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

BING HAN1*, YIZHONG WANG1,2* and MEI ZHENG1,2

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

Received December 21, 2020; Accepted May 25, 2021

DOI: 10.3892/etm.2021.10488

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 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-membranated autophagosomes, which fuse with lysosomes to form autolysosomes. The components within the autolysosomes are subsequently degraded by lysosomal hydrolases. In the process of autolysosome membrane formation, the cytoplasmic form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) conjugates with phosphatidylethanolamine to form LC3-II,
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 a 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 inflammmasome 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% CO₂. 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).

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'-GAG GAT ACCACTTCCAACAGACC-3' and reverse, 5'-AAG TGC ATC ATGTTGTTCATAC-3'; IL-8 forward, 5'-CTTGGCAGC TTCTCTGA-3' and reverse, 5'-TTTCCTTACTGCCTCTTGG CAAA-3'; β-actin forward, 5'-CCGACAGATGACAG AGGAG-3' and reverse, 5'-GTTGGGTTGGCTTTAGGA TG-3'. The cycle threshold or quantitation cycle (Cq) was detected, and the 2^ΔΔCq method (23) was used to determine the relative expression of the target RNAs with normalization to β-actin expression.

Western blot analysis. Cells 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; HAN et al: INHIBITION OF AUTOPHAGY PROMOTES INFLAMMATION AND APOPTOSIS
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.

**Statistical analysis.** All data were analyzed using GraphPad Prism 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 cell apoptosis 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 effect 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.
HAN et al: INHIBITION OF AUTOPHAGY PROMOTES INFLAMMATION AND APOPTOSIS

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

(Annexin V-FITC)/PI were living.
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
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-S6K/P70 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-S6K/P70 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-S6K/P70 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...
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.

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-S6K/P70/S6K P70 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; S6K P70, 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.
mTOR is a key regulator of autophagy, and inhibition of the mTOR-S6K70 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-S6K70, 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-S6K70 signaling pathway. However, the mechanism by which NS1 modulates the mTOR-S6K70 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.

Acknowledgements

The authors would like to thank Professor Zhao of Wuhan University Zhongnan Hospital for providing the NS1 plasmids and Medical Research Center for Structural Biology of Basic Medical Sciences, Wuhan University for technical help with the confocal fluorescence microscopy and flow cytometry.

Funding

No funding was received.

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.

References

1. Shi T, McAllister DA, O’Brien KL, Simoes EAF, Madhi SA, Gesner BD, Polack FP, Babes S, Acacio S, Aguayo C, et al: Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: A systematic review and modelling study. Lancet 390: 946-958, 2017.
2. Zhang W, Yang H, Kong X, Mohapatra S, San Juan-Vergara H, Hendriksen G, Bots L, Overduin M, Simon BD, Lockhart RF, and Mohapatra SS: Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat Med 11: 56-62, 2005.
3. Lo MS, Brazas RM and Holtzman MJ: Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness. J Virol 79: 9315-9319, 2005.
4. Sedeyn K, Schepens B and Saelens X: Respiratory syncytial virus nonstructural proteins 1 and 2: Exceptional disrupters of innate immune responses. Viruses Pathog 7: 2, 2015.
5. Murphy BR and Collins PL: Live-attenuated virus vaccines for respiratory syncytial and parainfluenza viruses: Applications of reverse genetics. J Clin Invest 110: 21-27, 2002.
6. Stoppenburg AJ, Salimi V, Hennus M, Plantinga M, Huis in’t Veld K, Wijburg J, Meerdinger C, Cuenen F, Bots L and Boes M: Local IL-17A potentiates early neutrophil recruitment to the respiratory tract during severe RSV infection. PLoS One 8: e78461, 2013.
7. Faber TE, Groen H, Welfing M, Jansen KJ and Bont LJ: Specific increase in local IL-17 production during recovery from primary RSV bronchiolitis. J Med Virol 84: 1084-1088, 2012.
8. Zamora MR, Budev M, Rolfe M, Gottlieb J, Humar A, Devincenzo J, Vaishnav A, Cehelsky J, Albert G, Nouchr’s, et al: RNA interference therapy in lung transplant patients infected with respiratory syncytial virus. Am J Respir Crit Care Med 183: 531-538, 2011.
9. Bitko V, Musiyenko A, Shulyayeva O and Barik S: Inhibition of respiratory viruses by nasally administered siRNA. Nat Med 11: 50-55, 2005.
10. Kong X, Zhang W, Lockey RF, Auais A, Piedimonte G and Mohapatra SS: Respiratory syncytial virus infection in Fischer 344 rats is attenuated by short interfering RNA against the RSV-NS1 gene. Genet Vaccines Ther 5: 4, 2007.
11. Boyapalle S, Wong T, Garay J, Teng M, San Juan-Vergara H, Mohapatra S and Mohapatra S: Respiratory syncytial virus NS1 protein colocalizes with mitochondrial antiviral signaling protein MAVS following infection. PLoS One 7: e29386, 2012.
12. Rostad CA, Stobart CC, Gilbert BE, Pickles RJ, Hotard AL, Meng J, Blanco JCG, Moin SM, Graham BS, Piedra PA and Moore ML: A recombinant respiratory syncytial virus vaccine candidate attenuated by a low-fusion F protein is immunogenic and protective against challenge in cotton rats. J Virol 90: 7508-7518, 2016.
13. Yang P, Zheng J, Wang S, Liu P, Xie M and Zhao D: Respiratory syncytial virus nonstructural proteins 1 and 2 are crucial pathogenic factors that modulate interferon signaling and Treg cell distribution in mice. Virology 485: 223-232, 2015.
14. Yan S, Yue YZ, Sun MM, Wu BS and Wang XP: Suppressive effect of Aurantii Fructus Immaturus and Atractylodis Macrocephalae Rhizoma on glutamic acid-induced autophagy of interstitial cells of Cajal. J Integr Med 18: 334-343, 2020.
15. Huang XP, Ding H, Lu JD, Tang YH, Deng BX and Deng CQ: Autophagy in cerebral ischemia and the effects of traditional Chinese medicine. J Integr Med 13: 289-296, 2015.
16. Heidevand V, Saitoh T and Akira S: Autophagy in infection, inflammation and immunity. Nat Rev Immunol 13: 722-737, 2013.
17. Tanida I, Ueno T and Kominami E: LC3 and autophagy. Methods Mol Biol 445: 77-88, 2008.
18. Shi B, Ma M, Zheng Y, Pan Y and Lin X: mTOR and Beclin1: Two key autophagy-related molecules and their roles in myocardial ischemia/reperfusion injury. J Cell Physiol 234: 12562-12568, 2019.
19. Jung CH, Ro SH, Cao J, Otto NM and Kim DH: mTOR regulation of autophagy. FEBS Lett 584: 1287-1295, 2010.
20. Reed M, Morris SH, Owczarczyk AB and Lukacs NW: Deficiency of autophagy protein Map1-LC3b mediates IL-17-dependent lung pathology during respiratory viral infection via ER stress-associated IL-1. Mucosal Immunol 8: 1118-1130, 2015.

21. Xu X, Zheng J, Zheng K, Hou Y, Zhao F and Zhao D: Respiratory syncytial virus NS1 protein degrades STAT2 by inducing SOCS1 expression. Intervirology 57: 65-73, 2014.

22. Zullo AJ, Jurcic Smith KL and Lee S: Mammalian target of Rapamycin inhibition and mycobacterial survival are uncoupled in murine macrophages. BMC Biochem 15: 4, 2014.

23. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-\Delta\Delta C(T)} method. Methods 25: 402-408, 2001.

24. Li M, Li J, Zeng R, Yang J, Liu J, Zhang Z, Song X Yao Z, Ma C, Li W, et al: Respiratory syncytial virus replication is promoted by autophagy-mediated inhibition of apoptosis. J Virol 92: e02193-e02217, 2018.

25. O'Donnell V, Pacheco JM, LaRocco M, Burrage T, Jackson W, Rodriguez LL, Borca MV and Baxt B: Foot-and-mouth disease virus utilizes an autophagic pathway during viral replication. Virology 410: 142‑150, 2011.

26. Pokharel SM, Shil NK and Bose S: Autophagy, TGF-β, and SMAD-2/3 signaling regulates interferon-β response in respiratory syncytial virus infected macrophages. Front Cell Infect Microbiol 6: 174, 2016.

27. Owczarczyk AB, Schaller MA, Reed M, Rasky AJ, Lombard DB and Lukacs NW: Sirtuin 1 regulates dendritic cell activation and autophagy during respiratory syncytial virus-induced immune responses. J Immunol 195: 1637-1646, 2015.

28. Zhang J, Ruan T, Sheng T, Wang J, Sun J, Wang J, Prinz RA, Peng D, Liu X and Xu X: Role of c-Jun terminal kinase (JNK) activation in influenza A virus-induced autophagy and replication. Virology 526: 1-12, 2019.

29. Pandey A, Ding SL, Qin QM, Gupta R, Gomez G, Lin F, Feng X, Fachini da Costa L, Chaki SP, Katepalli M, et al: Global reprogramming of host kinase signaling in response to fungal infection. Cell Host Microbe 21: 637-649.e6, 2017.

30. Gannage M, Dormann D, Albrecht R, Dengjel J, Torossi T, Ramer PC, Lee M, Strowig T, Arrey F, Conenello G, et al: Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. Cell Host Microbe 6: 367-380, 2009.

31. Nakashima A, Tanaka N, Tamai K, Kyuuma M, Ishikawa Y, Sato H, Yoshimori T, Saito S and Sugamura K: Survival of parvovirus B19-infected cells by cellular autophagy. Virology 349: 254-263, 2006.

32. Shrivastava S, Raychoudhuri A, Steele R, Ray R and Ray RB: Knockdown of autophagy enhances the innate immune response in hepatitis C virus-infected hepatocytes. Hepatology 53: 406-414, 2011.