International Immunopharmacology 74 (2019) 105737

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Autophagy is involved in the acute lung injury induced by H9N2 influenza virus

Rui-hua Zhang[a,b,1], Hong-liang Zhang[a,1], Pei-yao Li[b], Jing-ping Gao[b], Qiang Luo[b], Ting Liang[b], Xue-jing Wang[c], Yong-qing Hao[a,*], Tong Xu[b,*], Chun-Hong Li[b], Cun-Lian Wang[b], Hui-chen Zhang[d], Ming-Ju Xu[b], Shu-Fei Tian[b]

[a] Department of Veterinary Medicine, Inner Mongolia Agricultural University, Hohhot 010018, PR China
[b] Key Laboratory of Preventive Veterinary Medicine, Department of Veterinary Medicine, Animal Science College, HeBei North University, Zhangjiakou 075131, PR China
[c] The Animal Husbandry and Veterinary Institute of Heibei, Baoding 071001, PR China
[d] He He Animal Husbandry Development Co. Ltd, Zhenlai 137300, PR China

ARTICLE INFO

Keywords:
Autophagy
Acute lung injury
H9N2 influenza virus

ABSTRACT

Influenza A virus usually leads to economic loss to breeding farms and pose a serious threat to human health. Virus infecting tissues directly and influenza virus-induced excessive production of inflammatory factors play the key role in pathogenesis of the disease, but the mechanism is not well clarified. Here, the role of autophagy was investigated in H9N2 influenza virus-triggered inflammation. The results showed that autophagy was induced by H9N2 virus in A549 cells and in mice. Inhibiting autophagy by an autophagy inhibitor (3-methyladenine, 3-MA) or knockdown of Atg5(autophagy-related gene) by Atg5 siRNA significantly suppressed H9N2 virus replication, H9N2 virus-triggered inflammatory cytokines and chemokines, including IL-1β, TNF-α, IL-8, and CCL5 in vitro and in vivo, and suppressed H9N2 virus-triggered acute lung injury as indicated as accumulative mortality of mice, inflammatory cellular infiltrate and interstitial edema, thickening of the alveolar walls in mice lung tissues, increased inflammatory cytokines and chemokines, increased W/D ratio in mice. Moreover, autophagy mediated inflammatory responses through Akt-mTOR, NF-κB and MAPKs signaling pathways. Our data showed that autophagy was essential in H9N2 influenza virus-triggered inflammatory responses, and autophagy could be target to treat influenza virus-caused lung inflammation.

1. Introduction

Influenza A virus often causes economic loss to breeding farms and pandemics in humans and results in morbidity and mortality [1,2]. Most patients infected with influenza virus usually suffered from acute lung injury (ALI) with rapid progression to its severe form, acute respiratory distress syndrome (ARDS) [3–5]. The major pathogenic factors of influenza virus infection are virus infecting tissues directly and rapid viral replication [6]. Another major factor that contributes to virus-caused acute lung injury is virus-triggered increase in inflammatory cytokines and chemokines [5,7,8]. However, it is not known how virus infecting lung tissues directly and virus-triggered the inflammatory responses are involved in the pathogenic mechanism of acute lung injury [9].

It was reported that autophagy was induced by influenza A virus infection (IAV) and plays an important role in pathogenic mechanism [10]. Autophagy is a cellular degradation process that keeps the intracellular homeostasis by eliminating various cytoplasmic debris and controls microbe invasion [11,12]. Autophagy can be triggered by nutrient starvation, infection with pathogen and other factors and is initiated by the activation of Beclin-1 complex, then the microtubule-associated protein 1 light chain 3 (LC3) protein transforms its cytosolic non-lipidated form LC3-I to a phosphotidylamine-conjugated form LC3-II which attaches to the autophagosome membrane and serves as a marker for the formation of autophagosome [13–15]. It is reported that p62 can act as an autophagic adaptor protein interacted with LC3-II to facilitate the delivery of cytoplasmic cargos, such as nonfunctional organelles, misfolded proteins, and microorganisms, into the autophagosomes [16]. The fully formed and closed autophagosome will fuse with the lysosome for degradation [17]. It is reported that autophagy could be induced by different subtypes of influenza virus, such as H5N1, H3N2 and H1N1, playing the important role in the virus

* Corresponding authors.
E-mail addresses: haoyq1960@163.com (Y.-q. Hao), xutong1969@sohu.com (T. Xu).
[1] Ruihua Zhang and Hongliang Zhang contributed equally to this work.

https://doi.org/10.1016/j.intimp.2019.105737
Received 17 January 2019; Received in revised form 21 June 2019; Accepted 1 July 2019
Available online 06 July 2019

replication and pathogenic mechanism of influenza virus [18–21]. Research indicates that autophagy triggered by influenza virus is an important factor for viral replication [10]. In addition, it was shown that autophagy was critical for the production of inflammatory cytokines and chemokines in influenza virus-infected human macrophages [19]. Evodiamine (an autophagy inhibitor) treatment dramatically suppressed H1N1-induced cytokine production in A549 cells [22]. These data indicated that the autophagy promoted influenza virus-triggered inflammatory responses, however, it remains unclear how autophagy causes the lung inflammation induced by influenza virus.

In this study, the relationship between autophagy induced by influenza virus and lung inflammation was investigated. The results demonstrated that H9N2 influenza virus induced autophagy and inflammatory response in A549 cells and in mice. Inhibiting the autophagy by autophagy inhibitor and knockdown of Atg5 dramatically suppressed H9N2 virus-induced inflammatory response in A549 cells and in mice. Moreover autophagy mediated the inflammatory responses through Akt-mTOR, MAPKs and NF-κB signaling pathways. Our study showed that autophagy was essential for H9N2 virus-induced inflammatory responses, and autophagy could be target to treat influenza virus-caused lung inflammation.

2. Materials and methods

2.1. Virus strains and cell culture

The H9N2 influenza A/swine/HeBei/012/2008 virus (H9N2 virus) was provided by key Laboratory of Preventive Veterinary Medicine in HeBei North University. Human lung adenocarcinoma A549 cells were kindly provided by Nankai University, and cultured in DMEM medium with 10% fetal bovine serum in 5% CO_2 at 37 °C.

2.2. Cell treatments and virus infection

A549 cells were treated with 10 mM (final concentration) 3MA (a PI3K inhibitor), 50 μM (final concentration) CQ (an inhibitor of lysosome acidification) for 1 h and then infected with H9N2 virus at a multiplicity of infection (MOI) of 0.1, 2, 6 or 10 or an equal volume of vehicle, followed by further study. For RNA interference, A549 cells were plated and transiently transfected with 50 nM Atg5-specific siRNA or control siRNA. The sequence of Atg5-specific siRNA (Atg5 siRNA) was determined in accordance with references [20]. At 48 h post transfection, the protein levels of Atg5 were determined by Western blotting analysis.

2.3. Animals and intranasal inoculation

Animal experiments were approved by the Committee on the Animal Care and Use of the Hebei North University in accordance with the ethics guidelines for the Care and Use of Laboratory Animals of the National Research Council Guide. BALB/c mice (the Laboratory Animal Center of Beijing, China) were caged in a pathogen-free facility and fed standard rodent chow and water ad libitum.

Mice in H9N2 group were anesthetized by diethyl ether, and then were intranasal inoculated 50 μL H9N2 virus (10^7 $TCID_{50}$). Mice in control group were intranasal inoculated an equivalent dilution of noninfectious allantoic fluid. In the H9N2 + 3-MA group, 3-MA (15 mg/kg) was injected intraperitoneally 2 h before the infection of H9N2 virus. In the 3-MA group, only 3-MA (15 mg/kg) was injected intraperitoneally. In the Atg5 siRNA group and control siRNA group, mice were intratracheally treated with Atg5-specific siRNA or control siRNA (100 μg in 100 μL), respectively. In the H9N2 + Atg5 siRNA group and H9N2 + control siRNA group, mice were treated with Atg5-specific siRNA or control siRNA and intranasal inoculated 50 μL H9N2 virus (10^7 $TCID_{50}$). All mice were monitored throughout a 24-hour period of recovery, and knockdown efficiency of Atg5-specific siRNA was estimated by Western blotting. The sequence of Atg5-specific siRNA (Atg5 siRNA) was determined in accordance with references [20]. After H9N2 virus challenge, the survival rates of mice in each group (n = 18) were monitored for 14 days, and mice were sacrificed at 5 d after H9N2 virus infection in the other experiments.

2.4. Measurement of lung wet/dry ratio

Mice in all groups as described above were euthanized, and mice lungs were collected and evaluated for their wet weight 5 days after H9N2 virus infection and were dried in a 70 °C oven to obtain a dry weight. Then the lung wet/dry ratio was measured.

2.5. Histological staining

At 5 days postinfection, mice were euthanized and the lung tissues were fixed with formalin. After dehydration, the fixed lung tissues samples were embedded in paraffin and sectioned at 4 μm. Ultrathin sections were rehydrated, and stained with hematoxylin and eosin (H&E). The histopathological features of the mice lung tissues were detected by use of an optical microscopy. Lung injury, based on the inflammatory cell infiltration, alveolar and interstitial oedema, was assessed according to our previous research [23].

2.6. Titration of viruses

A549 cells grown in 12-well plates were treated with 3MA, control siRNA or Atg5-specific siRNA, and then infected with H9N2 virus at an MOI of 10. Cells were then incubated for 24 h, supernatants of infected culture were harvested and serially diluted in serum-free DMEM. Ten-fold dilutions were added to MDCK cells in a 96-wells plate, and infected cells were maintained in DMEM with 10% FBS for 72 h. Virus titers were determined by the Reed-Muench method and were expressed as $TCID_{50}$ per milliliter supernatant. BALB/C mice were treated with 3MA, control siRNA or Atg5-specific siRNA, and then the mice were infected with H9N2 virus (10^7 $TCID_{50}$). Mice were killed at 72 h after infection, and mice lungs were homogenized in serum-free DMEM. The homogenate supernatants of lung were harvested, then $TCID_{50}$ was measured as described above.

2.7. Real-time quantitative assay

A549 cells and BALB/C mice were treated with 3-MA, control siRNA or Atg5-specific siRNA, and then infected with H9N2 virus or an equal volume of vehicle. Total RNA in cells or lung tissues was extracted using Trizol reagent (Invitrogen) according to manufacturer's protocol. cDNA was synthesized from 1.5 μg of RNA with cDNA Reverse Transcription Kit (Applied Biosystems). PCR amplification assays were performed with a SYBR Premix Ex Taq II kit (TaKaRa) on an ABI 7300 Real-Time PCR system (Applied Biosystems). The expression of NP gene was normalized on the basis of the expression of β-Actin. The sequences of primers were listed in Table 1 [24,25].

2.8. Western blotting analysis

A549 Cells were collected, lysed and the protein concentration in supernatant was determined by a BCA protein assay kit (Thermo scientific) according to the manufacturer's protocol. The supernatant of homogenized mice lungs were harvested, and protein was also determined by a BCA Protein Assay Kit. Proteins from each sample were run on SDS polyacrylamide gel. Then protein bands were transferred onto nitrocellulose filter membranes. Membranes were incubated with the specific antibodies against LC3B, p62, Atg5, GAPDH, NF-κB p65, phosphorylated NF-κB p65, p38 MAPK, phosphorylated p38 MAPK, JNK, phosphorylated JNK, ERK, phosphorylated ERK, Akt, phosphorylated Akt, mTOR, phosphorylated mTOR (Cell Signaling Technology)

Table 1
The sequences of primers for real-time PCR reaction.

Primer	Sense (5′-3′)	Anti-sense (5′-3′)
Human β-Actin	CGCGAGAAGATGACCCAGATC	CATGAGGTAGTCAGTCAGGTCCC
Human IL-1β	CCCAGAGAGTCCTGTGCTGAATG	GAGAGCTGACTGTCCTGGCTGAT
Human TNF-α	TGGAGAAGGGTGACCGACTCAG	GTTTGGGAAGGTTGGATGTTCG
Human CCL5	AACCCAGCAGTCGTCTTTGTC	GGACAAGAGCAAGCAGAAACAG
Human IL-8	TGTGGAGAAGTTTTTGAAGAGGG	CCCTACAACAGACCCACACAATAC
Mouse β-Actin	GCTTCTTTGCAGCTCCTTCGT	CCTTCTGACCCATTCCCACC
Mouse IL-1β	GGTGTGTGACGTTCCCATTAGAC	AGGGTGGGTGTGCCGTCTT
Mouse TNF-α	GTCTACTGAACTTCGGGGTGATCG	TGGGCTACAGGCTTGTCACTCG
Mouse CCL5	ACTCCCTGCTGCTTTGCCT	CACACTTGGCGGTTCCTTCG
Mouse IL-8	GGTCTTCCTGCTTGAATGGCTTGA	GCGGTGTCCTGATTATCGTCCTC

and then with horseradish peroxidase-labeled IgG secondary antibodies (Cell Signaling Technology). Finally membranes were visualised with enhanced chemiluminescence system (Amersham Pharmacia Biotech).

2.9. Statistical analysis

All data are presented as means ± SD. Statistically significant differences among groups were calculated by one-way ANOVA analysis followed by Tukey's multiple comparison test. Survival data were analyzed by a Log-Rank (Mantel-Cox) test. $P < 0.05$ shows statistical significance.

3. Results

3.1. H9N2 virus induced autophagy in A549 cells and mouse lung tissues

The process of autophagy involves the formation of double-membraned autophagosome, the fusion of autophagosome and lysosome, and the degradation of sequestered cargoes in autolysosome [26]. When autophagy is induced, cytosolic LC3-I is converted to membrane-bound LC3-II by the accretion of phosphatidylethanolamine to LC-I [27,28]. In order to confirm whether H9N2 virus induced autophagy in A549 cells and mouse lung tissues, we assessed autophagosome formation by detecting the LC3-II expression using the Western blotting analysis according to the correlative references [28]. The data showed that LC3-II-to GAPDH ratio in H9N2 virus-infected A549 cells and mouse lung tissues increased relative to the controls. However, 3-MA treatment remarkably decreased LC3-II-to GAPDH ratio in A549 cells (Fig. 1A and C) and mouse lung tissues (Fig. 1F and H). The results implied that H9N2 virus successful induced autophagy in mouse lung tissues and human lung A549 cells. Since the increase of LC3-II expression could be due to increased formation of autophagosome or decreased fusion and degradation of autolysosome, we next blocked the fusion of autophagosome and lysosome by using CQ. The results showed that the CQ treatment increased the LC3-II expression in H9N2 virus-infected A549 cells (Fig. 1B and D) and mice lungs (Fig. 1G and I), which declared that accumulation of LC3-II was caused by formation of autophagosome.

P62 is another important protein which involved in autophagic degradation. In this study, the protein level of p62 in H9N2-infected A549 cells and mice lungs showed significant decrease, CQ treatment rescued p62 expression in A549 cells (Fig. 1B and D) and mice lung tissues (Fig. 1G and I). These results further confirmed H9N2 virus promoted autophagy. Moreover, the viral NP protein could be detected in the A549 cells and mice infected by H9N2 virus, and inhibition of autophagy with autophagy inhibitor decreased the accumulation of NP. The data showed that altering cellular autophagy has the effect on viral protein synthesis (Fig. 1A–I).

3.2. Autophagy induced by H9N2 virus was critical for the inflammatory responses in A549 cells

In the present study, we investigated the effect of autophagy in inflammatory responses caused by H9N2 virus. H9N2 virus infection dramatically increased the levels of inflammatory cytokines and chemokines, such as IL-1β, TNF-α, IL-8, and CCL5 in A549 cells at 24 h p.i., indicating the inflammatory effects of H9N2 virus. The 3-MA treatment significantly attenuated H9N2 virus-induced inflammatory cytokines and chemokines in A549 cells (Fig. 2A–D). As autophagy promotes to influenza virus replication [10], suppression of inflammatory responses is possibly due to lower viral loads in the cells after blockage of autophagy. That is to say, virus itself but not autophagy directly contributes to inflammatory responses. Hence, it is necessary to investigate if 3-MA suppresses inflammatory cytokine expression equally after infection of different viral loads. A549 cells were pretreated with 3-MA, followed by H9N2 virus infection at MOI of 2, 6 or 10 at 24 h p.i. and 36 h p.i., then the levels of inflammatory cytokines and chemokines was detected. The data showed that no significant difference in production of cytokines and chemokines was found in 3-MA treated A549 cells infected by H9N2 virus at an MOI of 2, 6, and 10 at 24 h p.i. (Fig. 2A–D). Similar results were obtained in the 3-MA treated A549 cells infected by H9N2 virus at an MOI of 2, 6, and 10 at 36 h p.i. (Fig. 2E–H). So, the results showed that autophagy inhibition, instead of lower viral loads after blockage of autophagy, was critical in the decreased inflammatory responses in A549 cells. To investigate further, autophagy-related gene Atg5 was knocked down with Atg5 siRNA to block the autophagic pathway, and inflammatory responses were detected. The results demonstrated that the Atg5 knockdown reduced H9N2 virus-induced autophagy as indicated as decreased expression of LC3-II (Fig. 2I and J) and alleviated the expression of inflammatory cytokines and chemokines caused by H9N2 virus. Moreover, there were no significant differences in the levels of inflammatory cytokines and chemokines in the Atg5 gene knockout cells infected by H9N2 virus at an MOI of 2, 6, and 10 at 24 h p.i. (Fig. 2K–N). By contrast, treatment with control siRNA has no effect on the expression of inflammatory cytokines and chemokines. So autophagy was critical for the inflammatory responses induced by H9N2 virus.

3.3. Autophagy induced by H9N2 virus was essential for the acute lung injury in mice

3.3.1. Inhibiting autophagy ameliorated histopathological lesions in mice infected with H9N2 influenza virus

We previously successfully established a mouse model of ALI (acute lung injury) by inoculation with H9N2 influenza virus to research the pathogenic mechanisms of the H9N2 virus [29]. In the present study, BALB/c mice inoculated intranasally with H9N2 virus showed severe lung inflammation including inflammatory cellular infiltrate and interstitial edema, thickening of the alveolar walls compared with the control mice (Fig. 3A and B). However, autophagy inhibitor 3-MA

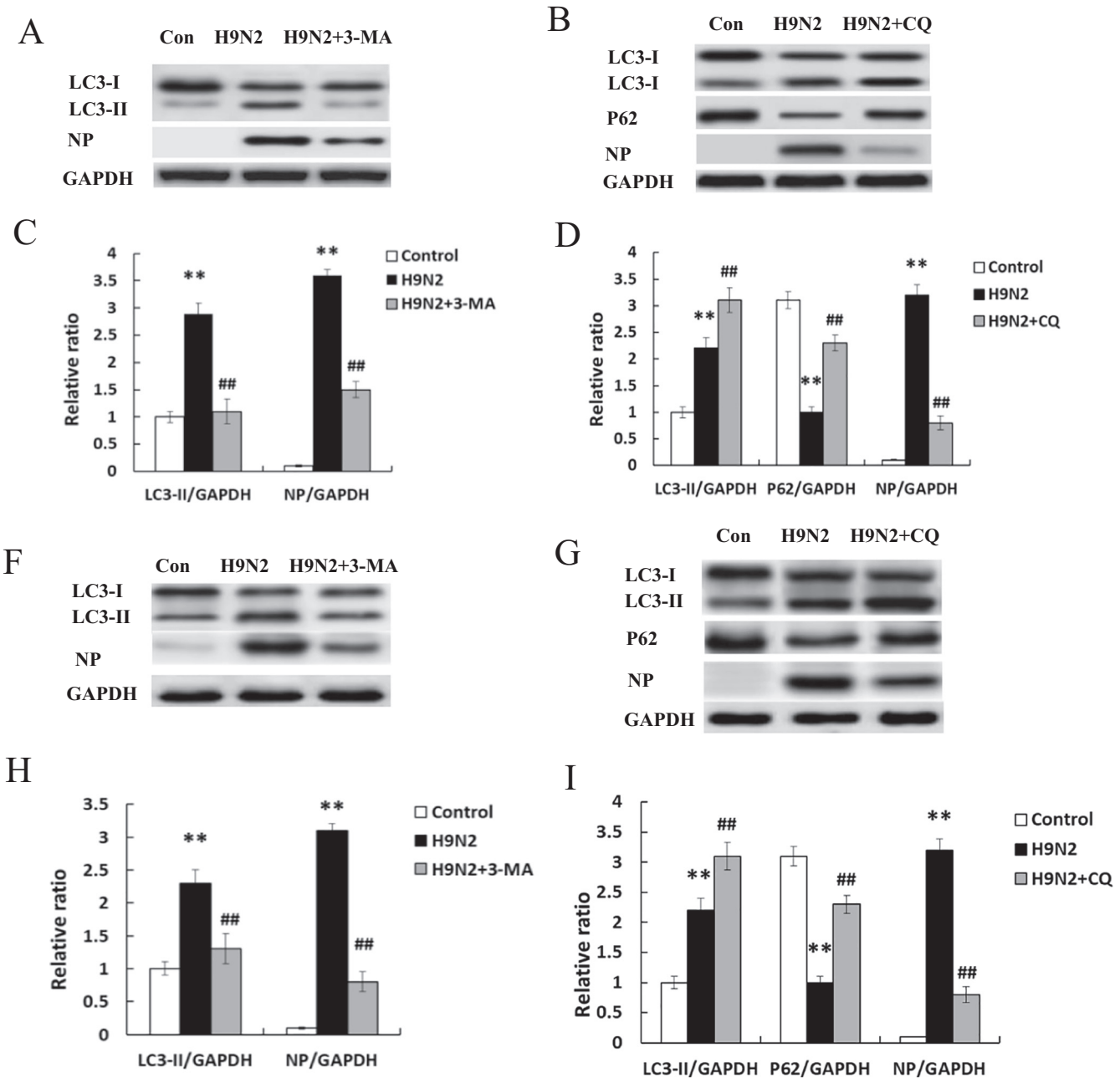

Fig. 1. H9N2 influenza virus induced autophagy in A549 cells and in mice.
A549 cells were pretreated with 3-MA or CQ, followed by infection with H9N2 influenza virus. Western blotting was used to detect the protein expression levels of LC3-II, p62 and NP in A549 cells (A and B). The protein levels of LC3-II, p62 and NP in A549 cells were analyzed as ratios of intensities of GAPDH bands (C and D). BALB/C mice were pretreated with 3-MA or CQ, followed by infection with H9N2 influenza virus. Mouse lung tissues were collected and the protein expression levels of LC3-II, p62 and NP were detected by Western blotting (F and G). The protein levels of LC3-II, p62 and NP in mouse lung tissues (H and I) were analyzed as ratios of intensities of GAPDH bands. The data were showed as means ± SD ($n \geq 3$). $^{**}P < 0.01$ relative to control. $^{\#\#}P < 0.01$relative to H9N2.

treatment relieved the pathological lesions (Fig. 3C). We also knocked down the autophagy-related gene Atg5 in mice by Atg5 siRNA treatment, and the treated mice were infected with H9N2 virus. As shown in the figure the Atg5 knockdown reduced H9N2 virus-induced autophagy as indicated as decreased expression of LC3-II (Fig. 3L and M). Pathological lesions in Atg5 siRNA treated mice infected with H9N2 influenza virus were ameliorated compared with the control siRNA treated mice infected with H9N2 virus (Fig. 3E and F). Additionally, no difference in pathological changes was found in H9N2 influenza virus-infected mice and virus-infected mice pretreated by control siRNA (Fig. 3B and E). The pathological changes showed that autophagy induced by H9N2 virus was essential for the acute lung injury in mice. The score of histopathological changes also showed that autophagy was involved in acute lung injury induced by H9N2 virus. The severity of inflammatory cellular infiltration, interstitial and alveolar edema induced by H9N2 influenza virus was significantly alleviated by 3-MA treatment or Atg5 siRNA treatment (Fig. 3G–J).

3.3.2. Inhibiting autophagy decreased the mortality of H9N2 influenza virus-infected mice

The accumulative mortality of mice infected with H9N2 virus was significantly high when compared to the mortality of control mice. Treatment with 3-MA reduced the mortality of mice caused by H9N2 virus (Fig. 4A). Similar to the 3-MA treatment, Atg5 siRNA treatment

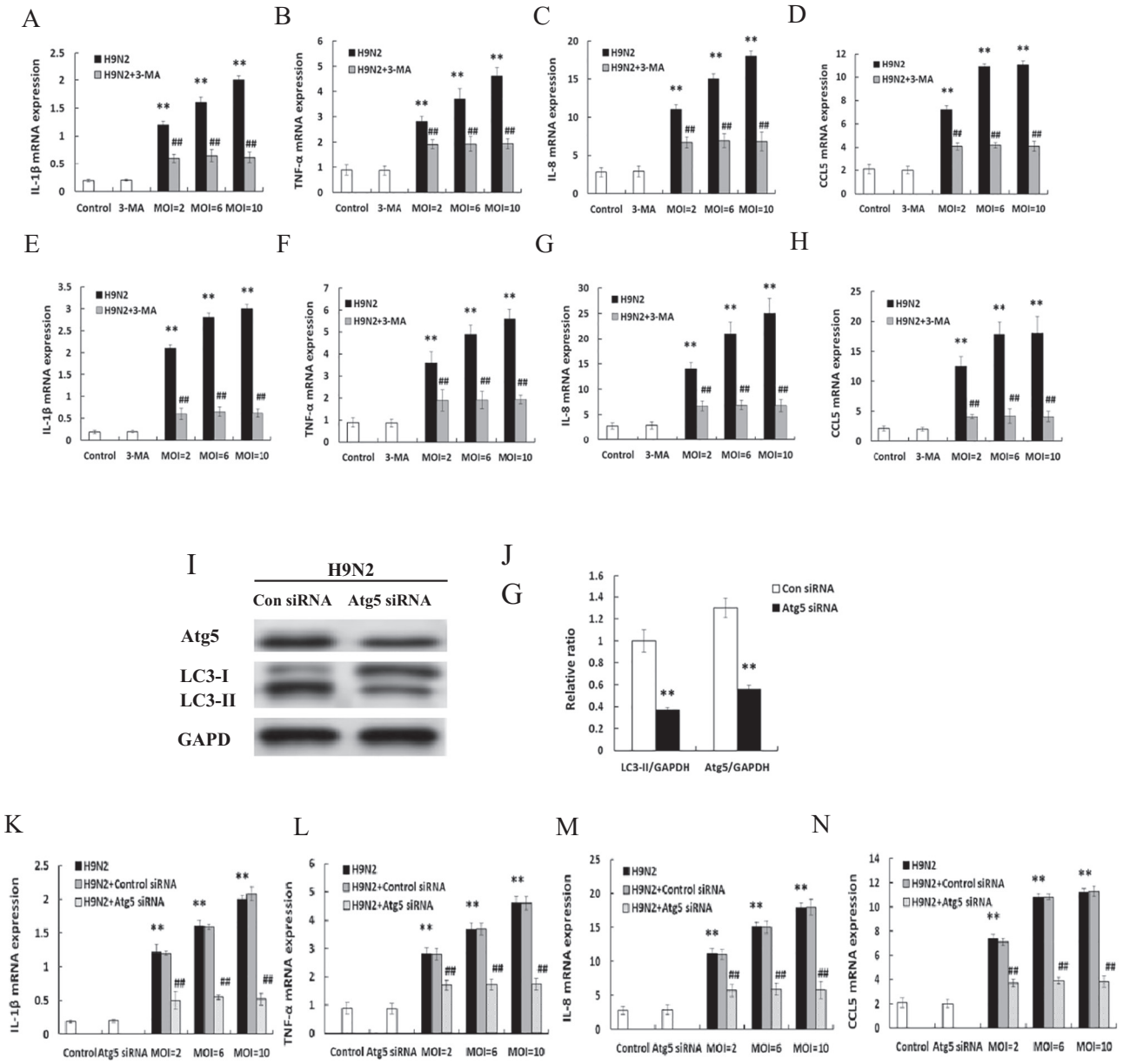

Fig. 2. Autophagy was essential for the inflammatory responses in A549 cells.
A549 cells were pretreated with 3-MA, followed by infection with H9N2 influenza virus at an MOI of 2, 6 or 10. Then cells were collected and the levels of inflammatory cytokines and chemokines were detected by real-time PCR at 24 h p.i. (A–D) and 36 h p.i. (E–H). A549 cells were transfected with control siRNA or Atg5 siRNA, followed by infection with H9N2 influenza virus at an MOI of 2, 6 or 10. Cells were collected and the protein expression levels of Atg5 and LC3-II were detected by Western blotting to detect whether Atg5 is knocked down and autophagy is inhibited (I). The protein levels were analyzed as ratios of intensities of GAPDH bands (J). The levels of inflammatory cytokines and chemokines were detected by real-time PCR at 24 h p.i. (K–N). The data were showed as means ± SD ($n \geq 5$). $^{**}P < 0.01$ relative to control. $^{\#\#}P < 0.01$ relative to H9N2.

also results in a decrease in the mortality of H9N2 infected mice (Fig. 4B).

3.3.3. Inhibiting autophagy attenuated lung edema in mice infected by H9N2 influenza virus

To evaluate the severity of lung edema, the ratio of the weight of wet lung to the weight of dry lung (W/D) was determined in our study. H9N2 influenza virus results in an increase in the W/D ratio. Treatment with 3-MA or Atg5 siRNA suppressed H9N2 virus-induced increase in the W/D ratio (Fig. 5A and B). So lung edema induced by H9N2 virus was prominently decreased in mice treated with the autophagy inhibitor 3-MA or Atg5 siRNA. These data showed that autophagy induced by H9N2 virus was essential for the acute lung injury in mice.

3.3.4. Inhibiting autophagy reduced production of cytokines and chemokines in mice infected by H9N2 influenza virus

To further evaluate the role of autophagy in H9N2 influenza virus-induced ALI, Inflammatory cytokines and chemokines in BALF in mice were detected by real-time PCR. The result showed that 3-MA treatment significantly reduced H9N2 virus-induced inflammatory cytokines and chemokines in mice (Fig. 6A–D). Similar to 3-MA, knockdown of autophagy-related gene Atg5 also alleviated the excessive expression of inflammatory cytokines and chemokines caused by H9N2 virus (Fig. 6E–H). We wanted to show that suppression of inflammatory responses is due to autophagy inhibition directly but not lower viral loads in the tissues after blockage of autophagy, mice treated with 3-MA were inoculated with different viral titers, then inflammatory cytokine

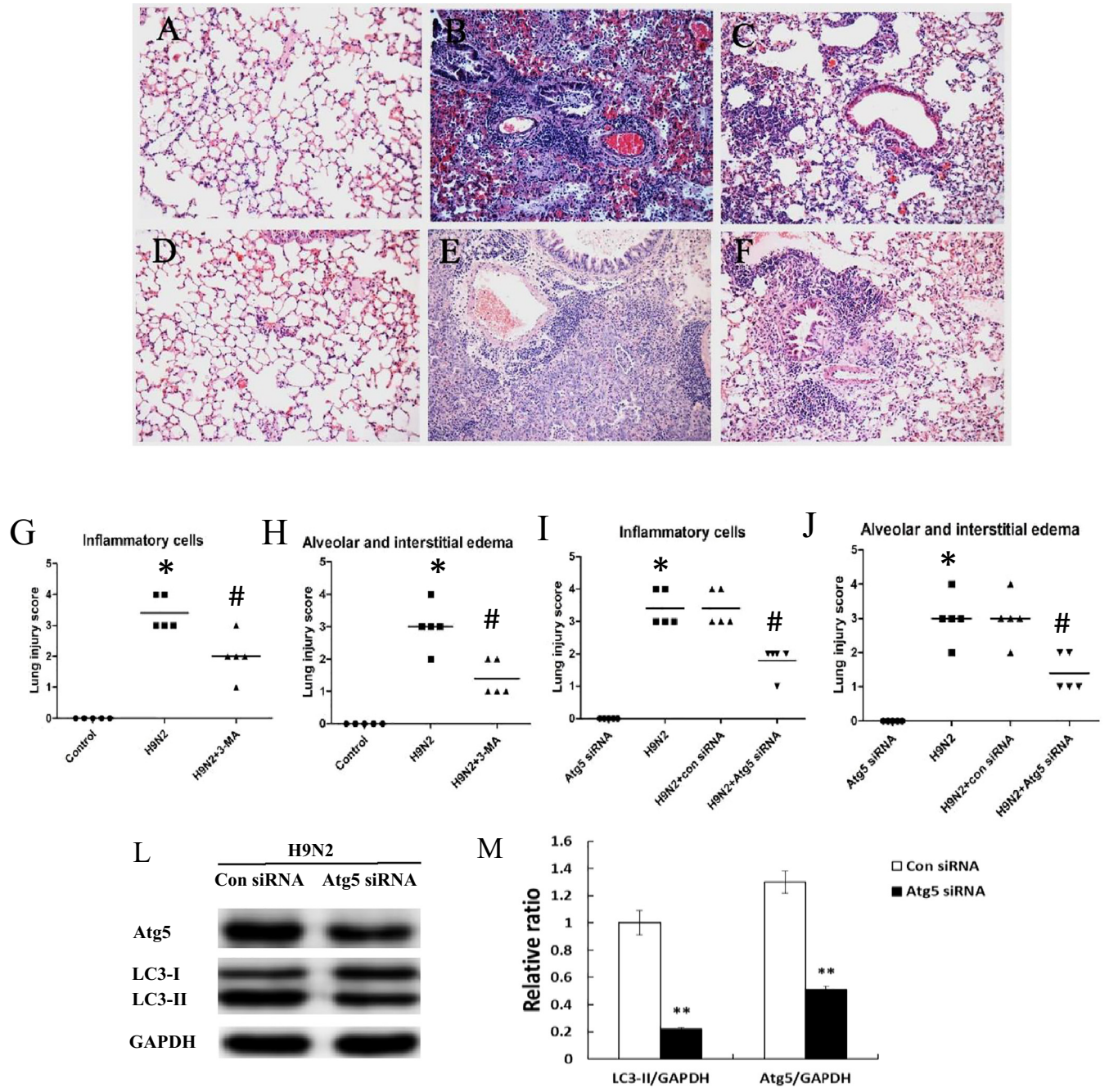

Fig. 3. Inhibiting autophagy ameliorated histopathological lesions in mice infected with H9N2 influenza virus.
BALB/C mice were pretreated with 3-MA, or control siRNA or Atg5 siRNA, followed by infection with H9N2 influenza virus. Mice were euthanized and mice lung tissues were collected and stained with H&E. A: Control. B: H9N2. C:H9N2 + 3-MA. D: Atg5 siRNA. E: H9N2 + control siRNA. F: H9N2 + Atg5 siRNA. Scale bar: 200 mm (A–F). The severity of lung injury was determined on the basis of inflammatory cell infiltration (G and I), alveolar and interstitial edema (H and J). The severity of lung injury was ranked from 0 (normal) to 4 (severe). $^{*}P < 0.05$ relative to control, $^{\#}P < 0.05$ relative to H9N2. Besides, the protein expression levels of LC3-II in supernatants in lung tissues were detected by Western blotting and analyzed as ratios of intensities of GAPDH bands (L and M).

expression was detected. The data showed that no significant difference in production of cytokines and chemokines was found in 3-MA (Fig. 6A–D) or Atg5 siRNA (Fig. 6E–H) treated mice infected by H9N2 virus at an MOI of 2, 6, and 10 at 24 h p.i. So, autophagy inhibition, but not lower viral loads after blockage of autophagy was critical in the decreased inflammatory responses in mice. So, autophagy was critical for the inflammatory responses induced by H9N2 virus.

3.4. Inhibiting autophagy decreased influenza A virus replication

It is reported that the virus (H1N1) titer was relative to autophagy [30] and autophagy activation is required for influenza A virus (H1N1) replication [31]. However, other studies reported that autophagy did not affect the H5N1 virus replication [20]. In this study, we detected the role of autophagy on H9N2 virus replication by treating the cells and mice with 3-MA or Atg5 siRNA. The virus titer was determined in supernatants of A549 cells and mouse lung tissue homogenates infected by H9N2 virus by measuring tissue culture infectious dose ($TCID_{50}$). To verify H9N2 virus replicated in A549 cell and mice, different multiplicity of infection (MOI = 0.1 and 10) were applied to infected cells and mice, and the $TCID_{50}$ was detected in this study. The results showed that H9N2 virus replicated in A549 cells and mice, and treated cells and

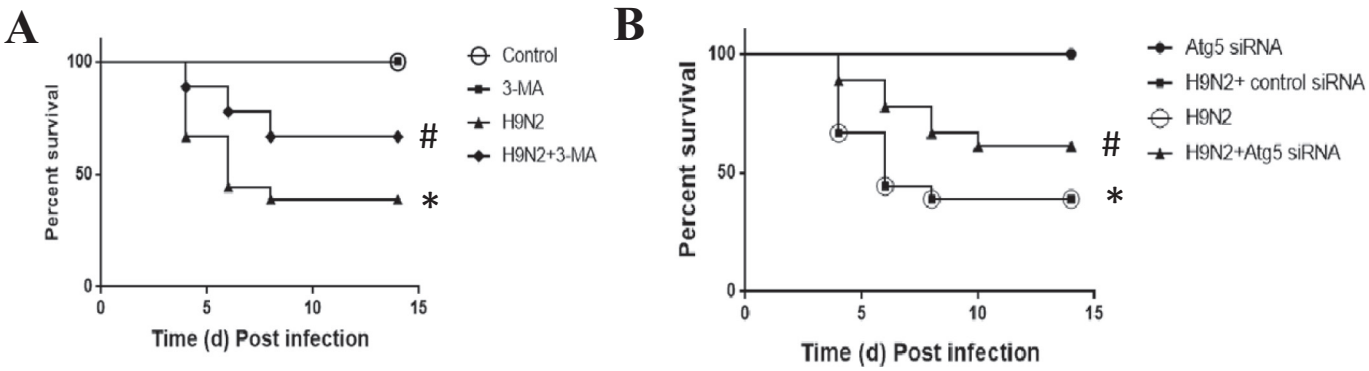

Fig. 4. Inhibiting autophagy decreased the mortality of H9N2 influenza virus-infected mice.
BALB/C mice ($n = 18$) were pretreated with 3-MA, or control siRNA or Atg5 siRNA, followed by infection with H9N2 influenza virus, and survival was calculated $^{*}P < 0.05$ relative to control. $^{\#}P < 0.05$ relative to H9N2.

mouse lung tissues with 3-MA showed lower H9N2 virus titer (Fig. 7A–D). To further explore the relevance between autophagy and H9N2 virus titer, we treated A549 cells and mice with Atg5 siRNA to knock down the Atg5. As shown in the Fig. 7A–D, Atg5 knockdown significantly resulted in a decrease in the virus titer. Control siRNA treatment has no effect on the H9N2 virus titer. The result above showed that inhibiting autophagy decreased H9N2 influenza virus replication.

3.5. Autophagy induced by H9N2 virus mediated the inflammatory responses through Akt-mTOR, MAPK, and NF-κB signaling pathways in A549 cells

Our previous research found that NF-κB signaling participates in the inflammatory responses induced by H9N2 influenza virus [23], here, we examined if NF-κB signaling involved in the inflammatory responses is relative to autophagy induced by H9N2 virus. The Western blotting analysis demonstrated that the phosphorylation of NF-κB p65 and the ratio of LC3II to GAPDH markedly increased in H9N2 virus-infected A549 cells, which were remarkably decreased by 3-MA (Fig. 8A and B) and siRNA of Atg5 treatment (Fig. 8C and D). Notably, H9N2 virus activated NF-κB signaling pathway and autophagy pathway, blocking autophagy with 3-MA and Atg5 siRNA remarkably reduced phosphorylation of NF-κB p65 in A549 cells. By contrast, control siRNA treatment did not lead to the decrease in level of phosphorylation of NF-κB p65. So the data showed that autophagy induced by H9N2 virus mediated the inflammatory responses through NF-κB signaling pathways in A549 cells.

Our previous research also reported that mitogen-activated protein kinases (MAPKs) signaling pathways participate in the inflammatory responses induced by H9N2 influenza virus [23]. In the present study, the phosphorylation of ERK, JNK, and p38 MAPK, and the ratio of LC3II to GAPDH significantly increased in A549 cells infected by H9N2 virus, which were remarkably decreased by 3-MA (Fig. 8A and B) and siRNA of Atg5 treatment (Fig. 8C and D). Notably, MAPKs and autophagy pathways were activated by H9N2 virus infection, and activated MAPKs and autophagy pathways induced by H9N2 virus were suppressed by blocking autophagy with 3-MA and Atg5 siRNA. By contrast, control siRNA treatment did not lead to the decrease in level of phosphorylation of ERK, JNK and p38 MAPK. So the autophagy induced by H9N2 virus mediated the inflammatory responses through ERK, JNK, and p38 MAPK signaling pathways in A549 cells.

It has been reported the induction of autophagy could be mediated by inhibition of Akt-mTOR signaling in IAV-infected HPMECs [25], we next examined whether Akt-mTOR signaling pathway plays a critical role in the attenuation of proinflammatory responses and inhibition of autophagy in H9N2 virus-infected A549 cells. The results showed that the phosphorylation of Akt and mTOR significantly decreased in A549 cells infected by H9N2 virus, which were remarkably increased by 3-MA treatment (Fig. 8A and B). So the autophagy induced by H9N2 virus mediated the inflammatory responses through Akt-mTOR signaling pathways in A549 cells. Taken together, our data suggest that Akt-mTOR, MAPKs, and NF-κB signaling pathways play the critical role in the attenuation of proinflammatory responses and inhibition of

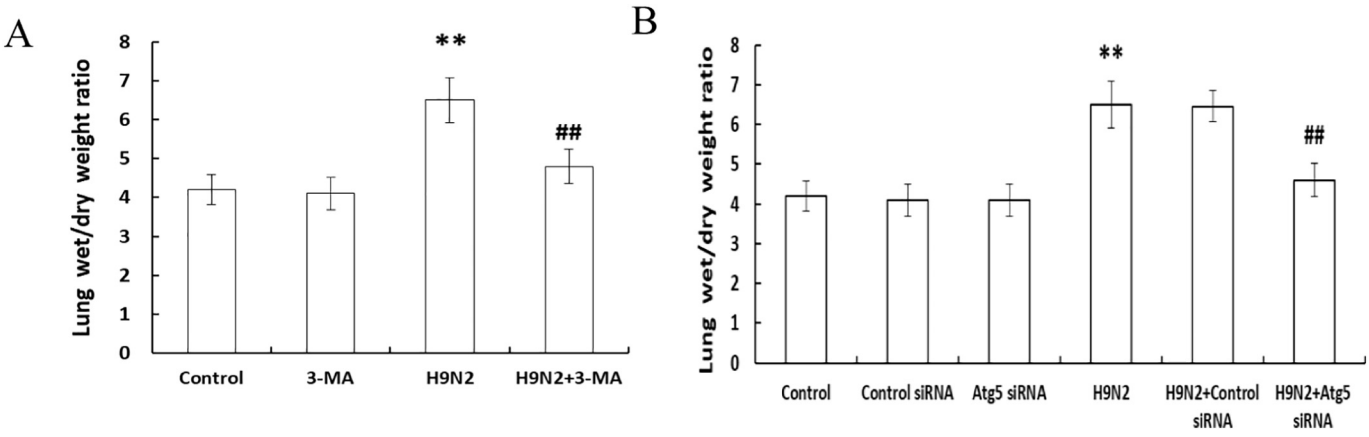

Fig. 5. Inhibiting autophagy attenuated lung edema in mice infected by H9N2 influenza virus.
BALB/C mice were pretreated with 3-MA, or control siRNA or Atg5 siRNA, followed by infection with H9N2 influenza virus. Mice lungs were collected, and lung W/D (wet weight-to-dry weight) ratios were measured (A and B). The data were showed as means ± SD ($n = 5$). $^{**}P < 0.01$ relative to control. $^{\#\#}P < 0.01$ relative to H9N2.

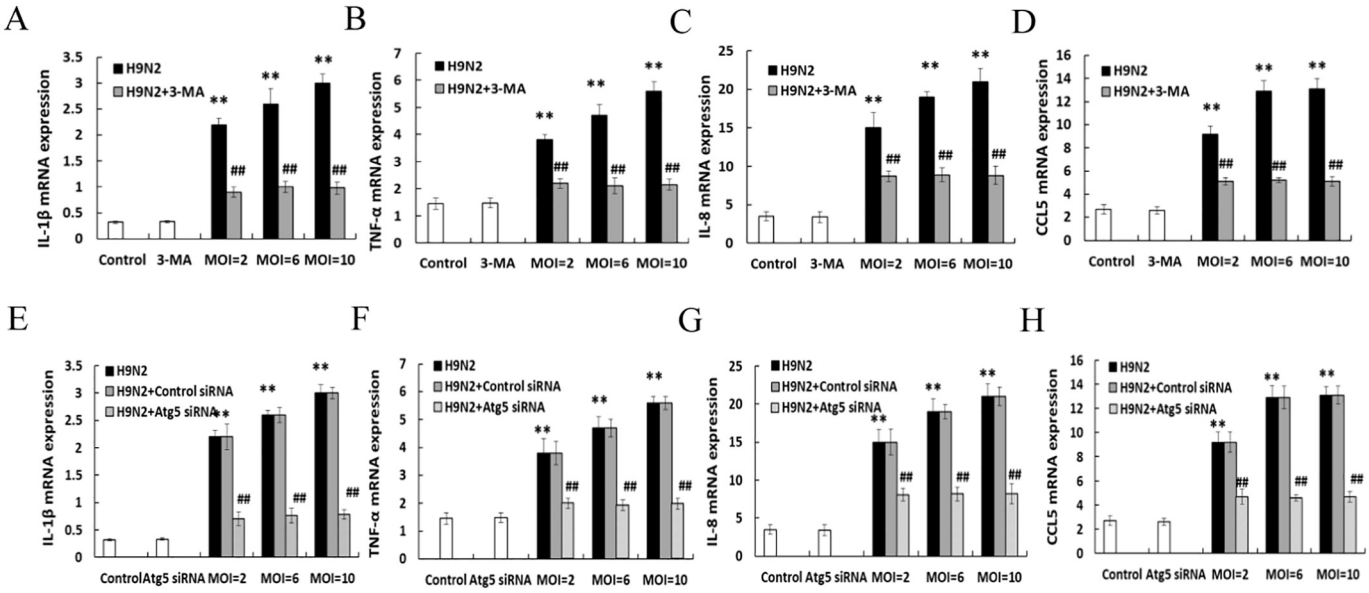

Fig. 6. Inhibiting autophagy reduced cytokines and chemokines in mice infected by H9N2 influenza virus.
BALB/C mice were pretreated with 3-MA, or control siRNA or Atg5 siRNA, followed by infection with H9N2 influenza virus at an MOI of 2, 6 or 10. Levels of inflammatory cytokines and chemokines in BALF were detected by real-time PCR (A–H). The data were showed as means ± SD ($n = 5$). $^{**}P < 0.01$ relative to control. $^{\#\#}P < 0.01$ relative to H9N2.

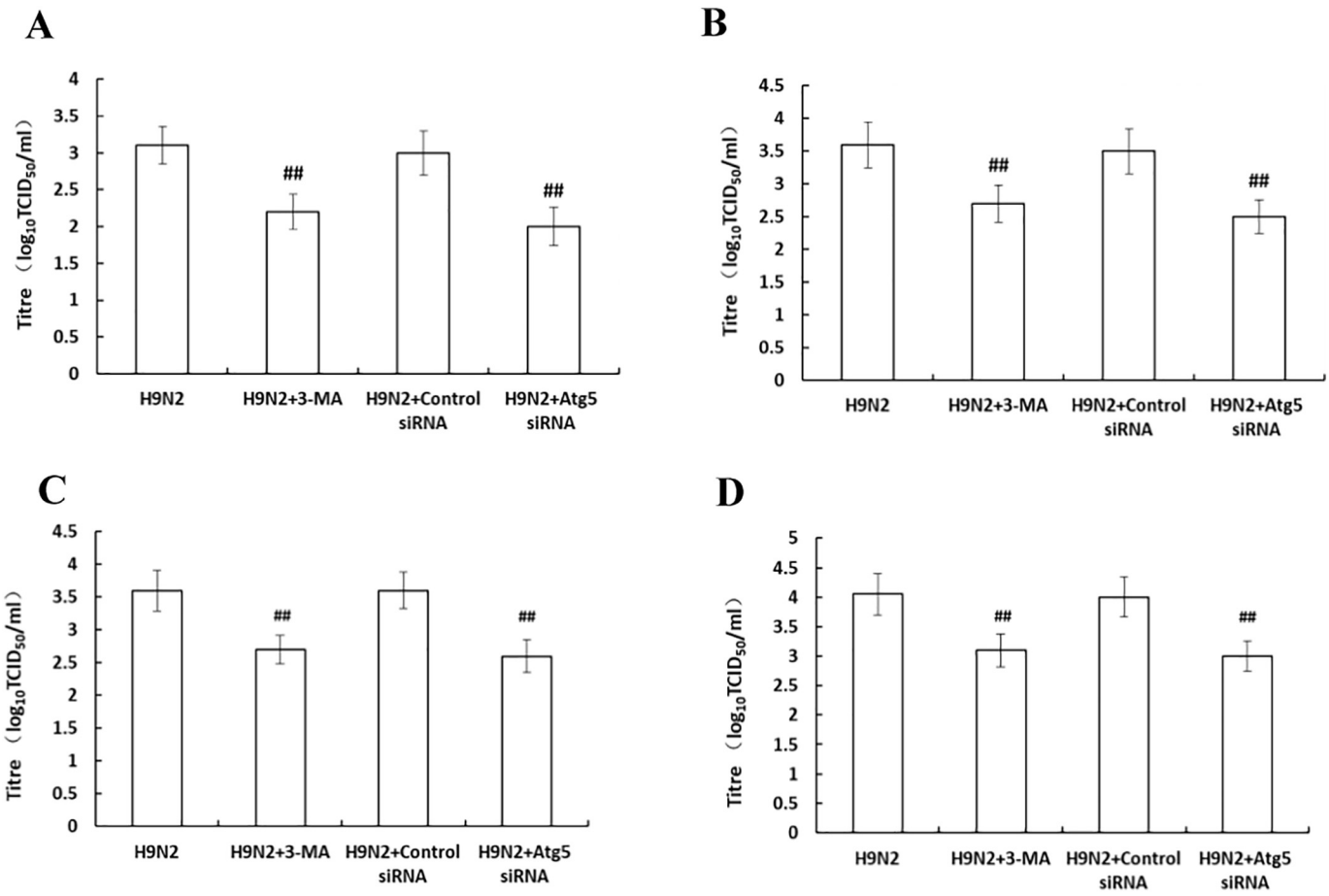

Fig. 7. Inhibiting autophagy decreased influenza A virus replication.
A549 cells and BALB/C mice were pretreated with 3-MA, or control siRNA or Atg5 siRNA, followed by infection with H9N2 influenza virus at MOI of 0.1 and 10. Supernatants from A549 cells (A and B, A, MOI = 0.1, B, MOI = 10) and lung tissue homogenates (C–D, C, MOI = 0.1, D, MOI = 10) were collected, and the virus titer was determined by measuring tissue culture infectious dose ($TCID_{50}$). The data were showed as means ± SD ($n = 5$). $^{\#\#}P < 0.01$ relative to H9N2.

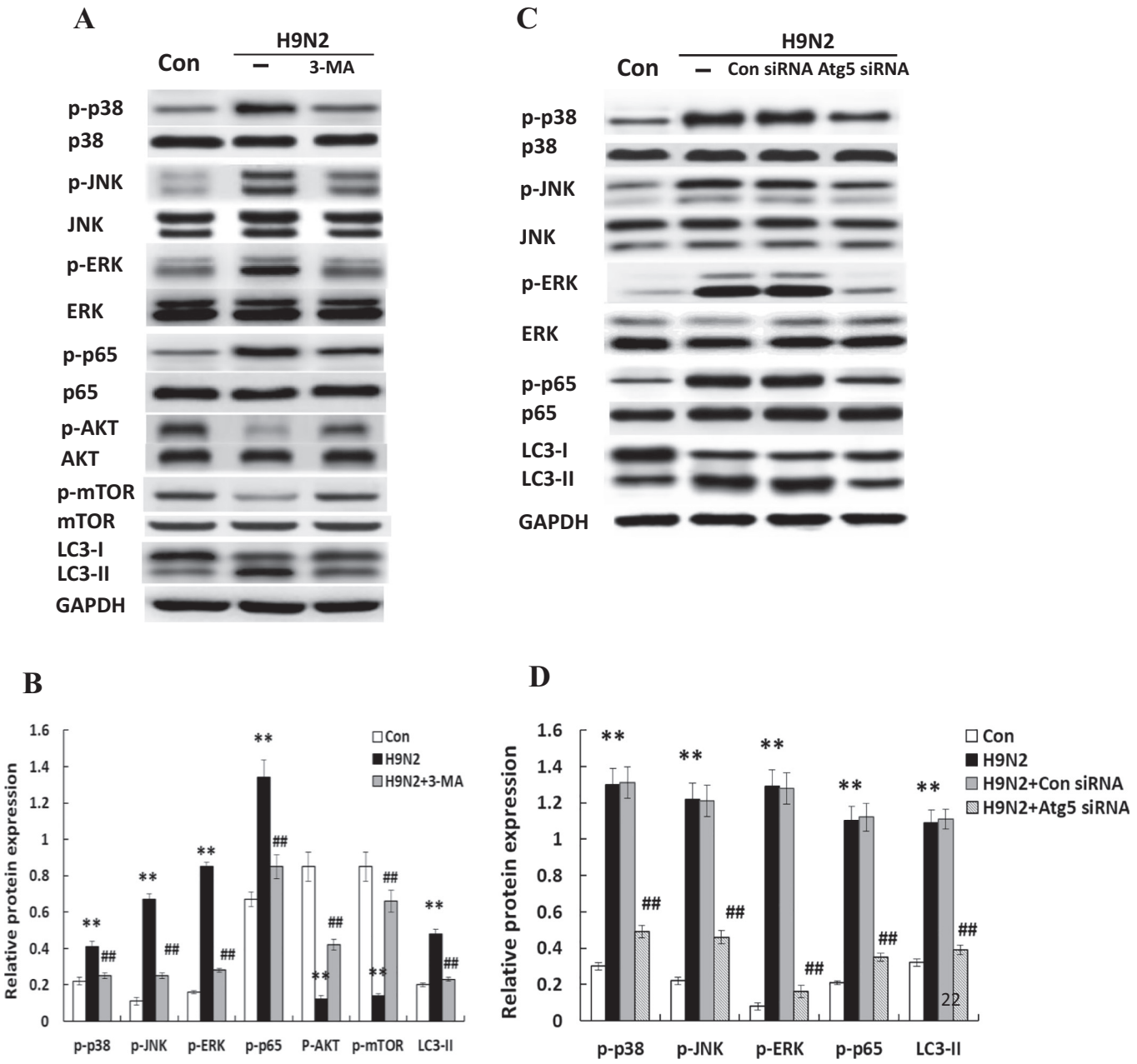

Fig. 8. Autophagy mediated the inflammatory responses through Akt-mTOR, MAPKs, and NF-κB signaling pathways in A549 cells.
A549 cells were pretreated with 3-MA (A and B), or transfected with control siRNA or Atg5 siRNA (C and D), followed by infection with H9N2 influenza virus. Then A549 cells were collected and the protein expression levels of LC3-II, phospho-pERK, phospho-pJNK, phospho-p38MAPK, phospho-pAkt, phospho-pmTOR, phospho-NF-κB p65, total ERK, JNK, p38MAPK, Akt, mTOR, and NF-κB p65 were detected by Western blotting. The protein levels of LC3-II were analyzed as ratios of intensities of GAPDH bands. The protein levels of phospho-pERK, phospho-pJNK, phospho-p38MAPK, phospho-pAkt, phospho-pmTOR, and phospho-NF-κB p65, were analyzed as ratios of intensities of total ERK, JNK, p38MAPK, Akt, mTOR and NF-κB p65 bands, respectively. The data were showed as means ± SD ($n \geq 5$). $^{**}P < 0.01$ relative to control. $^{\#\#}P < 0.01$ relative to H9N2.

autophagy in A549 cells infected by H9N2 virus.

3.6. Autophagy induced by H9N2 virus mediated the inflammatory responses through Akt-mTOR, MAPKs, and NF-κB signaling pathways in lung tissues

We further investigated autophagy induced by H9N2 virus mediated the inflammatory responses through Akt-mTOR, MAPKs and NF-κB signaling pathways in mice. H9N2 virus infection significantly results in an increase in the phosphorylation of ERK, JNK, p38 MAPK, and NF-κB p65 and a decrease in the phosphorylation of Akt and mTOR in lung tissues. H9N2 virus infection also increased the ratio of LC3II to GAPDH. Treatment with 3-MA markedly suppressed H9N2 virus-induced increase in the phosphorylation of ERK, JNK, p38 MAPK, and NF-κB p65 and restored the phosphorylation of Akt and mTOR (Fig. 9A and B). Similar to 3-MA, Atg5 siRNA treatment significantly reduced the increase in the phosphorylation of ERK, JNK, p38 MAPK induced by H9N2 virus (Fig. 9C and D). By contrast, control siRNA treatment did not lead to the decrease in the phosphorylation of ERK, JNK, p38 MAPK, and NF-κB p65. These data obviously demonstrated that the autophagy induced by H9N2 virus mediated the inflammatory responses through Akt-mTOR, MAPKs, and NF-κB signaling pathways in mice.

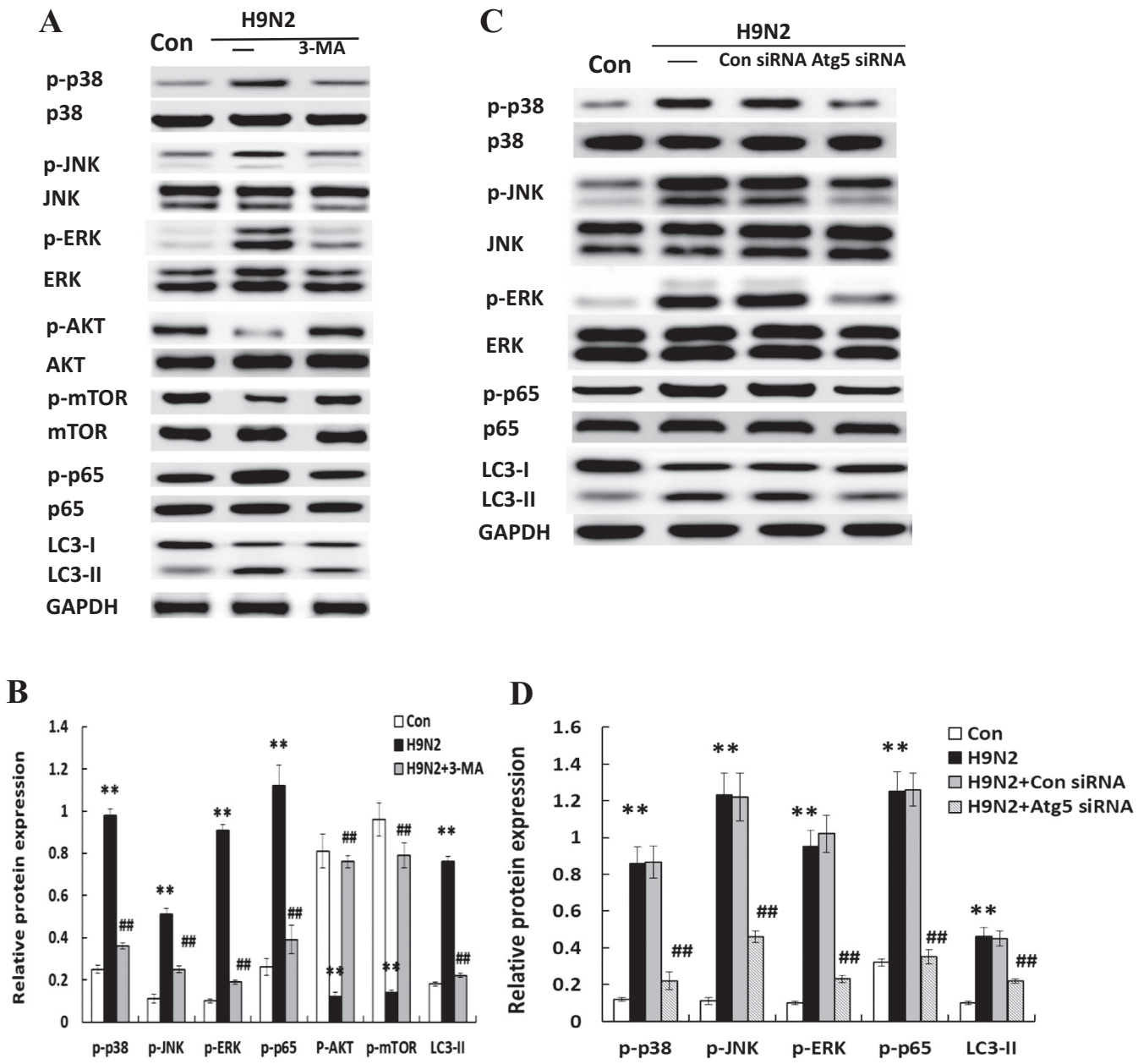

Fig. 9. Autophagy mediated the inflammatory responses through Akt-mTOR, MAPKs, and NF-κB signaling pathways in mice.
BALB/C mice were pretreated with 3-MA (A and B), or control siRNA or Atg5 siRNA (C and D), followed by infection with H9N2 influenza virus. Then mouse lung tissues were collected and the protein expression levels of LC3-II, phospho-pERK, phospho-pJNK, phospho-p38MAPK, phospho-pAkt, phospho-pmTOR, phospho-NF-κB p65, total JNK, ERK, p38MAPK, Akt, mTOR, and NF-κB p65 were detected by Western blotting. The protein levels of LC3-II were analyzed as ratios of intensities of GAPDH bands. The protein levels of phospho-pERK, phospho-pJNK, phospho-p38MAPK, phospho-pAkt, phospho-pmTOR, and phospho-NF-κB p65, were analyzed as ratios of intensities of total ERK, JNK, p38MAPK, Akt, mTOR and NF-κB p65 bands, respectively. The data were showed as means ± SD ($n = 5$). $^{**}P < 0.01$ relative to control. $^{\#\#}P < 0.01$ relative to H9N2.

4. Discussion

Influenza A viruses usually lead to economic loss to breeding farms and pose a serious threat to human health. However, the pathogenesis of influenza virus infection has not been well elucidated, which hindered the development of antiviral treatment. In this study, we have demonstrated that autophagy triggered by H9N2 influenza was essential for the inflammation in A549 cells and in mice, which functioned through Akt-mTOR, MAPKs, and NF-κB signaling pathways. Induction of autophagy is the major mechanism of H9N2 influenza virus-induced inflammation responses in vitro and in vivo, therefore, autophagy suppression may become effective therapeutic strategy to alleviate the pathogenesis of H9N2 influenza viruses.

Autophagy could be induced by different subtypes of influenza virus, such as H5N1, H3N2, and H1N1, playing the important role in the virus replication and pathogenic mechanism of influenza virus [18–21]. However, how autophagy involves in pathogenic mechanism of influenza virus has not been well elucidated yet. It is reported that autophagy did not affect the H5N1 virus replication, and inhibiting autophagy did not regulate the level of H5N1-induced cytokine in A549 cells [20]. However, in this study, the data showed that autophagy played an important role in regulating the H9N2 virus replication, and is essential for the inflammatory responses induced by H9N2 virus. Inhibiting autophagy by an autophagy inhibitor 3-MA or knockdown of

essential gene Atg5 by Atg5 siRNA treatment suppressed the H9N2 virus replication and the H9N2 virus-induced inflammatory responses in A549 cells and in mice. It was reported that viral infection of tissues and virus-induced increase in inflammatory cytokines and chemokines are two major factors in the pathogenic mechanism of influenza virus [5–8]. Our data showed that autophagy restricted the replication of H9N2 virus and inflammatory responses which are the two major factors in the pathogenic mechanism of influenza virus, so autophagy could be another important mechanism contributing to the pathogenesis of H9N2 influenza virus infection. Therefore autophagy could be target to treat influenza virus-caused inflammation.

Our previous study showed that NF-κB and MAPKs signaling pathways was involved in H9N2 influenza virus-triggered inflammatory responses [23]. H5N1 influenza virus infection resulted in a significant increase in inflammatory cytokines and chemokines through NF-κB signaling in human microvascular endothelial cells and in mice [32,33]. Moreover, previous studies showed that p38 MAPK was mediated the H5N1-triggered inflammatory cytokines in macrophages [34,35]. However, how the NF-κB and MAPKs signaling pathways mediates the inflammation induced by influenza virus remains unclear. The present study demonstrated that the autophagy was essential in H9N2 virus-induced inflammatory cytokines and chemokines in A549 cells and mice lung tissues, and inhibiting autophagy by an autophagy inhibitor 3-MA or knockdown of essential gene Atg5 suppressed the H9N2 virus induced inflammation. Besides, autophagy mediated virus-induced inflammation through MAPKs and NF-κB signaling, and autophagy inhibiting suppressed the virus-triggered activation of MAPKs and NF-κB signaling. It has been reported the induction of autophagy could be mediated by inhibition of Akt-mTOR signaling in IAV-infected HPMECs [25]. The present study demonstrated that Akt-mTOR signaling pathway plays a critical role in the attenuation of proinflammatory responses and inhibition of autophagy in A549 cells infected by H9N2 virus.

In conclusion, autophagy is essential in H9N2 influenza virus-triggered increase in inflammatory cytokines and chemokines in A549 cells and in mice. Autophagy induced by H9N2 influenza virus mediates inflammatory responses through Akt-mTOR, MAPKs and NF-κB signaling pathways. This study showed the definite connection between H9N2-triggered autophagy and inflammation responses, which supplements the pathogenesis of H9N2 virus. Suppressing autophagy could be target to treat influenza virus-caused inflammation responses.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31602030, No. 31672522), General Project Hebei North University (No. YB2018012). Postgraduate Research and Innovation Funding Project of Inner Mongolia Autonomous Region (B2018111941Z). Scientific and Technical Research Program of Zhangjiakou in HeBei province (1811037C). Special Funds for Layer and Broiler Innovation Team of the Second Phase of Agricultural Modernization Industry System of Hebei Province (Specialist Position for the Prevention and Control of Broiler Disease) (No. HBCT2018150207).

References

[1] G.R. Bai, M. Chittaganpitch, Y. Kanai, Y.G. Li, W. Auwanit, K. Ikuta, P. Sawanpanyalert, Amantadine- and oseltamivir-resistant variants of influenza A viruses in Thailand, Biochem. Biophys. Res. Commun. 390 (2009) 897–901.
[2] T.T. Bauer, S. Ewig, A.C. Rodloff, E.E. Muller, Acute respiratory distress syndrome and pneumonia: a comprehensive review of clinical data, Clin. Infect. Dis. 43 (2006) 748–756.
[3] A. Gambotto, S.M. Barratt-Boyes, M.D. de Jong, G. Neumann, Y. Kawaoka, Human infection with highly pathogenic H5N1 influenza virus, Lancet 371 (9622) (2008) 1464–1475.
[4] S. Herold, C. Becker, K.M. Ridge, G.R. Budinger, Influenza virus-induced lung injury: pathogenesis and implications for treatment, Eur. Respir. J (2015) 1463–1478.
[5] T.T. Thanh, H.R. van Doorn, M.D. de Jong, Human H5N1 influenza: current insight into pathogenesis, Int. J. Biochem. Cell Biol. 40 (2008) 2671e2674.
[6] A.C. Boon, D. Finkelstein, M. Zheng, G. Liao, J. Allard, K. Klumpp, R. Webster, G. Peltz, R.J. Webby, H5N1 influenza virus pathogenesis in genetically diverse mice is mediated at the level of viral load, MBio 2 (2011) e00171–11.
[7] H. Wang, C. Jiang, Avian influenza H5N1: an update on molecular pathogenesis, Sci China C Life Sci 52 (2009) 459–463.
[8] G. Neumann, H. Chen, G.F. Gao, Y. Shu, Y. Kawaoka, H5N1 influenza viruses: outbreaks and biological properties, Cell Res. 20 (2010) 51–61.
[9] C. Korteweg, J. Gu, Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans, Am. J. Pathol. 172 (2008) 1155–1170.
[10] Z. Zhou, X. Jiang, D. Liu, Z. Fan, X. Hu, et al., Autophagy is involved in influenza A virus replication, Autophagy 5 (2009) 321–328.
[11] D.J. Klionsky, S.D. Emr, Autophagy as a regulated pathway of cellular degradation, Science 290 (2000) 1717–1721.
[12] B. Levine, Eating oneself and uninvited guests: autophagy-related pathways in cellular defense, Cell 120 (2005) 159–162.
[13] D.J. Klionsky, The molecular machinery of autophagy: unanswered questions, J. Cell Sci. 118 (2005) 7–18.
[14] A. Orvedahl, B. Levine, Eating the enemy within: autophagy in infectious diseases, Cell Death Differ. 16 (2009) 57–69.
[15] J.S. Rossman, R.A. Lamb, Autophagy, apoptosis, and the influenza virus M2 protein, Cell Host Microbe 6 (2009) 299–300.
[16] T. Johansen, T. Lamark, Selective autophagy mediated by autophagic adapter proteins, Autophagy 7 (2011) 279–296.
[17] C.M. Fader, M.I. Colombo, Autophagy and multivesicular bodies: two closely related partners, Cell Death Differ. 16 (2009) 70–78.
[18] M. Gannage, D. Dormann, R. Albrecht, J. Dengjel, T. Torossi, P.C. Ramer, M. Lee, T. Strowig, F. Arrey, G. Conenello, M. Pypaert, J. Andersen, A. Garcia-Sastre, C. Munz, Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes, Cell Host Microbe 6 (2009) 367–380.
[19] A.H. Law, D.C. Lee, K.Y. Yuen, M. Peiris, A.S. Lau, Cellular response to influenza virus infection: a potential role for autophagy in CXCL10 and interferon-alpha induction, Cell Mol. Immunol. 7 (2010) 263–270.
[20] Y. Sun, C. Li, Y. Shu, X. Ju, Z. Zou, H. Wang, S. Rao, F. Guo, H. Liu, W. Nan, Y. Zhao, Y. Yan, J. Tang, C. Zhao, P. Yang, K. Liu, S. Wang, H. Lu, X. Li, L. Tan, R. Gao, J. Song, X. Gao, X. Tian, Y. Qin, K.F. Xu, D. Li, N. Jin, C. Jiang, Inhibition of autophagy ameliorates acute lung injury caused by avian influenza A H5N1 infection, Sci. Signal. 5 (2012) ra16.
[21] R. Wang, Y. Zhu, J. Zhao, C. Ren, P. Li, H. Chen, M. Jin, H. Zhou, Autophagy Promotes Replication of Influenza A Virus In Vitro, J Virol (2019) (pii:e01984-18).
[22] J.P. Dai, W.Z. Li, X.F. Zhao, G.F. Wang, J.C. Yang, L. Zhang, X.X. Chen, Y.X. Xu, K.S. Li, A drug screening method based on the autophagy pathway and studies of the mechanism of evodiamine against influenza A virus, PLoS One 7 (2012) e42706.
[23] R. Zhang, X. Ai, Y. Duan, M. Xue, W. He, C. Wang, T. Xu, M. Xu, B. Liu, C. Li, Z. Wang, R. Zhang, G. Wang, S. Tian, H. Liu, Kaempferol ameliorates H9N2 swine influenza virus-induced acute lung injury by inactivation of TLR4/MyD88-mediated NF-κB and MAPK signaling pathways, Biomed. Pharmacother. 89 (2017) 660–672.
[24] H. Pan, Y. Zhang, Z. Luo, P. Li, L. Liu, C. Wang, H. Wang, H. Li, Y. Ma, Autophagy mediates avian influenza H5N1 pseudotyped particle-induced lung inflammation through NF-κB and p38MAPK signaling pathways, Am. J. Physiol. Lung Cell Mol. Physiol. 306 (2) (2014) L183–L195.
[25] L. Wang, H. Jiang, S.M. Shen, C.X. Wen, Z. Xing, Y. Shi, Inhibition of autophagy and chemokine induction by sphingosine 1-phosphate receptor 1 through NF-κB signaling in human pulmonary endothelial cells infected with influenza A viruses, PLoS One 13 (10) (2018 Oct 10) e0205344.
[26] N. Mizushima, T. Yoshimori, Y. Ohsumi, The role of Atg proteins in autophagosome formation, Annu. Rev. Cell Dev. Biol. 27 (2011) 107–132.
[27] K. Yoshimura, M. Shibata, M. Koike, K. Gotoh, M. Fukaya, M. Watanabe, Y. Uchiyama, Effects of RNA interference of Atg4B on the limited proteolysis of LC3 in PC12 cells and expression of Atg4B in various rat tissues, Autophagy 2 (2006) 200–208.
[28] N. Mizushima, T. Yoshimori, How to interpret LC3 immunoblotting, Autophagy 3 (2007) 542–545.
[29] R.H. Zhang, C.H. Li, C.L. Wang, M.J. Xu, T. Xu, D. Wei, B.J. Liu, G.H. Wang, S.F. Tian, N-acetyl-l-cystine (NAC) protects against H9N2 swine influenza virus-induced acute lung injury, Int. Immunopharmacol. 22 (1) (2014) 1–8.
[30] N. Feizi, P. Mehrbod, B. Romani, H. Soleimanjahi, T. Bamdad, A. Feizi, E.O. Jazaeri, H.S. Targhi, M. Saleh, A. Jamali, F. Fotouhi, R.N. Nargesabad, A. Abdoli, Autophagy induction regulates influenza virus replication in a time-dependent manner, J. Med. Microbiol. 66 (4) (2017) 536–541.
[31] B. Yeganeh, S. Ghavami, M.N. Rahim, T. Klonisch, A.J. Halayko, K.M. Coombs, Autophagy activation is required for influenza A virus-induced apoptosis and replication, Biochim. Biophys. Acta Mol. Cell Res. 1865 (2) (2018) 364–378.
[32] K. Droebner, S.J. Reiling, O. Planz, Role of hypercytokinemia in NFkappaB p50-deficient mice after H5N1 influenza A virus infection, J. Virol. 82 (2008) 11461–11466.
[33] M. Schmolke, D. Viemann, J. Roth, S. Ludwig, Essential impact of NF-kappaB signaling on the H5N1 influenza A virus-induced transcriptome, J. Immunol. 183 (2009) 5180–5189.
[34] K.P. Hui, S.M. Lee, C.Y. Cheung, I.H. Ng, L.L. Poon, Y. Guan, N.Y. Ip, A.S. Lau, J.S. Peiris, Induction of inflammatory cytokines in primary human macrophages by influenza A virus (H5N1) is selectively regulated by IFN regulatory factor 3 and p38 MAPK, J. Immunol. 182 (2009) 1088–1098.
[35] D.C. Lee, C.Y. Cheung, A.H. Law, C.K. Mok, M. Peiris, A.S. Lau, p38 mitogen-activated protein kinase-dependent hyperinduction of tumor necrosis factor alpha expression in response to avian influenza virus H5N1, J. Virol. 79 (2005) 10147–10154.