable role in host immune responses in both plants and animals. Autophagy is also known to mediate a variety of host–virus interactions (Dong and Levine, 2013; Paul and Munz, 2016; Kushwaha et al., 2019; Leary et al., 2019).

Macroautophagy (hereinafter referred to as autophagy) is a conserved intracellular pathway in plants and animals through which cytosolic contents are encircled by specialized double-layered membrane vesicles, known as autophagosomes, that then transported them to lysosomes/vacuoles for degradation. Under normal conditions, autophagy functions primarily as a housekeeper in the maintenance of cellular homeostasis. Under conditions of stress such as starvation, aging, and microbial infections, autophagic activity is enhanced, and cellular homeostasis is maintained through the degradation of damaged cellular components in the cytoplasm, which, in turn, promotes cell and organism survival (Mizushima and Komatsu, 2011; Marshall and Vierstra, 2018). In animal cells, autophagy is involved in controlling viral infection not only by directly degrading viral components but also through regulating the intensity of the inflammatory response or promoting viral antigen presentation by major histocompatibility complexes (Choi et al., 2018). The role of autophagy in animal defenses against viral pathogens *in vivo* has been extensively studied; however, less is known about the function of autophagy in plant–virus interactions. During the long-term "arms race" between viruses and eukaryotes, autophagy is the "commanding height" that viruses must conquer, not only because it represents a cell-autonomous defense mechanism against microbial invasion, but also because it functions as a scaffold to promote viral replication. Autophagy also provides lipid membranes, and vectors for viral exit from cells, and can improve the survival rate of infected cells, thereby increasing the viral load (Lussignol and Esclatine, 2017; Wang et al., 2019; Keller et al., 2020). Indeed, many viruses have evolved a variety of strategies to disrupt or manipulate cellular autophagy to promote their own replication and spread.

Long-term mutual adaptation has resulted in an extremely complex interaction between hosts and viruses. In this review, we analyze the universal role of autophagy in antiviral defenses in animals and plants. We further discuss how viruses hijack host autophagic pathways to evade immune responses and promote self-replication and highlight a versatile virus–host autophagy interaction mechanism that exists in both plants and animals. This review provides novel insights into the role of autophagy in virus–host interactions and offers potential targets for the prevention and control of viral infection in both groups of eukaryotes.

## AUTOPHAGY MACHINERY

The core feature of the autophagy machinery is the formation of autophagosomes. This process is sequentially regulated by autophagy-related proteins (ATGs) that aggregate into complexes that hierarchically promote autophagy initiation, vesicle nucleation, phagosome expansion, cargo uptake, autophagosome closure, autophagosome–vacuoles/lysosome fusion, and content degradation (Dikic and Elazar, 2018; Ding et al., 2018). Many excellent reviews have focused on the molecular mechanism involved in autophagy (Chen and Klionsky, 2011; Michaeli et al., 2016; Dikic and Elazar, 2018; Ding et al., 2018; Soto-Burgos et al., 2018). In this section, we offer a context-dependent overview of autophagy, thus providing a basis for subsequent sections.

According to their function and physical interactions, core autophagy proteins can be divided into several functional units, namely, the ATG1/ULK1 (Unc-51-like kinase 1) complex; the ATG6/Beclin1-PI3K (phosphoinositide 3-kinase)/VPS34 (vacuolar protein sorting 34) complex; the ATG9 complex; the ATG12/ATG5/ATG16 ubiquitination-like conjunction system; and the ATG8/LC3 (microtubule-associated protein 1 light chain 3)-PE (phosphatidylethanolamine) ubiquitination-like conjunction system (Xie and Klionsky, 2007). The ATG1/ULK1 complex, consisting of the serine/threonine protein kinases ATG1/ULK1, ATG13, ATG101 and ATG11/FIP200 (FAK family-interacting protein of 200 kDa), is central to the initiation of autophagy (Hurley and Young, 2017). Multiple kinases upstream of autophagy can induce ATG1/ULK1 complex assembly and regulate autophagy initiation. For example, mammalian target of rapamycin kinase complex 1 (mTORC1) becomes inactivated once it senses stress associated with cellular energy and nutrient deprivation, resulting in the activation of the autophagy initiators ATG1/ULK1 and the promotion of the assembly of ATG1/ULK1, ATG13, and the accessory subunits ATG11 and ATG101 into an active complex (Hosokawa et al., 2009; Pu et al., 2017). This complex can stimulate several downstream phosphorylation-dependent autophagic steps, such as the delivery of lipids to constantly expanding phagocytic vesicles, as driven by the ATG9 complex composed of the transmembrane protein ATG9 as well as ATG2 and ATG18, which have been implicated in ATG9 recycling (Young et al., 2006; Zhuang et al., 2017). Another step, also phosphorylation-dependent, involves the activation of the ATG6/Beclin1-PI3K/VPS34 complex, which subsequently converts phosphatidylinositol (PI) from lipid molecules on the surface of phagocytic vesicles to phosphatidylinositol-3-phosphate (PI3P) (Russell et al., 2013). PI3P on isolation membranes is recognized by PI3P-binding factors (WD-repeat protein interacting with phosphoinositides [WIPIs, ATG18 homologous proteins]) that anchor to PI3P-decorated phagocytic vesicles and then recruit the ATG12-ATG5-ATG16 complex (Polson et al., 2010; Dooley et al., 2014; Proikas-Cezanne et al., 2015), which is composed of ATG12, ATG5, and ATG16 in a 2:2:2 ratio. Subsequently, ATG7 (E1-like enzyme) and ATG10 (E2-like enzyme) mediate the attachment of the C-terminal glycine of ATG12 to a conserved lysine residue within ATG5, yielding an ATG12-ATG5 conjugate, which then non-covalently binds to the dimeric scaffold protein ATG16 to form a hexameric complex with ligase activity (**Figure 1**)

(Ichimura et al., 2000; Fujita et al., 2008). The ATG8-PE ubiquitination-like conjunction system plays an important role in the expansion of phagocytic vesicles, cargo uptake, and autophagosome closure (Nakatogawa et al., 2007). Inactive ATG8/LC3 protein is cleaved by the protease ATG4 to expose the conserved C-terminal glycine residue, and ATG7 (E1) transfers the cleaved ATG8 to ATG3 (E2). With the help of ATG12-ATG5-ATG16, acting as an E3 ligase, the C-terminal glycine carboxyl group of ATG8 covalently binds to the N-terminus of phosphatidylethanolamine components of the autophagy bilayer membrane, yielding lipidated ATG8/LC3, which mediates autophagic bilayer membrane expansion, closure, and autophagic cargo uptake (**Figure 1**) (Geng and Klionsky, 2008; Nakatogawa, 2013). Once the autophagosome is formed, it is transported to the vacuole/lysosome *via* a microtubule network controlled by the endosomal sorting complexes required for transport (ESCRT) machinery (Vietri et al., 2020). The autophagosome then fuses with the vacuole/lysosomal membrane, a process that is mediated by factors such as vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) (Itakura et al., 2012).

Autophagy was initially thought to be a bulk catabolic process involving the re-mobilization of nutrients and the support of energy requirements, with cellular components being indiscriminately phagocytosed into the autophagosome. However, there is substantial evidence to indicate that autophagy can also degrade cytoplasmic cargo, such as misfolded/aggregated proteins, damaged organelles, and invading microorganisms, in a highly selective manner (Gatica et al., 2018; Marshall and Vierstra, 2018; Ran et al., 2020). ATG8/LC3 plays a key role in selective autophagy. Membrane-anchored ATG8/LC3 not only interacts with ATG1, ATG6/Beclin1, and other core autophagy proteins to synergistically regulate the initiation, extension, and maturation of autophagosomes, but also provides a platform for cargo receptors to selectively recruit their cargo (Johansen and Lamark, 2020). Autophagy cargo receptors typically recognize ubiquitinated substrates through a highly conserved ubiquitin-association domain (UBA) and anchor to the autophagosome *via* ATG8-interacting motifs (AIMs) (W/F/Y-x-x-L/I/W; also known as LC3-interacting regions [LIR] in animals), thereby recruiting cargo to the developing autophagosome for degradation (Johansen and Lamark, 2011; Birgisdottir et al., 2013). Notably, some autophagy receptors do not contain an AIM/LIR, but rather mediate selective cargo degradation through interaction with ATG8s *via* a ubiquitin interacting motif (UIM) (Marshall et al., 2019).

Selective autophagy can be subdivided into several types, including mitophagy [the removal of damaged or excessive

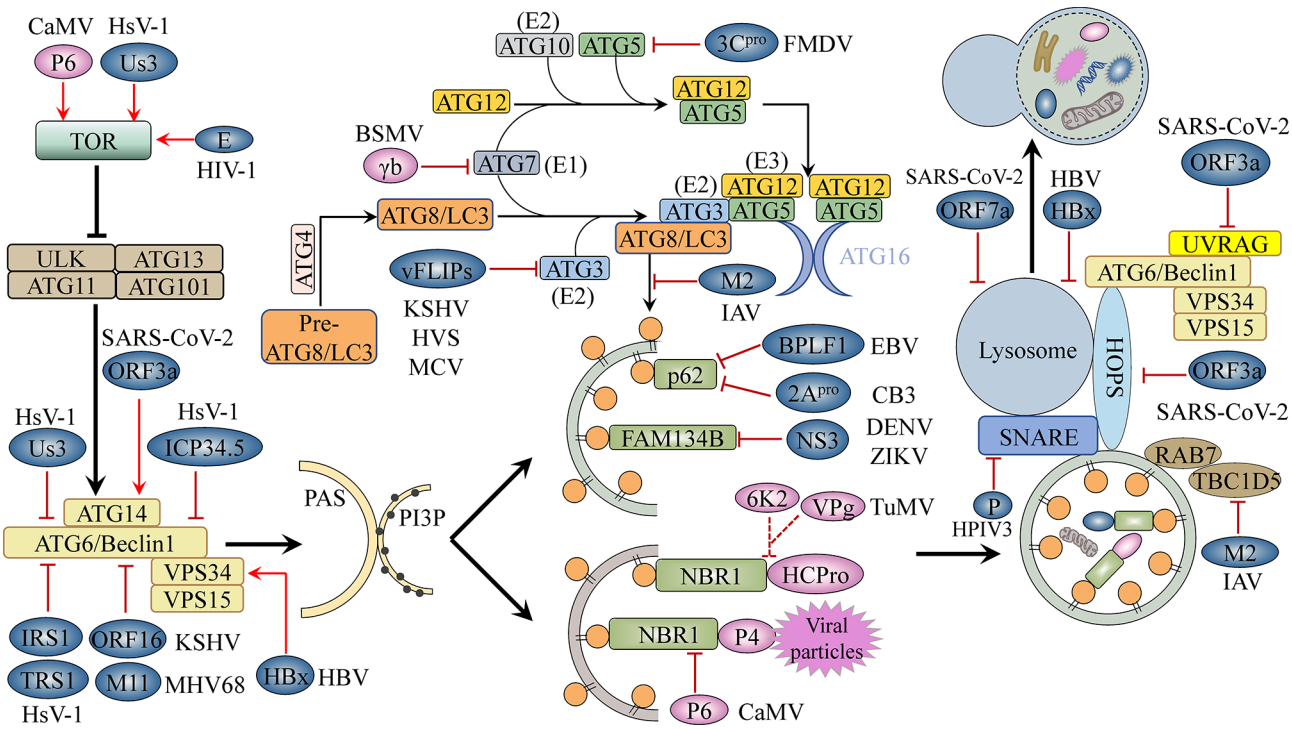


**FIGURE 1** | The autophagy machinery and its inhibition by viruses. The ATG1/ULK1 complex, the ATG6/Beclin1-PI3K/VPS34 complex, and the ATG12-ATG5-ATG16 and ATG8-PE conjugation systems, among others, are involved in key steps of the autophagy pathway, including initiation, elongation, completion, and fusion. Viral proteins block cellular autophagy and promote virus development by activating TOR, a conserved Ser/Thr kinase; interacting with autophagy-related proteins, thereby inhibiting or promoting their activity; targeting selective autophagy processes; and interfering with autophagosome–lysosome fusion or lysosomal acidification. Blue-grey ovals represent animal viral proteins. Plant viral proteins are shown in pink. TOR, target of rapamycin; PAS, pre-autophagosomal structure; PI3P, phosphatidylinositol-3-phosphate; E1/2/3, E1/2/3-like enzyme.

mitochondria), reticulophagy (degradation of endoplasmic reticulum (ER)], aggrephagy (the degradation of protein aggregates), and proteaphagy (degradation of inactive proteasomes), among others (Gatica et al., 2018). In addition to maintaining cellular homeostasis, autophagy also participates in pathogen clearance. Selective autophagy of intracellular pathogens is called xenophagy, while that of virions or viral components is known as virophagy (Yla-Anttila, 2021). Selective autophagy depends on receptor recognition of cargo and the initiation of autophagosome formation (Huang et al., 2020; Vo and Choi, 2021). Substrate ubiquitination is often a key intermediary step in the recognition and degradation of these cargoes. For instance, in both plants and animals, once protein aggregates have been labeled through ubiquitination, the autophagy cargo receptor neighbor of brca 1 (NBR1) acts as a ubiquitin-binding protein that interacts with ubiquitinated protein aggregates and the core autophagy protein ATG8, following which both ubiquitinated proteins and NBR1 are degraded by autophagy (Kirkin et al., 2009; Jung et al., 2020).

## AUTOPHAGY-MEDIATED ANTIVIRAL RESPONSES

Although studies have shown that autophagy plays distinct roles in host–virus interaction, corresponding to different viruses and host cell types, autophagy can be used to degrade viral components, viral particles, and even host factors required for viral replication; autophagy is therefore an important innate antiviral response (Choi et al., 2018; Ismayil et al., 2020; Yang et al., 2020). Many studies have confirmed that autophagy plays an antiviral defense role in host–virus interaction through silencing or mutating ATGs (Liu et al., 2005; Yordy et al., 2013).

### Autophagy as an Antiviral Strategy in Animal Cells

The autophagy protein ATG5 is essential for protecting the mouse central nervous system from lethal infection with Sindbis virus (SINV). Orvedahl et al. (2010) reported that *Atg5* deletion resulted in the delayed clearance of viral proteins, while also leading to an increase in neuronal cell death and the cellular accumulation of the adaptor protein p62 [also known as sequestosome 1 (SQSTM1)]. The authors further found that p62 acts as a cargo receptor mediating the selective clearance of SINV capsid proteins, thereby promoting cell survival (**Figure 2**). High-throughput, genome-wide, small interfering RNA (siRNA) screening subsequently identified the host ubiquitin ligase SMURF1 as an essential factor for the colocalization of p62 and SINV capsid proteins as well as virophagy. Following the silencing of SMURF1, p62 lost its ability to target SINV (**Figure 2**) (Orvedahl et al., 2011). The Fanconi anemia complementation group C (FANCC) protein was also found to mediate virophagy by interacting with the SINV capsid and promoting host antiviral defenses (**Figure 2**) (Sumpter et al., 2016). SMURF1 and FANCC also target HSV-1 for selective degradation, suggesting that these two proteins generally function as virophagy factors (Orvedahl et al., 2011; Sumpter et al., 2016).

A complex cell type- and infection status-dependent link exists between HIV-1 and autophagy. (Sagnier et al., 2015) reported that the HIV-1 reverse transcription activator Tat, a protein essential for viral transcription and virion production, is recognized by the adaptor protein p62/SQSTM1 in a ubiquitin-independent manner in $CD^{4+}$ T-cells, following which it is degraded *via* selective autophagy (**Figure 2**). HIV-1 is a "master" at manipulating host cellular mechanisms, including autophagy, and facilitates its own replication and infection through disrupting or hijacking host cellular autophagic mechanisms. Influenza A virus (IAV) is an important zoonotic pathogen, causing significant morbidity in humans and representing an ever-present threat to humanity. IAV achieves efficient cross-species transmission through reassortment or directing host adaptation processes (Taubenberger and Kash, 2010). When IAV containing avian PB2 infects mammalian cells, viral ribonucleoprotein (vRNP) forms aggregates that localize to the microtubule-organizing center in infected cells (Liu et al., 2021). Correspondingly, the selective autophagy receptor p62/SQSTM1 targets newly synthesized vRNPs through PB2, a viral polymerase subunit, inducing higher autophagic flux and greater autolysosome accumulation, which limits viral infection (Liu et al., 2021). p62 can also mediate the degradation of avibirnavirus proteins. Infectious bursal disease virus (IBDV) capsid protein VP2 is responsible for virus assembly, maturation, and replication. (Li et al., 2020c) revealed that p62 recognizes ubiquitinated VP2 proteins and specifically recruits them to autophagosomes (**Figure 2**). p62 lacking the UBA or LIR can no longer promote VP2 degradation, indicating that p62 promotes the selective autophagic degradation of VP2 in a ubiquitin-dependent manner (Li et al., 2020c). It has been suggested that the ER-resident protein SCOTIN may act as a cargo receptor for virophagy and recruits non-structural 5A (NS5A), a key factor in hepatitis C virus (HCV) replication, into autophagosomes for degradation (**Figure 2**) (Kim et al., 2016). In animal cells, the antiviral restriction factors tripartite motif-containing proteins (TRIMs) comprise a large family of pattern recognition receptors (PRRs) containing a RING domain, a B box domain, and a coiled-coil domain at the N-terminus; additionally, most TRIMs contain a variable C-terminal domain that plays a role in substrate binding (Kawai and Akira, 2011). TRIMs not only regulate autophagy initiation and nucleation, but also act as cargo receptors to mediate the selective autophagy of viral capsid proteins. For example, in Langerhans cells (LCs), TRIM5α mediates the assembly of autophagy-activating complexes to turn on the virophagy machinery (Ribeiro et al., 2016). In addition, TRIM5α recruits the HIV-1 capsid into autophagosomes for HIV-1 degradation through directly interacting with the capsid and ATG8s (**Figure 2**) (Mandell et al., 2014a; Mandell et al., 2014b) (**Table 1**).

### Autophagy Plays a Role in Resistance to Plant Viruses

Several studies have provided clear evidence that autophagy can also serve as an antiviral defense in plants, directly targeting viruses or individual viral components for degradation. The cotton leaf curl Multan virus (CLCuMuV) βC1 protein is a key

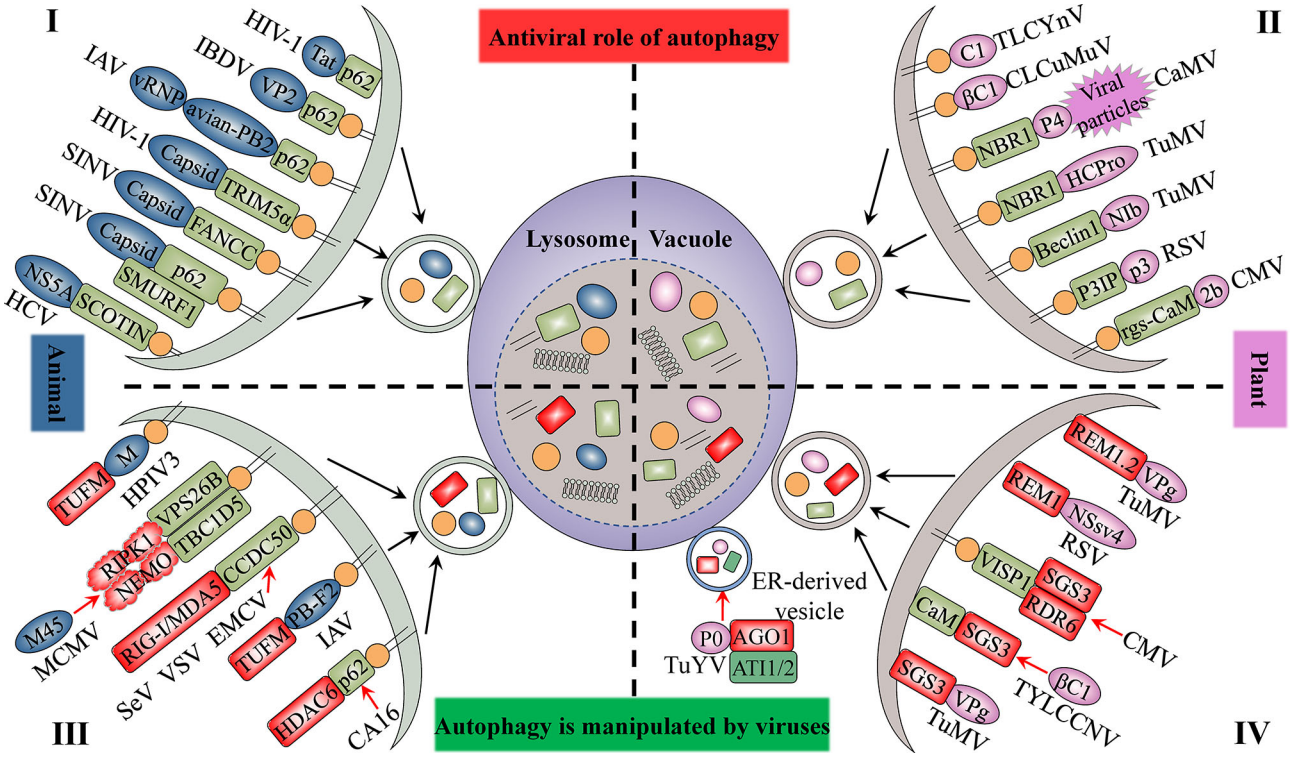


**FIGURE 2** | The antiviral role of autophagy and its manipulation by viruses. The upper panel indicates the antiviral aspects of cellular autophagy (I and II). Viral proteins that manipulate host autophagy are shown in the lower part (III and IV). The left (I and III) and right (II and IV) parts represent the interaction of animal viruses and plant viruses with host autophagy, respectively. Selective autophagy mediated by cargo receptors is an antiviral mechanism common to both animal and plant cells. Viruses derived from both plants and animals hijack autophagy to degrade factors that positively regulate host immune responses to enhance their self-proliferation. Blue-grey ovals represent animal viral proteins. Plant viral proteins are shown in pink. The grass-green rectangle with rounded corners represents host selective autophagy cargo receptors. Factors that positively regulate host immune responses are displayed in red.

**TABLE 1** | Autophagy-mediated antiviral immune responses.

| Host | Virus | Viral protein (s) | Host protein(s) | Functions | References |
|---|---|---|---|---|---|
| Animal | SINV | Capsid | p62; LC3 | p62 adaptor protein mediates autophagic viral protein clearance, thus promoting cell survival | (Orvedahl et al., 2010) |
| | SINV | Capsid | SMURF1 | Acts as a mediator of virophagy | (Orvedahl et al., 2011) |
| | SINV | Capsid | FANCC | Interacts with the capsid protein, facilitating virophagy | (Sumpter et al., 2016) |
| | HIV-1 | Tat | p62 | Selective degradation of Tat in a ubiquitin-independent manner | (Sagnier et al., 2015) |
| | IAV containing avian PB2 | PB2; vRNP | p62; LC3 | p62 targets vRNP to form an autophagosome through interaction with viral PB2 | (Liu et al., 2021) |
| | IBDV | VP2 | p62; LC3 | p62 mediates the selective autophagic degradation of VP2, thus targeting IBDV replication | (Li et al., 2020b) |
| | HCV | NS5A | Scotin; LC3 | Scotin recruits the NS5A protein to autophagosomes for degradation | (Kim et al., 2016) |
| | HIV-1 | Capsid | TRIM5α; ATG8s | TRIM5α functions both as a regulator of autophagy and as an autophagic cargo receptor mediating HIV-1 restriction | (Mandell et al., 2014a; Mandell et al., 2014b; Ribeiro et al., 2016) |
| Plant | CLCuMuV | βC1 | ATG8f | ATG8f targets βC1 for degradation | (Haxim et al., 2017) |
| | TLCYnV | C1 | ATG8h; XPO1 | ATG8h interacts with C1, directing it for degradation in an XPO1-mediated, nuclear export pathway-dependent manner | (Li et al., 2020a) |
| | CaMV | P4 and viral particles | NBR1; ATG8a | NBR1 targets P4 and viral particles, thus mediating their autophagy-dependent degradation | (Hafren et al., 2017) |
| | TuMV | HCPro | NBR1; ATG8a | HCPro is selectively degraded by the autophagy pathway through binding with NBR1 | (Hafren et al., 2018) |
| | TuMV | NIb | Beclin1; ATG8a | Beclin1 interacts with Nib, targeting it for selective degradation | (Li et al., 2018) |
| | RSV | p3 | P3IP; ATG8f | P3IP directs the selective autophagic degradation of p3 through interaction with ATG8, thereby limiting virus infection | (Jiang et al., 2021) |
| | CMV | 2b | rgs-CaM; ATG8 | rgs-CaM interacts with 2b for autophagy degradation | (Nakahara et al., 2012) |

factor in virus-induced disease symptoms and virus accumulation in plants (Jia et al., 2016). Haxim et al. (2017) showed that ATG8 directly targets βC1 for degradation, thereby protecting plants against this geminivirus (**Figure 2**). Interestingly, the V32A mutation in βC1 disrupts the interaction between ATG8 and βC1, preventing βC1 degradation through autophagy. In *Nicotiana benthamiana*, CLCuMuV carrying the $\beta C1^{V32A}$ mutation induces severe symptoms and viral DNA accumulation (Haxim et al., 2017). Similarly, tomato leaf curl Yunnan virus (TLCYnV) nucleoprotein C1 undergoes autophagic degradation through directly interacting with ATG8h (**Figure 2**). During virophagy, exportin1 (XPO1) participates in the transfer of C1 from the nucleus to the cytoplasm and mediates the binding of ATG8h to C1 (Li et al., 2020b). When the autophagy-related genes *ATG8h*, *ATG5*, and *ATG7* are separately knocked out in plants, the degradation of C1 is inhibited, thus promoting TLCYnV infection (Li et al., 2020b) (**Table 1**).

In plants, besides the direct targeting of the core autophagy protein ATG8 to the viral component, virophagy also requires cargo receptors as intermediaries. Hafren et al. (2017) reported that selective autophagy limits cauliflower mosaic virus (CaMV) infection during compatible interactions between CaMV and host plants. Autophagy-defective Arabidopsis mutants (*atg5* and *atg7*) develop more severe symptoms after CaMV infection than their wild-type counterparts. The viral capsid protein P4 specifically accumulates in *Atg5* and *Atg7* mutant strains, whereas the levels of other viral proteins remain unchanged. Furthermore, selective autophagy mediated by the cargo receptor NBR1 inhibits the accumulation of CaMV P4 (**Figure 2**) (Hafren et al., 2017). NBR1-dependent selective autophagy has also been reported to function as an antiviral mechanism targeting RNA viruses. NBR1 inhibits turnip mosaic virus (TuMV) accumulation by targeting the TuMV helper component protease HCpro, a suppressor of antiviral RNA silencing (**Figure 2**) (Hafren et al., 2018). Nevertheless, TuMV appears to antagonize NBR1-dependent autophagy during infection through the activity of different viral proteins, thus limiting its antiviral ability (see below). ATG6/Beclin1 reportedly acts as a selective autophagic cargo receptor and interacts with RNA-dependent RNA polymerase (RdRp) by targeting its GDD motif, which results in the autophagic degradation of RdRp and the inhibition of TuMV infection (**Figure 2**) (Li et al., 2018). Silencing ATG6/Beclin1 or ATG8 to block autophagy can promote RdRp accumulation and viral infection and *vice versa*. That the GDD motif is relatively conserved and is found in the RdRps of most plant and animal viruses suggests that ATG6/Beclin1 may be a general cargo receptor in virophagic processes (Li et al., 2018). Recently, Jiang et al. (2021) identified a novel cargo receptor, P3IP, which induces and mediates the autophagic degradation of the rice stripe virus (RSV)-encoded RNA silencing suppressor (RSS) P3 protein (**Figure 2**). The calmodulin-like protein rgs-CaM may also be a selective autophagic receptor that regulates viral infection. For example, tomato rgs-CaM can interact with and stimulate the autophagic degradation of a variety of viral RNA RSSs, including HCpro from potyvirus as well as the 2b protein from cucumber mosaic virus (CMV) and tomato aspermy virus (**Figure 2**) (Nakahara et al., 2012) (**Table 1**).

In summary, selective autophagy mediated by cargo receptors has been shown to play a key role in host protection against viral infection. Meanwhile, this antiviral mechanism is well conserved in animal and plant cells. The identification of receptors or adaptors for selective autophagy will greatly advance the understanding of virophagy and better reveal the biological processes underlying the role of selective autophagy in host–virus interactions.

## AUTOPHAGY IS SUBVERTED BY VIRUSES

In response to the limiting effects of autophagy on viral infection, persistent viruses have developed both specific and non-specific strategies to inhibit or disrupt multiple steps of the autophagy pathway for effective replication (**Table 2**) (Choi et al., 2018; Huang et al., 2020).

### Viruses Disrupt Autophagosome Initiation and Nucleation

The TOR kinase complex functions upstream of autophagy, controlling autophagosome biogenesis by negatively regulating the activity of the ATG1/ULK1 complex (**Figure 1**). Some animal and plant viruses can activate TOR kinase activity to inhibit virophagy (**Table 2**). For example, CaMV, a plant virus, binds to and activates TOR kinase through the multifunctional protein P6, which, in turn, blocks cellular autophagy and promotes CaMV infection (**Figure 1**) (Zvereva et al., 2016). Regarding animal viruses, Blanchet et al. (2010) reported that HIV-1 envelope activates the mTORC1 pathway in dendritic cells (DCs), leading to impaired autophagy and, consequently, the blocking of autophagosome-mediated degradation (**Figure 1**). Autophagy is a powerful inhibitor of herpes simplex virus 1 (HSV-1) pathogenesis in neurons (Yordy et al., 2012). However, HSV-1 serine/threonine kinase Us3 can antagonize autophagy in non-neuronal cells by activating mTORC1 activity and increasing the phosphorylation level of ATG1/ULK1 (**Figure 1**) (Rubio and Mohr, 2019). Us3 can also directly phosphorylate ATG6/Beclin1 and inhibit its activity, which further suppresses cellular autophagy (**Figure 1**) (Rubio and Mohr, 2019). Indeed, ATG6/Beclin1 is a target of many animal viral proteins (**Table 2**). The HSV-1-encoded neurotoxic protein ICP34.5 binds to ATG6/Beclin1 and inhibits its autophagic function (**Figure 1**) (Orvedahl et al., 2007). The HSV-1 ICP34.5 mutant lacking the ATG6/Beclin1 binding domain cannot inhibit autophagy in neurons or cause fatal encephalitis in mice (Orvedahl et al., 2007). Human cytomegalovirus (HCM1) expresses TRS1, a functional homolog of ICP34.5, which can also block autophagosome biogenesis (**Figure 1**) (Chaumorcel et al., 2012). The N-terminal domain of TRS1 contains the Beclin1 binding region, which is crucial for inhibiting Beclin1-mediated autophagy (Chaumorcel et al., 2012). Another HCMV protein, IRS1, has also been reported

**TABLE 2 |** Autophagy is subverted by viruses.

| Host | Virus(s) | Viral protein(s) | Host protein(s) | Effects on host–virus interactions | References |
|---|---|---|---|---|---|
| Animal | HIV-1 | Envelope | mTORC1 | The envelope protein activates the mTORC1 pathway, leading to autophagy exhaustion | (Blanchet et al., 2010) |
| | HSV-1 | Us3 | mTORC1 | Us3 activates mTORC1, which inhibits the ULK autophagy-promoting complex | (Rubio and Mohr, 2019) |
| | HSV-1 | Us3 | Beclin1 | Us3 associates with and phosphorylates Beclin1, thus limiting autophagy and promoting virus replication | (Rubio and Mohr, 2019) |
| | HSV-1 | ICP34.5 | Beclin1 | ICP34.5 interacts with Beclin1, thus inhibiting autophagy | (Orvedahl et al., 2007) |
| | HCMV | TRS1 | Beclin1 | TRS1 interacts with Beclin1, thus inhibiting autophagy | (Chaumorcel et al., 2012) |
| | HCMV | IRS1 | Beclin1 | IRS1 blocks host autophagy by interacting with Beclin1 | (Mouna et al., 2016) |
| | KSHV | ORF16 | Beclin1 | ORF16 mimics cellular Bcl-2 and attenuates autophagy through direct interaction with Beclin1 | (Pattingre et al., 2005) |
| | MHV68 | M11 | Beclin1 | M11 mimics cellular Bcl-2 and attenuates autophagy through direct interaction with Beclin1 | (Pattingre et al., 2005) |
| | KSHV; HVS; MCV | vFLIPs | ATG3 | vFLIPs suppresses autophagy by preventing ATG3 from binding and processing LC3 | (Lee et al., 2009) |
| | FMDV | $3C^{pro}$ | ATG5-ATG12 | $3C^{pro}$ suppresses autophagy *via* the degradation of the ATG5-ATG12 conjugate | (Fan et al., 2017) |
| | IAV | M2 | LC3 | M2 interacts with LC3 and promotes its relocalization to the host's plasma membrane | (Beale et al., 2014) |
| | DENV; ZIKV | NS3 | FAM134B | NS3 cleaves the FAM134B receptor, thereby suppressing the reticulophagy pathway | (Lennemann and Coyne, 2017) |
| | CB3 | $2A^{pro}$ | p62 | $2A^{pro}$ cleaves p62, resulting in disrupted selective autophagy | (Shi et al., 2013; Mohamud et al., 2019) |
| | EBV | BPLF1 | p62 | BPLF1 targets p62 and decreases its ubiquitination, thus inhibiting selective autophagy | (Yla-Anttila et al., 2021) |
| | SARS-CoV-2 | ORF3a | VPS39 | ORF3a interacts with VPS39 and prevents the assembly of the SNARE complex | (Hayn et al., 2021; Koepke et al., 2021; Miao et al., 2021) |
| | SARS-CoV-2 | ORF7a | Unknown | ORF7a interferes with autophagosome acidification | (Hayn et al., 2021; Koepke et al., 2021) |
| | SARS-CoV-2 | ORF3a | UVRAG | ORF3a interacts with UVRAG to inhibit PI3KC3-C2 and promote the formation of PI3KC3-C1 | (Qu et al., 2021) |
| | HPIV3 | P | SNAP29 | P binds to SNAP29 and prevents SNARE proteins from mediating autophagosome–lysosome fusion | (Ding et al., 2014) |
| | IAV | M2 | TBC1D5 | M2 abrogates TBC1D5-Rab7 binding through interaction with TBC1D5 | (Martin-Sancho et al., 2021) |
| | HBV | HBx | Unknown | HBx impairs lysosomal acidification | (Liu et al., 2014) |
| | PV | Unknown | Galectin 8; PLA2G16 | Galectin 8 initiates the autophagic degradation of viral RNA, the virus uses PLA2G16 to evade galectin 8-mediated detection | (Staring et al., 2017) |
| Plant | CaMV | P6 | TOR | P6 activates TOR kinase, which blocks cellular autophagy and promotes CaMV translation | (Zvereva et al., 2016) |
| | BSMV | γb | ATG7 | γb interacts with ATG7 and disrupts ATG7-ATG8 interaction, thus suppressing autophagy and promoting viral infection | (Yang et al., 2018) |
| | CaMV | P6 | NBR1 | P6 disrupts the interaction between P4 and host NBR1, which protects viral replication factory inclusions from autophagic degradation | (Hafren et al., 2017) |
| | TuMV | VPg; 6K2 | Unknown | VPg and 6K2 antagonize the antiviral capacity of NBR1-dependent autophagy by blocking NBR1 and HCpro degradation | (Hafren et al., 2018) |
| | RSV | NSvc4 | Type-I J-domain proteins | NSvc4 hijacks UPR-activated type-I J-domain proteins, thus preventing its autophagic degradation | (Li et al., 2021) |

to block autophagy by interacting with Beclin1 (**Figure 1**) (Mouna et al., 2016).

In yeast and mammalian cells, in addition to its anti-apoptotic role, B-cell lymphoma 2 (Bcl-2) also exerts anti-autophagic activity through its interaction with Beclin1 (Pattingre et al., 2005). Some animal viral proteins function in a manner similar to that of host cell Bcl-2, i.e., they attenuate autophagy by directly interacting with Beclin1 (**Table 2**). For example, Kaposi's sarcoma-associated herpesvirus (KSHV) and murine gammaherpesvirus 68 (MHV68) encode the Bcl-2 paralogs ORF16 and M11, respectively. These viral Bcl-2 (vBcl-2) proteins mimic their cellular counterparts (cBcl-2) and inhibit autophagosome formation (**Figure 1**) (Pattingre et al., 2005). Furthermore, structural and biochemical analysis has shown that vBcl-2 has a significantly higher affinity for Beclin1 and inhibits autophagosome formation to a greater extent than cBcl-2 (Ku et al., 2008). Because of the lack of a regulatory loop that can undergo phosphorylation by c-Jun N-terminal kinase (JNK), vBcl-2 can remain bound to Beclin1, indicating that vBcl-2 has evolved into a highly effective autophagy inhibitor (Wei et al., 2008). Whether plant viruses can inhibit the activity of ATG6/Beclin1 through phosphorylation, direct interaction, or any other mechanisms, thereby blocking autophagosome biogenesis, is unknown. Moreover, no homolog of yeast and mammalian

Bcl-2 has been identified in plants, and it is unclear whether a mechanism similar to the Bcl-2-mediated regulation of the autophagy pathway exists in plants. More studies investigating the regulation of host autophagy by plant viral proteins are needed to elucidate these possibilities.

## Viruses Disrupt Phagosome Expansion

The ATG12-ATG5-ATG16 and ATG8/LC3-PE ubiquitin-like conjugation systems are essential for autophagosome formation and may also be a driving force for vesicle membrane deformation or bending. Some animal and plant viral proteins bind to certain proteins of these two sets of ubiquitin-binding systems, thus interfering with autophagosome biogenesis (**Table 2**). The FLICE-like inhibitor protein (vFLIP) of KSHV, herpesvirus saimiri (HVS), and molluscum contagiosum virus (MCV) can inhibit autophagy by preventing ATG3 from binding and processing LC3 (**Figure 1**) (Lee et al., 2009). Foot-and-mouth disease virus (FMDV) mediates the degradation of the ATG5-ATG12 complex through viral $3C^{pro}$ (**Figure 1**) (Fan et al., 2017). The siRNA-mediated knockdown of ATG5-ATG12 significantly increases the FMDV load and *vice versa* (Fan et al., 2017). Plant viruses also adopt similar strategies to inhibit virophagy. For example, the γb protein of barley stripe mosaic virus (BSMV) interferes with the interaction between ATG7 and ATG8 in a competitive manner and disrupts autophagy-mediated antiviral defenses (**Figure 1**) (Yang et al., 2018). IAV targets ATG8/LC3, the core component of autophagy, through the multifunctional protein M2, thereby disrupting autophagy. Beale et al. (2014) found that the cytoplasmic tail of IAV M2 contains a highly conserved LIR that mediates a direct interaction between M2 and LC3 in virus-infected cells and thereby promoting the mislocalization of LC3 to the plasma membrane. Moreover, mutations in M2 abolish LC3 binding, interfere with virus budding, and reduce the stability of virus particles.

## Viruses Interfere With Selective Autophagy

Interference with cargo receptor-dependent selective autophagy is a commonly used strategy by animal and plant viruses to counteract host antiviral responses (**Table 2**). Some arboviruses, such as dengue virus (DENV) and Zika virus (ZIKV), use the ER as a source of membranes to establish replicative organelles and promote their assembly and final maturation along the secretory pathway (Welsch et al., 2009). Correspondingly, host cells have evolved reticulophagy to cope with this stress. For example, the ER-localized cargo receptor FAM134B can limit the replication of DENV and ZIKV (Khaminets et al., 2015). However, these virus-encoded NS3 proteases specifically block reticulophagy by cleaving FAM134B at a single site in its reticular homology domain (RHD) (**Figure 1**) (Lennemann and Coyne, 2017). p62/SQSTM1 plays an important role in mediating virophagy. For instance, p62/SQSTM1 can directly interact with the coxsackievirus B3 (CVB3) capsid protein VP1 and recruit it for autophagic degradation, which reduces intracellular viral protein production (Shi et al., 2013). Interestingly, CVB3 viruses use viral protease $2A^{pro}$ to cleave p62/SQSTM1, disrupting its function in selective autophagy (**Figure 1**) (Shi et al., 2013; Mohamud et al., 2019). In contrast, Epstein–Barr virus (EBV) targets p62/SQSTM1 *via* the deubiquitinase BPLF1, which inhibits selective autophagy and promotes EBV replication and transmission (**Figure 1**) (Yla-Anttila et al., 2021). Plant viruses employ a similar strategy to inhibit selective autophagy. For example, NBR1 targets the viral capsid protein P4 and mediates its autophagic degradation, which inhibits CaMV infection (Hafren et al., 2017). However, the P6 protein of CaMV can disrupt the interaction between host NBR1 and P4 and protect the inclusion bodies of viral replication factories from autophagic degradation (**Figure 1**) (Hafren et al., 2017). In addition, TuMV VPg and 6K2 can reportedly antagonize the antiviral ability of NBR1-dependent autophagy by blocking the degradation of HCpro by NBR1; however, the molecular mechanism underlying this inhibition of autophagy remains unclear (**Figure 1**) (Hafren et al., 2018).

## Viruses Block Autophagosome–Lysosome Fusion

In addition to activating the TOR kinase complex, inhibiting autophagic core protein activity, and disrupting selective autophagy, animal viruses can also interfere with autophagosome maturation and block autophagosome–lysosome fusion, thus suppressing antiviral autophagic activity (**Figure 1**). SARS-CoV-2 restricts autophagy-associated signaling and blocks autophagic flux. Cells infected with SARS-CoV-2 show an accumulation of key metabolites, the activation of autophagy inhibitors such as AKT and SKP2, and a reduction in the levels of several proteins responsible for processes spanning from autophagosome formation to autophagosome–lysosome fusion (Gassen et al., 2021). In a recent study, the effect of individual SARS-COV-2 proteins on autophagy was systematically analyzed, and the authors found that E, M, ORF3a, and ORF7a promoted autophagosome accumulation, while also reducing autophagic flux (Hayn et al., 2021; Koepke et al., 2021). Additionally, ORF3a and ORF7a were reported to block autophagy by respectively interfering with autophagosome–lysosome fusion and lysosomal acidification (Hayn et al., 2021; Koepke et al., 2021). Miao et al., (2021) conducted an in-depth analysis of the mechanism by which ORF3a prevents autophagosome–lysosome fusion. ORF3a strongly interacts with VPS39, a component of the tethering factor HOPS (homotypic fusion and protein sorting) complex. The binding of ORF3a to VPS39 disrupts the assembly of the HOPS complex, which is followed by the failure of STX17-SNAP29-VAMP8 SNARE complex assembly. As the SNARE complex mediates autophagosome–lysosome fusion, this effect of ORF3a leads to the inhibition of autophagosome–lysosome fusion and the complete blockage of autophagic flux (Miao et al., 2021; Yim and Mizushima, 2021). Qu et al. (2021) showed that ORF3a has another effect associated with host autophagy. The core autophagy protein ATG6/Beclin1 is known to regulate lipid kinase Vps34 (PI3KC3) and to interact with mammalian ATG14 or UVRAG to form two phosphatidylinositol 3-kinase complexes with significantly distinct functions. The PI3KC3-C1 (ATG6/Beclin1-Vps34-Atg14) complex positively regulates autophagosome formation while the PI3KC3-C2 complex

(ATG6/Beclin1-Vps34-UVRAG) mediates autophagosome maturation by promoting autophagosome–lysosome fusion (Levine et al., 2015). ORF3a interacts with the autophagy regulator UVRAG to selectively inhibit PI3KC3-C2 and promote the formation of PI3KC3-C1, thus inducing incomplete autophagy (Qu et al., 2021). It is not clear whether SARS-CoV-2 protein is normally targeted for autophagic degradation. If so, blocking fusion will allow SARS-CoV-2 to avoid lysosomal degradation and prevent the degradation products from being used for antigen presentation to T cells. If not, the accumulation of membrane-related components caused by incomplete autophagy may exert a positive effect on SARS-CoV-2 replication. Interestingly, several other intractable viruses adopt similar strategies to avoid autophagosome–lysosome fusion. Human parainfluenza virus type 3 (HPIV3) phosphoprotein (P) binds to SNAP29 and inhibits its interaction with STX17, thus preventing autophagosome–lysosome fusion mediated by these two host SNARE proteins (Ding et al., 2014). IAV utilizes M2 to block fusion, resulting in autophagosome accumulation. M2 physically interacts with TBC1D5 through its cytoplasmic tail, thereby abrogating TBC1D5-Rab7 binding, which is critical for autophagosome–lysosome fusion (Martin-Sancho et al., 2021). Hepatitis B virus (HBV) is one of the most successful human pathogens. Liu et al. (2014) showed that the HBV X protein (HBx) significantly impairs lysosomal acidification and affects lysosomal maturation, thereby inhibiting autophagic degradation. However, HBx can also bind to and enhance the enzymatic activity of PI3KC3, an enzyme vital for initiating autophagy, which, in turn, promotes autophagosome formation in infected cells (Sir et al., 2010). Consequently, inducing incomplete autophagy may allow HBV to both avoid autophagic degradation and promote its own replication through making use of the components of the autophagy machinery.

There are no reports to date of plant viruses interfering with the mechanism of autophagy similar to that seen with animal viruses; however, given that plant viruses may promote replication through autophagy biogenesis, and because they must avoid degradation *via* the autophagy pathway, plant viruses may "kill two birds with one stone" by blocking the last link in the autophagic process, that is, the autophagosome maturation and fusion steps.

Utilizing host proteins to evade being degraded by autophagy is a versatile mechanism adopted by viruses. Galectin-8 can detect nucleosomes containing picornaviruses (PVs) and mark them for autophagic degradation; meanwhile, PVs such as poliovirus can evade this detection with the aid of the host protein HRAS-like suppressor 3 (PLA2G16), thus evading clearance by autophagy and ensuring the delivery of viral genomes into the cytoplasm (**Table 2**) (Staring et al., 2017). Rice streak virus (RSV) can induce an unfolded protein response (UPR) in both rice and tobacco. In turn, RSV-induced UPR activates the host's autophagy pathway, targeting the RSV-encoded motor protein NSvc4 for autophagic degradation and inhibiting RSV movement between cells. Correspondingly, RSV NSvc4 hijacks UPR-activated type I J-domain proteins in plants to evade autophagic degradation (**Table 2**) (Li et al., 2021).

# AUTOPHAGY IS MANIPULATED BY VIRUSES

## Animal Viruses Hijack Autophagy, Leading to Weakened Immunity

Besides directly disrupting host autophagy, some plant- or animal-derived viruses can also hijack host cell autophagy, which leads to weakened host antiviral defense responses (**Table 3**). Recently, Hou et al. (2021) found that CCDC50 negatively regulates the type I interferon (IFN) signaling pathway that is activated by animal RNA viral sensor RIG-I-like receptors (RLRs). Interestingly, in human monocytes (THP-1) infected with RNA viruses such as Sendai virus (SeV), vesicular stomatitis virus (VSV), or encephalomyocarditis virus (EMCV), CCDC50 expression is significantly enhanced, and CCDC50 specifically recognizes polyubiquitinated RLRs, resulting in the delivery of activated RIG-I/MDA5 into autophagosomes for degradation (**Figure 2**) (Hou et al., 2021). Histone deacetylase 6 (HDAC6), a component of viral RNA-induced stress granules, acts as an antiviral immune complex and plays an active role in the type I IFN responses (Zheng et al., 2020). Coxsackievirus A16 (CA16) triggers p62-mediated selective autophagic degradation of HDAC6, inhibits type I IFN responses, and promotes viral replication (**Figure 2**) (Zheng et al., 2020). Some viral proteins act directly as cargo receptors and manipulate selective autophagy to inhibit host antiviral responses. For example, human parainfluenza virus type 3 (HPIV3) matrix protein (M) translocates to host mitochondria and induces mitophagy through interacting with Tu translation elongation factor, mitochondrial (TUFM) and the autophagy protein LC3 (**Figure 2**) (Ding et al., 2017). M-mediated mitophagy leads to the inhibition of type I IFN responses. The IAV PB1-F2 protein functions in a similar manner to HPIV3 M, simultaneously interacting with TUFM and LC3B to induce complete mitochondrial autophagy, which promotes the degradation of mitochondrial antiviral signaling protein (MAVS) and suppresses the host's innate immunity (Wang et al., 2021a). Muscolino et al. (2020) detailed a mechanism by which viruses hijack cellular autophagy to degrade host signaling proteins and thus evade immunity. The M45 protein of murine cytomegalovirus induces the degradation of nuclear factor κ-light-chain-enhancer of activated B-cells (NF-κB) essential modulator (NEMO) and receptor-nuclear protein kinase 1 (RIPK1) by first promoting their sequestration as insoluble protein aggregates and then recruiting the retromer component vacuolar protein sorting 26B (VPS26B) and the LC3-interacting adaptor protein TBC1D5 to promote the degradation of the aggregates through selective autophagy (**Figure 2**) (Muscolino et al., 2020). Like M45, the HSV-1 ICP6 protein also induces the aggregation and degradation of RIPK1 (Muscolino et al., 2020).

## Plant Viruses Manipulate Autophagy to Counteract Host Antiviral Defenses

siRNA-mediated post-transcriptional gene silencing (PTGS) is a well-characterized conserved antiviral defense mechanism in higher

**TABLE 3** | Autophagy is manipulated by viruses.

| Host | Virus(s) | Viral protein(s) | Host protein(s) | Effects on host–virus interactions | References |
|---|---|---|---|---|---|
| Animal | SeV; VSV; EMCV | Unknown | CCDC50; RIG-I/MDA5 | Enhances CCDC50 expression, which delivers activated RIG-I/MDA5 for autophagic degradation | (Hou et al., 2021) |
| | CA16 | Unknown | p62; HDAC6 | Triggers p62-mediated selective autophagic degradation of HDAC6 | (Zheng et al., 2020) |
| | HPIV3 | M | LC3; TUFM | M mediates mitophagy *via* interactions with TUFM and inhibits the type I interferon response | (Ding et al., 2017) |
| | IAV | PB1-F2 | LC3; TUFM | PB1-F2 interacts with TUFM and LC3B, thus inducing complete mitochondrial autophagy | (Wang et al., 2021a) |
| | MCMV | M45 | VPS26B; TBC1D5; NEMO; RIPK1 | M45 promotes NEMO and RIPK1 aggregation and recruits VPS26B and TBC1D5 to facilitate the degradation of the aggregates through selective autophagy | Muscolino et al., 2020) |
| | HBV | SHBs | LC3 | SHBs interacts with LC3 and induces autophagy *via* triggering UPR and ER stress | (Li et al., 2011) |
| Plant | TuYV | P0 | AGO1; ATI1/2 | P0 triggers AGO1 degradation by the autophagy pathway | (Derrien et al., 2012; Michaeli et al., 2019) |
| | TuMV | VPg | SGS3 | VPg mediates the degradation of SGS3 by autophagy and ubiquitination | (Cheng and Wang, 2017) |
| | TYLCCNB | βC1 | CaM; SGS3 | βC1 upregulates CaM expression and promotes CaM-mediated SGS3 degradation | (Li et al., 2014; Li et al., 2017) |
| | CMV | Unknown | VISP1; SGS3/RDR6 | CMV induces VISP1 expression, VISP1 interacts with SGS3 and mediates the autophagic degradation of SGS3/RDR6 | (Tong et al., 2021) |
| | RSV | NSsv4 | REM1 | NSsv4 interacts with REM1 and interferes with its S-acylation, inducing the autophagic degradation of unmodified REM1 | (Fu et al., 2018) |
| | TuMV | VPg | REM1.2 | VPg interacts with REM1.2 and mediates REM1.2 degradation through autophagy and ubiquitination pathways | (Cheng et al., 2020) |
| | TuMV | 6K2; NIb | NBR1; ATG8f | TuMV activates UPR-dependent NBR1-ATG8f autophagy to target the VRC to the tonoplast, thus promoting viral replication | (Li et al., 2020a) |

plants. Key components involved in the PTGS mechanism include ARGONAUTE1 (AGO1) and the host RNA-dependent RNA polymerase 6 (RDR6)/suppressor of gene silencing 3 (SGS3) complex (Ding, 2010). During interaction with their hosts, plant viruses target these proteins for autophagic clearance to counteract host-mediated RNA silencing. Derrien et al. (2012) found that polerovirus P0 triggers AGO1 degradation through the autophagy pathway (**Figure 2**). Subsequently, Michaeli et al. (2019) showed that P0 and AGO1 are associated with the ER, leading to their loading into ER-associated vesicles and, subsequently, their vacuolar degradation in an ATG5- and ATG7-dependent manner. In addition, ATG8-interacting proteins 1 and 2 (ATI1 and ATI2) are recruited to the ER and interact with AGO1 to promote the ER-associated autophagic degradation of AGO1 (Michaeli et al., 2019). TuMV infection has been reported to reduce the level of SGS3, which is essential for the biosynthesis of virus-derived small interfering RNA (vsiRNA) (Cheng and Wang, 2017). TuMV-encoded viral genomic connexin (VPg) interacts with SGS3 and induces its degradation and that of its interacting partner RDR6 through both 20S ubiquitin-proteasome and autophagic pathways (**Figure 2**) (Cheng and Wang, 2017). Li and co-workers reported that geminiviruses appear to indirectly utilize the plant endogenous RNA silencing suppressor calmodulin-like protein (CaM) to inhibit the siRNA mechanism by promoting the autophagic degradation of SGS3 (**Figure 2**) (Li et al., 2014; Li et al., 2017). Recently, Tong et al. (2021) identified a novel 71-amino acid virus-induced small peptide (VISP1) in plants that acts as an autophagy cargo receptor. VISP1 overexpression induces selective autophagy, which attenuates SGS3/RDR6-dependent viral siRNA amplification and enhances viral infection; meanwhile, VISP1 mutants display the opposite effect. Some plant viruses can induce the upregulation of VISP1 expression, thus mediating selective autophagic degradation of SGS3/RDR6 and, consequently, promoting self-replication and infection (**Figure 2**).

Remorins (REMs) are plant-specific membrane-associated proteins that play an important role in the interaction between plants and pathogens (Konrad et al., 2014). Fu et al. (2018) reported that S-acylation is a prerequisite for NbREM1 to target plasma membrane microdomains and is also required for its antiviral function, i.e., the inhibition of intercellular virus transport. Meanwhile, the RSV motor protein NSvc4 interacts with NbREM1 and interferes with its S-acylation; accumulated (untargeted) NbREM1 is degraded by autophagy, leading to the downregulation of NbREM1. In summary, RSV attenuates NbREM1-mediated antiviral activity, which promotes viral infection (**Figure 2**) (Fu et al., 2018). Similarly, Cheng et al. (2020) reported that TuMV VPg interacts with REM1.2 and mediates its degradation through the 26S ubiquitin-proteasome and autophagy pathways (**Figure 2**).

## Viruses Exploit the Autophagy Machinery for Replication

Viruses can also directly use the autophagy machinery to promote their own replication. For example, TuMV upregulates NBR1-mediated selective autophagy in a UPR-dependent manner and targets viral RdRp-containing virus replication complex (VRC) to the vacuolar membrane, which promotes viral replication and virion accumulation through cascades of protein–protein interactions (**Table 3**) (Li et al., 2020a). Animal viruses also induce autophagosome accumulation by activating the UPR;

however, they do not promote the degradation of autophagic proteins (Sir et al., 2008). Importantly, this autophagosome accumulation enhances HCV replication, suggesting that HCV uses an incomplete autophagic response to promote its replication (Sir et al., 2008). The production and envelopment of another animal virus, HBV, is also dependent on the host autophagy machinery. Unsurprisingly, HBV enhances the autophagy process in host cells without promoting protein degradation. This enhancement is mediated by HBV small surface protein (SHBs), which induces autophagy *via* triggering UPR and ER stress (Li et al., 2011).

In summary, plant and animal viruses employ several strategies to manipulate the autophagy machinery of their hosts. Viruses promote antiviral factor degradation by activating selective autophagy receptors or inducing the expression of negative immune regulators in their hosts. Additionally, virus-encoded proteins act as selective autophagy receptors and mediate autophagy, thus obstructing antiviral responses. Finally, some viruses use their host's autophagic process directly, but do not promote autophagic protein degradation to enhance viral replication.

## CONCLUSIONS AND PERSPECTIVES

The past decade has seen significant progress in the study of autophagy–virus interactions. It is well-established that this ancient and conserved catabolic pathway is a key element of antiviral immunity *via* mediating the selective elimination of viral proteins and particles. However, in the long-term "arms race" with their hosts, viruses have evolved a variety of strategies to inhibit and disrupt the autophagy pathway, thereby limiting the hosts' antiviral ability, and even manipulate and use autophagy to enhance infection. Here, we have reviewed the research progress related to the interaction between autophagy and viruses, and summarized the process of virophagy mediated by a variety of selective autophagy receptors. Moreover, we have described the similar strategies used by plant and animal viruses, including the autophagy-induced activation of negative regulatory signals, direct inhibition of key proteins involved in autophagosome biogenesis, interruption of selective autophagy, and use of host proteins to evade autophagic degradation, all of which disrupt antiviral responses. In addition, we compared the mechanisms used by plant and animal viruses to manipulate autophagy and promote self-replication and infection, highlighting that both plant and animal viruses can manipulate selective receptors in the host or produce proteins that act as cargo receptors, thus inhibiting antiviral immune responses.

Although numerous studies have investigated the control of viral infection by autophagy and how viruses counteract autophagy-induced adverse consequences, autophagy–virus interactions remain ill-defined. Additionally, the mechanisms involved in how viral material is specifically recognized and targeted for degradation *via* autophagy remain poorly understood. A key direction for future research will be to identify and characterize virophagy receptors that drive host defense responses as well as the role played by ubiquitination and/or other post-translational modifications in selectivity and cargo recognition. These will greatly improve our knowledge of the mechanisms and functions of autophagy in plant immunity. Additionally, an interesting balance exists in some viruses, especially RNA viruses, in that because they can neither survive nor escape autophagy, they have evolved a mechanism that blocks only some aspects of autophagy. For example, herpesviruses can effectively prevent autophagosome maturation; in turn, autophagosomes represent a source of their outer membrane. It remains unclear how the virus subtly regulates autophag signaling during its infection cycle such that it can simultaneously escape autophagic degradation while exploiting the structural benefits provided by autophagy.

Compared with plant viruses, substantially more is known about interactions between animal viruses and host autophagy. Some directions for research on plant autophagy–virus interactions can be garnished from our knowledge of animal viruses, which can be summarized as follows: (1) In animal cells, autophagy controls viral infection at three levels. At the first level, autophagy directly mediates the selective degradation of viral components or particles; the second level involves the autophagy-mediated initiation of the innate immune response through synergizing with pattern recognition receptor signaling to induce IFN production; at the third level, meanwhile, autophagy activates adaptive immunity by promoting antigen presentation. Although innate and adaptive immune response mechanisms do not exist in plant cells, factors such as hormones, PTI, and ETI play key roles in plant antiviral responses. Studies have shown that autophagy is associated with the salicylic acid signaling pathway and programmed cell death; however, the intrinsic crosstalk mechanisms have not been explored. In addition, it is not known whether the complex regulatory mechanism, including negative feedback, found between autophagy and immune receptors in animals also exists in plants. Yang et al. (2019) reported that autophagy is involved in the degradation of the plant immune receptor FLS2, suggesting that such a mechanism does indeed exist in plants. Revealing the relationship between autophagy and plant immune signaling will greatly increase our knowledge of the mechanisms and functions of autophagy in plant immunity and antiviral responses. (2) Accumulating evidence has suggested that within animal cells, many components of the autophagy machinery also mediate autophagy-independent antiviral functions (Galluzzi and Green, 2019). For example, ATG16L1-dependent targeting of LC3 to single-membrane, non-autophagosome compartments—referred to as non-canonical autophagy—protects mice from lethal IAV infection (Wang et al., 2021b). However, whether ATGs in plant cells also have autophagy-independent functions remains largely unknown. (3) There is a unique virophagy pathway controlled by SNX5 in animal cells. This pathway can activate the autophagy-related PI3KC3-C1 kinase complex and produce the key autophagy initiation signal PI3P, thus activating autophagy (Dong et al., 2021). Snx5-regulated virophagy has no effect on basic autophagy and autophagy induced by multiple classical or non-classical stimuli, and is not related to other cellular pathways, including

endocytosis and interferon signaling, but rather to a specific viral defense responses through autophagy (Dong et al., 2021).

It is not known whether plants have a similar virophagy-specific machinery. (4) Many animal viruses encode proteins that target ATG6/Beclin1 and inhibit its activity, thus blocking autophagosome nucleation and maturation. This mechanism of disrupting autophagosome biosynthesis has not been found in plant viruses. However, given the dual role of ATG6/Beclin1 in mediating antiviral responses, ATG6/Beclin1 should be an ideal target for plant viruses. (5) Animal viruses can also interfere with the maturation of host autophagosomes and their fusion with lysosomes, achieving the effect of "killing two birds with one stone", whereby they promote their own replication while also avoiding degradation *via* the autophagy pathway. No such reports exist regarding plant viruses. Further in-depth investigation of the autophagic mechanism in plants and the strategies used by plant viruses against autophagy may shed light on these questions. (6) The molecular basis underlying virus-induced autophagy activation in plants and the role of plant autophagy proteins and membranes in viral replication, which have been established in animal systems, is currently unknown. In summary, further investigation is required to reveal the specific role of autophagy in antiviral infection as well as the mechanism by which viruses manipulate autophagy.

## AUTHOR CONTRIBUTIONS

WW and XL prepared the original draft. WW and XL undertook editing. MR was involved in supervision. XL and MR acquired the funding. All authors contributed to the article and approved the submitted version.

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REVIEW
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# *LAPped in Proof:* LC3-Associated Phagocytosis and the Arms Race Against Bacterial Pathogens

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Cells of the innate immune system continuously patrol the extracellular environment for potential microbial threats that are to be neutralized by phagocytosis and delivery to lysosomes. In addition, phagocytes employ autophagy as an innate immune mechanism against pathogens that succeed to escape the phagolysosomal pathway and invade the cytosol. In recent years, LC3-associated phagocytosis (LAP) has emerged as an intermediate between phagocytosis and autophagy. During LAP, phagocytes target extracellular microbes while using parts of the autophagic machinery to label the cargo-containing phagosomes for lysosomal degradation. LAP contributes greatly to host immunity against a multitude of bacterial pathogens. In the pursuit of survival, bacteria have developed elaborate strategies to disarm or circumvent the LAP process. In this review, we will outline the nature of the LAP mechanism and discuss recent insights into its interplay with bacterial pathogens.

**Keywords: LC3-associated phagocytosis, macrophages, neutrophils, autophagy, innate immunity, intracellular pathogens, virulence mechanisms, immune evasion**

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## INTRODUCTION

Throughout evolution, microbial pathogens and animal immune cells have developed elaborate mechanisms to face and withstand each other. Understanding these mechanisms lies at the heart of improving medical interventions against microbial infections. Phagocytes, specialized cells of the innate immune system, are characterized by their ability to engulf and intracellularly destroy foreign particles and dying cells. Engulfment and subsequent degradation of microbes is key to our innate, and ultimately adaptive defenses. Central to the phagocytic elimination of microbial invaders is the fusion of the phagosome with lysosomes, a process called phagosome maturation. Unless inhibited by virulence factors, an engulfed microbe will be exposed to an array of lysosomal enzymes — killing it within minutes (Fountain et al., 2021) .

Three different vesicle trafficking mechanisms are known to direct microbial pathogens to lysosomal degradation: phagocytosis, autophagy and LC3-associated phagocytosis (LAP). All have overlapping characteristics but are initiated *via* distinct pathways, where cargo-containing vesicles form and mature by different mechanisms. Phagocytosis, which was recognized as early as the 19th century, targets extracellular microbes *via* receptor-mediated recognition (Rosales and Uribe-Querol, 2017). Several pathogens have evolved strategies to subvert the phagocytic process, allowing them to establish a niche for their own proliferation (Flannagan et al., 2009). Some microbes, like *Streptococcus pyogenes*, can arrest ingestion by producing toxins or expressing antiphagocytic

surface proteins (Brouwer et al., 2016), while others, like *Mycobacterium tuberculosis*, interfere with phagosome integrity to first establish an intravesicular replicative niche and subsequently escape the confines of the phagosomal vesicle (Simeone et al., 2021). Other microbes still, like *Listeria monocytogenes*, take advantage of the acidification of the phagosome, utilizing it to activate virulence-mediated disruption of the phagosomal membrane, leading to immediate invasion of the cytosol (Matereke and Okoh, 2020).

A second way for cells to effectively degrade microbial invaders is autophagy, strictly speaking macroautophagy. By definition, autophagy targets intracellular structures, such as protein aggregates or cytosolic bacteria, capturing them in a characteristic double-membrane vesicle (Boya et al., 2013). Mediated by a group of conserved autophagy-related proteins, a cup-shaped double-membrane complex is nucleated around a target structure. It extends to seal the target into a closed vesicle, the autophagosome. Similar to phagosomes, the transient autophagosomes mature by fusing with lysosomes. In recent decades, accumulating evidence has illustrated the extensive interactions between microbial pathogens and the host autophagic response, referred to as xenophagy (Deretic and Levine, 2009; Huang and Brumell, 2014). Intracellular pathogens have developed ingenious evasive mechanisms to avoid being killed in the autophagosome. Such strategies include the interference with autophagy-initiating signaling, disruption of lysosome function and proteolytical inactivation of the autophagic machinery (Jiao and Sun, 2019). Furthermore, microbes have even evolved means to turn host autophagy to their own advantage, utilizing it to foster their own nutrient supply, replication, cellular egress and virulence (Kimmey and Stallings, 2016).

Since 2007, it has become clear that phagocytes have a third degradation mechanism to their disposal, which is now commonly referred to as LAP (Sanjuan et al., 2007). LAP has been described to exist at the crossroads of autophagy and phagocytosis, combining the strengths of both processes to ensure enhanced degradation of the engulfed cargo (Martinez, 2018; Heckmann and Green, 2019). Given its intricate role in anti-microbial immunity and preservation of homeostasis, LAP has sparked much interest in recent years. During LAP, which is initiated by receptor signaling, select parts of the autophagic machinery – particularly the ubiquitin-like protein LC3 (microtubule-associated proteins 1 A/1B light chain) – are specifically recruited to the single-membrane phagosome (Sanjuan et al., 2007). Early in the maturation process, an NADPH oxidase complex is assembled that generates reactive oxygen species (ROS) within the vesicle. Soon after LC3 is conjugated onto the phagosomal membrane, the phagosome (now termed LAPosome) fuses with lysosomes, leading to rapid clearance of the internalized material. LAP is often referred to as a form of non-canonical autophagy, but strictly speaking the term autophagy applies only in relation to the vesicular uptake of cytoplasmic cargo, while LAP targets vesicles with material coming directly from the extracellular environment.

Multiple lines of evidence have demonstrated that LAP mediates a variety of immunological functions that go beyond the elimination of pathogens. The process has been deemed important for the immunotolerant processing of dying cells, regulation of inflammatory responses, establishment of signaling compartments, and even attenuating autoimmunity (Martinez et al., 2015; Heckmann et al., 2017; Wong et al., 2021). With regard to human disease, LAP has drawn particular attention for its role in immunity to different classes of microbial pathogens (Chamilos et al., 2016; Besteiro, 2019; Jiao and Sun, 2019; Akoumianaki et al., 2021). In this review, we focus on the role of LAP in bacterial infectious diseases. We discuss the molecular mechanisms that orchestrate LAP, and provide an overview of its significance in fighting bacterial infections as well as its fragility in view of pathogenic evasion.,

## MECHANISMS OF LAP INDUCTION AND MATURATION

While classical autophagy and LAP have significant overlap in their utilization of the molecular machinery, induction of these processes is fundamentally distinct. LAP and related single membrane LC3 lipidation processes are triggered by the engagement of various surface receptors (Sanjuan et al., 2007; Martinez et al., 2015), including Toll-like receptors (TLRs), Dectin-1, Dectin-2, but also immunoglobulin receptors such as Fc$\gamma$R and scavenger receptors such as TIM4 (Sanjuan et al., 2007; Huang et al., 2009; Martinez et al., 2011; Ma et al., 2014; Lamprinaki et al., 2017). In addition, activation of the cytosolic innate immune sensor STING induces LC3 lipidation of single-membrane vesicles (Fischer et al., 2020). How these different cargo engagements and consequent signaling pathways activate the machinery required for LAP remains unclear. However, it has been well documented that LAP proceeds independently of the pre-initiation complex containing ULK1, ATG13, ATG101 and FIP200, which is crucial for autophagy induction (Martinez et al., 2011; Heckmann and Green, 2019). Indeed, LAP typically appears unresponsive to nutrient starvation and other autophagic signals associated with ULK1 activation (Sanjuan et al., 2007). Similar to phagocytosis but unlike classical autophagy, pathogens targeted by LAP are engulfed in a single-membrane phagosome (Schille et al., 2018). This is one of the most significant ultrastructural differences that distinguishes LAPosomes from classical autophagosomes (Lai and Devenish, 2012).

After the pathogen is internalized, one of the first signaling complexes to associate with the budding phagosome is the class III phosphatidylinositol 3-kinase complex (PI3KC3), which ultimately delivers PI(3)P onto the phagosomal membrane (Matsunaga et al., 2009) (**Figure 1A**). The functional core of PI3KC3 is composed of VPS34 (the catalytic subunit), VPS15 and Beclin-1 (Backer, 2016). Following activation by VPS15 and Beclin-1, VPS34 generates PI(3)P from PI(3) *via* its kinase activity (Volinia et al., 1995; Petiot et al., 2000). The newly formed PI(3)P molecules disseminate throughout the phagosomal membrane, acting as a label for future LC3-conjugation (Martinez et al., 2011). Two critical proteins that specifically recruit PI3KC3 to the phagosome during LAP, are

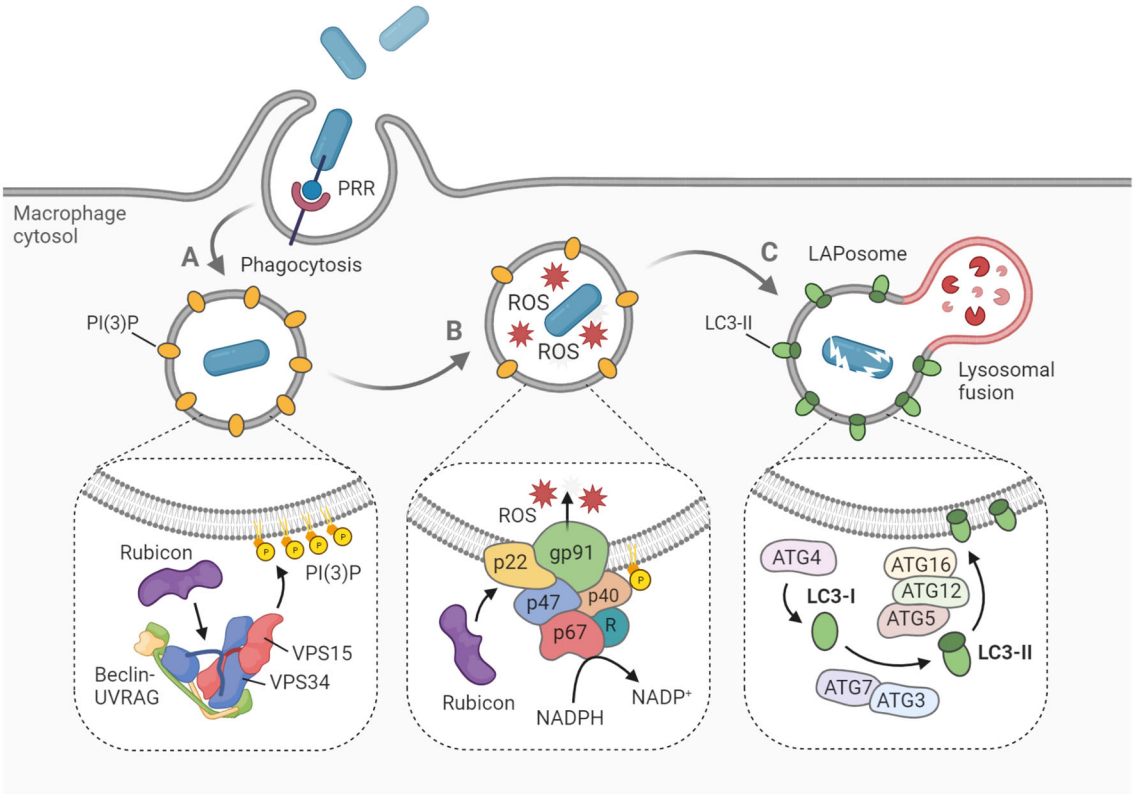


**FIGURE 1** | Hallmarks of LAP on the molecular level. LAP begins with pattern recognition receptor (PRR)-mediated phagocytosis of pathogens, dying cells and other particles. **(A)** The phagosome is marked with PI(3)P, a signaling lipid which is generated by the PI3KC3 complex, consisting of Beclin-1, UVRAG, VPS15, VPS34 and Rubicon. **(B)** Within the phagosome, ROS are produced by the NADPH oxidase complex. Rubicon stabilizes the complex *via* interaction with $p22^{phox}$, while $p40^{phox}$ interacts with PI(3)P to recruit the remaining components. **(C)** Cytosolic LC3 is lipidated by the conjugation machinery to form LC3-II on the phagosomal membrane. Soon after, the LAPosome fuses with an available lysosome resulting in rapid degradation of the engulfed cargo. Figure created with BioRender.com.

UVRAG and Rubicon (Martinez et al., 2015). While the PI3KC3 complex itself is non-specific for LAP, and is also involved in the activation of classical autophagy, Rubicon is essential for LAP maturation in contrast to its inhibitory role in autophagosome maturation (Martinez et al., 2015). In fact, Rubicon participates at multiple signaling steps relevant for LAP development through interaction with different binding partners (Matsunaga et al., 2009; Yang et al., 2012).

Another hallmark of LAP, which also depends strictly on Rubicon activity, is the generation of reactive oxygen species (ROS) within the phagosome lumen (Martinez et al., 2015) (**Figure 1B**). ROS are produced by the NADPH oxidase 2 complex (NOX2), the only NADPH oxidase complex expressed in phagocytes (Bedard and Krause, 2007). The activity of NOX2 is dependent on the recruitment of four cytosolic subunits, namely $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac1, to the two membrane-embedded subunits $p22^{phox}$ and $gp91^{phox}$, which form the catalytic center. The p40 protein is capable of direct interaction with the PI(3)P present on the phagosome, functioning as a docking site for the other cytosolic subunits (Ellson et al., 2006). Rubicon is able to stabilize the NOX2 complex *via* direct interaction with p22, resulting in maximal ROS production (Yang et al., 2012). The ROS may serve several roles in the LAP process. Aside from their putative oxidative activity against the pathogen (Slauch, 2011), ROS are needed for recruitment of downstream LAP components, such as ATG7 and LC3 (Lam et al., 2013; Martinez et al., 2015). Furthermore, ROS generation by NOX2 has been shown to cause oxidative inactivation of ATG4B, thereby inhibiting the proteolytic release of LC3 and thus stabilizing the LAPosome (Ligeon et al., 2021). Further aspects of the mechanistic interplay between ROS signaling and LAP maturation are incompletely understood, though it is hypothesized that lipid peroxidation within the phagosome could serve a regulatory function (Holmström and Finkel, 2014).

After the phagosomal membrane is marked by PI(3)P and ROS have been produced, two conjugation systems are activated that will mediate the processing and incorporation of LC3 onto the phagosomal membrane (**Figure 1C**). Cytosolic pro-LC3 is converted into LC3-I by ATG4. Then, LC3-I is lipidated by ATG7-ATG3 and ATG12-ATG5-ATG16L1 *via* covalent attachment on phagosomal surface to form LC3-II (Martinez

et al., 2015; Schille et al., 2018). Both LAP and classical autophagy are characterized by the association of LC3-II onto the target membrane. However, recruitment proceeds differently in both processes, as the target membrane is the phagophore in the case of autophagy, and the phagosome in the case of LAP (Herb et al., 2020). Furthermore, autophagy can maintain tissue homeostasis independent of LAP, which has been illustrated by the differential role of ATG16L1 in both processes. Specifically, autophagy requires the ATG5-binding and coiled coil domains of ATG16L1 but not the WD domain, whereas the WD domain is indispensable for LAP (Rai et al., 2019; Fischer et al., 2020; Wang et al., 2021). Following LC3-decoration, the LAPosome will rapidly fuse with lysosomes and acidify (Martinez et al., 2011). While it has been argued that LC3 family proteins play an important role in facilitating this lysosomal fusion, details about the vesicle fusion mechanism remain obscure (Martinez et al., 2015; McEwan et al., 2015; Nguyen and Yates, 2021).

## BIOLOGICAL FUNCTIONS OF LAP

The primary function of LAP is to facilitate the fusion of phagosomes with lysosomes, assuring rapid degradation of the engulfed cargo and regulation of the appropriate immune response (Martinez, 2018). LAP and related single membrane LC3 lipidation processes exhibit a surprising antimicrobial versatility as it is required for successful processing of a wide variety of pathogens across different kingdoms, with the fungal pathogen *Aspergillus fumigatus*, the bacterial pathogen *Listeria monocytogenes*, the parasite *Toxoplasma gondii*, and Influenza A virus as notable examples (Martinez, 2018; Schille et al., 2018; Besteiro, 2019; Herb et al., 2020; Wang et al., 2021). Consequences of aberrant LAP for human disease is now an active field of research (Martinez, 2018; Upadhyay and Philips, 2019). In recent decades, interest in uncovering novel antimicrobial strategies has grown steadily, mainly due to the alarming prevalence of antibiotic resistance leading to incurable bacterial infections (Aslam et al., 2018).

In addition to its antimicrobial functions, LAP has been shown to be relevant for many other immunological processes, such as the clearance of dying cells and apoptotic remnants – a process known as efferocytosis. LAP enables professional phagocytes to process cellular debris in a remarkable immunosilent manner, by keeping levels of pro-inflammatory cytokines and associated signaling pathways at bay (Heckmann et al., 2017). Indeed, Rubicon-deficient mice show a defective clearance of apoptotic cells, resulting in an exaggerated inflammatory phenotype and ultimately the formation of auto-antibodies (Martinez et al., 2016). For humans, proper processing of cellular debris has been shown to be crucial for averting autoimmune disorders, such as systemic lupus erythematosus (SLE) (Muñoz et al., 2010). Intriguingly, genome-wide association studies among SLE patients have found a polymorphism in the ATG5 protein, suggesting that LAP or autophagy might play a critical role in the development of this disorder (Harley et al., 2008; Gateva et al., 2009). Furthermore, defects in LAP have been linked to numerous other inflammatory abnormalities, including atherosclerosis, visceral adiposity, and insulin resistance (Heckmann and Green, 2019).

Contrary to intuition, LAP may be a contributing factor in tumorigenesis, as it has been implicated in the establishment of a conducive microenvironment for cancerous cells. In mice, an increased LAP activity has been associated with tumor growth and aggressiveness (Asare et al., 2020). Indeed, high expression of Rubicon in cancer tissues predicts an adverse survival rate of patients with various cancer types. It is thought that the immunosuppressive signaling networks associated with LAP could be hijacked by developing cancer cells to bypass the immune response, thereby promoting their progression and metastatic potential. The implications of this have been reviewed elsewhere (Asare et al., 2020).

## INTERACTIONS OF BACTERIAL PATHOGENS WITH LAP

The diversity of evasive strategies adopted by different species of pathogens is testament to the complexity and effectiveness of the LAP process. In many cases, evasion mechanisms are only beginning to be discerned on the molecular level. Some bacterial pathogens circumvent LAP altogether by expressing effectors that impair their targeting, while others orchestrate their own internalization and survive inside phagosome (**Figure 2**). Below, we describe some notable examples of LAP-targeted bacterial pathogens and discuss how LAPosome formation and maturation may be modulated by virulence mechanisms of these pathogens (**Table 1**). We have included also cases that may represent different forms of single membrane LC3 lipidation closely resembling LAP.

### *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* is the causative agent of acute or chronic manifestations of tuberculosis, the most lethal bacterial infectious disease today (WHO, 2020). *M. tuberculosis* is recognized and phagocytosed by macrophages *via* different surface receptors, including TLRs, mannose receptors and complement receptors (Schlesinger, 1993; Yu et al., 2014). Even though *M. tuberculosis* has been shown to be targeted by LAP, it is still not clear which fraction of phagosomes progresses to LAPosomes and whether this process enhances the ability of phagocytes to clear the pathogen or it is exploited by the pathogen for its intracellular survival (Köster et al., 2017; Köster et al., 2018).

LAP resistance of *M. tuberculosis* was found to be mediated by the virulence factor CpsA, which prevents recruitment of NOX2 to the pathogen-containing phagosome (Köster et al., 2017; Köster et al., 2018). While the inactivation of NOX2 by CpsA resulted in impaired lysosomal trafficking, reduced phagolysosome biogenesis and ultimately the survival and proliferation of intracellular *M. tuberculosis*, the deletion of CpsA in *M. tuberculosis* resulted in efficient degradation of the

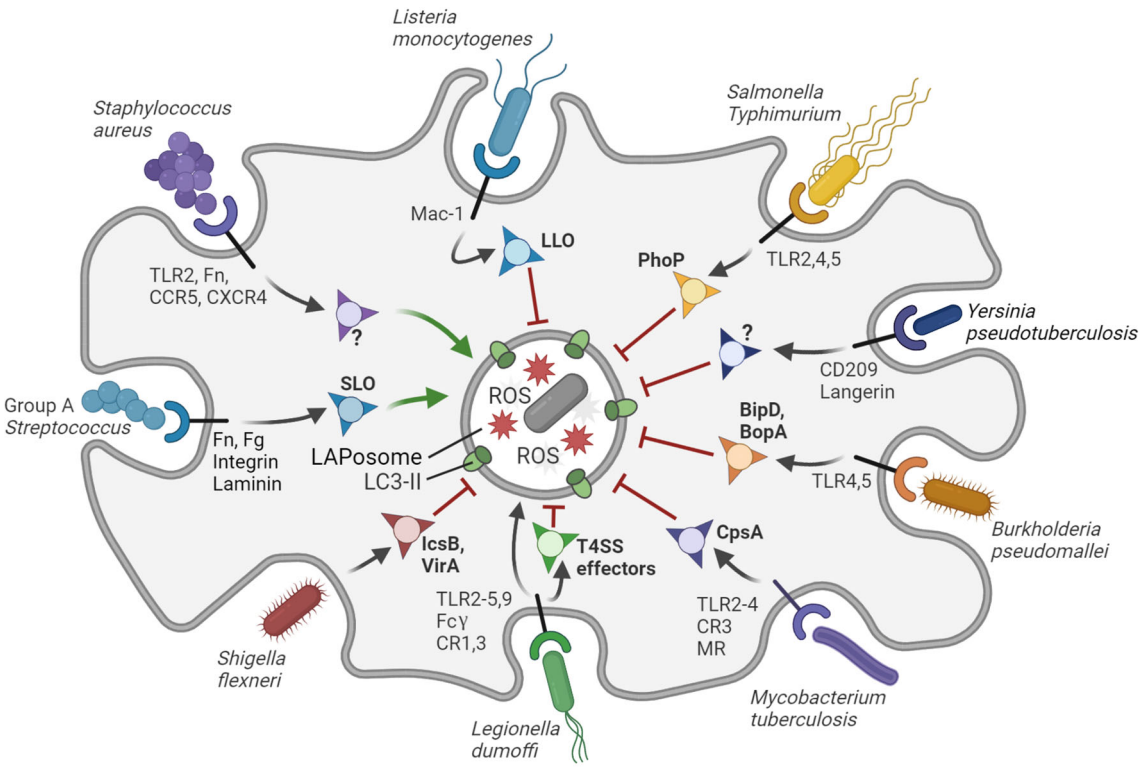


**FIGURE 2** | Interactions of bacterial pathogens with LAP. The LAPosome is a single membrane vesicle marked by LC3-II and producing ROS. LAP contributes to host defense, but bacterial pathogens have evolved diverse ways to inhibit (red arrows) or promote (green arrows) LAP to their own benefit. *S. aureus* promotes the formation of LAPosomes in neutrophils *via* an unknown virulence factor to establish a replicative niche. Group A Streptococcus promotes LAP *via* the virulence factor SLO to evade bactericidal xenophagy. *L. dumoffi* is effectively degraded in the LAPosome (black arrow), although it may inhibit LAP to some extent *via* T4SS effector proteins. All other bacterial pathogens shown in the figure can partially inhibit LAP in phagocytes or LAP-like processes in epithelial cells through the virulence factors indicated. Virulence factors that remain to be identified are indicated with question marks. The receptors that mediate entry and/or immune recognition by the host phagocytic cells are shown, except for *S. flexneri*, which attaches to surface proteins of M-cells in the gut epithelium. Figure created with BioRender.com.

**TABLE 1** | Overview of LAP-targeted bacterial pathogens and their evasion strategies.

| Pathogen | Mode of entry/innate immune recognition | Virulence factor | Evasion or exploitation of LAP or LAP-like processes | References |
|---|---|---|---|---|
| *Mycobacterium tuberculosis* | TLR2, TLR4, mannose receptor, complement receptor 3 | CpsA, PDIM | Inhibits recruitment of NAPDH oxidase to phagosome. Conceal TLR ligands triggering LAP | Stamm et al. (2015); Köster et al. (2017) |
| *Listeria monocytogenes* | Mac-1 | LLO | Upregulates mitochondrial calcium signaling to acetylate Rubicon | Gluschko et al. (2018); Li et al. (2021) |
| *Salmonella enterica* serovar *typhimurium* | TLR2, TLR4, TLR5 | PhoP, FlhD, SsrB | Inhibition of phagolysosomal fusion (PhoP), triggering TLR5 and LAP (FlhD), displaying Rubicon-independent virulence (SsrB) | Masud et al. (2019b) |
| *Legionella dumoffi* | TLR2,3,4,5,9, Fcγ, complement receptor 1,3 | Possibly T4SS | Unknown | Hubber et al. (2017) |
| *Burkholderia pseudomallei* | TLR4, TLR5 | BopA, BipD (T3SS efectors) | Escape from LAPosome *via* T3SS | Gong et al. (2011) |
| *Yersinia pseudotuberculosis* | C-type lectins: Langerin, CD209 | Unknown | Interference with LC3. Recruitment through VAMP3, VAMP7 | Ligeon et al. (2014) |
| *Shigella flexneri* | Surface proteins of M-cells | IcsB, VirA, IpaB, OspC3, IpgD | Inhibition LC3 recruitment | Baxt and Goldberg (2014); Campbell-Valois et al. (2015) |
| Group A *Streptococcus* | Fibronectin, fibrinogen, integrins, laminins | SLO | Evasion of xenophagy by inducing LAP | Lu et al. (2017) |
| *Staphylococcus aureus* | TLR2, CCR5, CXCR4, fibronectin | Unknown | Establishing a LAP-dependent replication niche | Prjasnar et al. (2021) |

pathogen by LAP. *M. tuberculosis* is known to secrete several virulence factors that interfere with phagosome maturation and thus, it is likely to evade LAP in different ways. In this regard, the virulence factor NdkA has been shown to contribute to intracellular survival by interfering with phagosome maturation (Sun et al., 2010). Moreover, the presence of NdkA has been shown to decrease the recruitment of $p67^{phox}$ and Rac1 to the phagosome, interfering with ROS production by the NADPH oxidase complex and presumably undermining LAP (Sun et al., 2013).

In addition to inhibiting phagosome maturation, *M. tuberculosis* is able to conceal its presence by manipulating TLR recognition and therefore preventing its phagocytosis. It is known that TLR4 recognizes lipids, glycoproteins, secreted proteins and other surface ligands from *M. tuberculosis*, leading to fast phagocytosis of the pathogen (Stamm et al., 2015). Absence of phthiocerol dimycocerosate lipids (PDIM) in the *M. tuberculosis* cell wall induced an increase of TLR-dependent recruitment of microbicidal macrophages, indicating the inhibitory role of PDIM on pathogen recognition (Cambier et al., 2014). It has been suggested that this TLR recognition inhibition is due to masking of the mycobacterial pathogen associated molecular patterns (PAMPs) by PDIM (Cambier et al., 2014). The study of PDIM adds another dimension by which evasion of the LAP mechanism by *M. tuberculosis* is possible.

A screen in planarian flatworms identified a protein MORN2, of which the human ortholog was shown to play an important role in the LAP response of macrophages to *M. tuberculosis*, and also *L. pneumophila* and S. aureus (Abnave et al., 2014). MORN2 promotes LC3 recruitment to *M. tuberculosis*-containing phagosomes and their maturation into phagolysosomes. The role of LAP was confirmed by demonstrating the single-membrane nature of the bacteria-containing vesicles as well as the requirement of Atg5 and Beclin1 for LC3 recruitment, but not Ulk1 and Atg13 (Abnave et al., 2014). Further in line with the proposed role of MORN2 in LAP, its function was shown to depend on ROS (Morita et al., 2020). Using *Escherichia coli* and zymosan as alternative LAP substrates, SNARE proteins like SNAP-23 and syntaxin11 were implicated in vesicle fusions during MORN2-mediated LAP (Morita et al., 2020). Altogether, MORN2 emerges from this work as a positive regulator of LAP, which warrants further studies with *M. tuberculosis* and other pathogens.

In conclusion, while there is evidence that *M. tuberculosis* is actively targeted by LAP, its modes of evasion are still starting to be understood. Evasion of LAP by *M. tuberculosis* is likely to occur during the maturation of the phagosome while, among others virulence factors, CpsA and NdkA are secreted. Considering the diversity of virulence factors known to affect phagosome maturation, it is expected that *M. tuberculosis* mutant screens will soon reveal additional effectors critical for phagosome maturation and anti-LAP virulence. Additionally, evasion of LAP initiation *via* phagocytosis has been observed, a process mediated by effector molecules such as PDIM cell wall lipids. MORN2 seems a useful addition to Rubicon for further study as a host factor specifically promoting LAP.

## *Listeria monocytogenes*

*Listeria monocytogenes* is an opportunistic bacterium that can cause severe food-borne diseases in immunocompromised individuals, pregnant women and newborns (Cossart and Lecuit, 1998; Lam et al., 2013). Clearance of *L. monocytogenes* is explicitly promoted by LAP and this host-pathogen interaction can be regarded as one of the most striking examples of the microbicidal power of the LAP pathway (Gluschko et al., 2018; Herb et al., 2018).

During infection, *L. monocytogenes* utilizes the virulence factors listeriolysin (LLO) and PlcA/B to escape from the phagosome and enter the cytosol, where it acquires actin-based motility (Cossart and Lecuit, 1998; Seveau, 2014). Within the cytosol, *L. monocytogenes* actively inhibits classical autophagy *via* IcsB, ActA and PlcA/B (Birmingham et al., 2008; Lam et al., 2013). LAP has become known as the key mechanism providing anti-Listeria immunity (Gluschko et al., 2018). Recognition of *L. monocytogenes via* the β2 integrin Mac-1 receptor activates LAP and the associated phagosomal ROS response. Interestingly, the same study also provided evidence that the execution of LAP is not influenced by virulence factors that inhibit classical autophagy and revealed a crucial role for acid sphingomyelinase, broadening our understanding of the LAP mechanism. The acid sphingmyelinase protein facilitates alterations in the lipid composition of the membrane, allowing the subsequent activation of the Nox2 complex, crucial for the ROS production and subsequent LC3 recruitment (Gluschko et al., 2018).

The possible evasion of LAP by *L. monocytogenes* is only beginning to be understood. Recent work showed that *L. monocytogenes* is able to suppress LAP by modulating mitochondrial calcium signaling (Li et al., 2021). After phagocytosis, *L. monocytogenes* induces mitochondrial calcium uptake by the mitochondrial $Ca^{2+}$ uniporter (MCU) transporter. This increased calcium uptake promotes the production of acetyl-coenzyme A (acetyl-CoA) by pyruvate hydrogenase. Outside the mitochondrion, Rubicon is acetylated by acetyl-CoA resulting in decreased activity of Rubicon in the LAP pathway, thus acting in favour of bacterial survival. In agreement, a knockout of the MCU transporter abolishes calcium uptake, allowing LAP to overpower *L. monocytogenes* infection (Li et al., 2021). Together, these results show that *L. monocytogenes* is able to inhibit LAP through eliciting mitochondrial signaling, which adds to the growing connective network between mitochondrial metabolism and innate immune defense mechanisms (Li et al., 2021).

Interestingly, there are also cases known in which *L. monocytogenes* induces a prolonged infection which LAP fails to control. This might be achieved due to the formation of certain compartments termed spacious Listeria-containing phagosomes (SLAPs) *via* manipulation of the LAP mechanism. It is believed that these single membrane compartments provide a niche in which the bacteria are able to replicate and proliferate. The formation of this niche is possible due to failure of LAP to clear the infection and a lack of the expression of virulence factors that mediate escape from phagosomes into the cytosol (Birmingham et al., 2008; Lam et al., 2013). However, it should be noted that

SLAPs were observed in immunodeficient or oncogenic transformed cells, and therefore their formation in healthy macrophages remains unclear.

Recent studies revealed that phagosome permeabilization by *L. monocytogenes* triggers another single membrane LC3 lipidation pathway, which has been named pore-forming toxin-induced non-canonical autophagy pathway (PINCA) (Mitchell et al., 2018; Gluschko et al., 2021). In bone marrow-derived macrophages, *L. monocytogenes* was shown to be targeted sequentially by multiple autophagic processes. The LLO-mediated perforation of phagosomes was shown to trigger LC3 recruitment in an ULK1-independent process. However, this PINCA response had no role in restricting bacteria growth, in contrast to subsequent xenophagy, which defends against *L. monocytogenes* bacteria upon invasion of the cytosol (Mitchell et al., 2018). PINCA is distinct from LAP, because it can occur in NOX2-deficient macrophages (Gluschko et al., 2021). Induction of LAP in PINCA-competent cells confirmed that LAP contributes to host defense, while no clear anti-*Listeria* function of PINCA could be identified (Gluschko et al., 2021).

To summarize, LAP provides anti-*Listeria* immunity in macrophages, while PINCA, the LC3 recruitment to permeabilized phagosomes, does not restrict bacterial growth (Gluschko et al., 2018; Herb et al., 2018; Mitchell et al., 2018; Gluschko et al., 2021). The host defense function of LAP is counteracted by bacterial LLO, the primary virulence factor that mediates invasion of the cytosol after phagocytosis, where *L. monocytogenes* has to defend itself against xenophagy (Seveau, 2014; Osborne and Brumell, 2017; Mitchell et al., 2018). In addition, it was recently reported that *L. monocytogenes* is able to suppress LAP by manipulating the MCU transporter and redirecting calcium signaling to inhibit the key LAP host factor, Rubicon (Li et al., 2021). Many cases of prolonged infections of *L. monocytogenes* are known, suggesting that bacterial virulence mechanism can modulate LAP to clear the way for SLAP biogenesis (Lam et al., 2013). The distinctive roles of LAP and PINCA and the mechanistic differences between these two processes require further dissection. It will be of great interest to investigate how these two mechanisms may also function side by side in infections with other pathogens that permeabilize phagosomes.

## *Salmonella typhimurium*

*Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is an intracellular pathogen that can invade both non-myeloid and phagocytic cells and is a major cause of gastroenteritis (Ibarra and Steele-Mortimer, 2009). Early studies on Salmonella infection in mouse macrophages and human epithelial cells already suggested that LAP could be a critical player in the immune response, because the triggering of TLR or Fc-gamma receptors induced LC3 recruitment on phagosomes in a manner dependent on ROS production (Huang et al., 2009). The requirement of phagocytic NADPH oxidase for LC3 recruitment to macrophage phagosomes was confirmed by knockdown of the Cyba component of NOX2 in a zebrafish embryo model of systemic *S. typhimurium* infection (Masud et al., 2019a). Furthermore, knockdown of Atg5 and Rubicon, but not the autophagy preinitiation factor Atg13, were shown to be required for LC3 recruitment and for the successful clearance of bacteria in the zebrafish model, providing *in vivo* evidence for the anti-Salmonella function of LAP (Masud et al., 2019a).

While LAP provides protection to *S. typhimurium* infection in zebrafish embryos, there is still a high mortality rate, indicating that the pathogen can resist LAP to a certain extent (Masud et al., 2019a). Several mutant *S. typhimurium* strains were screened to determine the possible role of virulence factors in LAP evasion (Masud et al., 2019b). None of the virulence factors tested, PhoP, PurA, FlhD, SipB and SsrB, appeared to be necessary for the host LAP response, as mutations in these factors did not abolish Rubicon-dependent GFP-LC3 recruitment (Masud et al., 2019b). However, quantitative differences in GFP-LC3 recruitment were observed between the wild type and mutant strains. The PhoP and PurA deficient strains, both attenuated in zebrafish and other animal models, respectively elicited higher and lower GFP-LC3 recruiment (Masud et al., 2019b; Garvis et al., 2001; Thompson et al., 2011; Dalebroux and Miller, 2014). The PhoP regulon has been reported to reduce TLR activation, serve a role in the inhibition of the phagolysosomal fusion, and mediate adaption to intra-macrophage stress (Garvis et al., 2001; Thompson et al., 2011; Dalebroux and Miller, 2014). Therefore, the higher levels of GFP-LC3 recruitment in infection with the Δ*phoP* mutant could suggest a role for PhoP in LAP evasion (Masud et al., 2019b). In contrast, in the case of Δ*PurA* mutant bacteria, a strongly reduced GFP-LC3 recruitment was observed, which might be explained by the virtually complete loss of virulence of this mutant, which could lead to rapid clearance of most of the bacterial population without inducing signals for LAP. (O'Callaghan et al., 1988; Masud et al., 2019b).

Mutation in the FlhD gene, which is crucial for flagella formation of *S. typhimurium*, strongly reduced GFP-LC3 recruitment in the zebrafish model. In line with results in mice, FlhD mutation also resulted in hypervirulence of the *S. typhimurium* pathogen in zebrafish (Fournier et al., 2009). An explanation for both the reduced GFP-LC3 recruitment and the hypervirulence could be that LAP induction is dependent on the recognition of flagellin by TLR5. However, to date no direct link between the signaling of TLR5 and LAP has been established, and therefore the role of the TLR ligand receptor interaction in LAP remains to be studied.

Finally, all the above-mentioned *S. typhimurium* strains displayed increased virulence in a Rubicon-deficient zebrafish host, with the notable exception of a Δ*SsrB* mutant (Masud et al., 2019b). SsrB is part of the bacterial regulatory system controlling expression of *Salmonella* Pathogenicity Island 2 (SPI2) effector molecules that are required for maintenance of the *Salmonella*-containing vacuole (Walthers et al., 2007). Knockdown of zebrafish Rubicon led to reduced GFP-LC3 recruitment towards Δ*SsrB* mutant bacteria, similar as observed with wild type bacteria or other virulence mutants. However, ΔSsrB survival was unaffected by Rubicon knockdown, suggesting that SPI2 effectors could be important for intracellular

replication of *S. typhimurium* under conditions where LAP is impaired (Masud et al., 2019b).

To sum up, *S. typhimurium* is a pathogen which is targeted by the LAP pathway that is crucial for proper pathogen clearance, possibly triggered by TLR5-mediated recognition of flagella. Although successful engulfment and degradation is observed, it is possible that virulence factors like the PhoP/Q operon contribute to LAP evasion. Unlike other wild type or mutant Salmonella strains, Δ*SrsB* mutants, impaired in the expression of SPI2 effectors, were unable to display increased virulence in a LAP-deficient zebrafish host. The specific SPI2 effector(s) responsible for this phenotype remain to be established.

## *Legionella dumoffii*

*Legionella dumoffii* is an intracellular pathogen which can reside in the vacuole after phagocytosis, and is closely related to the human lung disease pathogen, *Legionella pneumophila* (Horwitz, 1983). Phagocytosis of *Legionella* species is mediated by the CR1 and CR3 complement receptors and the Fcy receptor, and innate immune recognition of cell wall components, flagella and bacterial DNA is facilitated by among others the TLR2,3,4,5,9 receptors (Husmann and Johnson, 1992; Grigoryeva and Cianciotto, 2021). Upon phagocytosis, a subpopulation of *L. dumoffii*-containing single-membrane vesicles is decorated with LC3, which requires Rubicon and NOX2 activity, indicating that maturation of these vesicles occurs *via* the LAP pathway (Hubber et al., 2017). In addition, the initiation of the LAP response towards L. dumoffii requires pathogen recognition *via* TLR2 and diacylglycerol signaling. There was no interaction of *L. dumoffii* with ubiquitin receptors and LC3 decoration was independent of ULK1 kinase, thus arguing against a role for selective autophagy and supporting that a subpopulation of *L. dumoffii* resides in LAPosomes (Hubber et al., 2017).

Interestingly, the formation of *L. dumoffii*-containing LAPosomes is dependent on the presence of the bacterial type four secretion system (T4SS). However, independent of LAP, the majority of the bacteria-containing phagosomes are remodelled into a compartment that resembles the endoplasmatic reticulum, thereby inhibiting the fusion with lysosomes and allowing replication (Hubber et al., 2017). Similar to LAP this process is also mediated *via* the activity of T4SS, but it is not understood what determines if expression of T4SS leads to evasion of the immune system or directs bacteria to LAP-mediated degradation. To date *L. dumoffii* remains a relatively poorly studied pathogen compared to other pathogens. Further research involving *L. dumoffii* should be performed to create a more in depth understanding of the interaction between LAP and *L. dumoffi*.

## *Burkholderia pseudomallei*

*Burkholderia pseudomallei* is a soil-dwelling pathogen that causes pneumonia, skin changes and sometimes severe inflammatory cascades and lethal sepsis, a condition known as melioidosis (Wiersinga et al., 2007). It is phagocytosed by macrophages, neutrophils and dendritic cells, and capable of invading epithelial cells (Horton et al., 2012). The pathogen is recognized by TLR2 and TLR4, but TLR2 has been shown to impact negatively on the host defense function, suggesting that this TLR is responsive for severe dysregulation of the immune system and/or facilitates the creation of a bacterial replication niche (Wiersinga et al., 2007). The type III secretion system (T3SS) of *B. pseudomallei* is required for its escape from phagosomes, permitting replication in the cytosol (Cullinane et al., 2008).

*B. pseudomallei* was found to co-localize with LC3 during infection of mouse RAW 264.7 macrophages and resides in single-membrane compartments, characterized as LAPosomes (Cullinane et al., 2008; Gong et al., 2011; Li et al., 2013). Starvation but not rapamycin treatment enhanced the residence *B. pseudomallei* in these LAPsomes, a process requiring Beclin 1 activity (Li et al., 2013). Treatment of RAW264.7 macrophages with lipopolysaccharide (LPS) from *B. pseudomallei* increased GFP-LC3 puncta formation, while removal of LPS decreased this response. Considering that the effect of *B. pseudomallei* LPS is mediated by TLR4 and unexpectedly also TLR2, it was proposed that LPS induces LAP in a TLR-dependent manner during *B. pseudomallei* infection (Wiersinga et al., 2007; Gong et al., 2011). By mediating the escape from phagosomes, the T3SS facilitates evasion of the LAP mechanism (Cullinane et al., 2008; Gong et al., 2011). Mutant bacteria for the bopA and bipD proteins, both crucial for the T3SS, show diminished escape from the phagosome, indicating the importance of a proper functioning T3SS for evasion of LAP (Cullinane et al., 2008; Gong et al., 2011).

The role of T3SS could in theory be exploited to increase the susceptibility of *B. pseudomallei* to LAP. T3SS-associated ATPases are known to be crucial for the proper function of the TTSS3 and therefore represent possible targets for modulating the interaction of the pathogen with LAP. Small-molecule inhibitors for the T3SS ATPase have been identified and are used to study the effect on *B. pseudomallei* infection and LAP. One of the ATPase inhibitors counteracted the escape of bacteria from the phagosome, leading to increased targeting by LAP and reduced bacterial survival. These promising results could be important for the development of therapies aimed against *B. pseudomallei* infections (Gong et al., 2015).

## *Yersinia pseudotuberculosis*

*Yersinia pseudotuberculosis* is another food-born pathogen capable of causing an enteric illness. It can infect both epithelial cells and phagocytes by binding to integrins (Isberg and Leong, 1990; Pujol and Bliska, 2003). After invading a phagocyte, *Y. pseudotuberculosis* can survive inside the cell by manipulating the autophagy machinery and impairing the acidification of the autophagosome (Moreau et al., 2010). However, in epithelial cells *Y. pseudotuberculosis* was found to be captured in LC3-decorated, single-membrane and non-acidic vesicles. Despite that epithelial cells are non-phagocytic and lack the NOX2 complex required for LAP, the response of these cells to *Y. pseudotuberculosis* is reminiscent of LAP and could represent a related mechanism (Ligeon et al., 2014).

The study of *Y. pseudotuberculosis* in epithelial cells focused on the role of host derived, vesicle-associated membrane proteins

(SNARE proteins) in the LAP-like response (Ligeon et al., 2014). At least two of the SNARE family members, VAMP3 and VAMP7, were found to be involved in the recruitment of LC3 to the pathogen-containing vesicles. Overexpression of VAMP3 resulted in an increase of *Y. pseudotuberculosis* bacteria localized in single-membrane vesicles. Conversely a knockdown of VAMP3 resulted in an increase of *Y. pseudotuberculosis* bacteria localized into double-membrane vesicles. These results suggest that a high concentration of VAMP3 increases LAP-like activity and a low concentration of VAMP3 increases the activity of the classical autophagy. In other words, VAMP3 appears to function as a molecular checkpoint for commitment to the single membrane pathway (LAP-like) or to the double membrane pathway (classical autophagy), dependent on its expression level. VAMP7 associates with the single membrane vesicles after the recruitment of VAMP3. Knockdown of VAMP7 led to a decrease in LC3 decoration of the single membrane compartments, suggesting that VAMP7 protein mediates LC3 recruitment during the LAP-like process. It should be noted that the VAMP7 protein also participates in the recruitment of LC3 during the classical form of autophagy, thereby suggesting a double role for VAMP7 of which the mechanism still remains unknown (Ligeon et al., 2014).

Evasion of the LAP-like response by *Y. pseudotuberculosis* is presumably mediated by blocking the acidification of the phagosome, something which is also seen in classical autophagy (Ligeon et al., 2014). Both the LAP-like process and autophagy are manipulated to establish a non-acidic niche, which raises the question how the manipulation of these two mechanisms is mediated and which processes contribute to their development. Multiple studies showed that SNARE proteins like VAMP3 and VAMP7 could be key to determine the maturation of different vesicular pathways (Fader et al., 2009; Itakura et al., 2012; Moreau et al., 2013).

Concluding, single membrane LC3 lipidation mechanism similar to LAP seems to target the *Y. pseudotuberculosis* pathogen in epithelial cells, but evasion of this mechanism by inhibition of LAPosome maturation is observed, leading to the formation of a replication niche (Ligeon et al., 2014). The VAMP3 protein seems to be a molecular switch for commitment to the single membrane or double membrane pathways. Additional evidence indicated a role for VAMP7 in LC3 recruitment during the LAP-like response, similar as in classical autophagy (Ligeon et al., 2014). It remains to be established whether or not this response also plays a prominent role in other cell types, including phagocytes.

## *Shigella flexneri*

*Shigella flexneri* is a pathogen that invades epithelial cells and is targeted by a LAP-like mechanism early during infection, but is capable of effectively evading this host defense reponseby escaping into the cytosol and acquiring actin-based motility similar to *L. monocytogenes* (Baxt and Goldberg, 2014). It has been found that the presence of the T3SS is crucial to induce the uptake of *S. flexneri*, followed by the initiation of the LAP-like process (Campbell-Valois et al., 2015). IcsB and VirA are secreted effector proteins involved in the escape of the pathogen from the LC3-decorated vesicle into the cytosol, and therefore these virulence factors are also crucial for the evasion of the LAP-like pathway. (Baxt and Goldberg, 2014; Campbell-Valois et al., 2015).

Toca-1 is a host-derived protein required for the formation of actin tails that propel *S. flexneri* (Leung et al., 2008). The interaction of Toca-1 with IcsB was found to inhibit LC3 recruitment, presumably by inhibiting the ATG5 protein, which is crucial for the recruitment of LC3 to the phagosome (Baxt and Goldberg, 2014). Recent results also indicated that Toca-1, besides interacting with IcsB, also interacts with several other *S. flexneri* effectors, namely IpaB, OspC3 and IpgD. The function of these interactions and possible role in the evasion of the LAP-like response, autophagy and other aspects of *S. flexneri* pathogenesis remains to be further investigated (Miller et al., 2018).

## Group A *Streptococcus* and *Streptococcus pneumoniae*

Group A *Streptococci* (GAS), mostly belonging to the species *Streptococcus pyogenes*, are commonly found among the bacteria colonizing the throat and skin, but they can also cause a range of mild to severe infections, including the deathly toxic shock syndrome (Henningham et al., 2012). Similarly, *Streptococcus pneumoniae*, which is not classified under GAS, generally colonizes the nasopharynx, but can become a cause of pneumonia, septicemia and meningitis (Bogaert et al., 2004). *Streptococci* adhere to various host cell surface receptors, among which fibronectin, fibrinogen, integrins and laminins (Brouwer et al., 2016). Recent studies have implicated LAP in the innate immune defense against both GAS and *S. pneumoniae* (Lu et al., 2017; Cheng et al., 2019; Inomata et al., 2020; Ogawa et al., 2020; Shizukuishi et al., 2020).

GAS is able to survive and replicate in endothelial cells. While these cells are autophagy competent under starvation, they were unable to sequester GAS in autophagosomes, which could be attributed to defective ubiquitin recruitment (Lu et al., 2017). The endothelial cells did capture GAS inside single membrane, LC3-associated vesicles. However, these GAS-containing vesicles failed to properly acidify after fusion with lysosomes and therefore bacterial clearance was impaired (Lu et al., 2017). NOX2 but not ULK1 was found to colocalize with the LC3-positive GAS-containing vesicles, indicating that they arise by LAP (Cheng et al., 2019). Inhibition of ROS production *via* NOX2, restored the vesicle acidification, redirected LAP to conventional anti-bacterial autophagy, and thereby reduced the intracellular growth of GAS. Furthermore, it was shown that streptolysin O (SLO) induces LAP and associated ROS production *via* β1 integrin. Thus, GAS evades the conventional, bacteriostatic autophagy route and induces a largely ineffective LAP response *via* its virulence factor SLO.

In the case of *S. pneumoniae*, LC3 association was investigated both in non-myeloid cells (fibroblasts) and in macrophages (Inomata et al., 2020; Ogawa et al., 2020; Shizukuishi et al., 2020). In non-myeloid cells it was observed that a LAP-like process and canonical autophagy are deployed

sequentially, with the formation of LAPosome-like vesicles being indispensable for subsequent autophagosomes formation (Ogawa et al., 2020; Shizukuishi et al., 2020). In contrast to the LAP pathway, the *S. pneumoniae*-containing vesicles that resemble LAPosomes acquire LC3 independently of ROS. However, a feature shared with LAP is that their formation does not require FIP200, a component of the autophagy preinitiation complex. It was observed that that interactions between SQSTM1/p62 and ATG16L1 PcLV are required for the formation of the LAPosome-like vesicles and that LC3 and NDP52 (a member of the SQSTM1/p62 family) disappeared from these vesicles prior to the transition of the bacteria to autophagomes (Ogawa et al., 2020). What precisely distinguishes this LAP-like process from LAP, and whether the two processes can be operative simultaneously, requires further investigation.

In murine bone marrow-derived macrophages, a common LAP response to *S. pneumoniae* was observed where formation of LC3-positive, single membrane vesicles required Rubicon, NADPH oxidase, Atg5 and Atg7, but none of the autophagy preinitiation factors, Ulk1, FIP200, and Atg14 (Inomata et al., 2020). While highly efficient in macrophages from young mice, this LAP pathway was defective in macrophages from old mice, making them deficient in bacterial killing. Concomitant with the loss of LAP, macrophages from older mice also produced high levels of inflammatory cytokines. These interesting findings suggest that diminishing of LAP with age contributes to inflammation and infection susceptibility (Inomata et al., 2020).

### *Staphylococcus aureus*

*S. aureus* can cause a wide range of diseases, from local skin infections to fatal bacteremia, often associated with antibiotic resistance (Lowy, 1998). While known for its extensive extracellular growth ability in infected tissues, intracellular growth stages in host phagocytes were recently found to be crucial for *S. aureus* pathogenicity (Prajsnar et al., 2012; McVicker et al., 2014). The internalization of *S.aureus* and its recognition is mediated by several surface proteins and receptors, including fibronectin, TLR2, and chemokine receptors like CCR5 and CXCR4 (Edwards et al., 2010; Bi et al., 2015; Tam et al., 2016).

Studies into the autophagy response to *S. aureus* led to different outcomes, pointing either to a host-beneficial effect or suggesting that the pathogen takes advantage of the host autophagy machinery (reviewed in Munoz-Sanchez et al., 2020). Similarly, the host LAP pathway has been found to be exploited to the pathogen's benefit (Prajsnar et al., 2021). In a zebrafish systemic infection model, *S. aureus* was found to establish an intracellular niche in neutrophils. When internalized by these phagocytes, *S. aureus* was rapidly decorated by GFP-LC3, forming spacious GFP-LC3-positive vacuoles that did not acidify. Chemical and genetic disruption of NADPH oxidase prevented GFP-LC3 recruitment, indicating that the replication niche is formed by LAP, although the role of Rubicon was not addressed. Autophagy played an antagonistic role in this infection model, as GFP-Sqstm1 (p62) also decorated a subset of bacteria and Sqstm1 knockdown impaired host survival. Thus, despite a protective effect of selective autophagy, the prevailing LAP response in zebrafish neutrophils contributes to *S. aureus* pathogenesis and inhibition of this response improves host resistance (Prajsnar et al., 2021). The *S. aureus* virulence factors involved in generating the spacious LAPosomes and preventing acidification are yet to be uncovered.

## CONCLUSIONS AND PERSPECTIVES

When the LAP process was first described, its importance for microbial control was already underlined (Sanjuan et al., 2007). In the years that followed, it became clear that LAP constitutes a critical cornerstone for host defense against a variety of bacterial invaders. We now know that *M. tuberculosis*, *L. monocytogenes*, *L. dumoffi*, *S. Typhimurium*, *B. pseudomallei*, among other pathogens discussed in this review, are captured in a LC3-II-positive single-membrane phagosome and require Rubicon and NOX2-driven ROS production for their clearance. As the list of bacteria targeted by LAP continues to grow, efforts have been dedicated to determine how LAP affects the pathology of infectious disease. LAP has been best characterized in macrophages, yet LC3 lipidation of phagosomes has recently also been demonstrated in neutrophils, albeit as a mechanism of bacterial pathogenesis (Prajsnar et al., 2021). Most of the knowledge on LAP is based on genetic analyses of NOX2 and Rubicon, which also have LAP-independent roles in host defense and autophagy that complicate the interpretation of data. Furthermore, much remains to be discovered about the mechanisms downstream of NOX2 and Rubicon and about mechanisms independent of these two factors, especially because multiple pathways to single membrane LC3 lipidation seem to exist (Mitchell et al., 2018; Rai et al., 2019; Fischer et al., 2020, Gluschko et al., 2021 Wong et al., 2021).

Future research should lead to better understanding of the discrete mechanisms and functions of LAP and LAP-like processes, such as PINCA, which is triggered by phagosome permeabilization rather than by NOX2 activity (Mitchell et al., 2018; Gluschko et al., 2021). Another important area for future research is how LAP might work in concert with the closely related and recently discovered process, LC3-associated endocytosis (LANDO) (Heckmann and Green, 2019). LANDO has been shown to regulate the turnover of Aβ receptors in a murine model of Alzheimer's disease. It will be of great interest to explore if LANDO and LAP also control levels of pattern recognition receptors and thereby contribute to the regulation of the innate immune response and pathogen clearance.

Although the different evasive strategies that bacteria use to circumvent or take advantage of LAP are progressively being unraveled, many questions about the molecular mechanisms that undergird these strategies remain unexplored. Strikingly, most bacterial pathogens targeted by LAP have evolved ways to specifically interfere with NOX2, signifying the central importance of NOX-derived ROS in LAP maturation. It is still difficult to say if this importance arises from the microbicidal or rather from the signaling functions of ROS (Holmström and Finkel, 2014), although this seems to differ between bacterial species (Herb and Schramm, 2021). Redox regulation of ATG

proteins is indeed a prerequisite for the production of LC3-II during autophagy (Scherz-Shouval et al., 2007). Recently, it was discovered that NOX2 has a role in stabilizing the LAPosome itself by safeguarding LC3-II *via* redox regulation of ATG4B (Ligeon et al., 2021). Future studies should seek to answer how ROS contribute to pathogen clearance and engage parts of the LAP machinery, like the LC3 conjugation systems.

The ways in which LAP enhances phagosome-lysosome fusion are incompletely understood. Different bacterial effectors such as *Mycobacterium* CpsA and *Legionella* RavZ have been associated with impaired lysosomal trafficking during LAP (Choy et al., 2012; Köster et al., 2017). Such effectors may be critical for LAP evasion. However, as phagosome–lysosome fusion is a highly dynamic process that depends on membrane lipid composition and the coordinated action of Rab GTPases, tethering factors and SNAREs (Nguyen and Yates, 2021), details of the evasion strategies counteracting lysosomal fusion have yet to be substantiated.

At present, the machinery required for LAP can be specifically manipulated by various pharmacological or genetic means, such as the recently developed Rubicon inhibitor TIPTP (Kim et al., 2020), as well as Rubicon- and ATG16L1-deficient mouse lines (Martinez et al., 2016; Rai et al., 2019). Together, these techniques will be of great use to elucidate how bacterial species are targeted and killed by LAP, leaving aside the confounding effects of classical autophagy. Better knowledge about the antibacterial effects of LAP, and the comparison with antifungal and antiparasitic LAP mechanisms, could provide vital clues for developing novel intervention strategies in the ongoing battle against infectious diseases.

In most circumstances, cells that are proficient in LAP are generally well equipped to combat bacterial infection. However, some pathogens, with *S. aureus* as a notable example, are able to exploit LAP to generate a replication niche. In time, our understanding of LAP and its links with infectious disease will continue to increase in scope and diversity. It is, in the words of Shakespeare, a pathway *lapp'd in proof* – that is, clad in strong (proven) armor – when it comes to virulent bacteria that continue to undermine our vulnerable immune systems.

## AUTHOR CONTRIBUTIONS

BG and SK performed the literature research, wrote the manuscript, and designed the figures. MV made textual revisions. AM supervised the literature research and made textual revisions. All authors checked and approved the final version.

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# Shedding Light on Autophagy During Human Tuberculosis. A Long Way to Go

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Immunity against *Mycobacterium tuberculosis* (*Mtb*) is highly complex, and the outcome of the infection depends on the role of several immune mediators with particular temporal dynamics on the host microenvironment. Autophagy is a central homeostatic mechanism that plays a role on immunity against intracellular pathogens, including *Mtb*. Enhanced autophagy in macrophages mediates elimination of intracellular *Mtb* through lytic and antimicrobial properties only found in autolysosomes. Additionally, it has been demonstrated that standard anti-tuberculosis chemotherapy depends on host autophagy to coordinate successful antimicrobial responses to mycobacteria. Notably, autophagy constitutes an anti-inflammatory mechanism that protects against endomembrane damage triggered by several endogenous components or infectious agents and precludes excessive inflammation. It has also been reported that autophagy can be modulated by cytokines and other immunological signals. Most of the studies on autophagy as a defense mechanism against *Mycobacterium* have been performed using murine models or human cell lines. However, very limited information exists about the autophagic response in cells from tuberculosis patients. Herein, we review studies that face the autophagy process in tuberculosis patients as a component of the immune response of the human host against an intracellular microorganism such as *Mtb*. Interestingly, these findings might contribute to recognize new targets for the development of novel therapeutic tools to combat *Mtb*. Actually, either as a potential successful vaccine or a complementary immunotherapy, efforts are needed to further elucidate the role of autophagy during the immune response of the human host, which will allow to achieve protective and therapeutic benefits in human tuberculosis.

**Keywords: autophagy, tuberculosis, human, host-directed therapy, immunology & infectious diseases**

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## INTRODUCTION

*Mycobacterium tuberculosis* (*Mtb*) has killed nearly 1000 million people since the XIX century. And although an affordable and effective treatment is available to fight this pathogen, tuberculosis (TB), together with COVID19 in 2020-2021, is the leading cause of death from a single infectious agent. Therefore, improvement of treatment is included among the central aims of developing new strategies against this disease. Accordingly, it has been proposed that supplementing anti-TB therapy with host response modulators will augment standard TB treatment (Madhur et al., 2016). However, the immune response against *Mtb* is highly complex. The outcome of TB infection depends, at least in part, on several immune mediators that display critical temporal roles on the host microenvironment (Sodhi et al., 1997; Ottenhoff et al., 1998; Chen et al., 2008; Pasquinelli et al., 2009; Cooper, 2010; Jurado et al., 2012; Mayer-Barber et al., 2014; Pellegrini et al., 2021). It has been suggested that host-directed therapies (HDT) could be untapped strategies as complementary therapies against TB, augmenting the host defences and/or limiting tissue damage associated with infection (Martínez-Colón and Moore, 2018; Wan et al., 2018; Xiong et al., 2018). In this context, autophagy arises as an attractive therapeutic target, but currently available data on autophagy in TB patients and the potential clinical use of this cellular process remain insufficient. Here, we review the current knowledge of autophagy as a potential complement of anti-TB chemotherapy.

## AUTOPHAGY

Autophagy is an evolutionarily-conserved cellular process that mediates the lysosomal degradation of cytoplasmic components and damaged organelles, allowing eukaryotic cells to generate nutrients under starvation conditions and maintain cellular homeostasis. Three types of autophagy have been described: chaperone-mediated autophagy, microautophagy, and macroautophagy, herein referred to as autophagy (Jacomin et al., 2018). Importantly, autophagy participates in innate and adaptive immunity against intracellular pathogens, including *Mtb* (Gutierrez et al., 2004). Actually, increased autophagy in macrophages eliminates intracellular *Mtb via* lytic and antimicrobial mechanisms of the autolysosomes (Ponpuak et al., 2010). Notably, autophagy constitutes an anti-inflammatory mechanism that protects against endomembrane damage triggered by several endogenous components or infectious agents and precludes excessive inflammation (Castillo et al., 2012; Deretic and Levine, 2018). The autophagy process can be modulated by different immunological mediators (Djavaheri-Mergny et al., 2006). In particular, critical cytokines regulate both positively and negatively the autophagic response affecting survival of mycobacteria (Harris et al., 2007). Besides, the importance of the host autophagy process to manage an effective antimicrobial effect on mycobacteria during chemotherapy has been reported (Kim et al., 2012). A better understanding of the connections between autophagy and the immune response may have wide applications given that the pathology accompanying several diseases involves some form of inflammation (Deretic and Levine, 2018).

## AUTOPHAGY IN TB PATIENTS

Most of the studies that investigated autophagy as a defense mechanism against *Mtb* have been accomplished in murine cell lines, mouse models, primary culture cells, or human cell lines infected with the pathogen. However, very limited information regarding the study of the autophagic response in TB patients is available. During the past decade, we have studied *Mtb*-induced autophagy in two populations of patients with active disease, classified according to their T cell responses to the bacterium. Briefly, high responder (HR) TB patients displayed significant T cell proliferation and IFN-γ production against *Mtb*-antigen (Ag), while low responder (LR) TB patients displayed weak or no T cell responses to the antigen (Pasquinelli et al., 2004). Interestingly, we detected the highest autophagy levels in healthy donor (HD)′s monocytes whereas the lowest quantities were observed in monocytes from LR patients (Rovetta et al., 2014). Accordingly, it has been reported that Beclin-1, a signaling hub of autophagy, is increased in alveolar macrophages from TB patients and that those individuals with higher Beclin-1 levels achieve faster bacillary sterilization (Yu et al., 2016). Recently, we observed that autophagy levels decreased significantly in neutrophils from TB patients as compared to HD (Pellegrini et al., 2020). Moreover, a direct correlation between neutrophil numbers and TB severity was detected (Pellegrini et al., 2020). Given that during *Mtb* infection autophagy protects against massive inflammation (Deretic and Levine, 2018), the reduced levels of autophagy observed in neutrophils from TB patients could be related to the frequent harmful inflammatory responses that take place during active disease.

### Effect of the Diversity of *Mtb* Strains on the Autophagy Process

The immune response to *Mtb* is influenced by factors both from the host and the bacteria (Sousa et al., 2020). Accordingly, some studies have demonstrated a differential ability of different *Mtb* strains to modulate autophagy. In particular, Li et al. described that clinical isolates from *Mtb* differ in their ability to induce autophagosome formation (Li et al., 2016). The authors investigated the effect of more than 180 *Mtb* clinical isolates on the autophagy process in THP-1 macrophages. Interestingly, they observed that the capacity of inducing autophagy varied significantly among different isolates. Notably, patients infected with *Mtb* strains that displayed reduced autophagy-inducing ability showed more severe disease and displayed adverse treatment outcomes, suggesting that an autophagy deficiency induced by *Mtb* isolates augmented the risk of poor clinical outcomes in TB patients (Li et al., 2016).

The majority of the studies on the host immune response to *Mtb* infection have been performed using the laboratory strain H37Rv (see **Tables 1–3**). Moreover, most of the research

**TABLE 1** | Immunological mediators modulate the autophagy process during active tuberculosis.

| Immunological mediators | Effect on autophagy | Validation in human samples | *Mtb strain* | Host origin | Reference |
|---|---|---|---|---|---|
| TNF | Stimulation | Osteoarticular pathological tissues from TB patients. Validated with osteoclasts from HD | *Mtb* H37Rv and H37RvΔeis | Chinese men cohort from Wuhan | Liu G. et al. (2020) |
| IFN-γ | Stimulation | Monocyte-derived macrophages from HD | *M. bovis* BCG | Not detailed | Gutierrez et al. (2004) |
| | | Monocytes from TB patients and HD | *Mtb* H37Rv whole cell lysate | Argentine population cohort from Buenos Aires | Rovetta et al. (2014) |
| | | Monocytes from TB patients and HD | *Mtb* H37Rv, *Mtb* H37RvΔRD1 and *Mtb* H37Rv whole cell lysate | Argentine population cohort from Buenos Aires | Tateosian et al. (2017) |
| IL-4, IL-10, IL-13 | Inhibition | Human cell lines U937 and THP-1; Monocyte-derived macrophages | *M. bovis BCG* | Not detailed | Harris et al. (2007) |
| IL-17A | Stimulation | Monocytes from TB patients and HD | *Mtb* H37Rv, *Mtb* H37RvΔRD1 and *Mtb* H37Rv whole cell lysate | Argentine population cohort from Buenos Aires | Tateosian et al. (2017) |
| IL-26 | Stimulation | Monocyte-derived macrophages from HD | *Mtb* H37Ra and *M. leprae* | Not detailed | Dang et al. (2019) |
| SLAMF1 | Stimulation | Neutrophils from TB patients and HD | *Mtb* H37Rv whole cell lysate | Argentine population cohort from Buenos Aires (Caucasian, American Indian, Asian) | Pellegrini et al. (2020) |
| PGE2 | Stimulation | Monocytes and neutrophils from TB patients and HD | *Mtb* H37Rv whole cell lysate | Argentine population cohort from Buenos Aires (Caucasian, American Indian, Asian) | Pellegrini et al. (2021) |

*A summary of published studies on the modulation of autophagy by different immunological mediators such as cytokines, glycoproteins and lipid compounds with validation in human samples is shown. Mtb strains employed and host origin are detailed.*

performed with samples from TB patients does not consider the original *Mtb* strain that infected the host. Thus, investigation of the effect of *Mtb* genetic variability on the modulation of the autophagy process is of great interest.

Genome sequence analysis has identified seven phylogeographic *Mtb* lineages: four referred to as evolutionarily "ancient" and three as "modern". Interestingly, the "modern" strains were shown to display high virulence (Romagnoli et al., 2018). Therefore, Romagnoli et al. investigated the impact of the genetic diversity of *Mtb* strains on the host innate immune response by evaluating the autophagy response. Remarkably, the authors demonstrated that modern *Mtb* strains are able to avoid the autophagy machinery affecting the regulation of specific T-cell responses.

Together, the studies described above might suggest a possible limitation of using autophagy as a novel therapy against *Mtb*. However, on the other side, it was proposed that analyses of autophagosome formation by diverse clinical isolates of *Mtb* might contribute to the evaluation of TB outcomes (Li et al., 2016). Furthermore, the study of the genetic variability of *Mtb* on autophagy modulation was proposed to have translational implications for the design of HDT, which should consider both the autophagic and immunogenic properties of the lineage of the *Mtb* candidate. Accordingly, by studying 681 TB patients Sousa et al. showed that *Mtb* isolates from cases with mild disease stimulate strong cytokine responses in contrast to bacteria from patients with severe TB, indicating that *Mtb* strains manipulate host-pathogen interactions to drive variable TB severities. Then, they suggest to include *Mtb* genetic diversity in the development of HDT (Sousa et al., 2020). Finally, external autophagy modulators might act as adjuvants in *Mtb* treatment helping to overcome autophagy regulation/inhibition by pathogenic strains.

## Immunological Mediators

Autophagy is a process recognized to be regulated by cytokines and other immunological signals (Djavaheri-Mergny et al., 2006; Harris et al., 2007; Goletti et al., 2013; Chen H. et al., 2015; Pelissier-Rota et al., 2015; Martínez-Colón and Moore, 2018; Wan et al., 2018). TNF was originally shown to induce autophagy in Ewing sarcoma cells (Djavaheri-Mergny et al., 2006). Recently, TNF was demonstrated to promote the autophagy of *Mtb*-infected osteoclasts and constrain the apoptosis of mature osteoclasts (Liu W. et al., 2020). Furthermore, Liu et al. suggest that their data describe a novel osteoarticular TB-activated cytokine network where autophagy could have an important role in the pathogenesis of osteoarticular TB, pointing out the use of drugs such as TNF for treating this type of TB (Liu W. et al., 2020). Moreover, IFN-γ augments the autophagy process in macrophages and other cells (Gutierrez et al., 2004; Goletti et al., 2013), whereas IL-4, IL-10 and IL-13 inhibited autophagy in murine macrophages and human cell lines (Harris et al., 2007; Park et al., 2011). Accordingly, it has been demonstrated that autophagy participates in the immune response of TB patients against *Mtb*, in direct association with the specific IFN-γ levels secreted against the pathogen (Rovetta et al., 2014). By blocking *Mtb*-Ag-induced IFN-γ, a marked reduction of autophagy was measured in monocytes from HR patients. In contrast, the incorporation of small quantities of IFN-γ

**TABLE 2 |** Non-coding RNAs influence autophagy outcome during human tuberculosis.

| ncRNA | Target | Effect on autophagy | Validation in human samples | *Mtb* strain | Host origin | Reference |
|---|---|---|---|---|---|---|
| miR-30A | Beclin-1 | Inhibition | Expression in alveolar macrophages, association with clinical data and treatment | *Mtb* H37Rv (*in vitro* functional experiments) | Chinese cohort from Beijing | Chen Z. et al. (2015) |
| miR144* | DRAM2 | Inhibition | Expression in PBMCs and lung and lymph nodes biopsies from TB patients, functional experiments in human MDMs | *Mtb* H37Rv (*in vitro* functional experiments) miR144* expression confirmation upon infection with *Mtb* H37Ra, *M. bovis* BCG and *M. abscessus* | Samples from Korea Biobank Network | Kim et al. (2017) |
| miR-125b-5p | DRAM2 | not described | Expression in primary monocytes from TB patients | *Mtb* H37Rv | Chinese cohort from Xinjiang | Liu G. et al. (2020) |
| CircAGFG1 | miRNA1257 - Notch | Stimulation | Expression and correlation with autophagy/ apoptosis in alveolar macrophages | Not detailed | Chinese cohort from Chilin | Shi et al. (2020) |
| miRNA-27a | Cacna2d3 | Inhibition | miRNA expression profiles from PBMCs of patients with active pulmonary TB | *Mtb* H37Rv | Chinese cohort from Shanghai | Liu et al. (2018)* |
| lncRNA-EPS | – | Inhibition | Negative correlation with LC3 levels in monocytes from TB patients | – | Chinese cohort from Wuhan | Ke et al. (2020)** |
| PCED1B-AS1 | miR-155 | Stimulation | Expression in peripheral monocytes from TB patients, functional experiments in human MDMs | *Mtb* H37Rv (*in vitro* functional experiments) | Chinese cohort from Xinxiang | Li et al. (2019) |
| miR-155 | ATG3 | Inhibition | Expression and functional experiments in *Mtb*-infected human dendritic cells | *Mtb* H37Rv | Samples from Blood Bank of University "La Sapienza", Italy | Etna et al. (2018) |
| miRNA-889 | TWEAK | Inhibition | miRNA next-generation sequencing (NGS) analysis in PBMC of RA patients with latent TB infection, functional experiments in human PBMCs | *Mtb* H37Rv and *M. bovis* BCG (*in vitro* functional experiments) | Taiwanes cohort from Taichung | Chen et al. (2020) |

*A summary of published studies on the modulation of autophagy by non-coding RNAs with validation in human samples is shown. Mtb strains employed and host origin are detailed. *DOI: 10.1038/s41467-018-06836-4; **DOI: 10.1016/j.meegid.2019.104077.*

significantly augmented autophagy in LR patients (Rovetta et al., 2014). We also demonstrated that IL-17A increased autophagy in infected monocytes from HR patients (Tateosian et al., 2017). However, in severe LR TB patients' monocytes, a defect in the ERK1/2 signaling pathway prevented an augment in autophagy caused by IL-17A. Both IFN-γ and IL-17A increased the levels of autophagy in HR patients, promoting mycobacterial killing (Tateosian et al., 2017). Besides, Dang et al. demonstrated that addition of IL-26 to human *M. Leprae* infected monocytes induced autophagy (Dang et al., 2019). Furthermore, LC3-positive autophagosomes were mainly detected in lesions from T-lep (tuberculoid) as compared with L-lep (lepromatous) patients, indicating that *M. Leprae* dampened autophagy in human cells as an immune escape mechanism (Silva et al., 2017). It has been reported that type I glycoproteins such as SLAMF1 recruit molecules like Beclin-1 to the phagosome, participating in the connection to the cellular machinery that controls bacterial killing (Berger et al., 2010; Ma et al., 2012). Accordingly, we recently demonstrated that human neutrophils express SLAMF1 upon *Mtb*-stimulation, a protein that colocalized with $LC3B^+$ vesicles (Pellegrini et al., 2020). Furthermore, SLAMF1 activation augmented neutrophil autophagy induced by *Mtb*, and neutrophils from TB patients showed reduced levels of SLAMF1 and lower amounts of autophagy against *Mtb* as compared to HD (Pellegrini et al., 2020). The eicosanoids, a family of potent biologically active lipid mediators, modulate immune responses in *Mtb* infection and have been suggested as potential HDT targets. Actually, manipulation of PGE2 and/or 5-LO was suggested to potentially counteract the type I IFN response in patients with severe TB as a HDT against *Mtb* (Mayer-Barber et al., 2014). Recently, we reported that PGE2 promotes autophagy in monocytes and neutrophils cultured with *Mtb*. We demonstrated that PGE2 augmented the percentage of $LC3^+$ neutrophils and monocytes upon *Mtb*-Ag stimulation. Furthermore, the exogenous addition of this eicosanoid triggered a functional autophagy flux both in monocytes and lymphocytes from TB patients (Pellegrini et al., 2021). Thus, according to our results, PGE2 might be a new target for the development of novel therapeutic tools to fight *Mtb*. **Table 1** summarizes the data mentioned in this section.

## Non-Coding RNAs in Autophagy Modulation During Human Tuberculosis

In recent years, there was a growing body of evidence suggesting a critical role of non-coding RNAs (ncRNAs) in regulating host-pathogen interactions and immunity. A variety of pathogens, including *Mtb*, have been described to modulate the expression of these modulators by evading host responses and influencing the outcome of the infection (Staedel and Darfeuille, 2013; Zhang et al., 2019). Actually, some authors have proposed that miRNAs/lncRNAs regulation is an important strategy employed by *Mtb* to survive inside host cells (Kundu and Basu, 2021). Mycobacteria can alter the host miRNA expression profile for their benefit, affecting antimicrobial responses, cytokine production, metabolism and inflammation, among other

**TABLE 3 |** SNPs in autophagy-related genes associated with TB. List of SNPs in genes codifying for proteins involved in autophagy that have been found to be associated with increasing or decreasing susceptibility to active TB.

| Gene | SNP | Alleles | Consequence | Association with TB | *Mtb* strain involved | Host origin | Reference |
|---|---|---|---|---|---|---|---|
| **IRGM1** | rs9637876 | C>T | Non Coding Transcript Variant | Decreased susceptibility | *Mtb* Euro-American lineage (and not TB caused by by *M. africanum* or *M. bovis*) | Patients cohort from Ghana (Ahsanti, Eastern and Central regions) | Intemann et al. (2009) |
| **IRGM1** | rs10065172 | C>A,T | Missense Variant | Increased susceptibility among African Americans | Not determined | Caucasian and African American patients cohort from Boston, EEUU, | King et al. (2011) |
| **IRGM1** | rs10065172<br>rs10051924<br>rs13361189 | C>A,T<br>T>A,C<br>T>C | Missense Variant<br>Non Coding Transcript Variant | Increased susceptibility | Chinese patients cohort from Hubei Han region | Not determined | Lu et al. (2016) |
| **IRGM1** | rs4958846 | T>C | 2KB Upstream Variant | Decreased susceptibility | Not determined | Chinese patients cohort from Hubei Han region | Yuan et al. (2016) |
| **ULK1** | rs12297124<br>rs7300908 | G>T<br>C>T | Intron Variant<br>Intron Variant | Associated with latent TB | Not determined | Patients cohort from Seattle, EEUU, self-identified as black or Asian | Horne et al. (2016) |
| **LAMP1** | rs9577229 | C>T | Missense Variant | Increased susceptibility | *Mtb* Beijing genotype | Indonesian patients cohort from Jakarta and Bandung regions | Songane et al. (2012) |
| **MTOR** | rs6701524 | A>G | Intron Variant | Increased susceptibility | *Mtb* Beijing genotype | Indonesian patients cohort from Jakarta and Bandung regions | Songane et al. (2012) |
| **P2X7** | SNP000063002 (–762) | C>T | 762b Upstream Variant | Decreased susceptibility | Not determined | Patients cohort from the western region of Gambia | Li et al. (2002) |
| **P2X7** | 1513A-C | A>C | Missense Variant | Increased susceptibility | Not determined | Cohorts of refugee and australian patients with northern european and vietnamese ancestry | Fernando et al. (2007) |
| **VAMP8** | rs1010 | T>C / T>G | 3 Prime UTR Variant | Increased susceptibility | Not determined | Chinese patients cohort from Hubei Han region | Cheng et al. (2019) |

*Mtb strain corresponds to the specific strains or clinical isolates that originally infected the host.*

processes (Yang and Ge, 2018). Moreover, the differential miRNA and lncRNA profiles detected in clinical samples from TB patients have led to an increasing interest in their use as TB biomarkers (Sabir et al., 2018). Importantly, some of these TB-associated ncRNAs play a role in the regulation of autophagy during *Mtb* infection, although most of these studies have been performed in murine models or cell lines.

Few reports have explored the role of these intermediaries in autophagy by using primary human cells from TB patients (**Table 2**). Accordingly, by analyzing GSE 29190 and GSE34608 miRNA microarray datasets Kim et al. detected that only 10 miRNA were differentially expressed in peripheral blood mononuclear cells (PBMCs) and biopsies from lungs and lymph nodes from TB patients, for example, miR-144* (Kim et al., 2017). Importantly, the authors demonstrated that miR-144* targets DRAM2 (an interactor of Beclin 1 and UVRAG) in human monocytes/macrophages, thus affecting autophagosome formation (Kim et al., 2017). Consequently, DRAM2 levels were decreased in monocytes from TB patients as compared to HD (Liu G. et al., 2020).

Furthermore, some studies have used primary cells obtained directly from the site of infection. Accordingly, Chen et al. demonstrated that miR-30A suppresses the elimination of intracellular *Mtb* by inhibiting autophagy. In fact, a higher concentration of miR-30A in alveolar macrophages from bronchoalveolar lavage (BAL) of smear-positive patients were detected in comparison with smear-negative patients and HD. Moreover, the expression of this miRNA decreased upon anti-TB treatment (Chen Z. et al., 2015). Besides, circAGFG1 was found to upregulate autophagy in *Mtb*–infected alveolar macrophages by targeting miRNA-1257, which in turn suppresses Notch signaling pathway (Shi et al., 2020).

Notably, Li et al. observed that PCED1B-AS1, a 410-bp lncRNA, is down-regulated in TB patients, which is accompanied by increased autophagy (Li et al., 2019). This function is carried out through binding with miR-155 to control its expression. This observation is concordant with a previous work demonstrating that *Mtb* can manipulate cellular miR-155 expression to regulate Atg3 levels, decreasing autophagosome formation in human dendritic cells (Etna et al., 2018). Finally, one study has explored the role of miR-889 and autophagy to maintain latent TB status (Chen et al., 2020). Chen et al. observed an increased miR-899 expression in latent TB individuals as compared to HD, which was significantly restored after anti-TB therapy. The authors identified the cytokine TWEAK as the target of miR-899, which inhibits autophagy and maintains mycobacterial survival in a human TB granuloma model (Chen et al., 2020). In summary, the increasing evidence found in murine models and cell lines demonstrates that some miRNAs/lncRNAs directly

participate in the host response to *Mtb* by modulating autophagy. These studies were partially confirmed in human studies as we summarized here. Then, ncRNA-based therapeutics appear as an attractive target to directly modulate autophagy as novel HDT. However, an efficient drug design including ncRNA should be protected from degradation and successful delivery to the site of infection has to be ensured (Singh et al., 2021).

## Genetic Association Studies in Autophagy-Related Genes

Host and environmental factors have been shown to play a role in the pathogenesis and development of TB. For thousands of years, *Mtb* has co-evolved with humans, suggesting a powerful evolutionary pressure between the host and pathogen genomes, and therefore a strong impact of genetic factors on the development of different TB stages (Campbell and Tishkoff, 2008; Comas et al., 2013; Galagan, 2014). One particularly powerful approach to assess the role of some processes in humans is to investigate whether genetic variation influences susceptibility to infection. Single Nucleotide Polymorphisms (SNPs) are believed to be the true source of variability among humans (Pacheco and Moraes, 2009; Singh et al., 2017) and it is expected that the variants in genes involved in the pathogen-host interaction are influencing resistance/susceptibility to the disease.

Some reports have investigated SNPs in genes involved in the autophagy process, leading to important evidence about its role during human TB. For instance, IRGM1, a GTPase effector protein that plays an essential role in autophagy induction, has been studied in several populations. Rs9637876 IRGM1 SNP was associated with decreased susceptibility to TB caused only by *Mtb* Euro-American lineage in Ghana (Intemann et al., 2009). Moreover, the rs10065172 SNP within its coding region was associated with susceptibility to TB among African-Americans and in a Chinese population (King et al., 2011; Lu et al., 2016). Interestingly, this polymorphism was previously associated with mortality of patients with severe sepsis (Kimura et al., 2014). Furthermore, Yuan et al. identified three polymorphisms in the IRGM1 promoter region and found that CT genotype of rs4958846 decreased the risk of pulmonary TB in comparison with TT genotype (Yuan et al., 2016). In addition, Horne et al. selected ULK1 and GABARAP as candidate genes since they play fundamental roles in autophagy initiation and autophagosome maturation, respectively. Thus, they identified 2 SNPs in ULK1 (rs12297124 and rs7300908) in Asian participants that were significantly associated with latent TB. Moreover, ULK1-deficient cells had increased *Mtb* replication, decreased TNF response to stimulation, and impaired autophagy. Intriguingly, a previous work had investigated 22 polymorphisms of 14 autophagy genes in an Indonesian population. The authors found associations between SNPs in LAMP1 and MTOR genes and infection with *Mtb* Beijing genotype, but all those associations lost statistical significance after correction for multiple testing (Songane et al., 2012). Similarly, no associations were found in ATG5 (rs2245214, c.574-12777G>C) and NOD2 (rs2066844, c.2104C>T) genes in Romania (Cucu et al., 2017). Besides, 2 SNPs in the P2X7 gene coding for a plasma receptor that mediates ATP-induced autophagy, both in the promoter (Li et al., 2002) and the coding region (Fernando et al., 2007) were found to be associated with protection against TB. Finally, Cheng et al. found that rs1010 SNP in the VAMP8 gene is significantly associated with pulmonary TB in a Chinese Han population (Cheng et al., 2019). More comprehensive studies are required to evaluate the contribution of autophagy in different contexts because these studies are influenced by ethnicity, infection strains, polygenicity, among others. **Table 3** resumes the results cited in this section.

## Autophagy Activating Compounds for Human Host Directed Therapy in Tuberculosis

Autophagy modulation may signify a promising HDT strategy to fight human TB (Yuk et al., 2009). However, the clinical knowledge about HDT implementation is still widely deficient. HDT compounds combined with current TB drugs could shorten and improve treatments against *Mtb* infection. Therefore, autophagy activation by newborn drugs, soluble mediators or agents administered alone or in combination with anti-TB antibiotics still requires long-term clinical trials. Nevertheless, preclinical studies revealed that repurposing licensed drugs with a demonstrated potential to induce autophagy showed an effective therapeutic manipulation of host immunity against *Mtb* infection. These drugs displaying safe and pharmacokinetic profiles are promising for the evaluation of their effectiveness in randomized and controlled clinical trials. Accordingly, several clinical trials (clinicaltrilas.gov) have been conducted implementing dietary supplementation of the immunomodulator Vitamin D3. Innate immunity mediated by Vitamin D3 conferred protection against infection with *Mtb* (Yuk et al., 2009). Interestingly, Vitamin D3 and autophagy are physiologically linked *via* human cathelicidin (hCAP-18/LL-37), which activates transcription of autophagy-related genes such as Beclin-1 and Atg5 (Yuk et al., 2009). In the last ten years, numerous trials were performed supplementing Vitamin D3 as an adjunctive therapy. Nevertheless, differences in these trial outcomes have hampered the interpretation about Vitamin D3 efficacy as HDT for TB. Moreover, the impact of Vitamin D3 as an adjunctive therapy displayed no effect on culture conversion and sputum smear negativization (Ralph et al., 2013; Daley et al., 2015; Tukvadze et al., 2015; Ganmaa et al., 2017; Wu et al., 2018). However, genetic variation in the Vitamin D receptor gene was suggested to modify the effects of adjunctive Vitamin D3 in TB patients (Jolliffe et al., 2016). Additionally, multiple randomized trials suggested that adjunctive Vitamin D treatment has limited effect in improving clinical and immunologic outcomes during active *Mtb* infection despite evidence that specific VDR polymorphisms are predictive of sputum conversion time (Xia et al., 2014; Grobler et al., 2016; Zittermann et al., 2016). A phase 2 clinical study in TB patients (NCT02968927) assess the anti-inflammatory effects of Vitamin D3 in combination with 3 other adjunctive HDT compounds: CC-11050, Everolimus and Auranofin (Wallis et al., 2021). The CC-11050 is a type 4 phosphodiesterase inhibitor that

displays anti-inflammatory properties (Subbian et al., 2016a; Subbian et al., 2016b); Everolimus, a serine/threonine-protein kinase mTOR inhibitor, is an autophagy inducer (Cerni et al., 2019) and Auranofin is an anti-inflammatory gold salt with antimicrobial activity against *Mtb* (Harbut et al., 2015). The preliminary results confirmed that CC-11050 and Everolimus are safe and well tolerated indicating a potential benefit to current TB treatment (Wallis et al., 2021). In other studies, Metformin, the AMPK-activating antidiabetic drug, was shown to inhibit the intracellular replication of *Mtb*, restrict disease immunopathology and enhance conventional anti-TB drug efficacy (Singhal et al., 2014). Moreover, in a pre-clinical study metformin administration in combination with either isoniazid (INH) or ethionamide (ETH) was reported to decrease *Mtb* load in lungs of infected mice (Singhal et al., 2014). Besides, the combined therapy including metformin with standard TB antibiotics was associated with beneficial consequences on clinical outcomes in TB (Singhal et al., 2014). Furthermore, an ongoing randomized clinical trial (NCT-CTRI/2018/01/011176) is evaluating the safety and efficacy of metformin as an adjunct used with rifampicin (RIF), INH, ETO and pyrazinamide (PZA) in patients with pulmonary TB (Padmapriyadarsini et al., 2019). Moreover, a new clinical trial in TB/HIV co-infected patients (Phase II A randomized, open-label trial, NCT04930744) is analyzing the effect of metformin with standard anti-TB drugs plus anti-retroviral therapy (Sullivan and Ben Amor, 2012; Marupuru et al., 2017).

Besides, statins (cholesterol-lowering drugs that inhibit β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase) reduce the risk of coronary disorders and hypercholesterolemia. However, statins can also influence immunologic responses (Parihar et al., 2014). In pre-clinical models, statins such as Simvastatin, Rosuvastatin and Atorvastatin decreased *Mtb* load by enhancing autophagy, phagosomal maturation, and reducing pulmonary pathology, which suggests a potential role for statins as HDT in TB (Lobato et al., 2014; Parihar et al., 2014). Consequently, statins are among the most promising HDT agents for TB. The purpose of the numerous clinical trials that are currently undergoing is to assess the security, tolerance and pharmacokinetics of Pravastatin (NCT03882177) or Atorvastatin (NCT04147286) as adjunctive therapy when combined with standard TB drugs in adults infected with *Mtb*. There is still a long way to go by investigating many other repurposing licensed drugs with the ability to induce autophagy. For example, the mucokinetic Ambroxol (Choi et al., 2018), the antidiarrheal drug Loperamide (Lee, 2015; Juárez et al., 2016), the anti-protozoal drug Nitazoxanide (Lam et al., 2012), the anti-seizure drug Carbamazepine and Valproic acid (Schiebler et al., 2015), psychotropic or antidepressant drugs such as Nortriptyline, Fluoxetine and Prochlorperazine edisylate and Fluoxetine (Sundaramurthy et al., 2013; Stanley et al., 2014) are some of the drugs with potential use as HDT for TB treatment.

## Manipulating Autophagy to Improve Vaccination Against TB

The role of autophagy as a defense mechanism allows to hypothesize that vaccines that increase the autophagic response might be more effective in preventing the reactivation of latency or the acquisition of active TB. In fact, autophagy could be key in the development of effective TB vaccines since this process has the potential to improve the host immune response against *Mtb*. The attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is effective in protecting against pulmonary and extrapulmonary TB in children up to 10 years old (Sterne et al., 1998; Abubakar et al., 2013), but protection against the pulmonary form of TB in adults remains highly controversial (Hatherill et al., 2020). BCG is able to affect the activation of T cells by evading phagosome maturation, autophagy, and by reducing MHC-II expression of antigen-presenting cells (APCs) (Russell, 2001). To avoid these deficiencies, an autophagy-inducing, TLR-2 activating C5 peptide from *Mtb*-derived CFP-10 protein was overexpressed in BCG in combination with Ag85B. This recombinant BCG was shown to induce stronger and longer-lasting immunity, increasing protection in a TB murine model (Khan et al., 2019). Furthermore, overexpression of Ag85B in BCG induced autophagy in APCs and increased immunogenicity in mice, indicating that vaccine efficacy can be augmented by enhancing autophagy-mediated antigen presentation (Jagannath et al., 2009). Therefore, exacerbation of autophagy could contribute to increase the immune response conferred by BCG. Interestingly, BCG was also genetically modified to improve its immunogenicity by replacing the urease C encoding gene with the listeriolysin encoding gene from *Listeria monocytogenes*. As a result, BCGΔureC::hly (VPM1002) was demonstrated to promote apoptosis and autophagy and facilitate the release of mycobacterial antigens into the cytosol (Nieuwenhuizen et al., 2017). The use of VPM1002 vaccine in preclinical trials has been shown to be more effective and safer than BCG (Nieuwenhuizen et al., 2017).

# PERSPECTIVES

For most countries, the end of TB as an epidemic disease and a public health problem still remains an aspiration rather than a reality. Current treatments still depend on antibiotic therapy and, considering the increasing antibiotic resistance, additional therapeutic targets are becoming progressively essential. To this end, the modulation of the autophagy process arises as an attractive goal. However, a deeper study of the cellular mechanisms that operate in humans are required, especially in TB patients where the infective status of each subject might have a special impact on autophagy modulation. As described here, at present the information regarding the human autophagic response during *Mtb* infection is very limited and precludes a better understanding of the process. In fact, the patients' genetic background, among other factors, could be determinant in the development of their specific response to *Mtb* infection, influencing the effectiveness of a particular treatment. Furthermore, most of the existing studies are focused on autophagy in macrophages as the main target for *Mtb*, but this process is implicated in a wide variety of cell types. For example,

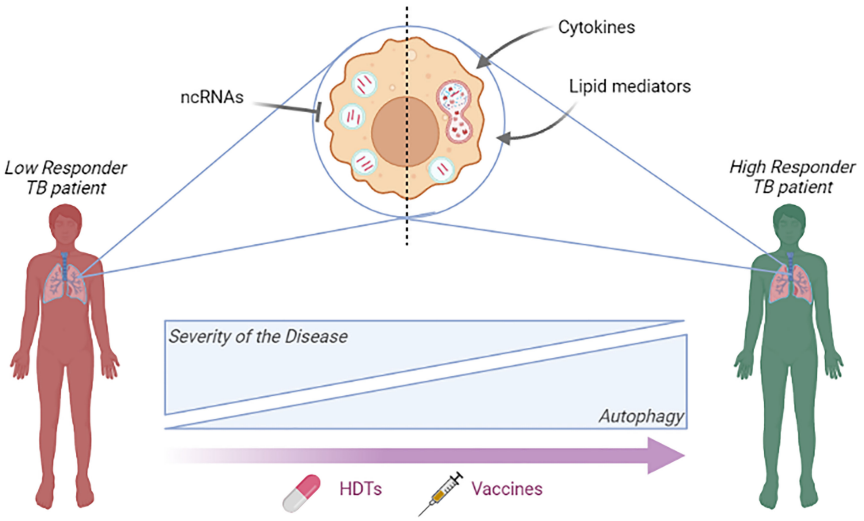


**FIGURE 1** | A simplified model of the role of autophagy in TB patients according to their immunological response to *Mtb*. The potential influence of immunological mediators and ncRNAs on autophagy is shown. High responder TB patients correspond to patients that display strong T cell immunity to *Mtb*, which correlates with milder manifestations of the disease (Pasquinelli et al., 2004; Jurado et al., 2012) and robust autophagic responses, as previously demonstrated (Rovetta et al., 2014; Tateosian et al., 2017). In contrast, patients with weak or no T cell responses to *Mtb* are associated with more severe disease (Pasquinelli et al., 2004; Jurado et al., 2012) and diminished autophagy (Rovetta et al., 2014; Tateosian et al., 2017). The purple arrow indicates the hypothetical impact of new HDT strategies (e.g.: autophagy as adjuvant therapy) in the treatment of TB patients and novel vaccines inducing an autophagic response. Figure created with BioRender.com..

autophagy has been shown to be critical during T cell activation and differentiation, central processes in TB immunity. Thus, an expanded analysis over the autophagy process in other cells such as lymphocytes, dendritic cells, neutrophils, basophils, among others would be necessary. Moreover, a broad examination of the immune responses of TB patients following an autophagy-modulating treatment would be extremely informative.

Based on the mentioned findings reported by several authors and our studies, we proposed a schematic summary of the potential role of autophagy in TB patients according to their immunological response to *Mtb* (**Figure 1**). Briefly, immunological mediators such as cytokines, lipid mediators or ncRNAs, influence autophagy in TB patients with different immunological response to the bacteria. Implementing novel HDT strategies such as the modulation of autophagy as adjuvant therapy or novel vaccines might improve the treatment of TB patients. Current and future studies on autophagy-based therapeutic candidates may contribute to possible therapeutic/prevention improvements against TB, directly impacting the lives of millions of individuals infected with *Mtb*.

## AUTHOR CONTRIBUTIONS

The writing-original draft preparation as well as the writing-review, and editing of the manuscript was in charge of JP, NT, MM, and VG. JP and VG were responsible for the conceptualization and organization of the present work. VG was in charge of supervision and project administration. All authors have read and agreed to the final version of the manuscript.

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# *Aolatg1* and *Aolatg13* Regulate Autophagy and Play Different Roles in Conidiation, Trap Formation, and Pathogenicity in the Nematode-Trapping Fungus *Arthrobotrys oligospora*

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Autophagy is a conserved cellular recycling and trafficking pathway in eukaryotes that plays an important role in cell growth, development, and pathogenicity. Atg1 and Atg13 form the Atg1–Atg13 complex, which is essential for autophagy in yeast. Here, we characterized the roles of the *Aolatg1* and *Aolatg13* genes encoding these autophagy-related proteins in the nematode-trapping fungus *Arthrobotrys oligospora*. Investigation of the autophagy process by using the AoAtg8-GFP fusion protein showed that autophagosomes accumulated inside vacuoles in the wild-type (WT) *A. oligospora* strain, whereas in the two mutant strains with deletions of *Aolatg1* or *Aolatg13*, GFP signals were observed outside vacuoles. Similar results were observed by using transmission electron microscopy. Furthermore, deletion of *Aolatg1* caused severe defects in mycelial growth, conidiation, conidial germination, trap formation, and nematode predation. In addition, transcripts of several sporulation-related genes were significantly downregulated in the Δ*Aolatg1* mutant. In contrast, except for the altered resistance to several chemical stressors, no obvious differences were observed in phenotypic traits between the WT and Δ*Aolatg13* mutant strains. The gene ontology analysis of the transcription profiles of the WT and Δ*Aolatg1* mutant strains showed that the set of differentially expressed genes was highly enriched in genes relevant to membrane and cellular components. The Kyoto Encyclopedia of Genes and Genomes analysis indicated that differentially expressed genes were highly enriched in those related to metabolic pathways, autophagy and autophagy-related processes, including ubiquitin-mediated proteolysis and SNARE interaction in vesicular transport, which were enriched during trap formation. These results indicate that *Aolatg1* and *Aolatg13* play crucial roles in the autophagy process in *A. oligospora*. *Aolatg1* is also involved in the regulation of asexual growth, trap formation, and pathogenicity. Our results highlight the importance of *Aolatg1* in the growth and development of *A. oligospora*, and provide a basis for

elucidating the role of autophagy in the trap formation and pathogenicity of nematode-trapping fungi.

**Keywords: *Arthrobotrys oligospora*, autophagy-related gene (*atg*), conidiation, trap formation, nematode predation, transcriptomic analysis**

## INTRODUCTION

Autophagy is a conserved degradation pathway that controls the homeostasis of the cellular environment by degrading organelles and proteins (Kroemer and Levine, 2008). Autophagy is induced in response to nutrient starvation and mediated by the cytoplasm-to-vacuole targeting (Cvt) pathway, which is responsible for specific sorting of proteins to vacuoles (Ying and Feng, 2019). Autophagy is tightly controlled by autophagy-related genes (*atg*), and approximately 42 Atg proteins have been identified in *Saccharomyces cerevisiae* (Zhu et al., 2019; Ying and Feng, 2019). Based on their specific functions, Atg proteins have been classified into six different groups: the Atg1 kinase complex, the Atg18–Atg2 complex, the phosphatidylinositol 3-kinase complex, the Atg12 conjugation system, the Atg8 conjugation system, and the Atg9 recycling complex (Nanji et al., 2017; Nakatogawa, 2020). The Atg1 complex is the initiator kinase complex for autophagy that serves as a scaffold to recruit downstream factors and regulate their functions *via* phosphorylation of serine or threonine residues (Nakatogawa, 2020). Atg1 is a serine/threonine protein kinase and the only enzymatic subunit of the Atg1 complex. Atg1 activity is essential for the turnover and recycling of other Atg proteins after the formation of autophagosomes (Cheong et al., 2008). Atg13 functions as a major positive regulator of Atg1 protein kinase and is highly phosphorylated under nutrient-rich conditions by protein kinase A and the target of rapamycin complex 1 (TORC1) (Kamada et al., 2000; Stephan et al., 2009). Under conditions of nutrient starvation or the presence of the specific inhibitor rapamycin, Atg13 is dephosphorylated, which allows its interaction with Atg1 and Atg17, resulting in the formation of the Atg1 complex and activation of the Atg1 kinase *via* its autophosphorylation (Memisoglu et al., 2019; Nakatogawa, 2020). In *S. cerevisiae*, the Atg1 complex consists of the protein kinase Atg1, the TORC1 substrate Atg13, and the trimeric Atg17–Atg31–Atg29 scaffolding subcomplex, which triggers autophagy when Atg1 and Atg13 assemble with the trimeric scaffold (Stjepanovic et al., 2014). The Atg1 complex mediates autophagosome formation by initiating phagophore assembly and localizing the downstream phosphatidylinositol 3-kinase complex, Atg9, and the ubiquitin-like conjugation systems to this membrane compartment (Chew et al., 2015).

In filamentous fungi, autophagy appears to be involved in nutrient recycling during starvation, and it has been suggested to regulate normal developmental processes. Several methods have been developed to visualize autophagy, such as transmission electron microscopy (TEM), GFP-Atg8 fusion protein, and probes for acidic compartment (Pollack et al., 2009). At present, the functions of Atg1 have been described in several filamentous fungi. For example, blockade of autophagy in the Δ*Mgatg1* mutant of *Magnaporthe oryzae* (syn. *Magnaporthe grisea*) impaired its ability to penetrate and infect the host (Liu et al., 2007). In *Aspergillus oryzae*, conidiation and development of aerial hyphae were suppressed in the Δ*Aoatg1* mutant, so AoAtg1 was deemed to be essential for nonselective autophagy and the Cvt pathway (Yanagisawa et al., 2013). Similarly, disruption of *Bbatg1* impaired autophagy, conidial yield, conidial germination, and virulence in *Beauveria bassiana* (Ying et al., 2016). Deletion of *Bcatg1* impaired autophagy and dramatically suppressed vegetative growth, conidiation, and sclerotium formation in the Δ*Bcatg1* mutant of *Botrytis cinerea* (Ren et al., 2017). Unlike Atg1, Atg13 has been characterized only in a limited number of fungi. In *M. oryzae*, the Δ*Moatg13* mutant displayed the phenotype similar to that of the wild-type (WT) strain (Dong et al., 2009), whereas in *A. oryzae*, the number of conidia was lower in the Δ*Aoatg13* mutant than in the WT strain (Kikuma and Kitamoto, 2011).

Nematode-trapping (NT) fungi are a specific filamentous group that can form unique mycelial structures (traps) for nematode predation, thus playing important roles in maintaining nematode population density in natural environments (Su et al., 2017). *Arthrobotrys oligospora* is a representative NT species that can live both saprophytically on organic matter and as a predator, by capturing tiny animals (Nordbring-Hertz et al., 2006). *A. oligospora* is used as the primary model for interactions between fungi and nematodes (Niu and Zhang, 2011). When stimulated by nematodes or other inducing factors, *A. oligospora* produces adhesive networks, indicating a switch from the saprophytic lifestyle to the predacious stage (Nordbring-Hertz, 2004; Yang et al., 2011). Since the sequencing of *A. oligospora* genome, an increasing number of studies has focused on the mechanism underlying trap formation, and several signaling proteins, such as regulators of G-protein, G-protein subunits and small GTPases, have been shown to regulate trap morphogenesis and lifestyle switch (Yang et al., 2018; Yang et al., 2020; Bai et al., 2021; Ma et al., 2021; Yang et al., 2021). Moreover, three orthologous Atg proteins have been identified in *A. oligospora*: deletion of *Aolatg8* blocked autophagy and abolished conidiation and trap formation (Chen et al., 2013), whereas deletion of *Aolatg4* and *Aolatg5* impaired autophagy and resulted in a reduction in conidia yields, cell nucleus number, and trap production (Zhou et al., 2020; Zhou et al., 2021). Despite their conserved features, autophagy proteins have different functions in fungi that are highly divergent morphology and lifestyle (Pollack et al., 2009; Ying and Feng, 2019).

In this study, to further probe biological impact of autophagy on the growth, development, and differentiation of NT fungi, the Atg1 and Atg13 homologs, which govern the first step of autophagy, were characterized in *A. oligospora* by gene disruption, phenotypic comparison, and transcriptomic

analysis. Our results showed that Atg1 (AolAtg1) plays a crucial role in autophagy and underpins multiple phenotypic traits, whereas Atg13 (AolAtg13) plays a conserved role in autophagy and has a limited impact on the growth and development in *A. oligospora*. In addition, we compared transcriptional profiles of the WT and Δ*Aolatg1* mutant strains obtained using RNA-seq technology, which provided insights into the regulation of autophagy in *A. oligospora* and other NT fungi.

# MATERIALS AND METHODS

## Strains and Media

The fungus *A. oligospora* (ATCC24927) and corresponding mutants were stored in the Microbial Library of the Germplasm Bank of wild species from Southwest China (Kunming, China). Potato dextrose agar (PDA), tryptone glucose (TG), and corn-maizena yeast extract (CMY) were prepared as described previously (Zhou et al., 2020; Zhou et al., 2021) and used to analyze fungal phenotypic traits. The complete medium (CM) and MM-N (0.5 g $L^{-1}$ KCl, 0.5 g $L^{-1}$ $MgSO_4$, 1.5 g $L^{-1}$ $KH_2PO_4$, 0.1% trace element, and 10 g $L^{-1}$ glucose; pH 6.5) were used to compare colony growth and induce autophagy under nitrogen starvation (Talbot et al., 1993). Plasmids pRS426 and pCSN44 were maintained in the *Escherichia coli* strain DH5α (Takara, Shiga, Japan). *S. cerevisiae* (FY834) was used to screen the correctly recombined construct, and the selectionwas performed on the SC-Ura medium (Park et al., 2011). *Caenorhabditis elegans* (strain N2) was incubated on the oatmeal medium at 26°C for trap induction and bioassays.

## Sequence Analysis of AolAtg1 and AolAtg13 Proteins

The homologous sequences of AolAtg1 (AOL_s00076g234) and AolAtg13 (AOL_s00215g74) were retrieved from the *A. oligospora* genome (Yang et al., 2011) using the sequences of the orthologous proteins Atg1 (NP_011335) and Atg13 (NP_015511) in *S. cerevisiae*. The molecular mass and isoelectric point of the proteins were calculated using the online tool Compute pI/Mw (https://web.expasy.org/compute_pi/), and the conserved protein domains were predicted using InterPro (http://www.ebi.ac.uk/interpro/). The orthologs of AolAtg1 and AolAtg13 from other fungi were examined by BlastP, and the similarity between Atg1 or Atg13 homologs was analyzed using DNAman software (Version 6). A neighbor-joining tree was constructed using Mega software (7.0) (Kumar et al., 2016).

## Deletion of *Aolatg1* and *Aolatg13* Genes

The *atg* genes of *A. oligospora* were deleted using the homologous recombination method (Tunlid et al., 1999; Park et al., 2011). The upstream and downstream sequences corresponding to the genes *Aolatg1* and *Aolatg13* in *A. oligospora* were amplified using paired primers (**Supplementary Table S1**). Subsequently, the *hph* cassette for hygromycin resistance was amplified using primers Hph-f and Hph-r (**Supplementary Table S1**). Then, three PCR fragments and a linearized pRS426 vector were co-transformed into the yeast strain FY834 *via* electroporation. The complete fragment for gene disruption was amplified from the recombinant plasmid pRS426-Atg-hph using primers AolAtg1-5f/AolAtg1-3r or AolAtg13-5f/AolAtg13-3r (**Supplementary Table S1**), and it was transformed into *A. oligospora* using the protoplast transformation method as described previously (Tunlid et al., 1999; Liu et al., 2021; Long et al., 2021). The putative transformants were selected on the PDAS medium containing 200 g $L^{-1}$ hygromycin B (Amresco, Solon, United States) (Liu et al., 2020; Xie et al., 2021). The successful deletions of the *Aolatg1* and *Aolatg13* genes were confirmed using PCR amplification and Southern blotting analyses, as described previously (Xie et al., 2019; Xie et al., 2020).

## Generation of the AoAtg8-GFP Fusion Protein

The pPK2-GFP-Sur (pPK2) vector harboring the green fluorescent protein (GFP) gene and the sulfonylurea resistance gene (*sur*) was used as a basic framework. The promoter fragment was amplified with primers AoP-f/AoP-r and inserted into the *Bsr*GI/*Spe*I sites of the pPK2 vector, and the cDNA fragment of *Aoatg8* (AOL_s00007g534) was amplified using the primer pair Atg8-f/Atg8-r (**Supplementary Table S1**) and then inserted into the *Bsr*GI/*Spe*I sites of the pPK2 vector. The resultant pPK2-GFP-AoAtg8 vector was inserted into the WT, Δ*Aolatg1*, and Δ*Aolatg13* mutant strains using the protoplast transformation method (Tunlid et al., 1999). The putative transformants were cultured on plates supplemented with 10 μg $mL^{-1}$ chlorimuron ethyl, and GFP signals were examined under a confocal laser scanning microscope.

## Comparison of Mycelial Growth and Stress Resistance

The WT and mutant strains were incubated on PDA plates at 28° C for 5 days, then transferred onto PDA, CMY, TG, CM, and MM-N plates to evaluate their growth rate under different nutritional conditions, and the diameters of colonies were determined at 24 h intervals (Zhou et al., 2020; Zhou et al., 2021). To determine the levels of stress resistance, the fungal strains were incubated on TG plates supplemented with or without (control) different concentrations of chemical stressors, including oxidative agents ($H_2O_2$ and menadione) and osmotic agents (NaCl and sorbitol) at 28°C for 7 days. Relative growth inhibition (RGI) values of the fungal strains were calculated as previously described (Zhen et al., 2018). To compare the lipid droplets (LDs) of fungal mycelia, the WT and mutant strains were incubated on PDA plates for 5 days and then stained with 10 μg $mL^{-1}$ BODIPY staining solution for 10 min. LDs were observed using a fluorescence microscope.

## Comparison of Conidiation and Transcription of Sporulation-Related Genes

To determine the spore yield, the WT and mutant strains were incubated on CMY plates at 28°C for 7 days, and then 5 mL of sterile water was added to each plate to harvest spores. Conidia were counted in 50 μL aliquots using a hemocytometer. To

analyze spore germination, 50 μL suspensions ($10^6$ spores per mL) of WT and mutant strains were added to the MM-N liquid medium at 28°C, and the number of germinated conidia was determined at 4, 8, and 12 h (Zhen et al., 2018).

To determine transcriptional levels of the sporulation-related genes, 50 μL conidial suspension aliquots ($10^6$ spores per mL) of fungal strains were spread on CMY plates at 28°C. The fungal samples were harvested from the cultures grown for 3, 5, and 7 days and stored at −80°C for subsequent quantitative real-time PCR (qRT-PCR) analysis. The primers (**Supplementary Table S2**) for the target genes were designed using online software Primer3 (v0.4.0, https://bioinfo.ut.ee/primer3-0.4.0/). The expression of the *A. oligospora* β-tubulin gene (AOL_s00076g640) was used as the reference, and qRT-PCR analysis was performed as previously described (Yang et al., 2013). The transcript levels of each gene were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

## Confocal Microscopy and TEM Assays

Hyphae of the WT and mutant strains were incubated in the CM medium at 28°C with gentle shaking at 180 r $min^{-1}$ for 2 days, then transferred into the MM-N medium (nitrogen starvation) and incubated for 6 h. Hyphae were collected to observe autophagosome formation using TEM and confocal microscopy (Lv et al., 2017). The lipophilic styryl dye FM4-64 (Invitrogen, Carlsbad, CA, USA) was used for vacuole staining of hyphal cells, as described previously (Ma et al., 2020).

## Trap Induction and Bioassay

To induce trap formation, the conidia of fungal strains were collected from 7-day-old cultures on CMY plates, and 50 μL suspensions ($10^6$ spores per mL) were incubated on water agar plates at 28°C for 3 days. Then, ~300 nematodes were added to each plate to induce trap formation, followed by microscopic observation of trap formation and nematode predation at 12 h intervals (Zhou et al., 2020).

## Transcriptomic Profile Analysis

To probe the mechanism by which AolAtg1 regulates autophagy, the WT and Δ*Aolatg1* mutant strains were incubated in the CMY medium at 28°C, and the spores were harvested 7 days post incubation. Next, $1\times10^5$ spores were incubated on water agar plates at 28°C for 48 h, and the hyphae were harvested. Two treatment groups with three independent biological replicates were collected at 0 h without nematodes and following 24 h incubation after the addition of 300–400 nematodes. The hyphae were sent to the Shanghai Meiji Biological Company (Shanghai, China) for RNA sequencing and data analysis.

High-quality RNA samples were used to construct a sequencing library that was sequenced on an Illumina HiSeq 4000 system (Illumina, San Diego, CA, USA). The data were analyzed using the Majorbio Cloud Platform (www.majorbio.com). To identify differentially expressed genes (DEGs), transcripts per kilobase million (TPM) values were calculated for each gene and compared between the WT and Δ*Aolatg1* mutant strains. Gene abundance following RNA-seq was quantified by the expectation-maximization algorithm (Li and Dewey, 2011). Based on the quantitative expression results, DEGs were identified based on the following thresholds: $|\log2 \text{ ratio}| \geq 1$ and adjusted $P < 0.05$. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to determine enrichment in GO terms of function classes and metabolic pathways in DEGs in comparison to the whole-transcriptome background. Sequence data were deposited in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under the accession number PRJNA784322.

## Statistical Analysis

All experimental data are presented as the mean ± standard deviation (SD) of three biological replicates. Group effects were assessed by one-way analysis of variance followed by the Tukey's honestly significant difference (HSD) test. Prism 5 (GraphPad, San Diego, CA, USA) was used to generate plots and perform statistical analyses. Effects were considered statistically significant if $P < 0.05$.

# RESULTS

## Analysis of AolAtg1 and AolAtg13 Protein Sequences

The sequences of the Atg1 and Atg13 homologs were retrieved from the *A. oligospora* genome. AolAtg1 was found to consist of 949 amino acids with a predicted molecular mass and isoelectric point of 103.6 kD and 8.83, respectively. AolAtg13 was shown to comprise 984 amino acids with a predicted molecular mass and isoelectric point of 105.8 kD and 9.43, respectively. AolAtg1 contains a protein kinase domain (IPR000719) at the N-terminal and a serine/threonine-protein kinase at the C-terminal (IPR022708). AolAtg13 contains the autophagy-related protein 13 N-terminal domain (IPR018731). AolAtg1 shares a highly conserved protein sequence with homologous proteins from various NT fungi, having 94.2% and 86.8% identity to orthologous Atg1 from *Arthrobotrys flagrans* (syn. *Duddingtonia flagrans*) and *Dactylellina haptotyla*, respectively. AolAtg1 has moderate similarity (42.4–48.6%) to orthologs from different filamentous fungi and 32.4% identity with Atg1 of *S. cerevisiae* (**Supplementary Table S3**). Relative to AolAtg1, AolAtg13 also has a high degree of identity (72.7–91.2%) to orthologs from NT fungi, whereas its identity to orthologs from other filamentous fungi is low (23.2–29.5%), and it has only 10.6% identity with Atg13 from *S. cerevisiae* (**Supplementary Table S3**). Phylogenetic analysis showed that orthologous Atg1 and Atg13 from filamentous fungi were divided into two clades, whereas orthologous Atg1 or Atg 13 from NT fungi were clustered together (**Supplementary Figure S1**).

## *Aolatg1* and *Aolatg13* Are Involved in Mycelial Growth and Lipid Metabolism

Two independent positive transformants for *Aolatg1* and *Aolatg13* were screened and confirmed (**Supplementary Figure S2**), and their growth in various media was observed. The Δ*Aolatg1* mutant displayed lower hyphal growth on CMY, TG, and PDA plates. The colony diameter of the WT strain was 7.75 ± 0.25 cm on CMY plates at day 6, whereas those of the

Δ*Aolatg1* and Δ*Aolatg13* mutants were 6.61 ± 0.26 cm and 6.94 ± 0.30 cm, respectively. Similarly, hyphal growth of the Δ*Aolatg1* mutant was lower than that of the WT strain in the CM and MM-N plates (**Supplementary Figure S3**; **Figures 1A, B**). After staining with BODIPY staining solution, the LDs in hyphal cells were visualized. The hyphal cells of the WT strain contained numerous LDs, whereas in Δ*Aolatg1* and Δ*Aolatg13* mutants displayed remarkably fewer LDs (**Figure 1C**).

## *Aolatg1* Regulates Sporulation and Spore Germination

*Aolatg1* deletion resulted in defective growth of aerial hyphae on CMY plates (**Figure 2A**), and the conidiophores of Δ*Aolatg1* mutants became sparse compared to their number in the WT strain (**Figure 2B**). Thus, the loss of *Aolatg1* caused a significant reduction in spore yield: the Δ*Aolatg1* mutant produced 1.16 × $10^6$ conidia $cm^{-2}$, which was 87.6% lower compared to the yield in the WT strain (9.40 × $10^6$ conidia $cm^{-2}$) (**Figure 2C**). Furthermore, *Aolatg1* deletion caused a reduction in the spore germination rate: 29.0%, 47.6%, and 53.0% of the Δ*Aolatg1* mutant spores germinated at 4, 8, and 12 h, respectively, whereas 43.5%, 56.4%, and 78.4% of the WT strain spores germinated at the same time points (**Figure 2D**). However, no obvious differences in the numbers of aerial hyphae and conidiophores, spore yield, and spore germination rate were observed between the WT strain and Δ*Aolatg13* mutant.

The transcript levels of ten sporulation-related genes were determined in the WT, Δ*Aolatg1*, and Δ*Aolatg13* mutant strains using qRT-PCR at different growth stages. The transcript levels of *flbA* and *medA* were remarkably upregulated in the Δ*Aolatg1* mutant on days 3 and 5, and the remaining eight analyzed genes, including *abaA*, *brlA*, *flbC*, *fluG*, *nsdD*, *velB*, *vosA*, and *wetA*, were downregulated on day 7 (**Figure 2E**). In the Δ*Aolatg13* mutant, *flbC*, *flbA*, and *medA* were significantly upregulated on day 3, five genes (*brlA*, *flbC*, *fluG*, *velB*, and *wetA*) were downregulated on day 5, and only one gene, *flbC* was downregulated on day 7 (**Figure 2F**).

## *Aolatg1* and *Aolatg13* Are Involved in Stress Resistance

The stress response of fungal strains was evaluated on TG plates. We observed that deletion of *Aolatg1* and *Aolatg13* altered sensitivity to oxidative and osmotic agents. For example, deletion of *Aolatg1* and *Aolatg13* increased RGI by oxidative agents. In particular, in the presence of 5 mM $H_2O_2$, the RGI values of the Δ*Aolatg1* (45.9%) and Δ*Aolatg13* (48.7%) mutant strains were higher than that of the WT strain (34.5%), although no significant differences were noted at 10 and 15 mM $H_2O_2$ (**Figures 3A, B**). Further, the Δ*Aolatg1* and Δ*Aolatg13* mutants had higher RGI values in the presence of several menadione concentrations (0.04–0.08 mM and 0.06–0.08 mM, respectively) compared to the RGI value of the WT strain (**Figures 3A, C**).

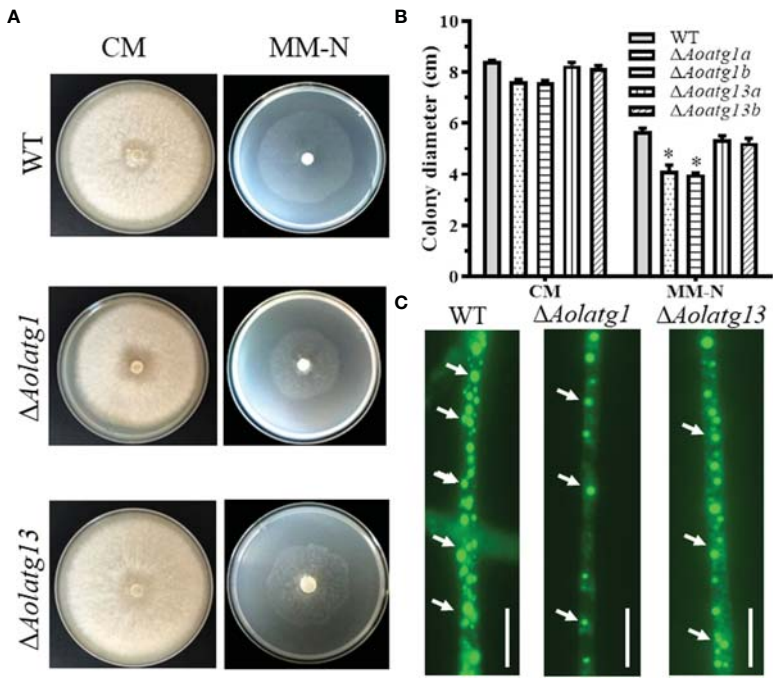


**FIGURE 1** | Comparison of mycelial growth in the WT and mutant strains of *Arthrobotrys oligospora* on CM and MM-N plates. **(A)** Colony morphology of fungal strains incubated on CM and MM-N plates at 28°C for 6 days. **(B)** Colony diameters of fungal strains incubated on CM and MM-N plates at 28°C for 6 days. Data are presented as the mean ± standard deviation. Statistical significance of differences between mutant strains and WT strain is indicated as follows: $^*P < 0.05$ (Tukey's HSD). **(C)** Comparison of lipid droplets (LDs) in the WT and mutant strains. The arrows indicate LDs stained with BODIPY staining solution. Scale bars = 10 μm.

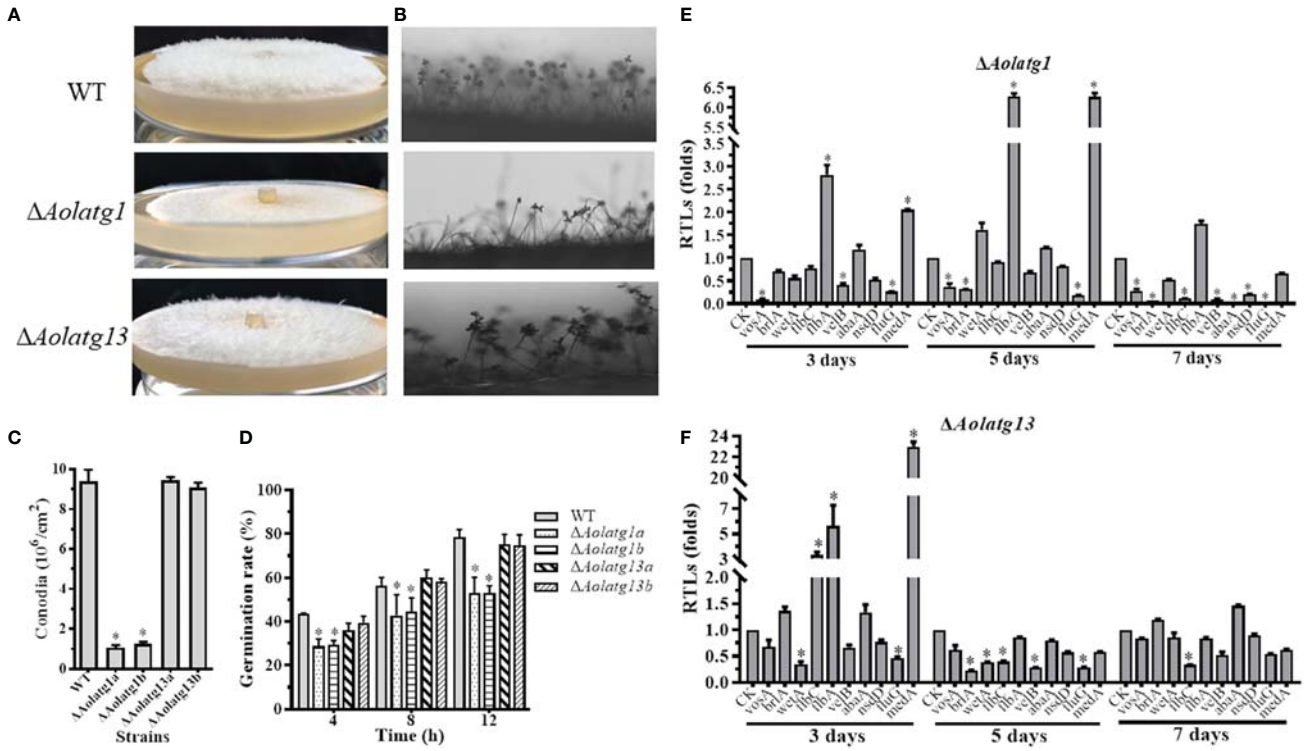


**FIGURE 2** | Comparison of conidiation and transcript levels of sporulation-related genes in the WT and mutant strains of *A. oligospora*. **(A)** Observation of aerial hyphae in the WT and mutant strains. **(B)** Observation of conidiophores in the WT and mutant strains. **(C)** Comparison of conidia yields in the WT and mutant strains. **(D)** Comparison of the germination rate in the WT and mutant strains. **(E)** The relative transcription levels (RTLs) of the sporulation-related genes in the WT and Δ*AolATG1* mutant strain. **(F)** The RTLs of sporulation-related genes in the WT and Δ*AolATG13* mutant strain. RTLs of the sporulation-related genes were assessed by comparing the levels of their transcription in the mutant strain with that of the WT strain. CK is the standard used in statistical analysis of the RTL of each gene in the deletion mutant compared to that in the WT strain under any given condition. Data are presented as the mean ± standard deviation. Statistical significance of differences between mutant strains and WT strain is indicated as follows: *$P < 0.05$ (Tukey's HSD).

In addition, deletion of *Aolatg1* and *Aolatg13* altered sensitivity to osmotic agents. The Δ*Aolatg13* mutant had a higher RGI value at 0.2–0.3 M NaCl compared to that of the WT strain, whereas the sensitivity of the Δ*Aolatg1* mutant to NaCl was not changed significantly (**Supplementary Figure S4A, B**). Growth of both mutants was more strongly inhibited by sorbitol (0.5 M for Δ*Aolatg13* and 0.3 M for Δ*Aolatg1*) compared to the effect of sorbitol on the WT strain (**Supplementary Figure S4A, C**).

## *Aolatg1* and *Aolatg13* Regulate Autophagosome Formation

To probe the effect of *Aolatg1* and *Aolatg13* deletion on autophagy, we constructed a GFP-Atg8 fusion protein and expressed it in the WT, Δ*Aolatg1*, and Δ*Aolatg13* mutant strains. The WT and mutant strains were cultured in the CM medium for 24 h, and then transferred to the MM-N medium and incubated for 6 h. GFP-Atg8 signals were observed in the vacuoles of the hyphae in the WT strain (**Figure 4A**), whereas punctate GFP signals were observed near the vacuoles in the Δ*Aolatg1* and Δ*Aolatg13* mutants. We then used TEM to visualize autophagosomes and confirmed that when fungal strains were cultured in the MM-N medium for 6 h, autophagosomes were clearly observed in the vacuoles of the WT strain, whereas there were few autophagosomes or autophagosome-like structures in the vacuoles of the Δ*Aolatg1* and Δ*Aolatg13* mutants (**Figure 4B**).

## *Aolatg1* Regulates Trap Formation and Pathogenicity

The WT and mutant strains were incubated on water agar plates at 28°C, followed by the addition of nematodes to induce trap formation. The WT and Δ*Aolatg13* mutant strains produced more traps than the Δ*Aolatg1* mutant (**Figure 5A**). At 12, 24, and 36 h, the WT strain produced 1,893, 2,953, and 3,804 traps per plate, respectively. The Δ*Aolatg13* mutant generated 1,606, 2,690, and 3,998 traps per plate at the same time points, whereas the Δ*Aolatg1* mutant produced only 582, 756, and 971 traps, respectively (**Figure 5B**). Accordingly, upon the formation of traps, 27%, 59.7%, and 99.5% of nematodes were captured by the WT strain at 12, 24, and 48 h, respectively, and for the Δ*Aolatg13* mutant the corresponding fractions were similar: 26.7%, 56.7%, and 90.9%. In contrast, only 19.5%, 41%, and 50% nematodes were captured by the Δ*Aolatg1* mutant at the corresponding time points (**Figure 5C**).

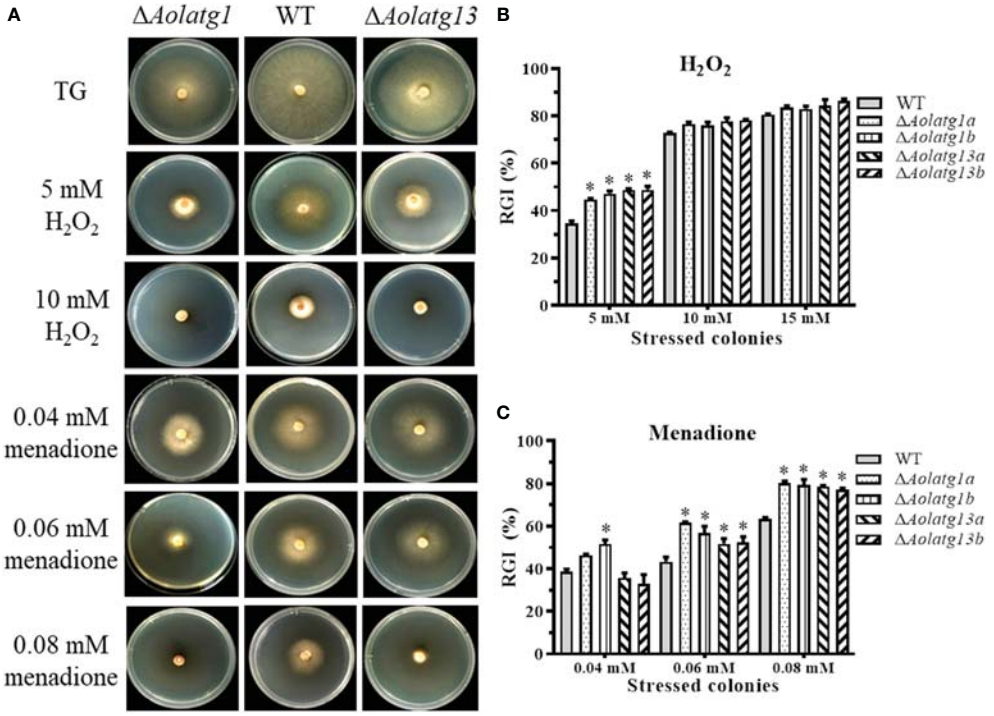


**FIGURE 3** | Comparison of tolerance to oxidative stress in the WT and mutant strains of *A. oligospora*. **(A)** Colonial morphology of the WT and mutant strains on TG plates or plates containing $H_2O_2$ and menadione at 28°C for 7 days. **(B)** Relative growth inhibition (RGI) of WT and mutant strains grown on the TG medium containing 5–15 mM $H_2O_2$ for 6 days. **(C)** RGI of WT and mutant strains grown on the TG medium containing 0.04–0.08 mM menadione for 6 days. Data are presented as the mean ± standard deviation. Statistical significance of differences between mutant strains and WT strain is indicated as follows: $^*P < 0.05$ (Tukey's HSD).

## Transcriptomic Analysis of the WT and Δ*Aolatg1* Mutant Strains

The transcriptomic profiles of the WT and Δ*Aolatg1* mutant strains were compared by RNA-seq. The raw and clean RNA-seq reads are shown in **Supplementary Table S4**. The percentage of phred-like quality scores at the Q30 level ranged from 91.5% to 92.9%, and the GC content ranged from 47.7% to 50.3% (**Supplementary Table S4**). The principal component analysis results showed that the WT and Δ*Aolatg1* mutant strains were located in different quadrants, suggesting that their transcription profiles were significantly different, whereas the independent samples at each time point were in close proximity, indicating high similarity and good reproducibility of the three repeats (**Supplementary Figure S4**).

A total of 2,545 and 4,103 DEGs were identified at 0 and 24 h, respectively, between the WT and Δ*Aolatg1* mutant strains, whereas expression levels of 1,632 genes were similar at these time points (**Figure 6A**). At 0 h, 1,410 genes were upregulated and 1,135 were downregulated in the WT compared to theΔ*Aolatg1* mutant (**Figure 6B**). The upregulated genes were enriched in 164 GO terms and 29 KEGG pathways (**Supplementary Figure S5B, C**). In particular, membrane (intrinsic/integral component of membrane, membrane part, and membrane), catalytic activity, and ion binding were the highly enriched terms in the GO analysis (**Supplementary Figure S6A**). In the KEGG analysis, the following metabolic pathways were highly enriched: carbohydrate metabolism, amino acid metabolism, and lipid metabolism (**Supplementary Figure S5C** and **Figure 6C**). Fold sorting and degradation, and translation were enriched in genetic information processing; transport and catabolism and cell growth and death were enriched in cellular processes (**Figure 6E**). The downregulated genes were enriched in 132 GO terms and 24 KEGG pathways (**Supplementary Figure S5B**). The catalytic activity was highly enriched in the GO analysis (**Supplementary Figure S6B**). In the KEGG analysis, metabolic pathways and biosynthesis of secondary metabolites were highly enriched, such as carbohydrate metabolism, amino acid metabolism, lipid metabolism, energy metabolism, nucleotide metabolism, and metabolism of terpenoids and polyketides (**Supplementary Figure S7**).

After induction with nematodes for 24 h, 1,976 genes were upregulated and 2,127 were downregulated in the WT strain compared to their levels in the Δ*Aolatg1* mutant (**Figure 6B**). The upregulated genes were enriched in 97 GO terms and 26 KEGG pathways (**Supplementary Figure S5B, C**), membranes were highly enriched in GO terms (**Supplementary Figure S6C**), whereas amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, MAPK signaling pathway, autophagy, ubiquitin-

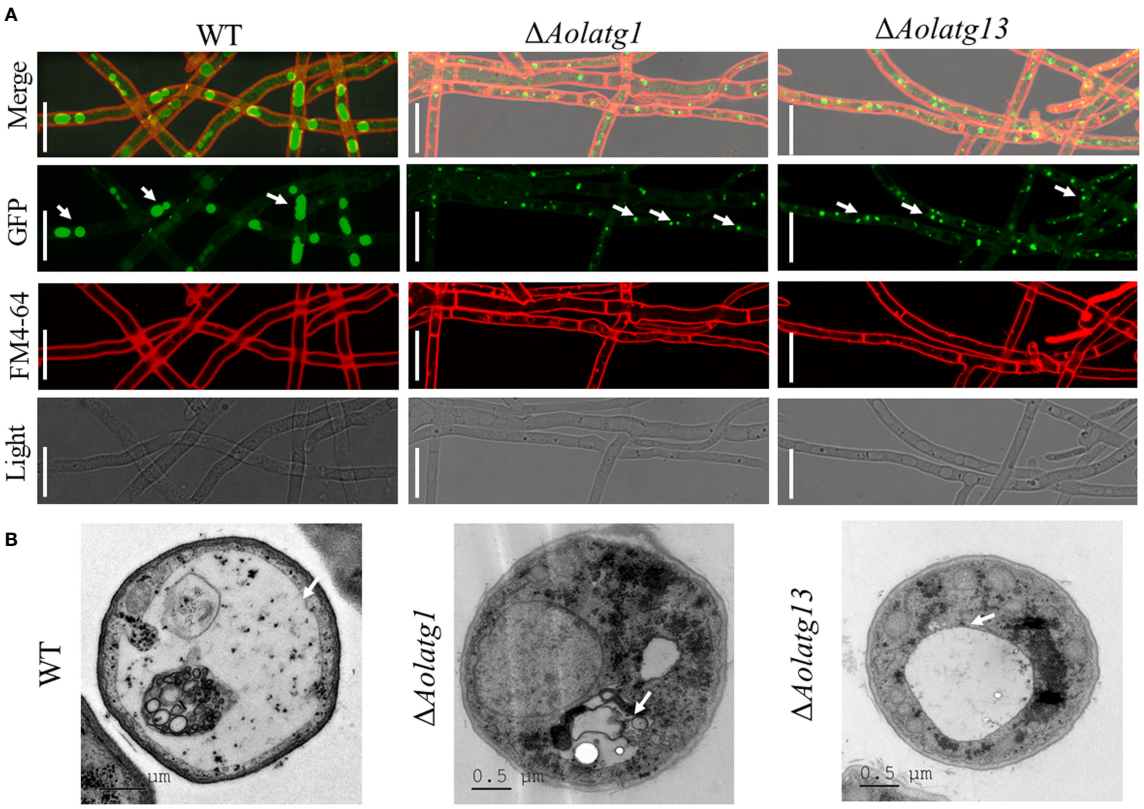


**FIGURE 4** | Comparison of the autophagy process in the WT and mutant strains of *A. oligospora*. **(A)** Expression of the GFP-Atg8 protein in the WT and mutant strains. The *A. oligospora* strains were grown in the liquid CM medium at 28°C for 24 h, and then transferred to the liquid MM-N medium for 6 h. The vacuoles were stained by FM4-64 and examined by fluorescence microscopy. White arrow: GFP signals. Scale bars = 10 μm. **(B)** The vacuoles of hyphal cells were observed using transmission electron microscopy. Arrows indicate the vacuole. Scale bars = 0.5 μm.

mediated proteolysis, and SNARE interaction in vesicular transport were enriched in KEGG pathways (**Figure 6D**), and carbohydrate metabolism, amino acid metabolism, and lipid metabolism were highly enriched in metabolism (**Figure 6F**). The downregulated genes were enriched in 198 GO terms and 28 KEGG pathways (**Supplementary Figure S5B, C**), cellular components were highly enriched in GO terms, such as the cytoplasm, ribosome, mitochondrion, and non-membrane-bounded organelle (**Supplementary Figure S6D**). In the KEGG pathway analysis, metabolic pathways and biosynthesis of secondary metabolites were enriched; with carbohydrate metabolism, amino acid metabolism, energy, and lipid metabolism being the particularly highly enriched processes (**Supplementary Figure S8**).

The comparison of the transcript levels of the genes associated with trap formation (**Table 1**) revealed that the expression of *flbA* was significantly increased by 5.69 folds in Δ*Aolatg1* mutant compared to the WT at 24 h post-induction (hpi) with nematode, and its expression was unaltered at 0 h. The expression of *hog1* in the Δ*Aolatg1* mutant was augmented by 1.92 folds at 24 hpi. However, the expression levels of *slt2* and *fus3* remained unperturbed in both WT and Δ*Aolatg1* mutant strains. The *atg8* expression level increased by 2.72 and 2.50 folds in the Δ*Aolatg1* mutant at 0 h and 24 hpi, respectively. Similarly, the expression of *ubr1* was elevated by 2.14 and 1.83 folds in the Δ*Aolatg1* mutant at 0 h and 24 hpi, respectively. Additionally, the expression levels of genes involved in oxidative stress response was also evaluated (**Table 1**). The comparative analysis demonstrated that the expression of genes *per* and *nox1* was enhanced by 2.80 and 3.71 folds, respectively in the Δ*Aolatg1* mutant compared to that in the WT at 24 hpi,. The expression of *cat2* was enriched by 4.15 and 12.84 folds in the Δ*Aolatg1* mutant at 0 h and 24 hpi, respectively. Similarly, the expression of *sod-2*, a gene encoding a superoxide dismutase was increased by 2.0 and 5.81 folds in the Δ*Aolatg1* mutant at 0 h and 24 hpi, respectively. In contrast, the expression of the *cat* gene (AOL_s00173g374) was decreased by 2.08 folds in the Δ*Aolatg1* mutant at 0 h. However, the expression of another *cat* gene (AOL_s00188g243) was reduced by 4.76 and 5.73 folds in the Δ*Aolatg1* mutant at 0 h and 24 hpi, respectively. In addition, the expression of *noxR* was also decreased by 2.54 folds in the Δ*Aolatg1* mutant at 24 hpi.

## DISCUSSION

Autophagy is an evolutionarily conserved physiological process in eukaryotic cells that regulates programmed cell fate, tissue and

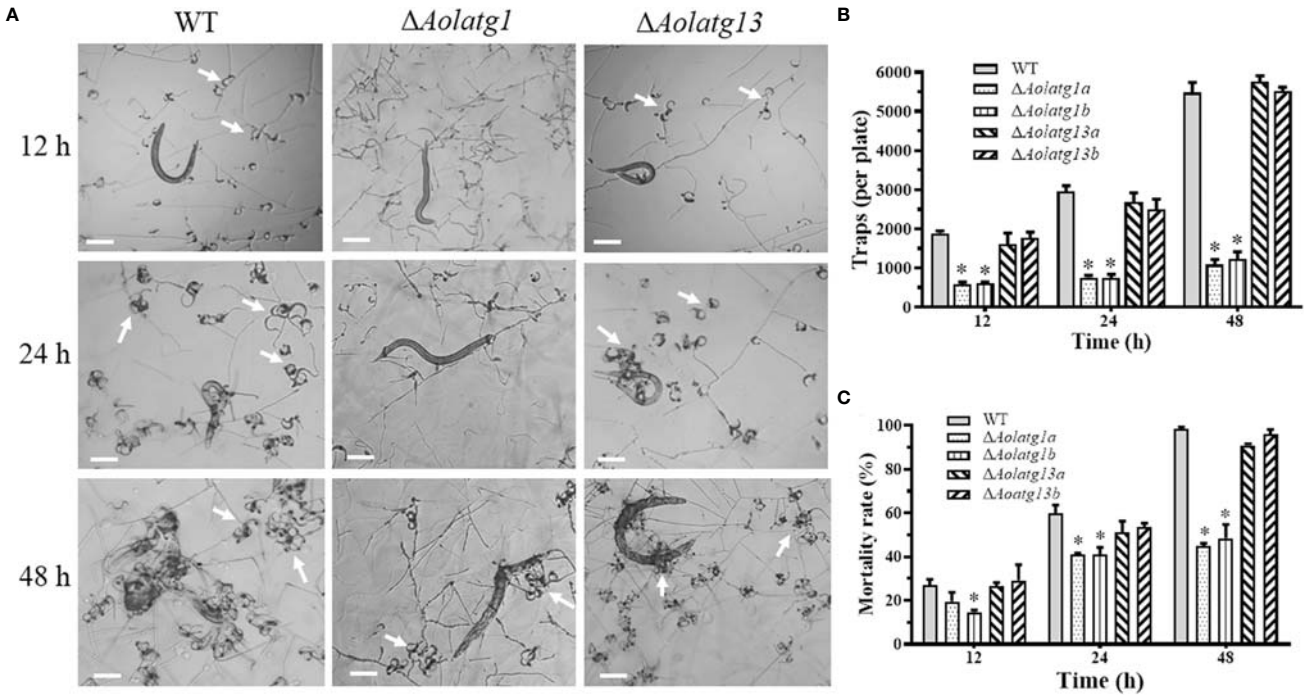


**FIGURE 5** | Comparison of trap formation and nematicidal activity in the WT and mutant strains of *A. oligospora*. **(A)** Traps (indicated by white arrows) were induced by nematodes at 12, 24, and 48 h. Scale bars = 100 μm. **(B)** Traps produced by the WT and mutant strains at 12, 24, and 48 h. **(C)** Nematode mortality rate (%) at 12, 24, and 48 h. Data are presented as the mean ± standard deviation. Statistical significance of differences between mutant strains and WT strain is indicated as follows: $^*P < 0.05$ (Tukey's HSD).

cellular remodeling, and development (Pollack et al., 2009). Atg1 and Atg13 are core Atg proteins involved in the initial nucleation step of the phagophore formation (Itakura and Mizushima, 2010). Recently, Atg1 has been shown to be involved in the Cvt pathway and to play an essential role in the regulation of mycelial growth, conidiation, and virulence in filamentous fungi (Zhu et al., 2018; Ying and Feng, 2019). In this study, orthologs of Atg1 and Atg13 were characterized in the typical NT fungus *A. oligospora*, and their roles in autophagy, asexual development, trap formation, and nematode predation were comprehensively compared.

We used the GFP-Atg8 fusion protein to visualize autophagy and observed that in the WT strain, GFP signals accumulated in the vacuole, whereas in the Δ*Aolatg1* and Δ*Aolatg13* mutants, the majority of GFP signals were dispersed outside the vacuole in the hyphae, suggesting that the absence of the *Aolatg1* and *Aolatg13* genes blocked the autophagy pathway. Similar results have been reported for other fungi. For example, autophagic bodies were observed in the vacuoles of the WT strain of *Fusarium graminearum*, whereas no autophagic bodies or a small number of autophagosome-like structures were observed in the vacuoles of a Δ*Fgatg1* mutant (Lv et al., 2017). In *A. oryzae*, AoAtg1 is essential for nonselective autophagy and the Cvt pathway (Yanagisawa et al., 2013), and only a slight accumulation of EGFP-AoAtg8 in the vacuoles of the Δ*Aoatg13* mutant was observed (Kikuma and Kitamoto, 2011). Deletion of *Atg1* abolished autophagosome accumulation in the vacuoles of carbon-starved *Ustilago maydis* cells (Nadal and Gold, 2010). In addition, deletion of *Bcatg1* inhibited autophagosome accumulation in the vacuoles of nitrogen-starved *B. cinerea* cells (Ren et al., 2017). Similarly, autophagy was blocked in the Δ*Mgatg1* (Liu et al., 2007) and Δ*Bbatg1* mutants (Ying et al., 2016). These findings suggest that orthologs of Atg1 and Atg13 are indispensable for autophagy in various fungi.

Mycelial growth was impaired in the absence of *Aolatg1* and *Aolatg13* compared to that in the WT strain. The colony size and aerial mycelia of the Δ*Aolatg1* mutant were remarkably lower, whereas the mycelial growth of the Δ*Aolatg13* mutant was slightly reduced on the PDA, TG, and CMY media, but not on the CM and MM-N media. Moreover, deletion of *Aolatg1* and *Aolatg13* caused a remarkable reduction in LDs in hyphal cells. In *A. oryzae*, deletion of *Aoatg1* and *Aoatg13* did not affect colony size, but developed aerial hyphae were scarcely observed in the Δ*Aoatg1* mutant (Yanagisawa et al., 2013). Deletion of *Fgatg1* reduced the hyphal growth of *F. graminearum* (Lv et al., 2017), but colonies of the Δ*Fgatg13* mutant were the same as those of the WT strain when cultured on PDA plates (Lv et al., 2017). In *B. cinerea*, the mycelial radial growth rate of the Δ*Bcatg1* mutant was broadly similar to that of the WT, but the former produced significantly fewer aerial hyphae with more of

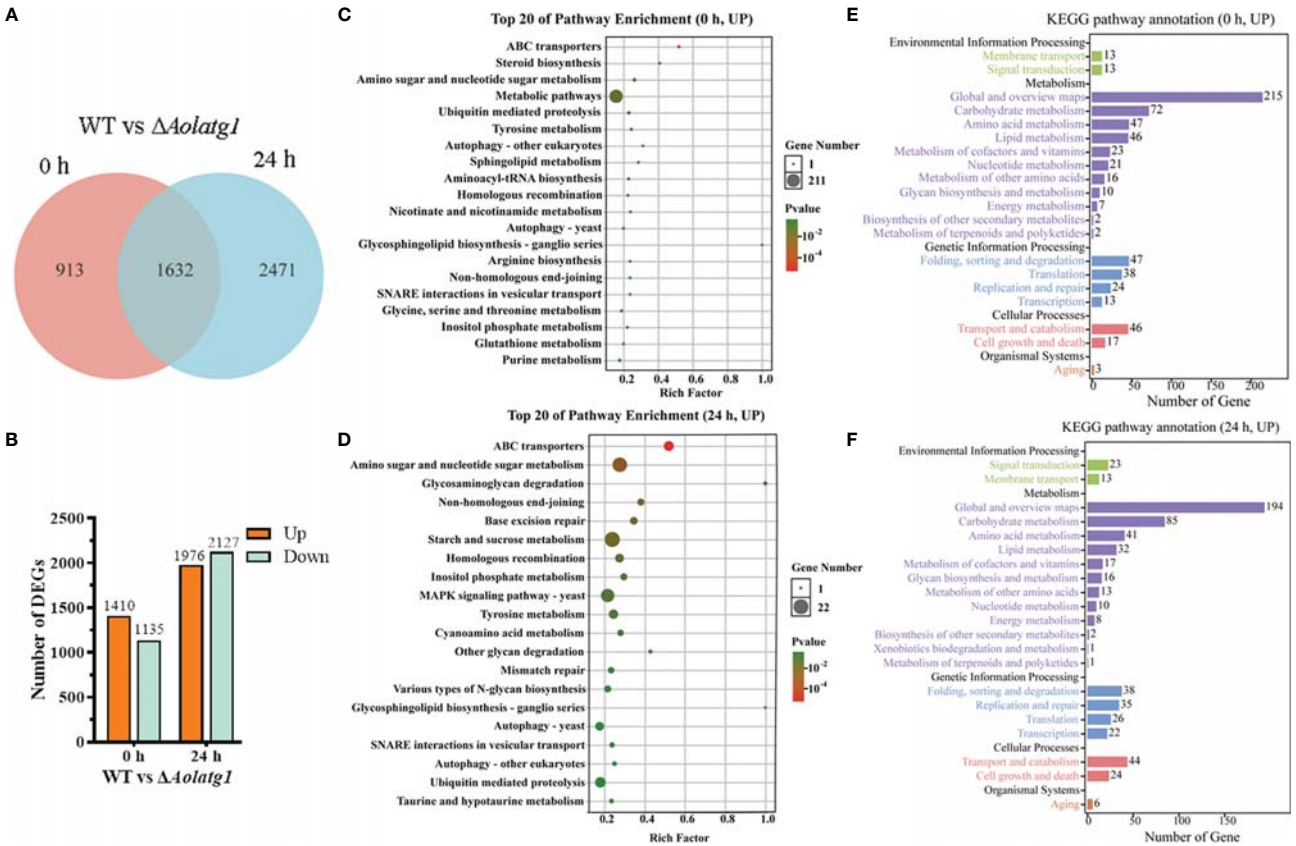


**FIGURE 6** | Comparison of differentially expressed genes (DEGs) between WT and the Δ*Aolatg1* mutant strain of *A. oligospora*. **(A)** Venn analysis of DEGs. **(B)** The number of upregulated and downregulated DEGs in the WT versus the Δ*Aolatg1* mutant strains. **(C)** The top 20 pathways that are upregulated in the WT strain compared to the Δ*Aolatg1* mutant strain at 0 h. **(D)** The top 20 pathways that are upregulated in the WT strain compared to the Δ*Aolatg1* mutant strain at 24 h. **(E)** Annotation of the upregulated KEGG pathways in the WT strain compared to the pathways in Δ*Aolatg1* mutant at 0 h. **(F)** Annotation of the upregulated KEGG pathways in the WT strain compared to the pathways in Δ*Aolatg1* mutant at 24 h.

them being fused (Ren et al., 2017). As in the Δ*Bcatg1* mutant of *B. cinerea*, the Δ*Mgatg1* mutant had sparse aerial hyphae on both CM and MM plates, although growth of its colonies was not affected (Liu et al., 2007). These findings suggest that orthologs of Atg1 play an essential role in mycelial development, especially in the growth of aerial hyphae, whereas Atg13 has only a minor role in these processes.

Deletion of *Aolatg1* caused a remarkable reduction in spore yield and spore germination rate, whereas deletion of *Aolatg13* did not affect the sporulation of *A. oligospora*. Transcription of several sporulation-related genes, such as *fluG*, *abaA*, *brlA*, and *velB*, was significantly downregulated in the Δ*Aolatg1* mutant compared to the WT strain on day 7, whereas in the Δ*Aolatg13* mutant, transcription levels of these genes showed no obvious change. Similarly, deletion of *Aoatg1* strongly inhibited the conidiation in *A. oryzae* (Yanagisawa et al., 2013), and the number of conidia was lower in the Δ*Aoatg13* mutant than in the WT strain (Kikuma and Kitamoto, 2011). In *B. cinerea*, the Δ*Bcatg1* mutant produced significantly fewer conidia than the WT strain, and most conidia showed an aberrant shape with many vacuoles (Ren et al., 2017). In *F. graminearum*, the conidiation was significantly reduced in the Δ*Fgatg1* and Δ*Fgatg13* mutants (Lv et al., 2017). Conidiogenesis in the Δ*Mgatg1* mutant was reduced significantly, and the conidia of Δ*Mgatg1* mutants germinated more slowly than those of the WT strain (Liu et al., 2007). Moreover, a mutant with disrupted *Bbatg1* had impaired conidial yield and conidial germination under starvation stress (Ying et al., 2016). These findings suggest that Atg1 plays a conserved and important role in the conidiation of many filamentous fungi, whereas the role of Atg13 in conidiation varies among fungi.

Apart from mycelial growth and conidiation, deletion of *Aolatg1* and *Aolatg13* impaired stress resistance, as the Δ*Aolatg1* and Δ*Aolatg13* mutants were more sensitive to oxidative stress caused by menadione and $H_2O_2$ than the WT strain. Furthermore, the Δ*Aolatg13* mutant was also sensitive to osmotic pressure (NaCl and sorbitol). It has been shown that deletion of *Aolatg4* and *Aolatg5* in *A. oligospora* also altered sensitivity to oxidative and osmotic stresses (Zhou et al., 2020; Zhou et al., 2021). In *B. bassiana*, the Δ*Bbatg8* mutant exhibited enhanced sensitivity to

**TABLE 1** | Transcriptional response to *Aolatg1* deletion by the genes involved in trap formation and oxidative stress response in comparative transcriptome analysis.

| Locus | Function annotation | Expressional levels | | | | References |
|---|---|---|---|---|---|---|
| | | TPM-0 h | | TPM-24 h | | |
| | | WT | Δ*Aolatg1* | WT | Δ*Aolatg13* | |
| **Genes involved in trap formation and pathogenicity** | | | | | | |
| AOL_s00215g516 | *flbA*, developmental regulator | 109.05 | 101.09 | 29.29 | 166.74 | (Ma et al., 2021) |
| AOL_S00215g7 | *ras2*, Ras family | 480.70 | 601.97 | 130.02 | 75.16 | (Yang et al., 2021) |
| AOL_s00054g446 | *rab-7A*, Rab small GTPase | 221.4 | 241.79 | 186.05 | 263.07 | (Yang et al., 2018) |
| AOL_s00054g68 | *glo3*, Arf GAP | 119.8 | 138 | 100.8 | 127.71 | (Ma et al., 2020) |
| AOL_s00110g154 | *fus3*, MAP kinase | 89.59 | 86.69 | 119.39 | 109.09 | (Chen et al., 2021) |
| AOL_s00109g23 | *hog1*, MAP kinase | 146.71 | 148.25 | 137.99 | 265.83 | (Kuo et al., 2020) |
| AOL_s00173g235 | *slt2*, MAP kinase | 169.76 | 150.98 | 191.17 | 186.58 | (Zhen et al., 2018) |
| AOL_s00083g25 | *stuA*, APSES transcription factor | 51.04 | 37.8 | 48.2 | 45.78 | (Xie et al., 2019) |
| AOL_s00007g534 | *atg8*, autophagy-related protein 8 | 769.28 | 2096.53 | 596.99 | 1493.93 | (Chen et al., 2013) |
| AOL_s00112g56 | *hex1*, woronin body major protein | 1590.13 | 2597.71 | 3231.56 | 2343.38 | (Liang et al., 2017) |
| AOL_s00080g296 | *ubr1*, E3 ubiquitin-protein ligase | 40.98 | 87.82 | 25.94 | 47.47 | (Zhang et al., 2021) |
| AOL_s00054g811 | *velB*, developmental regulator | 119.08 | 73.47 | 103.64 | 62.76 | (Zhang et al., 2019) |
| **Genes involved in oxidative stress response** | | | | | | |
| AOL_s00109g143 | *per*, peroxidase | 79.47 | 113.59 | 70.81 | 198.26 | (Zhu et al., 2013) |
| AOL_s00173g374 | *cat*, catalase | 304.47 | 146.51 | 355.82 | 304.33 | (Michán et al., 2003) |
| AOL_s00188g243 | *cat*, catalase | 1.19 | 0.25 | 1.49 | 0.26 | (Wang et al., 2007) |
| AOL_s00006g411 | *cat2*, catalase | 1.48 | 6.14 | 3.00 | 38.53 | (Sun et al., 2019) |
| AOL_s00193g69 | *nox-1*, NADPH oxidase | 120.89 | 178.27 | 67.51 | 250.78 | (Li et al., 2017) |
| AOL_s00007g557 | *nox-2*, NADPH oxidase | 38.43 | 53.72 | 81.44 | 96.65 | (Cano-Domínguez et al., 2008) |
| AOL_s00054g538 | *noxR*, NADPH oxidase regulator | 29.19 | 16.85 | 186.17 | 73.22 | (Sun et al., 2019) |
| AOL_s00007g292 | *sod*, superoxide dismutase | 53.73 | 48.84 | 34.25 | 63.31 | (Zhu et al., 2013) |
| AOL_s00054g687 | *sodB*, superoxide dismutase | 465.97 | 289.02 | 426.03 | 394.27 | (Zhu et al., 2013) |
| AOL_s00170g93 | *sod-2*, superoxide dismutase | 603.01 | 1206.07 | 359.61 | 2089.07 | (Zhu et al., 2013) |

*WT, wild-type strain; ΔAolatg1, Aolatg1 deletion mutant; -0 h, samples of the WT and ΔAolatg1 mutant strains in vegetative growth stage; -24 h, samples of the WT and ΔAolatg1 mutant strains after induced with nematodes for 24 h. Locus numbers and function were annotated according to the A. oligospora genome assembly (https://www.ncbi.nlm.nih.gov/). TPM, transcripts per kilobase million.*

oxidative stress, whereas the Δ*Bbatg1* mutant did not (Ying et al., 2016). These results show that the autophagy pathway is also involved in regulating the fungal stress response.

Several studies have established that the asexual development of fungi is correlated to oxidative stress response. This is validated by the observation that Δ*cat-3* mutant of *Neurospora crassa* produces six times more aerial hyphae and conidia compared to the WT strain (Michán et al., 2003). Moreover, the depletion of *cat-1* resulted in a significant reduction in the rate of conidial germination (Wang et al., 2007). Recently, a Zn(II)2Cys6-type transcription factor, ADA-6 was identified in *N. crassa*. Characterization of *ada-6* revealed that its deletion impaired conidial production and induced female sterility. In addition, RNA-seq analysis demonstrated that ADA-6 modulates the transcription of *cat-3* and other genes participating in the production of reactive oxygen species during conidiation (Sun et al., 2019). In this study we found that the expressions of several genes associated with oxidative stress response were altered. The *cat-2* and *sod-2* genes were significantly enhanced in the Δ*Aolatg1* mutant compared to the WT strain at 0 and 24 hpi. However, *noxR* and the other two *cat* genes were downregulated in the Δ*Aolatg1* mutant. These findings suggest that defect in conidiation of Δ*Aolatg1* mutant might be connected to the oxidative stress response.

Trap formation in NT fungi is a complex cellular process that was suppressed by deletion of Atg4, Atg5, and Atg8 orthologs, which suggested that autophagy plays an important role in trap development in *A. oligospora* (Chen et al., 2013; Zhou et al., 2020; Zhou et al., 2021). In this study, we characterized the role of *Aolatg1* and *Aolatg13* in trap formation and nematode predation. Our results showed that trap formation and nematicidal activity were remarkably decreased in the Δ*Aolatg1* mutant. Autophagy has been recently demonstrated to be closely associated with fungal virulence. For example, deletion of *Mgatg1* caused lower turgor pressure of the appressorium, and the Δ*Mgatg1* mutant lost its ability to penetrate and infect host plants (Liu et al., 2007). However, the lack of *Mgatg13* did not have any influence on the pathogenicity of *M. oryzae* (Kershaw and Talbot, 2009). In *F. graminearum*, the pathogenicity of the Δ*Fgatg1* and Δ*Fgatg13* mutants was lower than that of the WT strain, as these mutants had decreased abilities to infect wheat spikelets and to spread to new spikelets following the original infection (Lv et al., 2017). In *B. cinerea*, most conidia of the Δ*Bcatg1* mutant lost the capacity to form the appressorium infection structure and failed to penetrate the onion epidermis, and pathogenicity assays showed that the virulence of Δ*Bcatg1* tested in different host plant tissues was drastically impaired (Ren et al., 2017). Moreover, the virulence of the Δ*Bbatg1* mutant was considerably weaker than that of the WT strain, as indicated by lower infectivity in the topical and intrahemocoel injection assays (Ying et al., 2016). These findings suggest that Atg1 plays a conserved and crucial role in the virulence of many pathogenic

fungi, whereas Atg13 effect on virulence is prominent only in few fungi (e.g., *F. graminearum*).

Transcriptomic analysis showed that more DEGs were identified after the fungi were induced with nematodes compared to the transcriptomic differences between intact fungi. This finding suggests that many genes were mobilized during trap formation. In the GO analysis, the upregulated genes were highly enriched in the membrane-related terms at 0 h and 24 h, indicating that membrane trafficking plays a crucial role in autophagy, mycelial growth, and trap formation. In turn, the downregulated genes were enriched in catalytic activity at 0 h and in cellular components at 24 h, including ribosome, mitochondrion, and organelles, suggesting that there are multiple organelles involved in trap formation. In the KEGG analysis, the upregulated genes were highly enriched in metabolic pathways at 0 h; in contrast, except for metabolism, MAPK signaling pathways, autophagy, and autophagy-related processes such as ubiquitin-mediated proteolysis and ANARE interaction in vesicular transport were also enriched at 24 h. These findings suggest that MAPK pathways and autophagy play an important role in trap formation in *A. oligospora*. In fact, several MAPK signaling proteins have been proved to regulate trap formation in *A. oligospora*, such as Slt2 (Zhen et al., 2018; Xie et al., 2021), Hog1 (Chen et al., 2021), and Ime2 (Xie et al., 2020). Autophagy-related proteins such as AolAtg4 (Zhou et al., 2020), AolAtg5 (Zhou et al., 2021), and AolAtg8 (Chen et al., 2013) have also been shown to influence trap formation in *A. oligospora*, and deletion of *AolAtg1* significantly reduced of the number of traps. The set of downregulated genes was highly enriched in genes relevant to metabolic pathways and biosynthesis of secondary metabolites at 0 h and 24 h. Moreover, genes involved in lipid metabolism were enriched at 0 h and 24 h, suggesting that autophagy may regulate lipid metabolism. In *M. oryzae*, deletion of *the Mgatg1* gene influenced the number of lipid bodies, and lipid storage in conidia of the Δ*Mgatg5* mutant was lower than in the WT strain (Liu et al., 2007; Lu et al., 2009). Similarly, LDs accumulation was significantly reduced in the conidia of Δ*Bcatg1*, but the glycerol content was increased in Δ*Bcatg1* mutant (Ren et al., 2017). In addition, DEGs at 0 h and 24 h were enriched in genes involved in energy metabolism. Energy is required for trap development, as evidenced by the fact that deletion of the malate synthase gene led to a defect in trap formation (Zhao et al., 2014). Moreover, DEGs at 0 h and 24 h were enriched in genes relevant to metabolism of terpenoids and polyketides, which are involved in the biosynthesis of arthrobotrisins, a special group of metabolites identified in *A. oligospora* and other NT fungi (Anderson et al., 1995; Wei et al., 2011). Recently, 6-methylsalicylic acid, an intermediate in the biosynthesis of arthrosporols produced by NT fungi, was found to be a morphogen for spatiotemporal control of trap formation and a chemoattractant that lured *C. elegans* into fungal colonies (Yu et al., 2021). Therefore, transcriptomic analysis provides a good basis for understanding the mechanisms of mycelial growth, development, and pathogenicity.

G-protein signaling plays an indispensable role in trap formation of *A. oligospora*. There are several evidences corroborating this hypothesis, such as deletion of *flbA*, which encodes a regulator of G-protein signaling, abrogates trap formation in *A. oligospora* (Ma et al., 2021). Moreover, *hog1* deletion caused a reduction in trap formation and predation efficiency in *A. oligospora* (Kuo et al., 2020). In this study, we observed that the transcripts of *flbA* and *hog1* were markedly upregulated in Δ*Aolatg1* mutant when induced with nematodes. In addition, the expressions of *atg8* and *ubr1* were upregulated in Δ*Aolatg1* mutant during the stages of vegetative growth and trap formation. Atg8 has been shown to be indispensable for trap formation in *A. oligospora* (Chen et al., 2013). Additionally, the Δ*ubr1* mutant of *A. oligospora* exhibits a substantial reduction in vegetative growth and trap formation (Zhang et al., 2021). Therefore, according to these observations, it is evident that AolAtg1 regulates trap formation and pathogenicity of *A. oligospora* by promoting G-protein signaling and regulating protein ubiquitination.

At present, orthologs of Atg1 have been identified in many different fungi and shown to play conserved and indispensable roles in autophagy, mycelial growth, conidiation, lipid metabolism, and pathogenicity. In contrast, Atg13 has been identified only in a limited number of fungal species, and although it plays an essential role in autophagy, its deletion seemed to influence few if any phenotypic traits in most fungi, with the notable exception of *F. graminearum*. Here, we characterized Atg1 and Atg13 in *A. oligospora*, a typical NT fungus, and showed that AolAtg1 and AolAtg13 play crucial roles in autophagy, whereas their contributions to mycelial growth, conidiation, trap formation, and nematode predation are different. However, the underlying molecular mechanisms by which AolAtg1 and AolAtg13 regulate diverse phenotypes need to be further investigated using detailed comparative analysis of the transcriptome and by various other methods. Meanwhile, because of the lack of available resistance markers for *A. oligospora*, we failed to construct a double deletion mutant of *Aolatg1* and *Aolatg13*, which could help to understand the interaction between these two proteins in this fungus. Nonetheless, our results provide a solid basis for further investigation of the roles and regulatory mechanisms of *atg* genes in the growth, development, and pathogenicity of NT fungi.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA784322.

## AUTHOR CONTRIBUTIONS

JY and K-QZ conceived and designed the study. DZ and YZ conducted the experiments. DZ, YZ, NB, and MX analyzed the data. JY, DZ, and YZ wrote and revised the manuscript. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.824407/full#supplementary-material

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# Interferon Alpha Induces Cellular Autophagy and Modulates Hepatitis B Virus Replication

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Hepatitis B virus (HBV) infection causes acute and chronic liver diseases, including severe hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Interferon alpha 2a (IFNα-2a) is commonly used for treating chronic HBV infection. However, its efficacy remains relatively low. Yet, the immunological and molecular mechanisms for successful IFNα-2a treatment remain elusive. One issue is whether the application of increasing IFNα doses may modulate cellular processes and HBV replication in hepatic cells. In the present study, we focused on the interaction of IFNα signaling with other cellular signaling pathways and the consequence for HBV replication. The results showed that with the concentration of 6000 U/ml IFNα-2a treatment downregulated the activity of not only the Akt/mTOR signaling but also the AMPK signaling. Additionally, IFNα-2a treatment increased the formation of the autophagosomes by blocking autophagic degradation. Furthermore, IFNα-2a treatment inhibited the Akt/mTOR signaling and initiated autophagy under low and high glucose concentrations. In reverse, inhibition of autophagy using 3-methyladenine (3-MA) and glucose concentrations influenced the expression of IFNα-2a-induced ISG15 and IFITM1. Despite of ISGs induction, HBV replication and gene expression in HepG2.2.15 cells, a cell model with continuous HBV replication, were slightly increased at high doses of IFNα-2a. In conclusion, our study indicates that IFNα-2a treatment may interfere with multiple intracellular signaling pathways, facilitate autophagy initiation, and block autophagic degradation, thereby resulting in slightly enhanced HBV replication.

**Keywords: Hepatitis B virus, IFNα-2a, Akt/mTOR signaling, AMPK, autophagy**

## INTRODUCTION

Hepatitis B virus (HBV) infection causes acute and chronic infections that results in the death of over 887,000 people every year due to severe hepatitis, liver cirrhosis, and cancer despite of effective vaccination (Polaris Observatory, 2018). Current standard HBV therapeutic strategies include treatment using interferon alpha (IFNα) (Ferir et al., 2008) and nucleoside/nucleotide analogs (NAs) (Terrault et al., 2018). IFNα-2a is a cytokine with immunomodulatory and antiviral effects and is one of the first-line drugs for clinical treatment of chronic hepatitis B (CHB) (Cho and Kelsall, 2014). Nevertheless, the performance of IFNα-2a-based therapies has been limited, with a response rate of only approximately 30% (Ferir et al., 2008). The mechanisms underlying the limited response to IFNα in CHB patients are not completely understood.

The major human type I IFN includes three subtypes: α, β, and ω, primarily exerting antiviral, antiproliferative, and immunomodulatory functions (Wittling et al., 2020). IFNα binds to its specific receptors and activates intracellular signaling cascades (Pestka et al., 2004). A great number of cellular genes are up-or downregulated by IFNα and many of those so-called IFN-stimulated genes (ISGs) have antiviral functions (Schoggins and Rice, 2011; Schneider et al., 2014). The antiviral effect of ISGs in the context of HBV infection has been extensively studied over decades (Wieland et al., 2003). Overexpression of selected ISGs may lead to HBV suppression in cell models (Pei et al., 2014; Leong et al., 2016). Though many ISGs may contribute to suppression of HBV replication, their actions were rather ineffective (Rang et al., 2001; Kim et al., 2008). For example, IFIT1 and -2 are the most strongly upregulated genes, however, they rather play a role in limiting HBV replication at a high level (Pei et al., 2014). IFNα subtypes differ in their antiviral and immunomodulatory functions (Chen et al., 2021). IFNα-14 has been shown to be superior in HBV inhibition compared to other IFNα subtypes including IFNα-2a (Shen et al., 2018; Chen et al., 2021). Mechanistically, IFNα-14 is able to trigger a concerted IFNα and –γ response and presumably a broader antiviral activity in hepatocytes. Besides the JAK/STAT, which are called canonical signaling pathways (Mazewski et al., 2020), IFNα is able to modulate multiple intracellular signaling pathways through non-canonical, including MAP kinase and phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathways (Mazewski et al., 2020). Specifically, IFNα activates PI3K/Akt/mTOR signaling pathway (Uddin et al., 1995; Lekmine et al., 2004) and therefore initiates cellular autophagy.

Autophagy is an evolutionarily conserved cellular process that can be induced by various stimuli, including starvation, hypoxia, and treatment with cytokines (such as IFN and transforming growth factor (TGF)) (Lekmine et al., 2004; Schmid and Munz, 2007; Chang et al., 2010). Autophagy is initiated by mTOR or AMPK-ULK1 signaling to regulate ULK1-Atg13-FIP200 complex formation (Jung et al., 2009). Recent evidence suggests that type I IFN can induce autophagy by canonical and non-canonical pathways and participate in many pathological processes (Degtyarev et al., 2008; Zhu et al., 2013; Gu et al., 2017; Perot et al., 2018; Ma et al., 2019), which is correlated with cellular apoptosis and proliferation (Degtyarev et al., 2008; Zhu et al., 2013), cytokines networks (Gu et al., 2017; Ma et al., 2019), and antiviral activities (Perot et al., 2018).

Our and others' previous studies showed that autophagy plays a key role in HBV replication and pathogenesis (Tian et al., 2011; Lin et al., 2017). Therefore, it is important to investigate whether the intracellular crosstalk induced by IFNα-2a can enhance cellular autophagy and change HBV replication in hepatic cells. In the present study, we tested the changes in intracellular signaling pathways and HBV replication after IFNα-2a treatment in hepatoma cells, HepG2.2.15 and Huh7 cells, which are commonly used cell models for HBV replication, and primary human hepatocytes (PHHs). The results illustrate that IFNα-2a induced autophagy and blocked autophagic degradation, resulting in a slight increase in HBV replication.

## MATERIALS AND METHODS

### Cell Culture

All the cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. The HBV-producing HepG2.2.15 hepatoma cell line, which harbors the integrated HBV genomic dimers (Sells et al., 1987) (provided by American Type Culture Collection), was cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and 1% nonessential amino acids (NEAAs), 1% HEPES, and 500 μg/ml G418 (Merck Millipore). Different concentrations of glucose were used in HepG2.2.15 culture medium, based on glucose-free RPMI-1640 medium (11879020; Gibco), supplemented with the indicated concentrations of glucose (5 and 25 mM), 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 1% NEAAs, 1% HEPES, and 500 μg/ml G418 (Merck Millipore). Huh7 cells were cultured in DMEM medium, supplemented with 10% inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 1% NEAA, and 1% HEPES. Primary human hepatocytes (PHHs) were cultured in Williams E medium, supplemented with 250 μL insulin, 2% DMSO, and 125 μL hydrocortisone hemisuccinate. The HBV particles harvested from HepG2.117 cells were used for PHHs infection. For HBV infection, as described previously (Wan et al., 2017). PHHs were cultured in primary hepatocyte maintenance medium (PMM) for 24 hours and then inoculated with a 30 multiplicity of genome equivalents of HBV in PMM with 4% PEG 8000 at 37°C for about 24 hours. One day after infection, the cells were washed with warm PBS three times to remove residual viral particles and refreshed the PMM medium containing 2% DMSO and treated with the indicated concentrations of IFNα-2a. The medium was refreshed every other day.

### Chemical Reagents

Human interferon alpha 2a (11100) was purchased from PBL assay science. Glucose (G8270), chloroquine (C6628), insulin (I-034), and rapamycin (R8781) were purchased from Sigma-Aldrich. MHY1485 (S7811) and AICAR (S1802) were purchased from Selleck Chemicals.

**Abbreviations:** HBV, Hepatitis B virus; ISGs, IFN-stimulated genes; ISG15, Interferon Stimulated Gene 15; IFITM, interferon-induced transmembrane proteins; LC3, microtubule-associated protein 1 light chain 3 beta.

## Western Blotting Assay

Western blotting assay was carried out as described previously (Lin et al., 2020). Antibodies we used in the experiment were as following: anti-AMPK (2532S), anti-phospho-AMPK (2531S), anti-Akt (9272S), anti-phospho-Akt (Ser473; 9271S), anti-mTOR (2972S), anti-phospho-mTOR (2971S), anti-p70 S6K (9202S), anti-phosphop70 S6K (S371; 9234S), anti-p62 (5114S), anti-LC3B (3868S), anti-IFITM1 (13126S), anti-ISG15 (2758S) and anti-beta-actin (Sigma, A5441). The signals were visualized with an enhanced chemiluminescence (ECL) system (GE Healthcare, UK). The relative levels of indicated proteins were determined by quantifying the gray scales of bands using ImageJ software, using beta-actin as a loading control.

## Immunofluorescence Staining

HepG2.2.15 cells were grown on coverslips and treated as indicated in each experiment. After treatment, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Then, the cells were incubated with anti-LC3B antibodies, staining with Alexa Fluor 488-conjugated Goat anti-Rabbit IgG (H + L). The nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI). The distribution of LC3B protein was visualized with a confocal microscope (LSM710, Carl Zeiss). For quantification the number of LC3B puncta in cells, around 3 cells were recorded and analyzed by ImageJ software.

## Dye Quenched-Bovine Serum Albumin (DQ-BSA) Degradation Assay

HepG2.2.15 cells were grown on coverslips and treated as indicated in each experiment. After treatment, cells were incubated with DQ Red BSA (10 μg/mL; Invitrogen, D-12051) for 30 minutes. The fluorescent signal produced by lysosomal proteolysis of DQ Red BSA was quantified with an LSM 710 confocal microscope (Zeiss, Germany).

## Detection of HBV Gene Expression and Replication

The levels of HBsAg and HBeAg in cell lysates and in the supernatant were measured by chemiluminescent microparticle immunoassay (CMIA, Abbott Laboratories, Chicago, IL, USA). HBV replicative intermediates (RIs) from intracellular core particles were extracted and detected by Southern blotting as described previously (Guan et al., 2007).

## Statistical Analyses

All experiments were repeated independently at least 3 times. The data were expressed as the means ± SEM. Data were analyzed for statistical significance by ANOVA with two-tailed Student's t test or by one-way ANOVA with a Tukey post-test using Prism 8 software. $p < 0.05$ was considered significant and indicated by asterisks.

# RESULTS

## IFNα-2a Interferes Intracellular Signal Crosstalk

Previously, it has been shown that IFNα-2a was able to interfere with multiple cellular signalings. We firstly asked whether IFNα-2a interferes with Akt/mTOR and AMPK activities. HepG2.2.15 cells were treated with IFNα-2a at different concentrations (1000 U/ml and 6000 U/ml), and harvested after 48 hr. The baseline expressions of total AMPK, Akt, mTOR, and their phosphorylated forms were detected by western blotting. As the **Figure 1A** shows, the levels of phosphorylated Akt and mTOR were decreased with the increasing IFNα-2a concentrations. Interestingly, the levels of total and phosphorylated AMPK were decreased (**Figure 1B**). These data indicate that IFNα-2a attenuates Akt/mTOR signaling and AMPK signaling.

Previous studies have shown that insulin physiologically activates the Akt/mTOR pathway (Molinaro et al., 2019). Insulin was added into the culture medium. Indeed, the levels of phosphorylated Akt and mTOR were increased after insulin treatment. Simultaneous addition of 6000 U/ml IFNα-2a counteracted the increased activities of Akt and mTOR stimulated by insulin (**Figure 1C**), confirming the suppressive effect of IFNα-2a on Akt/mTOR signaling.

To further examine the effect of IFNα-2a on mTOR signaling pathway, HepG2.2.15 cells were treated with 6000 U/ml of IFNα-2a combined either with rapamycin, an inhibitor of mTOR, or MHY1485, an agonist of mTOR, respectively. The results showed that the level of phosphorylated mTOR decreased after rapamycin treatment and was further reduced following the combined treatment with IFNα-2a and rapamycin. On the other hand, the inhibitory effect of IFNα-2a on mTOR phosphorylation was attenuated in cells co-treated with MHY1485 (**Figure 1D**). These results confirm that IFNα-2a treatment inhibits the mTOR signaling pathway in hepatoma cells.

IFNα-2a treatment resulted in a significant decrease of AMPK phosphorylation at the baseline expression. When HepG2.2.15 cells were grown in the presence of AICAR, an AMPK agonist, for 48 hr, AMPK phosphorylation markedly increased. Again, IFNα-2a co-treatment abolished increased phosphorylation of AMPK by AICAR activation (**Figure 1E**). Thus, IFNα-2a treatment also diminishes AMPK signaling in hepatoma cells.

Besides the hepatoma cells, we also used PHHs in our experiment. However, uninfected PHHs had initially very low levels of phosphorylated Akt and mTOR. PHHs were incubated in medium and treated with indicated concentrations of IFNα-2a (1000 U/ml and 6000 U/ml) for 48 hr. Differed from HepG2.2.15 cells, IFNα-2a treatment caused increased levels of phosphorylated Akt, mTOR, and AMPK in PHHs (**Figure 1F**). HBV-infected PHHs cell model is a transient and *de novo* infection model and is notably different from the stable HBV-transfected cells, such as HepG2.2.15 cells.

## IFNα-2a Induces Autophagy Through Inhibiting Akt/mTOR Pathway and Blocks Autophagic Degradation in Hepatoma Cells

The Akt/mTOR signaling pathway is known to regulate autophagy (Degtyarev et al., 2008; Jung et al., 2009). Thus, we addressed the question whether IFNα-2a treatment changes the autophagic activity in hepatoma cells. The levels of LC3II after IFNα-2a treatment were determined using IF staining and western blotting. IF staining showed that the numbers of endogenous

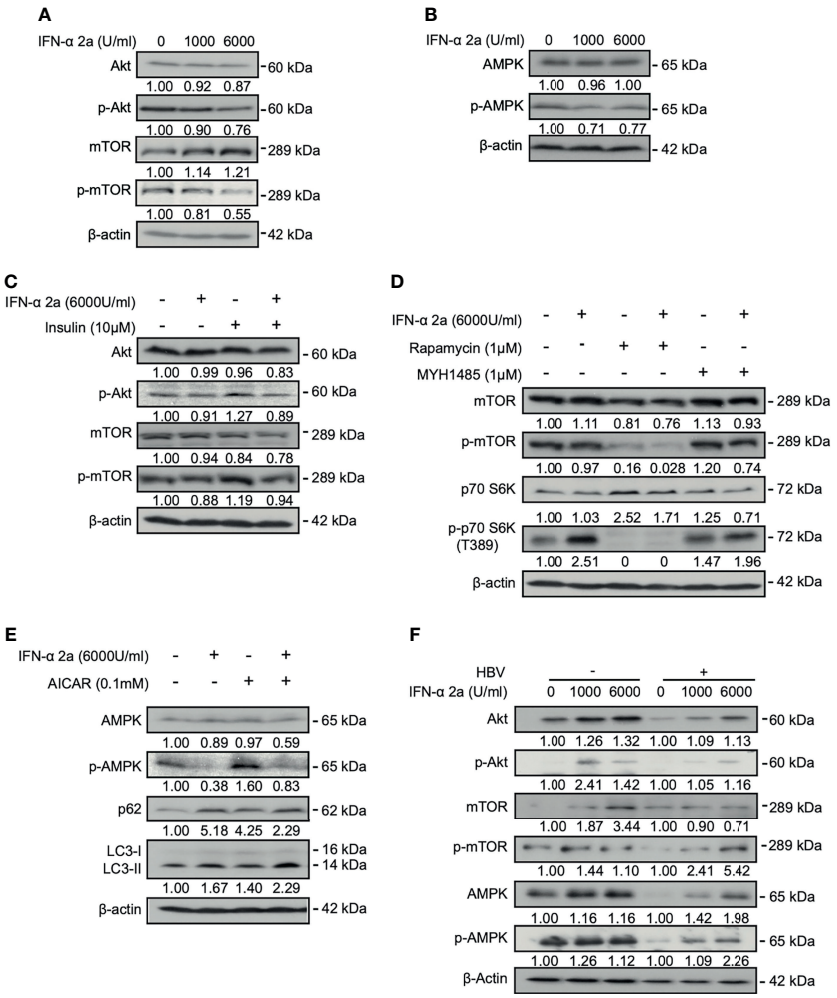


**FIGURE 1** | IFNα-2a interferes with intracellular signal crosstalk. **(A, B)** HepG2.2.15 cells were treated with the indicated concentrations of IFNα-2a and harvested after 48 h. The total and phosphorylated Akt, mTOR, and AMPK were detected by western blotting and their relative levels were determined by quantifying the gray scales of bands, using beta-actin as a loading control. **(C)** HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a in combination with 10 μM insulin for 48 hr. The total and phosphorylated Akt, and mTOR were detected by western blotting and their relative levels were determined by quantifying the gray scales of bands, using beta-actin as a loading control. **(D)** HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a with or without 1 μM MTOR inhibitor rapamycin or activator MHY1485 for 48 h The total and phosphorylated mTOR and p-p70 S6K were detected by western blotting and their relative levels were determined by quantifying the gray scales of bands, using beta-actin as a loading control. **(E)** HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a with or without AMPK activator 0.1 mM AICAR for 48 hr. AMPK, phosphorylated AMPK, p62 and LC3 were analyzed by western blotting, using beta-actin as a loading control. **(F)** PHHs were infected with HBV virions (multiplicity of infection = 30). 4 days post infection, PHHs were treated with IFNα-2a twice (1000 U/ml and 6000 U/ml). After 48 hr, The total and phosphorylated Akt, mTOR, and AMPK were detected by western blotting and their relative levels were determined by quantifying the gray scales of bands, using beta-actin as a loading control.

LC3-positive autophagic puncta increased in IFNα-2a-treated cells (**Figure 2A**). Consistently, the IFNα-2a treatment also markedly increased the levels of LC3II, as shown by western blotting analysis (**Figure 2B**). Next, HepG2.2.15 cells were cultured in the presence of rapamycin with and without IFNα-2a, respectively. The level of LC3II was higher after rapamycin treatment and further increased by IFNα-2a co-treatment. Interestingly, the level of p62 also increased markedly after IFNα-2a treatment, similar to chloroquine (CQ) treatment. These results indicate that IFNα-2a treatment blocked autophagic degradation and led to accumulation of LC3 puncta. To confirm this assumption, HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a for 48 hr and then incubated with dye quenched-bovine serum albumin (DQ-BSA) for 30 min. Cells cultured in Earle's balanced salt solution (EBSS) was used as a positive control and CQ treatment as a negative control. Fluorescence microscopic analysis showed that the fluorescent signal of DQ-BSA decreased upon IFNα-2a treatment, while increased upon incubation with Earle's balanced salt solution (EBSS), suggesting that IFNα-2a treatment blocks autophagic degradation in hepatoma cells (**Figure 2C**).

Next, we explored whether IFNα-2a treatment induce autophagy in PHHs. Interestingly, consistent with the previous

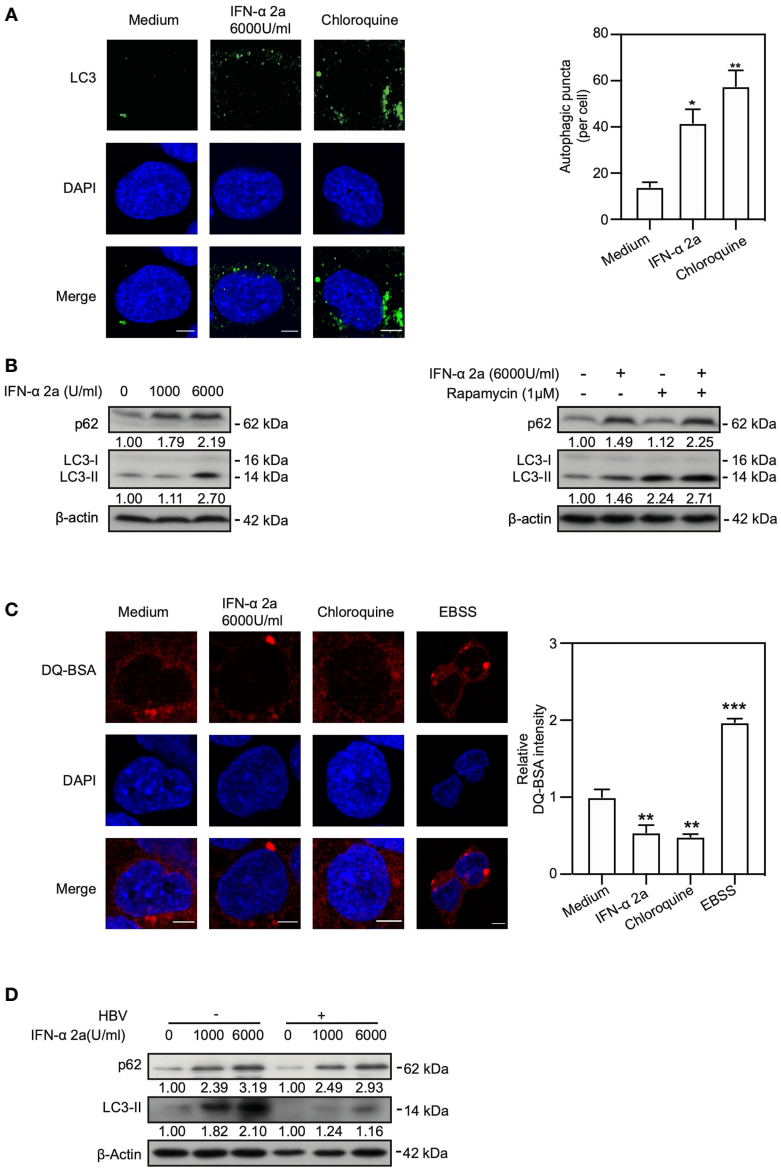


**FIGURE 2** | IFNα-2a induces autophagy through inhibiting Akt/mTOR pathway and blocks autophagic degradation. **(A)** HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a. After 48 hr, cells were fixed, incubated with rabbit anti-LC3 antibodies, followed by staining with Alexa Fluor 488-conjugated anti-rabbit secondary antibody IgG. Finally, the distribution of LC3 was imaged by confocal microscopy. Cells treated with 10 nM chloroquine (CQ) were used as a positive control. LC3 puncta in cells were quantified as described previously (Lin et al., 2020). Scale bar: 5 μm. **(B)** HepG2.2.15 cells were treated with the indicated concentrations of IFNα-2a and harvested after 48 hr. HepG2.2.15 cells were cultured in the medium either with 6000 U/ml IFNα-2a or rapamycin (1 μM) for 48 hr. p62 and LC3 were analyzed by western blotting using beta-actin as a loading control. The relative levels were determined by quantifying the gray scales of bands, using beta-actin as a loading control. **(C)** HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a for 48 hr, followed by incubation with 10 μg/mL DQ-BSA for 30 minutes. The fluorescent signal of DQ-BSA was detected by confocal microscopy. Cells cultured with EBSS for 2 hours were used as a positive control and CQ treatment was as a negative control. Scale bar: 5 μm. **(D)** PHHs were infected with HBV virions (multiplicity of infection = 30). 4 days post infection, PHHs were treated with IFNα-2a twice (1000 U/ml and 6000 U/ml). After 48 hr, p62 and LC3 were analyzed by western blotting using beta-actin as a loading control. *P < 0.05; **P < 0.01; ***P < 0.001.

results, the levels of p62 and LC3 were upregulated after being posed to IFNα-2a, indicating an increased level of autophagy and a decreased autophagic degradation in PHHs (**Figure 2D**).

Taken together, these results demonstrate that IFNα-2a treatment promotes the formation of autophagosomes and blocks the autophagic degradation in lysosomes.

## IFNα-2a Inhibits Akt/mTOR Activation and Enhances Autophagy Independently on Glucose Concentrations

Cellular energy metabolism can modulate cellular autophagy (Meijer and Codogno, 2011). Our previous study showed that high glucose concentration reduced autophagy by activating Akt/

mTOR pathway. On the contrary, autophagy is enhanced by activation of AMPK and ULK1 and inhibition of Akt, mTOR and p70 S6K at low glucose concentration (Wang et al., 2020). Therefore, we asked whether glucose concentrations also influence IFNα-2a-mediated Akt/mTOR inhibition and enhancement of autophagy. HepG2.2.15 cells were cultured with the indicated glucose concentrations (5 mM and 25 mM, later called low glucose and high glucose conditions, respectively) and treated with 6000 U/ml IFNα-2a for 48 hr. Western blotting was used to detect the levels of total Akt, mTOR, and AMPK proteins and their phosphorylated forms. Consistent with our previous results, the levels of the phosphorylated Akt and mTOR were increased under the high glucose condition (**Figures 3A** and **S1**) but decreased in hepatoma cells in the presence of IFNα-2a in hepatoma cells. In addition, AMPK was inactivated under the high glucose condition and much less active when additionally treated with IFNα-2a (**Figure 3B**). These data suggest that IFNα-2a-mediated Akt/mTOR inhibition occurred independently on glucose concentrations.

Consistently, independent of the glucose concentrations, the levels of LC3II and p62 and the numbers of LC3-positive autophagic puncta increase in IFNα-2a-treated HepG2.2.15 cells (**Figures 3C, D**) and Huh7 cells (**Figure S1**).

Taken together, IFNα-2a inhibits Akt/mTOR activation and enhances autophagy independently on glucose concentrations in hepatoma cells.

## IFNα-2a Induced ISGs Expression Is Dependent on Autophagy and Glucose

Interferon-stimulated 15 kDa protein (ISG15) and interferon-induced transmembrane protein1 (IFITM1) are the typical antiviral proteins under the control of IFN signaling and highly expressed after IFN stimulation. IFN-α treatment activates the JAK–STAT pathway and upregulates the expression of ISGs (Darnell et al., 1994; van Boxel-Dezaire et al., 2006). Activation of the JAK-STAT pathway could be verified by the detection of increased levels of STAT1 and phosphorylated STAT1 in our cell model (**Figure S2**). Further, emerged evidence implicated that PI3K/Akt/mTOR pathway is also involved in the induction of ISGs (Kaur et al., 2008; Kaur et al., 2012). We asked whether PI3K/Akt/mTOR signal pathways-related autophagy may also participate in the regulation of ISG15 and IFITM1 expression. Western blotting analysis showed that the expressions of ISG15 and IFITM1 were upregulated after IFNα-2a treatment (**Figure 4A**). When using an autophagy inhibitor 3-Methyladenine (3-MA), ISG15 and IFITM1 were reduced (**Figure 4B**), suggesting a positive effect of autophagy on ISG15 and IFITM1 induction.

As glucose can modulate Akt/mTOR/ULK1 signaling pathway and regulate autophagy (Wang et al., 2020), we tested whether glucose-mediated autophagy also influences ISG15 and IFITM1 expression. HepG2.2.15 cells were cultured in medium with low and high glucose concentrations, respectively, and then treated with IFNα-2a. Interestingly, IFNα-2a differently promoted ISG15 and IFITM1 expression at high glucose concentration, if compared with their corresponding expression levels at the low glucose concentration (**Figure 4C**).

Moreover, HepG2.2.15 cells were treated with 3-MA at the indicated glucose concentrations, combined with IFNα-2a treatment. Consistently, both the expressions of ISG15 and IFITM1 were significantly suppressed by the co-treatment of 3-MA and IFNα-2a. It can be further verified ISG15 and IFITM1 expression were dependent on autophagy. These findings illustrate that IFNα-2a-induced autophagy and glucose concentrations influence ISGs expression, however, the accurate mechanisms need to be investigated in the future.

## High IFNα-2a Concentrations Do Not Inhibit HBV Replication and Gene Expression in Hepatoma Cells

Cellular autophagy is an important process for HBV life cycle by regulating HBV transcription, assembly, and release (Lin et al., 2017; Lin et al., 2020). Thus, we explored whether IFNα-2a-induced autophagy has an impact on HBV replication. PHHs were infected with HBV and treated with IFNα-2a post infection. The levels of HBsAg and HBeAg in the culture supernatants were detected by CIMA. After the treatment at the concentration of 6000 U/ml IFNα-2a, the HBsAg increased significantly (**Figure 5**). A short-term (24 h) IFNα-2a treatment of transiently pSM2-transfected Huh7 did not significantly change the HBsAg production, likely due to the short time period (**Figure S3A**).

HepG2.2.15 cells were treated with IFNα-2a at different concentrations (1000 U/ml and 6000 U/ml), and harvested after 72 hr. The levels of secreted and intracellular HBsAg and HBeAg were measured by chemiluminescent microparticle immunoassay. HBV replicative intermediates (RIs) were detected by Southern blotting. The levels of secreted and intracellular HBsAg but not HBeAg as well as the amount of HBV RIs increased after IFNα-2a treatment (**Figure 6A**).

These data suggest that IFNα-2a has a poor antiviral response in HepG2.2.15 cells and infected PHHs, and has a slight but measurable virus-promoting effect at high concentrations.

Then, to test whether IFNα-2a-mediated enhancement of HBV replication is related to autophagy, HepG2.2.15 cells were co-treated with 3-MA and IFNα-2a. 3-MA clearly blocked the positive effect of IFNα-2a on HBsAg production and secretion (**Figure 6B**).

Next, HepG2.2.15 cells were grown at the indicated glucose concentrations and treated with IFNα-2a. The results showed that the secreted and intracellular HBsAg increased after IFNα-2a treatment both at low and high glucose conditions (**Figure 6C**). The amount of HBV RIs decreased at the high glucose concentration, but it increased after IFNα-2a treatment independent on glucose concentrations (**Figure 6D**). Consistently, the secreted and intracellular HBsAg decreased after co-treatment with 3-MA (**Figure 6E**). All these results indicate that IFNα-2a enhances HBV replication at a high dose and this enhancement is dependent on autophagy.

# DISCUSSION

In the present study, we demonstrated that IFNα-2a interferes with multiple intracellular signaling pathways, including

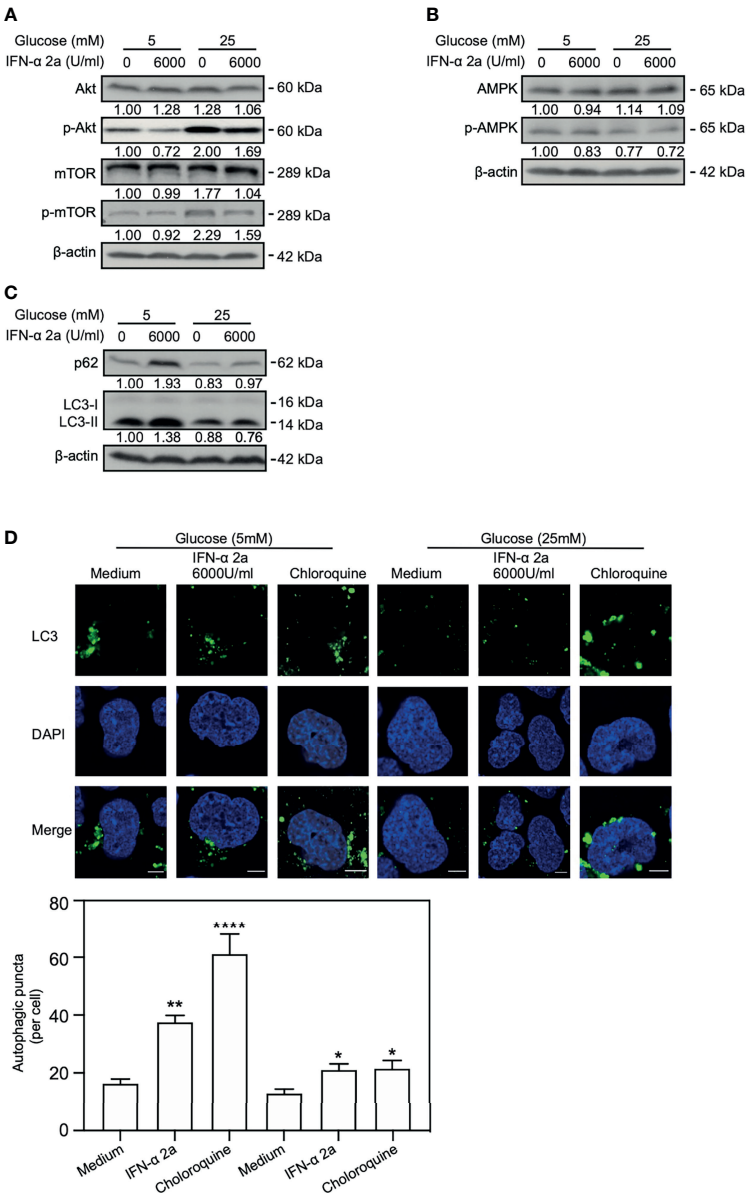


**FIGURE 3** | IFNα-2a inhibits Akt/mTOR activation and enhances autophagy independently on glucose concentrations. HepG2.2.15 cells were cultured in medium with the indicated glucose concentrations (5 and 25 mM) with or without 6000U/ml IFNα-2a and harvested after 48 hr. **(A)** The total and phosphorylated Akt, mTOR, **(B)** AMPK and **(C)** p62 and LC3 were detected by western blotting and their relative levels were determined by quantifying the gray scales of bands, using beta-actin as a loading control. **(D)** The cells were fixed, incubated with rabbit anti-LC3 antibodies, followed by staining with Alexa Fluor 488-conjugated anti-rabbit secondary antibody IgG. Finally, the distribution of LC3 was imaged by confocal microscopy. The results presented in the graphs were calculated from at least five cells. Scale bar: 5 μm. *P < 0.05; **P < 0.01; ****P < 0.001.

inhibiting Akt/mTOR and AMPK signaling pathways, promoting the autophagosomes formation, and blocking autophagic degradation. This action of IFNα-2a resulted in enhanced HBV replication. Additionally, the induction of ISGs, ISG15 and IFITM1 by IFNα-2a is dependent on Akt/mTOR signaling and autophagy, as examined in the present study.

Type I IFN (IFN-I) can modulate JAK-STAT and PI3K/Akt/mTOR pathways, which induce autophagy and drive downstream biological activities. IFNα induces rapid tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1) and then activates PI3'-kinase was firstly demonstrated in U-266 and in Daudi cells (Uddin et al., 1995). Lekmine et al. (2004) found that type I IFN activated the proteins downstream of PI3K pathway including p70 S6K and 4E-BP1. Schmeisser et al. confirmed that type I IFN induces autophagy and particularly, the autophagosomes were induced by type I IFNs in Daudi and

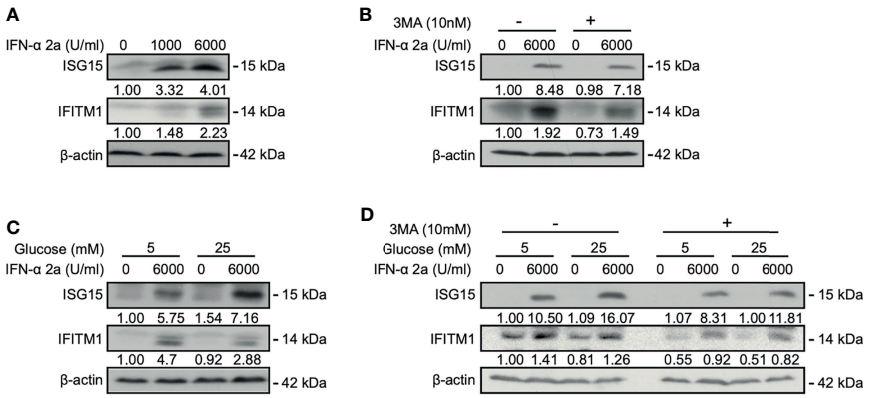


**FIGURE 4** | IFNα-2a induced ISGs expression is dependent on autophagy and glucose concentrations. **(A)** HepG2.2.15 cells were treated with the indicated concentrations of IFNα-2a and harvested after 48 hr. **(B)** HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a with or without 10 nM 3-MA and harvested after 48 hr. **(C)** HepG2.2.15 cells were cultured in medium with the indicated glucose concentrations (5 and 25 mM) with or without 6000 U/ml IFNα-2a and harvested after 48 hr. **(D)** HepG2.2.15 cells were cultured at the indicated glucose concentrations and 6000 U/ml IFNα-2a with or without 10 nM 3-MA. The levels of ISG15 and IFITM1 were detected by western blotting, and their relative levels were determined by quantifying the gray scales of bands, using beta-actin as a loading control.

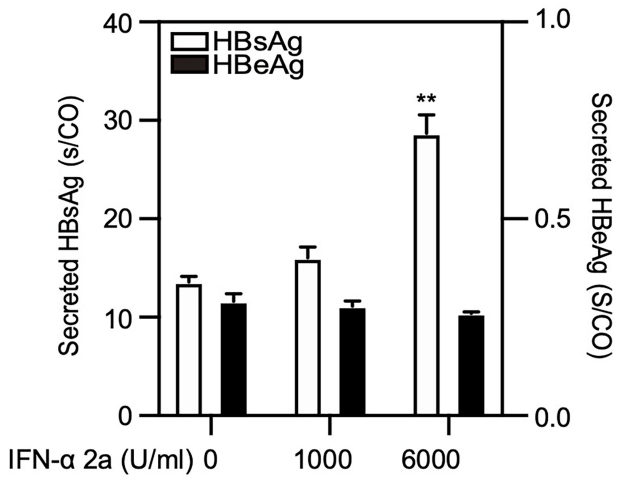


**FIGURE 5** | High IFNα-2a concentrations promote the yield of HBsAg in PHHs. PHHs were infected with HBV virions (multiplicity of infection = 30). 4 days post infection, PHHs were treated with IFNα-2a twice (1000 U/ml and 6000 U/ml). After 48 hr, the HBsAg and HBeAg levels in the culture supernatants were harvested and quantified by chemiluminescent microparticle immunoassay (CMIA). $^{**}P < 0.01$.

T98G cells as shown by electron microscopy (Schmeisser et al., 2013). IFNs induces autophagy through IGF1–PI3K/Akt/mTOR signaling pathways and participates in controlling tumor growth, inflammatory reactions and antiviral activities (Degtyarev et al., 2008; Gu et al., 2017; Perot et al., 2018; Ma et al., 2019). Leukocyte IFN failed to increase autophagosomes formation after JAK1 or STAT1 knockout, which highlighted the role of JAK/STAT pathway in IFN-α-induced autophagy in antitumor activity (Zhu et al., 2013). Therefore, IFNα triggers autophagy *via* both canonical and non-canonical signaling pathways and may contribute to the outcome of IFNα treatment in different diseases. Our results verified that IFNα-2a induces autophagy by Akt/mTOR in hepatic cells.

In our study, as shown in **Figures 1** and **3**, IFNα-2a treatment decreased the phosphorylation of Akt/mTOR and increased the autophagosome formation in hepatocytes. IFNα-2a downregulated the phosphorated form of Akt, and it cannot be recovered by insulin-mediated Akt activation, suggesting that IFNα-2a may also interfere with some other upstream proteins expression and functions.

Additionally, cellular autophagy process can be modulated by different glucose concentrations through AMPK signaling pathways (Wang et al., 2020). Low cellular energy metabolism activates AMPK and initiates autophagy. In our study, even though IFNα-2a treatment downregulated the phosphorylated form of AMPK, it did not interfere with LC3II expression. In addition, using AICAR to upregulate the activity of AMPK increased the expression of LC3II in IFNα-2a and AICAR-cotreated cells. Thus, IFNα-2a may also significantly disturb AMPK phosphorylation in HepG2.2.15 cells. Besides, blocking autophagic degradation by suppressing lysosomal acidification can promote the expression of LC3 (Lin et al., 2020). In this study, the expression of p62, the cargo for autophagic degradation, was increased in IFNα-2a-treated cells. Consistently, the DQ-BSA assay indicated reduced autophagic degradation after IFNα-2a treatment, leading to the accumulation of autophagosomes.

However, the situation in PHHs was completely different. Since PHHs are not growing cells when cultured in the medium, thus with very low basal levels of phosphorylated mTOR and Akt. The levels of phosphorylated Akt and mTOR increased rapidly in response to stimulation of different doses of IFNα-2a, indicating that IFN activated the non-canonical pathways. Upon HBV infection, the levels of phosphorylated Akt and mTOR are increased accompanied with IFNα-2a treatment. Several studies indicated that HBV infection leads to the accumulation of HBsAg in the endoplasmic reticulum and thereby activates the PI3K/Akt/mTOR pathway (Yang et al., 2009). We previously

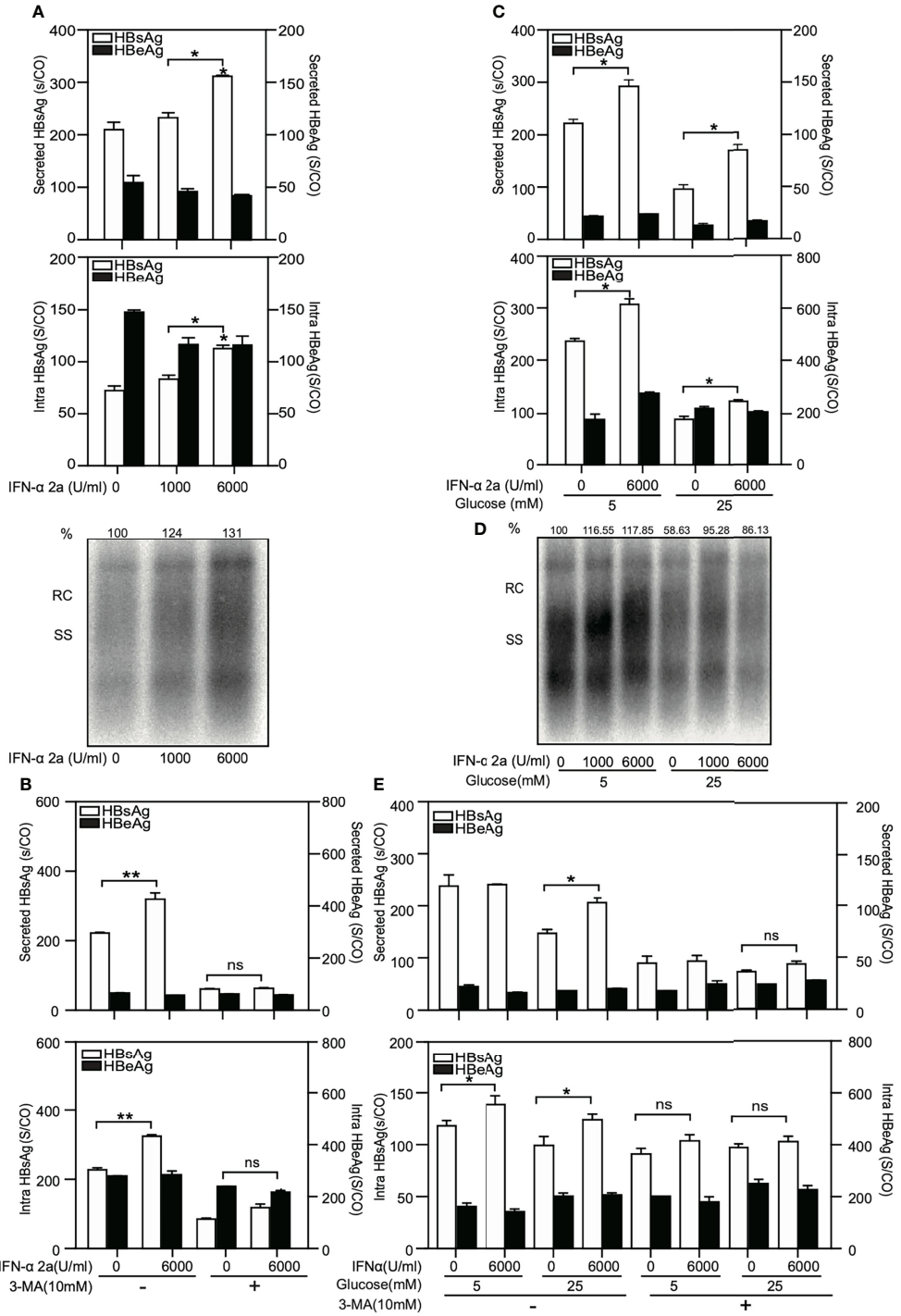


**FIGURE 6** | High IFNα-2a concentrations do not inhibit HBV replication and gene expression in HepG2.2.15 cells. **(A)** HepG2.2.15 cells were cultured in medium with the indicated concentrations of IFNα-2a. **(B)** HepG2.2.15 cells were treated with 6000 U/ml IFNα-2a with or without 10 nM 3-MA. **(C)** HepG2.2.15 cells were cultured in medium with the indicated glucose concentrations (5 and 25 mM) and treated with 6000 U/ml IFNα-2a. After 72 hr, cells were harvested and the HBsAg and HBeAg levels in the culture supernatants and intracellular HBsAg and HBeAg from cell lysates were quantified by chemiluminescent microparticle immunoassay Å(CMIA). **(D)** HepG2.2.15 cells were cultured at the indicated glucose concentrations (5 and 25 mM) and IFNα-2a (1000 U/ml and 6000 U/ml). Encapsidated HBV RIs were detected by Southern blotting. **(E)** HepG2.2.15 cells were cultured at the indicated glucose concentrations (5 and 25 mM) and 6000 U/ml IFNα-2a with or without 10 nM 3-MA. After 72 hr, cells were harvested and the HBsAg and HBeAg levels in the culture supernatants and intracellular HBsAg and HBeAg from cell lysates were quantified by chemiluminescent microparticle immunoassay (CMIA). *P < 0.05; **P < 0.01; ns, not significant; RC, relaxed circular DNA; S/CO, signal to cutoff ratio; SS, single-stranded DNA.

reviewed that mTOR pathway is a central regulator of cell growth, metabolism, proliferation, survival and autophagy and how this pathway is regulated in HBV infection (Wang et al., 2021). AMPK is a sensitive indicator of the cytosolic AMP/ATP ratio (Towler and Hardie, 2007). Treatment with interferons may cause a reduction in cellular ATP levels (Lewis et al., 1996), thus, IFN-α 2a likely induces an increased level of phosphorylated AMPK indirectly through changes in the AMP/ATP ratio in PHHs. Our previous study revealed AMPK positively regulates autophagy and thereby increased HBV replication in PHHs (Wang et al., 2020), which is consistent with this finding in IFNα-2a treated PHHs. Overall, naïve or transiently infected PHHs differently respond to IFNα treatment than persistently infected cells with high levels of HBV replication and gene expression.

Previously, the antiviral activity against IFNα-2a was tested in different cell systems (Hayashi and Koike, 1989; Caselmann et al., 1992; Rang et al., 2001; Shen et al., 2018; Chen et al., 2021). IFNα subtype 14 showed a higher potency to reduce HBV replication by simultaneously eliciting IFN-α and -γ signaling in PHHs. Thus, type I IFNs are able to modulate other cellular signaling pathways and thereby exert antiviral activities.

A relatively high dose of IFNα-2a was required to suppress HBV replication in the primary human hepatocytes (PHHs) (Chen et al., 2021). In hepatoma cells, HBV suppression could be achieved if the cells were treated with IFNα-2a prior to or early after transfection with replication-competent HBV genomes (Rang et al., 1999). This is reproducible in our own experiments (data not shown). Moreover, suppression of established HBV replication in hepatic cells by IFNα-2a is rather ineffective (Hayashi and Koike, 1989; Caselmann et al., 1992). In our cell culture system, HBV replication was slightly enhanced when treated with 1000 or 6000 U/ml of IFNα-2a. We also attempted to achieve stronger antiviral activities by increasing IFNα-2a concentrations up to 24000 U/ml to HepG2.2.15 cells. The high doses of IFNα-2a increased ISGs expression but did not reduce HBV replication and gene expression in HepG2.2.15 cells (**Figure S4**). Thus, it is not likely to achieve stronger HBV suppression by escalating doses of IFNα-2a in this cell system. It is consistent with Hayashi et al. (Hayashi and Koike, 1989) and Caselmann's (Caselmann et al., 1992) studies that HBV replication and HBsAg production were not changed after treatment with IFNs in HBV-producing cell lines. Thus, IFNα-2a may preferentially protect uninfected hepatocytes against the establishment of HBV infection while it does not effectively clear HBV from persistently infected cells. Recently, Wu et al. investigated the hepatic gene expression profiles in patients received IFNα treatment (Wu et al., 2016). They found that non-responder patients have elevated ISGs expression prior to the treatment. It is generally assumed that HBV clearance is not achieved by the direct antiviral activity of IFNs rather by their ability for immune modulation (Pei et al., 2014).

Further, the interaction of HBV and IFN signaling is also very complex. A recent study showed that HBV may escape from the host innate immune system by promoting a complex composed of HK2, MAVS and VDAC1, which required Akt activity (Zhou et al., 2021). Besides, Li et al. (2021) reported that IFN-α inhibited the expression of MAVS in HepG2.2.15, thereby weakening its anti-HBV effect.

IFNs can activate a signal transduction cascade and induce the expression of ISGs. In the present study, it was demonstrated that the expression of ISGs, ISG15 and IFITM1, was attenuated by blocking autophagy. These results indicate that induction of ISG15 and IFITM1 is dependent on the initiation of autophagy. However, IFNα-2a-induced ISG15 and IFITM1 expressions are differently regulated at low and high glucose conditions. The activation of the Akt/mTOR pathway may also allow ISG15 expression (Kaur et al., 2008; Kaur et al., 2012). Our results revealed that the high glucose concentration modulated higher expression levels of Akt and mTOR, which may contribute to the expression of ISG15.

In conclusion, our findings demonstrated that IFNα-2a can inhibit Akt/mTOR signaling pathway, resulting in the initiation of autophagy and blockade of autophagic degradation. This non-canonical IFNα signaling may positively modulate HBV replication in hepatic cells.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

ML contributed to the conception of this study. JL performed the experiment and data analyses and wrote the manuscript. TK offered excellent technical support. RB provided PHHs. JC and ZY helped perform the analysis with constructive discussions. XW aided in interpreting the results and worked on the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.804011/full#supplementary-material

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# When the Phagosome Gets Leaky: Pore-Forming Toxin-Induced Non-Canonical Autophagy (PINCA)

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Macrophages remove bacteria from the extracellular milieu *via* phagocytosis. While most of the engulfed bacteria are degraded in the antimicrobial environment of the phagolysosome, several bacterial pathogens have evolved virulence factors, which evade degradation or allow escape into the cytosol. To counter this situation, macrophages activate LC3-associated phagocytosis (LAP), a highly bactericidal non-canonical autophagy pathway, which destroys the bacterial pathogens in so called LAPosomes. Moreover, macrophages can also target intracellular bacteria by pore-forming toxin-induced non-canonical autophagy (PINCA), a recently described non-canonical autophagy pathway, which is activated by phagosomal damage induced by bacteria-derived pore-forming toxins. Similar to LAP, PINCA involves LC3 recruitment to the bacteria-containing phagosome independently of the ULK complex, but in contrast to LAP, this process does not require ROS production by Nox2. As last resort of autophagic targeting, macrophages activate xenophagy, a selective form of macroautophagy, to recapture bacteria, which evaded successful targeting by LAP or PINCA through rupture of the phagosome. However, xenophagy can also be hijacked by bacterial pathogens for their benefit or can be completely inhibited resulting in intracellular growth of the bacterial pathogen. In this perspective, we discuss the molecular differences and similarities between LAP, PINCA and xenophagy in macrophages during bacterial infections.

**Keywords: non-canonical autophagy, macrophages, ULK complex, pore-forming toxins, macroautophagy, xenophagy, LC3-associated phagocytosis, PINCA**

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## PHAGOCYTOSIS: MAIN WEAPON OF MACROPHAGES

The most prominent, characteristic feature of macrophages is their ability to phagocytose extracellular material ranging from cellular debris to whole cells (Fadok et al., 1998; Erwig and Henson, 2008; Kono and Rock, 2008; Suzanne and Steller, 2013; Kourtzelis et al., 2020), but also invading pathogens (Djaldetti et al., 2002; Haas, 2007; Lemke, 2019). With this ability to separate foreign invaders like bacteria, fungi or parasites from the rest of the organism, they represent one of the first lines of defense against invading pathogens (Rosales and Uribe-Querol, 2017). After induction by various cell surface receptors, such as the mannose receptor, Fc-receptors and

scavenger receptors (Freeman and Grinstein, 2014; Uribe-Querol and Rosales, 2020), the cargo is enclosed in a single-membrane structure called phagosome. Several factors, like vacuolar-type $H^+$-ATPase (V-ATPase)-mediated acidification (Sun-Wada et al., 2009; Dragotakes et al., 2020; Westman and Grinstein, 2021), production of reactive oxygen species (ROS) (Craig and Slauch, 2009; Slauch, 2011; Wink et al., 2011; Herb and Schramm, 2021) and exposure to hydrolases after fusion with lysosomes (del Cerro-Vadillo et al., 2006; Schramm et al., 2014; Weiss and Schaible, 2015) lead to the formation of a highly antimicrobial environment for engulfed pathogens and, in most cases, result in their degradation (Haas, 2007). However, several bacterial pathogens have established strategies to evade this degradative fate in the phagosome (Mitchell et al., 2016; Grijmans et al., 2022), e.g. *Staphylococcus aureus* (*S. aureus*) (Fraunholz and Sinha, 2012; Moldovan and Fraunholz, 2019; Rao et al., 2020), *Salmonella typhimurium* (*S. typhimurium*) (Eriksson et al., 2003; Fenlon and Slauch, 2014; Burton et al., 2014; Rhen, 2019; Rao et al., 2020) or *Mycobacterium tuberculosis* (Queval et al., 2017; Koster et al., 2017), which can alter the phagosomal composition and structure for their benefit and do not only remain unharmed, but also replicate inside the phagosome.

## LC3-ASSOCIATED PHAGOCYTOSIS

Since several bacterial pathogens can evade the degradative fate of the phagosome, macrophages activate a non-canonical autophagy pathway called LC3-associated phagocytosis (LAP), which can enhance phagolysosomal fusion. For example, phagosomes containing Toll-like receptor ligand-coated latex beads (Sanjuan et al., 2007), dead cells (Martinez et al., 2011) or pathogens, such as *Legionella dumoffii* (Hubber et al., 2017), *Listeria monocytogenes (L. monocytogenes)* (Gluschko et al., 2018) and *Aspergillus fumigatus* (Martinez et al., 2015) show increased fusion with lysosomes during LAP, resulting in enhanced degradation of the cargo. Notably, LAP can also delay phagolysosomal fusion, leading to prolonged antigen presentation by major histocompatibility complex (MHC) class II (Romao et al., 2013; Ma et al., 2014; Fletcher et al., 2018). LAP is induced by various surface receptors found on macrophages (Sanjuan et al., 2007; Ma et al., 2012; Tam et al., 2014; Gluschko et al., 2018; Hayashi et al., 2018) and results in the decoration of phagosomes with microtubule-associated protein 1 light chain-3 (LC3) family proteins, resulting in so called LAPosomes. (Sanjuan et al., 2007; Martinez et al., 2011; Durgan et al., 2021). LAP and macroautophagy share some but not all components of the autophagic machinery, e.g. parts of the class III phosphatidylinositol 3-kinase (PI3KC3) complex or the two ubiquitin-like conjugation systems, i.e. the autophagy-related protein (ATG) 12 conjugation system and the LC3 lipidation system (Florey et al., 2011; Lystad et al., 2019) (**Figure 1**). Generation of LAPosomes requires the production of the membrane lipid phosphatidylinositol 3-phosphate (PI3P) (by components of the PI3KC3 complex) and ATG16L1 recruitment to the PI3P-containing target membrane (Kim et al., 2013; Martinez et al., 2015). Comparable to macroautophagy, the PI3KC3 complex, which is involved in LAPosome formation, also contains Beclin-1 (BECN1) (Sanjuan et al., 2007; Martinez et al., 2015; Backer, 2016), vacuolar protein sorting-associated proteins (VPS) 15 and 34, as well as UV radiation resistance-associated gene protein (UVRAG), but lacks ATG14 and activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) (Martinez et al., 2015; Backer, 2016). In contrast to macroautophagy, LAP requires a specific component of the PI3KC3 complex called Rubicon, which facilitates VPS34 activity and sustains PI3P presence on the LAPosome (Martinez et al., 2015). Another difference is the dispensability of WD repeat domain phosphoinositide-interacting proteins (WIPI) and ATG2, which are required for macroautophagy but not for LAP (Martinez et al., 2015; Fischer et al., 2020). Recently, it was shown that the WD40 domain of ATG16L1 is required for its recruitment to the PI3P-containing target membrane during LAP. Mice lacking the WD40 domain of ATG16L1 are deficient for LAP but not for macroautophagy (Rai et al., 2019). This implicates that a complete different factor than WIPI is required for recruitment of ATG16L1 to the PI3P-containing membrane on the LAPosome. Some studies have shown that the V-ATPase can recruit ATG16L1 onto single-membrane vesicles *via* its WD40 domain (Florey et al., 2015; Fletcher et al., 2018; Xu et al., 2019; Fischer et al., 2020). Moreover, activity of V-ATPase can be induced by osmotic imbalances caused by pore-forming toxins e.g. by the *Helicobacter pylori (H. pylori)* pore-forming toxin vacuolating cytotoxin A (VacA) (Florey et al., 2015). V-ATPase activation and ATG16L1 recruitment lead to LC3 lipidation onto single-membrane vacuoles, a mechanism which is independent of the upstream macroautophagy machinery, e.g. the unc-51-like kinase (ULK) complex (Fletcher et al., 2018; Xu et al., 2019), which resembles LAP. A recent preprint study by Hooper et al. indicates that also during LAP, V-ATPase is responsible for ATG16L1 recruitment and subsequent conjugation of LC3 onto the phagosomal membrane (Hooper et al., 2021) (**Figure 1**). LC3 lipidation resembles the conjugation of ubiquitin to proteins (Slobodkin and Elazar, 2013), therefore similar terms were used for the enzymes of the two ubiquitin like conjugation systems, which carry out the process (Ichimura et al., 2000). For both complexes E1-like ATG7 and E2-like ATG3 catalyze the reactions during LC3 lipidation. The ATG12–ATG5–ATG16L1 complex carries out an analogous function as the ubiquitin E3 ligase and mediates transfer of LC3 from ATG3 to phosphatidylethanolamine (PE) in the phagosomal membrane (Hanada et al., 2007; Sakoh-Nakatogawa et al., 2013).

Notably, Durgan and colleagues have shown that single-membrane structures during non-canonical autophagy, e.g. LAP, show a different LC3 lipidation pattern. In addition to PE-conjugated LC3, the study demonstrated that LC3 is also conjugated to phosphatidylserine (PS). This is in contrast to autophagosomes during macroautophagy, which exclusively display PE-conjugated LC3 (Durgan et al., 2021). However, the major difference beyond LC3 conjugation onto single- vs. double-membrane vesicles is that LAP, in contrast to macroautophagy, is completely independent of the ULK complex (Martinez et al., 2011;

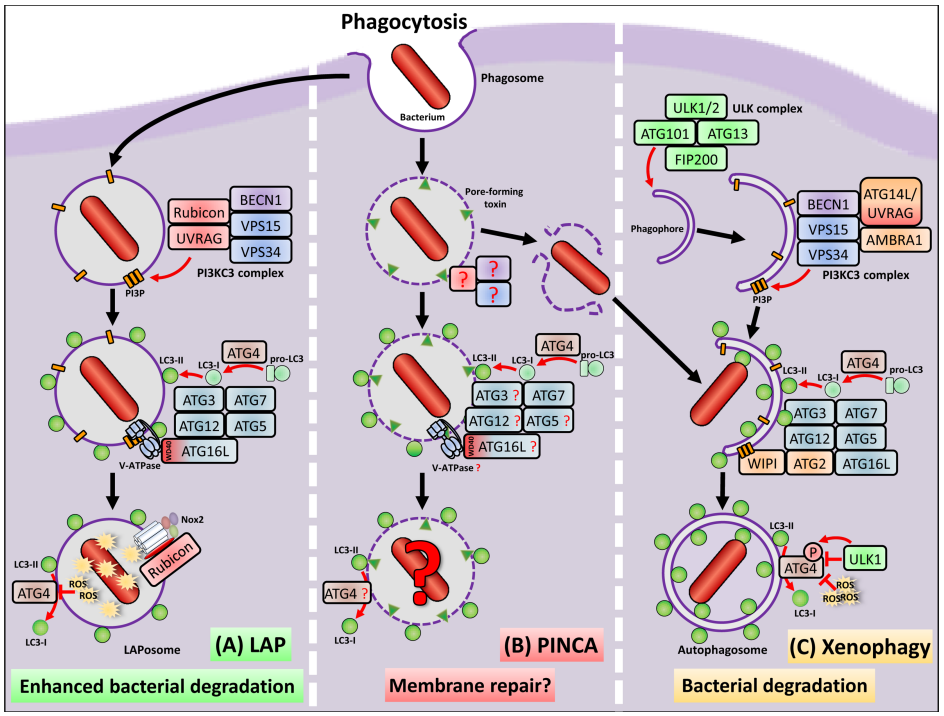


**FIGURE 1** | Macrophages remove bacteria from the extracellular milieu via phagocytosis, which is the engulfment and enclosure in a single membrane vesicle, called phagosome. Most of the engulfed bacteria are degraded due to the highly antimicrobial environment of the phagosome. However, several bacterial pathogens have evolved virulence factors, which allow escape from the phagosome into the cytosol. **(A)** Macrophages activate LAP to inactivate and destroy the bacterial pathogen before phagosomal escape. LAP shares some, but not all components of the autophagy machinery to conjugate LC3 to the phagosomal membrane. Importantly, LAP is completely independent from the ULK complex, defining it as non-canonical autophagy pathway. The PI3KC3 complex, which is activated during LAP, shares the core components BECN1, VSP15 and VSP34 but exclusively contains UVRAG and Rubicon, which is not only important for recruitment of the PI3KC3 complex, but also stabilizes the $p22^{phox}$ subunit of Nox2. To the PI3P platforms formed by the PI3KC3 complex, a so far unknown factor binds to which ATG16L1 is recruited via its WD40 domain together with the LC3 conjugation machinery consisting of ATG3, ATG7, ATG5 and ATG12. A preprint study (Hooper et al., 2021) marks the V-ATPase as possible candidate, which recruits ATG16L1 and the LC3 conjugation machinery to the phagosome during LAP. ROS production by Nox2 leads to oxidative inactivation of the protease ATG4, which prevents ATG4-mediated deconjugation of LC3 and stabilization of the LAPosome. LAPosomes show enhanced fusion with lysosomes and enhanced degradation of the bacterial pathogen. **(B)** During PINCA, damage induced by bacteria-derived pore-forming toxins induces LC3 recruitment to the phagosome independent of the ULK complex components FIP200 and ULK1/2. With the exception of ATG7, the factors for induction and execution, which are necessary for LC3 recruitment during PINCA, as well as its precise functions remain to be resolved. Since pore-forming toxins lead to V-ATPase and ATG16L recruitment during other non-canonical pathways, this might also be the case during PINCA. **(C)** To recapture bacterial pathogens that had managed to escape from phagosomes, either during conventional phagocytosis, LAP or PINCA, macrophages activate xenophagy, a selective form of macroautophagy. Initiation of xenophagy depends on the ULK complex, i.e. ULK1/2, FIP200, ATG13 and ATG101, which generates a double-membrane structure called phagophore. Recruitment of the proteins BECN1, VSP15, VSP34, AMBRA1 and either UVRAG or ATG14L to the phagophore leads to formation of the PI3KC3 complex, which then generates PI3P in the growing phagophore membrane. These PI3P platforms serve as docking station for the WIPI-ATG2 complex, which subsequently recruits the machinery for LC3 conjugation, facilitating degradation of the recaptured bacterial pathogen in autophagolysosomes. During xenophagy, inactivation of ATG4 can be mediated by cytotosolic ROS production or phosphorylation of ATG4 by ULK1 both preventing deconjugation of LC3 from the autophagosome.

Martinez et al., 2015). Most importantly, LC3 recruitment to phagosomes by LAP requires production of ROS by the NADPH oxidase 2 (Nox2) (Huang et al., 2009; Yang et al., 2012; Martinez et al., 2015; Gluschko et al., 2018; Gluschko et al., 2021; Herb and Schramm, 2021; Ligeon et al., 2021). The Nox2 complex consists of two integral membrane subunits, $gp91^{phox}$, and $p22^{phox}$, and the cytosolic subunits $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac1/2 (Herb and Schramm, 2021). For induction of ROS production by Nox2, the cytosolic subunits are recruited to the integral membrane subunits. Thereby, Rubicon plays an important role beyond its involvement in generation of PI3P by the PI3KC3 complex, by stabilizing $p22^{phox}$ *via* direct binding (Yang et al., 2012; Martinez et al., 2015). However, why Nox2-derived ROS are crucial for LAP induction is not understood in detail. Notably, it was recently shown that ROS production by Nox2 mediates oxidative inactivation of the protease ATG4 thereby preventing deconjugation of LC3 from the phagosome (Ligeon et al., 2021).

## PINCA – A NEW NON-CANONICAL AUTOPHAGY PATHWAY

We recently described a new variant of non-canonical autophagy in macrophages, which we termed pore-forming toxin-induced non-canonical autophagy (PINCA) (Gluschko et al., 2021).

Similar to other forms of non-canonical autophagy, e.g. LAP, PINCA also is independent of the ULK complex components focal adhesion kinase family interacting protein of 200 kD (FIP200) and ULK1/2. Importantly, in contrast to LAP, PINCA did also not require Nox2-derived ROS production, which is crucial for LC3 decoration of phagosomes during LAP (Sanjuan et al., 2007; Martinez et al., 2015; Gluschko et al., 2018; Herb et al., 2020; Ligeon et al., 2021). Thus, we observed induction of PINCA in Nox2-deficient peritoneal macrophages (PM) and in wildtype bone marrow-derived macrophages (BMDM), which fail to produce sufficient levels of ROS for induction of LAP due to low expression of Nox2 (Gluschko et al., 2021). Instead, damage induced by the pore-forming toxin listeriolysin O (LLO) of *L. monocytogenes* or by the several pore-forming toxins of *S. aureus* were necessary to induce LC3 recruitment to the damaged phagosomes, i.e. PINCA (**Figure 1**).

Notably, damage induced by the needle-like Type three secretion system (T3SS) of *Shigella flexneri (S. flexneri) or S. typhimurium* did not induce PINCA in macrophages. A possible reason for this is the expression of bacterial virulence factors such as Salmonella outer protein F (SopF) of *S. typhimurium*, which inhibits the vacuolar V-ATPase and thereby prevents ATG16L recruitment and LC3 lipidation onto *S. typhimurium*-containing vacuoles (Xu et al., 2019). Interestingly, vacuolar damage caused by SopF-deficient *S. typhimurium* triggered ATG16L recruitment and LC3 lipidation onto *S. typhimurium*-containing vacuoles in epithelial cells (Xu et al., 2019), which was independent of FIP200 and resembled PINCA. Thus, it is likely that also in macrophages T3SS-induced damage can trigger PINCA, when this process is not actively inhibited by bacterial virulence factors such as SopF. Notably, this T3SS-induced LC3 lipidation on damaged, but still intact vacuoles/phagosomes should not be mistaken with xenophagy, a selective form of macroautophagy, which targets ruptured vacuoles, membrane remnants or cytosolic bacteria and involves exposure of glycans and the recruitment of galectins and several other factors such as TANK-binding kinase 1 (TBK1) (Thurston et al., 2012; Ravenhill et al., 2019; Bell et al., 2021). As already mentioned, in mouse embryonic fibroblasts (MEFs) pore-forming toxins can induce osmotic imbalances within endolysosomal compartments, which are sensed by V-ATPase and result in ATG16L recruitment and LC3 conjugation (Florey et al., 2015). Due to this, it is plausible that bacterial toxin-induced pore formation during PINCA can also induce osmotic imbalances within phagosomes in macrophages. Wether these osmotic imbalances can trigger V-ATPase-coupled ATG16L recruitment and LC3 lipidation during PINCA, as observed during other non-canonical autophagy pathways (Florey et al., 2015; Fletcher et al., 2018; Xu et al., 2019; Hooper et al., 2021), are interesting topics for future studies.

The functional purpose of PINCA seems to be related to LAP during *L. monocytogenes* infection of wildytpe PM. We observed that during PINCA, LC3-positive phagosomes fused more often with lysosomes than conventional, LC3-negative phagosomes indicating that also PINCA promotes phagolysosomal fusion (Gluschko et al., 2021). However, in sharp contrast to LAP, which clearly promotes the anti-listerial activity of tissue macrophages, e.g. PM (Gluschko et al., 2018), LC3 recruitment to phagosomal membranes by PINCA and subsequently increased phagolysosomal fusion did not substantially contribute to anti-listerial activity of BMDM (Gluschko et al., 2021). Both LAP and PINCA require ATG7 for LC3 conjugation (Herb et al., 2020). Therefore, the question whether PINCA contributes to anti-listerial activity could unfortunately not be answered by the sole use of ATG7-deficient macrophages (Gluschko et al., 2021), since these cells can induce neither LAP nor PINCA. In addition, Nox2-deficient PM are not well suited to investigate the functional purpose of PINCA, since Nox2-derived ROS are not only necessary for LAP induction but also fulfill a plethora of other antimicrobial functions (Canton et al., 2021; Herb and Schramm, 2021). Moreover, ROS have been shown to inactivate ATG4, thereby preventing deconjugation of LC3 from the phagosome (Ligeon et al., 2021). It is conceivable that during PINCA, LC3 is continuously deconjugated from the phagosome in the absence of ROS production, except there is a, yet unknown, ROS-independent mechanism, which inactivates ATG4 during PINCA. Due to this, a possible antimicrobial function of PINCA could be easily overlooked, when PINCA is induced in the absence of ROS, which not only prevent LC3-deconjugation (Ligeon et al., 2021), but also substantially contribute to other antimicrobial functions independent of autophagic targeting of any kind (Canton et al., 2021; Herb and Schramm, 2021). Otherwise, when PINCA is induced in the presence of ROS, it is likely that also LAP is induced in parallel to PINCA, making it difficult to distinguish between these two pathways. The identification of a mechanistic component, which exclusively activates PINCA in the presence of functional ROS production, but without activating LAP, will be necessary to address this unanswered question.

Notably, in addition to enhanced phagolysosomal fusion, we found that LC3-positive phagosomes formed by PINCA were damaged less often than conventional, LC3-negative phagosomes (Gluschko et al., 2021). This indicates that either targeting by PINCA impedes the damage to the phagosomal membrane, or that LC3-decorated phagosomes are pre-assigned for membrane damage repair, as observed during autophagy induced in *S. typhimurium*-infected epithelial cells (Thurston et al., 2012; Kreibich et al., 2015). Thus, PINCA might represent an attempt of macrophages to repair damaged phagosomal membranes as last resort against the bacteria, which have not yet escaped from the phagosome.

## ANTIBACTERIAL XENOPHAGY IN MACROPHAGES

Several bacteria manage to escape form the phagosome *via* rupture of the phagosomal membrane prior to degradation or targeting by LAP or PINCA (Fernandez-Prada et al., 2000; Hamon et al., 2012; Jamwal et al., 2016; Bell et al., 2021). Escape into the cytosol not only means the evasion from

degradation, but provides also a rich pool of nutrients for the escaped pathogen, which enables cytosolic replication within the cell without being detected by other phagocytes. However, macrophages have established a counter measure to recapture cytosolic bacteria, namley xenophagy (Sharma et al., 2018) (**Figure 1**). During xenophagy, the cargo for the autophagic machinery is a bacterial pathogen that is escaping or has already escaped from the phagosome/vacuole (Pao and Rape, 2019). The cytosolic bacterium can be tagged with ubiquitin and recognized by various autophagy receptors, e.g. Sequestosome-1 (SQSTM1)/p62 or calcium-binding and coiled-coil domain-containing protein 2 (CALCOCO2)/NDP52 (Thurston et al., 2009; Johansen and Lamark, 2011), which recruit the autophagic components to the target. Thereby, the cargo is enclosed and isolated from the rest of the cell by formation of a double-membrane structure, called phagophore (Yla-Anttila et al., 2009; Lamb et al., 2013; Chang et al., 2021). Initiation of phagophore formation is, in contrast to LAP and PINCA, dependent on activation of the ULK complex (Suzuki et al., 2007; Jung et al., 2009; Koyama-Honda et al., 2013; Fujioka et al., 2014; Shi et al., 2020; Mercer et al., 2021), which is composed of ULK1 or ULK2 (ULK1/2), FIP200, ATG13, as well as ATG101 (Chang and Neufeld, 2009; Ganley et al., 2009; Hara and Mizushima, 2009; Hosokawa et al., 2009; Mercer et al., 2009; Lin and Hurley, 2016; Hurley and Young, 2017; Chang et al., 2021). ULK complex activity, which is not required for LAP (Martinez et al., 2011; Martinez et al., 2015) or PINCA (Gluschko et al., 2021), leads to the generation of PI3P at the membrane of the forming phagophore *via* one of the two PI3KC3 complexes (Russell et al., 2013; Backer, 2016). The two PI3KC3 complexes activated during macroautophagy contain the same core components, namely VPS34 and VPS15, BECN1 and AMBRA1 (Yu et al., 2015; Young et al., 2019), but can either recruit ATG14 (found in the PI3KC3 complex 1) or UVRAG (found in the PI3KC3 complex 2) (Itakura et al., 2008), (**Figure 1**). The PI3P generated at the membrane serves as a platform for recruitment of a complex consisting of WIPI proteins and ATG2, which are dispensable for LAP (Martinez et al., 2015; Fischer et al., 2020). After recruitment to the forming membrane of the phagophore, the WIPI-ATG2 complex itself recruits the LC3 conjugation machinery (Kabeya et al., 2000; Martens, 2016; Schaaf et al., 2016). Notably, ULK1 inhibits the catalytic activity of ATG4 by phosphorylation, thereby preventing the deconjugation of LC3 from the autophagosome (Pengo et al., 2017). In addition, cytosolic ROS can also inhibit ATG4 deconjugation activity (Scherz-Shouval et al., 2007), similar to Nox2-mediated oxidative inactivation of ATG4 during LAP (Ligeon et al., 2021). Finally, the closed autophagosome subsequently fuses with lysosomes, which leads to degradation of the recaptured bacterial pathogen in an autophagolysosome (Sharma et al., 2018). Xenophagy therefore plays a crucial role in the cellular defense against invading bacteria (Levine et al., 2011), not only in macrophages (Niu et al., 2008; English et al., 2009; Moreau et al., 2010; Travassos et al., 2010; Starr et al., 2012; Park et al., 2016; Ganesan et al., 2017; Zhou et al., 2017), but also in non-immune cells (Gutierrez et al., 2004; Birmingham et al., 2006; Thurston et al., 2009; Zheng et al., 2009; Wild et al., 2011; Thurston et al., 2012; von Muhlinen et al., 2012; Xu et al., 2019).

However, bacteria have also evolved mechanism to avoid degradation by xenophagy (Huang and Brumell, 2014). Some bacteria can reside in autophagosomes or autophagosome-like structures for replication, such as *H. pylori* (Wang et al., 2009; Hu et al., 2019), *Legionella pneumophila* (Amer and Swanson, 2005; Joshi and Swanson, 2011) or *Yersinia pseudotubercolosis* (Moreau et al., 2010), while others completely inhibit xenophagy and freely replicate in the cytosol. *L. monocytogenes*, for example, inhibits xenophagy *via* the virulence factors actin assembly-inducing protein (ActA) (Yoshikawa et al., 2009) and the two phosphatidylinositol-specific phospholipases C PlcA and PlcB (Mitchell et al., 2015), *S. flexneri* inhibits binding of ATG5 during xenophagy *via* the virulence factor IscB (Ogawa et al., 2005) and *S. typhimurium* secrets several virulence factors, such as SseL (Mesquita et al., 2012), SseF and SseG (Feng et al., 2018) to counter xenophagic targeting (Casanova, 2017).

## CONCLUSIONS

Macrophages are among the first line of defense against invading pathogens. Due to distinct virulence factors, some bacterial pathogens can evade the destruction in the phagosome, either by re-modulation of the phagosomal milieu or *via* escape into the cytosol. Activation of LAP, a highly microbicidal non-canonical autophagy pathway (Herb et al., 2020; Grijmans et al., 2022), enhances the degradative capacity of macrophages. We recently described another non-canonical autophagy pathway termed PINCA (Gluschko et al., 2021), which is triggered by perforation of bacteria-containing phagosomes, independent of the ULK complex components ULK1/2 and FIP200 and also independent of Nox2-derived ROS, therefore representing a non-canonical autophagy pathway distinct from LAP. Pore-forming toxins can induce osmotic imbalances, which are sensed by V-ATPase and result in ATG16L recruitment and LC3 conjugation (Florey et al., 2015). It is very likely that LC3 recruitment to perforated phagosomes during PINCA is also activated by the V-ATPase-ATG16L1-axis, which might represent a general pathway to recruit LC3 to damaged, yet not ruptured compartments. Furthermore, it is reasonable that also during LAP, V-ATPase is responsible for ATG16L1 recruitment and subsequent LC3 conjugation, since ROS production by Nox2 is not sufficient to induce LAP, when V-ATPase is inhibited (Hooper et al., 2021). It is tempting to speculate that LC3 conjugation during LAP is not triggered by Nox2-generated ROS but by V-ATPase-induced ATG16L1 recruitment. Instead, ROS production during LAP only prevents deconjugation of LC3 through oxidative inactivation of ATG4 as shown by Ligeon et al. (2021), which resembles redox-dependent inactivation of ATG4 during autophagy (Scherz-Shouval et al., 2007; Pérez-Pérez et al., 2016). While LAP and xenophagy have clear degradative functions, despite some bacterial pathogens exploiting autophagosomes as a replicative niche (Huang and Brumell, 2014; Siqueira et al., 2018; Riebisch et al., 2021), the functional purpose of PINCA remains

unclear. It is possible that PINCA might represent an emergency repair mechanism for damaged phagosomes, similar to membrane repair mechanisms in *S. typhimurium*-infected epithelial cells (Thurston et al., 2012; Kreibich et al., 2015). Alternatively, LC3 on the perforated phagosome may recruit an entire spectrum of proteins containing a LC3-interacting region (Johansen and Lamark, 2020), which in turn may accelerate phagolysosomal fusion, or exert another, yet unknown function of PINCA.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

Conceptualization, MH. writing—original draft preparation, MH, AG and MK. writing—review and editing, MH, AG, AF and MK. visualization, MH. supervision, MK. project administration, MK. funding acquisition, MH, AG and MK. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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# Autophagy Modulators in Coronavirus Diseases: A Double Strike in Viral Burden and Inflammation

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Coronaviruses are the etiologic agents of several diseases. Coronaviruses of critical medical importance are characterized by highly inflammatory pathophysiology, involving severe pulmonary impairment and infection of multiple cell types within the body. Here, we discuss the interplay between coronaviruses and autophagy regarding virus life cycle, cell resistance, and inflammation, highlighting distinct mechanisms by which autophagy restrains inflammatory responses, especially those involved in coronavirus pathogenesis. We also address different autophagy modulators available and the rationale for drug repurposing as an attractive adjunctive therapy. We focused on pharmaceuticals being tested in clinical trials with distinct mechanisms but with autophagy as a common target. These autophagy modulators act in cell resistance to virus infection and immunomodulation, providing a double-strike to prevent or treat severe disease development and death from coronaviruses diseases.

**Keywords: coronaviral infection, viral replication, inflammation, tissue damage, autophagy**

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## INTRODUCTION

Infectious diseases have been responsible for pandemics and caused billions of deaths during human history. The emergence of antimicrobial therapies and sanitary improvements dramatically contributed to increased life expectancy worldwide (Ventola, 2015). In contrast to antibacterial agents, antiviral pharmaceuticals are radically scarce due to viral dependence on host metabolism, making it more difficult to find highly active and selective compounds. This problem becomes especially critical during significant pandemics such as that caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic agent of COVID-19, originated in Wuhan, Hubei Province, China, which has affected around 350 million people with 5,61 million deaths worldwide.

SARS-CoV-2 is an enveloped positive single-strand RNA virus (ssRNA+) that belongs to the betacoronavirus (βCoV) genus and is related to SARS-CoV and the Middle East respiratory syndrome-coronavirus (MERS-CoV), the causative agents of recent outbreaks in 2003 and 2012,

respectively (Zhu et al., 2020). COVID-19 clinical presentations are highly variable, ranging from pauci- or asymptomatic to critical infections characterized by respiratory failure, shock, or multiorgan dysfunction, requiring hospitalization in intensive care units, jeopardizing private and public health systems worldwide.

Although the response to COVID-19 has led to unparalleled research efforts for the development and distribution of vaccines leading to an essential reduction in COVID-19 cases and disease severity, currently, breakthrough infection still occurs amongst fully vaccinated subjects. Thus, urgent and complementary therapeutic strategies to fight the disease are necessary.

Antiviral therapies are mainly targeted to interfere with viral structures. A major drawback frequently encountered is the emergence of drug resistance as a consequence of the selection of mutants due to the inherent viral variability, especially in respiratory viruses (Yan et al., 2014). An alternative to circumvent virus resistance is the design of strategies aiming to regulate host immune responses to infection. This strategy is associated with host protection, reducing the viral burden to promote neutralization, or controlling the detrimental effects of exacerbated inflammation and the resulting tissue damage. As an example, the use of host immune response modulators combined with antiviral agents has been successfully applied, compared to non-combined therapeutic strategies, for the treatment of co-infected patients with hepatitis C virus (HCV) and hepatitis B virus (HBV) (Shih and Liu, 2020). In this case, interferon-alpha (IFNα), an essential cytokine for host resistance, is combined with nucleoside analogs, such as ribavirin and lamivudine (Mavilia and Wu, 2018). Interestingly, SARS-CoV infection also benefits from interferon treatment (Booth et al., 2003). Besides that, corticosteroid treatment of critically ill patients with covid-19 exerts a beneficial effect, controlling inflammatory tissue damage and improving disease tolerance (Sterne et al., 2020).

The modulation of innate immunity, for example after IFNα treatment, can be used against different viruses and could inhibit viral replication, allowing the proper onset of adaptative immune responses. Innate immune recognition of virus initiates several responses to viral molecules such as genomic DNA and RNA or double-stranded RNA (dsRNA) released/formed during viral replication (Wu et al., 2020). These molecules are sensed by pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), zinc-finger antiviral protein (ZAP), RIG-I-like receptors (RLRs), and cyclic GMP–AMP synthase- Stimulator of interferon genes (cGAS-STING) pathway to trigger effective antiviral responses, including the production of a myriad of cytokines to induce inflammation and progression of adaptive immune responses (Luo et al., 2020; Wu et al., 2020). In this context, type I interferons (IFN-I), which include IFNα and IFNβ, mediate the induction of both innate and adaptative immune responses through dendritic cells (DCs) maturation promoting increased levels of co-stimulatory molecules, such as the cluster of differentiation 80 (CD80), CD86, and CD40. Mature DCs migrate to draining lymph nodes and present antigens *via* major histocompatibility complex I (MHC-I) to CD8+T cells and MHC-II to CD4+ T cells. CD8+ T cells secrete several pro-inflammatory cytokines and induce cell death of virus-infected host cells, reducing an important source of virus replication. CD8+ T cells induce cell death after recognition of the MHC-I-viral peptide complex expressed by the target cell. CD4+ T cells orchestrate antiviral cellular and humoral immunity. CD4+ T cells, through secretion of distinct cytokines, like IFNγ, increase the microbicidal ability of phagocytes, like macrophages and neutrophils, and promote antibody class-switch and affinity maturation of activated B cells. B cells are the adaptive immunity cells responsible for antibodies secretion, promoting viral neutralization, opsonization, and complement activation (Hoffman et al., 2016).

Regulation of inflammation-mediated tissue damage during viral infections is also an attractive therapeutic target. Inflammatory damage plays an essential role in coronavirus diseases, and anti-inflammatory approaches have been used, especially in the "inflammatory phase" of the infection in which neutralizing antibodies and low extracellular viral loads are found (Desai et al., 2020; Zhang Y. Y. et al., 2020). In these settings, the inflammatory-mediated tissue damage and epithelial cells metaplasia are thought to be the key players in disease progression (Schaefer et al., 2020). Inactivated viruses will not lead to direct cell damage and release of DAMPs (danger-associated molecular patterns). However, it will be responsible for activating several PRRs that are engaged in response to viral pathogen-associated molecular patterns (PAMPs), and, in combination with virus-antibody complexes, it will keep monocytes and neutrophils Fc receptors activated.

## CORONAVIRUS DISEASES PATHOGENESIS

There are several similarities in the pathogenesis of COVID-19 and the diseases caused by SARS-CoV and MERS-CoV, as reviewed elsewhere (Zhang Y. Y. et al., 2020). These viruses cause lower respiratory tract infections and, as such, possess several common symptoms and signs, like non-productive cough (in a small proportion of patients, there is hemoptysis), fever, myalgia, chills, malaise, and shortness of breath. Interestingly, gastrointestinal symptoms can also be present, like vomiting and diarrhea, a feature associated with the diversity of cells infected by these viruses (Zhang Y. Y. et al., 2020), including kidneys and immune cells, like T lymphocytes and macrophages. Thus, lymphopenia can also be a common finding associated with the cytopathic effects of the viruses and a dysregulated immune response. Severe disease caused by these coronaviruses was positively correlated with increased levels of inflammatory cytokines, especially after ten days of symptoms, a phase in which the viral titers are usually decreasing (Lee et al., 2020). A complex interplay between the kinetics and levels of IFN-I and proinflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin 6 (IL-6), IL-8, IL-1β, and interferon gamma-induced protein 10 (IP-10), seems crucial for resolution or progression to severe disease at this stage

(Lau et al.,2013; Zhou et al., 2014; Channappanavar et al., 2016). Thus, severe disease is associated with late high levels of IFN-I, probably a consequence of both viral evasion and interferon antagonism in the early times of infection (Totura and Baric, 2012). In this sense, early IFN-I treatment is associated with MERS-CoV disease protection in mice, and IFN-I therapy was used with promising results for SARS-CoV patients (Booth et al., 2003; Channapanavar et al., 2019), and as we discuss throughout this text, IFN signaling and cellular effects are affected and mediated in part by autophagy. In addition, samples obtained from the lungs of severe MERS-CoV-infected patients presented more than 50 times the levels of IL-1α, IL-1β, and IL-8 than those found in healthy individuals (Alosaimi et al., 2020). Once more, autophagy can be an exciting target in this setting. Autophagy restrains IL-1β release and may contribute to control immunopathology, as this cytokine is also involved in SARS-CoV and SARS-CoV-2 diseases (Rodrigues et al., 2020; Zhang Y. Y. et al., 2020). The kinetics and type of adaptive immune response, including T cell and B cell-mediated immunity, are also crucial for coronavirus diseases' progression. Severe disease is associated with a more prominent response of the CD4+ T cell subtype Th2 compared to Th1 (Li et al., 2008; Alosaimi et al., 2020; Pavel et al., 2021). Furthermore, increased levels of CD8+ T cells responses were found to be associated with mild disease, highlighting the importance of this cytotoxic T cell population (Zhao et al., 2017; Mallajosyula et al., 2021). Interestingly, hyperactivated CD8+ T cells responses were found in severe patients, indicating that a fine-tuning of the immune responses is crucial for coronavirus disease pathogenesis (Shin et al., 2019; Kang et al., 2020). As we will also explore later in this text, autophagy can influence the activation of both CD4+ and CD8+ T cells (Valečka et al., 2018). Similarly, the levels, kinetics, and type of post-translational modifications in antibody classes are also crucial for coronavirus diseases, and proper regulation will be a central feature for disease progression or control (Hoepel et al., 2021; Zhou et al., 2021). Even though there are many similarities between the diseases caused by SARS-CoV, SARS-CoV-2, and MERS-CoV, some significant differences should be highlighted. MERS-CoV spike (S) protein, which is crucial for coronavirus invasion, binds to the dipeptidyl peptidase 4 (DDP4) receptor (Wang et al., 2013), while S proteins from SARS-CoV and SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor (Li et al., 2003; Yang et al., 2020). DDP4 receptor is expressed at higher levels than ACE2 in many different cells such as monocytes and dendritic cells, in which MERS-CoV generates productive infections differently from SARS-CoV (Channappanavar and Perlman, 2017). Epithelial cells from the kidneys, liver, intestines, and prostate also express higher levels of DDP4 compared to ACE2 (van Doremalen et al., 2014). This differential expression between DDP4 and ACE2 might be associated with a higher dissemination rate of MERS-CoV throughout the body and the prevalence of systemic events, like septic shock and multiorgan failure. All these features contribute to the higher mortality of MERS-CoV disease compared to other coronaviruses diseases (Zhang Y. Y. et al., 2020).

## AUTOPHAGY: A CONTRIBUTOR TO IMMUNE RESPONSES

More recently, it has been demonstrated that the immune system deploys distinct pathways to fight viral infections. One of these strategies is macroautophagy, a crucial stress-induced response (Schmeisser et al., 2014; Caldwell, 2016; Laing et al., 2019). Autophagy is a term derived from the Greek, in which auto means "self" and phagy means "eat." In cell biology, autophagy describes the ability of cells to degrade its components. Three types of autophagy targets cell components for lysosomal degradation: microautophagy stands for the degradation of macromolecules captured by invaginations or protrusions of the lysosome membrane in a process mediated by the interaction of charged phosphatidylserine in the lysosomal membrane with chaperone heat shock cognate 71KDa protein (Hsc70) (Schuck, 2020); chaperone-mediated autophagy (CMA) stands for a more refined mechanism, in which cargo is not directly sequestered in membranous structures but possesses a five peptide chaperone binding motif that allows its interaction with Hsc70, and consequent interaction with the lysosome receptor lysosomal-associated membrane protein 2A (LAMP2A) (Kaushik and Cuervo, 2018); and macroautophagy, which is a complex multistep process that starts with the formation of the autophagosome, as we will describe later, here referred simply as autophagy.

Autophagy regulates several aspects of host immune response, such as antigen presentation for CD4+ and CD8+ T cells (Valečka et al., 2018); T and B cell development and homeostasis (Arbogast and Gros, 2018); and the secretion of inflammatory mediators (Dai et al., 2017; Tian et al., 2019) and their effects on target cells (Schmeisser et al., 2014; Prieto et al., 2015). For instance, autophagy can restrain intracellular pathways responsible for cytokines secretion, including intracellular PRRs, like RLRs and TLRs, responsible for IFN-I release (Reed et al., 2015; Cotzomi-Ortega et al., 2020). In this context, autophagy negative regulation of mitochondrial reactive oxygen species (ROS) and degradation of macromolecules seems to restrain IFN-I release after RLRs signaling (Tal et al., 2009), while possessing a dichotomic role for TLRs, depending on the circumstances and type of TLR activated (Henault et al., 2012; Zhou et al., 2012; Song et al., 2018). For example, TLR7 endosomal signaling is optimized by viral delivery to endosomes, a process that autophagy seems to contribute (Zhou et al., 2012). On the other hand, acceleration of endosome degradation *via* autophagy can contribute to TLR7-signaling restriction, a feature explored by some viruses like coxsackievirus 16, to circumvent host resistance provided by IFN-I release (Song et al., 2018). Interestingly, autophagy is also induced after IFN-I and lipid mediators (15-epi-lipoxin A4 and

resolving D1) signaling as an essential stress response mechanism regulated by inflammatory mediators, leading to viral clearance by xenophagy, in the case of IFN-I, and resolution by 15-epi-lipoxin A4 and resolving D1 (Schmeisser et al., 2014; Prieto et al., 2015; Tian et al., 2019). Thus, different studies demonstrated increased inflammatory conditions in mice with genetic deletions of autophagy-related genes (Reed et al., 2015; Cotzomi-Ortega et al., 2020), reinforcing the importance of autophagy for an appropriate immune response, with an anti-inflammatory role.

## AUTOPHAGY: PATHWAYS AND FUNCTIONS

Autophagy was initially described as an adaptive process to starvation, promoting energy maintenance; the recycling of senescent or disabled macromolecules and organelles; and providing the building blocks for *de novo* synthesis of macromolecules. Autophagy is highly conserved among eukaryotes. Several aspects and proteins involved in the autophagy process were first described in yeast, with many orthologs conserved until vertebrates. Nowadays, autophagy functions have been expanded to many distinct aspects of cell biology, such as cell death, signaling regulation, and cell resistance to infections after targeting microorganisms for lysosomal degradation (Bustos et al., 2020).

The first step of autophagy is the formation of the autophagosome that, once matured, fuse with lysosomes where acidic hydrolases degrade their components. The formation of the double membrane structure of the autophagosomes (pre-autophagosomal structure-PAS) will require a cluster formed by the proteins Unc-51 like autophagy activating kinase (ULK1), autophagy-related protein 13 (Atg13), focal adhesion kinase family interacting protein of 200 kDa (FIP200), and Atg101 (Lahiri et al., 2019). This complex will phosphorylate and activate several proteins that form another cluster composed of Beclin-1, vacuolar protein sorting 15 (Vps15), Atg14L, and Vps34. This cluster forms the class III PI3K complex that is crucial for autophagosome formation through phosphorylation of the membrane lipid phosphatidylinositol (PI), involved in the recruitment of several adaptor proteins for autophagosome elongation (Lahiri et al., 2019). The elongation of the autophagosome structure is mediated by two ubiquitin-like systems composed by the complex Atg12-Atg5-Atg16L and light chain 3 (LC3) (Lahiri et al., 2019). Atg12 is activated by the protein Atg7 (with a function similar to E1 ubiquitin enzymes) and binds to Atg5 in an Atg10-dependent manner (similar to E2 ubiquitin transferase). The complex Atg12-Atg5 binds Atg16L1 and attaches to the outer membrane of the forming autophagosome, where it recruits the lipidated form of LC3 (LC3-II) (Walczak and Martens, 2013). LC3-II is formed after cleavage of cytosolic LC3 by Atg4, generating LC3-I. LC3-I is activated by Atg7 and transferred to Atg3, which is crucial for binding LC3 to phosphatidylethanolamine (PE) in the autophagosome membrane, forming LC3-II (Tanida et al., 2004). The last step of the autophagy process, the lysosome fusion, is also regulated by another protein complex composed of Beclin-1, Vps34, Vps15, and UV radiation resistance-associated gene protein-UVRAG (Morris et al., 2015). All the proteins in these clusters can be regulated by several other proteins, like Atg9, AMP-activated protein kinase (AMPK), mammalian target of rapamycin complex 1 (mTORC1), E1A Binding Protein P300 (EP300), and others, as described herein another section. In addition, the intracellular trafficking and fusion of autophagosomes with lysosomes are finely coordinated by Rab GTPases and N-ethylmaleimide-sensitive factor attachment receptors (SNAREs) proteins (Ao et al., 2014; Wang et al., 2016). Thus, modulators that affect distinct steps of the autophagic machinery have been described and will also be discussed here (**Figure 1**).

The macromolecules and organelles directed to autophagy must also be tightly regulated. In this sense, autophagy-adaptor proteins act like molecular linkers, allowing interaction between targeted molecules and LC3-II from the autophagosomes for later lysosome degradation. The best-characterized adaptor proteins are p62; nuclear dot protein 52 (Ndp52); histone deacetylase 6 (Hdac6); optineurin (Opt); Neighbor Of BRCA1 Gene 1 Protein (Nbr1); and Tax1 binding protein 1 (Tax1bp1). These adaptors possess multiple domains that allow their interaction with LC3 (LC3 interacting domains-LIR), ubiquitin, and distinct macromolecules, such as the active lipid phosphorylated phosphatidylinositol (PIP) (Johansen and Lamark, 2011).

## THE INTERPLAY BETWEEN AUTOPHAGY AND VIRUS INFECTIONS

Viral replication is a major cause of cellular stress, causing the misbalancing of cellular metabolism to produce a considerable number of infectious viral particles (Sanchez and Lagunoff, 2015). Not surprisingly, numerous studies have described an interplay between viruses and the autophagic machinery, with the first report dating from 1965, when the group of George Palade demonstrated the presence of "autolytic vesicles," later known as autophagosomes, containing poliovirus particles during infection (Carneiro and Travassos, 2016). It is clear that many viruses induce an autophagic response in the infected cell, but the contribution of this pathway to either host antiviral defenses and immune responses or viral replication is variable.

As a piece of fundamental machinery that rapidly responds to diverse types of stress, it is expected that autophagy plays a significant role in viral restriction, through the degradation of viral particles and their components or even host proteins used for viral replication, in a process termed virophagy (Choi et al., 2018). In addition, as previously described here, autophagy impacts host immune responses, which also influences viral replication.

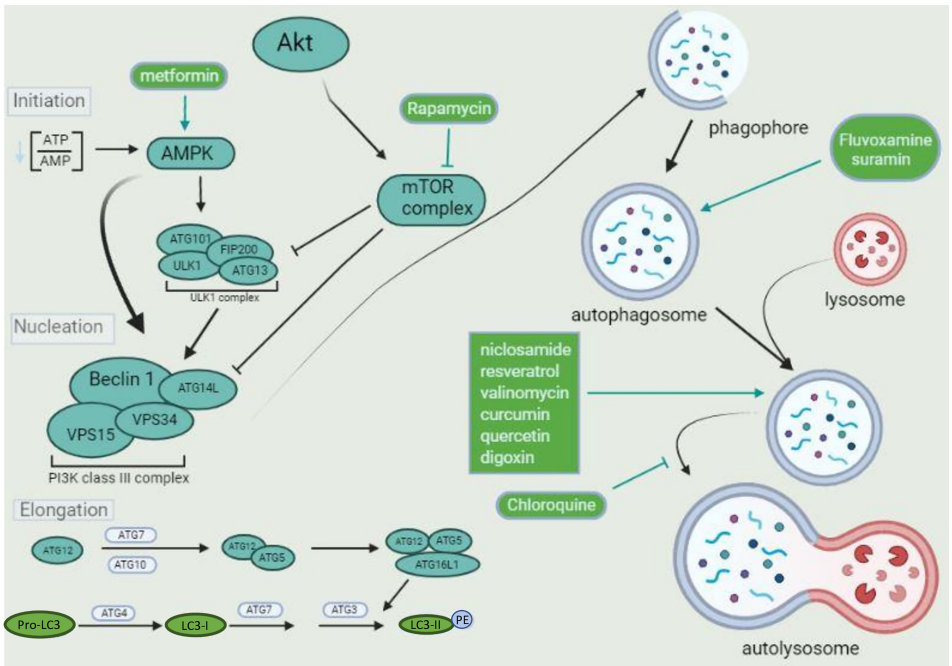


**FIGURE 1** | The autophagic flux and its components. Diverse complexes tightly regulate the autophagic pathway. Two kinase complexes are involved in phagophore formation. The ULK1 complex activates the class III PI3K that performs the phospholipid 3-phosphatidyl inositol (PI3) phosphorylation, generating PI3P, which is crucial for phagophore nucleation. Both complexes can be oppositely modulated by distinct kinases, like AMPK, associated with activation, and mTOR complex, associated with inhibition, depending on the amino acid residue targeted for phosphorylation by each kinase. The pharmaceuticals metformin and rapamycin drive autophagosome formation after activating AMPK and inhibition of mTOR complex, respectively. The kinase Akt (or protein kinase B-PKB) is upstream from the mTOR complex, activated by various stimuli. Once a phagophore is formed, it is elongated by different proteins, especially LC3. Pro-LC3 is converted to LC3-I by ATG4. ATG7, ATG3 and the complex formed by ATG12-ATG5-ATG16L1 are crucial for LC3-II formation and binding to the phagophore lipid phosphatidylethanolamine (PE). LC3-II promotes phagophore maturation and closure. The mature autophagosomes are fused with lysosomes and degraded by acid proteases, leading to macromolecules and organelles recycling. Pharmaceuticals that induce lysosome biogenesis, like niclosamide, resveratrol, and valinomycin, promote autophagosomes degradation. Chloroquine is an alkalinizing lysosomotropic agent, inhibiting acid proteases activity and autolysosome degradative function, leading to autophagosome accumulation.

## INHIBITION OF AUTOPHAGY BY VIRUSES

As an essential resistance factor for host cells against some viruses, it is expected autophagy to be inhibited by several viruses in different steps of its pathway to favor virus replication and spread. In agreement with the critical role of mTORC1 in autophagy suppression, some viruses present sophisticated strategies to inhibit autophagy through mTORC1 increased activity. This is the case for Kaposi's sarcoma-associated herpesvirus (KSHV), a double-stranded DNA virus (dsDNA) that has been shown to induce mTOR activation through its Viral-G protein-coupled receptor (v-GPCR), promoting an important increase in cellular protein synthesis, especially viral ones, and inhibiting autophagy as well (Bhatt and Damania, 2012). Interfering with early steps of autophagy also seems to be a strategy deployed by other viruses such as HIV-1. This is achieved by activating mTOR by the viral envelope protein during entry in dendritic cells, leading to inhibition of immunoamphisomes, which are intermediate/hybrid organelles formed after the fusion of endosomes and autophagosomes. These organelles possess immunostimulatory properties (Blanchet et al., 2010). HIV-1 can also inhibit early steps of autophagy activation by interacting with the viral protein Nef and Beclin-1, resulting in mTORC1 activation, transcription factor EB (TFEB) phosphorylation, and cytosolic sequestration, inhibiting autophagosome maturation (Campbell et al., 2015). Other viruses seem to aim at vesicle nucleation to dampen the autophagic pathway. Human cytomegalovirus (HCMV), a dsDNA virus, encodes TRS1 protein that interacts with Beclin-1 to suppress autophagy (Chaumorcel et al., 2012). Furthermore, Viral B-cell lymphoma 2 (vBCL-2), a viral counterpart of cellular Bcl-2 (cBcl-2), encoded by several viruses directly interacts with Beclin-1, leading to sequestration of this molecule and blunting of autophagy initiation (Yamamoto et al., 1999; Pattingre et al., 2005; Ku et al., 2008). Interestingly, some virus, like Chikungunya virus (CHIKV), a ssRNA+, inhibits mTORC1 after induction of oxidative and endoplasmic reticulum (ER) stresses. In this case, mTORC1 inhibition by CHIKV was associated with increased viral replication and viral protein translation through an eIF4E activation-dependent mechanism, while mTORC1-independent (Joubert et al., 2015). These studies demonstrate that mTORC1 inhibition by CHIKV can promote viral replication, despite a possible induction of autophagy. Thus, in the case of CHIKV, the complex network of virus-host

interactions targeting a protein complex (mTORC1) related to different signaling pathways, in the referred case, autophagy and protein translation, will determine the outcome of the infection.

## AUTOPHAGY AS AN INHIBITORY FACTOR FOR VIRAL INFECTIONS

The interplay of autophagy and CHIKV is complex. A differential role of two autophagy-related adaptor proteins seems crucial for the balance between virophagy and viral replication, with opposite outcomes concerning host cell death. While cellular Ndp52 promoted viral replication, after interaction with the CHIKV nonstructural protein 2 (Nsp2), p62 promoted virophagy and increased survival of the host cells after interaction with viral ubiquitinated capsid. Ndp52 ability to bind Nsp2 is present in permissive human cells but absent in non-permissive cells from mice (Judith et al., 2013). These results highlight critical molecular mechanisms that explain the difference in virus permissivity in two different mammalian cells (Judith et al., 2013). In line with an inhibitory role of autophagy for CHIKV infection, Joubert et al. (2012) showed that autophagy induction inhibits cell death and limits viral propagation. In Sindbis virus infection (SINV), another ssRNA+ virus, Beclin-1 and Atg5 were shown to protect the host against encephalitis (Liang et al., 1998). In the same direction, Smad ubiquitin regulatory factor 1 (SMURF1), an E3-ubiquitin ligase, was demonstrated to drive SINV viral capsid for autophagosomal degradation (Orvedahl et al., 2010). Autophagy-dependent restriction is also observed during Picornavirus (ssRNA+) infection when galectin-8 senses the virus to trigger autophagy and degrade the viral RNA genome (Staring et al., 2017).

It is important to highlight that autophagy-related protein possesses other functions not associated with the autophagic process. Thus, autophagy-independent effects of Atg13 and FIP200 have been described to inhibit viral replication of encephalomyocarditis virus (EMCV), a picornavirus (Mauthe et al., 2016). The authors demonstrated that knockdown of Atg13 and FIP200 in permissive cells, but not other components of the ULK1 complex or autophagy-related proteins like Atg7, increased EMCV replication. Furthermore, no additive effect was demonstrated with the knockdown of both Atg13 and FIP200, suggesting that both proteins are in the same pathway. Though the authors could not determine the exact manner by which Atg13 and FIP200 control viral replication, they did rule out an interplay between IFN mediated virus restriction and these autophagy proteins (Mauthe et al., 2016)

## AUTOPHAGY PROMOTES ANTIVIRAL IMMUNE RESPONSES

The protective role of autophagy to host cells can also be achieved independently of virophagy through the induction of immune responses. For example, viral recognition by plasmacytoid dendritic cells relies on autophagy for anti-viral cytokine secretion (Lee et al., 2007), such as IFN-I that hampers viral protein translation and assembly, after inducing interferon-stimulated genes (ISGs), like myxovirus resistance 1 (Mx1) (Verhelst et al., 2012). Curiously, type I interferons also rely on virophagy to exert their antiviral effects (Tian et al., 2019), in a compelling positive feedback loop in the case of TLR7-mediated antiviral signaling. Autophagy also seems to facilitate MHC-II antigen presentation by APCs and activation of CD4+ T cells (Rey-Jurado et al., 2015), and viruses have evolved strategies to inhibit autophagy and restrict recognition by specific CD4+ T cell clones, as demonstrated for Epstein-Barr virus nuclear antigen 1 (EBNA-1) (Paludan et al., 2005).

The effector functions of T cells are also influenced by autophagy, likely due to alterations in mitochondrial metabolism (Macian, 2019). At last, autophagy also affects antigen presentation and activation of CD8+ T cells in the context of the MHC-I-peptide complex. Different studies reported conflicting results in this context, showing both positive and negative effects (Van Kaer et al., 2019; Øynebråten, 2020). For instance, autophagy seems to decrease the surface expression of MHC-I, after internalization and degradation. In this regard, genetically deficient DCs for autophagic related proteins (Atg5, Atg7, or Vps34) possess an increased surface expression of MHC-I and viral antigen presentation ability (Mintern et al., 2015; Loi et al., 2016; Parekh et al., 2017). On the other hand, autophagy promotes antigen cross-presentation (Li et al., 2011; Dasari et al., 2016), in which exogenous antigens are presented in the context of MHC-I (Embgenbroich and Burgdorf, 2018). Hence, autophagy induction, after herpes simplex virus 1 (HSV-1) infection, is involved in the increased presentation of viral antigens to CD8+ T cells (English et al., 2009). In conjunction, different studies demonstrate that autophagy interplay with MHC-I peptide antigen presentation is complex, but can be an interesting target to modulate adaptive immunity activation.

## AUTOPHAGY AS A PROMOTER OF VIRAL INFECTION

Autophagy has also been widely reported as a mechanism that promotes some virus replication. The fact that autophagosomes harbor key molecules and provide protection from immune detection makes these compartments a target for creating a replicative niche for many RNA viruses. The formation of double-membrane vesicles (DMVs) has been widely reported during viral infection, which is the case for Picornaviruses. These small RNA viruses use autophagosomes as membrane scaffolds to assemble and replicate their genomic RNA. How these viruses escape virophagy is unclear, and controversial studies about a possible inhibition of autophagosomes and lysosomes fusion do not allow proper conclusions (Kemball et al., 2010; Shi et al., 2016). Picornaviruses have also been shown to use autophagy to induce their non-lytic release. Growing evidence from the literature demonstrates that poliovirus (PV) and coxsackievirus can spread without lysing the cell, through extracellular

microvesicles, including autophagosome-derived ones (Bird et al., 2014; Robinson et al., 2014; Granato et al., 2015). In line with a beneficial role for autophagy in PV replication, rapamycin, an inducer of autophagy, strongly up-regulates PV replication *in vitro*. Corroborating these findings, silencing autophagy in HeLa cells dampened PV replication (Jackson et al., 2005).

Arboviruses such as Dengue virus (DENV) and Zika virus (ZIKV), ssRNA+, have also been shown to induce the formation of membranes decorated with LC3 (Samsa et al., 2009; Liang et al., 2016). The mechanism relying upon autophagy-dependent DENV replication seems to involve the use of fatty acids generated during lypophagy, a specialized form of autophagy in which lipid droplets (LDs) are broken down for use in mitochondrial metabolism. DENV is known to increase LDs, which harbor viral capsid proteins. Thus, LDs provide a platform for viral replication (Samsa et al., 2009), a feature that also seems to occur in the case of SARS-CoV 2 (Dias et al., 2020). The pro-viral role of autophagy is elegantly illustrated in a study analyzing ZIKV vertical transmission. *Atg16l1*-deficient mice infected with ZIKV showed limited vertical transmission and placental and fetal damage (Cao et al., 2017). Furthermore, ZIKV promotes autophagy and dampens host efforts to induce another specialized form of autophagy, reticulophagy (or ER-phagy). The ER is a source of membrane for the establishment of viral replication. During ZIKV infection, reticulophagy is enhanced to restrain viral maturation, mediated by a protein called FAM134B. ZIKV-encoded NS3 cleaves FAM134B to suppress the formation of ER and viral protein-enriched autophagosomes, suggesting that the cleavage of FAM134B serves to specifically suppress the reticulophagy pathway (Lennemann and Coyne, 2017; Bhaskara et al., 2019). Recent findings showed that ZIKV, DENV, and PV regulate different subsets of autophagy initiation components for efficient viral growth in a non-canonical way. In common, all three viruses utilize the lipid scavenger protein Atg9 and recruit LC3 directly to membranes, bypassing the need for Atg5-mediated lipidation (Abernathy et al., 2019).

The induction of autophagosome formation and its maturation arrest has been demonstrated for human parainfluenza virus type 3 (HPIV3), and Influenza A virus (IAV), both ssRNA- viruses. This feature is also observed for some coronaviruses, as we will discuss later. The proposed mechanism involves the binding of viral proteins, such as phosphoprotein (P) of HPVI3 and matrix protein 2 (M2) of IAV, to cellular regulators of autophagy. In the case of HPVI3, SNARE domains of Synaptosome Associated Protein 29 (SNAP29) is bound to P, while Atg6/Beclin-1 and UVRAG containing PI-3 kinase complex is bound to M2 in the case of IAV (Gannage et al., 2009; Ding et al., 2014).

Once again, it is important to highlight that autophagy-related proteins might possess autophagy-independent effects. For example, LC3 and SNAP29 have been described to promote viral replication independently of the autophagic process (Monastyrska et al., 2013; Sharma et al., 2014; Alirezaei et al., 2015; Sarkar et al., 2021). For instance, non-lipidated LC3 is essential for double-membrane vesicles (DMVs) formation and replication of the ssRNA+ viruses, equine arteritis virus (EAV) and Japanese encephalitis virus replication (JEV) (Monastyrska et al., 2013; Sharma et al., 2014; Sarkar et al., 2021). Interestingly, coxsackievirus B3 replication is dependent on LC3 and autophagy but can still occur if non-lipidated LC3 is present and autophagy is absent (Alirezaei et al., 2015), showing surprising plasticity in terms of LC3-dependent processes of viral replication.

Viruses also impair viral recognition through autophagy modulation. Data from the literature demonstrate that HPIV3 suppresses innate immune responses after enhancing mitophagy to dampen viral recognition by mitochondria-located sensors, which blunts the production of IFN-I. More specifically, the matrix protein (M) of HPIV3 interacts with mitochondrial translation factor Tu (EF-TU) and binds to LC3 to promote autophagosome formation and mitochondrial degradation in a Parkin-PINK1 independent manner (Ding et al., 2017). A similar strategy of autophagic trafficking remodeling is used by enterovirus 65, a ssRNA+ virus, to replicate and exit from the cell without being degraded after lysosome fusion (Corona et al., 2018).

## AUTOPHAGY AND CORONAVIRUSES INFECTION (COVS)

Much of our current knowledge on the interaction between autophagy machinery and CoVs rely on studies using mouse hepatitis virus (MHV) as a model, possibly due to its ability to be used in BSL-2 facilities and to infect multiple cell types and host species (de Haan et al., 2005). MHV infection leads to the formation of DMVs, closely resembling autophagosomes. In contrast to a well-established role of autophagy during infection with several viruses, its implication in the replication and pathogenesis of CoVs is still under investigation.

Initial studies on the interaction of autophagy and CoVs using MHV as a model demonstrated conflicting results. Prentice et al. (2004) showed that Atg5 was required to support viral replication in embryonic stem (ES) cells. The authors evaluated the replication of MHV in ATG5-deficient ES cells and observed a significant decrease in the number of plaque-forming units (PFU) (Prentice et al., 2004). A subsequent study revealed that ATG5 was dispensable for MHV replication in bone marrow macrophages and primary mouse embryonic fibroblasts (Prentice et al., 2004). Apart from the different cell types in these two studies, it is not possible to rule out a non-canonical role for Atg5. The hypothesis of non-canonical roles of ATG proteins is supported by findings that non-lipidated LC3-I localized to MHV-induced DMVs. For a long time, the origin of membranes composing the DMVs remained obscure. Currently, several pieces of evidence suggest the ER as the source. Two non-structural proteins (nsps 3 and 4) which have been suggested to be part of the replication-transcription complex (RTC), were shown to be N-glycosylated.

Interestingly, when ectopically expressed, nsp4 locates to the ER and moves to DMV upon infection (Oostra et al., 2007). In addition, ultrastructural studies report that DMVs are

interconnected through their outer membranes as part of the reticulovesicular network (Knoops et al., 2008). Corroborating the hypothesis of an autophagy-independent origin of DMVs, Reggiori et al. (2010) demonstrated that DMVs originate from ER vesicular export containing non-lipidated LC3 and short-lived chaperones ER Degradation Enhancing Alpha-Mannosidase Like Protein 1 (EDEM-1) and OS9. One aspect against the idea of an ER origin for DMV membranes is that fragmentation of the Golgi apparatus contributes to DMV formation, a feature in which autophagy-related proteins also participate (Cortese et al., 2020).

Some shared features can be observed among SARS-CoV, SARS-CoV-2, and MERS-CoV infections *in vitro* concerning autophagy regulation, as extensively reviewed elsewhere (Shojaei et al., 2020; Zhao et al., 2021). Autophagy seems to be induced by all SARS-CoV, MERS-CoV, and SARS-CoV-2, with a potential role in viral replication (Chen et al., 2014; Yang et al., 2014; Gordon et al., 2020; Gassen et al., 2021). Direct regulation of autophagy by viral proteins, such as ORF3a and nsp6, is associated with autophagy induction (Cottam et al., 2011; Gassen et al., 2019; Qu et al., 2021). At the same time, the last step of autophagy seems to be negatively regulated by viral proteins, causing an accumulation of autophagosomes due to impaired fusion with lysosomes, both *in vitro* and *in vivo*, in the case of lung samples from deceased SARS-CoV-2 patients (Gassen et al., 2019; Gassen et al., 2021; Qu et al., 2021; Zhang et al., 2021). Altogether, these reports suggest that these viruses manipulate autophagy at multiple levels to its benefit, and specific autophagy modulators must overcome viral regulation to allow virophagy. In addition, as discussed earlier, autophagy is both regulated and an essential player in inflammation and resolution. Interestingly, autophagy is involved in the reduction of IL-17 secretion and NLRP3-dependent signaling (Reed et al., 2015; Cotzomi-Ortega et al., 2020), both possibly involved in coronavirus pathogenesis, and, pointed out as important mechanisms that govern bats, animals known for their resilience to viruses, disease tolerance (Ahn et al., 2019; Pacha et al., 2020; Rodrigues et al., 2020). Furthermore, autophagy restrains PRRs activation through a negative feedback loop (Zhu et al., 2019). Thus, autophagy modulators are promising drugs to restrain coronavirus pathogenesis by their anti-inflammatory effects and reduction of the intracellular viral load (Laing et al., 2019).

## AUTOPHAGY PATHWAYS: DIFFERENT TARGETS FOR MODULATION

Autophagy can be regulated at both transcriptional and post-translational levels, and several proteins are known to coordinate the process, opening several new roads for specific targets modulation along the autophagy pathway.

The primary transcription factors involved in autophagosome formation and vesicular transport are TFEB, cyclic AMP response element-binding protein (CREB), and Forkhead box proteins (FOXOs 1, 3, 4, and 6), which are positive regulators of genes involved in autophagosome and lysosome biogenesis, and the negative ones, farnesoid x receptor (FXR) and Zinc Finger Protein With KRAB And SCAN Domains 3 (ZKSCAN3) (Di Malta et al., 2019). TFEB is a member of the helix-loop-helix leucine zipper (bHLH-Zip) family of transcription factors. Generally, under nutrient availability, TFEB is localized in the cytoplasm in its phosphorylated form (Sardiello et al., 2009). TFEB phosphorylation is mediated by different kinases, such as mTORC1, extracellular-signal-regulated kinase 2 (ERK2), glycogen synthase kinase 3 beta (GSKB3), Akt, and protein kinase C beta (PKCβ) (Martina et al., 2012; Li et al., 2016). mTORC1, ERK2, GSKB3, and Akt are responsible for TFEB retention on the cytoplasm. In contrast, PKCβ is associated with TFEB stability, supporting its activation after nuclear translocation (Peña-Llopis et al., 2011; Martina et al., 2012; Settembre et al., 2012; Ferron et al., 2013; Li et al., 2016; Palmieri et al., 2017). This indicates that specific phosphorylation sites are crucial for TFEB proper regulation. ZKSCAN3 is also regulated by mTORC1, opposing TFEB transcriptional activity under nutrient availability (Chauhan et al., 2013). TFEB can also be targeted by different phosphatases, favoring nuclear translocation and autophagy induction. For example, protein phosphatase 2A and calcineurin are responsible for TFEB activation after oxidative stress and calcium signaling, respectively (Medina et al., 2015; Martina and Puertollano, 2018; Xu et al., 2020). Once activated, TFEB translocates to the nucleus, where it binds to specific regulatory sequences (coordinated lysosomal expression and regulation- CLEAR) in the promoter regions of several genes that code for autophagy and lysosomal proteins, such as Atg9B, Beclin-1, Atg5, p62, and lysosomal transmembrane proteins and hydrolases, driving their transcription (Xu et al., 2020). TFEB is the target of different drugs, such as resveratrol, digoxin and curcumin (Xu et al., 2020). These drugs are being used in clinical trials against many diseases, in which their efficacy is being evaluated for their pleiotropic effects, which includes but is not restricted to autophagy induction, as discussed later.

FOXO transcription factors are also translocated to the nucleus once activated in the cytoplasm, promoting autophagy and lysosome-related genes transcription (Mammucari et al., 2007; Zhao et al., 2007; Sanchez et al., 2012). FOXO activity is modulated by Akt, which phosphorylates and restricts FOXO cytoplasmic localization (Brunet et al., 1999). Thus, different pharmaceuticals that target Akt can also impact FOXO activity, such as MK-2206 and BAY1125976, among others (Song et al., 2019). FOXO activity can also be regulated by AMPK (Song et al., 2019). Furthermore, sirtuins can positively regulate FOXO transcriptional activity, and the activity of Atg5, 7, and 8 are associated with autophagy induction (Ng and Tang, 2013). Interestingly, some of these modulators like MK-2206 and BAY1125976, and suramin, have been tested in clinical trials against tumors with good safety assessments (NCT01307631; Schneeweiss et al., 2019; NCT01671332).

CREB interacts with the coactivator CREB regulated transcription coactivator 2 (CRTC2) to induce Atg7, ULK1, and TFEB coding genes transcription, among other autophagy-related genes (Di Malta et al., Seok et al., 2014). FXR inhibits CREB interaction with CRTC2, restraining autophagy induction under

nutrient availability (Seok et al., 2014). Both FXR and CREB are targeted by pharmaceuticals in different diseases (Barco et al., 2003; Wang et al., 2018; Sapio et al., 2020), with distinct proposed mechanisms of action. Among them, the FXR agonist, obeticholic acid, has been tested in different clinical trials, for example, in alcoholic liver disease, in which FXR agonists are thought to decrease the P450 2E1 enzyme responsible for oxidative stress-mediated injury in hepatocytes (Ali et al., 2015). CREB inhibitors have also been tested in anti-cancer therapies, aiming at the effects of CREB on cell proliferation and tumorigenesis-associated genes (Sapio et al., 2020).

At the post-translational level, different enzymes, kinases, and acetylases regulate protein complexes that coordinate the formation, elongation, and fusion of autophagosomes with lysosomes. As already discussed, the major complexes involved in the autophagic process are ULK1, ATG13, FIP200, and ATG101, which are responsible for the activation of the PI3K class III complex (Mercer et al., 2018), composed of Beclin-1, Vps15, Atg14L, and Vps34, which are responsible for autophagosome nucleation; two ubiquitin-like systems composed of the complex Atg12-Atg5-Atg16L and LC3 (Lahiri et al., 2019); and the complex that mediates lysosome fusion with autophagosomes, composed of Beclin-1, VPS34, VPS15 and UVRAG (Matsunaga et al., 2009). Several kinases and acetylases influence the activity of each complex. The major enzymes targeted by pharmacological modulators are AMPK, a positive regulator of Beclin-1 and ULK-1, activated by metformin (Nazarko and Zhong, 2013); mTORC1, a negative regulator of ULK-1 complex and TFEB, inhibited by rapamycin (Kim et al., 2011); and S-phase kinase-associated protein 2 (Skp2) and Akt that also affect different steps of the autophagic process. Akt can phosphorylate multiple targets, including mTORC1 and the transcription factor FOXO1, mediating autophagy inhibition (Dan et al., 2014; Pan et al., 2017). Skp2 is downstream from mTORC1 signaling, drives Beclin-1 degradation by ubiquitination (as an E3 ligase), and is also involved in epigenetic changes preventing TFEB transcriptional activity (Shin et al., 2016; Gassen et al., 2019). Many other players also affect autophagy, such as cyclic AMP (cAMP) and calcium. Calcium can induce or inhibit autophagy, depending on its levels and subcellular localization (Sun et al., 2016). In general, constitutive calcium release from the ER inhibits autophagy. It keeps mitochondrial tricarboxylic acid enzymes active and, consequently, high levels of ATP, which restrains AMPK activity (Ivanova et al., 2017). Calcium release from the ER can be mediated by the Inositol triphosphate (IP3) receptor (IP3R), linking phospholipase C (PLC) activity and autophagy (Putney and Tomita, 2012). PLC generates IP3 from phosphatidylinositol biphosphate (PIP2), and it is dependent on free inositol levels as an essential substrate for its enzymatic activity (Berridge, 2016). PLC activity is also dependent on cAMP levels that keep Exchange protein directly activated by cAMP (EPAC) activated and, consequently, Rap2b. Rap2b is a GTPase that interacts and activates PLC and calpain (Schmidt et al., 2001; Mestre et al., 2010). Calpain is a calcium-activated cysteine protease that inhibits autophagy through Beclin-1 degradation (Russo et al., 2011). Thus, PLC activity affects calcium release and is affected by cAMP. IP3R also directly inhibits the autophagic process through the recruitment of Beclin1, reducing its availability for the initiation complexes of autophagy (Vicencio et al., 2009). On the other hand, increased cytoplasmic calcium levels have also been associated with calmodulin activation (Keller et al., 2008), an upstream activator of AMPK and autophagy (Fogarty et al., 2010). Intriguingly, this process can also be activated by cAMP and EPAC1 (Laurent et al., 2015) (**Figure 2**). Altogether, as a complex and multiple-step process, autophagy can be manipulated in diverse ways, and the outcome will depend on the sum of positive and negative stimuli. Next, we will further discuss different pharmaceuticals that can modulate autophagy. Some of these drugs have already been approved for use in many different diseases with distinct proposed mechanisms of action, associated or not with autophagy modulation, and have been tested for coronavirus diseases. Thus, the United States Food and Drug Administration (FDA) approved the clinical use of statins, rapamycin, sirolimus, hydroxychloroquine, metformin, niclosamide, valinomycin, quercetin, digoxin, fluvoxamine, verapamil, clonidine, geldanamycin, loperamide, and miconazole in different diseases. Interestingly, the only drug approved by the FDA for treating viral infections (hepatitis B virus and hepatitis C virus) that is known to modulate autophagy (and many other anti-viral effects) is IFN-I. As already mentioned, IFN-I treatment has been tested in clinical trials for coronavirus diseases.

## AUTOPHAGY MODULATORS FOR CORONAVIRUS DISEASES TREATMENT

Different autophagy modulators are available or under investigation for their therapeutic effects in infectious and non-infectious diseases. Here, we discuss several studies that described the effects of these modulators in SARS-CoV, MERS-CoV, and SARS-CoV2 infections.

### Lysosome Alkalinizing Agents

Chloroquine and its analogs, like hydroxychloroquine, possess pleiotropic effects and have been used for years as antiparasitic and anti-inflammatory drugs to treat malaria and different inflammatory conditions, respectively. Chloroquine is known for its ability to inhibit lysosome acidic hydrolases, as a lysosomotropic alkalinizing agent. It also affects many processes within the cells and in mammalian physiologic and pathological events, for example inhibiting heme-mediated inflammation (Silva et al., 2021). Interestingly, chloroquine and hydroxychloroquine impaired *in vitro* viral replication by a not fully determined mechanism. It is believed that these pharmaceuticals drive a catastrophic accumulation of autophagosomes, leading to cell death and eliminating viral particles (Shojaei et al., 2020). However, *in vivo*, chloroquine reduces lung pathology in mice from SARS-CoV infection without affecting viral burden, suggesting an effect independent of viral replication (Barnard et al., 2006; Weston et al., 2020). Further studies are needed to confirm and better comprehend the impact of chloroquine on coronavirus infections.

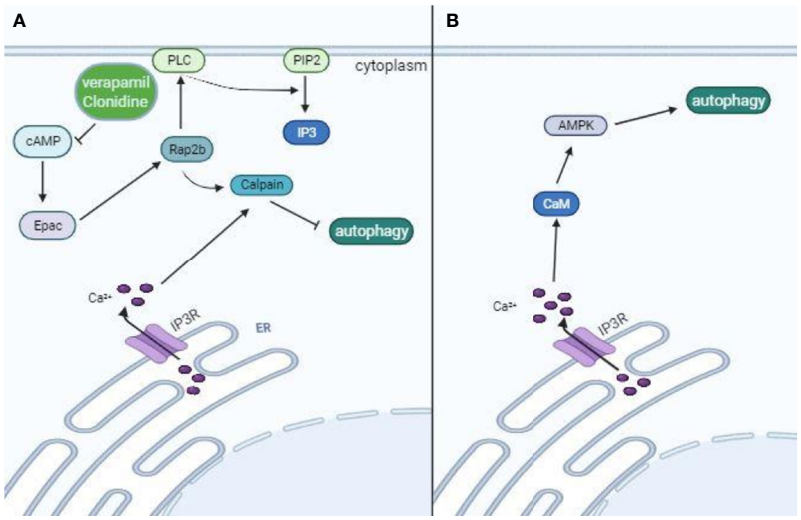


**FIGURE 2** | Opposing effects of intracellular $Ca^{+2}$ in autophagy modulation. Calcium ions can inhibit or induce autophagy, depending on its levels and interactive proteins. Calcium release from the ER is mediated by IP3R, which links PLC activity to autophagy. IP3 is generated from PIP2 by PLC, which is dependent on free inositol levels. PLC activity also depends on cAMP levels that keep Epac and Rap2b activated. Rap2b-mediated activation of calpain drives Beclin-1 degradation, restraining phagophore formation. IP3R also recruits Beclin-1, which further restrains Beclin-1 availability from forming the class III PI3K complex. Thus, pharmaceuticals like verapamil and clonidine that lead to reduced cAMP levels are associated with autophagy induction **(A)**. On the other hand, high levels of intracellular $Ca^{+2}$ lead to calmodulin activation and subsequent AMPK-induced autophagy after phosphorylation of ULK-1 complex and class III PI3K **(B)**.

## mTORC1 Inhibitors and AMPK Activators

Rapamycin and its analogs, inhibitors of mTORC1, are known inducers of autophagy affecting many different aspects of mammalian physiology, such as immune responses (Janes and Fruman, 2009). Rapamycin acts on cellular replication of SARS-CoV, MERS-CoV and SARS-CoV-2 in a specific manner, which correlates with the viral regulation of mTORC1 and related kinases. Similar to SARS-CoV-2, SARS-CoV infection seems to inhibit mTORC1, resulting in increased autophagic flux, contrary to MERS-CoV, which is associated with increased mTORC1 phosphorylation and kinase activity. Thus, rapamycin restrains viral replication in MERS-CoV infected hepatocytes (Kindrachuk et al., 2015) but increases viral replication in kidney cells infected with SARS-CoV-2 (Qu et al., 2021). In agreement with the effects of rapamycin in SARS-CoV-2 infection, AMPK activators, which also lead to autophagy assembly and initiation, were associated with increased viral replication (Gassen et al., 2021). Surprisingly, metformin (an AMPK activator)-treated patients seem to have a lower mortality rate (Bramante et al., 2021), but the role of autophagy in this supposed beneficial effect was not evaluated.

Sorafenib is a multikinase inhibitor that induces autophagy. It inhibits mTOR signaling and promotes the phosphorylation of AMPK, though this latter does not correlate with autophagy induction (Prieto-Domínguez et al., 2016). Sorafenib is approved for the treatment of hepatocellular carcinoma, as an inhibitor of growth factors signaling and, probably, as a regulator of autophagy as well. Interestingly, sorafenib has been described to inhibit both SARS-CoV-2 and MERS-CoV replications *in vitro* and is a compelling drug to be further evaluated in future clinical trials (Kindrachuk et al., 2015; Klann et al., 2020).

Recently, statins have been described as autophagy inducers (Zhang et al., 2013). The mechanism by which statins induce autophagy is not completed understood, but they do inhibit mTORC1 activity (Wei et al., 2013). Statins are currently used for the treatment of coronary heart diseases, with pleiotropic effects, including the improvement of endothelial function and the ability to lower low-density lipoprotein (LDL)-cholesterol levels. Interestingly, long-time users of statins seem to possess a lower risk for severe covid-19 development, despite increased ACE2 expression levels (Zhang X. J. et al., 2013). It is believed that the anti-inflammatory effects of statins are crucial for this protection, a feature that can be directly associated with autophagy induction (Peng et al., 2018).

Another interesting pharmaceutical that induces autophagy is ivermectin. It does so through regulation of mTOR/AMPK pathway (Liu et al., 2019; Zhang P. et al., 2020). Ivermectin is an antiparasitic drug with *in vitro* anti-viral properties, including against SARS-CoV-2 (Mastrangelo et al., 2012; Caly et al., 2020). Besides its effects on autophagy, ivermectin affects different cellular and physiologic processes, and, *in silico* analysis indicated a possible effect on viral enzymes as well, such as RNA-dependent RNA polymerase and 3-chymotrypsin like protease ($3CL^{pro}$) (Heidary and Gharebaghi, 2020; Eweas et al., 2021; Mody et al., 2021). Thus, the mechanisms by which ivermectin inhibits viral replication (at least *in vitro*) are not fully established and its efficacy against covid-19 is being evaluated in distinct clinical trials with different results (Krolewiecki et al., 2021; Lim et al., 2022).

## Activators of Lysosomal Biogenesis

Modulators that target lysosomal biogenesis, like niclosamide and resveratrol, showed promising results *in vitro* regarding SARS-CoV-2 and MERS-CoV replication (Lin et al., 2017; Bramante et al., 2021). In addition, as previously discussed, autophagosome-lysosome fusion can restrain pro-inflammatory signaling and reduce inflammatory damage. Thus, several modulators that act on this step of autophagy and are known anti-inflammatory agents, such as valinomycin, curcumin, resveratrol, quercetin, digoxin, and niclosamide, are described as potential therapies against coronavirus diseases (Shen et al., 2019; Cho et al., 2020; Marinella, 2020; Pindiprolu and Pindiprolu, 2020; Zhang D. et al., 2020; Di Pierro et al., 2021; Ter Ellen et al., 2021; Thimmulappa et al., 2021). Niclosamide and quercetin have already been tested in clinical trials that confirmed both niclosamide safety, warranting further tests, and quercetin positive effects in patients, preventing severity (Backer et al., 2021; Di Pierro et al., 2021) (**Figure 3**). It is important to mention that these autophagy modulators have pleiotropic effects and may act on multiple targets across host cells and coronaviruses (Gibellini et al., 2015; Pandey et al., 2020). For example, valinomycin can also affect potassium equilibrium inside host cells, affecting viral replication (Sandler et al., 2020); quercetin, curcumin, and resveratrol showed *in silico* ability to bind S-ACE2 receptor complex, involved in SARS-CoV-2 entry, possibly inhibiting viral invasion (Pandey et al., 2020). Besides that, these compounds also affect mitochondrial metabolism and intracellular ROS generation (Ungvari et al., 2011; Sandler et al., 2020); niclosamide can affect SARS-CoV-2 entry as well, targeting the viral endocytosis process (Pindiprolu and Pindiprolu, 2020); and digoxin might impact on viral RNA synthesis through ion homeostasis misbalance (Cho et al., 2020).

## Suramin: A Sirtuin Activator

Suramin is an antiparasitic drug used to treat African sleeping sickness (Wiedemar et al., 2020). Suramin acts as an inducer of autophagy by deacetylating regulatory proteins (Trapp et al., 2007) and has been described to inhibit SARS-CoV-2 infection *in vitro* (Salgado-Benvindo et al., 2020). Thus, suramin repurposing to treat coronavirus diseases might be an interesting strategy to pursue. It seems that suramin modulates the early steps of viral replication cycle, probably viral binding and entry into the host cells (Salgado-Benvindo et al., 2020), and the exact role of autophagy modulation for the anti-viral effects of suramin were not established, but instigates further studies.

## Cyclic AMP and Calcium Modulators Driving Autophagy

Verapamil and clonidine are used as antihypertensive drugs, with distinct mechanisms of action. Clonidine acts as an agonist of the presynaptic alpha$_{2A}$ receptor, depending on the autonomic nervous system for its effects (Szabo, 2002). Verapamil inhibits L-type calcium channels leading to vasodilation and relaxation of vascular smooth muscle cells, in an autonomic nervous system

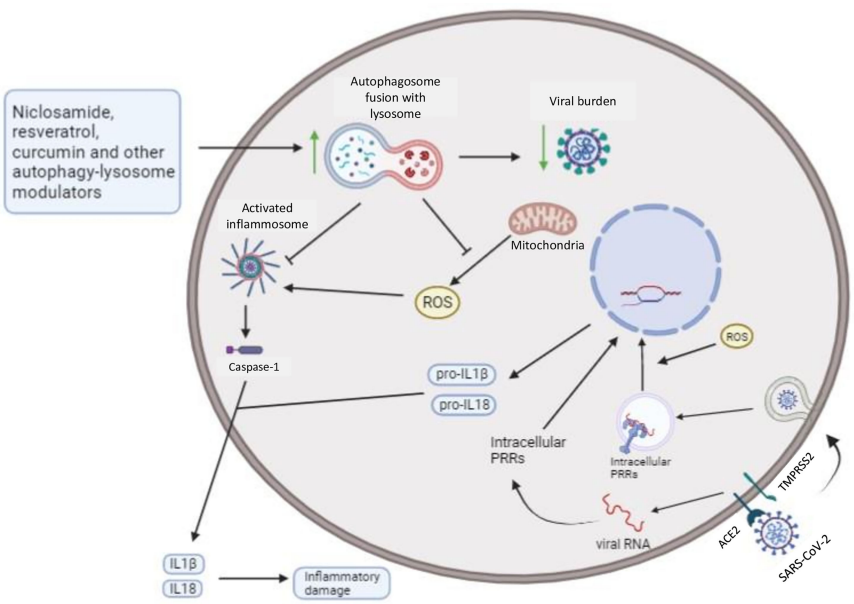


**FIGURE 3** | Autophagy can restrain intracellular viral burden and secretion of pro-inflammatory cytokines by infected host cells: Autophagy modulators that increase lysosome biogenesis and fusion with autophagosomes lead to degradation of viral particles, organelles, and macromolecules, like NLRP3, a known cytoplasmic PRR. Mitochondrial ROS also promotes both cytoplasmic, such as RLRs, and endosomal, such as TLR7 and TLR3, PRRs signaling, and mitophagy-mediated restriction of ROS can further restrain intracellular inflammatory pathways (from activated PRRs). Thus, niclosamide, resveratrol, and curcumin potentially drive increased resistance and disease tolerance, after autophagy induction, towards coronavirus diseases, reducing the intracellular viral burden and inflammatory mediators associated with tissue damage. Additionally, resveratrol and curcumin might also contribute to disease tolerance through its anti-oxidant activities, restraining mitochondrial ROS, a critical second messenger in PRRs signaling (Forman et al., 2010).

independent manner (Lee and Tsien, 1983). Clonidine inhibits cyclic AMP, while verapamil modulates intracellular calcium levels, leading to autophagy induction (Williams et al., 2008). A limited study made with only three patients showed a possible benefit associated with clonidine treatment (Hyoju et al., 2021). Though the mechanisms were not determined, the authors believe that sympathetic norepinephrine interference might reduce the deleterious inflammation associated with covid-19 (Hyoju et al., 2020). Verapamil is also believed to be an interesting pharmaceutical for the treatment of coronavirus diseases, since calcium entry during the initial life cycles of several viruses, including MERS-CoV, are very important (Straus et al., 2020). A clinical trial to determine the effects of verapamil treatment in covid19 was initiated, but suspended due to limited funding and number of patients (NCT04330300).

### Cellular Stressors as Inducers of Autophagy

Plumbagin and tunicamycin are stressors that induce autophagy as a response to ROS and ER stresses, respectively (Kuo et al., 2006; Ogata et al., 2006). Though both have been described as promising pharmaceuticals for the treatment of coronavirus diseases, they possess important toxicity, and, clinical trials associated with new formulations are needed. Tunicamycin inhibits crucial glycoproteins E2, S and M involved in SARS-CoV-2 replication and assembly (Dawood and Alnori, 2020), while plumbagin influences ROS levels, also supposedly impacting on viral replication (Nadhan et al., 2021).

### Autophagy activators with poorly determined mechanisms of action

The mechanisms by which some pharmaceuticals, like fluvoxamine, geldanamycin, miconazole, loperamide and rottlerin, activate autophagy are not completely understood (Cho et al., 2016; Qing et al., 2006; Qi et al., 2016; Tong et al., 2020; Ho et al., 2021). These drugs are used to treat many different diseases. Interestingly, they have been pointed out as exciting pharmaceuticals for the treatment of coronavirus diseases (Dawood and Alnori, 2020; Jayaseelan and Paramasivam, 2020; Karunakaran and Ganapathiraju, 2020; Klann et al., 2020; Lenze et al., 2020; Zhang D. et al., 2020; Lubkowska et al., 2021; Sanders et al., 2021). Once more, many of the mechanisms proposed for the beneficial effects of these pharmaceuticals were not related to autophagy modulation, as discussed next. Thus, the exact role of autophagy in the inhibitory activity of these pharmaceuticals still needs further investigation.

Fluvoxamine, a diffused antidepressant, was tested in a clinical trial to treat Covid-19 patients with positive effects, lowering the likelihood of clinical deterioration (Lenze et al., 2020). The proposed mechanism of the beneficial effects of fluvoxamine was the interference with serotonin-mediated thrombogenesis (Sukhatme et al., 2021).

Geldanamycin, an inhibitor of the chaperone Hsp90, presents promising results for cancer therapy in pre-clinical tests (Ochel et al., 2001). Though geldanamycin toxicity and instability precluded clinical trials, its analogs are showing important improvements concerning deleterious side-effects (Dey and Cederbaum, 2006; Kim et al., 2009). Several studies showed an important role of Hsp90 in different virus replication, including coronaviruses (Li et al., 2004; Li et al., 2020; Lubkowska et al., 2021). Hsp90 is crucial for appropriate viral proteins folding, making it a compelling molecular target (Li et al., 2020). Thus, the inhibitory effects of geldanamycin in MERS-CoV and SARS-CoV-2 replication *in vitro* were recently evaluated and proved (Li et al., 2020). In addition, geldanamycin also possesses anti-inflammatory effects (Kasperkiewicz, 2021), placing it as an important alternative for coronavirus diseases treatment.

Miconazole is used to treat fungal infections (Heel et al., 1980) and has been recently described, *in silico*, for its ability to bind and probably inhibit SARS-CoV-2 main protease $CL^{pro}$, also called $M^{pro}$ (Ferraz et al., 2020). Further studies are necessary not only to confirm a possible inhibitory effect of miconazole during viral replication but also to establish if autophagy induction contributes to this inhibition. In addition, other viral $M^{pro}$ inhibitor, nelfinavir, which restrains SARS-CoV-2 replication *in vitro*, is also able to induce autophagy (Gills et al., 2008).

Loperamide is used as an agonist of opiate receptors reducing intestinal motility. As discussed earlier, coronavirus diseases might induce gastrointestinal symptoms and loperamide can be used to treat diarrhea. Unexpectedly, loperamide showed inhibitory activity *in vitro* for MERS-CoV and SARS-CoV-2 infection. The authors proposed that loperamide inhibition of $3C^{pro}$ and papain-like protease ($PL^{pro}$) was responsible for the viral replication impairment (Kuo et al., 2021).

At last, rottlerin acts as an inhibitor of different kinases (like PKC and calcium modulator kinase III) that affect multiple cellular processes, including autophagy (Williams et al., 2008; Xiang et al., 2020). Thus, rottlerin possesses pleiotropic effects, suppressing *de novo* lipogenesis in adipocytes and tumor growth *in vitro* (Ma et al., 2018; Kim and Go, 2021). Computational analysis indicated rottlerin as a promising drug that targets SARS-CoV-2 protease $M^{pro}$ (Glaab et al., 2021). However, rottlerin inhibited the anti-MHV-1 activity of IFNα (Zorzitto et al., 2006), and many more studies are required to validate this drug for use in covid-19 patients.

## DISCUSSION

We reinforce that autophagy modulators are exciting candidates for drug-repurposing to treat coronavirus diseases, mainly if used in combination with other treatment modalities. Multifactorial diseases, such as viruses, usually require combinatorial therapies in order to be effective. Thereby, pharmaceuticals that block coronavirus replication, such as the ribonucleoside analog MK4482 (Rosenke et al., 2021), can be combined with several auto-lysosome modulators, like curcumin and resveratrol, restraining both viral replication and increasing viral macromolecules degradation by xenophagy. Furthermore, inhibitors of viral proteases that are crucial for viral proteins maturation after polyproteins catalysis, such as PF-07321332 (clinical test NCT04960202), can also be combined with auto-

lysosome activators. Another promising strategy is to combine autophagy modulators with IFNs administration. As discussed earlier, it has been described that autophagy is induced by IFNs, a feature that can contribute to the antimicrobial effects of IFNs (Tian et al., 2019). Thus, the combination of IFNs and autophagy modulators would allow an additive effect, increasing host resistance while providing an essential control to inflammatory damage, as autophagy can also exert anti-inflammatory effects. During the late inflammatory phase of SARS-CoV-2 disease, characterized by immune dysfunction and increased inflammatory damage, a combination of antibiotics, like azithromycin, corticosteroids, and autolysosome activators, can protect against secondary bacterial infections and inflammatory damage. Autolysosome modulators and antibiotics can restrain bacterial infections through increased resistance due to induction of xenophagy and bacterial metabolism blockage, depending on the type of antibiotics used. In addition, autolysosome inducers are possible regulators of inflammatory mediated damage through PRRs signaling and inflammasome restriction, also contributing to corticosteroid control of inflammation-mediated tissue damage.

## CONCLUDING REMARKS

Autophagy is a critical stress response mechanism that has a back-and-forth interplay with the immune system and can impact pathological processes associated with inflammation. In addition, many viruses can manipulate autophagy machinery to support viral replication or prevent viral components degradation by lysosomes. Different studies support the idea that coronavirus modulates the autophagic machinery positively while inhibiting autophagosome fusion with lysosomes. Thus, autophagy modulation is an attractive therapeutic strategy to increase disease tolerance and resistance to coronavirus infections. The central purpose of these modulators is to increase autophagosome fusion with lysosomes, allowing proper virophagy, while restraining NLRP3-dependent cytokines (IL-18 and IL-1β) release, mitochondrial ROS-PRRs signaling pathways, and promoting the anti-inflammatory effects of lipid mediators, leading to increased disease tolerance. Thus, these autophagy modulators would reduce pathological inflammation and viral replication within the cells. Interestingly, several pharmaceuticals under clinical tests for coronavirus diseases have shared the ability to induce autophagy. These compelling findings might not be fortuitous but indicate that autophagy can contribute to or even be the central mechanism behind the supposed efficacy of certain pharmaceuticals.

## AUTHOR CONTRIBUTIONS

RS contributed with text writing and figures elaboration; JR contributed with figures elaboration and text review and edition; GdS contributed with text review, edition and elaboration; LdC contributed with text review, edition and elaboration; LT contributed with text writing, edition and review, and figure elaboration. All authors contributed to the article and approved the submitted version.

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# Monitoring Autophagy at Cellular and Molecular Level in *Crassostrea gigas* During an Experimental Ostreid Herpesvirus 1 (OsHV-1) Infection

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Mortality outbreaks of young Pacific oysters, *Crassostrea gigas*, have seriously affected the oyster-farming economy in several countries around the world. Although the causes of these mortality outbreaks appear complex, a viral agent has been identified as the main factor: a herpesvirus called ostreid herpesvirus 1 (OsHV-1). Autophagy is an important degradation pathway involved in the response to several pathologies including viral diseases. In *C. gigas*, recent studies indicate that this pathway is conserved and functional in at least haemocytes and the mantle. Furthermore, an experimental infection in combination with compounds known to inhibit or induce autophagy in mammals revealed that autophagy is involved in the response to OsHV-1 infection. In light of these results, the aim of this study was to determine the role of autophagy in the response of the Pacific oyster to infection by virus OsHV-1. For this purpose, an experimental infection in combination with a modulator of autophagy was performed on Pacific oysters known to have intermediate susceptibility to OsHV-1 infection. In haemolymph and the mantle, the autophagy response was monitored by flow cytometry, western blotting, and real-time PCR. At the same time, viral infection was evaluated by quantifying viral DNA and RNA amounts by real-time PCR. Although the results showed activation of autophagy in haemolymph and the mantle 14 hours post infection (after viral replication was initiated), they were also indicative of different regulatory mechanisms of autophagy in the two tissues, thus supporting an important function of autophagy in the response to virus OsHV-1.

**Keywords: autophagy, Pacific oyster (Crassostrea gigas), herpesvirus, innate immunity, invertebrate**

## INTRODUCTION

Increased hatchery production and livestock translocation contribute to the increasing occurrence and spread of infectious diseases among bivalves (Barbosa Solomieu et al., 2015). Recently, disease outbreaks have significantly affected farmed Pacific oysters in Europe and in other parts of the world (Barbosa Solomieu et al., 2015). Since 1990, mortality of spat of *Crassostrea gigas*, has been observed

due to a virus called ostreid herpesvirus 1 (OsHV-1) in France and in other European and world regions (Hine et al., 1992; Nicolas et al., 1992; Renault et al., 1994a; Renault et al., 1994b; Friedman et al., 1997; Cherr and Friedman, 1998; Lynch et al., 2012; Peeler et al., 2012). This double-stranded DNA enveloped virus is currently the only known characterized member of the Malacoherpesviridae family, and its reference genotype was published in 2005 (Davison et al., 2005). In 2008, the emergence of a specific genotype of this virus called µVar was associated with mass mortality outbreaks among spat and juvenile *C. gigas* (Segarra et al., 2010). All the French oyster production areas were affected, and between 40% and 100% of Pacific oyster spat died.

Other studies have generally focused on the identification of antiviral compounds to expand the knowledge about the mechanisms underlying the resistance of the Pacific oyster to a viral infection (Bachère et al., 1990; Olicard et al., 2005; Renault et al., 2011; Green et al., 2014). The recent publication of *Crassostrea gigas* genome (Zhang et al., 2012) has allowed identifying several pathways involved in immune-system mechanisms (He et al., 2015; Moreau et al., 2015; Rosani et al., 2015). These studies suggest that several mammal innate immune pathways exist in this specie. It has been suggested that *C. gigas* can control a viral infection by means of the RNA interference (RNAi) pathway, an interferon-like pathway, apoptosis, and *via* autophagy (Zhang et al., 2011; Green and Montagnani, 2013; Segarra et al., 2014a; Segarra et al., 2014c; Green et al., 2015; He et al., 2015; Moreau et al., 2015; Martenot et al., 2017).

Macroautophagy, which is more commonly simply called autophagy, is a pathway widely conserved among eukaryotes. This process involves engulfment of a portion of the cytoplasm with components of the cell (from proteins to whole organelles) for their degradation by fusion with lysosomes (Levine and Deretic, 2007). Autophagy participates in key cellular processes including cellular homeostasis, starvation adaptation, cell death, and immune response to pathogens (Klionsky and Emr, 2000; Mizushima, 2005; Deretic, 2006; Schmid and Münz, 2007). This cellular mechanism can block the replication of (or infection by) different pathogens including viruses, bacteria, and parasites.

In *C. gigas*, autophagy has previously been characterized in the mantle and haemocytes (Picot et al., 2020). In these two oyster compartments, autophagy has been successfully modulated after exposing oysters to molecules well known to modulate the autophagy pathway in mammals (Moreau et al., 2015; Picot et al., 2019). The mantle has been reported to be a target organ of OsHV-1 (Renault et al., 1994a; Renault et al., 2001; Schikorski et al., 2011a; Segarra et al., 2016). The presence of viral mRNA is detected earlier in the mantle compared to the other organs (Segarra et al., 2014b; Segarra et al., 2014c). Haemocytes are the principal effectors of the oyster immune system (Allam and Raftos, 2015). It has been suggested that haemocytes are carrier cells responsible for the transport of OsHV-1 to target organs during the first stages of viral infection (Schikorski et al., 2011a; Segarra et al., 2016; Morga et al., 2017). One study showed that 20 h post infection (hpi), the autophagy pathway is implying in the presence of virus OsHV-1 in the mantle of *C. gigas* (Moreau et al., 2015).

To clarify the role of autophagy in the response of the Pacific oyster to OsHV-1 infection, experimental infections were carried out using respectively a known inhibitor of autophagy ($NH_4Cl$). Both autophagy and the development of the virus were monitored concurrently in the mantle and haemolymph. Autophagy was measured using cellular (flow cytometry and western blotting) and complementary molecular approaches (real-time PCR) previously developed and applied by (Picot et al., 2019; Picot et al., 2020). The viral DNA load and expression of three viral genes were monitored by real-time PCR during the experimental infection. Thanks to an integrated approach, this study has revealed that autophagy is activated in the mantle and haemolymph of *C. gigas* after the initiation of OsHV-1 replication. Interestingly different autophagy regulatory mechanisms seem to occur in the two tissues in response to OsHV-1.

# MATERIALS AND METHODS

## Oyster Production

*Crassostrea gigas* spat were produced at the Ifremer facilities in La Tremblade (Charente-Maritime, France) from one family. This family was selected for its intermediate susceptibility to the viral infection when tested under experimental conditions as described by Segarra et al. (2014b). Spawn occurred in May 2016, and larval and spat cultures were performed as described by Dégremont et al. (2005) and Azéma et al. (2017). All growth steps involved filtered and UV-treated seawater to prevent contamination with pathogens naturally present in the environment, including OsHV-1 and *Vibrio aestuarianus*.

Prior to the experiment, spat were acclimated *via* a constant flow of filtered and UV-treated seawater enriched in phytoplankton (*Skeletonema costatum*, *Isochrysis galbana*, and *Tetraselmis suecica*) in 120 L tanks at 19°C for at least 2 weeks.

## Experimental Design Including Pharmacological Agent and Virus OsHV-1

Seven hundred and fifty oysters (3-4cm) were chloride induced a myorelaxing for 4 h in a solution containing magnesium chloride ($MgCl_2$, 50 g/L) in seawater mixed with distilled water (1:4, v/v) (Schikorski et al., 2011b). Four conditions were tested, each replicated by 12 tanks, and each tank containing 15 oysters: oysters either injected with 100 µL of an OsHV-1 suspension at $1 \times 10^4$ copies of viral DNA/µL or injected with seawater, which were subsequently either kept in seawater or kept in seawater supplemented with $NH_4Cl$ at 1 mM). Two tanks of each condition were sampled at 6, 10, 14, 18, 24, and 30 h post infection. At T0 (time before oysters were incubated under the different tested conditions), two pools of 15 oysters were sampled to determine the basal level of autophagy in the mantle and haemolymph. At each sampling time and for each condition, pieces of mantle were collected from six oysters to quantify viral DNA and measure viral and autophagy gene expression, and western blotting. In parallel,

haemolymph was withdrawn from the adductor muscle of oysters with a 1 mL syringe equipped with a needle (0.6 × 32 mm) as described by Picot et al. (2019). The haemolymph of the 15 oysters in each tank was pooled for viral DNA quantification, analysis of viral and autophagy genes expression and flow cytometry.

Survival was monitored for 7 days after injection (three additional tanks of 15 oysters per condition). Percentages of cumulative survival were determined daily for the different conditions. Dead oysters were removed from tanks in the course of the experiment.

## DNA Extraction

Total DNA was extracted from the mantle or haemolymph using the QiaAmp DNA Mini Kit (Qiagen), according to the manufacturer's protocol. Elution was performed in 200 µL (for mantle extraction) and 50 µL (for haemolymph extraction) of AE buffer provided in the kit. The DNA quality and quantity were determined on a NanoDrop 2000 instrument (Thermo Scientific, http://www.nanodrop.com).

## OsHV-1 DNA Quantification by Real-Time PCR

OsHV-1 DNA quantification was estimated by real-time PCR in duplicate according to Pepin et al. (2008) on a Mx3000 Thermocycler sequence detector (Agilent). Amplification reactions were carried out in a total volume of 20 µL. Each well contained 5 µL of genomic DNA (5 ng/mL), 10 µL of Brillant III Ultra-Fast SYBR Green Master Mix (Agilent), 2 µL of each primer (5.5 µM: OsHV-1 DPFor 5′-ATTGATGATGTGGATAATCTGTG-3′, 5.5 µM OsHV-1 DPRev 5′-GGTAAATACCATTGGTCTTGTTCC-3′) (Webb et al., 2007), and 1 µL of distilled water. Real-time PCR cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 20 s. The results were expressed as $\log_{10}$ of virus DNA copy numbers per nanogram of total DNA.

## Total RNA Extraction and cDNA Synthesis

From each tissue, total RNA was extracted with the TRIzol™ Reagent (Ambion) according to the manufacturer's recommendations. Total RNA was treated with TURBO™ DNase (Invitrogen) to remove genomic DNA. The quality and quantity of the RNA were determined on the NanoDrop 2000 (Thermo Scientific). Mock reverse transcription was performed after each DNase treatment to verify the absence of oyster and/or viral genomic DNA. First-strand cDNA synthesis was carried out by means of SuperScript® III Reverse Transcriptase (Invitrogen) with 500 ng of the treated RNA.

## Expression of Viral Genes

Real-time PCR was carried out to monitor the expression of three viral genes (ORFs 80, 87, and 99). These three ORFs were selected based on their protein functions and early expression during the viral infection (Davison et al., 2005; Segarra et al., 2014b; Morga et al., 2017). ORF 80 encodes a putative membrane protein and ORFs 87 and 99 apoptosis inhibitors. The expression of the three viral genes was evaluated following the protocol described by Segarra et al. (2014a) with 5 µL of cDNA dilution (1/30) instead of genomic DNA. Normalized relative viral gene expression levels were calculated for each sample with the formula: Delta $C_t$ = $C_t$ ORF − $C_t$ Elongation factor 1alpha (EF1-α). The gene expression level (Delta $C_t$) of the initial array data was transformed as follows: [1 − (Delta $C_t$/$C_t$ EF1-α)]/100. $C_t$ (threshold cycle) corresponds to the PCR cycle number.

## Expression of Oyster Immunity Genes

Moreover, the relative expression of seven immunity genes in *C. gigas* spat was analyzed during the OsHV-1 experimental infection at T0, 6, 10, 14, 18, 24, and 30 hpi. The relative quantification value (ratio R) was calculated by the method described by Pfaffl (2001):

$$R = \left[ \left(E_{target}\right)^{.\Delta CTtarget(control-sample)} \right] / \left[ \left(E_{ref}\right)^{.\Delta CTref(control-sample)} \right]$$

The efficiency of each primer pair was determined by constructing a standard curve from serial dilutions (**Table 1**). These five genes of the Pacific oyster were (i) sequestosome 1 (*SQSTM1*), (ii) microtubule-associated protein 1A/1B light chain 3A (*MAP1LC3A*), (iii) beclin-1 (*BECN1*), (iv) serine/threonine protein kinase ULK2 (*ULK2*), and (v) autophagy-related protein 7 (*ATG7*; **Table 1**). Host gene expression was normalized to EF1-α, because no significant differences in $C_t$ values were observed for this housekeeping gene among several conditions in the course of the experiment. The calibrator in this experiment was individuals sampled at T0.

**TABLE 1** | List of primer for viral ORF and C. gigas autophagy genes expressions.

| Categories | Gene name/ ORFs | Forward | Reverse | Effeciency | Protein |
|---|---|---|---|---|---|
| **Autophagy genes** | BECN1 | AATGCTGCTTGGGGTCAGA | CGGAATCCACCAGACCCATA | 102.2 | PI3KC3 complex |
| | ULK2 | CTGACTTTGGCTTTGCTCGT | TTTGAGCTGTTGAGGGGTCT | 103.9 | Atg1/ULK1 complex |
| | MAP1LC3A | CCGATGCTTGACAAGACCAA | CCGTCCTCGTCTTTCTCCTG | 98.2 | LC3 conjugation system |
| | P62/SQQTM1 | AGGGAATGAGAAGGCCGAAA | CCTCAAGCAACTCCTCTCCA | 96.5 | Delivers ubiquitinated cargoes for autophagic degredation |
| | ATG7 | CGCCCCTTGTAAACAAAATG | ATTCTGCAAGGCATTCCAAC | 104.8 | LC3 and ATG12 conjugation systems |
| **OsHV-1 genes** | ORF80 | AAGAGGATTTGGGTGCACAG | TTGCATCCCAGGATTATCAG | 98.5 | Membrane protein |
| | ORF87 | CACAGACGACATTTCCCCAAA | AAAGCTCGTTCCCACATTGGT | 98.7 | Inhibitor of apoptosis protein |
| | ORF99 | GGTGGAGGTGGCTGTTGAAA | CCGACTGACAACCCATGGAC | 96.3 | Inhibitor of apoptosis protein |

## Flow Cytometry

Before autophagic activity was investigated in haemocytes, haemocyte mortality was determined. As described by Gagnaire (2005), haemocyte mortality was measured in 200 µL of a cell suspension sampled from each condition (two replicates) and at each sampling time point. The cells were incubated in the dark for 30 min at 4°C with 10 µL of propidium iodide (PI, 1 mg/mL; Thermo Fisher Scientific, cat. # P3566).

Then, percentages of haemocytes with autophagic activity were quantified with the commercial Cyto-ID® autophagy detection kit (ENZO Life Sciences, ENZ-51031-K200) as described by Picot et al. (2020).

For each sample, 5000 events were acquired on an EPICS XL 4 cytometer (Beckman Coulter, USA). Size discrimination was implemented to ensure that small particles or bacteria were not counted, so that only haemocytes were taken into account when cell activity was measured. The results were depicted as cell cytograms and reported as log scale fluorescence levels of each marker tested. The results were expressed as differences between the percentage of haemocytes that positively presented autophagosomes for each condition and the percentage of haemocytes labeled in the artificial seawater condition at each sampling time point. Flow cytometry data were analyzed in Flowing software 2.5.1 (University of Turku, Finland).

## Western Blot

Pieces of mantle were collected from Pacific oysters (20 to 25 mg). Mantle protein extraction and western blot were performed as reported by Picot et al. (2020). Thirty micrograms of each pool of the mantle protein extracted was loaded onto an SDS polyacrylamide gel (Bio-Rad). Primary antibodies against Actin (A4700, Sigma-Aldrich), SQSTM1 (P0067, Sigma-Aldrich) were respectively diluted at 1/1000, 1/500, and 1/6000.

MAP1LC3-II/actin and SQSTM1/actin ratios were calculated based on densitometry analysis of the bands in the ImageJ software (v. 1.51q). Each sample was normalized to actin and calibrated in comparison with the control condition for each experiment.

## Data Analysis

All analyses were conducted in the R studio software (version 3.3.2). First, normality of all the datasets was tested by the Shapiro–Wilk test, and homogeneity of variances was assumed because of the results of Bartlett's test.

Kaplan–Meier survival curves and the logrank test were used to characterize and compare survival between oyster conditions (packages survival, v2.39-5, and survminer, v. 0.4.3).

OsHV-1 DNA amounts were compared between groups "virus" and "virus+$NH_4Cl$" for each tissue by the Kruskal–Wallis test (package PMCMR, v. 4.1). In haemolymph, the sampling time points were subdivided into two categories. The first category consisted of the early sampling time points (6, 10, and 14 hpi), and the second of the late sampling time points (18, 24, and 30 hpi). For the mantle, results of each sampling time point were tested separately. Scatterplots and trend curves were built with package ggplot2 (version 2.2.1).

Relative expression of oyster and viral genes are presented as scatterplots with trend curves (package ggplot2, v 2.2.1). The Kruskal–Wallis test was carried out to determine whether significant differences exist between experimental treatments at different sampling time points. In haemolymph, the difference was tested at early (6, 10, and 14 hpi) and late time points (18, 24, and 30 hpi) of the experimental infection. For the mantle, results of each sampling time point were tested separately.

Flow cytometry data were compared between the conditions tested and the artificial seawater condition at early (6, 10, and 14 hpi) and late (18, 24, and 30 hpi) time points of the experimental infection by Student's *t* test. Scatterplots and trend curves were constructed using package ggplot2.

Western blot data were compared by the Mann–Whitney test between the conditions tested and the artificial seawater group at early and late time points of the experimental infection. Bar plots were built by means of package ggplot2.

# RESULTS

## Mortality

To assess the effects of OsHV-1 and $NH_4Cl$ on Pacific oyster spat, survival was monitored for 7 days post infection (dpi). No oyster mortality was detected in the artificial seawater group (**Figure 1**). In the $NH_4Cl$ group, the mean survival rate was 83% at 7 days post exposure. The presence of virus OsHV-1 and bacterium *V. aestuarianus* was investigated by real-time PCR in dead animals. The results did not allow us to detect the bacterium or the virus in any dead animal. The mean survival rates in the virus group (60%) and virus+$NH_4Cl$ group (20%) were significantly different from the results obtained for the seawater group at 7 dpi ($p \leq 0.05$). At the same time, significant differences in the mean survival rate were observed between the $NH_4Cl$ and virus+$NH_4Cl$ groups ($p \leq 0.05$) and the virus and virus+$NH_4Cl$ groups ($p \leq 0.05$).

## Detection of OsHV-1 DNA and RNA in the Mantle and the Haemolymph of *C. gigas*

Viral DNA detection was conducted in the haemolymph and mantle of Pacific oysters during the process of infection (**Figure 2A**). Viral DNA and RNA amounts were monitored at each sampling time point (T0, 6, 10, 14, 18, 24, and 30 hpi).

In haemolymph and the mantle, no viral DNA was detected in artificial seawater and $NH_4Cl$ groups (**Figure 2A**). OsHV-1 DNA could be detected as early as 6 hpi in both tissues tested in the virus group and virus+$NH_4Cl$ group. In haemolymph (**Figure 2A**), the viral DNA amount increased from 6 to 18 hpi in the virus group ($1.03 \times 10^6 \pm 6.21 \times 10^5$ viral DNA copies/µL of haemolymph) to 24 hpi in the virus+$NH_4Cl$ group ($4.02 \times 10^5 \pm 1.91 \times 10^5$ viral DNA copies/µL of haemolymph). After that, the viral DNA amount tended to stay stable until the end of the experiment in the two groups. No significant difference was observed between the virus group and virus+$NH_4Cl$ group at early (6 to 14 hpi) and late (18 to 30 hpi) time points of the experimental infection in haemolymph. In the mantle

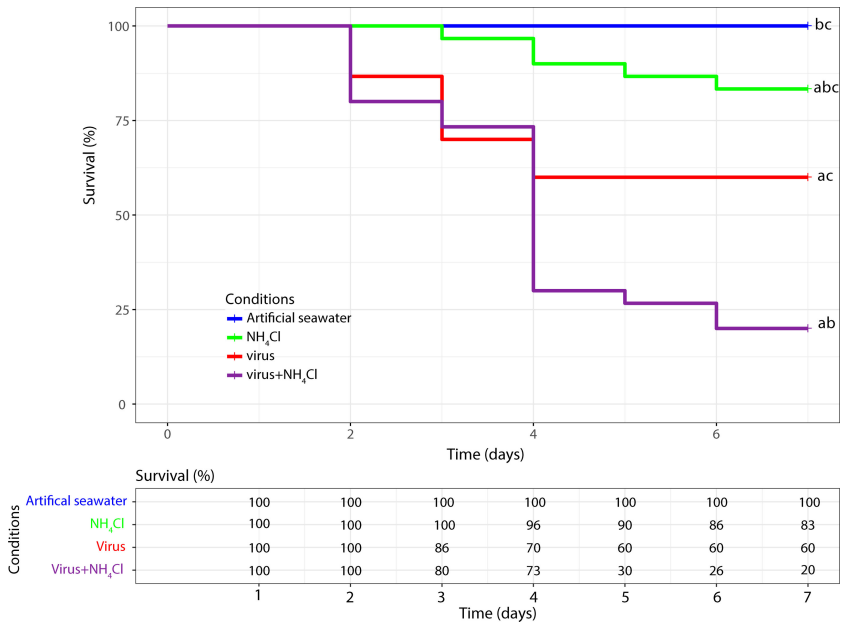


**FIGURE 1** | Kaplan-Meier survival curves of the pacific oyster *Crassostrea gigas* injected with the virus OsHV-1, exposed to $NH_4Cl$ or injected with OsHV-1 and exposed to $NH_4Cl$. The control condition corresponds to pacific oysters injected with artificial sea water. In each condition the mortality was monitored on n=30 oysters. a: significant difference with the artificial seawater condition ($p < 0.05$). b: significant difference with the virus condition ($p < 0.05$). c: significant difference with the virus+$NH_4Cl$ condition ($p < 0.05$).

(**Figure 2A**), viral DNA amounts increased from 6 until 30 hpi in virus and virus+$NH_4Cl$ groups. The maximal viral DNA amounts were detected at 30 hpi in the virus group ($6.57 \times 10^4 \pm 1.54 \times 10^5$ viral DNA copies/µL of haemolymph) and in the virus+$NH_4Cl$ group ($1.26 \times 10^5 \pm 2.89 \times 10^5$ viral DNA copies/µL of haemolymph, respectively). No significant difference was observed between the virus group and virus+$NH_4Cl$ group at any sampling time point in the mantle.

The expression of three viral ORFs was monitored by real-time PCR (**Figure 2B**) to estimate viral replication in the haemolymph and mantle under the different conditions at each sampling time point. In the mantle and haemolymph, the first detection of viral RNA of the three genes was observed at 6 hpi in the virus group and virus+$NH_4Cl$ group (**Figure 2B**). In haemolymph, the viral RNA amount increased exponentially at the beginning of the experiment and tended to stay stable until the end of the experiment in the virus group and virus+$NH_4Cl$ group (**Figure 2B**). In the mantle, the viral transcripts of the three ORFs were detected from 6 until 30 hpi, but their expression manifested high variation among individuals (**Figure 2B**). No significant differences in the relative expression of each ORF was thus detected between the two groups in the course of the experiment in haemolymph and the mantle.

## Monitoring of Haemocyte Mortality in Oyster Haemolymph

Prior to monitoring the autophagic activity by flow cytometry, haemocyte mortality was evaluated with PI. In all the tested groups at all sampling time points, the mean cell mortality never exceeded 11.9%.

## Monitoring of Autophagic Activity in Oysters Exposed to OsHV-1

In the oyster haemolymph from the virus group, the autophagic activity was determined by calculating the difference in the percentages of cells containing autophagosomes between the virus group and artificial seawater group (**Figure 3A**). At late (18 to 30 hpi) time points of the experimental infection, the difference significantly increased and stayed positive in comparison with early (6 to 14 hpi) time points of the experimental infection ($p \leq 0.05$). These results indicated that the percentage of cells containing autophagosomes was higher in the virus group between 18 and 30 hpi. The relative expression of five autophagy genes was monitored in the oyster haemolymph from the virus group (**Figure 3B**). The results revealed that the relative expression of *ULK2*, *SQSTM1*, and *MAP1LC3A* was significantly higher in the virus group in comparison with the artificial seawater group at later time points of the experimental infection (18 to 30 hpi; $p \leq 0.05$).

In the mantle, *MAP1LC3A* and *ULK2* relative expression levels were significantly higher at two time points (14 and 30 hpi; $p \leq 0.05$) in the virus group (**Figure 3B**). The relative expression of *SQSTM1* significantly increased from 24 to 30 hpi ($p \leq 0.05$). Relative expression of *BECN1* significantly increased at 30 hpi ($p \leq 0.05$). *ATG7* relative expression did not seem to vary during the experiment. At the protein level, the SQSTM1/actin ratio was determined by western blotting to follow the protein quantity of

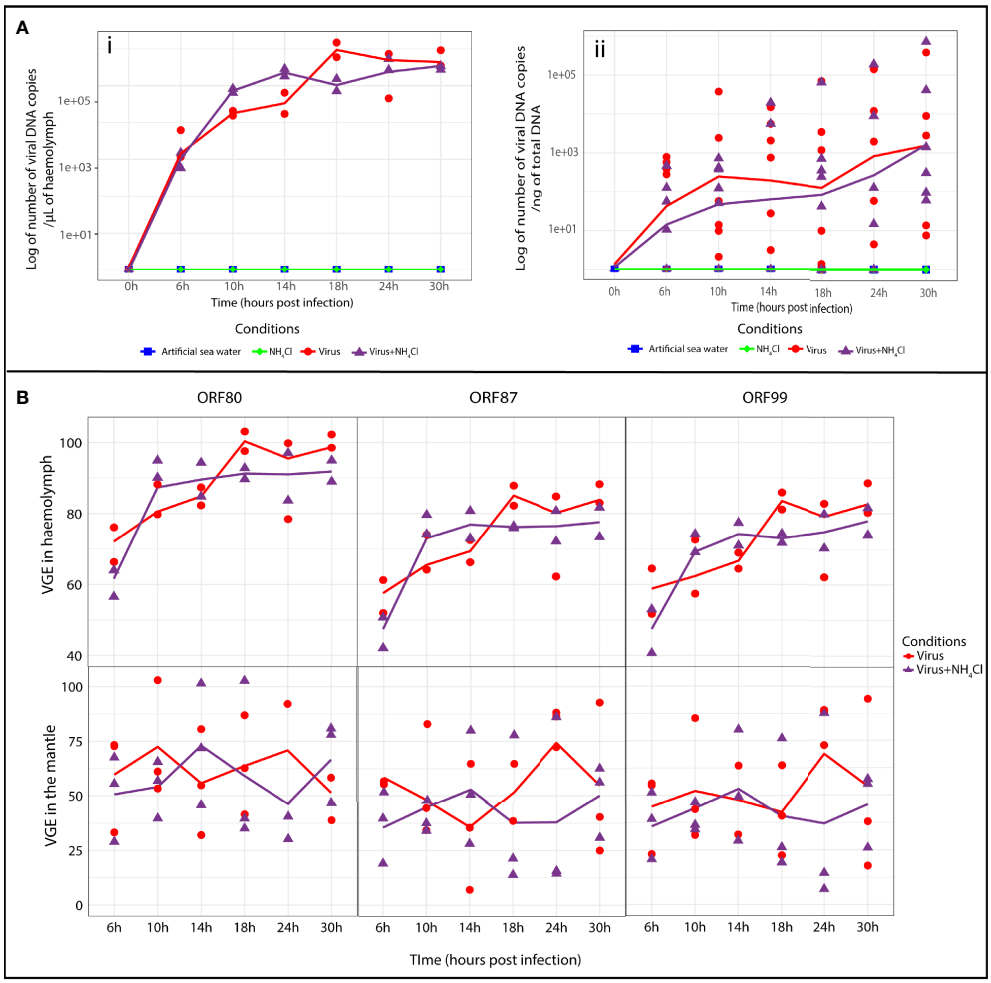


**FIGURE 2** | Detection of OsHV-1 DNA and RNA in haemolymph and mantle of *Crassostrea gigas*. **(A)** Viral DNA amounts detected by real time PCR in Pacific oysters of the four different tested conditions (artificial seawater, $NH_4Cl$, virus and virus+$NH_4Cl$) after injection by OsHV-1 (i) in haemolymph (n=2 pools of 15 animals) and (ii) in the mantle. (n=6 animals) The line represents the trend curve of viral DNA amount in the virus condition (red line) and the virus+$NH_4Cl$ condition (purple line). No significant difference was observed between the virus and virus+$NH_4Cl$ conditions in the mantle and haemolymph. **(B)** Relative genes expressions of ORF 80, ORF 87 and ORF 99 estimated by RT-PCR at different time of exposure for the virus and virus+$NH_4Cl$ condition in haemolymph (n= 2 pools of 15 animals) and mantle (n=3 animals). The line represents the trend curve of the expression of each gene in the virus condition (red line) and the virus+$NH_4Cl$ condition (purple line). No significant difference was observed between the virus and virus+$NH_4Cl$ condition in haemolymph and the mantle of the Pacific oyster. VGE, viral gene expression.

SQSTM1 in the mantle of *C. gigas* (**Figure 3C**). The results indicated that the SQSTM1 protein quantity significantly increased in the virus group relative to the seawater group at the later time points of the experiment (18 to 30 hpi).

## Monitoring of Autophagic Activity in Oysters Exposed to $NH_4Cl$

The monitoring of autophagic activity by flow cytometry revealed a significant increase in the percentage of cells containing autophagosomes in the $NH_4Cl$ group in comparison with the artificial seawater group at late time points [18 to 30 h post exposure (hpe)] of the experimental exposure to $NH_4Cl$ ($p \leq 0.05$; **Figure 4A**). On the other hand, in the $NH_4Cl$ group, the relative expression of *MAP1LC3A*, *ULK2*, and *SQSTM1* was higher at early (6 to 14 hpe) and late time points (18 to 30 hpe) of the experimental infection in comparison with the artificial seawater group ($p \leq 0.05$). *ATG7* and *BECN1* expression showed no significant difference between the $NH_4Cl$ group and artificial seawater group.

In the mantle, genes *MAP1LC3A* and *ULK2* were significantly upregulated respectively from 10 to 30 hpe and from 6 to 30 hpe ($p \leq 0.05$) in the $NH_4Cl$ group (**Figure 4B**). *SQSTM1* was significantly upregulated from 10 to 30 hpe ($p \leq 0.05$). The relative expression of *ATG7* and *BECN1* stayed weak in comparison with their expression levels in the seawater group. Nevertheless, *ATG7* was significantly upregulated from 6 to 24 hpe, and *BECN1* was upregulated at 30 hpe ($p \leq 0.05$) in the presence of $NH_4Cl$. Moreover, we observed an increase in the protein SQSTM1 amount by western blot analysis. In fact, the results indicated that

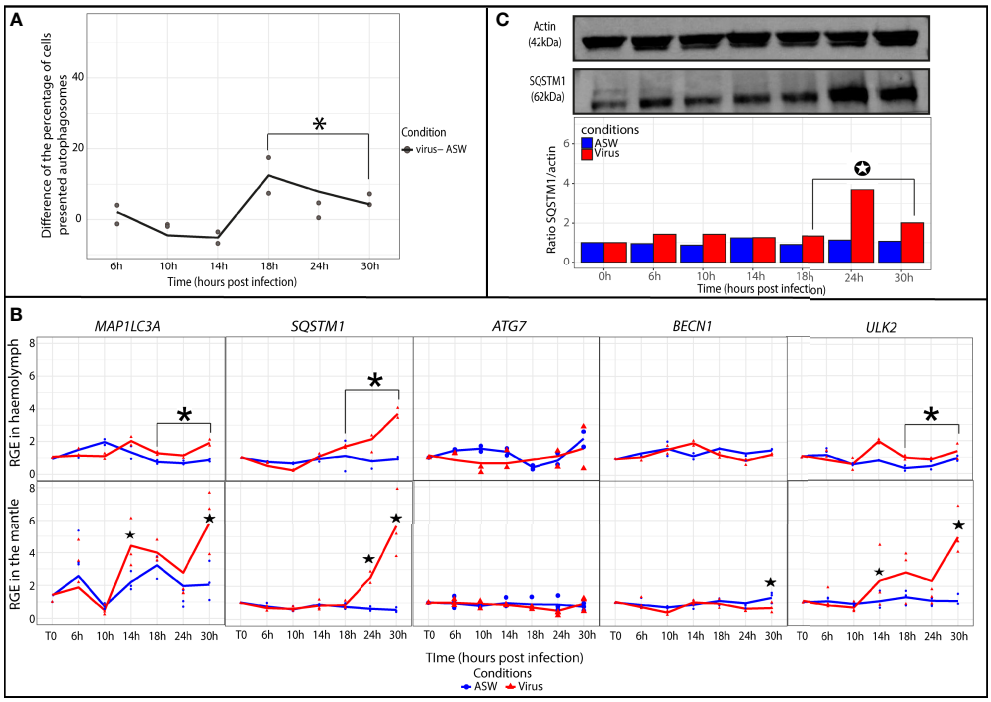


**FIGURE 3** | Modulation of the autophagy in *Crassostrea gigas* exposed to OsHV-1. **(A)** Monitoring of autophagy in haemocytes of *C. gigas* by flow cytometry. Scatterplot of the difference of the percentage of haemocytes presented autophagosomes between the seawater condition (ASW) and the virus condition from 6 to 30 hpi (n=2 pools of 15 animals). The line represents the trend curve of the difference of percentage of cells between the two conditions. *Significant increase of the difference of the percentage of cells presented autophagosomes between the virus and seawater condition ($p < 0.05$). **(B)** Relative gene expression of key autophagy genes in haemolymph and the mantle of the Pacific oysters, *C. gigas*, in ASW condition (blue) and virus condition (red) at each sampling time point (T0, 6, 10, 14, 18, 24 and 30 hpi) detected by real time PCR (haemolymph: n=2 pools of 15 animals; mantle: n=3 animals). The line represents the trend curve of the expression of each gene in the virus condition (red) and the ASW condition (blue). Significant difference between the virus and ASW condition at early (6-14 hpi) or late time points (18-30 hpi) of the experimental infection ($p < 0.05$). Significant difference between the virus and ASW conditions of the same time point ($p < 0.05$). RGE, relative gene expression. **(C)** Detection of the autophagy protein SQSTM1 in the mantle of *C. gigas* during the kinetic of infection by OsHV-1 in the virus condition at each sampling time point (for each time point n=1 pool of 3 animals). Significant differences between the virus and ASW condition at early (6-14 hpi) or late time points (18-30 hpi) of the experimental infection ($p < 0.05$).

the SQSTM1 protein quantity was significantly higher in the $NH_4Cl$ group than the artificial seawater group during early (6 to 14 hpe) time points of the experimental infection ($p \leq 0.05$; **Figure 4C**).

## Monitoring of Autophagic Activity in Oysters Exposed to Virus+$NH_4Cl$

The monitoring of the autophagic activity by flow cytometry suggested that the percentage of cells containing autophagosomes was significantly higher in the virus+$NH_4Cl$ group than in the artificial seawater group at late (18 to 30 hpi) time points of the experimental infection ($p \leq 0.05$; **Figure 5A**). At the molecular level, the relative expression of genes *MAP1LC3A*, *SQSTM1*, and *ULK2* was significantly upregulated at early (6 to 14 hpi) and late (18 to 30 hpi) time points in the virus+$NH_4Cl$ group in comparison with the artificial seawater group ($p \leq 0.05$; **Figure 5B**). *BECN1* and *ATG7* expression showed no significant differences between the two tested groups.

In the mantle, relative expression of *MAP1LC3A* was significantly higher from 14 to 24 hpi in the virus+$NH_4Cl$ group in comparison with the seawater group ($p \leq 0.05$; **Figure 5B**). *ULK2* was significantly upregulated from 6 to 30 hpi ($p \leq 0.05$). *SQSTM1* was significantly upregulated in comparison with the seawater group from 6 to 30 hpi ($p \leq 0.05$). *ATG7* was statistically but weakly upregulated at 6, 10, and 18 hpi ($p \leq 0.05$). Relative expression of *BECN1* did not vary during the experiment. The protein ratio SQSTM1/actin confirmed the increase in the quantity of the SQSTM1 protein in the virus+$NH_4Cl$ group relative to the seawater group at early (6 to 14 hpi) and late (18 to 30 hpi) time points of the experiment ($p \leq 0.05$; **Figure 5C**).

# DISCUSSION

*C. gigas* is the most important aquaculture farming resource in France. This specie is widely cultivated due to its good growth capacity and resistance to environmental factors (FAO, 2018b; FAO, 2018a). Nonetheless, since 1990, virus OsHV-1 has been responsible for mortality events among Pacific oyster spat

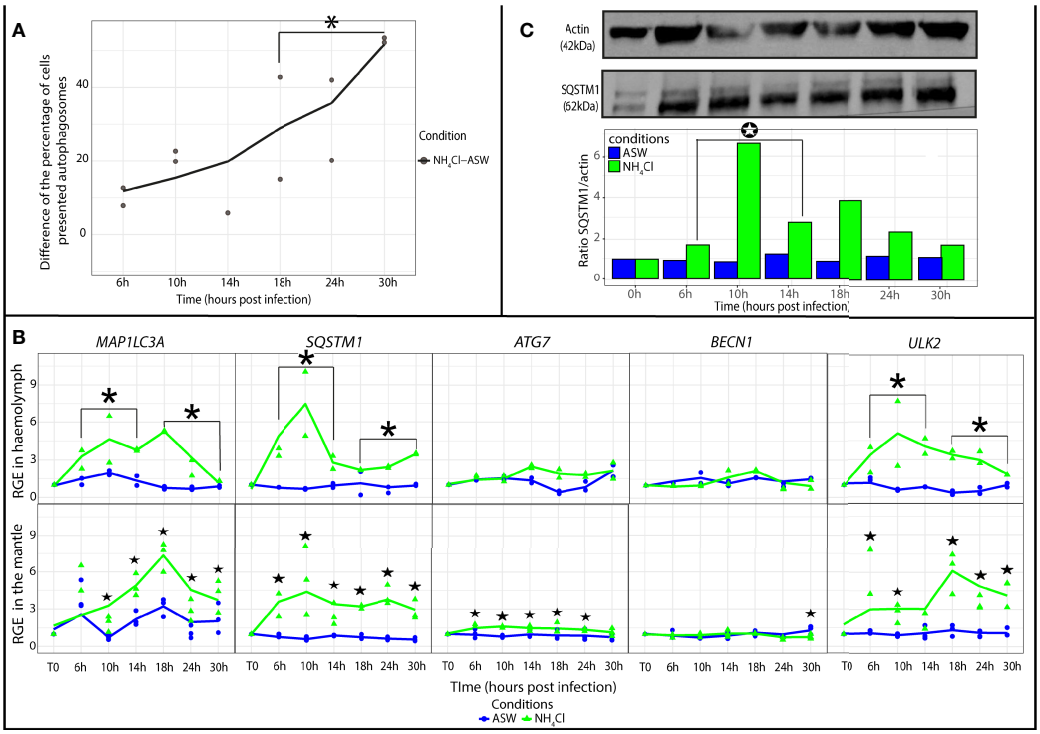


**FIGURE 4** | Modulation of autophagy in *Crassostrea gigas* exposed to $NH_4Cl$. **(A)** Monitoring of autophagy in haemocytes of *C. gigas* by flow cytometry. Scatterplot of the difference of the percentage of haemocytes presented autophagosomes between the seawater condition (ASW) and the $NH_4Cl$ condition from 6 to 30 hpi (n=2 pools of 15 animals). The line represents the trend curve of the difference of percentage of cells between the two conditions. *Significant increase of the difference of the percentage of cells presented autophagosomes between the $NH_4Cl$ and ASW condition between 18 to 30hpi ($p < 0.05$). **(B)** Relative gene expression of key autophagy genes in haemolymph and the mantle of the Pacific oysters, *C. gigas*, in ASW condition (blue) and $NH_4Cl$ condition (green) at each sampling time point (T0, 6, 10, 14, 18, 24 and 30 hpi) detected by real time PCR (haemolymph: n=2 pools of 15 animals; mantle: n=3 animals). The line represents the trend curve of the expression of each gene in the $NH_4Cl$ condition (green) and the ASW condition (blue). Significant difference between the $NH_4Cl$ and ASW conditions at early (6-14 hpi) or late time points (18-30 hpi) of the experimental infection ($p < 0.05$). Significant difference between the $NH_4Cl$ and ASW conditions of the same time point ($p < 0.05$). RGE, relative gene expression. **(C)** Detection of the autophagy protein SQSTM1 in the mantle of *C. gigas* during the kinetic of exposition to $NH_4Cl$ at each sampling time point (for each time point n=1 pool of 3 animals). Significant differences between the $NH_4Cl$ and ASW condition at early (6-14 hpi) or late time points (18-30 hpi) of the experimental infection ($p < 0.05$).

(Nicolas et al., 1992; Renault et al., 1994a; Renault et al., 1994b). Despite the impact of the mortality caused by this virus on the aquaculture economy, few countermeasures are available. The innate immunity mechanisms of *C. gigas* involved in the response to OsHV-1 need to be documented more thoroughly. Recently, autophagy, one of the innate immunity pathways of the Pacific oyster, was investigated in the mantle of *C. gigas* and was demonstrated to participate in the response to OsHV-1 infection (Moreau et al., 2015). In that study, autophagy was investigated in a single tissue of the Pacific oyster, the mantle, and at a single time point during the viral infection.

In the present study, we investigated the role played by autophagy in *C. gigas* during OsHV-1 infection using an integrated approach. An experimental infection was carried out in combination with a known inhibitor of the autophagy pathway, ammonium chloride ($NH_4Cl$). During this experiment, autophagy kinetic was monitored in the mantle and haemolymph by different cellular (flow cytometry, western blot) and molecular (real-time PCR) approaches.

The first experimental infection was performed by intramuscular injection of a viral suspension into *C. gigas* spat. Oyster survival was monitored for 7 dpi. Higher mortality rates were observed in oysters injected with the virus and exposed to $NH_4Cl$ and to a lesser extent in oysters injected only with the virus. Similar results were obtained by Moreau et al. (2015). Nevertheless, these authors did not detect mortality in oysters exposed to $NH_4Cl$ alone. In our experiment, a low mortality rate (17%) was observed among the oysters exposed to $NH_4Cl$ alone. Because OsHV-1 or *V. aestuarianus* DNA were not detected, these deaths appeared to be unrelated to these pathogens generally responsible for *C. gigas* mortality. On the other hand, the presence of other pathogens that may kill *C. gigas* was not investigated here. Moreover, the animals used in the experiment were mature. *C. gigas* is highly sensitive to changes in biotic and abiotic factors during gametogenesis (Berthelin et al., 2000; Li et al., 2007; Enríquez-Díaz et al., 2008). Perhaps sexually mature animals are more susceptible to $NH_4Cl$ exposure than immature oysters. Finally, exploration of autophagy in

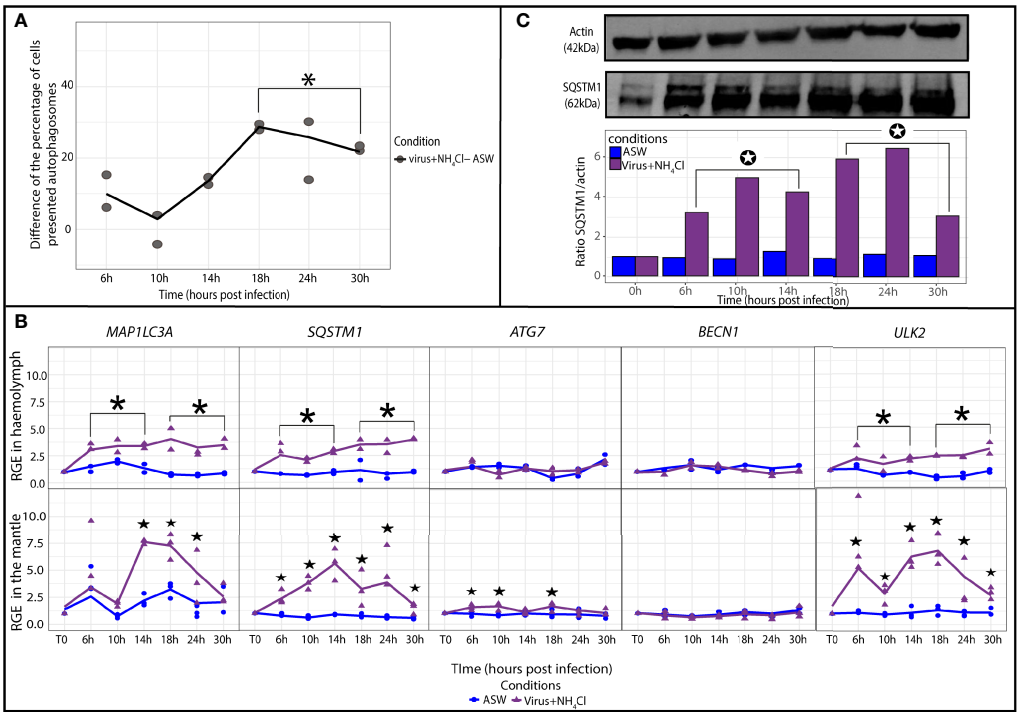


**FIGURE 5** | Modulation of autophagy in *Crassostrea gigas* exposed to virus+$NH_4Cl$. **(A)** Monitoring of autophagy in haemocytes of *C. gigas* by flow cytometry. Scatterplot of the difference of the percentage of haemocytes with autophagosomes between the seawater condition (ASW) and the virus+$NH_4Cl$ condition from 6 to 30 hpi (n=2 pools of 15 animals). The line represents the trend curve of the difference of percentage of cells between the two conditions. *Significant increase of the difference of the percentage of cells presented autophagosomes between the virus+$NH_4Cl$ and ASW condition between 18 to 30hpi ($p < 0.05$). **(B)** Relative gene expression of key autophagy genes in haemolymph and the mantle of the Pacific oysters, *C. gigas*, in seawater condition (blue) and the virus+$NH_4Cl$ condition (purple) at each sampling time point (T0, 6, 10, 14, 18, 24 and 30 hpi) detected by real time PCR (haemolymph: n=2 pools of 15 animals; mantle: n=3 animals). RGE, relative gene expression. The line represents the trend curve of the expression of each gene in the virus+$NH_4Cl$ condition (purple) and the ASW condition (blue). Significant difference between the virus+$NH_4Cl$ and ASW conditions at early (6-14 hpi) or late time points (18-30 hpi) of the experimental infection ($p < 0.05$). Significant difference between the virus+$NH_4Cl$ and ASW conditions of the same time point ($p < 0.05$). **(C)** Detection of the autophagy protein SQSTM1 in the mantle of *C, gigas* during the kinetic of infection by OsHV-1 in the virus+$NH_4Cl$ condition at each sampling time point (for each time point n=1 pool of 3 animals). Significant differences between the virus+$NH_4Cl$ and ASW condition at early (6-14 hpi) or late time points (18-30 hpi) of the experimental infection ($p < 0.05$).

relation to viral infection was performed before first deaths were observed.

First, the modulation of autophagy was investigated in Pacific oysters exposed to $NH_4Cl$. This reagent was employed in the experiment owing to its capacity to inhibit autophagy, namely, specific suppression of autophagosome degradation (Sharifi et al., 2015; Klionsky et al., 2016). In haemolymph and the mantle, our results uncovered modulation of autophagy genes starting from 6 hpe. $NH_4Cl$ induced a modulation of the autophagy genes. Moreover, the percentage of cells containing autophagosomes and expressing protein SQSTM1 significantly increased respectively since 18 and 6 hpe. SQSTM1 is a protein required for the formation and degradation of polyubiquitin-containing bodies *via* autophagy (Pankiv et al., 2007). This protein is a marker used to study autophagy flux (Bjørkøy et al., 2009). Our results indicated that $NH_4Cl$ induced accumulation of the SQSTM1 protein in the mantle. In the haemolymph, accumulation of autophagosomes was observed through an increase in the percentage of haemocytes containing autophagosomes. This analysis indicates that in the two tissues, autophagy was functional and was inhibited by $NH_4Cl$. Similar results were already obtained in the haemocytes and mantle of *C. gigas* by means of the same reagent (Moreau et al., 2015; Picot et al., 2019). In haemocytes, the percentage of cells containing autophagosomes significantly increased in oysters exposed to $NH_4Cl$ at 24 hpe (Picot et al., 2019). In the mantle, accumulation of the MAP1LC3-II protein (another key protein of the autophagy pathway) revealed inhibition of autophagy at 20 hpe as determined by western blotting (Moreau et al., 2015). The results of our experiment suggest that in *C. gigas*, expression of autophagy genes and proteins can be induced earlier than previously reported in the literature in the presence of $NH_4Cl$.

The autophagy modulation was also investigated in oysters injected with OsHV-1. Several key genes of the autophagy pathway were significantly upregulated at 14 and 24–30 hpi in the mantle, and at late time points (18 to 30 hpi) of the experimental infection, in haemolymph. These results suggest that the virus can induce a modulation of autophagy genes.

Upregulation of several *ATG* genes has already been reported at the transcriptional level after influenza virus infection (Klionsky et al., 2016). In the mantle of low-susceptibility Pacific oysters injected with OsHV-1, upregulation of *BECN1* at 8 to 12 hpi was reported (Moreau et al., 2015). In an analysis at different time points, we demonstrated here that several autophagy genes were upregulated at two time points in the mantle, whereas in the haemolymph, they were upregulated at one time point. These results point to different modulation of autophagy at the transcriptional level in the two tissues. Moreover, at the protein level, the expression of SQSTM1 significantly increased between 18 and 30 hpi in the mantle. In haemolymph, the percentage of cells containing autophagosomes significantly increased from 18 to 30 hpi. These results confirmed that OsHV-1 induced a modulation of the autophagy flux in the two tissues tested. In the mantle of *C. gigas*, a similar result was obtained by Moreau et al. (2015). Accumulation of the MAP1LC3-II protein was also demonstrated by western blotting, indicating that the autophagy flux was modulated at 20 hpi by OsHV-1. These findings are in agreement with the existing literature. For instance, induction of autophagy flux during the Sindbis virus infection in mouse embryonic fibroblasts was reported (Orvedahl et al., 2010; Chiramel et al., 2013).

In parallel, the viral replication was monitored. Viral DNA and RNA were detected in the two tissues starting from 6 hpi, indicating early replication of the virus in Pacific oysters. It has already been demonstrated that viral DNA can be detected in the mantle and the haemolymph of Pacific oyster spat since 6 hpi (Schikorski et al., 2011a). Moreover, viral transcripts of some OsHV-1 ORFs can be detected starting from 2 hpi in the mantle and from 1 h post contact in the haemolymph (Segarra et al., 2014b; Morga et al., 2017). By contrast, the viral replication kinetic seems to be different depending on the tissue considered. In haemolymph, the amounts of viral DNA and RNA strongly increased and reached a plateau, whereas in the mantle, the amount of viral DNA increased, and the expression levels of viral genes were stable. All our results indicate that the virus seems to start to replicate in the two tissues of *C. gigas* before the autophagy flux is modulated. Nonetheless, the autophagy modulation and the virus response are different between haemolymph and the mantle. It could be hypothesized that the two compartments regulate autophagy differently due to their different physiological functions. Dissimilar modulation of autophagy across different tissues of *Caenorhabditis elegans* in response to stress (starvation or anoxia) or aging has already been observed (Chapin et al., 2015). Moreover, it is also possible that in our study, the virus did not target the two compartments with the same aim and strategy. In fact, the mantle of *C. gigas* is an organ targeted by the virus, whereas in haemolymph, the haemocytes can serve as the cells transporting the virus OsHV-1 to target organs (Segarra et al., 2016; Morga et al., 2017).

Next, the autophagy modulation was followed in Pacific oysters exposed to $NH_4Cl$ and injected with the virus. In haemolymph and the mantle, autophagy gene expression levels increased starting from 6 hpi. In the presence of the virus and $NH_4Cl$, this result means early modulation of autophagy genes in the two tissues. Moreover, the expression of protein SQSTM1 increased earlier and more strongly. The SQSTM1 protein quantity was found to be significantly higher in the virus+$NH_4Cl$ group than in the artificial seawater group at early (6 to 14 hpi) and late time points (18 to 30 hpi) of the experimental infection. In haemolymph, the percentage of cells containing autophagosomes significantly increased in the virus+$NH_4Cl$ group relative to the artificial seawater group at later time points of the experiment (18 to 30 hpi). In the two tissues, there was earlier and/or stronger induction of autophagy flux in the presence of the virus and $NH_4Cl$ than in the presence of the virus alone. This earlier and stronger autophagy induction can be partially due to the inhibition of autophagy by $NH_4Cl$ and an interaction with the infection process. It is important to remember that $NH_4Cl$ acts quite late in the autophagy process, inhibiting degradation of autophagosomes and promoting autophagosome accumulation. Moreover, autophagy is a process that is involved in the response to viral infections. This process can exert an antiviral action by degrading viral particles or viral proteins *via* its cellular mechanism (Tallóczy et al., 2006; Orvedahl et al., 2010; Judith et al., 2013; Sagnier et al., 2015). Nevertheless, it is known that the autophagy mechanism can be hijacked by viruses, e.g., herpesvirus among others, to enhance their replication, to be transported, or to exit the cell (Cavignac and Esclatine, 2010; Miszczak and Cymerys, 2014; Jackson, 2015; Lussignol and Esclatine, 2017).

In the mantle and haemolymph, no significant difference in viral DNA and RNA was detected between virus and virus+$NH_4Cl$ groups. In another study carried out at 20 hpi, no significant difference in viral DNA was observed in the mantle of Pacific oysters exposed to the virus and to virus+$NH_4Cl$ (Moreau et al., 2015). Unexpectedly, in these tissues, stronger and earlier modulation of autophagy does not induce a change in virus development. On the other hand, Moreau et al. (2015) demonstrated that inhibition of the autophagy pathway by $NH_4Cl$ increases the rate of mortality during OsHV-1 infection. Two hypotheses can be proposed to explain these results. Because the oyster family used here manifested an intermediate level of susceptibility to viral infection, it is possible that at the individual level, oysters can present a high level of variability in the severity of infection associated with high variability of viral DNA and RNA. This variability can conceal the effect of autophagy inhibition on viral replication. Besides, these results can suggest that inhibition of the degradation of autophagosomes by lysosomes has no effect on viral development in the two tissues. It is possible that the use of $NH_4Cl$ does not allow us to determine the role played by autophagy in the response to a viral infection. Perhaps the reason is that this compound does not inhibit the autophagic sequestration step to work.

Nevertheless, using other known inhibitors of the autophagy pathway to block autophagosome formation could be an alternative strategy to study the role play by autophagy during a viral infection. Several pharmacological agents are available to inhibit the nucleation step of autophagy (Galluzzi et al., 2017). Wortmannin and 3-methyladenine, two inhibitors of PIK3C3 function (class III phosphatidylinositol-3-kinase) are good candidates (Toogood, 2002; Rubinsztein et al., 2007).

Nevertheless, the majority of these pharmacological agents serving to modulate the autophagy pathway have low pharmacological specificity for their target and can influence several other cellular pathways as well (Klionsky et al., 2016; Galluzzi et al., 2017). Therefore, in addition to the tested autophagy modulators, the role of autophagy in the response to viral infection needs to be investigated *via* other approaches (Klionsky et al., 2016).

## CONCLUSION

In this study, we demonstrated that autophagy is active during infection by OsHV-1. The results showed that viral replication was initiated before autophagy was activated (**Figure 6**). Nevertheless, the autophagy modulation differs depending on the tissue being considered. Using a known inhibitor of autophagy, called $NH_4Cl$, we found that autophagy can be inhibited beforehand in two

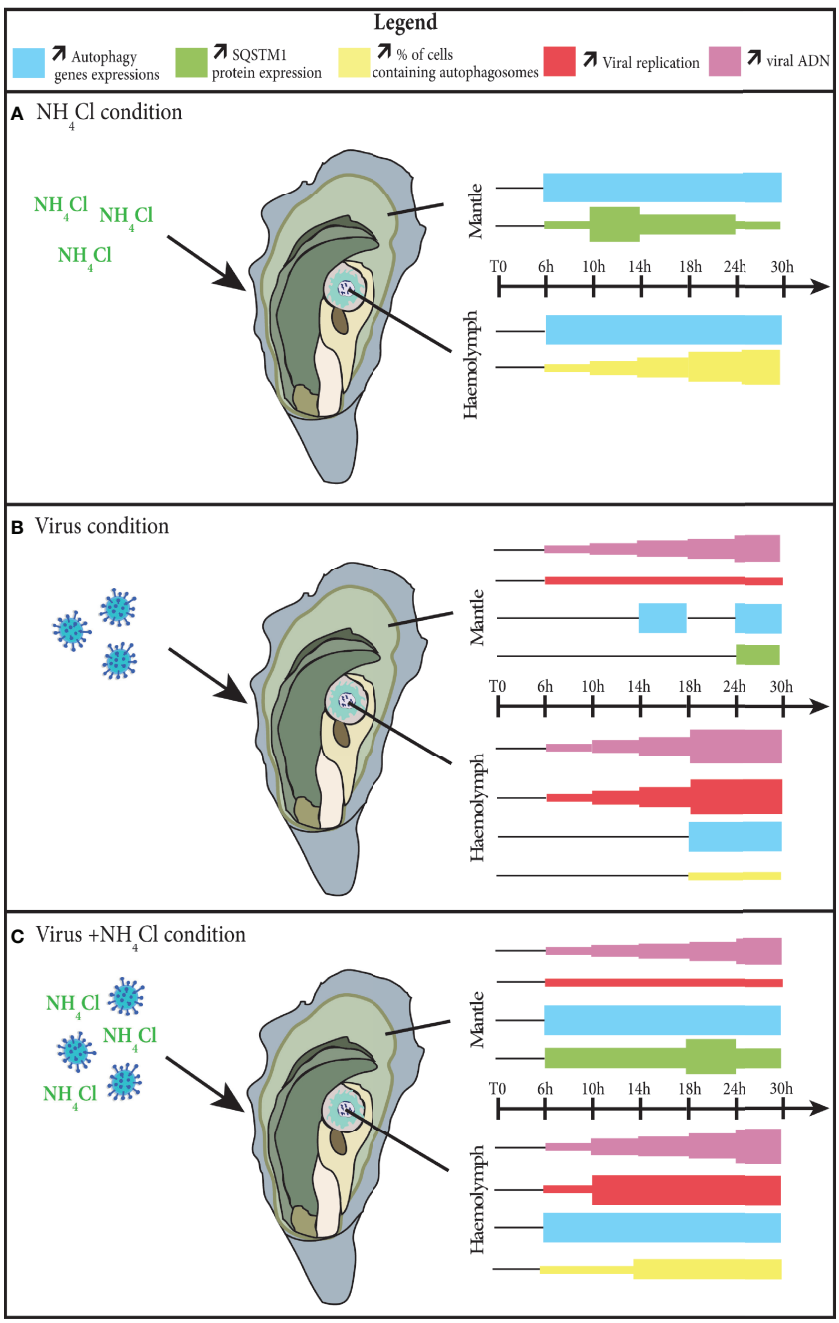


**FIGURE 6** | Modulation of autophagy in the mantle and haemolymph of the *Crassostrea gigas*, during the process of an infection by the virus OsHV-1. **(A)** in the $NH_4Cl$ condition; **(B)** in the virus condition; **(C)** in the virus+$NH_4Cl$ condition. The thickness of each color square represents an increase of the parameter considered.

tissues, the mantle and haemolymph, during the experiment. Because of the additive effects of $NH_4Cl$ and OsHV-1, earlier and stronger inhibition of autophagy was observed during the viral infection. Nevertheless, in the two tissues, inhibition of autophagy does not seem to be related to viral replication. Further research is needed to determine whether autophagy has an antiviral function or is manipulated by the virus for its own benefit.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

This study is the result of a collective work. SP, NF, and BM conceived this study and participated in its design. SP and NF performed the sample preparation for cellular and molecular analysis. SP, BM, NF, LD, IA, BC, and TR interpreted the results. SP and BM drafted the manuscript. All authors contributed to the article and approved the submitted version.

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# Computational prediction and experimental validation of *Salmonella* Typhimurium SopE-mediated fine-tuning of autophagy in intestinal epithelial cells

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Macroautophagy is a ubiquitous homeostasis and health-promoting recycling process of eukaryotic cells, targeting misfolded proteins, damaged organelles and intracellular infectious agents. Some intracellular pathogens such as *Salmonella enterica* serovar Typhimurium hijack this process during pathogenesis. Here we investigate potential protein-protein interactions between host transcription factors and secreted effector proteins of *Salmonella* and their effect on host gene transcription. A systems-level analysis identified *Salmonella* effector proteins that had the potential to affect core autophagy gene regulation. The effect of a SPI-1 effector protein, SopE, that was predicted to interact with regulatory proteins of the autophagy process, was investigated to validate our approach. We then confirmed experimentally that SopE can directly bind to SP1, a host transcription factor, which modulates the expression of the autophagy gene *MAP1LC3B*. We also revealed that SopE might have a double role in the modulation of autophagy: Following initial increase of *MAP1LC3B* transcription triggered by *Salmonella* infection, subsequent decrease in *MAP1LC3B* transcription at 6h post-infection was SopE-dependent. SopE also played a role in modulation of the autophagy flux machinery, in particular MAP1LC3B and p62 autophagy proteins, depending on the level of autophagy already taking place. Upon typical infection of epithelial cells, the autophagic flux is increased. However, when autophagy was chemically induced prior to infection, SopE dampened the autophagic flux. The same was also observed when most of the intracellular *Salmonella* cells were not associated with the SCV (strain lacking

*sifA*) regardless of the autophagy induction status before infection. We demonstrated how regulatory network analysis can be used to better characterise the impact of pathogenic effector proteins, in this case, *Salmonella*. This study complements previous work in which we had demonstrated that specific pathogen effectors can affect the autophagy process through direct interaction with autophagy proteins. Here we show that effector proteins can also influence the upstream regulation of the process. Such interdisciplinary studies can increase our understanding of the infection process and point out targets important in intestinal epithelial cell defense.

KEYWORDS

***Salmonella* Typhimurium, autophagy, SopE, network biology, MAP1LC3B, Host-microbe interactions**

## Introduction

Invasion and survival of intracellular bacterial pathogens within mammalian cells results from the timely expression of an arsenal of virulence factors, often horizontally acquired (Marcus et al., 2000; Hansen-Wester and Hensel, 2001; Nieto et al., 2016). The zoonotic *Salmonella enterica* subsp. *enterica* serovar Typhimurium pathogen (*S.* Typhimurium for short) is no exception to that rule (Srikumar et al., 2015; Colgan et al., 2016; Powers et al., 2021). Like many other enteric pathogens, *S.* Typhimurium hijacks the host cell machinery to enter the host cells, to hide from the innate immune system and ultimately to survive and spread to the next host (Alpuche Aranda et al., 1992; Lucas and Lee, 2000; Kröger et al., 2013). For that, *Salmonella* expresses and coordinates the secretion of its effectors directly into the host cell cytosol through type three secretion systems (T3SS), encoded on two *Salmonella* Pathogenicity Islands (SPIs) (Marcus et al., 2000; Schlumberger and Hardt, 2005; Lou et al., 2019).

Upon entry, *S.* Typhimurium will reside in a membrane-bound vacuole called the *Salmonella*-containing vacuole (SCV), where it may replicate. *Salmonella* entry is mediated by molecular mimics of host proteins (such as Guanine Exchange Factors (GEFs)) through effectors, including SopE, SopE2 and SopB, that activate host Rho-GTPases, RAC1, CDC42 (Schlumberger and Hardt, 2005), and the GTPase activating protein (GAP) SptP that subsequently deactivates Rho-GTPases, resolving host cell apical changes (Srikanth et al., 2011). In 20% of the cases entry into non-phagocytic cells is followed by an escape of *Salmonella* from the SCV (Brumell et al., 2002; Beuzón et al., 2002; Castanheira and García-Del Portillo, 2017). The resulting cytosolic *Salmonella* population must then adapt to the cytosol environment by mechanisms that are not fully elucidated, although SPI-1 effectors, including SopB, SptP, SipA, SopA, SopB, SopD and SopE may play additional roles impacting cytosolic *Salmonella* (Kubori and Galán, 2003; Drecktrah et al., 2005; Giacomodonato et al., 2007). Consistent with the idea that SopE could play a critical role in the adaptation of the pathogen to the host cell cytosol, SopE and SopE2 remain detectable on the SCV membrane up to 6 hours post-infection (Vonaesch et al., 2014).

Autophagy is a ubiquitous process crucial for cell homeostasis and stress survival of eukaryotic cells. Double membrane structures called autophagosomes are generated inside the cells and engulf superfluous organelles and proteins as well as invading pathogens. Autophagosomes then fuse with lysosomes, leading to degradation of the content (Feng et al., 2014; Cicchini et al., 2015; Sorbara and Girardin, 2015). By eliminating intracellular pathogens, such as bacteria and viruses (Sorbara and Girardin, 2015), autophagy assists the immune system in fighting infectious agents.

Over 38 proteins are involved in the autophagy process, each being temporally regulated throughout the different stages of the process: initiation, cargo recognition by the ATG ubiquitination system, membrane nucleation permitting the double membrane autophagosome formation, maturation of the compartment and fusion with the lysosome (Quan and Lee, 2013; Cicchini et al., 2015; Türei et al., 2015). We have previously described a computational pipeline allowing identification of interactions between secreted effector proteins of bacterial pathogens and autophagy core proteins (Sudhakar et al., 2019). In another previous study, we had generated and manually curated an autophagy protein interaction network in which we grouped the core autophagy proteins based on the following phases of autophagy: induction; cargo recognition and packaging; Atg protein cycling; vesicle nucleation; vesicle expansion and completion; transport of autophagosome; fusion with the lysosome (Kubisch et al., 2013).

Among those proteins, the MAP1LC3B (LC3-II) receptor, a ubiquitin-like protein, plays a critical role in the capture of the

cargo into the autophagosome. When the autophagy process is activated, MAP1LC3B gets lipidated and associated with the double membrane of the autophagosome. In concert with autophagy adaptors (e.g. p62, NDP52, OPTN), MAP1LC3B binds to the targeted protein, ensuring its capture for lysosomal degradation (Shaid et al., 2013; Khaminets et al., 2016).

Although autophagy is a robust clearing process against intracellular pathogens, some infectious agents, including *S.* Typhimurium have developed ways to escape or hijack autophagy for their own benefit. *S.* Typhimurium can subvert host autophagy at several stages of this process (Baxt et al., 2013; Baxt and Xavier, 2015; Sorbara et al., 2018). *Salmonella* secretes several effectors that have been proposed to interact with the ubiquitin pathway, such as the E3 ligases SopA, SspH1, SspH2 and SlrP, and the deubiquitinases SseL and AvrA (Baxt et al., 2013; Herhaus and Dikic, 2018). SopB can prevent fusion of the autophagosome with the lysosome (Weigele and Alto, 2010). Furthermore, cytosolic *S.* Typhimurium interacts with autophagy proteins, particularly MAP1LC3B and p62 proteins (Yu et al., 2014). Autophagy can also promote bacterial growth by sealing damaged SCVs maintaining a suitable environment for replication (Kreibich et al., 2015). It is therefore apparent that *Salmonella*-mediated modulation of autophagy is a more complicated and dynamic process that remains to be fully elucidated.

In this study, we present a network approach and describe its application to predict interactions of *Salmonella* effectors with host transcription factors, potentially resulting in changes in expression of key autophagy genes. We experimentally validate this prediction for the GEF-mimicking effector SopE and show that it can also modulate the flux of autophagy later on during infection.

# Materials and methods

## Computational predictions of interactions between Salmonella effectors and core autophagy genes

Protein-protein interactions (PPIs) were inferred by domain-domain interaction (DDI) prediction using the MicrobioLink pipeline (Andrighetti et al., 2020). Briefly, DDIs with high confidence values (interactions predicted by at least two different *in silico* methods or using multiple sources) were collected from the DOMINE resource (Yellaboina et al., 2011). We assumed that *Salmonella* and human proteins are connected if the interacting domains were represented in the database. The interaction prediction was merged with already existing predictions (Krishnadev and Srinivasan, 2011; Kshirsagar et al., 2012) and experimentally validated transcription factor-gene interactions related to autophagy.

Scripts for processing the interaction predictions, databases and other tables were written in R (See Table S1 for a list of host transcription factors and bacterial effectors found to interact). For the network analysis the Cytoscape network visualization program was used (Shannon et al., 2003). Autophagy core genes and their direct transcriptional interactions were downloaded from the following databases: Autophagy Regulatory Network (ARN, http://autophagyregulation.org/, (Türei et al., 2015), HTRIdb (http://www.lbbc.ibb.unesp.br/htri, (Bovolenta et al., 2012) and TRRUST (https://www.grnpedia.org/trrust/, (Han et al., 2015). All scripts are available at https://github.com/korcsmarosgroup/HMIpipeline.

## SopE-SP1 immunoprecipitation assay

HEK293 cells grown to 50% confluence were transfected with plasmids for overexpression of BirA-Myc-SP1 (gift from Markku Varjosalo; Addgene plasmid #167726) and GFP or GFP-SopE (Yuki et al., 2019) using GeneJuice transfection reagent according to the manufacturer's recommendations. Protein extracts were recovered 24-hour post-transfection in lysis buffer (20mM Tris ph7.5, 0.5% Triton-X100, 150mM NaCl, 2mM EDTA) supplemented with EDTA-free proteases inhibitors cocktail (04693132001, Roche) and benzonase (1.03773.1010, Millipore). Co-immunoprecipitations were performed on cleared lysates with GFP-Trap Magnetic beads (gtd, Chromotek) overnight at 4°C. Four consecutive washes with the lysis buffer were performed before the bead suspension was added in sample loading buffer (Sigma) and incubated for 5-10 min at 95°C. Inputs and IP samples were loaded onto 4-20% polyacrylamide gels and were transferred onto PVDF membranes (cold wet transfer in 10% ethanol for 1h at 100V). Membranes were blocked in 5% non-fat milk in TBST (0.1% Tween-20 in TBS) for 1 h. Primary antibodies anti-Myc (Cell Signaling #2276) and anti-GFP (sc-9996, Santa Cruz Biotechnology) diluted in TBST were incubated overnight at 4°C with gentle agitation. HRP-coupled secondary antibodies binding was done at room temperature (RT) for 45 min in 1% non-fat milk dissolved in TBST. All washes of the membranes were performed for 10 min in TBST at RT. Probed membranes were developed using a Chemidoc imaging system (BioRad) and signal intensity quantification was performed by measuring the densitometry of appropriate bands on not overexposed membranes using FiJi/ImageJ.

## Bacterial strains and culture conditions

All bacterial strains are listed below in Table 1. Gene deletion mutant strains were developed from the JH3009 (here referred to as wild type, wt) strain. The *sopE* gene was replaced in *S.* Typhimurium with the *aphII* gene conferring resistance to

TABLE 1 *Salmonella enterica* Typhimurium strains, generated and used in this study.

| Strain | Description | Reference |
|---|---|---|
| SL1344 | 4/74 *hisG rpsL* | Hoiseth and Stocker, 1981 |
| JH3009 (named wt in this manuscript) | SL1344, ϕ (*ssaG'-gfp*$^+$), $Cm^R$ | Hautefort et al., 2003 |
| SL1344Δ*sopE* | SL1344Δ*sopE*, $Km^R$ | This study |
| TK0014 | JH3009 ϕ(*ssaG'-gfp*$^+$), Δ*sopE*, $Km^R$, $Cm^R$ | This study |
| TK0019 | JH3009 ϕ(*ssaG'-gfp*$^+$), Δ*sifA*, $Km^R$, $Cm^R$ | Sudhakar et al., 2019 |
| TK0021 | SL1344, Δ*sifA*, $Km^R$ | Sudhakar et al., 2019 |
| TK0026 | JH3009 ϕ(*ssaG'-gfp*$^+$), Δ*sifA* Δ*sopE*, $Km^R$, $Cm^R$ | This study |

kanamycin, using a method LambdaRed recombination method (Datsenko and Wanner, 2000), with the exception that the red recombinase was supplied on the pSIM18 plasmid (Chan et al., 2007). The *aphII* gene from pKD4 was amplified using oligonucleotide primers 5'- CCTGCTATCTATATATAA ATGAATTATGTACATATAAAAGGATCATTACCgtgtagg ctggagctgcttcg-3' and 5'- GGTTCATATTAATCAGGAAG AGGCTCCGCATATTTTTTGGTTTTTCAGTGTcatatgaa tatcctccttagt-3' and introduced into strain SL1344 containing the pSIM18 plasmid. Recombinant transformants were selected on LB agar plates containing 50 μg ml-1 kanamycin. The *sopE* gene deletion was reintroduced into SL1344 by P22 transduction to minimise off-target mutations, as described previously (Kingsley et al., 1999). The genotype was confirmed by PCR amplification across the *sopE* locus using oligonucleotides 5'-CAGATGGACATAGCATTTGC-3' and 5'-ATGACGGTTTA GCTCCGGAG-3'.

Combination of the ϕ(*ssaG'-gfp*$^+$) intracellular reporter fusion with both the *sifA* and *sopE* gene deletions was carried out as follows in the SL1344 genetic background. The Kanamycin resistance cassette replacing the *sifA* gene in TK0021 was excised from the chromosome using the yeast Flp recombinase expressed from the thermosensitive replicon pCP20. The pCP20 replicon was subsequently removed from TK0021 after culture at 40°C in non-selective medium. The *sopE*, $Km^R$ deletion and the ϕ(*ssaG'-gfp*$^+$), $Cm^R$ transcriptional fusion were transduced into the *sifA*, $Km^S$ new strain by P22 phage transduction, generating the strain TK0026 used in this study (See Table 1) (Gemski and Stocker, 1967). The generated the strain TK0026, lacking both SifA and SopE and carrying a GFP reporter system to indicate intracellular *Salmonella* location in infected epithelial cells.

Bacterial strains were grown in 5 ml of LB broth overnight (Sambrook and Russell, 2001) at 37°C at 250 rpm. For invasion assays, a 1:100 dilution of the overnight bacterial culture was grown in 25 ml of LBS (LB containing a total of 0.3 M NaCl) in 250 ml conical flasks until an optical density of 1.2 was obtained at 600 nm (A600). Antibiotics were added as required at the following final concentrations (kanamycin, 50 mg ml-1; chloramphenicol, 10 mg ml-1).

## HT-29 cell culture and invasion assays

HT-29 human colon cancer epithelial line (HTB-38, ATCC) was cultured in DMEM supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 2 mM L-Glutamine at 37°C, 5% $CO_2$. HT-29 epithelial cells were seeded into 6- and 24-well cell culture plates at a density of $3.2x10^6$ and $2x10^5$ cells/well, respectively, to obtain 80% confluency by the day of the invasion assays (48h later). Six well-plates were used to generate enough infected epithelial cells for RNA extraction and qPCR autophagy gene expression analysis. Twenty four-well plates were used for immunofluorescence microscopy monitoring of autophagy flux upon infection. In those plates, HT-29 cells were seeded on 13mm diameter glass round coverslips. Three biological replicates were used for each condition and time point. Where necessary, autophagy was induced by treating the cells with 30 μg ml-1 rapamycin or DMSO only prior to and during the 6h long infection (17h total). On the day of the invasion assay, cells were washed twice in non-supplemented DMEM followed by the infection. Bacterial suspensions were prepared in DMEM from the LBS sub-culture of the *Salmonella* strains (see Bacterial strains and culture conditions) at a multiplicity of infection (MOI) of 10 bacterial cells per mammalian cell. Infected cells were incubated for 30 minutes at 37°C, 5% $CO_2$. The infection medium was then replaced with a complete medium containing 30 μg ml-1 gentamicin for 30 minutes to kill the remaining extracellular *Salmonella* cells. For the rest of the experiment, the gentamicin concentration was then reduced to and maintained at 5 μg ml-1.

For the assay allocated to autophagy flux bioimaging, the medium was removed at 6h post infection (p.i.). Cells were washed twice in Dubelcco's phosphate-buffered saline (DPBS; D8537, Sigma Aldrich), fixed in 4% paraformaldehyde at room temperature for 20 min, and washed twice for 5 min at room temperature in DPBS prior to immunofluorescence labelling.

## Cell sorting

At 2h and 6h post-infection, the medium was removed and cells were washed twice in DPBS. Each sample well was

trypsinised in 50% Trypsin-Versene (EDTA), 49.6% DPBS, 2mM EDTA and 5 µg ml-1 gentamicin for 5 minutes at 37°C. The trypsin reaction was stopped in 89.6% DPBS, 10% FBS, 2mM EDTA and 5 µg ml-1 gentamicin (i.e. FACS buffer). Single-cell suspensions were obtained by pipetting several times. Cells were washed and resuspended in FACS buffer. Infected cells and bystanders were separated by Fluorescence-Activated Cell Sorting on a BD FACSMelody machine (BD Biosciences). Sorting gates were set based on negative cells (from an uninfected well) and positive control cells (from an infected well) based on the level of GFP present in each cell. Eight pools of 50 epithelial cells were sorted from each condition and time point (4 x GFP- as non-infected cells or "bystanders" and 4 x GFP+ as "*Salmonella*-containing epithelial cells") into 96-well plates containing 2 µl of lysis buffer (0.2% Triton X-100 and 2 U µl-1 RNase inhibitor). Samples were then processed as detailed in the RNA samples extraction and processing section.

## RNA samples extraction and processing

The low input RNA extraction and reverse transcription were carried out following the SmartSeq2 protocol previously described (Picelli et al., 2013). Reverse transcription was performed as in the following steps. First, Oligo-dT30VN primer was added to the sample lysates at 2.5 µM, 2.5 nM each dNTP final, and priming reactions were incubated at 72°C for 3 mins. First strand synthesis was subsequently initiated by addition of 1.03 µM custom template switching oligo, 6.18 µM MgCl2, 1.03 M Betaine, 5.12 mM DTT, 1.03x Superscript First Strand Buffer, 0.52 U µl-1 RNase Inhibitor, 10.3 U µl-1 SuperScript II reverse transcriptase in nuclease-free water. The reverse transcription reactions followed the successive steps: 42°C 90 mins, 10×(50°C 2 mins, 42°C 2 mins), and 70°C 15 mins. Finally, 15 ul PCR mastermix (1.6x KAPA HiFi Hotstart Readymix, KAPABIOSYSTEMS, 0.16 µM IS primers, nuclease-free water) was added to each sample. PCR cycles were as follows, 98°C 3 mins, 21×(98°C 20 secs, 67°C 15 secs, 72°C 6 mins), 72°C 5 mins). PCR products were cleaned up with 0.8x volumes of Ampure XP and 80% ethanol. Samples were then eluted in 20 µl 10 mM Tris-HCl.

## qPCR

Taqman gene expression analysis was conducted on the SmartSeq2 amplified cDNA. In brief, TaqMan™ Fast Advanced Master Mix (4444557, ThermoFisher Scientific) was used for all qPCR reactions, assay probes and samples were dispensed into 384-well Roche-style qPCR plates (4titude, 4ti-1381) using a Mosquito HV automated liquid handling instrument (SPT Labtech), to a final reaction volume of 1.6µl (80nl 20× TaqMan® Gene Expression Assay, 800nl 2× TaqMan® Gene Expression Master Mix, 720nl normalised cDNA). Samples were then analysed on a Roche Lightcycler 480. The Taqman gene expression assay probes were used for the *MAP1LC3B* test gene and the beta-2-microglobulin *B2M* reference gene (Hs00797944_s1 4453320 and Hs00984230_m1 4453320, respectively, ThermoFisher Scientific).

Gene expression was normalised to the B2M internal reference gene and plotted as log2^-deltaC$_T$ (Rao et al., 2013). First, technical replicates with the smallest and largest C$_T$ values (raw C$_T$ in case of potential reference gene and normalised C$_T$ values in case of genes of interest) were excluded for each gene within each condition.

## Immunohistochemistry

MAP1LC3 and p62 were labelled as previously done (Sudhakar et al., 2019). In brief, for MAP1LC3B/LC3B immunostaining, cells were quenched at room temperature in 50 mM NH4Cl in DPBS for 10 min, then permeabilized in methanol for 5 min and washed in DPBS 3 times for 5 min. This was followed by blocking the samples at room temperature in 1% bovine serum albumin (BSA) Fraction V (05479, Sigma-Aldrich) in DPBS for 30 min. The rabbit anti-MAP1LC3B/LC3B (ab48394, Abcam) antibody was applied overnight at 4°C. The antibody labelling solution was diluted at a 1:2000 dilution in DPBS containing 1% BSA fraction V (05479, Sigma Aldrich). SQSTM1/p62 immunolabeling was performed as follows. Fixed cells were permeabilized and blocked in a solution containing 1% BSA and 0.1% saponin (84-510, Fluka), in DPBS for 30 min at room temperature. The rabbit anti-SQSTM1/p62 antibody (ab91526, Abcam) was applied overnight at 4°C at a f 1:6000 dilution in DPBS containing 1% BSA and 0.1% Saponin.

The FITC-conjugated anti-GFP antibody (ab6662, Abcam) was applied overnight at 4°C in all samples at a 1:200 dilution in all primary solutions.

All primary antibodies were washed 3 times in either 1% BSA in DPBS (MAP1LC3B/LC3B) or 1% BSA and 0.1% saponin in DPBS (SQSTM1/p62). Alexa Fluor 594-conjugated anti-rabbit secondary IgG (ab91526, Abcam) was diluted 1 in 1000 and applied to all samples at room temperature for 1 h. All samples were counterstained with DAPI at a dilution of 1:2000 in the buffer respective to each primary antibody. Samples were then washed 3 times in their respective buffers, once in water and finally mounted on microscopy glass slides. Coverslips were mounted in Aqua-poly/mount anti-fading compound (18606, Polysciences Inc.). Coverslips were left to set, sealed using nail varnish and stored at -20°C until observation. Slides were imaged on a Zeiss LSM710 microscope, using a 100x Apochromat (100x/1.4 Oil DIC plan Apo) oil immersion objective. Focal plan and laser power/gain was kept constant throughout the acquisition process. Over 500 epithelial cells were imaged per condition tested. The acquisition was semi-blind with conditions revealed post-analysis.

## Image analysis

The analysis of SQSTM1/p62 and LC3 staining was done using semi-automated macros within FiJi (Image J2) software. To avoid unconscious-bias, imaged areas were chosen randomly based solely on the DAPI staining. DAPI staining was used for the identification of nuclei and individual cells were identified by extension of the nuclei mask. Segmented cells touching the edge of the images or artefacts (small objects) were eliminated. Individual regions of interest (ROIs) were saved for each image and subsequently used for quantification of SQSTM1/p62 and LC3 staining. Intensity and puncta information for individual cells in each fluorescent channel was exported into Excel spreadsheet and used for statistical analysis.

## Statistical tests

Distribution normality of data points was determined by the Shapiro-Wilk test and the equality of variance was determined by the Levene's test. To compare 3 or more groups, one-way ANOVA (for normal distribution) or Kruskal-Wallis (for non-normal distribution) tests were performed using R. On statistically different samples the appropriate *post hoc* test was applied: Tukey following ANOVA and kruskalmc following Kruskal-Wallis. T-tests and kruskalmc tests were performed to compare LC3 and p62 protein levels in wild type *Salmonella*-infected cells and cells infected by the Δ*sopE* deletion strain derivative.

# Results

## Network analysis of potential pathogen-host interactions affecting autophagy

The potential effect of *S.* Typhimurium effector proteins on autophagy gene expression was analysed using a network of predicted and experimentally validated interactions. We predicted pathogen-host interactions based on known domain-domain interactions and supplemented this with two predictions previously described (Krishnadev and Srinivasan, 2011; Kshirsagar et al., 2012) making up our merged list of interactions (See Table S1). The source proteins were filtered for secreted *S.* Typhimurium effectors (Miao and Miller, 2000; Ehrbar and Hardt, 2005; Haraga et al., 2008; Figueira et al., 2013), and the target proteins were filtered for those that had previously been validated experimentally as transcription factors of autophagy-core genes (Bovolenta et al., 2012; Han et al., 2015; Türei et al., 2015). The core autophagy network was extended with an additional gene, *ATG4D* and its connection to *GABARAPL1* (Betin and Lane, 2009). As further filtering steps, transcription factors were kept if they were predicted to connect to only one or few *Salmonella* effectors (eliminating some potential false positives) while at the same time regulating more than one of the core autophagy genes.

The first layer of the constructed network was centred around the interactions between *S.* Typhimurium virulence proteins (excluding SlrP) and human autophagy core-regulating transcription factors. Regarding these interactions there was no overlap between the three sources, and only three interactions of SlrP with CTCF, YY1 and SP1 were overlapping between the prediction of Kshirsagar et al. and our prediction. This layer of the final network contains 71 connections from Kshirsagar, 0 connections from Krishnadev and 4 connections from our prediction (Krishnadev and Srinivasan, 2011; Kshirsagar et al., 2012). On the human side there were 154 transcription factor-autophagy core interactions from ARN, 105 from HTRI and TRRUST database, with altogether 33 transcription factors, 35 core autophagy genes and 159 regulatory interactions between them.

Our network analysis highlights that several *Salmonella* effectors can impact several transcription factors. For example, effectors such as SseI, SseL, SlrP and SspH2 target 9, 10, 16 and 33 transcription factors, respectively (Figure 1A). This reflects the multiple routes *Salmonella* has evolved to subvert host intracellular clearance mechanisms, here by affecting all stages of the autophagy process (from induction, through to the autophagosome formation to the fusion with lysosomes; Figure 1). We selected six *S.* Typhimurium proteins that were not predicted to connect to too many of the human transcription factors (except SspH2) to simplify our further analysis. One of these selected effectors is SlrP. SlrP, that can be secreted through the SPI-1 and -2 apparatus as well, and is an E3 ubiquitin ligase, hence potentially able to interact with ubiquitin-mediated autophagy. Moreover, it has overlapping predicted interactions in two of the PPI predictions, highlighting the power of our computational approach. Two additional effectors, SopE and SopE2, are SPI-1 effectors and mimic host GEFs. They are particularly important in the membrane ruffling associated with *Salmonella* entry into the non-phagocytic epithelial cells. Yet, they are also expressed in later stages of the invasion and present in our network possible interactions with two autophagy transcription factors. The remaining of the selected proteins, SseI, SseL and SspH2 are SPI-2 virulence effectors, which means that they are probably secreted in the later stages of invasion and SseL and SspH2 are also among the proteins that can alter ubiquitination.

Considering SopE's role in altering RhoGTPases downstream effect and persisting long enough in the host cells to be exposed to the host cell machinery, including the autophagy process (Schlumberger and Hardt, 2005; Vonaesch et al., 2014), it was subsequently selected for experimental validation of our predictions. When focusing on the SopE potential interactions with autophagy regulators, we predicted

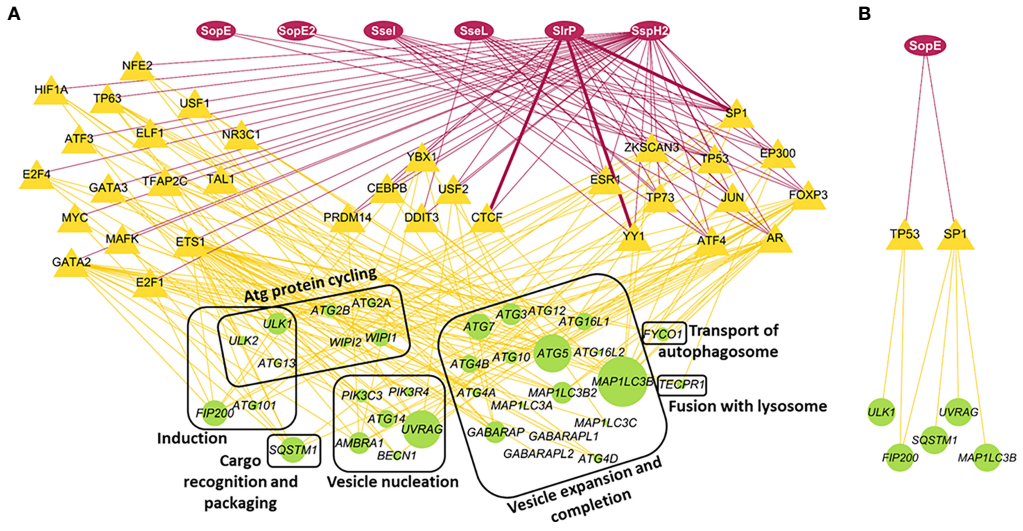


FIGURE 1
Interaction network between selected *Salmonella* Typhimurium effectors, host cell transcription factors and autophagy core genes. **(A)** Network analysis of potential interactions between *Salmonella* and host autophagy. Red ovoid nodes are the selected *Salmonella* effector proteins. Red edges are PPI predictions. Thin edges were predicted by one of the three methods. Thick edges were predicted by two of the three methods. Yellow triangular nodes are host transcription factors that *Salmonella* effectors can influence. These are clustered according to the number of *Salmonella* effectors they are targeted by. Yellow edges reflect transcriptional regulation of core autophagy genes (round green nodes) by the transcription factors. The size of the green nodes is proportional to the number of transcription factors they are connected to. **(B)** Subnetwork illustrating the potential interaction of the *Salmonella* effector SopE with transcription factors affecting specific autophagy core genes. The same layout was used here as for the large network.

that SopE can interact with only two key transcription factors, TP53 regulating genes in the autophagy induction phase, and SP1, controlling the formation and expansion of the autophagosome compartment (Figure 1B). These two steps of autophagy are likely to be influenced differently depending on the location of the intracellular *Salmonella* cells, either within a damaged SCV or cytosolic. We investigated further if the GEF-mimicking SopE influenced the autophagy process at different levels, focusing only on the SopE and SP1 potential interaction.

## SopE can directly interact with the host SP1 transcription factor

Our network analysis suggests that one way *Salmonella* can modulate autophagy flux is through altering the regulation of autophagy-related gene expression. For example, we predicted that SopE can interact with SP1 and TP53 (Figure 1B). Our study focuses later on the modulation of the autophagy process through MAP1LC3B, so we centred our experimental validation on the potential SopE-SP1 interaction. To validate this hypothesis, we performed a co-immunoprecipitation assay between GFP-SopE and BirA-Myc-SP1 recombinant proteins, ectopically expressed in HEK293 cells (Figure 2A). We observed that SP1 is enriched over sixfold with GFP-SopE compared to GFP alone (Figure 2B), confirming the direct interaction of the *Salmonella* SopE protein with the SP1 autophagy regulator. This suggests that through interaction with SP1, SopE can alter the expression of SP1-target genes, such as *MAP1LC3B*.

## SopE contributes to down-regulating autophagy MAP1LC3B expression

SopE was shown to be essential in the SCV formation during invasion and it was also shown that some of the intracellular SopE protein remains associated with the SCV membrane at later time point during infection at a time when autophagy process is induced (Schlumberger and Hardt, 2005; Vonaesch et al., 2014). We first tested the functional importance of the direct interaction of SopE with SP1 by monitoring whether SopE influences the expression of the key *MAP1LC3B* gene, directly downstream of the SP1 transcription factor. For that, we infected HT-29 epithelial cell monolayers with either a *sopE*$^+$ strain of *S.* Typhimurium (=wt), or its Δ*sopE* deletion strain derivative. Both strains contain a ϕ(*ssaG'-gfp*$^+$) transcriptional fusion, allowing intracellular GFP$^+$ *Salmonella* containing HT-29 cells identifiable in a pool of infected and non-infected cells. At 2 and 6h p.i., *MAP1LC3B* RNA levels were quantified in *Salmonella*-containing cells or in epithelial cells that did not

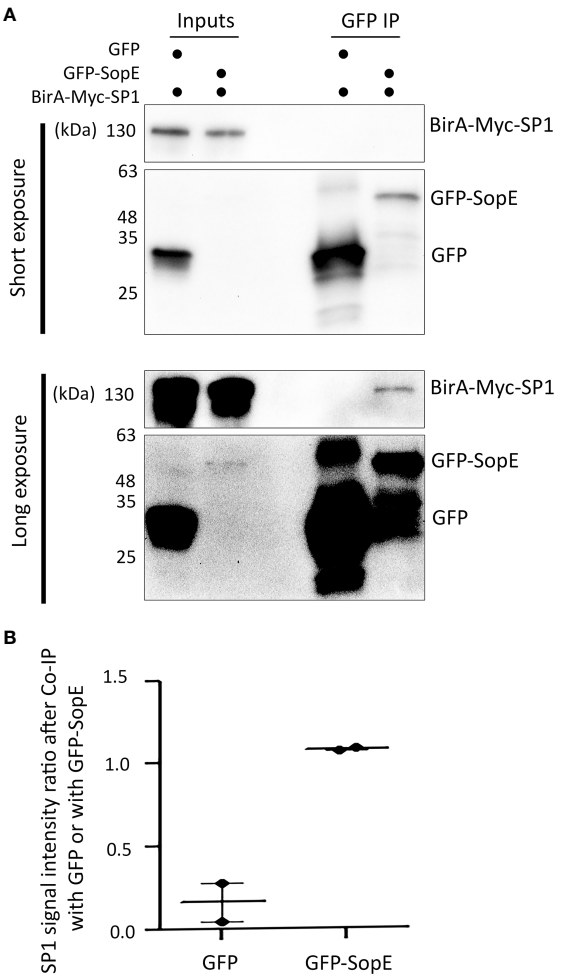


FIGURE 2
The *Salmonella* effector SopE can bind to the human transcription factor SP1, as predicted. **(A)** Co-immunoprecipitation of GFP-SopE and BirA-Myc-SP1 ectopically expressed in HEK293 cells. **(B)** SP1 signal intensity ratio after Co-IP with GFP or with GFP-SopE showing enrichment of SP1 when GFP-SopE is used compared with GFP alone, based on two independent measurements.

contain *Salmonella* despite being part of the infected cells (from then on called "bystanders"). *MAP1LC3B* RNA levels revealed that SopE impacts on the transcriptional levels of *MAP1LC3B* compared with non-infected cells (Figure 3), consistent with this pathogen-derived protein interacting with SP1 transcription factor (Figure 2).

Surprisingly, although SopE is essential for the early internalisation of *Salmonella* inside epithelial cells, it is not required for upregulating autophagy early during infection, e.g. 2h p.i. (Figure 3A). Epithelial cells upregulated *MAP1LC3B* expression irrespective of the presence or absence of SopE. Interestingly, *MAP1LC3B* gene expression was upregulated significantly even in bystander epithelial cells, suggesting that either the bystander cells respond to *Salmonella*-derived compounds that are sensed when the pathogen is not internalised or that they respond to mediators produced by the *Salmonella*-containing epithelial cells directly (Figure 3B).

However, at a later time point, when *Salmonella* started proliferating intracellularly (6h p.i.), we observed that the level of *MAP1LC3B* expression returned to that of non-infected cells in a SopE-dependent manner. This was particularly the case in HT-29 cells that contained *Salmonella* and not so for bystander epithelial cells that did not contain *Salmonella* cells (Figure 3B).

## SopE also modulates autophagy flux

As well as modulating the regulation of core autophagy gene expression through specific transcription factors, we hypothesise that SopE could also influence the autophagy flux as it is retained at the SCV membrane site several hours after *Salmonella* internalisation. MAP1LC3, once lipidated and recruited to the membrane nucleation site, connects the cargo to the vesicle membrane through the SQSTM1/p62 adaptor protein. We chose to follow MAP1LC3 and SQSTM1/p62 associated with the autophagosome as these two proteins are good indicators of the autophagy flux taking place in a mammalian cell (Xu et al., 2018). To address this, LC3 number of puncta and p62 intensity were assessed by immunofluorescence confocal bioimaging 6h p.i., at a similar time when SopE is still retained at the SCV membrane (See Materials & Methods section).

When autophagy is not pre-induced before infection by *Salmonella*, no significant difference in the number of LC3 puncta is observed at 6h p.i. (Figure 4A). However, the p62 dots intensity is significantly greater in cells infected with *Salmonella* that lack *sopE* (Figure 4B). This suggests that SopE, although dampening the expression of autophagy core genes at 6h p.i., simultaneously modulates the autophagy flux locally, at intracellular autophagy sites.

Whether SopE acts on the autophagy flux, before or after that process is induced, is not yet clear. LC3 puncta number and p62 dots intensity were therefore monitored also after autophagy was experimentally triggered. Figures 4C, D clearly show that both LC3 puncta (C) and p62 dot intensity (D) increase significantly when SopE is absent, confirming that the dampening effect SopE normally has on autophagy by the time *Salmonella* starts to replicate intracellularly. SopE's effect is even amplified when autophagy is already activated.

## SopE modulates the autophagy flux only when Salmonella is within SCV and not when it has escaped the vacuole and is cytosolic.

Finally, we questioned whether the fraction of SopE retained at the SCV membrane implies that *Salmonella* benefits from the SopE-dampening autophagy only when residing within an SCV.

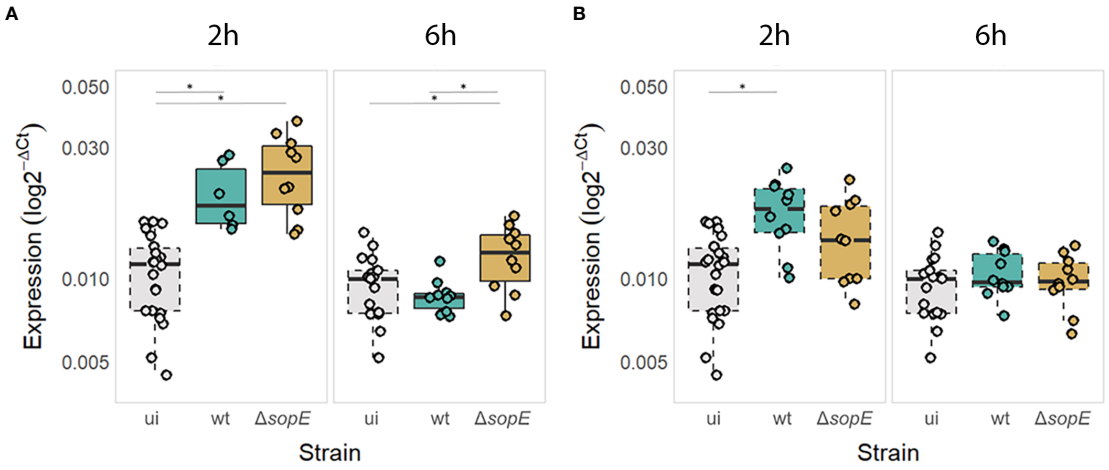


FIGURE 3
*MAP1LC3B* gene expression is increased at the early stages of *Salmonella* infection but decreased at later stages in a SopE-dependent manner in *Salmonella*-containing epithelial cells **(A)** but not in bystander cells **(B)**. *MAP1LC3B* expression levels expressed as log2-ΔCT so that uninfected cells (ui) are also displayed. Continuous borders = infected cells containing *Salmonella* (wt or mutant) and dashed borders = bystander epithelial cells not containing *Salmonella* or uninfected cells. * p<0.05.

To address the impact of *Salmonella* intracellular localisation on the role of SopE as modulator of the autophagy flux, we constructed a strain lacking SopE that would essentially be located in the epithelial cell cytosol. SifA effector protein of *Salmonella* is well known for being instrumental to the evolution and maintenance of the SCV (Beuzón et al., 2002), recruiting vacuolar ATPase to the SCV, permitting the SCV luminal environment to acidify (Beuzón et al., 1999; Martin-Orozco et al., 2006), and the formation and extension in certain host cells to *Salmonella*-induced Tubules (SITs) from the endosomal system for the intracellular survival of *Salmonella* in the host (Liss and Hensel, 2015). It was shown in diverse studies that *Salmonella* strains lacking SifA escape the SCV and start proliferating anarchically in the cytosol of non-phagocytic cells such as epithelial cells. We therefore constructed the strain TK0026 carrying the double Δ*sifA* and Δ*sopE* genes deletion (See Materials & Methods). Similarly done to the experiment shown on Figure 4, HT-29 epithelial cell monolayers were infected this time with either the strain lacking SifA only or the strain lacking both SifA and SopE. Figure 5 shows a mild, yet significantly different increase in the number of LC3 puncta in cells infected with *Salmonella* lacking SifA and SopE compared with the cells infected with *Salmonella* lacking SifA only, suggesting SopE could mildly influence MAP1LC3B lipidation and recruitment to the autophagosome membrane. However, the autophagy flux does not seem to be differing whether SopE is present or not once *Salmonella* is cytosolic, emphasising the specificity of SopE's modulatory effect.

## Discussion

Our study predicts that several *Salmonella* virulence effectors proteins interact directly with some transcription factors involved in regulating autophagy, impacting the subsequent expression of core autophagy genes, such as *MAP1LC3B*. We also observed that *Salmonella* influences the autophagy flux at certain stages of infection; it does so in epithelial cells where *Salmonella* is still associated with the SCV. We had previously shown through protein-protein interaction network analysis that autophagy preferentially targets virulence effectors secreted by intracellular pathogens, and that pathogens have evolved mechanisms that conversely compromise the autophagy process. Autophagy-associated proteins are directly targeted by pathogens at different stages of the process (Sudhakar et al., 2019). Here we asked whether *S.* Typhimurium virulence effector proteins can influence this clearing process by acting on the regulatory level above the core autophagy genes, i.e. on the transcription factors that normally regulate them. Using protein-protein interaction predictions and transcription factor-gene interaction databases we built a network that links several *S.* Typhimurium virulence effectors to key transcription factors and predicted that *S.* Typhimurium can modulate the regulation of autophagy core gene expression. We showed that six SPI-1 effectors could interact with overall 33 transcription factors that normally regulate the expression of core autophagy genes (Figure 1A). As expected, SPI-2 SseL and SspH2 effectors showed a greater number of interactions with transcription factors, with 10 and all 33 regulatory targets, respectively. This

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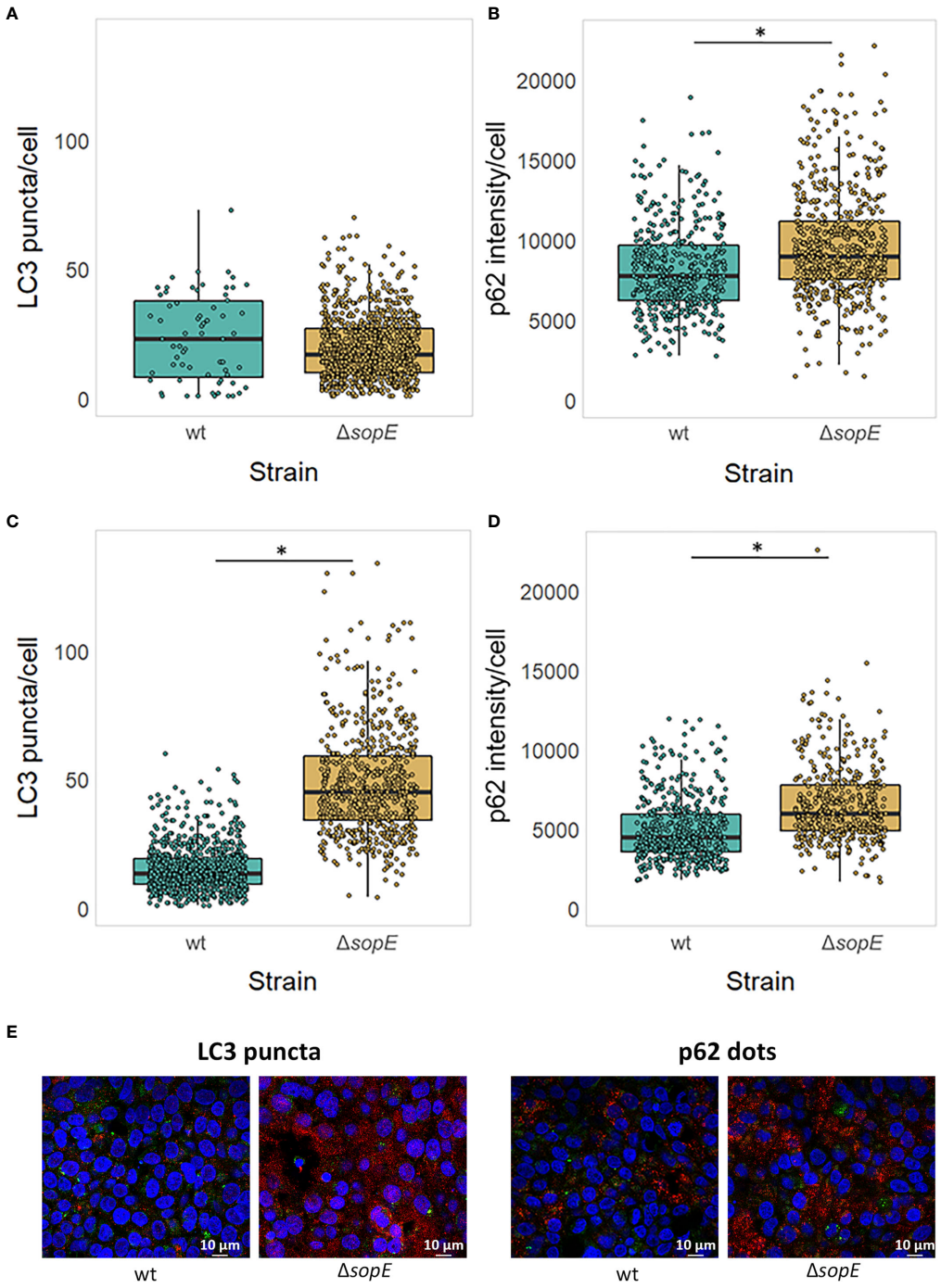


FIGURE 4
SopE dampens the autophagic flux by 6h post infection. LC3 puncta number and p62 dot intensity in HT-29 epithelial cells infected for 6h with either wt *Salmonella* strain or its Δ*sopE* gene deletion derivative. **(A, B)** HT-29 epithelial cells were infected with wt *Salmonella* and its Δ*sopE* gene deletion derivative strain as indicated before. **(C, D)** HT-29 cells were pre-treated with Rapamycin for 11h prior to and during the 6h-long infection (Maximum 17h). LC3 puncta **(A, C)** and p62 dot intensity **(B, D)** was quantified from HT-29 cells containing *Salmonella*. *p=0.05. **(E)** Micrographs of HT-29 cells pre-treated with Rapamycin showing LC3 puncta (red, left) and p62 dots (red, right), intracellular *Salmonella* cells (green) and nuclei (blue) illustrating panels **(C, D)**.

reflects the adaptation of *Salmonella* to the host cell intracellular environment where SPI-2 secreted effectors are the main contributors to *Salmonella* survival and virulence (Haraga et al., 2008; Srikanth et al., 2011). Yet our study also highlighted new autophagy interactors among the *Salmonella* effector arsenal; for example, we showed that the *Salmonella* SseI [also called SrfH; (Thornbrough and Worley, 2012)] cysteine hydrolase can interact with 9 transcription factors modulating autophagy gene expression in epithelial cells. This effector might play an important role later in the infection, affecting the migration of macrophages and DCs (Jennings et al., 2017) although a single nucleotide polymorphism in the *sseI* gene that occurs naturally in some *Salmonella* strains prevents SseI from stimulating monocyte migration (Thornbrough and Worley, 2012). Our study suggests that SseI essentially impacts autophagy through its interaction with SP1 and TP53 transcription factors. We can envisage that, in addition to its regulatory role, it could also interfere with proteolytic processes taking place within epithelial cells through its cysteine hydrolase activity; initiation, execution, or inhibition of the autophagy process being highly dependent on proteases activity (Kaminskyy and Zhivotovsky, 2012).

Fewer SPI-1 effectors seem to interact with autophagy regulators (Figure 1A). This is in agreement with SPI-1 effectors being mostly involved in the entry of the pathogen into non-phagocytic mammalian cells (Lou et al., 2019). However, certain SPI-1 effectors have been shown to persist within the host cells, even past the point when *Salmonella* starts replicating. For instance, the SPI1 SlrP E3 ligase can also bind to a large number of transcription factors (Figure 1A) highlighting the importance of interfering with the ubiquitin pathway of the host, and possibly assisting the interaction of E3 ligases normally secreted through SPI-2 T3SS (e.g. SspH2; (Herhaus and Dikic, 2018).

The two *Salmonella* SPI-1 GEFs-mimicking effectors SopE and SopE2 seem to have a more specific impact on autophagy gene expression as they can interact with much fewer transcription factors (Figures 1A, B). SopE and SopE2 effectors can both target two transcription factors, TP53 and SP1, affecting autophagy induction through ULK1 and FIP200, respectively, and the autophagosome formation through SQSTM1/p62, UVRAG and MAP1LC3B, rather than the late lysosome-mediated clearance of the cargo. This restricted number of transcription factors potentially interacting with SopE and SopE2, as shown here for SP1 and SopE, suggests a very specific role of these effectors in the hijacking of autophagy by *Salmonella*. TP53 was also predicted to be a binding target of SopE and, although the role of this interaction was not studied here, its role in *Salmonella*-mediated autophagy gene expression regulation should be further explored. Indeed, when the transcription factor TP53 is inhibited or absent, increased autophagosome formation and overall autophagy flux is observed, suggesting that cytosolic TP53 reduces autophagy initiation. TP53 transcription factor acts upon autophagy modulation even when *Salmonella* remains cytosolic (Tasdemir et al., 2008; Kroemer et al., 2015). We suggest that

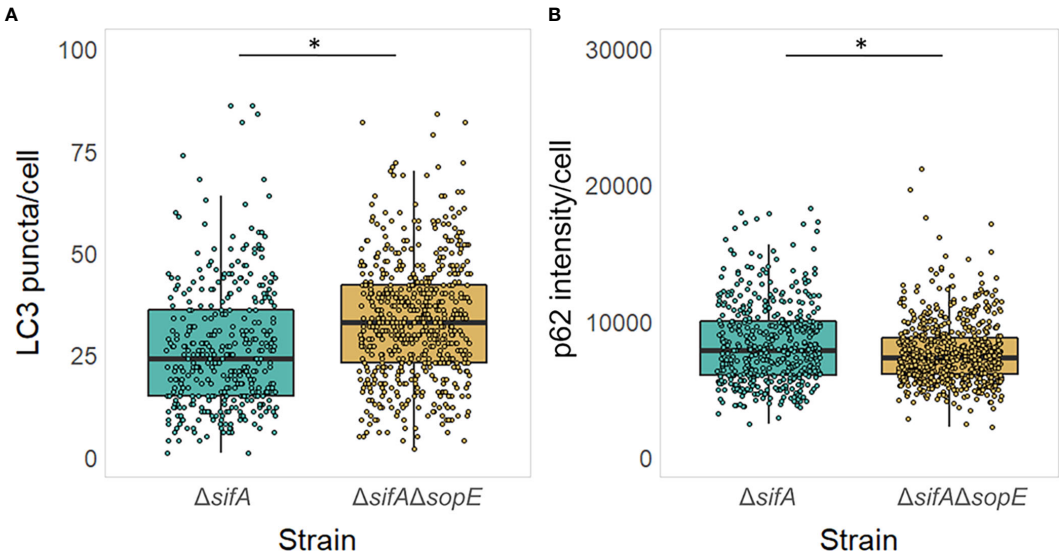


FIGURE 5
Cytosolic *Salmonella* no longer can dampen the autophagy flux in a SopE-dependent manner. **(A, B)** Autophagy was pre-induced with Rapamycin in HT-29 epithelial cell monolayers and maintained during the 6h infection with *Salmonella*, as it exacerbates the impact of SopE on modulating autophagy, making it easier to visualise. LC3 puncta **(A)** and p62 intensity **(B)** was quantified from HT-29 cells containing *Salmonella*. *p=0.05.

SopE inhibits autophagy induction through its binding to TP53, maintaining this transcription factor in the cytosol and exacerbating its autophagy dampening effect. *sopE2* is present in all pathogenic strains of *Salmonella*, while *sopE* is assumed to have appeared later in the *Salmonella* evolution by duplication of *sopE2* and is present only in a subset of strains such as SL1344 used in this study (Table 1) (Bakshi et al., 2000; Mirold et al., 2001), suggesting a gained function in these strains. Indeed, SopE was also obtained from the strain responsible for a major epidemic in the 1970s and 1980s (Mirold et al., 1999). We focussed particularly on SopE to study its added impact on core autophagy gene expression and autophagy flux modulation by these *Salmonella* strains.

We first validated experimentally that SopE can bind to the SP1 transcription factor regulating MAP1LC3B expression (Figure 2), validating our prediction (Figure 1B). We then showed that, following the early increase in autophagy associated with the infection by *Salmonella* (2h p.i.), SopE contributes to the dampening of *MAP1LC3B* gene expression (Figure 3), and of the autophagy flux (Figure 4) in the HT-29 cell line. Indeed, at 6h p.i., this effect observed with the wildtype strain was no longer observed when SopE was missing. Acting on key transcription factors directly is likely to be an evolutionary selected mechanism for *Salmonella* to control host clearance function. We propose that SopE induces autophagosome formation through SP1. Our regulatory network analysis showed that SP1 transcription factor regulates several core autophagy genes, including *MAP1LC3B* and p62/*SQSTM1* (Figures 1A, B). SP1 has also been shown to repress the autophagy process in malignant epithelial cells by dampening *p62* expression (Xu et al., 2018). Here, we show that SopE dampens the autophagy flux. Indeed, accumulation of p62 protein is a commonly used indication for autophagy impairment or decrease. Here, we observed a decrease in p62/SQSTM1 protein associated with autophagosomes that depended on SopE being present (Figures 4C, D, E). We suggest that SopE, through its interaction with the SP1 transcription factor, negatively modulates the autophagy flux, protecting vacuolar *Salmonella* from lysosomal degradation.

We observed however that the effect of SopE in dampening autophagy only happens when *Salmonella* is associated with the SCV. Conversely, when *Salmonella* was mostly cytosolic due to the deletion of *sifA*, SopE no longer seemed to have the striking effect on autophagy dampening we saw when *Salmonella* remained mostly associated with the SCV compartment (Figures 4 vs 5). A previous study showed that infection-induced autophagy also contributes to the healing of damaged SCV by SPI1 proteins secreted through theT3SS, helping the endosomal/SCV maturation (Kreibich et al., 2015). This benefits consequently intravacuolar *Salmonella* cells, as these can proceed with SPI-2 effector-mediated intracellular survival and proliferation. In our study, the SopE protein no longer dampens autophagy flux in host cells where *Salmonella* has escaped the vacuolar compartment or resides within damaged SCV that will require part of the autophagy flux to repair the endosomal/SCV membrane. This process could take place in parallel to epithelial cell compartment size regulation which determines whether the pathogens will escape the vacuole or trigger enlargement of the vacuole by fusion with infection-associated macropinosomes as previously described (Stévenin et al., 2019).

The prediction that both SopE and SopE2 effectors can similarly affect autophagy highlights even more the importance of these molecular mimics across a broad spectrum of *S.* Typhimurium strains. In strains expressing both *sopE* and *sopE2* genes, modulation of autophagy induction and autophagosome formation might play a key role in strongly hijacking host functions that respond to autophagy modulation, such as antimicrobial production, possibly to help maintaining intestinal *Salmonella* populations and ensure spreading of the pathogen. However, as harbouring the evolutionarily newer *sopE* suggests a gained function in these strains compared to having *sopE2*, it is crucial to do follow up experiments confirming or contradicting the predictions.

Our experimental strategy to physically separate bystanders from epithelial cells that contain *Salmonella* revealed clear differences between these two categories of host cells as well as between bystanders and uninfected cells. Indeed, bystanders seem to participate in the host response to *Salmonella* infection as already shown for other pathogens such as *Shigella flexneri*, where bystanders were responding to infection in an effector-independent manner (Lippmann et al., 2015). Cross-talk between infected and uninfected neighbouring cells has previously been described for many bacterial or viral pathogens, for example through cytokines signalling (Milivojevic et al., 2017; Bost et al., 2020), or directly through uptake of pathogen effector proteins from infected to uninfected cells (Guidi et al., 2013). *S.* Typhimurium was also shown to cause a reprogramming of microRNAs in infected cells, affecting also bystander cells (Aguilar et al., 2021). Endoplasmic reticulum stress response is activated in bystander cells, affecting many genes' expression; in particular downregulating key transcription factors, such as E2F1. E2F1 normally activates autophagy and was shown to work in synergy with the SP1 transcription factor (Lin et al., 1996) propose that SP1 is one target of the *Salmonella*-derived SopE GEF-mimicking protein, affecting autophagy regulation, possibly in combination with E2F1 down-regulation, although to a lesser degree in bystanders cells. This is in agreement with SopE acting locally within infected cells without affecting the surrounding cells, possibly as a hiding mechanism from host innate defense. It is also possible that bystanders would follow the same pattern at a later time point if there is a delay in their response to what is happening in *Salmonella*-containing cells.

The HT-29 cell line we used is a cancer-derived cell line, and like many lines it carries several mutations that affect the normal

functioning of the cells. However, along with other cell lines used to study *Salmonella*-host cell interactions, HT-29 cells carry no mutation in core-autophagy genes (unpublished data). Cancer cell lines present the advantage to study the pathogen's behaviour in a very homogenous host cell population, yet they only partially mirror what is happening in native tissue. With the growing evidence of how useful intestinal organoid-based models are to study host-microbe interactions, including *Salmonella*-host interactions, using them for validation of network-based predictions of how pathogens' effectors interfere with host cellular pathways is the obvious next step to pursue.

Overall, our study emphasises the power of network analysis approach in identifying potential interactions between pathogen effector proteins and host cellular machinery regulation. We had previously demonstrated that protein-protein interactions between secreted pathogen effectors and core autophagy proteins is a conserved strategy for many intracellular pathogens (including *Salmonella*) to modulate the host autophagic clearance mechanisms (Sudhakar et al., 2019). Here, using a similar approach, we showed that *Salmonella* targets also the regulation of those core autophagy genes. Our experimental validation emphasises the possible role of SopE effector protein as such a local regulator of autophagy flux. Future complementary work investigating the mechanism behind SopE/SP1 or SopE/TP53 interactions will add to our understanding of the complexity and fine tuning of how host-pathogens crosstalk has developed.

## Data availability statement

The list of Salmonella effectors, relevant host transcription factors and their first neighbours used in the study have is available as supplementary data. The scripts used in the study are available at: https://github.com/korcsmarosgroup/HMIpipeline.

## Author contributions

AD has conducted the majority of the computational and experimental work. She contributed to the data analysis as well as the writing and editing of the manuscript. A-CJ has performed all microscopy work, image analysis for this study and the writing of the relevant methods section. LG has contributed to the computational work of this study. IM, JL and AL have contributed to the Flow cytometry work, RNA extraction from low input samples and the writing of the corresponding method section. PB, RI and RK have generated the initial Salmonella sopE gene deletion strain used in this study, and to the writing of the manuscript. IN has contributed to the writing and editing of the manuscript. TK contributed to the conception of the study and the writing and editing of this manuscript. Finally, IH contributed to the conception of the study, to some experimental work, to the data analysis. She led the writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.834895/full#supplementary-material

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