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Autophagy protects type II alveolar epithelial cells from *Mycobacterium tuberculosis* infection

Xu-Guang Guo [a,b,1], Tian-Xing Ji [c,1], Yong Xia [b,*], Yue-Yun Ma [a,*]

[a] Center for Clinical Laboratory Medicine of PLA, Xijing Hospital, Fourth Military Medical University, Xi'an, China
[b] Department of Laboratory Medicine, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China
[c] Department of Laboratory Medicine, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

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ABSTRACT

This study was designed to investigate the protective effect of the autophagy signaling pathway against *Mycobacterium tuberculosis* infection in type II alveolar epithelial cells. An *in vitro M. tuberculosis* system was established using human A549 cells. Infection-induced changes in the expression of the autophagic marker LC3 were assessed by reverse transcription-PCR and Western blotting. Morphological changes in autophagosomes were detected by transmission electron microscopy (TEM). The function of the autophagy signaling pathway during infection was assessed by measuring the level of cell death and the amount of lactate dehydrogenase (LDH) released in the presence or absence of the inhibitor 3-methyladenine (3-MA). In addition, effects on LDH release were assessed after the siRNA-mediated knockdown of the essential autophagosomal structural membrane protein Atg5. LC3 mRNA expression was significantly reduced in *M. tuberculosis*-infected A549 cells (16888.76 ± 1576.34 vs. uninfected: 12744.29 ± 1089.37; $P < 0.05$). TEM revealed *M. tuberculosis* bacilli-containing compartments that were surrounded by double membranes characteristic of the autophagic process. *M. tuberculosis*-infected A549 cells released more LDH (1.45 ± 0.12 vs. uninfected: 0.45 ± 0.04; $P < 0.05$). The inhibition of autophagy signaling significantly enhanced *M. tuberculosis*-induced necrosis (3-MA: 75 ± 5% vs. untreated: 15 ± 1%; $P < 0.05$) and LDH release (3-MA: 2.50 ± 0.24 vs. untreated: 0.45 ± 0.04; Atg5 knockdown: 3.19 ± 0.29 vs. untreated: 1.28 ± 0.11; $P < 0.05$). Our results indicate that autophagy signaling pathway prevents apoptosis in type II alveolar epithelial cells infected with *M. tuberculosis* and may represent a molecular target for promoting cell survival during infection by respiratory pathogens.

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), remains a global health threat, causing an estimated two million annual deaths worldwide [1]. Many studies have focused on the role of the immune system during *M. tuberculosis* infection because it is believed that immune responses represent the first and most robust challenge to the bacilli establishing a successful infection in the body [2]. Recently, alveolar epithelial cells were identified as important mediators of the early immune response to respiratory pathogens.

There are two types of alveolar epithelial cells: type I and type II cells, both of which contribute to the host response to and the pathogenesis of *M. tuberculosis* infection [3]. Several *in vitro* studies have demonstrated that type II alveolar pneumocytes are capable of internalizing *M. tuberculosis* bacilli but at a much slower rate than that of macrophages. Once inside the type II alveolar cells, the bacteria have been shown to replicate extensively [4–6]. The replicated bacteria may then escape the cell following bacteria-induced apoptosis or necrosis, facilitating systemic dissemination. Sato et al. reported that the human type II alveolar epithelial cultured cell line A549 secretes tumor necrosis factor-alpha (TNF-α) following infection by *M. tuberculosis*, and the secretion of TNF-α subsequently contributes to the activation of alveolar macrophages [7]. Another study demonstrated that mycobacterial movement through the alveolar epithelial cells promotes phenotypic changes that are related to the strain's virulence [8]. Thus, alveolar epithelial cells appear to play an important role in *M. tuberculosis* infection. In addition, alveolar epithelial cells are known to have critical roles in infections with other bacterial pathogens, including *Legionella pneumophila* and *Burkholderia cepacia* [9], and have been shown to release monocyte chemoattractant factors that stimulate the response of macrophages to *Pseudomonas aeruginosa* [10]. However, the precise role of these cells in *M. tuberculosis* infection remains to be elucidated.

The autophagy signaling pathway is a well-studied host immune response to intracellular pathogens and includes microauto-

* Corresponding authors. Fax: +86 20 81292243; +86 29 82527499.
E-mail addresses: gysyxy@gmail.com (Y. Xia), cmbmayy@fmmu.edu.cn (Y.-Y. Ma).
[1] These authors contributed equally to this work.

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phagy, chaperone-mediated autophagy, and macroautophagy (hereafter referred to as autophagy) [11]. Autophagy is considered an ancient cellular response to starvation that facilitates the recycling of amino acids and the sequestering of damaged organelles and unused proteins [12]. In pathogenic conditions in mammals, autophagy also plays a role in the immune response against intracellular pathogens by breaking down pathogen components even if these organisms have overcome the typical phagosome/lysosome fusion mechanism [13]. Studies of *M. tuberculosis* have revealed that the macrophage-mediated killing of the bacilli involves autophagy [12] and that the activation of the autophagy signaling pathway in phagocytic cells promotes the lysosomal killing process [14]. It is possible, however, that autophagy may also play a significant role in the *M. tuberculosis* infection of non-phagocytic cells.

Autophagy is a complicated multi-stage process. The newly developed vesicle membrane fuses with a lysosome to form an autolysosome, and the molecules and/or organisms sequestered in this vesicle are subjected to enzymatic degradation and subsequent recycling. This process is different from the endocytosis-mediated lysosomal degradation of extracellular proteins. Autophagosomes form from the elongation of autophagosome precursors, a process that is initiated by class III phosphoinositide 3 kinase (PI3K). A complex containing the autophagy-related (Atg) genes Atg15, Atg14, Vps34, and Beclin-1 (an ortholog of yeast Atg6) forms to trigger the nucleation of the pre-autophagosome [15]. In addition, two ubiquitin-like conjugates are required for the elongation of the isolation membrane and the completion of enclosure. One conjugate is the Atg12 and Atg5 conjugate, which forms a dimeric complex together with Atg16L1. The other conjugate is a phosphatidyl-ethanolamine-conjugated Atg8 homolog (e.g., LC3, GATE16, or GABARAP). LC3 is transferred to the autophagosomal membrane by the Atg12–Atg5 complex [15]. The soluble LC3 form, LC3-I, is then converted into the lipid-bound form, LC3-II, which is integrated into the functional autophagosome membrane [15,16].

In 2004, the autophagy pathway was shown to be an important intracellular response to bacterial infection [12,17]. More recently, the autophagy signaling pathway in human and murine macrophages was demonstrated to be critical during *M. tuberculosis* infection [18]. Because type II alveolar epithelial cells may play a role in the process of *M. tuberculosis* infection, we hypothesized that autophagy in type II alveolar epithelial cells is protective during infection. Testing this hypothesis would provide great insight into this bacterium's pathogenesis and may allow the identification of targets for molecular therapies. The A549 cell line was selected for use as the *in vitro* infection system due to its typical type II alveolar epithelial cell characteristics, including the ability to perform absorptive endocytosis and the existence of lamellar bodies [19]. The expression of LC3, an important marker and effector of autophagy, was investigated, along with characteristic morphological changes in autophagosomal compartments. In addition, the role of autophagy in *M. tuberculosis*-induced apoptosis and necrosis was studied. This study provided evidence of a previously unrecognized protective function of autophagy in type II alveolar epithelial cells against *Mycobacterium spp*., including the common Bacille Calmette–Guérin (BCG) vaccination strain.

2. Methods

2.1. Cell line and bacterial strains

The human type II alveolar epithelial cell line A549 (Institute of Biochemistry and Cell Biology, China) was maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen) at 37 °C. The *Mycobacterium bovis* BCG strain was from an in-house collection (Third Affiliated Hospital of Guangzhou Medical University). The *Mycobacteria* were grown as plated cultures on Middlebrook 7H10 agar enriched with 10% Middlebrook OADC growth supplement. A549 cells were seeded in 10 cm dishes and grown to 80–90% confluency, at which point the cells were infected with bacteria by replacing the medium with fresh medium containing one of the three strains (at a 10:1 bacterium-to-cell ratio).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

A549 cells were collected at various time points post-infection for extraction of the total RNA using the Trizol Reagent (Invitrogen). Complementary DNA was generated from 1 μg of total RNA using a cDNA synthesis kit (Promega, USA). Amplification of the LC3 gene (300 bp) was performed with the following gene-specific primers: forward, 5′-GCGGAGAAATAACAGCGTCTTCCG-3′, and reverse, 5′-CGCTATTCAGCCTCTCTGCTCTGC-3′. Amplification of the β-actin gene (250 bp; internal control) was performed with the following primers: forward, 5′-TCGTCGACAACGGCTCCGGCATGT-3′, and reverse, 5′-CATTGTAGAAGGTGTGGTG-3′. Each PCR was performed in a 25 μL reaction system composed of 2.5 μL of 10× PCR buffer, 2 μL of 2.5 mM dNTPs, 0.5 μL of *Taq* DNA polymerase, 0.5 μL of forward primer, 0.5 μL of reverse primer, 5 μL of cDNA, and water. A negative control reaction was run without template cDNA. The thermal cycling conditions for both LC3 and β-actin were as follows: initial denaturation at 95 °C for 10 min; 30 amplification cycles at 94 °C for 50 s, 55 °C for 50 s, and 72 °C for 50 s; and a final extension at 72 °C for 7 min. The quality of the PCR products was assessed by 1% agarose gel electrophoresis and ethidium bromide staining, and the gray values of the resolved bands were analyzed using ImageJ software. All samples were run in triplicate. LC3 expression was calculated relative to β-actin expression.

2.3. Western blotting

A549 cells were lysed by incubation in lysis buffer supplemented with 10 μL PMSF on ice for 30 min. The total protein concentration was measured using a bicinchoninic acid kit (Beyotime, China). Cell lysates (40-μg protein) were then separated by SDS/polyacrylamide gel electrophoresis and transferred to polyvinylidine fluoride membranes (Sigma, USA). After blocking with dry milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) for 2 h, the membrane was incubated with rabbit polyclonal antibodies against LC3 or β-actin (1:1000; Abcam, UK) overnight at 4 °C, followed by incubation with a goat anti-rabbit IgG antibody (1:3000; Invitrogen) for 1 h. Immunoreactive protein bands were detected using a diaminobenzidine (DAB) solution. β-Actin protein served as a loading control.

2.4. Atg5 knockdown by siRNA

Two siRNA sequences targeting the *Atg5* gene were synthesized: 5′-GCAACTCTGGATG-GGATTG-3′ and 5′-ATGGCATTATCCAATTGGT-3′. Each sequence was subcloned into a pSilencer3.1H1 vector to generate pSilencer3.1H1-D and pSilencer3.1H1-V, respectively. A549 cells were seeded into 6-well plates (3×10^5/mL) and cultured overnight to achieve 90–95% confluency. The culture medium was replaced with serum-free Opti-MEM (Invitrogen) containing 4 μg of control siRNA, pSilencer3.1-H1-RNAi-D, or pSilencer3.1-H1-RNAi-V coated with Lipofectamine 2000 (Invitrogen). After 6 h of incubation, RPMI-1640 containing 10% FCS was added to the transfection cultures, which were then incubated for an additional 48 h. The transfection efficiency of A549 cells was approximately 60%.

2.5. Transmission electron microscopy (TEM)

A549 cells were grown on plastic coverslips in 6-well plates. Before and after *M. tuberculosis* infection, the cells were fixed with 2.5% (v/v) glutaraldehyde in phosphate-buffered saline (PBS) overnight at 4 °C. Post-fixation was carried out with 1% (v/v) osmium tetroxide for 90 min, after which the cells were dehydrated by passage through a graded ethanol series. The cells were then impregnated with resin by incubating them in ascending dilutions of resin in propylene oxide, followed by incubation in pure resin overnight. The next day, the cells were embedded and polymerized by incubation at 60 °C for 48 h. The coverslips were peeled off to expose the cell monolayer, and thin sections (~60 nm) were generated using an ultramicrotome. The slices were placed on 200 mesh copper grids and stained with uranyl acetate and lead citrate for contrast. The grids were viewed using a JEOL 1210 transmission electron microscope at 80 kV, and images were captured using a digital camera. Vesicles were identified as autophagosomes if a double membrane was present, ribosomes were absent from the cytosolic side of the vacuole, the densities of the luminal side of the vesicle and the cytosol were similar, or organelles were observed inside the vesicle.

2.6. Lactate dehydrogenase (LDH) assay

A549 cells at 80–90% confluency in 96-well plates were infected with *M. tuberculosis* using a 10:1 bacterium-to-cell ratio. The infected cells were designated as the MTB cells and divided into two groups: the untreated infection control group (MTB group), and infected cells pre-treated with 3-MA, a pharmacological inhibitor of the autophagy signaling pathway (3-MA + MTB group). Uninfected and untreated cells were used as negative controls (PBS group). Uninfected cells with a pharmacologically inhibited autophagy signaling pathway were used as the treatment control (3-MA group). Cells were harvested at 4, 8, 16, and 24 h post-infection to measure the level of released LDH in the medium using the BioVision LDH-Cytotoxicity Assay Kit II (USA). The LDH reaction results were determined by reading the absorbance at 450 nm using a LAMBDA 35 UV/vis Spectrophotometer (Perkin Elmer, USA). The percentage of cytotoxicity was calculated using the following formula: [(Experimental release − spontaneous release)/(maximum LDH release − spontaneous release)] × 100, where spontaneous release was the level of LDH measured for the PBS group.

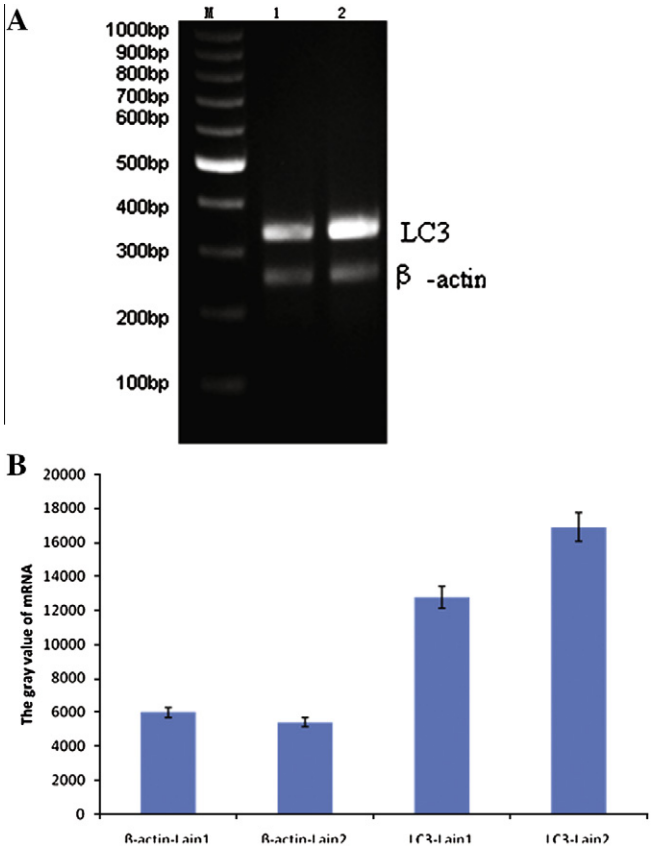

Fig. 1. *M. tuberculosis* infection reduces LC3 mRNA expression in A549 cells. RT-PCR detection of LC3 and β-actin mRNA expression in MTB-infected cells (lane 1) and uninfected control cells (lane 2) (A). The expression of LC3 ($n = 2$) was normalized to the expression of β-actin (internal control) and is presented as the mean ± SD. Lane M, marker. The amplified LC3 mRNA values of untreated A549 cells and A549 cells treated with MTB were 16888.76 ± 1576.34 and 12744.29 ± 1089.37, respectively (gray). These values were significantly lower than those in the uninfected control group ($P < 0.05$) (B).

2.7. Necrosis assays

A549 cells at 80–90% confluency in 6-well dishes were divided into four groups: two groups for infection with *M. tuberculosis* (10:1 bacterium-to-cell ratio) and two uninfected groups. The cells were then treated with 3-MA, as described above, and the four groups were designated PBS, 3-MA, MTB, and 3-MA + MTB. After 24 h, the cells were assessed for necrosis by incubating them with 0.5 mL of binding buffer and propidium iodide (PI) (all from Biyuntian, China) at 18–24 °C for 20 min and then observing them under a microscope, according to the manufacturer's instructions.

2.8. Statistical analysis

All statistical analyses were performed using SPSS v10.0 software (USA). All data are shown as the mean ± SD. Student *t*-test was used to analyze the differences between two groups. *P*-values lower than 0.05 were considered to be significant.

3. Results

3.1. Mycobacterium tuberculosis infection increases LC3 expression in A549 cells

RT-PCR revealed that the LC3 mRNA expression level in A549 cells was significantly increased after 24 h of *M. tuberculosis* infection (16888.76 ± 1576.34 vs. uninfected: 12744.29 ± 1089.37; $P < 0.05$) (Fig. 1A). Furthermore, TEM analysis showed that *M. tuberculosis*-infected cells had double-membrane compartments

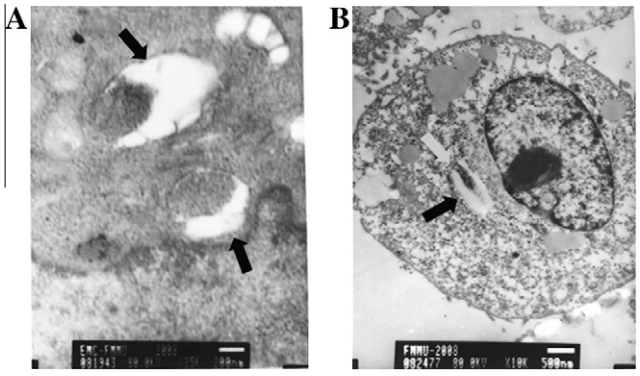

Fig. 2. The response of A549 cells to *M. tuberculosis* infection involves autophagosomes. TEM images showing autophagosomal compartments in (A) uninfected cells and (B) MTB-infected cells. The number of autophagosomes was higher in infected cells, and the double membrane vacuoles (black arrows) contained bacilli (white arrows). Magnification 1:10,000.

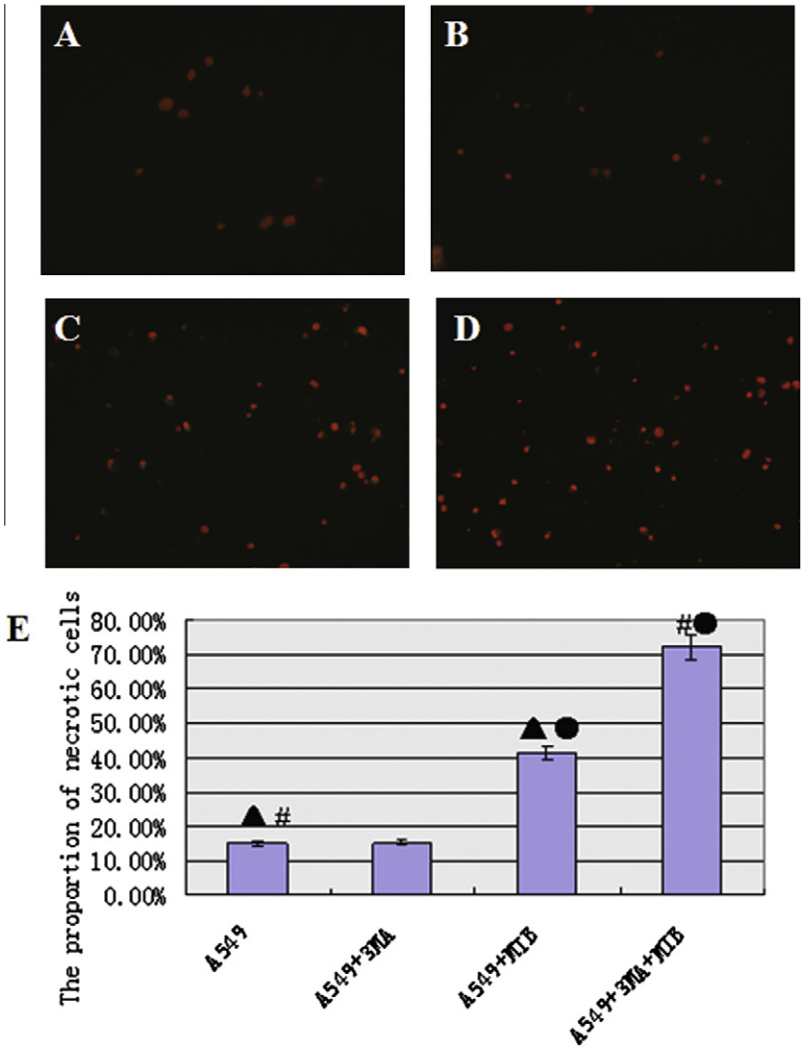

Fig. 3. Inhibition of the autophagy signaling pathway promotes necrosis in *M. tuberculosis*-infected A549 cells. PI staining (red) of PBS-treated uninfected cells (A), 3-MA pre-treated uninfected cells (B), MTB-infected cells (C), and 3-MA pre-treated MTB-infected cells (D). The 3-MA inhibitor had no effect on cell necrosis in uninfected cells (B) but did enhance MTB-induced necrosis (D). (E) Quantification of the number of necrotic cells in each group. $^{\blacktriangle}P < 0.05$, the control group vs. the MTB group; $^{\#}P < 0.05$, the 3-MA + MTB group vs. the MTB group; $^{\bullet}P < 0.05$, the control group vs. the 3-MA + MTB group. (For interpretation of color in this figure, the reader is referred to the web version of this article.)

containing bacilli, which were absent in uninfected cells (Fig. 2A and B). These findings suggest that the autophagy pathway might play a role in the response of type II alveolar epithelial cells to *M. tuberculosis* infection.

3.2. Autophagy prevents *M. tuberculosis*-induced necrosis in A549 cells

There were few to no necrotic A549 cells in the PBS-treated control group and the 3-MA treated control group (Fig. 3A and B). However, *M. tuberculosis* infection led to a significant increase in apoptotic A549 cells (vs. the PBS group, $P < 0.05$; Fig. 3C). Inhibition of the autophagic signaling pathway by 3-MA prior to infection significantly enhanced the levels of *M. tuberculosis*-induced apoptosis and necrosis in A549 cells (vs. the MTB group, $P < 0.05$; Fig. 3D and E).

3.3. Knockdown of Atg5 increases LDH release from *Mycobacterium spp.*-infected A549 cells

To further examine whether the inhibition of the autophagy signaling pathway affects the survival of type II alveolar epithelial cells during mycobacterium infection, we manipulated the autophagy pathway by knocking down Atg5 expression. Immunoblotting showed that the knockdown of Atg5 dramatically reduced the LC3 II protein level in A549 cells (Fig. 4A). LDH release experiments were performed to determine the effects of the autophagy pathway on host cell survivability after infection.

M. tuberculosis-infected A549 cells showed a higher level of LDH release (1.45 ± 0.12 vs. uninfected: 0.45 ± 0.04; $P < 0.05$). The inhibition of autophagy signaling significantly enhanced *M. tuberculosis*-induced necrosis (3-MA: 75 ± 5% vs. untreated: 15 ± 1%; $P < 0.05$) and LDH release (3-MA: 2.50 ± 0.24 vs. untreated: 0.45 ± 0.04, Atg5 knockdown: 3.19 ± 0.29 vs. untreated: 1.28 ± 0.11; $P < 0.05$).

BCG is a widely used vaccine against *tuberculosis* and is generated from a live attenuated strain of bovine *tuberculosis* that has lost virulence in humans. In A549 cells, BCG infection significantly increased LDH release (vs. the empty vector, $P < 0.05$; Fig. 4B and C). The siRNA-mediated knockdown of Atg5 significantly enhanced the BCG-induced LDH release from A549 cells at 12 and 24 h post-infection (vs. the empty vector, $P < 0.05$; Fig. 4B and C).

Our findings suggested that the autophagy signaling pathway plays important roles in the survival of type II alveolar epithelial cells during infection with *Mycobacterium spp*.

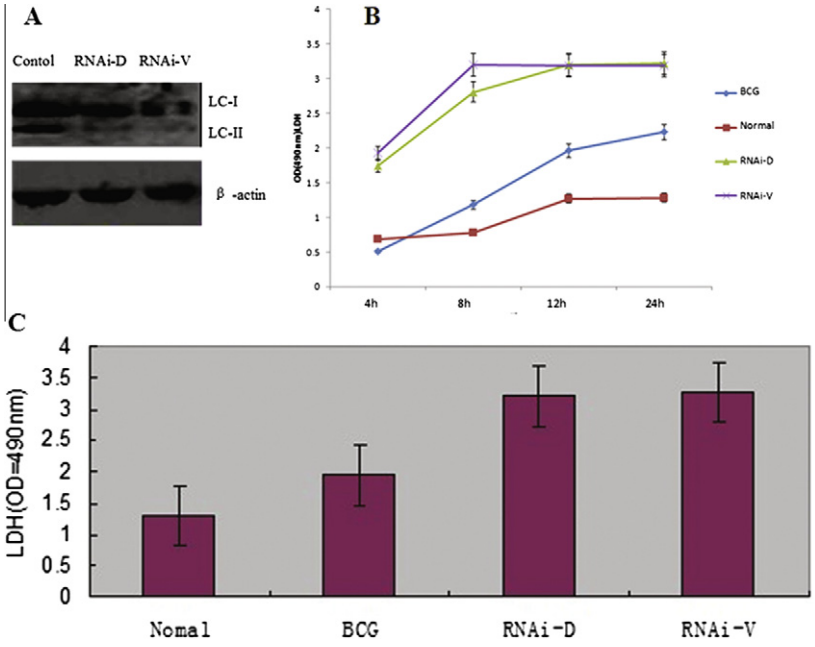

Fig. 4. The siRNA-mediated knockdown of Atg5 enhances LDH release from BCG-infected A549 cells. (A) Western blot showing that the knockdown of Atg5 using pSilencer3.1-H1-RNAi-D and pSilencer3.1-H1-RNAi-V significantly reduced LC3 II protein expression in A549 cells. β-Actin was detected as a loading control. (B and C) BCG-infected A549 cells (blue line) released more LDH at the indicated time points than uninfected cells (orange-yellow line). Atg5 knockdown by siRNA (green and purple lines) significantly enhanced LDH release from BCG-infected A549 cells ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, we determined that the autophagy signaling pathway has an important role in the infection of type II alveolar epithelial cells by *M. tuberculosis*. We showed that the LC3 mRNA level was reduced and that the autophagic biological process was induced upon infection in A549 cells. Pharmacological and genetic inhibition of the autophagy signaling pathway revealed that this mechanism protects type II alveolar epithelial cells from apoptosis induced by intracellular *M. tuberculosis* bacilli. Taken together, the results clearly show that there is an interaction between *Mycobacterium spp.* respiratory pathogens and alveolar epithelial cells *in vitro* and that the autophagy signaling pathway is indispensable for the survival of alveolar epithelial cells after infection.

For the RT-PCR experiments, we used the common 10:1 bacterium-to-cell ratio to infect A549 cells, and we detected a reduced LC3 mRNA level in the infected cells. The gray value of amplified LC3 mRNA from A549 cells infected with *M. tuberculosis* was obviously lower than that of the uninfected control group. Intriguingly, this finding is different from that of a previous study that showed that *M. tuberculosis* infection increased autophagy pathway activation in macrophages [20]. It is possible that the autophagy signaling pathway plays different roles in different types of immune cells, specifically macrophages and type II alveolar epithelial cells. Thus, certain factors that inhibit the autophagosome maturation process may exist in alveolar epithelial cells. A subsequent in-depth study using quantitative methods, such as real-time PCR, is merited.

In mammalian cells, the PI3K inhibitors wortmannin, 3-MA, bafilomycin A1 and LY294002 have been shown to effectively inhibit autophagy [21–23]. Autophagy is a dynamic process that is controlled by a precise set of autophagy-related genes that are tightly regulated by the upstream serine/threonine kinase, mammalian target of rapamycin (mTOR). mTOR has been characterized as a regulator of multiple biological functions, such as cell growth, cell death, cell motility, protein synthesis, and autophagy. Among the upstream factors that control mTOR activation, PI3K is the key factor that is responsive to different stimuli, including growth factors. Three PI3K classes have been recognized, among which class III PI3K/hVps34 has been shown to positively regulate autophagy [24]. Class III PI3K catalyzes the phosphorylation of the hydroxyl group at the third position of the inositol ring of phosphatidylinositol to produce phosphatidylinositol-3-phosphate (PI3P). Evolutionarily conserved from yeast to humans, class III PI3K consists of a core complex with PI3KC3 (Vps34), p150 (Vps15), and Beclin-1 (Vps30/Atg6) [24]. Autophagy has been implicated in the pathogenesis of diverse disease processes, including certain types of neuronal degeneration, cancer, viral infection, and aging [16]. The autophagy inhibitor 3-MA was identified through a screen of purine-related substances in hepatocytes isolated from starved rats. Since then, various studies have shown that 3-MA is able to block autophagosome maturation by increasing the lysosomal pH, reducing the lysosomal density, and inhibiting transport from late endosomes to lysosomes [25].

The autophagy-related proteins play a critical role in resistance to bacterial and viral infections in metazoan organisms [26]. Previous studies have shown that the Atg genes control autophagosome formation through Atg12–Atg5 and LC3 II complexes [16]. Atg5 conditional knockout mice with targeted depletion in macrophages and neutrophils were found to have decreased resistance to *Listeria monocytogenes* and *Toxoplasma gondii*, whereas neuron-specific Atg5 knockout mice exhibited increased susceptibility to infection of the central nervous system by *Sindbis* virus [27,28]. Therefore, we knocked down Atg5 in A549 cells to further evaluate the role of autophagy in *M. tuberculosis* infection. The knock down of this protein led to a significant reduction in LC3 II protein expression in normal (uninfected) cells, confirming the role of Atg5 in autophagosome maturation. Following BCG infection, the Atg5-depleted A549 cells showed a dramatic reduction in resistance to BCG infection. These findings suggest that the autophagy signaling pathway protects type II alveolar epithelial cells from BCG infection and that the inhibition of the autophagy pathway affects the infection process. We showed that autophagy can protect epithelial cells from *M. tuberculosis* infection. However, the receptor that

mediates this effect and is expressed on the epithelial cell remains unknown. Heparin-binding haemagglutinin (HBHA) is a candidate receptor for this process because several previous reports have demonstrated its role in the extrapulmonary dissemination of *M. tuberculosis*. Future studies will address whether HBHA is capable of inhibiting autophagy in *M. tuberculosis*-infected cells.

In summary, the data from our current *in vitro* study suggest that *M. tuberculosis* infection inhibits the autophagy signaling pathway in type II alveolar epithelial cells. TEM analysis revealed that these cells eliminate intracellular *M. tuberculosis* by autophagy. In addition, pharmacological and genetic inhibition of the autophagy pathway, *via* PI3K blockade and the siRNA-mediated silencing of Atg5, respectively, decreased the cellular resistance to *Mycobacterium spp*. infection and increased the sensitivity to infection-induced apoptosis, respectively. Collectively, these findings provide novel insights into the interactions between *Mycobacterium spp*. bacilli and human type II alveolar epithelial cells. Further studies of the mechanisms by which autophagy protects epithelial cells from killing by *Mycobacterium spp*. will help to uncover manipulable factors and processes, facilitating the development of more effective treatments for respiratory infections.

Acknowledgments

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