Autophagy plays a protective role during *Pseudomonas aeruginosa*-induced apoptosis via ROS–MAPK pathway

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Abstract

Pseudomonas aeruginosa infection can induce alveolar macrophage apoptosis and autophagy, which play a vital role in eliminating pathogens. These two processes are usually not independent. Recently, autophagy has been found to interact with apoptosis during pathogen infections. Nevertheless, the role of autophagy in *P. aeruginosa*-infected cell apoptosis is unclear. In this study, we explored the impact of *P. aeruginosa* infection on autophagy and apoptosis in RAW264.7 cells. The autophagy activator rapamycin was used to stimulate autophagy and explore the role of autophagy on apoptosis in *P. aeruginosa*-infected RAW264.7 cells. The results indicated that *P. aeruginosa* infection induced autophagy and apoptosis in RAW264.7 cells, and that rapamycin could suppress *P. aeruginosa*-induced apoptosis by regulating the expression of apoptosis-related proteins. In addition, rapamycin scavenged the cellular reactive oxygen species (ROS) and diminished p-JNK, p-ERK1/2 and p-p38 expression of MAPK pathways in RAW264.7 cells infected with *P. aeruginosa*. In conclusion, the promotion of autophagy decreased *P. aeruginosa*-induced ROS accumulation and further attenuated the apoptosis of RAW264.7 cells through MAPK pathway. These results provide novel insights into host–pathogen interactions and highlight a potential role of autophagy in eliminating *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa, a Gram-negative bacterium, is commonly found in moist environments, and can cause acute and chronic infectious diseases.[1] Severe *P. aeruginosa* infections, such as urinary tract infections, ventilator-associated pneumonia and digestive tract infections, are determinants of mortality for hospitalized patients.[2] Notably, antibiotics are often used to prevent complications in *P. aeruginosa*-infected patients, an approach challenged by drug resistance. Thus, there is an urgent need to identify novel therapeutic targets for *P. aeruginosa* infection. According to previous studies, therapies based on autophagy and the innate immunity defence system of the host is an effective way to solve this problem.[3,4] Macrophages, being the host of *P. aeruginosa* and typical APCs of the innate immune system, play a substantial role in the development of *P. aeruginosa* infection.[5] Therefore, it is particularly essential to elucidate the interaction between macrophages and *P. aeruginosa* infection.

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Macrophages can kill invading pathogens through phagocytosis and destruction of intracellular microorganisms. In addition to phagocytosis and intracellular killing, autophagy and apoptosis are also involved in innate immune response processes.[6] A process of cell self-digestion, autophagy is responsible for protein and organelle degradation to maintaining intracellular homeostasis. Thus, activation of autophagy in macrophages is generally regarded as a protective strategy against intracellular pathogens. However, autophagy dysfunction is associated with metabolic diseases, cancer, neurodegenerative diseases and microbial infection.[7] In this regard, autophagy is a double-edged sword, serving either pro-survival or pro-death functions. Different from autophagy, apoptosis is another type of programmed cell death that plays critical roles in maintenance of organism homeostasis by eliminating damaged cells.[8] However, several researchers uncovered that pathogens such as *P. aeruginosa* possessed the ability to interfere and evade the host immune system by inducing targeted cell apoptosis.[9] Accordingly, preventing excessive pathogen-induced cell apoptosis should be beneficial for eliminating bacteria. Interestingly, cellular contexts required a balanced interplay between autophagy and apoptosis, highlighting the relevance and impact of this crosstalk in normal development and in pathology.[10] For instance, etoposide, a general inducer of apoptosis, has been demonstrated to activate autophagy in mouse embryonic fibroblasts.[11] Moreover, autophagy also can activate caspase-dependent apoptosis.[12] Autophagy acts as a cellular cleanser, functioning to degrade misfolded proteins and attenuate endoplasmic reticulum stress, which further inhibits the activation of apoptosis.[13] These findings may imply that autophagy plays a modulatory role in apoptosis of host cells in response to a pathogen invasion. However, the mechanism of modulating the dynamic balance between autophagy and apoptosis during *P. aeruginosa* infection remains obscure. Thus, this study aimed to investigate whether autophagy is involved in regulating apoptosis in RAW264.7 cells infected with *P. aeruginosa*.

Materials and methods

Cell lines and P. aeruginosa strains

The macrophage mouse cell line RAW264.7 was purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), cultured at 37°C and 5% CO_2, in DMEM (Gibco, CA, USA) supplemented with 10% heated-inactivated FBS (Thermo Fisher) and 2 mM L-glutamine.

P. aeruginosa strains were purchased from the American Type Culture Collection (ATCC, 9027) and cultured in LB broth.

Materials and reagents

The following reagents were used: Abs against LC3, ATG5, BAX, Bcl-2, caspase-8, and β-actin were purchased from Proteintech (CA, USA). Abs against JNK, phosphor-JNK, p38, phosphor-p38, ERK1/2, phosphor-ERK1/2, P62/SQSTM1 and cleaved caspase-3 were purchased from Cell Signaling Technology (Boston, USA). Abs against caspase-9 and poly (ADP-ribose) polymerases (PARP) were purchased from Abcam (Cambridge, United Kingdom). Goat anti-mouse IgG (H+L) cross-adsorbed secondary Ab Alexa Fluor 555 was purchased from Invitrogen (Massachusetts, USA). Immunofluorescence reagents were obtained from Zhongshan Golden Bridge Biotechnology (Beijing, China). An Annexin V/PI kit was purchased from KeyGen Biotech (Jiangsu, China). Reactive oxygen species (ROS) and JC-1 kits were purchased from Thermo Fisher Scientific (Massachusetts, USA). CytoID autophagic detection kit was purchased from Enzo Life Sciences. Rapamycin (RAPA), 3-Methyladenine (3-MA), *N*-acetylcysteine (NAC) and SB-202190 were purchased from MedChemExpress (New Jersey, USA).

Infection of the RAW264.7 macrophages with P. aeruginosa

The bacteria were cultured at 37°C (180 rpm/min) for 12 h and then centrifuged at 5000 *g* for 5 min at room temperature (20–25°C). The supernatant was discarded, and the cells were washed once with sterile saline (0.85% NaCl) and then re-suspended in saline to a concentration of approximately 2.0×10^{10} CFU/ml using a standard curve relating viable counts to the OD. The RAW264.7 cells were infected with *P. aeruginosa* at MOI of 20 under the indicated conditions before being harvested for analysis.

Western blot analysis

The cells were washed three times with PBS at 4°C, and the entire population was extracted and lysed with lysis buffer for 30 min on ice. Next, the lysates were centrifuged at 14000 *g* for 15 min at 4°C, and the precipitates and supernatants were collected. Then, the total protein (20–50 μg) was separated by SDS-PAGE, and the separated proteins were transferred by the wet method to PVDF membranes. Subsequently, the membranes were blocked with Superblock (Thermo Fisher Scientific, USA) for 2 h at room temperature. Next, the membranes were incubated with primary Abs

overnight at 4°C and then with the appropriate secondary Abs for 2 h at room temperature. Finally, the proteins were visualized by an enhanced chemiluminescence (Advansta Inc., San Jose, CA, USA) system. The results were analysed by ImageJ software, and β-actin was used as the internal control.

Immunofluorescence detection

RAW264.7 cells were plated into 24-well plates at 1×10^5 cells/well, and adherent monolayers were infected with *P. aeruginosa* (MOI = 10). The cells were washed three times with ice-cold PBS and fixed with 4% paraformaldehyde for 20 min. The cells were then washed three times and permeabilized with TritonX-100 solution (0.01% in PBS). Next, the cells were re-washed, blocked with 5% normal goat serum for 30 min, and incubated with LC3 and caspase-3 Abs (1:50 dilution) for 12 h at 4°C. Finally, the cells were incubated with fluorescent secondary Abs for 1 h at room temperature, and nuclei were stained with DAPI.

Detection of apoptosis by flow cytometry

RAW264.7 cells were plated into six-well plates at 1×10^6 cells/well, treated with rapamycin (100 nM) for 2 h and then infected with *P. aeruginosa* (MOI = 20) for 4 h. The apoptotic cells were measured by FACS using an Annexin V/PI kit (KeyGENBioTECH, Jiangsu, China). The cells were washed with PBS at 4°C and centrifuged for 5 min at 1000 r/min; the supernatant was discarded, and the cell pellet was suspended in 400 μl of $1 \times$ binding buffer. Next, 5 μl of Annexin V-FITC and 5 μl of PI staining solution were added to the cells in each treatment group, after which the cells were gently mixed at incubated at room temperature in the dark for 20 min. The samples were analysed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) within 1 h.

Measurement of mitochondrial membrane potential

RAW264.7 cells were seeded into 6-well plates at 1×10^6 cells/well, pre-treated with rapamycin (100 nM) for 2 h or 3-methyladenine (3-MA, 5 mM) for 6 h and then treated with *P. aeruginosa* (MOI = 20) for 4 h. The mitochondrial membrane potential (MMP) was measured by FACS using a JC-1 kit (Thermo, USA). The JC-1 working solution was prepared by mixing 500 μl of PBS with 1 μl of JC-1 and mixing eddies. The cells were mixed with 500 μl of JC-1 working fluid, incubated at room temperature for 20 min and centrifuged for 5 min; the supernatant was discarded, and the cell pellet was re-suspended in PBS. Finally, the cells were detected by flow cytometry within 1 h. The results were determined by flow cytometry and a BD FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA), and the data were analysed with Cell Quest software.

Detection of ROS by flow cytometry

Intracellular ROS levels were evaluated by flow cytometry. RAW264.7 cells were seeded in 6-well plates at a density of 1×10^6 cells/well, treated with rapamycin (100 nM) for 2 h and infected with *P. aeruginosa* for 4 h. The cells were then harvested and incubated with Cell ROX Orange Reagent (ThermoFisher Scientific, USA) for 20 mins. The cells were washed, harvested from the wells by scraping and analysed for intracellular fluorescence using a BD-FACS can instrument (BD, USA) and Cell Quest software.

Detection of autophagic vacuoles by flow cytometry

RAW264.7 cells were seeded in 6-well plates at a density of 1×10^6 cells/well, treated with rapamycin (100 nM) for 2 h or 3-MA (5 mM) for 6 h and infected with *P. aeruginosa* for 4 h. The formation of autophagic vacuoles was detected by CytoID detection kit (Enzo Life Sciences, USA). Briefly, CytoID working solution was prepared by mixing 500 μl of PBS with 1 μl of CytoID and mixing eddies. The cells were suspended with 500 μl CytoID working solution and incubated at room temperature for 30 min. The results were determined by flow cytometry.

Statistical analyses

Data statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, USA). The data are presented as the mean ± SD, and all data collected were obtained from at least three independent experiments for each condition. The data were statistically evaluated to compare differences between two groups by one-way ANOVA; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Results

P. aeruginosa *infection induced autophagy in RAW264.7 cells*

RAW264.7 cells were infected with *P. aeruginosa* at various time and different MOI. As expected, *P. aeruginosa* infection increased the numbers of LC3-positive cells (Figure 1a). In addition, the expression of LC3 was in a time and dose-dependent manner (Figure 1b, c).

Importantly, autophagy-related (ATG) proteins known to regulate autophagy, such as ATG5 and ATG7, are involved in the extension and closure of
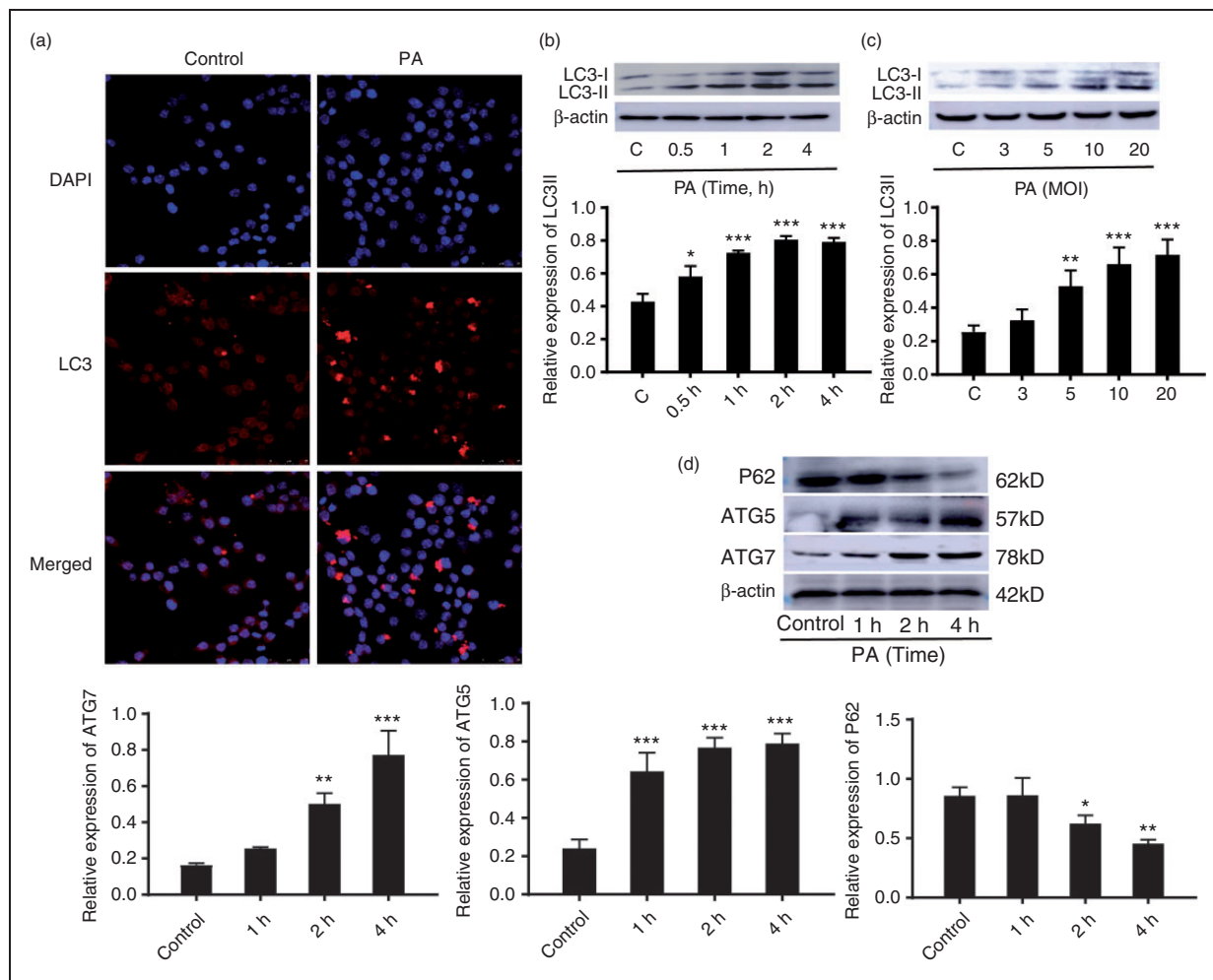
Figure 1. *P. aeruginosa* infection induced autophagy of RAW264.7 cells. (a) Immunofluorescence was used to detect intracellular LC3 puncta in RAW264.7 cells infected with *P. aeruginosa* (PA) for 2 h. (b,c) The expression of LC3II in RAW264.7 cells after infection with *P. aeruginosa* at different time and MOI were analysed by Western blot. (d) The expression of ATG5, ATG7 and P62 in RAW264.7 at different time points after *P. aeruginosa* infection. The protein ratio was calculated by ImageJ densitometry analysis. Data are shown as mean ± SEM, and one representative experiment from three independent experiments is shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

autophagic vesicles. However, P62, a lysosomal substrate, is degraded when autophagosomes fuse with lysosomes. To further confirm *P. aeruginosa*-induced autophagy, Western blot was used to detect the expression of the autophagy-related proteins ATG5, ATG7 and P62 in RAW264.7 cells.

Compared with uninfected cells, the expression of ATG5 and ATG7 was significantly increased, accompanied with a decreasing P62 production in *P. aeruginosa*-infected RAW264.7 cells (Figure 1d). Furthermore, the formation of autophagic vacuoles was measured by flow cytometry. As shown in the Supplemental material (Figure S1), *P. aeruginosa* infection increased the formation of autophagic vacuoles in RAW264.7 cells. These results unambiguously suggested that *P. aeruginosa* could induce RAW264.7

cells autophagy, which was consistent with the previous finding from other groups.[14]

P. aeruginosa *infection activated apoptosis in RAW264.7 cells*

To confirm whether *P. aeruginosa* infection could induce apoptosis, RAW264.7 cells were infected with *P. aeruginosa* at a MOI of 20 in the indicated conditions. As shown in Figure 2a, *P. aeruginosa* infection increased the numbers of caspase-3-positive cells. In addition, the expression of cleaved caspase-3 and PARP detected by Western blot were up-regulated in a time-dependent manner in RAW264.7 cells upon *P. aeruginosa* infection (Figure 2b). The results indicated that *P. aeruginosa* infection could induce cell apoptosis.
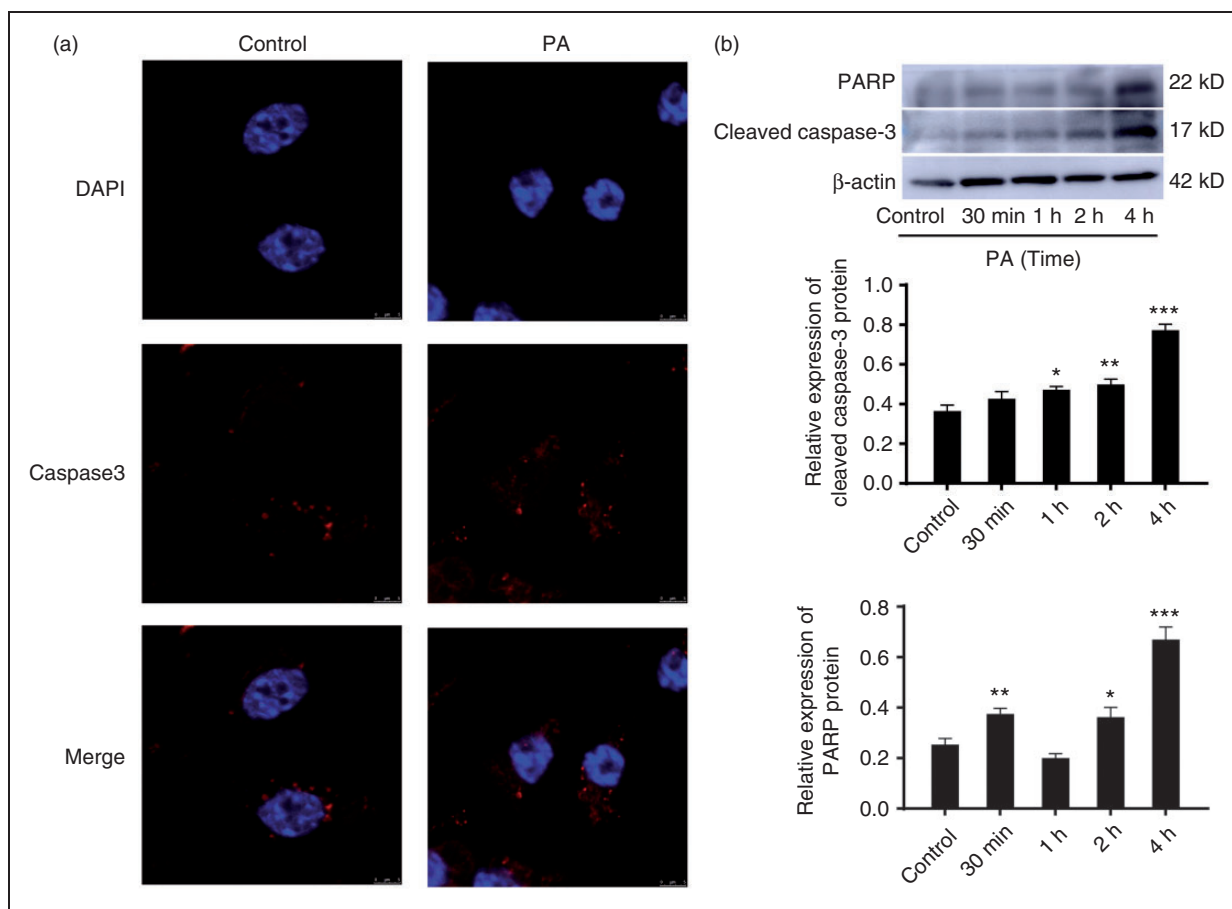
Figure 2. *P. aeruginosa* infection activated apoptosis in RAW264.7 cells. (a) Fluorescence microscopy was used to detect intracellular caspase-3 expression (red puncta) of RAW264.7 cells infected with *P. aeruginosa* (PA) for 2 h. (b) Time-dependent expression of cleaved caspase-3 and PARP in the RAW264.7 cells infected with *P. aeruginosa* (top panel). Western blot was used to analyse the expression of the proteins. The protein ratio was calculated using ImageJ densitometric analysis (bottom panel). The data are presented as mean $\pm$ SEM, and one representative experiment of three independent experiments is shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with the control group.

The promotion of autophagy inhibits P. aeruginosa-induced apoptosis in RAW264.7 cells

As both autophagy and apoptosis occurred in macrophages during *P. aeruginosa* infection, we hypothesized that apoptosis could be regulated by autophagy. To confirm the hypothesis, RAW264.7 cells were pre-treated with rapamycin or 3-MA following with *P. aeruginosa* infection for 4 h. The flow cytometry results showed *P. aeruginosa* infection significantly increased the apoptosis rate (Figure 3a, b). Meanwhile, compared with *P. aeruginosa* infection alone, rapamycin treatment effectively reduced the number of apoptotic cells. In contrast, 3-MA increased the number of apoptotic cells (Figure S2). The previously mentioned results indicated that the activation of autophagy attenuated the apoptosis rate of RAW264.7 cells infected with *P. aeruginosa*.

Subsequently, to further provide definitive evidence, we detected the expression of cleaved caspase-3, PARP

and LC3II in RAW264.7 cells by Western blot. Figure 3c shows that *P. aeruginosa* infection alone resulted in a marked increase the expression of PARP and cleaved caspase-3 in RAW264.7 cells. In contrast, rapamycin exhibited a capacity to suppress, while 3-MA promoted, *P. aeruginosa*-induced PARP and cleaved caspase-3 expression (Figure S3). Taken together, these results demonstrated that autophagy could reduce *P. aeruginosa*-induced RAW264.7 cells apoptosis.

Autophagy alleviated P. aeruginosa-induced apoptosis via the intrinsic apoptotic pathway

According to whether caspase-8 or caspase-9 is activated, apoptosis is divided into extrinsic and intrinsic pathways, respectively.[15] We detected caspase-8 and caspase-9 expression to explore which pathway was involved in the process. The results showed that
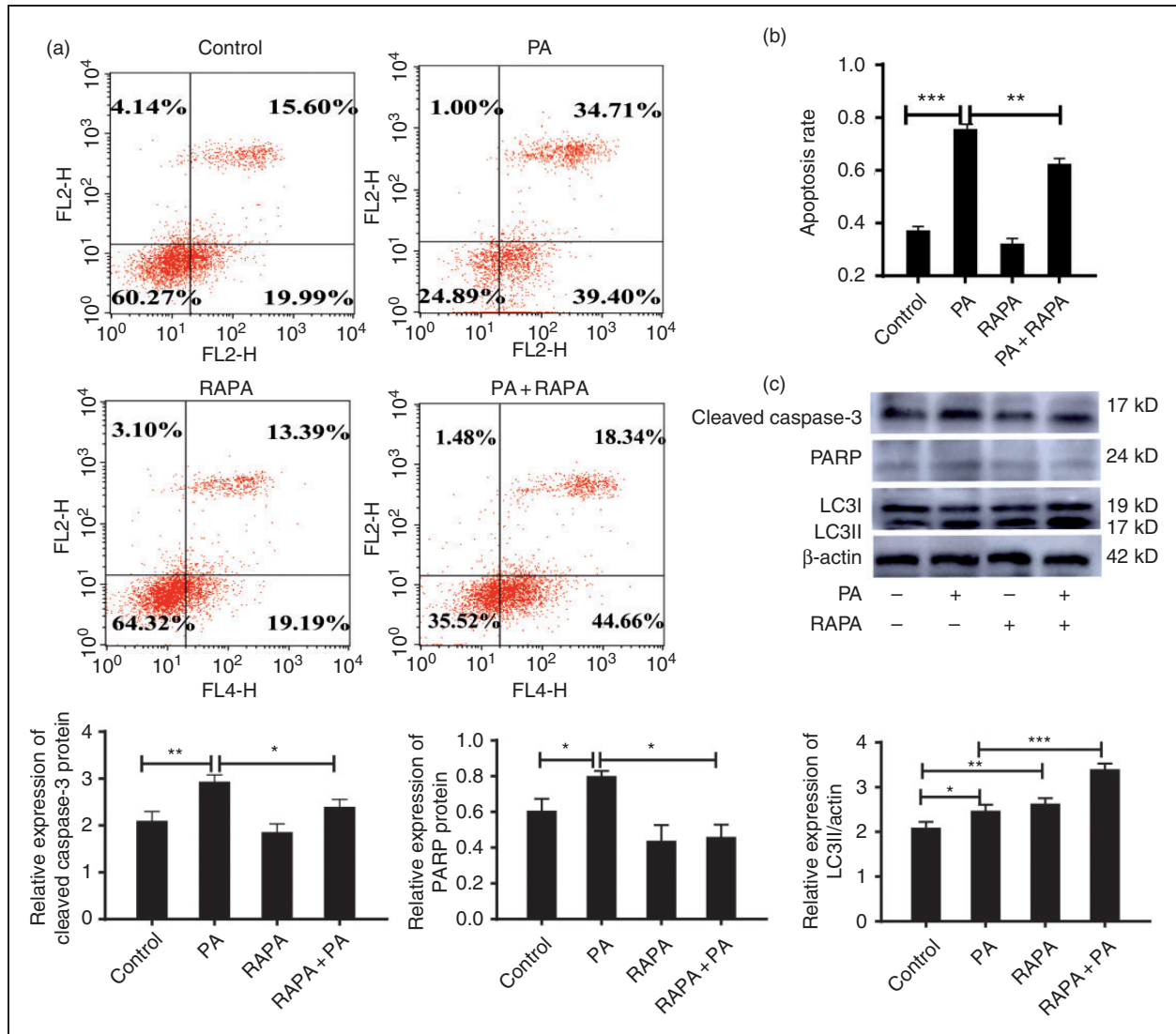
Figure 3. Autophagy prevents *P. aeruginosa*-induced apoptosis in RAW264.7 cells. (a) RAW264.7 cells were treated with rapamycin (RAPA) for 2 h and then infected with *P. aeruginosa* (PA) for 4 h. The apoptosis rates were determined by AnnexinV-FITC (AV) and PI staining. (b) Histogram of the RAW264.7 cell apoptosis rate as determined by the FACS assay. (c) The protein expression levels of PARP, cleaved caspase-3 and LC3 in RAW264.7 cells treated with *P. aeruginosa* or/and 10 μM rapamycin were determined by Western blot analysis. The data are represented as mean ± SD, and one representative experiment of three independent experiments is shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the control group.

P. aeruginosa infection dramatically up-regulated the expression of caspase-8 and caspase-9 in RAW264.7 cells (Figure 4a). Interestingly, rapamycin decreased the *P. aeruginosa*-induced caspase-9 expression, but not that of caspase-8. These results indicated that autophagy alleviated *P. aeruginosa*-induced apoptosis through the intrinsic apoptotic pathway.

Subsequently, we examined intrinsic apoptotic pathway-related proteins (Figure 4a). As expected, *P. aeruginosa* remarkably increased the expression of the pro-apoptotic proteins Bax and cytochrome C (cytc) in RAW264.7 cells and down-regulated the expression of the anti-apoptotic protein Bcl-2. In contrast, rapamycin reversed the associated protein expression induced by *P. aeruginosa* infection. Furthermore, loss of the integrity of the MMP was observed by flow cytometry assay based on JC-1 staining. As shown in Figure 4b and 4c, *P. aeruginosa* infection moderately decreased the MMP of RAW264.7 cells, while rapamycin partially reversed the loss of MMP induced by *P. aeruginosa* infection. Together, these data unambiguously indicated that autophagy could partially alleviate mitochondrial-mediated intrinsic apoptosis of RAW264.7 cells promoted by *P. aeruginosa* infection.
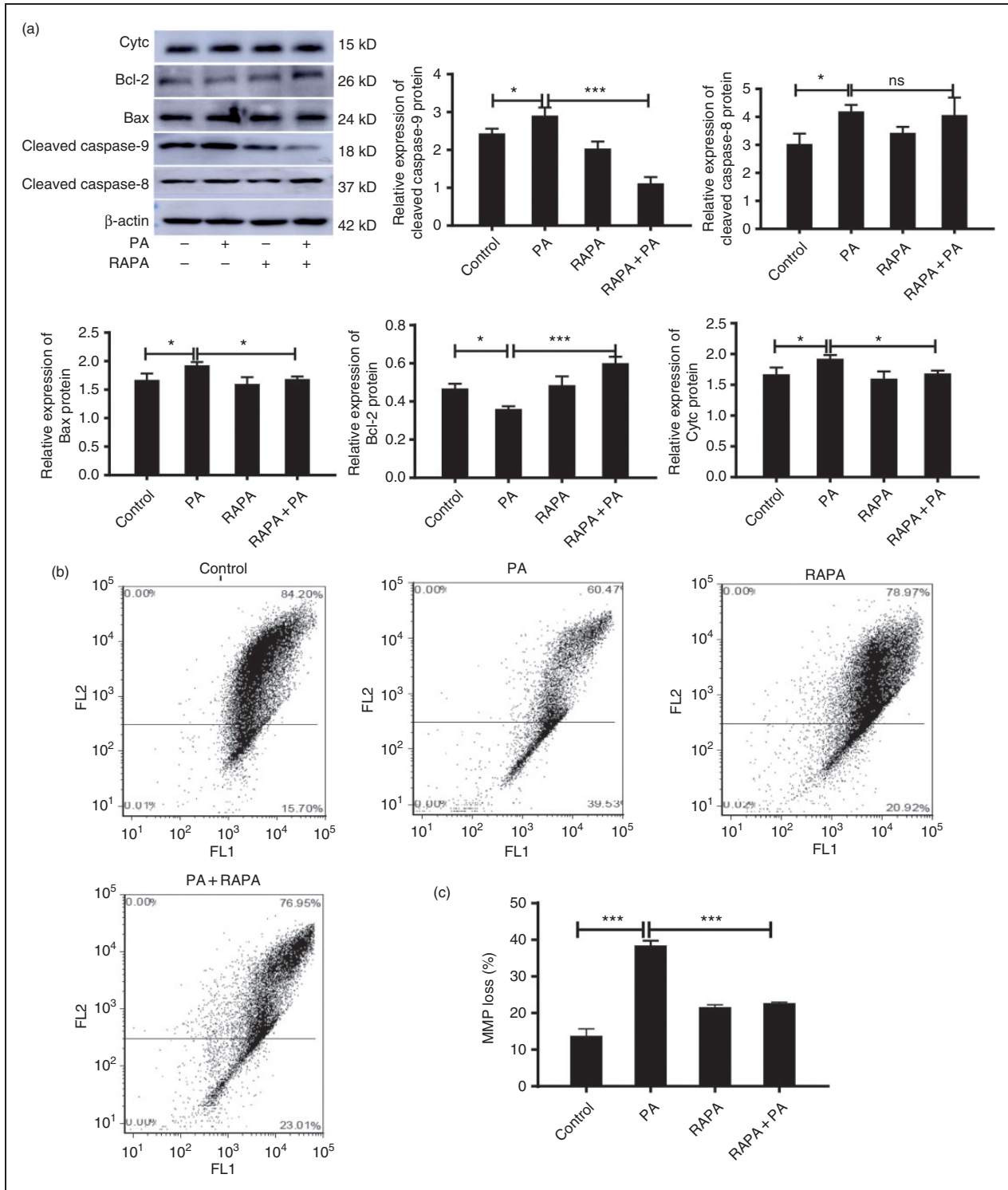
Figure 4. Autophagy alleviated *P. aeruginosa*-induced intrinsic apoptosis. (a) RAW264.7 cells were treated with *P. aeruginosa* (PA) or/ and 10 μM rapamycin (RAPA). The expression of cleaved caspase-8, cleaved caspase-9, cytc, Bax and Bcl-2 were measured by Western blot. (b) Mitochondrial membrane potential (MMP) was determined by flow cytometry. (c) Quantitative analysis of MMP in RAW264.7 cells by the FACS assay. The data are shown as mean ± SEM of three independent experiments. NS, no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Autophagy attenuated *P. aeruginosa*-induced apoptosis via ROS-dependent MAPK pathway

Autophagy can depress the production of ROS which play key roles in mediating the intrinsic apoptotic pathway.[10] Therefore, we hypothesized that the decreased apoptosis rate induced by autophagy is achieved via scavenging cellular ROS. To confirm the hypothesis, we explored the effect of autophagy on the potential of -mediated ROS production to determine the biological significance of autophagy in *P. aeruginosa* infection. We first used flow cytometry to detect ROS levels in RAW264.7 treated with rapamycin for 2 h and then infected with *P. aeruginosa* for 2 h. The results are shown in Figure 5a and 5b. *P. aeruginosa* infection significantly accumulated ROS production while rapamycin partial reversed *P. aeruginosa*-induced ROS accumulation in RAW264.7 cells. Collectively, these data implied that autophagy attenuates the ROS production induced by *P. aeruginosa* infection.

According to a previous study, autophagy may alter the activity of MAPKs, including JNK, ERK1/2 and p38, and affect cell proliferation, survival and apoptosis under various conditions.[16] Therefore, to further verify whether autophagy prevents *P. aeruginosa*-induced RAW264.7 cell apoptosis by MAPK pathway, we detected p-JNK, p-ERK1/2 and p-p38 protein expression of RAW264.7 cells treated with rapamycin for 2 h or 3-MA for 6 h and then exposed to *P. aeruginosa* for 4 h. The results showed that rapamycin potently suppressed the activation of p-JNK, p-ERK1/2 and p-p38 production in RAW264.7 cells infected with *P. aeruginosa* (Figure 5c). However, 3-MA promoted the activation of p-JNK and p-p38 (Figure S3). To further address our conclusion, NAC, which is a ROS scavenger, was employed to block ROS accumulation. As shown in Figure S4-a, NAC depressed the activation of MAPK pathways and abolished the 3-MA-induced MAPK pathway activation in *P. aeruginosa*-infected cells. What is more, we also employed SB-202190 to block p38 phosphorylation. The expression of apoptosis-related factors were detected by Western blot (Figure S4-b). The results showed that the inhibition of MAPK pathways by SB-202190 decreased the expression of apoptosis and alleviated 3-MA induced apoptosis in *P. aeruginosa*-infected cells. Taken together, these results clearly suggested that activated autophagy attenuated *P. aeruginosa*-induced cells apoptosis by scavenging the cellular ROS and blocking MAPK pathway.

Discussion

P. aeruginosa is a highly infectious bacterium which can cause host cell death, tissue and organ damage, and destroy the body's immune mechanisms.[17] Exploring the interaction between pathogenic bacteria and host cells is very important for a comprehensive understanding of the pathogenic mechanism of *P. aeruginosa* infection. In addition, this is increasingly critical in developing strategies to prevent *P. aeruginosa* infection.[18]

Accumulating evidence has shown that autophagy is considered an essential pathway for eliminating intracellular pathogenic bacteria infections.[19–22] Moreover, apoptosis is also involved in the process for bacterial infection. However, cell fate is neither determined by autophagy nor by apoptosis. To date, a thorough comprehension of multifaceted relationship between autophagy and apoptosis has been established in many diseases.[23,24] A previous study reported that autophagy plays a protective role in high Glc-induced apoptosis in adipose tissue-derived stem cells.[25] On the contrary, once apoptosis is blocked, autophagy can induce cell death. Meanwhile, elimination of damaged mitochondria reduces ROS accumulation, creating a feedback loop that causes inhibition of autophagy.[26] Thus, exploring the role of autophagy in apoptosis will contribute to understanding the pathogenesis of *P. aeruginosa*. With this in mind, we initially hypothesized that autophagy could be involved in regulating the apoptosis induced by *P. aeruginosa* infection.

We first determined the impact of *P. aeruginosa* infection on autophagy. The expression of LC3 in RAW264.7 cells was detected by Western blot and immunofluorescence. The results indicated that *P. aeruginosa* infection increased the numbers of LC3-positive cells and expression of LC3II as well in RAW264.7 cells. Consistent with the LC3II result, ATG7 and ATG5 expression was elevated in a time- and dose-dependent manner by *P. aeruginosa* infection followed by a decreased P62 expression. This implied that *P. aeruginosa* infection induced autophagy in RAW264.7 cells. Subsequently, we confirmed that *P. aeruginosa* infection induced RAW264.7 cell apoptosis with an increased expression of cleaved caspase-3 and PARP. Thus, consistent with other findings, *P. aeruginosa* infection can induce cell autophagy and apoptosis.[14,27]

In an effort to evaluate the role of autophagy on apoptosis, we used rapamycin or 3-MA to regulate autophagy. Flow cytometry results showed that rapamycin effectively reduced the apoptosis rate of RAW264.7 cells upon *P. aeruginosa* infection followed by decreasing cleaved caspase-3 and PARP expression. However, 3-MA exhibited a totally reversed function. These findings were consistent with a previous model, in which the authors determined that rapamycin protects cell death by activating autophagy, inhibiting oxidative stress, and mitochondria apoptosis.[28]
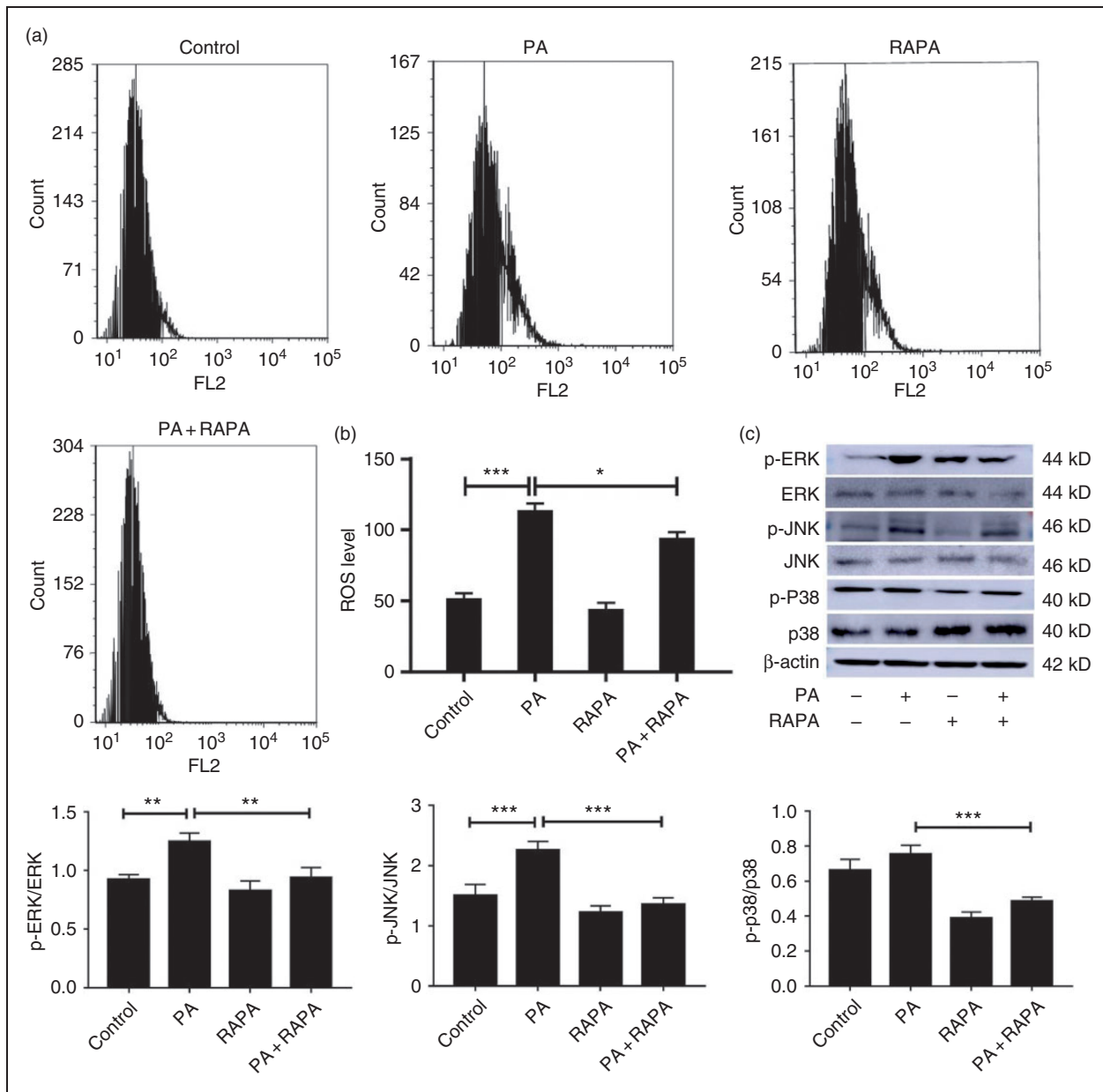
Figure 5. Autophagy blocked *P. aeruginosa*-induced activation of ROS-MAPK pathways in RAW264.7 cells. (a) ROS accumulation was determined by flow cytometry in RAW264.7 cells which were treated with rapamycin (RAPA) for 2 h, followed by exposure to *P. aeruginosa* (PA) for 4 h. (b) Quantitative analysis of ROS levels in RAW264.7 cells by FACS assay. (c) The expression of p-JNK, p-ERK1/2 and p-p38 was analysed by Western blot after exposure to rapamycin and *P. aeruginosa* alone or combined. The data are shown as mean ± SEM of three independent experiments. NS, no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

The intrinsic and extrinsic pathways are considered to be two different apoptosis pathways. One characteristic of intrinsic apoptosis is mitochondrial dysfunction, which occurs upon the release of cytc from the mitochondrion into the cytoplasm. These molecules lead to the self-splicing of caspase-9, which triggers caspase-9 activation, and enhances the catalytic properties of other caspases, such as caspase-3 maturation. This process is tightly regulated by Bcl-2 family

proteins, which function in the mitochondrial membrane.[29] According to our study, rapamycin decreased the *P. aeruginosa*-induced expression of the caspase-9, Bax and cytc proteins, but increased the expression of Bcl-2. Furthermore, the activation of mitochondrial apoptosis was also associated with a change in MMP. A flow cytometry assay also showed that rapamycin partially reversed the loss of MMP induced by *P. aeruginosa* infection. However, rapamycin did not change
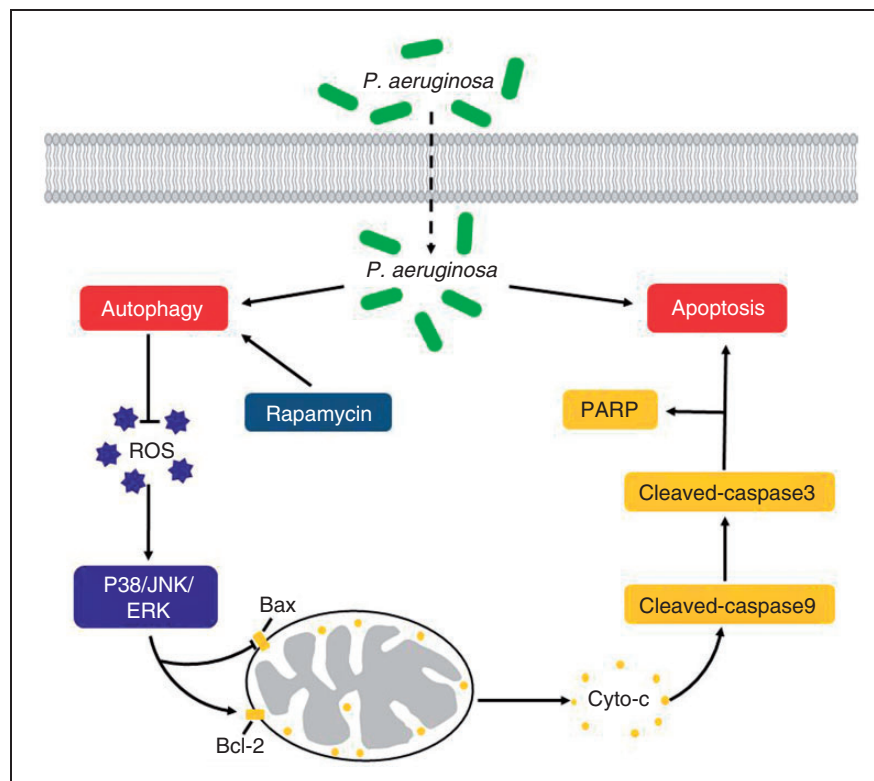
Figure 6. Proposed model for suppressed *P. aeruginosa*-induced apoptosis in RAW264.7 cells by activation of autophagy. The invasion of *P. aeruginosa* markedly increases abundance of apoptosis and autophagy in RAW264.7 cells. Rapamycin stimulates autophagy effectively and eliminates the accumulation of ROS which would reduce cellular damage caused by oxidative. Then the activated MAPK signalling components and their down-stream target gene further regulate apoptosis associated proteins Bax, Bcl-2, Cytc, caspase-9, caspase-3 and PARP production. Consequently, the activated autophagy suppresses *P. aeruginosa*-induced apoptosis in RAW264.7 cells.

the expression of the extrinsic apoptotic pathway-related protein caspase-8 in RAW264.7 cells infected with *P. aeruginosa*. Collectively, our results indicated that autophagy inhibited *P. aeruginosa*-induced apoptosis in RAW264.7 cells via the intrinsic apoptotic pathway, and not by the extrinsic apoptotic pathway.

Increasing evidence has demonstrated that during pathogen infection, ROS-induced oxidative stress could cause cell injury through mitochondrial dysfunction and reduced activity of antioxidant enzymes.[30–32] Multiple studies have revealed that exposure to *P. aeruginosa* can initiate apoptotic cell death via excessive ROS generation.[33,34] Given this evidence, we investigated the effect of rapamycin treatment on *P. aeruginosa*-induced ROS accumulation. The flow cytometry results showed that rapamycin effectively diminished ROS production in response to *P. aeruginosa* infection, indicating that autophagy inhibits *P. aeruginosa*-induced ROS production. As mentioned previously, ROS have been shown to induce apoptosis, therefore, decreased ROS accumulation may be the potential mechanism by which autophagy regulates apoptosis during *P. aeruginosa* infection.

The MAPK pathway, including the critical members of JNK, p38 and ERK, is a pivotal cellular signalling pathway implicated in cell survival and apoptosis.[35] Mechanistically, activation of the MAPK pathway is tightly related to ROS-induced apoptosis.[36] Moreover, *P. aeruginosa* infection can activate all three MAPK members, further leading to apoptosis through ROS accumulation.[37] In addition, many lines of evidence confirmed that rapamycin can alter the activity of MAPKs, which further affects cell proliferation, survival and apoptosis.[38–40] We proposed that the autophagy-mediated regulation of MAPK signalling pathway and scavenging ROS should be critical for inhibiting *P. aeruginosa*-induced apoptosis.

Finally, we examined the expression of p-JNK, p-ERK1/2 and p-p38 by Western blot analysis. The result strongly confirmed that the activation of autophagy blocked the MAPK signalling pathway by down-regulating JNK, ERK1/2 and p38 expression in *P. aeruginosa*-infected RAW264.7 cells. In contrast, the inhibition of autophagy by 3-MA activated them. The previously mentioned findings were further confirmed by ROS scavenger and p38 MAP kinase inhibitor.

The results showed that ROS scavengers abolished 3-MA-induced MAPK pathways activation and the inhibition of MAPK pathways alleviated 3-MA induced apoptosis in *P. aeruginosa*-infected cells. Conclusively, we defined a mechanism by which autophagy activated by rapamycin suppressed *P. aeruginosa*-induced oxidative damage and apoptosis in RAW264.7 cells.

As illustrated in Figure 6, *P. aeruginosa* infection could induce RAW 264.7 cells apoptosis and autophagy. Rapamycin could eliminate ROS accumulation, which will further suppress endogenous apoptosis through MAPK pathways in RAW264.7 cells infected with *P. aeruginosa*. This study suggested that autophagy plays a protective role in modulating apoptosis during *P. aeruginosa* infection. It also may provide a novel insight in further understanding the molecular mechanism of *P. aeruginosa* infection.

Declaration of conflicting interests

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Supplemental material

Supplemental material for this article is available online.

References

1. Garai P, Berry L, Moussouni M, et al. Killing from the inside: Intracellular role of T3SS in the fate of *Pseudomonas aeruginosa* within macrophages revealed by mgtC and oprF mutants. *PLoS Pathog* 2019; 15(6): e1007812.

2. Ruffin M and Brochiero E. Repair process impairment by in epithelial tissues: Major features and potential therapeutic avenues. *Front Cell Infect Microbiol* 2019; 9: 182.

3. Awasthi S, Singh B, Ramani V, et al. TLR4-interacting SPA4 peptide improves host defense and alleviates tissue injury in a mouse model of *Pseudomonas aeruginosa* lung infection. *PLoS ONE* 2019; 14(1): e0210979.

4. Kume S and Koya D. Autophagy: A novel therapeutic target for diabetic nephropathy. *Diabetes Metab J* 2015; 39(6): 451–460.

5. Nouari W, Ysmail-Dahlouk L, Aribi M, et al. Vitamin D3 enhances bactericidal activity of macrophage against Pseudomonas aeruginosa. *Int Immunopharmacol* 2016; 30: 94–101.

6. Thakur A, Mikkelsen H, Jungersen G, et al. Intracellular pathogens: Host immunity and microbial persistence strategies. *J Immunol Res* 2019; 2019: 1356540.

7. Mizushima N, Levine B, Cuervo AM, et al. Autophagy fights disease through cellular self-digestion. *Nature* 2008; 451(7182): 1069–1075.

8. Bai L, Chen Y, Zheng S, et al. Phenotypic switch of human and mouse macrophages and resultant effects on apoptosis resistance in hepatocytes. *Innate Immun* 2019; 25(3): 176–185.

9. Broquet A and Asehnoune K. Apoptosis induced by *Pseudomonas aeruginosa*: A lonely killer? *Microb Biotechnol* 2015; 8(1): 49–51.

10. Andreyev AY, Kushnareva YE, Murphy AN, et al. Mitochondrial ROS metabolism: 10 years later. *Biochemistry Mosc* 2015; 80(5): 517–531.

11. Feng Z, Zhang H, Levine A J, et al. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci USA* 2005; 102(23): 8204–8209.

12. Laussmann M A, Passante E, Düssmann H, et al. Proteasome inhibition can induce an autophagy-dependent apical activation of caspase-8. *Cell Death Differ* 2011; 18(10): 1584–1597.

13. Bernales S, McDonald K L, Walter P, et al. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. *PLoS Biol* 2006; 4(12): e423.

14. Wu Y, Li D, Wang Y, et al. *Pseudomonas aeruginosa* promotes autophagy to suppress macrophage-mediated bacterial eradication. *Int Immunopharmacol* 2016; 38: 214–222.

15. Chen K W, Demarco B, Heilig R, et al. Extrinsic and intrinsic apoptosis activate pannexin-1 to drive NLRP3 inflammasome assembly. *EMBO J* 2019; 38(10): e101638.

16. Barutcu S A, Girnius N, Vernia S, et al. Role of the MAPK/cJun NH-terminal kinase signaling pathway in starvation-induced autophagy. *Autophagy* 2018; 14(9): 1586–1595.

17. Wonnenberg B, Jungnickel C, Honecker A, et al. IL-17A attracts inflammatory cells in murine lung infection with *P. aeruginosa*. *Innate Immun* 2016; 22(8): 620–625.

18. Lyczak JB, Cannon CL and Pier GB. Establishment of *Pseudomonas aeruginosa* infection: Lessons from a versatile opportunist. *Microbes Infect* 2000; 2(9): 1051–1060.

19. Lane J D, Korolchuk V I, Murray J T, et al. Host-pathogen interactions and subversion of autophagy. *Essays Biochem* 2017; 61(6): 687–697.

20. Amano A, Nakagawa I and Yoshimori T. Autophagy in innate immunity against intracellular bacteria. *J Biochem* 2006; 140(2): 161–166.

21. Jo E K, Yuk J M, Shin D M, et al. Roles of autophagy in elimination of intracellular bacterial pathogens. *Front Immunol* 2013; 4: 97.

22. Hu W, Chan H, Lu L, et al. Autophagy in intracellular bacterial infection. In: *Seminars in Cell & Developmental Biology*. Elsevier, 2019.

23. Pei J, Deng J, Ye Z, et al. Absence of autophagy promotes apoptosis by modulating the ROS-dependent RLR signaling pathway in classical swine fever virus-infected cells. *Autophagy* 2016; 12(10): 1738–1758.

24. Ravegnini G, Sammarini G, Nannini M, et al. Gastrointestinal stromal tumors (GIST): Facing cell death between autophagy and apoptosis. *Autophagy* 2017; 13(3): 452–463.

25. Li Q, Yin Y, Zheng Y, et al. Inhibition of autophagy promoted high glucose/ROS-mediated apoptosis in ADSCs. *Stem Cell Res Ther* 2018; 9(1): 289.

26. Kaminskyy V O and Zhivotovsky BJA. Free radicals in cross talk between autophagy and apoptosis. *Antioxid Redox Signal* 2014; 21(1): 86–102.

27. Wood S J, Goldufsky J W, Bello D, et al. *Pseudomonas aeruginosa* ExoT induces mitochondrial apoptosis in target host cells in a manner that depends on its GTPase-activating Protein (GAP) domain activity. *J Biol Chem* 2015; 290(48): 29063–29073.

28. Dai C, Ciccotosto G D, Cappai R, et al. Rapamycin confers neuroprotection against colistin-induced oxidative stress, mitochondria dysfunction, and apoptosis through the activation of autophagy and mTOR/Akt/CREB signaling pathways. *ACS Chem Neurosci* 2018; 9(4): 824–837.

29. Li Z R, Ma T, Guo Y J, et al. Sanggenon O induced apoptosis of A549 cells is counterbalanced by protective autophagy. *Bioorg Chem* 2019; 87: 688–698.

30. Zhang J, Fan J, Li Y, et al. Porcine parvovirus infection causes pig placenta tissue damage involving Nonstructural Protein 1 (NS1)-induced intrinsic ROS/mitochondria-mediated apoptosis. *Viruses* 2019; 11(4): 389

31. Kammouni W, Wood H, Saleh A, et al. Rabies virus phosphoprotein interacts with mitochondrial Complex I and induces mitochondrial dysfunction and oxidative stress. *J Neurovirol* 2015; 21(4): 370–382.

32. Ren JHX, Chen K, Zhou Let al. Protective role of Sirtuin3 (SIRT3) in oxidative stress mediated by hepatitis B virus X protein expression. *PLoS One* 2016; 11(3): e0150961.

33. Xu Y, Duan C, Kuang Z, et al. *Pseudomonas aeruginosa* pyocyanin activates NRF2-ARE-mediated transcriptional response via the ROS-EGFR-PI3K-AKT/MEK-ERK MAP kinase signaling in pulmonary epithelial cells. *PloS One* 2013; 8(8): e72528.

34. Manago A, Becker K A, Carpinteiro A, et al. *Pseudomonas aeruginosa* pyocyanin induces neutrophil death via mitochondrial reactive oxygen species and mitochondrial acid sphingomyelinase. *Antioxid Redox Signal* 2015; 22(13): 1097–1110.

35. Raman M, Chen W and Cobb MH. Differential regulation and properties of MAPKs. *Oncogene* 2007; 26(22): 3100–3112.

36. Liu J, Wei X, Wu Y, et al. Giganteaside D induces ROS-mediated apoptosis in human hepatocellular carcinoma cells through the MAPK pathway. *Cell Oncol (Dordr)* 2016; 39(4): 333–342.

37. Lee C W, Wu C H, Chiang Y C, et al. Carbon monoxide releasing molecule-2 attenuates *Pseudomonas aeruginosa*-induced ROS-dependent ICAM-1 expression in human pulmonary alveolar epithelial cells. *Redox Biol* 2018; 18: 93–103.

38. Benoit M, Dormond-Meuwly A, Demartines N, et al. Targeting the JNK signaling pathway potentiates the antiproliferative efficacy of rapamycin in LS174T colon cancer cells. *J Surg Res* 2011; 167(2): e193–e198.

39. Kato H, Katoh R and Kitamura M. Dual regulation of cadmium-induced apoptosis by mTORC1 through selective induction of IRE1 branches in unfolded protein response. *PLoS ONE* 2013; 8(5): e64344.

40. Shi Y J, Sharma A, Wu H, et al. Cyclin D1 and c-myc internal ribosome entry site (IRES)-dependent translation is regulated by AKT activity and enhanced by rapamycin through a p38 MAPK- and ERK-dependent pathway. *J Biol Chem* 2005; 280(12): 10964–10973.