Inhibition of autophagy promotes human RSV NS1-induced inflammation and apoptosis *in vitro*

BING HAN[1*], YIZHONG WANG[1,2*] and MEI ZHENG[1,2]

[1]Department of Pediatrics, Xiangyang No. 1 People's Hospital, Hubei University of Medicine, Xiangyang, Hubei 441000; [2]Department of Pediatrics, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, P.R. China

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Abstract. Human respiratory syncytial virus (RSV) is a major health challenge due to the lack of a safe and effective vaccine and antiviral drugs. RSV non-structural protein 1 (NS1) is the main inhibitor of antiviral signaling pathways in RSV infection; however, the underlying mechanism is unclear. The aim of the present study was to investigate of the role of NS1 and its relationship with autophagy. NS1-Flag plasmid was transfected into A549 cells and the levels of inflammatory cytokines, autophagy markers and apoptosis were detected. In addition, the cells were treated with an autophagy inhibitor, 3-methyladenine for 12 h prior to transfection with the NS1 plasmid to explore the role of autophagy in NS1-transfected cells. The results showed that the production of inflammatory cytokines and autophagy was induced in NS1-transfected cells, and indicated that autophagy prevents the production of cytokines and the activation of apoptosis. Furthermore, the results demonstrated that NS1 activated autophagy partly through the mTOR-p70 S6 kinase signaling pathway. The results suggest that autophagy induced by NS1 transfection through the mTOR pathway can hinder the production of inflammatory cytokines and interferon-α and inhibit cell apoptosis, which may help to explain why autophagy has been shown to be beneficial to viral replication in most studies.

Introduction

Respiratory syncytial virus (RSV) is the most common cause of pneumonia and bronchiolitis in children worldwide (1). Although RSV has a significant clinical impact on global human health, no vaccines have yet been approved and effective antiviral treatments are lacking. Furthermore, while RSV infection induces humoral and cellular immune responses to clear the infection, these responses do not provide strong protection against subsequent RSV infection. The 11 proteins encoded by the RSV genome include non-structural protein 1 (NS1) and NS2, which are key in mediating the ability of the virus to evade immune surveillance (2,3). It has been reported that a series of antiviral mechanisms evoked by RSV infection in the host may be hampered by the NS proteins (4). RSV infection is severely attenuated *in vitro* and *in vivo* when either NS1 or NS2 is deleted, indicating that the NS proteins serve a key role in viral replication (4,5). In addition, it has been reported that the expression of inflammatory cytokines IL-6 and IL-8 in the lung is associated with the severity of disease in infants and adults infected with RSV (6,7). The silencing of NS1 provides substantial protection against RSV infection-induced inflammation and airway reactivity (2). RNA interference targeting the RSV nucleocapsid (8) and NS1 has been investigated as a new approach for RSV antiviral therapy (2,9,10). The NS proteins also prevent cells from undergoing apoptosis and prolong cell life, thus increasing the amount of viral replication (3). NS1 has been shown to inhibit the interaction between mitochondrial antiviral signaling protein (MAVS) and retinoic acid-inducible gene-I by binding to MAVS, and thereby decrease the production of type-I interferon (IFN) (11). RSV viruses lacking NS1 or/and NS2 are more sensitive to IFN, which leads to increased apoptosis and decreased viral replication efficiency in cell and animal models (12,13). We hypothesize that in the absence of RSV infection, the overexpression of NS1 alone may induce cellular inflammation and aim to explore the underlying mechanisms.

Autophagy is a highly conserved process that involves the encapsulation of cytoplasmic contents in a bilayer membrane, fusion with a lysosome and degradation. Autophagy plays important role in antigen presentation, bacterial and viral infection, and cell death (14-16). During autophagy, intracellular components, including organelles such as mitochondria, are phagocytized in double-membraned autophagosomes, which fuse with lysosomes to form autophagosomes. The components within the autophagosomes are subsequently degraded by lysosomal hydrolases. In the process of autophagosome membrane formation, the cytoplasmic form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) conjugates with phosphatidylethanolamine to form LC3-II,

Correspondence to: Dr Mei Zheng, Department of Pediatrics, Xiangyang No. 1 People's Hospital, Hubei University of Medicine, 15 Jiefang Road, Xiangyang, Hubei 441000, P.R. China
E-mail: zhengmei2018@163.com

*Contributed equally

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a lipid-modified form of LC3-I, via two consecutive ubiquitylation-like reactions. This intra-autophagosomal LC3-II is degraded by lysosomal proteases when autophagosomes and lysosomes fuse. Therefore, the cellular level of LC3-II can be regarded as a marker of autophagic activity. Monitoring LC3-I and LC3-II using immunofluorescence and western blotting is important when investigating the mechanism of autophagy (17). Beclin1, the first-described mammalian autophagy protein, serves an key role in the initiation of autophagy. The abundance of Beclin1 can also act as a determinant of autophagic activity (18).

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase, which serves as a sensor of cellular nutritional status, stress and growth factor signals and thereby serves a key role in the occurrence of autophagy (19). mTOR pathway-mediated autophagy is closely associated with apoptosis and inflammation. Reed *et al* (20) reported that the production of innate cytokines was decreased upon the blockade of autophagy, while RSV infection induced inflammasome activation and IL-1β secretion in autophagy-deficient cells. At present, the majority of studies have indicated that NS1 suppresses the IFN response signal to reinforce RSV infection. However, there have been few reports concerning the relationship between NS1 and autophagy or the mechanism of autophagy in NS1-induced inflammation. Therefore, the present study aimed to explore the role of autophagy in NS1-transfected cells with the aim of gaining an improved understanding of the role of NS1 and its relationship with autophagy. The findings of this study also may assist in the identification of strategies for viral vaccine development.

Materials and methods

Cell culture and treatment. The A549 type II human lung epithelial cell line was obtained from the American Type Culture Collection and cultivated with high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in an incubator containing 5% CO_2. The cells were passaged when they had grown to a dense monolayer. NS1 plasmid tagged with Flag (pNS1-Flag) was donated by the laboratory of Professor Zhao at Wuhan University Zhongnan Hospital (21). When the A549 cells reached a density of 60-70% they were transfected with pNS1 for 24 h at 37˚C. For the pNS1 transfection, a liposomal formulation (Lipofectamine® 2000; Invitrogen; Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions. Briefly, 2.5 μg plasmid, 7.5 μl Lipofectamine 2000 and 5 μl p3000 (Invitrogen; Thermo Fisher Scientific, Inc.) complex were used to transfect the cells in each well of 6-well plates. Cells were collected for subsequent experiments after 24 h. The autophagy inhibitor 3-methyladenine (3MA; MedChemExpress) dissolved in normal culture medium at a final concentration of 5 mM was used to treat the cells for 12 h prior to transfection with pNS1. The mTOR-specific inhibitor Torin-1 (Selleck Chemicals) dissolved in DMSO and diluted to a final concentration of 1 μM in the medium was used to treat the cells for 3 h prior to transfection with pNS1 as previously described (22).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT (Sigma-Aldrich; Merck KGaA) colorimetric assay was used to determine cell viability. Briefly, cells were seeded in 96-well plates ($5x10^3$ cells per well), and then subjected to different treatments (transfected with pNS1 for 24 h at 37˚C, treated with 3MA for 12 h and then transfected with pNS1 for 24 h at 37˚C, or treated with Torin-1 for 3 h at 37˚C). After the treatments, the cells were washed with PBS and MTT solution was added to the cells, which were then incubated for 2.5 h. DMSO was used to dissolve the purple formazan. The absorbance was measured at 490 nm with a microplate reader (BioTek Instruments, Inc.) to determine the cell viability.

Reverse transcription-quantitative PCR (RT-qPCR). The total RNA of A549 cells was separated using RNAiso Plus (Takara Biotechnology Co., Ltd.) following the manufacturer's protocol. A RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) was used to reverse transcribe the purified RNA into cDNA according to the manufacturer's protocol. The levels of IL-6, IL-8 and β-actin were examined using SYBR™ Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions for PCR was as follows: 50˚C for 2 min, 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, followed by annealing and extension at 60˚C for 30 sec. The primer sequences for IL-6, IL-8 and β-actin were purchased from Tianyi Huiyuan Biotech Co., Ltd. The sequences of the primers were as follows: Human IL-6 forward, 5'-GAGGATACCACTCCCAACAGACC-3' and reverse, 5'-AAGTGCATCATCGTTGTTCATAC-3'; IL-8 forward, 5'-CTTGGCAGCCTTCCTGA-3' and reverse, 5'-TTCTTTAGCACTCCTTGGCAAAA-3'; β-actin forward, 5'-CCGACAGGATGCAGAAGGAG-3' and reverse, 5'-GTGGGGTGGCTTTTAGGATG-'3. The cycle threshold or quantitation cycle (Cq) was detected, and the $2^{-\Delta\Delta Cq}$ method (23) was used to determine the relative expression of the target RNAs with normalization to β-actin expression.

Western blot analysis. *C*ells were washed with ice-cold PBS and then lysed for 30 min on ice with RIPA lysis buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor cocktail. A BCA Assay kit (Beyotime Institute of Biotechnology) was then used for protein quantification. Total proteins (30-40 μg/lane) were loaded and isolated by 12.5 or 10.0% SDS-PAGE and blotted onto PVDF membranes (EMD Millipore). The membranes were then blocked with 5% non-fat milk for 1 h at room temperature (26˚C) and incubated overnight at 4˚C with primary antibody. The primary antibodies used were as follows: Rabbit anti-Flag (1:1,000; ##14793; Cell Signaling Technology, Inc.), rabbit anti-IL-6 (1:1,000; #12153, Cell Signaling Technology, Inc.), mouse anti-IL-8 (1:400; sc-376750; Santa Cruz Biotechnology, Inc.), rabbit anti-LC3 (1:1,000; #4108; Cell Signaling Technology, Inc.), mouse anti-Bcl-2 (1:500; sc-7382; Santa Cruz Biotechnology, Inc.), mouse anti-Bax (1:500; sc-7480; Santa Cruz Biotechnology, Inc.), mouse anti-Beclin1 (1:500, sc-48381; Santa Cruz Biotechnology, Inc), anti-mTOR (1:1,000; #2983, Cell Signaling Technology, Inc.), anti-phosphorylated (p-)mTOR (1:1,000; #5536;

Cell Signaling Technology, Inc.), anti-p70 S6 kinase (S6KP70) (1:1,000; #2708; Cell Signaling Technology, Inc.), anti-p-S6KP70 (1:1,000; #9234; Cell Signaling Technology, Inc.) and rabbit anti-β-actin (1:10,000; AC026; ABclonal Biotech Co., Ltd.). Then the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG, 1:5,000, cat. no. AS014; goat anti-mouse IgG, 1:5,000; cat. no. AS003; both ABclonal Biotech Co., Ltd.) for 1 h at room temperature and the protein bands were visualized using ECL HRP substrate (PerkinElmer, Inc.). A ChemiDoc™ Image Analyzer (Bio-Rad Laboratories, Inc.) was used to detect the antibody binding signals. The results were quantified using ImageJ (version 7.0; National Institutes of Health) based on the analysis of three independent bands.

Immunofluorescence staining. A549 cells cultivated in a Nunc™ Petri dish (Thermo Fisher Scientific, Inc.) were washed three times with 1X PBS, fixed in 4% paraformaldehyde for 20 min at 4˚C and washed again three times with PBS. The cells were then permeabilized with 0.2% Triton X-100 in PBS and blocked for 30 min at 4˚C with 3% bovine serum albumin (Thermo Fisher Scientific, Inc.). The cells were then incubated overnight at 4˚C with anti-LC3 primary antibodies (1:400 dilution; SAB4300571, Sigma-Aldrich; Merck KGaA) in blocking buffer. After washing with PBS, the cells were incubated with Alexa Fluor 555 fluorescent secondary antibody (1:100; cat. no. bs-0295G-AF555; BIOSS) for 60 min at room temperature. The nuclei were then stained with 4',6-diamidino-2-phenylindole at 1:5,000 dilution for 5 min at room temperature. The cells were washed twice with PBS and fluorescence images were captured using a confocal microscope (Smartproof 5; Carl Zeiss AG). The intensity of staining was determined by measuring the integrated optical density (IOD) in 10 different fields for each sample.

Apoptosis detection using flow cytometry. The apoptosis of cells was detected using an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol and detected by flow cytometry (BD FACSAria™ III; BD Biosciences). Briefly, the cells were washed with PBS and resuspended; then Annexin V-FITC and PI binding solution was added to the resuspension and the cells were incubated at room temperature for 20 min in the dark. Cells were then placed in an ice bath prior to detected by flow cytometry. The results were analyzed using FlowJo v.8.0 software (Tree Star, Inc.).

Measurement of IFN-α level. The concentration of IFN-α in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA). A commercially available human IFN-α ELISA kit (cat. no. #41100; R&D Systems, Inc.) was used according to the manufacturer's instructions. The results were normalized to the amount of IFN-α in conditioned media and expressed in units of pg/ml.

Statistical analysis. All data were analyzed using GraphPadPrism 8 (GraphPad Software, Inc.). Quantitative data are expressed as the mean ± standard error of the mean. One-way ANOVA followed by Bonferroni's multiple comparisons post hoc test was used for comparisons among more than two groups; two-tailed Student's t-test was used for comparisons of two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NS1 induces inflammatory cytokine production and autophagy in A549 cells. The viability and expression of NS1 in A549 cells transfected with the plasmid pNS1-Flag were examined. As shown in Fig. 1A, NS1 transfection did not affect the viability of the cells (Fig. 1A). In addition, the expression level of NS1 protein in the cells 24 h after transfection with pNS1-Flag was higher than that in the control cells transfected with empty plasmid (Fig. 1B). The mRNA expression levels of the inflammatory cytokines IL-6 and IL-8 were increased significantly following transfection with NS1 (Fig. 1C and D), as were the respective protein expression levels (Fig. 1E and F). Furthermore, it was observed that following transfection with NS1, the protein levels of the autophagy markers LC3-II and Beclin1 were significantly increased (Fig. 1E and F). The results of immunofluorescence staining also showed that the fluorescence intensity of LC3 increased markedly after NS1 transfection (Fig. 1G). These results indicate that NS1 transfection increases the production of inflammatory cytokines and autophagy levels in A549 cells.

Inhibition of autophagy increases inflammatory factors and IFN-α production in NS1-transfected cells. To explore the role of autophagy in the expression of inflammatory factors induced by NS1, cells were pretreated with the autophagy inhibitor 3MA for 12 h prior to transfection with the NS1-expressing vector. Firstly, the MTT assay results demonstrated that cell viability was not affected by transfection or the 3MA treatment (Fig. 2A). Western blotting results showed that 3MA further elevated the increase in the protein expression levels of IL-6 and IL-8 induced by NS1, while the cells treated with 3MA alone exhibited no significant difference in the expression of inflammatory factors compared with the control group (Fig. 2B-D). The pretreatment with 3MA also attenuated the NS1-induced increase in the expression of autophagy markers LC3-II and Beclin1, while 3MA treatment alone had no significant effect on cell autophagy markers or NS1 expression (Fig. 2B and E-G). A significant weakening of the NS1-induced increase in LC3-II fluorescence intensity was also observed following 3MA treatment in the immunofluorescence assay (Fig. 2H and I). The levels of IFN-α were also measured. The results showed that transfection with NS1 downregulated the IFN-α level and this affect was attenuated by 3MA, while 3MA alone failed to induce IFN-α (Fig. 2J). These results suggest that the inhibition of autophagy increased inflammatory factors and IFN-α production in the cells transfected with NS1.

Inhibition of autophagy increases cell apoptosis in NS1-transfected cells. The apoptosis of the cells was tested following transfection with the NS1 overexpression vector by flow cytometry using Annexin V-FITC and PI. In Fig. 3A, cells in Q1 (Annexin V-FITC)$^-$/PI$^+$ were necrotic, in Q2 (Annexin V-FITC)$^+$/PI$^+$ were late apoptotic, in Q3 (Annexin V-FITC)$^+$/PI$^-$ were early apoptotic and in Q4

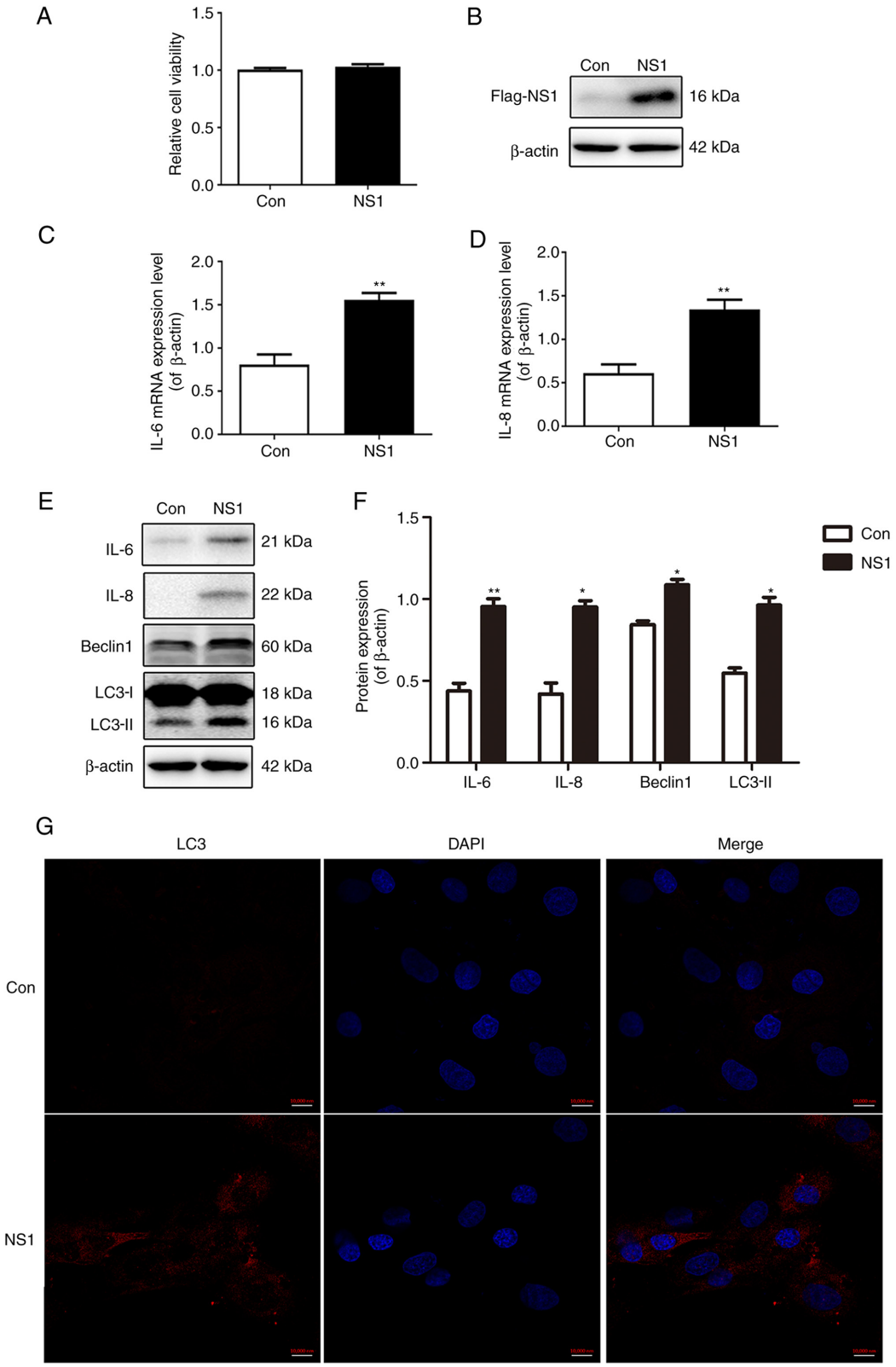

Figure 1. NS1 induces inflammatory cytokine production and autophagy in A549 cells. A549 cells were transfected with plasmid pNS1-Flag for 24 h and the cells were collected. (A) Cell viability measured by MTT assay. (B) Representative immunoblots for Flag-NS1 in A549 cells detected by western blotting. The mRNA expression of (C) IL-6 and (D) IL-8 in A549 cells was measured by reverse transcription-quantitative PCR assay. (E) Representative immunoblots and (F) quantification of IL-6, IL-8, Beclin1 and LC3-II/I in the cells. (G) Immunofluorescence staining of LC3 in the cells. Scale bar, 10 μm. Values are presented as the mean ± SEM and are derived from three independent experiments. *P<0.05, **P<0.01. NS1, non-structural protein 1; LC3-I, cytoplasmic microtubule-associated protein 1A/1B-light chain 3; LC3-II, lipid-modified LC3-I; Con, control.

(Annexin V-FITC)$^{-}$/PI^{-} were living. The results showed that the apoptosis of NS1-transfected cells was increased compared with that in the control group and 3MA increased the apoptosis induced by NS1, whereas 3MA treatment alone had no

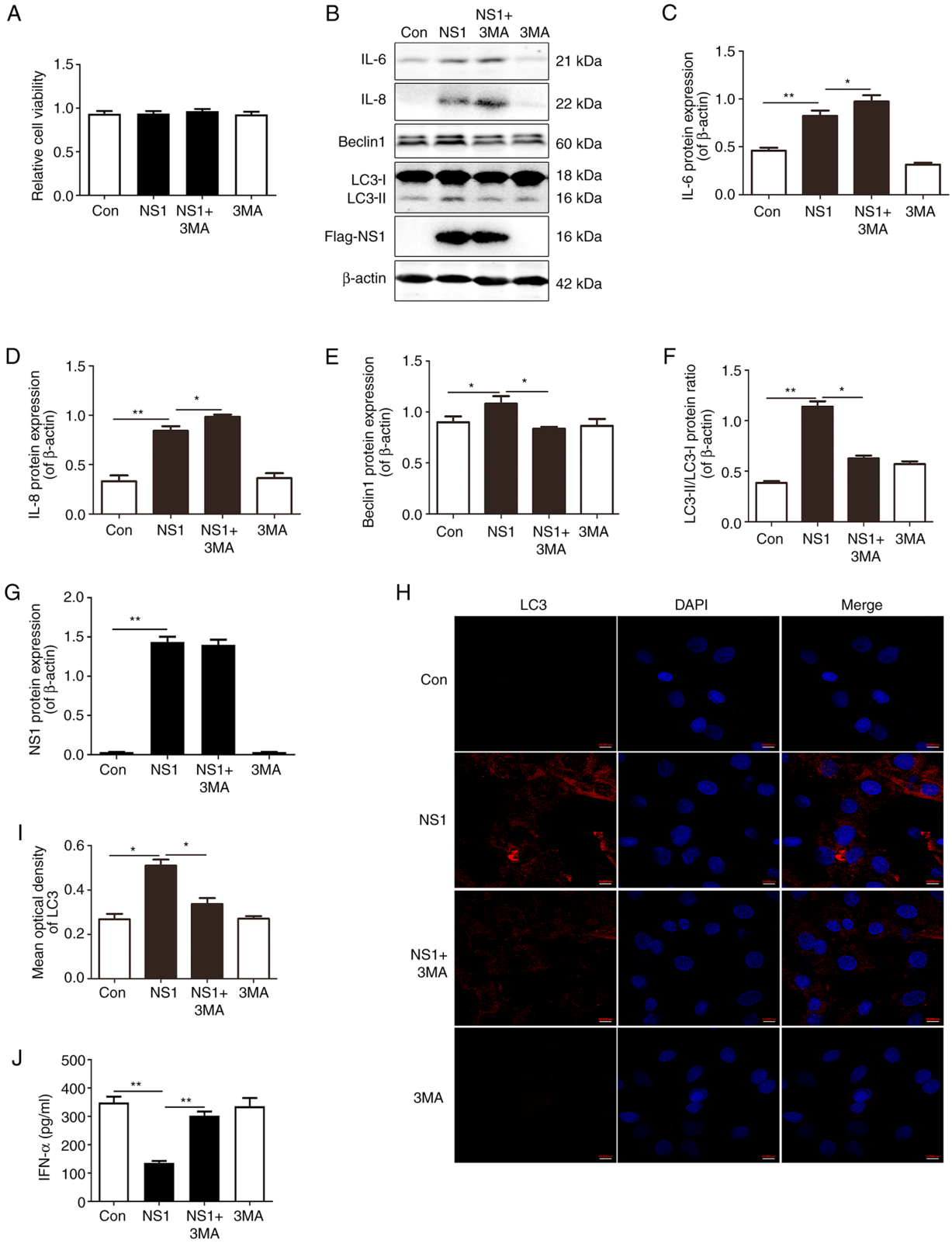

Figure 2. Inhibition of autophagy increases the production of inflammatory factors and IFN-α in NS1-transfected cells. A549 cells were treated with 3MA for 12 h prior to transfection with plasmid pNS1-Flag. (A) Cell viability of various treatment groups measured by MTT assay. (B) Representative immunoblots and quantification of (C) IL-6, (D) IL-8, (E) Beclin1 and (F) the LC3-II/LC-I ratio, (G) NS1 in the cells. (H) Cell immunofluorescence staining and (I) quantification of the optical density value of LC3 in the cells. Scale bar, 10 μm. (J) IFN-α production in the cell supernatant was determined by enzyme-linked immunosorbent assay. Values are presented as the mean ± SEM and are derived from three independent experiments. *P<0.05, **P<0.01. IFN-α, interferon-α; NS1, non-structural protein 1; 3MA, 3-methyladenine; LC3-I, cytoplasmic microtubule-associated protein 1A/1B-light chain 3; LC3-II, lipid-modified LC3-I; Con, control.

significant effect on apoptosis (Fig. 3A and B). The detection of apoptosis-associated proteins by western blotting showed that the expression of anti-apoptotic protein Bcl-2 decreased and pro-apoptotic protein Bax increased significantly following NS1

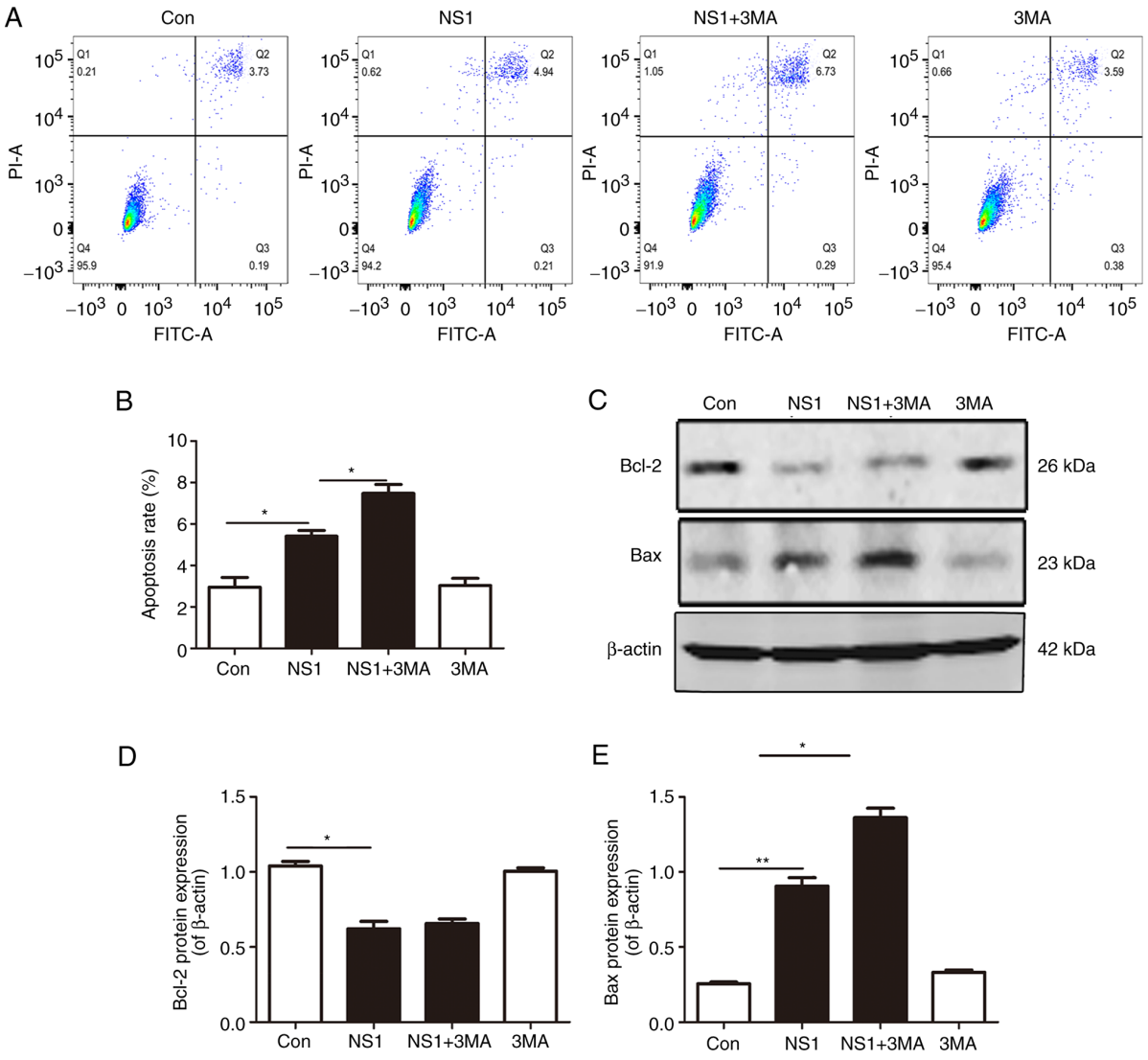

Figure 3. Inhibition of autophagy increases the apoptosis of NS1-transfected cells. A549 cells were treated with 3MA for 12 h prior to transfection with plasmid pNS1-Flag. (A) Cell apoptosis was measured by flow cytometry; representative plots are shown. (B) Quantification of apoptosis rate. (C) Representative immunoblots and quantification of (D) Bcl-2 and (E) Bax in the cells. Values are presented as the mean ± SEM and are derived from three independent experiments. *P<0.05, **P<0.01. NS1, non-structural protein 1; 3MA, 3-methyladenine; Con, control; PI, propidium iodide; FITC-A, FITC-Annexin V.

transfection. When compared with the NS1 transfection group, the Bcl-2 expression level was not significantly changed in the 3MA-pretreated NS1-transfected cells, while Bax expression was significantly upregulated. 3MA treatment alone had no significant effect on these two proteins (Fig. 3B-D). These results demonstrate that transfection with NS1 increases apoptosis and the inhibition of autophagy exacerbated this phenomenon.

NS1 activates autophagy through the mTOR pathway. To further explore the mechanism by which autophagy occurs in NS1-transfected cells, the classic mTOR pathway of autophagy was examined. Western blotting results showed that compared with cells that were not transfected with NS1, the p-mTOR/mTOR and p-S6KP70/S6KP70 protein ratios were significantly reduced in the NS1-transfected cells. In addition, pretreatment with 3MA blocked the NS1-induced reduction in these protein ratios, while the administration of 3MA alone did not change the p-mTOR/mTOR and p-S6KP70/S6KP70 protein ratios significantly (Fig. 4A-C). The cells were pretreated with the mTOR-specific inhibitor Torin-1 to further confirm the links between autophagy and the mTOR pathway. Firstly, the results of an MTT assay showed that treatment with 1 μM Torin-1 alone had no significant effect on cell viability (Fig. 4D), and Torin-1 significantly inhibited the activation of mTOR by phosphorylation (Fig. 4E and F). When mTOR activity was inhibited by Torin-1, the LC3-II level was upregulated in the NS1-transfected cells compared with the control and untreated NS1-transfected cells. Treatment with Torin-1 alone also significantly increased the level of LC3-II compared with that in the control group (Fig. 4E and G). These results suggested that NS1 may induce autophagy through the mTOR-S6KP70 pathway.

Discussion

RNA and DNA viral infections can induce non-identical autophagy responses, and a recent study demonstrated that the inhibition of autophagy by 3MA decreased RSV replication and ameliorated lung pathology in the lungs of mice following

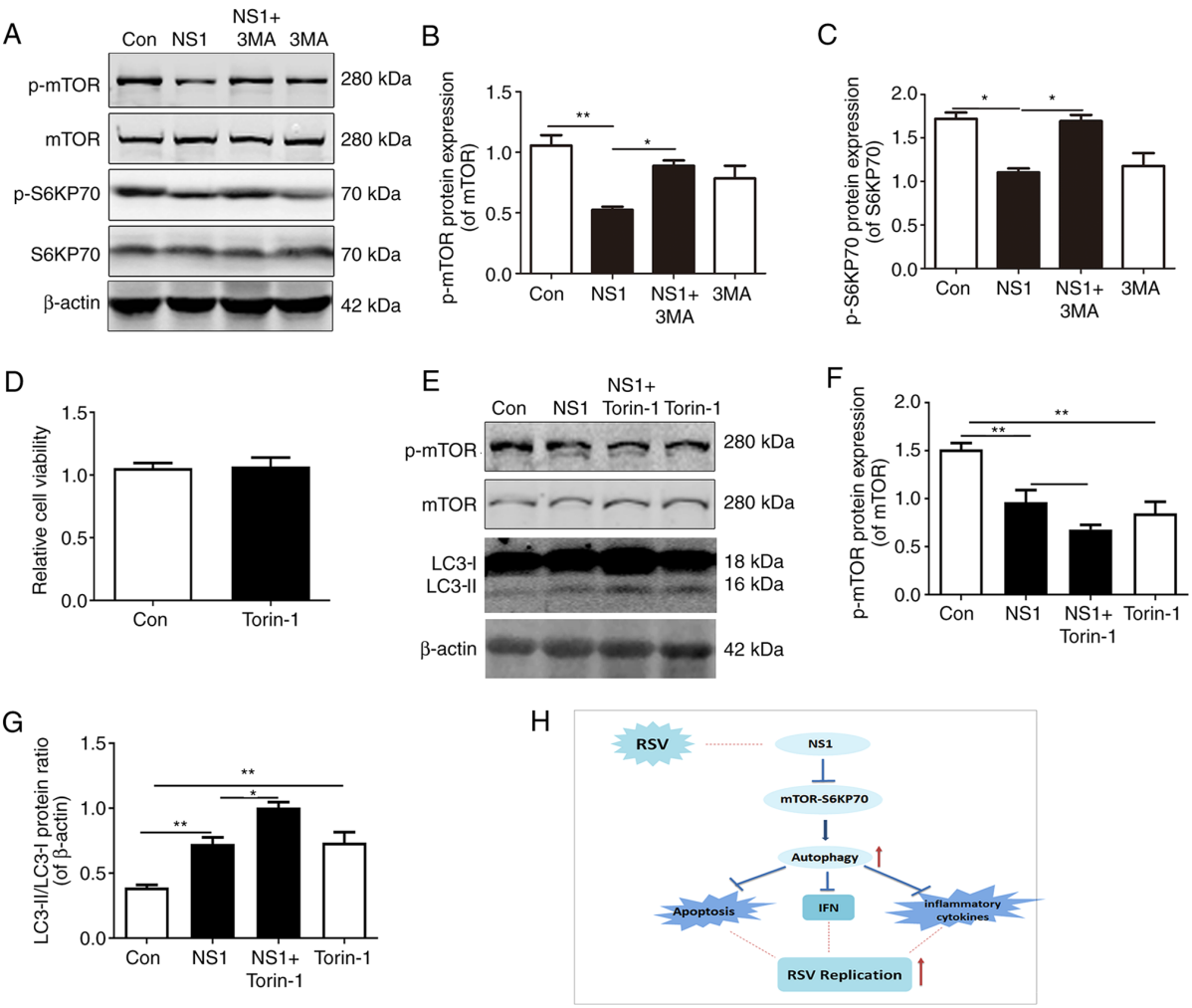

Figure 4. NS1 activates autophagy through the mTOR pathway. (A-C) A549 cells were treated with 3MA for 12 h prior to transfection with plasmid pNS1-Flag. (A) Representative immunoblots and quantification of (B) p-mTOR/mTOR and (C) p-S6KP70/S6KP70 ratios in cells. (D-G) Cells were treated with Torin-1 for 3 h prior to transfection with plasmid pNS1-Flag, then (D) an MTT assay and (E) western blot analysis of p-mTOR and LC3 levels were performed. (F) p-mTOR/mTOR and (G) LC3-II/LC3-I ratios were quantified (H) Scheme of the potential mechanism for the beneficial effect of NS1 in RSV-infected epithelial cells via autophagy. Values are presented as the mean ± SEM and are derived from three independent experiments. *P<0.05, **P<0.01. NS1, non-structural protein 1; mTOR, mammalian target of rapamycin; 3MA, 3-methyladenine; p-, phosphorylated; S6KP70, p70 S6 kinase; LC3-I, cytoplasmic microtubule-associated protein 1A/1B-light chain 3; LC3-II, lipid-modified LC3-I; Con, control; RSV, respiratory syncytial virus; IFN, interferon.

RSV infection (24). It also has been shown that the induction of autophagy is beneficial for the replication of viruses, but the detailed mechanisms remain elusive (25-27). Although it is known that NS1 attenuates the production of IFN during RSV infection, the precise role of the RSV NS1 protein in the early host-virus interaction during the infection is poorly understood. Zhang *et al* (28) suggested that JNK activation regulates virus replication via the induction of autophagy. On the basis of these previous findings, it was hypothesized that autophagy may play a role in RSV viral replication. This hypothesis was supported by the observation of increased autophagic markers, notably LC3-II and Beclin1, when cells were transfected with NS1, and the increase of inflammatory cytokine and IFN-α production when autophagy was inhibited. The present study suggests that when cells are transfected with NS1, cell autophagy is activated to prevent the production of cytokines and IFN-α and the activation of apoptosis, which may be conducive to RSV viral replication.

Autophagy serves a dual role as it mediates both cell survival and cell death. Autophagy limits the spread of a virus from infected to healthy tissues and regulates the programmed death of adjacent uninfected cells, thereby acting as a cell survival mechanism during viral infection (29). The influenza A virus induces the apoptosis of autophagy protein-deficient cells, which suggests that autophagy is beneficial for the survival of infected cells (30). Autophagy is also thought to prolong the survival of erythroid cells infected with human parvovirus B19 (31). The impairment of autophagy in cells may mediate apoptotic cell death. The results from a study by Shrivastava *et al* (32) demonstrated that hepatitis C virus infection in cells with autophagy-associated protein knockdown induced apoptotic cell death. In the present study, the findings of increased IL-6 and IL-8 secretion, decreased Bcl-2 and increased Bax levels when autophagy was inhibited concur with a previous report of RSV infection inducing cytokine secretion in dendritic cells via an LC3-dependent mechanism (20). The current study also documented increased apoptosis in NS1-transfected cells, which was further increased by the inhibition of autophagy. This suggests that autophagy may promote cell survival in response to NS1 by preventing apoptosis.

mTOR is a key regulator of autophagy, and inhibition of the mTOR-S6KP70 signaling pathway is one of the main mechanisms by which autophagy is activated (33). Torin-1 is a specific mTOR inhibitor, which has been shown to induce autophagy by inhibiting mTOR activity (22). The results of the present study showed that transfection with NS1 decreased the levels of p-mTOR and p-S6KP70, and the effects of NS1 were blocked by 3MA. Furthermore, LC3-II levels increased in cells with or without NS1 transfection when mTOR activity was inhibited by Torin-1. These results suggest that NS1 induced autophagy through the mTOR-S6KP70 signaling pathway. However, the mechanism by which NS1 modulates the mTOR-S6KP70 signaling pathway remains unknown. To summarize, the postulated mechanism of the beneficial effect of NS1-induced autophagy in RSV-infected epithelial cells mediated through the mTOR pathway is illustrated by the scheme in Fig. 4H.

In conclusion, the present study demonstrated that the induction of autophagy by NS1 transfection via the mTOR pathway may hinder the production of inflammatory cytokines and IFN-α, and inhibit cell apoptosis, which may help to explain why autophagy has been found to be beneficial to viral replication in most studies. The findings of the present study suggest that autophagy may be a novel and effective therapeutic target against RSV infections.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MZ co-designed the study, performed experiments, analyzed data and co-wrote and revised the manuscript. BH co-designed the study, analyzed data and co-wrote the manuscript. YW performed experiments, analyzed data and co-wrote the manuscript. BH and YW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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