Autophagy mediates avian influenza H5N1 pseudotyped particle-induced lung inflammation through NF-κB and p38 MAPK signaling pathways

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H5N1 viruses are highly pathogenic avian influenza A viruses (IAV) causing severe economic loss to breeding farms. In the past decades, H5N1 viruses have been reported to cross the species barrier to infect humans and to result in over 60% mortality of human infections (14). The patients infected with H5N1 viruses usually suffered from severe pneumonia with rapid progression to respiratory failure due to the acute respiratory distress syndrome, which is primarily attributable to the alveolar epithelial cell death and severe lung tissue injury. The major pathogenic mechanism could lie in the high viral loading and active viral replication (1). On the other hand, H5N1-infected patients showed significantly increased proinflammatory cytokines and chemokines both in serum and lung tissues (10), suggesting that virus-induced hypercytokemia is another key mechanism contributing to H5N1-caused lung tissue injury (50). H5N1-induced hypercytokemia can be related with several viral components, such as PA, PB1, and PB2 proteins (36, 45). More recently, hemagglutinin (HA) protein of H5N1 viruses has been reported as a major viral component responsible for the production of cytokines and chemokines in H5N1-infected cells (4). In addition, administration of HA protein of H5N1 viruses by intratracheal instillation caused severe lung inflammation in mice, including thickening of the interalveolar septum and neutrophil infiltration (55). However, the underlying molecular mechanism by which H5N1 triggers the inflammatory responses remains unclear.

Autophagy is the highly conservative cellular degradation machinery that maintains the cellular homeostasis by eliminating unwanted proteins and damaged organelles as well as controls microbe invasion (26, 30). Autophagy can be induced by various stimuli (e.g., nutrient starvation and pathogen infection) and is initiated by the inhibition of the mammalian target of rapamycin (mTOR) and activation of Beclin1-phosphatidylinositol-3 kinase (PI3K) complexes, followed by the formation of double-membraned autophagosomes (35). The elongation of autophagosomes requires the involvement of two ubiquitin-like conjugation systems, including Atg12-Atg5 and Atg8-PE (LC3-II in mammalian cells). The latter is assembled on the matured autophagosomes, therefore serving as a marker for autophagosome formation. P62/SQSTM1 (p62) has been reported as an autophagic adaptor protein that interacts with LC3-II and facilitates the delivery of cytoplasmic cargos, including misfolded proteins, organelles, bacteria, and viruses (xenophagy), into the elongating autophagosomes (22). When the autophagosome is fully formed and closed, it could fuse with the endosome/lysosome system for degradation (12).

Previous studies demonstrated that autophagy not only participated in many physiological processes, such as protecting cells from stress and infection, regulating cell survival, and death (18, 39), but also in the pathogenesis of human diseases (6). Especially in lung tissues, accumulating data revealed a strong association between elevated autophagy and several lung diseases, such as chronic obstructive pulmonary disease and cigarette smoke-induced emphysema (38). More recent studies have shown that autophagy could be induced by a group of IAVs, such as H1N1, H3N2, and H9N2, participating in the replication and pathogenesis of IAVs (15, 28, 48, 57). Law et al. (28) reported that autophagy was required for the

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production of proinflammatory cytokines and chemokines in H1N1- or H9N2-infected human blood macrophages. Administration of evodiamine, an autophagy inhibitor, significantly attenuated H1N1-induced cytokine production and cell death in A549 human lung epithelial cells (9). These data indicated the involvement of autophagy in IAV-induced lung inflammation. On the other hand, although the induction of autophagy has been reported to mediate cell death in H5N1-infected cells and tissues (32), its role in H5N1-triggered lung inflammation remains unclear.

Pseudotyped viral particles are a reliable model to mimic authentic influenza viruses and study surface protein-related antigenicity and pathogenesis (16). In the present study, we generated H5N1 pseudotyped viral particles (H5N1pps) as previously reported (51) to investigate the relationship between autophagy and H5N1-induced lung inflammation. The results showed that H5N1pps not only induced proinflammatory cytokines and chemokines but also triggered autophagy both in human lung epithelial A549 cells and in mouse lung tissues. Inhibiting the autophagy significantly attenuated H5N1pp-induced cytokines and chemokines as well as lung inflammation, indicating the critical role of autophagy in H5N1-caused inflammation. Autophagy mediated the inflammatory responses through NF-κB and p38 MAPK signaling pathways, which required the presence of clathrin but did not rely on p62 or autophagosome-lysosome fusion. Moreover, the activation of NF-κB signaling also promoted H5N1pp-induced autophagosome formation. The positive feedback loop between autophagy and NF-κB signaling cascade could be an important mechanism contributing to H5N1-induced hypercytokinemia. Our data demonstrated a central role of autophagy in H5N1pp-triggered inflammatory responses, and targeting the autophagy could be a potential strategy to treat H5N1 virus-caused lung inflammation.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents. The plasmids of pSV-Mo-MLV-Gag-pol, pMP71-eGFP-pre, pHCMV-H5, and pHCMV-N1 were prepared as described previously (51). The tandem mRFP-GFP-LC3B plasmid was provided by Dr. Hongchang Li from Shenzhen Institution of Advanced Technology, Chinese Academy of Science. The polyclonal or monoclonal antibodies against LC3B, p62, Beclin1 (D40C5), Atg5 (D1G9), phospho-p38 MAPK (D3F9), and NF-κB p65 subunit (C22B4) were purchased from Cell Signaling Technology (Beverly, MA). The rabbit anti-H5N1 HA (H5) polyclonal antibody was obtained from Sino Biological (Beijing, China), 3-Methyladenine (3-MA), chloroquine (CQ), SB203580, and BAY11-7082 were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture and plasmid transfection. Human embryonic kidney (HEK293T) cells and human lung epithelial cell line (A549) were purchased from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Hyclone, Thermo Scientific, Rockford, IL) medium supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37°C in a humidified atmosphere with 5% CO2. In some experiments, A549 cells were transiently transfected with plasmids of pSV-Mo-MLV-Gag-pol, pMP71-eGFP-pre, pHCMV-H5, and/or pHCMV-N1 by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Preparation of H5N1pps in HEK293T cells. The H5N1pps were prepared in HEK293T cells by transfection with the envelope plasmids and package plasmids as previously described (51). Briefly, HEK293T cells with 80% confluence were transiently transfected with plasmids including pSV-Mo-MLV-Gag-pol, pMP71-eGFP-pre, pHCMV-wH5, and pHCMV-N1 using calcium phosphate transfection kits (Promega, Madison, WI) as previously described (49). Six hours later, the cells were cultured in fresh DMEM medium with 2% FBS for another 48 h. The culture supernatants were carefully collected and H5N1pps in the supernatants were harvested by ultracentrifugation through a 20% sucrose cushion in SW28 Ti Rotor (Beckman Coulter, Brea, CA) at 25,000 rpm for 2 h. The protein concentration of purified H5N1pps was determined by use of a BCA protein assay kit (Pierce, Rockford, IL). To determine the titer of H5N1pps, HEK293T cells were incubated with 100 μl of serially diluted H5N1pps for 24 h. The titer was calculated as clone forming units (CFU)/ml according to the following formula: CFU/ml = the number of green fluorescent protein (GFP)-positive cells per well × dilution folds × 10.

Viral infection. A549 cells were incubated with H5N1pps [multiplicity of infection (MOI) = 10] in the DMEM medium supplemented with 2% FBS. In some experiments, A549 cells were pretreated with a variety of inhibitors, including 5 mM 3-MA (a PI3K inhibitor), 50 μM CQ (an inhibitor of lysosome acidification), 20 μM SB203580 (a p38 inhibitor), or 10 μM BAY11-7082 (a NF-κB inhibitor) for 0.5 or 1 h, followed by H5N1pp infection (MOI = 10).

Fluorescence microscopy and immunofluorescence staining. A549 cells were seeded into eight-well Lab-Tek chamber slides (Thermo Fisher Scientific, Waltham, MA) and transiently transfected with plasmids of EGFP-LC3, mRFP-LC3, mRFP-GFP-LC3, or DsRed-Rab5 with Lipofectamine 2000. At 24 h posttransfection, the cells were infected with H5N1pps (MOI = 10) for 3 or 8 h, followed by washing three times with PBS and fixated by 10.220.32.246 on April 6, 2017 http://ajplung.physiology.org/ Downloaded from

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for 15 min. The fluorescent images were recorded by use of a confocal laser scanning microscope (TCS SP5II, Leica, Ernst-Leitz-Strasse, Germany) or a fluorescence microscope (Leica).

To detect the intracellular H5N1pps, LC3 puncta, p62 aggregates, or NF-κB in A549 cells, cells were fixed and permeabilized with 0.25% Triton X-100 in PBS for 10 min, followed by blocking with 10% donkey serum albumin for 30 min. After blocking, the cells were incubated with rabbit antibodies against H5, LC3B, p62, or NF-κB p65 subunit for 2 h, followed by incubation with Alexa 488-conjugated donkey anti-rabbit antibody for another 1 h. Cells were labeled with 75 nM lysoTracker Red (a lysosomal probe, Life Technologies) or Hoechst 33258 (Sigma) to identify lysosomes and nucleus. The fluorescent images were recorded by confocal microscopy.

Single luciferase reporter assay of AP-1 and NF-κB activity. A549 cells were transiently transfected with AP-1 reporter plasmid (pAP-1-luc, Stratagene, La Jolla, CA) or NF-κB reporter plasmid (pNF-κB-luc, Stratagene) by using Lipofectamine 2000, followed by H5N1pp infection. At 24 h postinfection (p.i.), the cells were harvested and lysed with cell culture lysis buffer (Promega, Madison, WI) on ice for 30 min with agitation at 100 rpm. The supernatant was collected by centrifugation at 14,000 g for 10 min, and the total protein concentrations were determined by use of a BCA protein assay kit. The firefly luciferase levels in the supernatant were determined by use of luciferase reporter system (Promega) with a multifunction microplates reader (BioTek, Winooski, VT), and the final firefly luciferase activity was normalized to the total protein concentration.

Real-time PCR. Total RNA was extracted from A549 cells or lung tissues by using a multisource total RNA miniprep kit (Axygen) or TRIzol reagent (Life Technologies), respectively, according to the manufacturer’s instructions, followed by reverse transcription into cDNA. Real-time PCR was performed by using Thunder Bird SYBR qPCR mix (Toyobo) in a Lightcycler 480II instrument (Roche Applied Sciences). The sequences of primers for IL-1β, IL-6, TNF-α, CCL2, CCL5, IL-8, and CXCL2 were listed in Table 1.

Western blotting analysis. Cells were lysed in RIPA buffer (Beyotime, China) and the total protein concentration in supernatant was determined by a BCA protein assay kit. In some experiments, the cytoplasmic and nuclear fractions of cell lysates were separated, using...
nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer’s instructions. Equal amount of lysates was separated by 12% SDS-PAGE gel and transferred to PVDF membranes. Immuno blotting was performed by incubation with anti-LC3B, anti-p65, anti-p-p38, or anti-p62 primary antibodies, followed by horseradish peroxidase-coupled goat anti-mouse or anti-rabbit secondary antibody (KPL, Gaithersburg, MD), and finally visualized with enhanced chemiluminescence system (Pierce).

**RNA interference.** A549 cells were transiently transfected with small interfering RNAs (siRNAs) of Beclin1, Atg5, the heavy chain of clathrin, or p62 using Lipofectamine 2000 as described above. The siRNA sequences of human Beclin1 (siBeclin1), Atg5 (siAtg5), clathrin (siHC), and p62 (sip62) were 5′-AACAGTTGGCACAATCAATA-3′, 5′-AACACATCTGGATGGGATTG-3′, 5′-AAGCTGGGAAAACTTCTCAGA-3′, and 5′-GCAATGGATTGATATCGAT-3′, respectively. At 48 h posttransfection, the protein levels of Beclin1, Atg5, clathrin, and p62 were determined by Western blotting analysis.

**Animals and intranasal inoculation.** Six-week-old female C57/BL6 mice were purchased from the Laboratory Animal Center of Guangdong province and housed under specific pathogen-free conditions. All animal procedures and protocols were approved by the institution Animal Care and Use Committee at Chinese Academy of Sciences. All animal procedures and protocols were approved by the institution Animal Care and Use Committee at Chinese Academy of Sciences. The mice were pretreated with 3-MA (15 mg/kg) for 1.5 h via intraperitoneal injection and then inoculated intranasally with H5N1pps (1 × 10^7 CFU) as previously described (48).

**Histological staining of lung tissues.** At 3–4 days p.i., mice were euthanized and the lungs were perfused with 10% formalin or homogenized for extraction of total RNA and protein. After dehydration in 25% sucrose solutions for 24 h, the fixed lung tissues were embedded in OCT medium (Sakura Finetek USA, Torrance, CA) and sectioned at 10 μm. GFP expression in lung tissues was examined using fluorescence microscopy as described above. Hematoxylin and eosin staining was performed as previously described (2). Briefly, the sections were rehydrated in gradient ethanol solutions (from 100% to 75%), followed by staining with hematoxylin and eosin, and finally mounted with neutral balsam after dehydration. The images were analyzed by use of an inverted microscope (Olympus, Japan). Lung pathology was assessed by the histopathological scoring system as previously described (3, 54).

**Statistical analysis.** Data were represented as means ± SE from at least three independent experiments. The differences among groups were calculated by paired Student’s t-test or one-way ANOVA analysis followed by Tukey’s posttest (GraphPad Prism, GraphPad Software, La Jolla, CA).

**RESULTS**

H5N1pps induced inflammatory responses in both A549 cells and mouse lung tissues. The H5N1pps were generated in HEK293T cells by incorporating the H5N1 HA (A/swine/Anhui/ca/2004) and H1N1 neuraminidase (NA; A/Puerto Rico/8/34) onto the surface of murine leukemia virus (MLV) Gag-pol core particles containing an EGFP reporter gene, as described previously (51). The expression of H5N1 HA on the H5N1pps was examined by Western blotting using anti-H5N1 HA polyclonal antibody or the anti-serum of H5N1pp-immunized mice (Fig. 1A). To evaluate the in vitro infectivity of H5N1pps, A549 human lung epithelial cells were infected with H5N1pps followed by immunofluorescent staining using anti-HA antibody. The results showed that H5N1pps were internalized by most of A549 cells at 3 h p.i. (Fig. 1B). Moreover, the EGFP reporter gene of H5N1pps was expressed in A549 cells and mouse lung tissues at 24 h p.i. and 96 p.i., respectively, further confirming the infectivity of H5N1pps (Fig. 1, C and D).

The inflammatory responses in lung tissues have been reported as a major mechanism contributing to H5N1-caused acute lung injury (17, 47). In the present study, C57/BL6 mice intranasally inoculated with H5N1pps showed significant infiltration of immune cells and thickening of epithelial cells in lung tissues 4 days p.i. (Fig. 1E), indicating the potent proinflammatory effects of H5N1pps in vivo. Moreover, H5N1pp...
infection significantly upregulated the mRNA expression of proinflammatory cytokines and chemokines, such as IL-1β, TNF-α, IL-6, IL-8, CCL2, CCL5, and CXCL2 in A549 cells (Fig. 1F), which probably contributed to the proinflammatory effects of H5N1pps in vivo. It was reported that wild-type H5N1 virus induced greater amounts of chemokines in HUVEC cells and more serious lung inflammation in mice than what we observed in this study (48, 52), which could be attributable to both the defect of H5N1pps in virus replication and other contributors such as PA, PB1, and PB2 proteins of H5N1 virus involved in the inflammatory responses.

To identify which viral component contributed to H5N1pp-induced inflammatory responses, A549 cells were transiently transfected with plasmids expressing H5N1 HA, NA, or MLV Gag-pol core particles. The results showed that the mRNA levels of proinflammatory cytokines and chemokines were significantly elevated by HA but not by NA or the components of MLV core particles (Fig. 1G), indicating the critical role of HA in H5N1-induced inflammation. Overall, H5N1pps were capable of not only infecting human lung epithelial cells but also triggering inflammatory responses both in vitro and in vivo.

H5N1pp-induced autophagy was essential for the inflammatory responses in A549 cells. The dynamic process of autophagy involves autophagosome formation, autophagosome-lysosome fusion, and the degradation of sequestered cargoes by lysosomal hydrolases (35). We first evaluated the autophagosome formation by examining the LC3-II expression and GFP-LC3 puncta as previously described (25, 34). The results showed that both GFP-LC3 puncta and LC3-II expression were significantly induced in A549 cells by H5N1pps and rapamycin, an mTOR inhibitor and autophagy inducer (Fig. 2, A–C).

Fig. 3. Autophagy mediated the production of cytokines and chemokines induced by H5N1pps in A549 cells. A549 cells were pretreated with 3-MA (A), or transfected with control siRNA (siCtrl) or siRNA sequences of human Beclin1 (siBCN1) or Atg5 (siAtg5) (C) prior to H5N1pp infection [multiplicity of infection (MOI) = 10], mRNA levels of cytokines and chemokines in A549 cells were determined at 12 h p.i. by real-time PCR. Data shown are means ± SE from 4 independent experiments. Differences between groups were analyzed by 1-way ANOVA followed by Tukey’s posttest or by paired t-test. *P < 0.05. Expression levels of Beclin1, Atg5, or LC3B in A549 cells transfected with siCtrl, siBCN1, or siAtg5 were determined at 3 h p.i. by Western blotting (B).

Fig. 4. Clathrin played an essential role in H5N1pp-induced autophagy and inflammatory responses. A549 cells were transfected with siCtrl or siRNA of clathrin (siCHC) prior to H5N1pp infection (PPs, MOI = 10). Cells were labeled with anti-HA antibody at 3 h p.i., and the intracellular H5N1pps was detected by confocal imaging (A). Scale bar, 5 μm. Cells were harvested at 3 h p.i., and expression of clathrin (CHC) (B) and LC3B (C) in cell lysates was determined by Western blotting. mRNA levels of cytokines and chemokines in A549 cells were determined at 12 h p.i. by real-time PCR (D). Data shown are means ± SE (n = 5), and differences between groups were analyzed by paired t-test. *P < 0.05.
indicating enhanced autophagosome formation. Pretreatment with 3-MA, an inhibitor of classic III PI3K, effectively attenuated the induction of autophagy by H5N1pps (Fig. 2, A–C). Since accumulated LC3-II could be attributable to increased autophagosome formation or decreased lysosomal fusion and degradation, we next blocked autophagosome-lysosome fusion by using CQ, an inhibitor of lysosome acidification. The results showed that the pretreatment of CQ further promoted the accumulation of LC3-II in H5N1pp-infected A549 cells (Fig. 2D), which excluded the possibility of lysosomal dysfunction-caused LC3-II accumulation. P62 is another multifunctional autophagic adaptor protein involved in autophagic degradation. In the present study, H5N1pp infection reduced the protein level of p62 in A549 cells, while blocking the autophagosome-lysosome fusion with CQ rescued p62 level (Fig. 2E). These results further confirmed H5N1pp-promoted autophagic degradation.

To further determine which viral component contributed to H5N1pp-induced autophagy, A549 cells were transfected with equal amount of plasmids expressing H5N1 HA, NA, or MLV Gag-pol core particles. As expected, HA alone most potently triggered autophagosome formation and elevated the LC3-II expression in A549 cells. In contrast, MLV Gag-pol plus GFP barely induced autophagy. Compared with HA alone, HA plus NA appeared to induce autophagy less effectively. These data indicated HA as the major contributor in H5N1pp-triggered autophagy (Fig. 2, F–H).

We next investigated the role of autophagy in H5N1pp-induced inflammatory responses. The pretreatment of 3-MA, an inhibitor of classic III PI3K and autophagy, significantly attenuated H5N1pp-induced proinflammatory cytokines and chemokines (such as IL-1β, IL-6, TNF-α, CCL2, and CCL5) in A549 cells. These results suggested the potential involvement of autophagy in virus-induced inflammation (Fig. 3A). To further block the autophagic pathway, autophagy-related gene atg5 or beclin1 was deleted with siRNA. The results showed that the gene knockdown of atg5 or beclin1 not only attenuated H5N1pp-triggered autophagosome formation but also dramat-

![Image](http://apjplung.physiology.org/)
ually decreased the induction of proinflammatory factors by viral infection (Fig. 3, B and C). Hence autophagy played an essential role in H5N1pp-induced inflammatory responses.

Autophagy-mediated inflammatory responses relied on clathrin but not on the fusion between autophagosomes and lysosomes in H5N1pp-infected A549 cells. The invasion of influenza H5N1 virus to host cells is initiated by the interaction between HA and sialic acid receptor on host cell surface, followed by clathrin-mediated endocytosis, and finally fused into lysosomes (53). However, it is unknown whether clathrin is involved in H5N1-induced autophagy. In the present study, deletion of clathrin with siRNA not only reduced the internalization of H5N1pp but also attenuated H5N1pp-elevated LC3-II expression (Fig. 4, A–C). Moreover, deletion of clathrin dramatically diminished the production of proinflammatory cytokines and chemokines induced by H5N1pp (Fig. 4D). These results demonstrated an essential role of clathrin in H5N1pp-induced autophagy and inflammatory responses.

Upon internalization, H5N1 viruses have been reported to be processed through the endosome-lysosome pathways (23). In the present study, over 70% of H5N1pp were found to be colocalized with DsRed-Rab5+ early endosomes at 3 h p.i. (Fig. 5, A and B), consistent with previous reports (27). In the meantime, about 60% of H5N1pp were observed to be colocalized with autophagosomes (LC3 puncta). In addition, majority of H5N1pp-induced autophagosomes were also colocalized with endosomes. These data implied that autophagosomes fused with endosomes containing H5N1pp after clathrin-dependent endocytosis. At 6 h p.i., H5N1pp were observed to be located in lysosomes, indicating the viral particles were delivered into lysosomes for degradation (Fig. 5, A and B).

To determine whether autophagosomes were involved in the delivery of viral particles into lysosomes, A549 cells were transfected with a tandem reporter plasmid (mRFP-GFP-LC3B), which expresses stable mRFP but pH-sensitive GFP protein (24). As expected, H5N1pp-infected A549 cells only showed bright GFP-LC3 puncta at 6 h p.i., suggesting the GFP fluorescence of LC3-II puncta was quenched by the low pH in lysosomes (Fig. 5C). In contrast, blocking autophagosome-lysosome fusion with CQ rescued the GFP fluorescence of LC3-II puncta. These data suggested that autophagosomes participated in the delivery of H5N1pp to lysosomes for degradation. Interestingly, the pretreatment of CQ did not decrease the production of proinflammatory cytokines induced by H5N1pp (Fig. 5D). These data implied that H5N1pp-triggered inflammatory responses should occur prior to the fusion of autophagosomes and lysosomes and not rely on lysosomal degradation. Overall, clathrin-dependent H5N1pp internalization triggered autophagosome formation, which in turn facilitated the delivery of virus-containing endosomes into lysosomes for degradation. H5N1pp-induced inflammation relied on the involvement of clathrin but did not involve autophagosome-lysosome fusion.

H5N1pp-induced autophagy mediated the inflammatory responses through NF-κB and p38 MAPK signaling pathways. NF-κB signaling was reported to participate in the immune responses induced by H5N1 virus (46). In the present study, H5N1pp infection significantly induced the nuclear transloc-
tion of NF-κB p65 subunit in A549 cells (Fig. 6, A and B). Moreover, the Western blotting analysis demonstrated that the expression of NF-κB p65 was significantly increased in the nuclear fraction of H5N1pp-infected A549 cells (Fig. 6 C). These data indicated that H5N1pps activated NF-κB signaling pathway. In contrast, blocking autophagy with 3-MA and siRNA of **atg5** or **beclin1** significantly attenuated the nuclear translocalization of NF-κB (Fig. 6, A–C). More importantly, BAY11-7082 administration, a NF-κB inhibitor, completely abolished the mRNA expression of proinflammatory cytokines and chemokines induced by H5N1pps (Fig. 6D). Therefore, autophagy mediated inflammatory responses through NF-κB signaling in H5N1pp-infected A549 cells. Notably, the administration of BAY11-7082 also dramatically abolished H5N1pp-elevated LC3-II expression and autophagosome formation (Fig. 7). These data suggested a positive feedback loop between autophagy and NF-κB signaling pathway in H5N1pp-infected A549 cells.

In addition to NF-κB signaling pathway, p38 MAPK has also been reported to participate in H5N1-induced inflammatory responses (19, 29). In the present study, H5N1pps significantly induced the phosphorylation of p38 MAPK in A549 cells, whereas blocking the autophagic pathway with 3-MA, **Atg5** siRNA, or Beclin1 siRNA attenuated p38 MAPK phosphorylation (Fig. 8, A and B). These data suggested an essential role of autophagy in H5N1pp-triggered p38 MAPK activation. As is known, the MAPK signaling cascades can lead to the activation of the activator protein 1 (AP-1), a transcription factor that consequently promotes the transcription of proinflammatory cytokines and chemokines (31). Therefore, the AP-1 activity was further evaluated by luciferase reporter gene assay. The results showed that the transcription activity of AP-1 was significantly elevated by H5N1pps, whereas the presence of 3-MA significantly diminished AP-1 activity (Fig. 8 C). Furthermore, the administration of SB203580, a p38 MAPK inhibitor, partially abolished the elevated mRNA expression of cytokines including IL-1β, IL-6, and TNF-α induced by H5N1pps (Fig. 8D). Hence p38 MAPK/AP-1 pathway contributed to autophagy-mediated inflammatory responses in H5N1pp-infected A549 cells.

P62 was not involved in autophagy-mediated inflammatory responses. Since p62, an autophagic adaptor protein, has been reported to interact with TRAF6 and activate NF-κB signaling cascades (37), it would be interesting to determine whether p62 was involved in autophagy-dependent inflammatory responses...
in H5N1pp-infected cells. The confocal imaging showed that p62 was colocalized with autophagosomes in H5N1pp-infected cells (Fig. 9A), consistent with previous studies (20, 28). However, deletion of p62 with siRNA neither affected autophagosome formation nor attenuated H5N1pp-induced NF-κB activation as well as the mRNA levels of proinflammatory cytokines/chemokines (Fig. 9, B–D). Hence p62 appeared not to participate in H5N1pp-induced NF-κB activation and downstream proinflammatory cytokine/chemokine production in A549 cells.

Autophagy mediated the inflammatory responses in H5N1-infected lung tissues. The role of autophagy in H5N1pp-induced inflammatory responses was further investigated in vivo. Six-week-old C57/BL6 mice were inoculated intranasally with H5N1pps (1 × 10^7 CFU) or PBS. Three days later, H5N1pps caused immune cell infiltration into alveolus, diffuse alveolar damage, and thickening epithelial cells in lung tissues, indicating acute lung inflammation (Fig. 10, A and B). H5N1pps also dramatically increased the mRNA expression of proinflammatory cytokines/chemokines and activated NF-κB and p38 MAPK in lung tissues (Fig. 10, C and D), which probably contributed to the inflammatory responses in lung tissues. Notably, administration of 3-MA by intraperitoneal injection prior to H5N1pp infection not only dramatically suppressed virus-triggered autophagy but also significantly attenuated the lung inflammation caused by H5N1pps as well as the activation of NF-κB and p38 MAPK (Fig. 10, A–D). These data clearly demonstrated an essential role of autophagy in H5N1pp-triggered lung inflammation in vivo, which involved NF-κB and p38 MAPK/AP-1 signaling pathways.

**DISCUSSION**

Since the antigenic shift and drug resistance of influenza viral infection remain the major obstacles for the antiviral treatment, discovering the general mechanism of influenza viral pathogenesis is highly desirable to antiviral therapy, especially for highly virulent H5N1 influenza viruses. Although hypercytokemia has been reported to play an important role in the pathogenesis of H5N1 infection (5, 43), its underlying molecular mechanism has not been well elucidated yet. In the present study, we demonstrated that autophagy played a central role in H5N1pp-induced proinflammatory cytokines and chemokines both in vitro and in vivo. Autophagy mediated inflammatory responses through NF-κB and p38 MAPK signaling cascades, and required the presence of clathrin. Blocking autophagy effectively attenuated H5N1pp-induced lung inflammation. Hence our data suggested that targeting autophagy could be an effective therapeutic strategy to ameliorate the pathogenesis of H5N1 viruses.
Recent studies have shown that autophagy, as an important cellular homeostatic machinery, plays a crucial role in regulating both adaptive immunity [e.g., major histocompatibility complex (MHC) class I and II antigen presentation] and innate immunity (e.g., inflammatory responses) (31). Autophagy also serves as a key mechanism contributing to the inflammatory responses induced by several IAV, such as H1N1 and H9N2 (28). However, the role of autophagy in H5N1-triggered lung inflammation has not been elucidated yet. Sun et al. (48) showed that autophagy mediated alveolar epithelial cell death caused by H5N1 infection, but inhibiting autophagy did not affect H5N1-induced cytokine productions in A549 cells. In the present study, we observed that H5N1pp infection not only induced cytokines and chemokines in A549 cells but also led to acute lung inflammation as indicated by increased leukocyte infiltration, epithelial thickening, and elevated proinflammatory cytokines/chemokines in mouse lung tissues. Moreover, H5N1pp-triggered inflammatory responses required the involvement of the autophagic pathway, and inhibition of autophagy dramatically attenuated virus-induced proinflammatory cytokines/chemokines and ameliorated acute lung inflammation. Therefore, in addition to inducing the alveolar epithelial cell death, autophagy also might mediate H5N1-triggered inflammatory responses, which could be another important mechanism contributing to the pathogenesis of H5N1 infection. Notably, H5N1pp-triggered autophagy and downstream inflammatory responses were both primarily attributable to HA protein rather than NA or MLV core particles, suggesting the central role of HA in pathogenesis of H5N1 infection. In addition to HA, multiple viral components of IAVs have been reported to differentially regulate autophagy (56). For example, M2 protein of IAVs was observed to inhibit autophagy by blocking autophagosome fusion with lysosomes (15). However, since autophagy-mediated inflammation in H5N1pp-infected cells did not rely on the fusion of autophagosome with lysosomes, the presence of M2 protein would unlikely interfere with HA-induced inflammatory responses. Nevertheless, our study demonstrated a key role of autophagy in H5N1pp-trigger lung inflammation and might shed a light on the mechanism of H5N1-caused acute lung injury.

NF-κB and p38 MAPK have been reported as the major signaling pathways involved in H5N1-triggered inflammatory responses. Previous studies reported that H5N1 virus induced proinflammatory cytokines and chemokines in NF-κB-dependent manner in both human microvascular endothelial cells and mice model (11, 46). HA protein of H1N1 virus was also capable of activating NF-κB signaling in human A3.01 T cells (13). Moreover, Lee et al. (29) and Hui et al. (19) reported that p38 MAPK was involved in the H5N1-induced proinflammatory cytokines in human macrophages. Although autophagy mediated H5N1pp-triggered lung inflammation, its role in the regulation of NF-κB and p38 MAPK signaling cascades re-
mains unknown. In the present study, we demonstrated that the activation of NF-κB and p38 MAPK signaling pathways was essential for H5N1pp-induced proinflammatory cytokines and chemokines in A549 cells and mice lung tissues, consistent with previous reports. Moreover, H5N1ppps activated NF-κB and p38 MAPK signaling cascades in an autophagy-dependent manner. Interestingly, inhibition of NF-κB signaling dramatically attenuated H5N1pp-induced autophagosome formation, whereas inhibition of p38 MAPK did not have such effect. These data indicated a positive feedback loop between NF-κB activation and autophagy, which would further exacerbate downstream inflammatory responses caused by H5N1 infection. Copetti et al. (7) demonstrated that NF-κB subunit p65 could bind to beclin1 promoter and directly promote Beclin1 expression. In addition, IKK activation was shown to be required for both autophagy induction and NF-κB activation (8). Nonetheless, the cross talk between autophagy and NF-κB signaling could be an important mechanism contributing to serious lung inflammation triggered by H5N1 viral infection.

Currently, the mechanism by which autophagy activates the inflammatory signaling cascades remains unclear. P62 has been reported as an autophagic adaptor protein involved in the regulation of the NF-κB signaling cascade (40, 41). In the present study, although p62 was colocalized with autophagosomes in H5N1pp-infected cells, deletion of p62 by siRNA failed to suppress either NF-κB activation or mRNA levels of cytokines and chemokines in H5N1pp-infected A549 cells. Hence H5N1pp-induced NF-κB activation and proinflammatory cytokines/chemokines did not require the involvement of p62.

As is known, clathrin-dependent endocytosis is essential for viral internalization and intracellular processing and downstream inflammatory responses/signaling cascades (33). After internalization, viral particles enter the early endosomes, followed by trafficking into late endosomes/lysosomes for degradation or releasing to the cytoplasm to evade the lysosome degradation (42). Previous studies showed that autophagosomes fused with endosomes or multivesicular bodies to generate amphipathics, which consequently fused with lysosomes for degradation (21). Autophagosome was also reported to facilitate the delivery of Epstein-Barr virus into lysosomes for degradation and MHC II antigen presentation (44). However, the relationship between autophagy and clathrin-dependent endocytosis of H5N1 viral particles is unknown. More importantly, whether autophagy is involved in the intracellular processing of H5N1 remains unclear. In the present study, depleting clathrin by siRNA not only blocked the internalization of H5N1ppps by A549 cells, but also inhibited H5N1pp-triggered autophagy flux. Moreover, clathrin depletion completely abolished H5N1pp-induced cytokines and chemokines in A549 cells. These data indicated that clathrin-dependent endocytosis was required for H5N1pp-triggered autophagy and downstream inflammatory responses. Furthermore, H5N1ppps were observed to be mostly colocalized with both the early endosome and autophagosome at 2–3 h p.i. and then located in late endosomes and lysosomes at 6 h p.i., suggesting that autophagosomes could fuse with endosomes to deliver H5N1ppps into lysosomes. In addition, mRFP-GFP-LC3, which should be assembled onto the autophagosomes, was found to be degraded by lysosomal hydrolysases. These data further confirmed that autophagosomes participated in the delivery of H5N1ppps to lysosomes for degradation. Interestingly, H5N1pp-induced proinflammatory cytokines and chemokines did not rely on lysosomal fusion, further indicating direct involvement of autophagy in triggering downstream inflammatory signalings.

In conclusion, autophagy played a crucial role in H5N1pp-induced proinflammatory cytokines and chemokines in human lung epithelial cells and mouse lung tissues. Autophagy-mediated inflammatory responses relied on the presence of clathrin and involved NF-κB and p38 MAPK signaling cascades. The positive feedback loop between NF-κB signaling and autophagy induction could be a potential mechanism contributing to H5N1-caused hypercytokemia. The present study demonstrated a close relationship between H5N1-induced autophagy and inflammation, which will shed a light on the molecular mechanism of H5N1 viral pathogenesis. Targeting the autophagic pathway could be an effective strategy for treating H5N1 virus-caused lung inflammation.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
H.P., Z.L., P.L., L.L., and C.W. performed experiments; H.P. analyzed data; H.P. and Y.Z. interpreted results of experiments; H.P., Y.Z., and Y.M. prepared figures; H.P., Y.Z., and Y.M. drafted manuscript; Y.Z., H.W., H.L., and Y.M. conception and design of research; Y.Z. and Y.M. edited and revised manuscript; Y.M. approved final version of manuscript.

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