Autophagy maintains the integrity of endothelial barrier in LPS-induced lung injury

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1 INTRODUCTION

Acute lung injury (ALI) is a common syndrome with high morbidity and mortality. One of the main pathological characteristics of this syndrome is endothelial hyper-permeability, which promotes lung inflammation and injury (Johnson & Matthay, 2010). LPS, or endotoxin, released from the bacterial cell wall is considered to be an important eliciting factor in the development of ALI, it results in the activation of a number of inflammatory pathways and then induces increased pulmonary endothelial permeability (Chignard & Balloy, 2000). Although many a research has been established in exploring and understanding the pathogenesis of ALI in recent years, the therapeutic effect has not been improved obviously. Therefore, it is essential to further clarify the mechanisms for regulating LPS-induced lung microvascular endothelial barrier dysfunction.

Macroautophagy (referred to hereafter as autophagy) is an intracellular lysosome-dependent degradation system by which the misfolded proteins and dysfunctional organelles are delivered to autophagosomes, then autophagosomes fuse with lysosomes and cytosolic contents are recycled and digested (Glick, Barth, & Macleod, 2010). Recently, activation of autophagy has been observed and pointed to a protective role in a variety of lung injury including COPD, ischemia-reperfusion induced lung injury, ventilator-induced lung injury and so on (Gao, Liu, Du, Sun, & Zhao, 2013; Ryter, Lee, & Choi, 2010). Recently, activation of autophagy has been observed and pointed to a protective role in a variety of lung injury including COPD, ischemia-reperfusion induced lung injury, ventilator-induced lung injury and so on (Gao, Liu, Du, Sun, & Zhao, 2013; Ryter, Lee, & Choi, 2010; Zhang et al., 2015). However, inappropriate stimulation of autophagy may facilitate cell death, referred to as type II programed...
cell death. Although LPS has also been reported to stimulate autophagy in some lung tissue cells (Aguirre et al., 2014), the exact role and mechanisms of autophagy in LPS-induced lung microvascular endothelial barrier damage are still unknown. One of our previous works has confirmed the protective role of autophagy in maintaining the integrity of microvascular barrier under ischemia-reperfusion induced lung injury (Zhang et al., 2015). Based on the previous observations, we will detect the status and the role of autophagy in LPS-induced lung microvascular barrier damage.

To characterize the functional implication of autophagy in LPS-LI, we (1) establish the in vitro and in vivo models of autophagy in LPS-challenging HPMVECs and mice lungs; (2) examine the effect of LPS on autophagy; and (3) define the influence of autophagy in LPS-LI. Our research and findings have the potential to provide a new insight for exploring the mechanism and new treatments for LPS-LI.

### 2 | MATERIALS AND METHODS

#### 2.1 | Chemicals and antibodies

The following chemicals and reagents, including LPS (from Escherichia coli) (L-2630), CLQ (C6628), monodansylcadaverine (MDC, 30432), pentobarbital sodium salt (P3761), FITC-dextran (53379), and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, 5655) were purchased from Sigma–Aldrich. Additionally, endothelial cell growth medium (ECM, 1001) was purchased from Sciencell (Carlsbad, CA). LDH test kit (A020-1) was purchased from Nanjing Jiancheng Bioengineering Institute of China. siATG7, siATG5, and siRNA transfection reagent system and antibodies against ATG7 and ATG5 were purchased from Santa Cruze Biotechnology, Santa Cruz, CA (sc-41447, sc-41445, sc-45064, sc-33211, and sc-133158). The anti-microtubule-associated protein 1-light chain 3 (LC3) B (3868), anti-sequestosome 1/p62 protein (p62) (5114), anti-GAPDH (2118), anti-microtubule-associated protein 1-light chain 3 (LC3) B (3868), anti-GAPDH (2118) and anti-rabbit IgG (7074) were purchased from Cell Signaling Technology (Danvers, MA). The anti-zonula occludens-1 (ZO-1) was purchased from Abcam, Cambridge, MA (ab59720). Rhodamine-conjugated phalloidin was purchased from Invitrogen, Carlsbad, CA (Molecular Probe, R415). The following enzyme-linked immunosorbent assay (ELISA) kits were purchased from Uscn Life Science Inc., Wuhan, China: Tumor necrosis factor (TNF)-α (SEA133Mu) and interleukin (IL)-1β (SEA563Mu).

#### 2.2 | Experimental cells and animals

In vitro experiments, HPMVECs (Sciencell, 3000) were grown to confluence (usually 3–5 days) in ECM, and then detached with trypsin/ethylene diamine tetraacetic acid and transferred to new dishes with split ratio of 1: 2 for further propagation. In our present research, cells (3–5 passages) were harvested and analyzed. In in vivo experiments, 8–10 weeks old wild-type C57BL/6J mice (Animal Center, Fudan University, Shanghai, China) were used. The experimental protocol was approved by the Committee of Animal Care in Fudan University. All animals were handled in accordance with the Guideline for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

#### 2.3 | Prohibiting ATG7 or ATG5 expression by siRNA

For siRNA transfection, 2 × 10⁶ cells were transfected with 50 nM siATG7 or siATG5 through using siRNA transfection reagent system. After 36 hr, cells were treated with LPS and then the autophagy level and damage degree were detected.

#### 2.4 | In vitro CLQ preconditioning

According to our previous research (Zhang et al., 2015), for CLQ preconditioning, the cells were treated with 2 μg/ml CLQ for 4 hr before LPS challenge.

#### 2.5 | In vitro LPS challenge

HPMVECs were grown to confluence. For imitating LPS-induced lung injury experiment, the cells were seeded onto 12-well plates and were then stimulated with LPS in a medium consisting of ECM with 5% FBS. We have incubated the cells with 100 ng/ml LPS for 6 hr, these were the lowest concentration of LPS in significantly affecting cells viability and the shortest period of action time in triggering the highest autophagy level in HPMVECs (Supplementary Figure S1).

#### 2.6 | Protein preparation and immunoblotting

After LPS challenge, the cells were harvested and rinsed twice in ice cooled PBS. And then the cells were homogenized in RIPA lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM phenylmethanesulfonyl fluoride. Moreover, the homogenate was incubated on ice for 45 min and then centrifuged at 4°C (12,000 rpm for 5 min). Finally, the supernatant protein concentration was determined using the BCA Protein Assay Kit according to manufactory’s instruction. Furthermore, approximately 30 μg protein was loaded on sodium dodecyl sulfate polyacrylamide gels to facilitate electrophoresis at 120 V for 2 hr. And then proteins in gels were transferred onto a polyvinylidene difluoride membrane. Target proteins were probed with primary antibodies against LC3B, p62, ATG7, ATG5, and ZO-1, respectively; meanwhile, GAPDH was used as a control for protein gel loading. A horse radish peroxidase-conjugated secondary antibody was applied for 1 hr followed by three washings. The blots were developed using Pierce ECL Western blotting substrate and quantified by Quantity One 4.6 software.

#### 2.7 | MDC staining

The cells were incubated with 0.05 mM MDC in PBS at 37°C for 60 min. After being washed three times with 0.1 M PBS, the cells were fixed with 4% paraformaldehyde for 15 min and then washed with 0.1 M PBS for three times. Finally, patterns of punctate green fluorescence were determined using a Nikon A1 R laser confocal...
microscope at an excitation wavelength of 492 nm and a detecting emission wavelength of 520 nm.

2.8 | Autophagy detection using mRFP-GFP adenoviral vector

HPMVECs were plated on 6-well plates and allowed to reach 50–70% confluence at the time of transfection. mRFP-GFP-LC3 adenoviral vectors were purchased from Han Bio Technology Co. Ltd. (HanBio, Shanghai, China). Adenoviral infection was performed according to the manufacturer’s instructions. The cells were incubated in growth medium with the adenoviruses at a MOI of 100 for 2 hr at 37°C, and were then grown in medium with addition of 100 ng/ml LPS or vehicle at 37°C for another 6 hr. The mRFP-GFP-LC3 adenoviral vectors enables the detection of LC3B positive, neutral pH autophagosomes in green fluorescence (GFP), and LC3B positive acidic pH autophagolysosomes in red fluorescence (RFP). Autophagic structures were observed under a Nikon A1 R laser confocal microscope. Autophagic flux was determined by evaluating the number of GFP and mRFP puncta (puncta/cell were counted).

2.9 | Transmission electron microscopy analysis of autophagy ultrastructures

After LPS treatment, HPMVECs were prefixed with 2.5% glutaraldehyde in 0.1 M PBS at 4°C overnight, post-fixed in 1% buffered osmium tetroxide, dehydrated in graded alcohols, embedded in Epon 812, sectioned with ultramicrotome, and stained with uranyl acetate and lead citrate. Autophagy precursors, autophagosomes, autophagolysosomes of the cells were observed with a transmission electron microscopy, Philips, Amsterdam, The Netherlands (CM120). A total of 30 electron microscopical sections were prepared and autophagy structures of each group were examined in 200 cells.

2.10 | Transendothelial permeability assay

FITC-dextran (1 mg/ml, MW 40,000) was added into the medium in upper wells. Samples were taken at 0 and 6 hr after LPS treatment from the lower compartment of the transwell chambers followed by refilling of an equal volume of the medium. The amount of FITC-dextran in the upper and lower wells was determined with a fluorescence microplate reader (BioTek Instruments Inc., Winooski, VT, FLX800TBID) at an excitation wavelength of 492 nm and a detecting emission of 520 nm. The final values were deduced through subtracting the values of fluorescence at 0 hr. The results were presented as percentage of the fluorescence in control group.

2.11 | MTT assay for cell viability

HPMVECs viability was determined with MTT assay. Each group was assigned with three wells and cells at a density of 3000 ~ 4000 cells per well. The cells were incubated with 5 mg/ml MTT during the last 4 hr of LPS challenge. After that, the medium was removed and formazan salts dissolved with 150 μl of dimethylsulfoxide. The absorbance values were determined at 570 nm with an automatic multi-well spectrophotometer (Bio-Rad Laboratories, 168–9520). The experiment was repeated three times in each group.

2.12 | The leakage rate of intracellular LDH

Supernatant of the cell culture was reserved after LPS treatment while cells were rinsed with PBS and lysed with 1% triton X-100 at 37°C for 30 min. Both samples were prepared following instructions of a LDH test kit. The absorbance value at 440 nm was determined with an automatic multi-well spectrophotometer. LDH leakage was calculated as following formula: LDH leakage (%) = absorbance value of LDH in the culture medium/total LDH absorbance value (LDH in culture medium and cell lysate).

2.13 | Filamentous (F)-actin labeling

To monitor the effects of LPS and autophagy on cytoskeletal protein, rhodamine-conjugated phalloidin molecular probe was used to detect F-actin according to manufacturer’s instructions. After being treated with 100 ng/ml LPS, the cells were fixed with 3.7% paraformaldehyde for 10 min, permeabilized with 0.5% triton X-100, then stained with rhodamine-conjugated phalloidin. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI), mounted and imaged with a Nikon A1 R laser confocal microscope.

2.14 | Establishing LPS-induced acute lung injury mice models and study grouping

LPS-induced acute lung injury mice models were established as previous reported (Wu et al., 2014). Mice were randomly grouped as animals undergoing a vehicle operation (sham); animals undergoing a vehicle operation and pre-treated with CLQ (CLQ + sham); animals undergoing LPS operation (LPS); animals undergoing LPS operation; and pre-treated with CLQ (CLQ + LPS). These intervention agents were pretreated as following doses and approaches. Both mice were anaesthetized and orally intubated with a sterile plastic catheter. CLQ at 10 mg/kg was intraperitoneally injected into animals 4 hr prior to LPS instillation. The animals were then challenged with intratracheal instillation of LPS at 2 mg/kg. Naive mice (without LPS instillation) were challenged with intratracheal instillation the same volume of PBS to serve as sham groups. Subsequently, they were allowed to recover from anesthesia and had free access to water and chow for 6 hr in a warm chamber before death. After killing the mice, fresh left lung tissues and BALF from the right lungs were harvested and then stored at −80°C for future detection.

2.15 | Lung tissue morphology and edema detection

Mice were sacrificed by cervical dislocation. Fresh left lung tissues were harvested and fixed in 10% formalin immediately and then embedded in paraffin. A total of 4 μm thick paraffin sections were stained with hematoxylin and eosin. Lung injury was assessed in four categories: interstitial inflammation, neutrophil infiltration, congestion, and edema. The assessing results were shown as scores on a 0–4 point scale: no injury = score of 0; injury in 25% of the field = score of 1; injury in 50% of
the field = score of 2; injury in 75% of the field = score of 3; and injury throughout the field = score of 4 (Smith et al., 1997). Ten microscopic fields from each slide were analyzed and the sums of tissue slides were averaged to evaluate the severity of lung injury. All microscopic sections were interpreted in a blind fashion by a pulmonary pathologist.

In addition, the lung edema was assessed by examining the lung wet to dry weight ratio. After wet weight was measured, the same lung was dried at 56°C for 72 hr. Then the wet to dry weight ratio was calculated.

2.16 | ELISA

BALF was collected from the right lungs, which were lavaged with 0.5 ml saline via tracheotomy. This procedure was performed three times and the fluid was pooled together. An average of 1.2 ml total BALF was collected from each mouse and centrifuged at 4°C (1000 rpm for 15 min). The supernatant was collected and stored at –80°C and concentrations of TNF-α and IL-1β in BALF were determined using mouse TNF-α and IL-1β ELISA kits according to the manufacturer’s instructions.

2.17 | Statistical analyses

Data were drawn from at least three separate experiments performed in triplicate. The data were presented as mean ± SD and analyzed using SPSS version 12.0 statistical software. Comparisons between multiple groups were performed by one-way analysis of variance procedures. The histologic semiquantitative analysis was compared by the nonparametric Mann–Whitney test. p-value < 0.05 was considered significant.

3 | RESULTS

3.1 | LPS-induced autophagy of HPMVECs

Both ATG7 and ATG5 are essential molecules involving in the autophagosome formation. We have applied specific siRNAs to reduce autophagy activity by blocking the expression of ATG7 or ATG5, respectively, so that to testify the effect of LPS on autophagy and make clear the influence of autophagy in LPS-induced HPMVECs injury. As shown, compared with the control group, LPS increased expression level of LC3-II, but decreased that of p62. However, these effects were significantly inhibited by blocking the expression of ATG7 or ATG5, respectively (Figure 1a,b). In addition, we have further testified the effects of LPS on autophagy through interfering autophagy with specific inhibitor CLQ. Consistently, CLQ had significantly inhibited LPS-induced autophagy (Figure 1c).

MDC staining has been applied to detect autophagy level. As shown, the number of cells containing MDC-labeled autophagosomes in LPS-challenging group was much more than that in control group, siATG7 treatment effectively inhibited autophagosomes formation under LPS challenging condition (Figure 1d).

The effect of LPS on autophagy markers could be related to protein accumulation due to impaired clearance of autophagosomes or could be an indication of an augmented autophagolysosomes formation which suggests an increased autophagy flux. For discriminating these, Ad-mRFP-GFP-LC3, a specific marker for autophagosomes and autolysosomes, was transfected to HPMVECs. As expected, we found the cells treated with LPS showed typically dense accumulation of GFP-LC3 and mRFP-LC3 puncta in the perinuclear region and cytoplasm (Figure 1e). Compared with those in control cells, the numbers of mRFP-GFP-LC3 puncta in LPS-exposed cells were much more. However, this effect was effectively lowered by inhibiting autophagy with siATG7 (Figure 1f).

In another experiment, autophagic ultrastructures were examined by transmission electron microscopy. Autophagosomes were double-membraned structures and engulfed cytoplasm fractions. In autolysosomes, the lysosome contains one or more autophagosomes (Figure 1g). Statistically, the number of autophagosomes in LPS groups was significantly higher than that in the control group (Figure 1h).

3.2 | Inhibiting autophagy aggravated LPS-induced high permeability of HPMVECs

For detecting the effects of autophagy on the integrity of lung microvascular, we have detected the permeability of HPMVECs. As shown, inhibiting ATG7 or ATG5 expression did not affect the permeability of HPMVECs under normal growth condition. Compared with that in the control group, the permeability of HPMVECs in LPS-challenging group was much higher. Inhibiting autophagy by blocking ATG7 or ATG5 expression further aggravated LPS-induced high permeability of HPMVECs (Figure 2a,b). In another experiment, CLQ was used to inhibit autophagy to further testify the effect of autophagy on LPS-induced high permeability. As shown, inhibiting autophagy by CLQ did not affect the permeability of HPMVECs under normal growth condition but further exacerbated permeability in LPS-stimulating HPMVECs (Figure 2c).

3.3 | Inhibiting autophagy affected the viability and induced damage of LPS-challenging HPMVECs

In normal growth condition, inhibiting autophagy by prohibiting ATG7 or ATG5 expression did not do extra influence in cells viability. Compared with the control group, LPS significantly lowered the cells viability, which was apparently aggravated by inhibiting the expression of ATG7 or ATG5 (Figure 3a,b). Similarly, inhibiting autophagy with CLQ had no remarkable effect on the viability of control cells but significantly lowered the viability of LPS-challenging cells (Figure 3c). In another experiment, the leakage rate of LDH was applied to assess cell damage: It increased from 17.52 ± 2.34% in control cells to 47.91 ± 3.77% in LPS-challenged cells. Prohibiting the expression of ATG7 further increased this index to 76.31 ± 4.98% in LPS-stimulating cells (Figure 4a). In addition, CLQ preconditioning did not significantly affect the LDH leakage rate in control cells but significantly soared that in LPS-stimulating cells from 44.11 ± 3.34% to 71.24 ± 4.01% (Figure 4b).
FIGURE 1  Effects of LPS on autophagy of HPMVECs. (a) Representative western blot analyses of LC3B, p62, and ATG7 in HPMVECs blocked ATG7 expression and treated with LPS or vehicle. (b) Representative western blot image for LC3, p62, and ATG5 from LPS-stimulating HPMVECs with siATG5 treatment. (c) Representative western blot image for LC3 and p62 from LPS-challenging HPMVECs with chloroquine preconditioning. (d) MDC staining to detect autophagosomes in HPMVECs, the green puncta revealed MDC-labeled autophagosomes. (e) Representative images of cells transiently overexpressing mRFP-GFP-LC3, The neutral pH LC3B positive autophagosomes (green fluorescence), and acidic pH LC3B positive autophagolysosomes (red fluorescence) were detected, respectively using a confocal microscope. (f) Quantification of LC3B positive autophagosomes and autophagolysosomes in at least 10 cells per condition. (g) Photographs from transmission electron microscopy showing characteristic autophagic ultrastructures in the cells. (h) Quantitative analysis of the number of autophagosomes in different groups. Autophagosomes and autolysosomes were indicated by thick arrows and double thin arrows, respectively. Results are mean ± SD. The experiment was repeated three times in each group. Molecular mass in kDa.
3.4 Inhibiting autophagy affected ZO-1 expression in LPS-stimulating HPMVECs

In normal growth condition, inhibiting autophagy by prohibiting ATG7 or ATG5 expression had no extra effects on the expression of ZO-1. Compared with that in the control group, LPS significantly lowered the ZO-1 expression, which was further aggravated by inhibiting autophagy level with siATG7 or siATG5. (c) LPS stimulation increased the permeability of the cells, which was further exacerbated by inhibiting autophagy level with CLQ. Results are mean ± SD. The experiment was repeated three times in each group.

3.5 Inhibiting autophagy promoted the stress fiber formation

F-actin can be induced to polymerize to form transcytoplasmic cables that generate tension between cell-cell junctions and focal adhesions on the extracellular matrix. Subsequent disruption of cell-cell junctions can lead to increased paracellular permeability (Su et al., 2007). For testing whether LPS-induced stress fiber formation could be regulated by autophagy, we inhibited autophagy and then treated the cells with LPS. As shown, compared with that in control cells, LPS significantly caused...
increase in actin stress fiber formation. Suppressing autophagy in advance had no significant effect on stress fiber formation in control cells but markedly augmented that effect in LPS-stimulating cells (Figure 6).

3.6 Inhibiting autophagy aggravated LPS-induced lung injury in mice

Compared with that in sham-operated mice, the severity of lung injury, characterized by lung tissue edema, leukocyte infiltration, and hemorrhage, was more obvious in LPS-treated mice, it was further exacerbated by inhibiting autophagy with siATG7 (Figure 7a). Severity of lung injury was also assessed by using a semiquantitative histopathology score system, the score in LPS-induced animals was much higher than that in sham-operated ones, and was further increased by CLQ pretreatment (Figure 7b).

In addition to morphologic evidence, the lung wet-to-dry weight ratio was detected to show the degree of lung edema. Compared with that in sham-operated mice, LPS increased the wet-to-dry weight ratio, which was further elevated by inhibiting autophagy with CLQ (Figure 8).

To further evaluate the inflammation reaction, levels of pro-inflammatory cytokines TNF-α and IL-1β were measured in BALF. Compared with those in sham-operated animals, concentrations of both the pro-inflammatory cytokines were elevated in response to LPS challenge. Pre-treatment with CLQ further promote the releasing of TNF-α and IL-1β into BALF (Figure 9).

4 DISCUSSION

Although activated autophagy has been proved in LPS-challenge conditions, the role of it in LPS-induced lung microvascular barrier damage remains unknown. Our study has investigated the role of autophagy in LPS-challenging HPMVECs and lung injury. Present research demonstrated that LPS could induce autophagy in HPMVECs. Prohibiting autophagy aggravated HPMVECs injury. Consistently, in vivo experiment revealed that blocking autophagy in LPS-challenging mice triggered much more serious leak in lung microvascular barrier and lung injury. Our findings suggested that autophagy played a protective role in LPS-LI through resisting the damage of pulmonary microvascular endothelial cells, so that to maintain the integrity of the lung microvascular barrier.

In present study, we found LPS soared autophagy level of HPMVECs, which was reflected by increased expression of LC3-II, decreased p62 and the corresponding increasing number of autophagosomes. The up-regulation in autophagy level has been considered a
response of the cells to the apoptosis induced by LPS challenge. Zou et al. (2014) found autophagy was activated in LPS-treated cardiomyocytes, and it played an essential role in preventing apoptosis in the cells. Blockade of autophagy through pharmacological or genetic approach sensitized LPS-challenge peritoneal mesothelial cells to apoptosis (Li et al., 2011). Similarly, loss of autophagy might promote hepatocyte caspase-8 activation to increase Bid cleavage and mitochondrial death pathway activation, so that to promote the LPS and D-galactosamine-induced apoptosis (Amir et al., 2013). The mechanisms of LPS inducing autophagy are not consistent till now.

**FIGURE 6**  The effect of autophagy on stress fiber formation was detected with F-actin staining, nuclei were stained with DAPI. The experiment was repeated three times in each group.

**FIGURE 7**  The effect of autophagy on LPS-LI. (a) Representative lung histopathological slices stained with hematoxylin and eosin. (b) Microscopic injury of the lung was statistically scored. Results are mean ± SD (n = 6/group for hematoxylin and eosin staining and pathological scores).
Mitochondrial damage, ROS generation and the excessive inflammatory cytokines, all these factors have been suggested the role of promoting autophagy under LPS challenging condition. Future researches should further focus on clarifying the exact mechanisms of LPS inducing autophagy in lung microvascular endothelial cells.

In the present study, it has been found that LPS treatment lowered the viability and induced high LDH release rate of HPMVECs. However, these effects were further strengthened after inhibiting autophagy. These findings suggested that autophagy was helpful of resisting the cells damage induced by LPS. The specific characters of autophagy reasonably make clear the protective functions of it in LPS induced lung microvascular endothelial cells injury. On the one hand, autophagy is activated to rapidly remove damaged organelles especially damaged mitochondria, so that to limit the cells death and damage (Hickson-Bick, Jones, & Buja, 2008). In addition, some organs injury induced by LPS or endotoxin could be partially due to the insufficient energy supply (Giannone, Nankervis, Richter, Schanbacher, & Reber, 2009; Kang et al., 2015). autophagy has been shown the function in maintaining cells energetic metabolism balance and is helpful of promoting cells survival through temporarily maintaining amino acid generation, tricarboxylic cycle fueling, and ATP energy production in some stresses. All these characteristics of autophagy are contributed to avoid the initiating of apoptosis. However, in other cases, autophagy or autophagy-relevant proteins can promote LPS-induced injury in some kinds of cells or organs, inhibiting autophagy level, reversely, effectively alleviated the injury. Wu, Zhang, Wang, Diao, and Liu, 2015 found that impairment of autophagy ameliorated LPS-induced inflammation and decreased acute kidney injury. In another side, autophagy might excessively degrade cellular components so that to activate the apoptosis machinery indirectly (Gump & Thorburn, 2011).

We deduced that autophagy might have converse effects under different stress challenges or pathological conditions, further research should be conducted to determine the exact mechanism for mediating autophagy in LPS-induced lung injury.

We have found LPS-induced high permeability, and this effect was significantly aggravated by inhibiting autophagy. These findings suggested that autophagy was participated in mediating endothelial barrier integrity. As known, intact cell tight junctions are essential to maintain the integrity of microvascular barrier. For testing the effect of autophagy on tight junction, we have detected the expression of ZO-1, one of the vital members in composing tight junction construction (Tornavaca et al., 2015). We found LPS treatment decreased the expression of ZO-1, which was further exacerbated by inhibiting autophagy in advance. Based on this, we consider autophagy regulated tight junction function partly through mediating the expression of ZO-1. Our previous study has also suggested that inhibiting autophagy further decreased ZO-1 expression in HPMVECs under mimic ischemia and reperfusion condition. Improving autophagy in advance, however, lowered the decrease of ZO-1 expression (Zhang et al., 2015). Similarly, Li et al. (2014) found rapamycin pretreatment significantly reversed the decreased level of ZO-1 induced by oxygen-glucose deprivation/reoxygenation and promoted the distribution of ZO-1 on cell membranes. In contrast, 3-methyladenine pretreatment exerted opposite effects on ZO-1. Additional studies are required to identify the exact mechanisms of autophagy in regulating ZO-1 expression and distribution.

Cytoskeletal F-actin stress fiber is well-described contributor to induce increase in pulmonary endothelial permeability. Some reports have linked regulation of the actin stress fibers formation to endothelial barrier function (Gabryś et al., 2007), but the precise mechanism for this regulation remains unclear. Our studies have identified autophagy as a specific regulator of suppressing the formation of actin stress fibers under LPS challenging condition. This was consistent with other previous reports. Qianying Lv, Yang, Chen, and Zhang, (2016) found inhibition of autophagy by 3-methyladenine significantly increased sublytic complement attack-induced stress fiber formation in MPC5 podocytes. In contrast, promotion of autophagy by rapamycin mitigated the effect. These findings suggested that autophagy might regulate changes in vascular permeability by coordinating interactions with the actin cytoskeleton. Further studies are essential to establish for identifying the exact molecular mechanisms by which autophagy regulates stress fiber formation.
LPS is one of the known culprits in the production of reactive oxygen species and pro-inflammatory cytokines including TNF-α, IL-1β, and so on (Kim et al., 2014). Production of these cytokines contributes to acquisition of neutrophils in the lung, leading to ALI (Bhatia & Moochhala, 2004; Qiu et al., 2011). In our study, we found LPS-induced the production of pro-inflammatory factors TNF-α and IL-1β, inhibiting autophagy with CLQ further promoted the production of these cytokines. In the later experiment, we have indeed detected CLQ pretreatment induced more serious lung neutrophil infiltration and edema in LPS-treated mice. Similarly, in other types of cells or organs, autophagy has also found the effects on regulating inflammation. Macrophages deleted autophagy related 7 or 16-like 1 genes increased release of IL-1β and IL-18 in response to LPS stimulation (Saitoh et al., 2008). TNF-α and IL-6 were decreased after activation of autophagy in ischemia/reperfusion challenged rats kidney tissue (Ling et al., 2016). Some evidences can be used to account for the anti-inflammatory role of autophagy in LPS-LI. On the one hand, autophagy is involved in the production, processing, and secretion of several pro-inflammatory cytokines, such as IL-1β (Nakahira et al., 2011). On the other hand, autophagy potentially captures bacteria that have escaped from phagosomes into the cytoplasm (Yuan et al., 2012), thereby restricting the pro-inflammation effects of LPS in the lung. Moreover, autophagy plays a vital role in restricting the initiating of inflammation through removing some damaged organelles, such as injured mitochondria, which will initiate inflammation through releasing reactive oxygen species (Lee, Giordano, & Zhang, 2012). These findings suggest the protective effects of autophagy on LPS-induced lungs are at least in part due to the anti-inflammation character.

5 | CONCLUSIONS

We have shown in present study that LPS challenge soared the autophagy level, inhibiting autophagy aggravated LPS-induced lung microvascular endothelial cells injury, further broke the integrity of lung microvascular endothelial barrier, and exacerbated inflammation reaction. These results provided new insights into the role of autophagy in LPS-LI and suggested that regulating autophagy may be a potential strategy in the treatment of LPS-related lung injury.

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6 | DISCLOSURE

None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this article.


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.