miRNA-344b-1-3p modulates the autophagy of NR8383 cells during *Aspergillus fumigatus* infection via TLR2

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Abstract. Autophagy serves a pivotal role in host defense during fungal infections, and the contribution by toll-like receptor 2 (TLR2) has been well demonstrated. It has been reported that microRNA-344a-1-3p (miR-344a-1-3p) can directly target TLR2. However, the expression of TLR2 significantly decreases during *Aspergillus fumigatus* infection. Therefore, the specific role of miR-344a-1-3p in the host defense against *A. fumigatus* infection remains to be elucidated. In the present study, *A. fumigatus* infection increased the expression of TLR2 and induced autophagy, which was indicated by increasing expression levels of autophagy-related protein 5 (ATG5), Beclin-1, light chain 3 (LC3)-1 and LC3-II, as measured by western blot analysis, and an increased number of GFP-LC3 puncta, as measured by fluorescence. Following transfection with miR-344a-1-3p mimics and/or TLR2, miR-344b-1-3p significantly inhibited the expression of TLR2, Beclin-1, ATG5, LC3-I and LC3-II, whereas the overexpression of TLR2 significantly attenuated the inhibitory effect on autophagy by miR-344b-1-3p. Collectively, these findings demonstrate that *A. fumigatus* can be controlled by the induction of autophagy following de-repression of the expression of TLR2, mediated by miR-344a-1-3p.

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Introduction

Human airways are subjected to constant exposure to *Aspergillus fumigatus*, resulting in the inhalation of several hundred spores per day (1). However, components of the innate immune system, particularly macrophages, efficiently remove *A. fumigatus* once the spores enter alveoli within the lungs, therefore, adverse effects are rare (2-4). In immunocompromised individuals, *A. fumigatus* has been shown to be a causative agent of multiple forms of aspergillosis, including saprophytic aspergillosis, allergic aspergillosis and invasive aspergillosis, a life-threatening systemic fungal infection (5-7). In addition, *A. fumigatus* is currently the most prevalent airborne fungal pathogen, responsible for ~90% of systemic infections (8-10).

The inhalation of *A. fumigatus* spores initiates an antifungal immune response by pattern recognition receptors (PRRs). In particular, the toll-like receptors (TLRs) are a class of PRRs that recognize pathogen-associated molecular patterns and activate the innate immune response to eliminate invading pathogens and mediate the adaptive immune response. As a member of the TLR family, TLR2 serves an important role in recognizing various *Aspergillus* cell wall components and modulating host defense responses, including autophagy (11-13). Furthermore, TLR2 is critical to activating autophagy against fungi in macrophages (14).

Autophagy maintains cellular homeostasis through targeted degradation. Multiple roles for autophagy have been reported in the inflammatory response and the defense against infections; a recent study suggested an important role in the clearance of bacterial, viral and parasitic pathogens (15). Regarding the response to fungal infection, autophagy has been linked to several processes important to immunity, including pathogen recognition, phagocytosis, microbial killing, cytokine release, antigen presentation and inflammation regulation. Several proteins are involved in autophagy, including light chain 3 (LC3), Beclin-1 and autophagy-related protein 5 (ATG5) (16-18).

Autophagy can also be regulated by microRNAs (miRNAs), a class of endogenous short, non-coding RNAs involved in the regulation of gene expression (19). Our previous study demonstrated that miR-344b-1-3p is an effective direct modulator of TLR2 (20). However, any correlation

between the levels of miRNA-344b-1-3p and autophagy remain to be elucidated.

Therefore, in the present study, the expression of miRNA-344a-1-3p was observed during *A. fumigatus* infection, and its role and potential mechanism in regulating infection-induced autophagy by macrophages was investigated to provide a better understanding of host defense mechanisms following *Aspergillus* infection.

Materials and methods

Cell culture. A rat alveolar macrophage cell line, NR8383, was purchased from the Shanghai Institute of Biochemistry and Cell Biology. The cells were maintained in Ham's F-12K medium (Sigma-Aldrich; Merck KGaA) containing 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2.

Microbes. *A. fumigatus* was prepared using the air from a moisture box and cultured on 1.5% malt extract agar plates (Biokar Diagnostics; Beuvais, France) placed in the dark at 25˚C for 7 days. Thee spores were harvested using a sterile loop, which were then suspended in Hank's balanced salt solution containing 0.0001% Triton X-100. The spores were counted under a light microscope using a Bürker chamber to calculate their concentration and their strain was identified using an identification service (Central Bureau of Schimmelcultures, Utrecht, Netherlands). The collected spores were stored at -20˚C until use.

Exposure to A. fumigatus. The NR8383 cells were seeded in triplicate into 6-well plates ($5x10^4$ cells per well) and incubated at 37˚C in an atmosphere containing 5% CO_2. Following overnight culture, the cells were exposed to *A. fumigatus* (10^7 spores per well) for the indicated durations (0, 30, 45, 60, 90 or 120 min) at 37˚C. Following treatment, the cells were harvested, following which total RNA was extracted and stored at -80˚C until examination.

Cell transfection. The miR-344b-1-3p mimics, negative control (NC), and inhibitor were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection, according to the manufacturer's protocol. The NR8383 cells were seeded into 24-well plates ($1x10^4$ cells per well) and transfected with the miR-344b-1-3p mimics, NC or inhibitor to a final concentration of 25 nM. *Tlr2* cDNA was cloned into the *Bam*HI and *Asc*I sites of the pLenti6/V5-DEST vector (Invitrogen; Thermo Fisher Scientific, Inc.). Then, 2 μl solution was added into 100 μl competence XL-BLUE *Escherichia coli* (Invitrogen; Thermo Fisher Scientific, Inc.) and absorbed for 15 min on ice. After being placed in a water bath for 50 sec at 42˚C and rapidly cooled for 2 min on ice, the cell solution was pipetted into 1 ml LB culture medium (Merck KGaA), oscillated for 1 h at 37˚C and 180 rpm and then centrifuged for 3 min 2,012.4 x g and 37˚C. The pellet was mixed with 200 ml LB culture medium, coated onto the LB culture plates containing 100 μg/ml ampicillin and cultured overnight at 37˚C. A total of 10 clones were picked out and inoculated in 3 ml LB culture medium. After culturing overnight at 37˚C on a shaker at 250 rpm, the vectors were extracted and then verified by nucleotide sequencing by GenScript Corporation (Nanjing, China). Subsequently, the NR8383 cells were stably transfected with the vector using Lipofectamine 2000 and incubated in Ham's F-12K medium containing 0.1 mg/ml blasticidin (Invitrogen; Thermo Fisher Scientific, Inc.). The transfected cells were then cultured for 24 h prior to treatment with *A. fumigatus* (10^7 spores per well) for 60 min at 37˚C, and then harvested for examination.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The samples (500 ng) were reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) through incubating for 5 min at 25˚C followed by 60 min at 42˚C. qPCR was performed using the SYBR Green PCR Master Mix kit (Takara Biotechnology Co., Ltd.), with the following steps: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 40 sec. The primers for miR-344b-1-3p, *Tlr2*, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and *U6* were designed and obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences are listed in Table I. The expression levels of the *Tlr2* mRNA and miR-344b-1-3p were normalized to *Gapdh* and *U6*, respectively, using the $2^{-\Delta\Delta Ct}$ method (21).

Western blot assay. Western blot analysis was performed using samples from whole-cell lysates processed using a Total Protein Extraction kit (Beyotime Institute of Biotechnology; Beijing, China) and quantified with a BCA Protein Assay kit. Following heat denaturation at 95˚C for 5 min, the samples of total protein (20 μg) were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (5%), electrophoresed and transferred onto polyvinylidene difluoride membranes (EMD Millipore; Billerica, MA, USA). The membranes were blocked in 5% nonfat milk for 2 h at room temperature, and were then incubated overnight at 4˚C with one of the following primary antibodies: TLR2 (cat. no. EPR20303; Abcam, Cambridge, MA, USA), ATG5 (cat. no. 12994), Beclin-1 (cat. no. 3738), LC3 (cat. no. 4108) and GAPDH (cat. no. 5174) from Cell Signaling Technology, Inc. (Danvers, MA, USA). The primary antibodies were used in a 1:1,500 dilution. Following washing three times with TBST, the membranes were incubated with secondary antibody in a 1:10,000 dilution (cat. no. A1949; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Following a final wash, signal was detected using enhanced chemiluminescence reagents (GE Healthcare Life Sciences). The band intensities were quantified and normalized to GAPDH using ImageJ 1.47 software (National Institutes of Health).

Examination of autophagy by GFP-LC3 detection. The NR8383 cells were transfected in the manner described above with the inclusion of GFP-LC3 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells transfected only with GFP-LC3 were incubated with *A. fumigatus* (10^7 spores per well) for the indicated duration (0, 30, 45, 60, 90 or 120 min).

Table I. Primer pairs for the reverse transcription-quantitative polymerase chain reaction analysis.

Name	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	AATGCATCCTGCACCACCAA	GATGGCATGGACTGTGGTCA
U6	CGCTTCACGAATTTGCGTGTCAT	GCTTCGGCAGCACATATACTAAAAT
miR-344b-1-3p	GGGGTGATATAACCAAAGCC	GTGCGTGTCGTGGAGTCG
TLR2	TGCAGGGACCTTTGCTATGATG	ACAAAGTCCCGCTTGTGGGA

miR, microRNA; TLR2, toll-like receptor 2.

The co-transfected cells were incubated with *A. fumigatus* spores for 60 min. The cells were then incubated with DAPI (5 μg/ml; Beyotime Institute of Technology) for 10 min. Following fixing of the cells in 4% paraformaldehyde for 30 min at room temperature, the cells were visualized by confocal microscopy.

Statistical analysis. Statistical analyses were performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± SEM, with each assay performed in triplicate. The statistical significance was estimated by using one-way analysis of variance followed by Bonferroni's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

A. fumigatus spores induce a reduction in miR-344b-1-3p and an increase in TLR2, LC-I, LC-II, beclin-1 and ATG-5. To confirm the association between miR-344b-1-3p and TLR2, their expression at the transcriptional level was determined by RT-qPCR analysis. As shown in Fig. 1A, the levels of miRNA-344b-1-3p decreased in a time-dependent manner following exposure to the spores, reaching a plateau after 90 min of treatment. By contrast, a corresponding time-dependent increase was observed in the mRNA expression of *Tlr2* following exposure to the spores, reaching a plateau after 90 min of treatment (Fig. 1B). A similar effect on the protein expression of TLR2 was observed (Fig. 1C and D). These data show that the inverse correlation between the mRNA levels of *Tlr2* and miR-344b-1-3p may indicate that TLR2 is a target of miR-344b-1-3p.

The immunofluorescence analysis showed that the number of GFP-LC3 puncta increased in a time-dependent manner following infection (Fig. 2), indicating that the presence of *A. fumigatus* spores induced autophagy time-dependently within 120 min of exposure. Furthermore, the expression levels of each of the above-mentioned proteins were negatively and positively associated with the levels of miR-344b-1-3p and TLR2, respectively. These data indicate that the expression of autophagy-related proteins and autophagy itself may be mediated by miR-344b-1-3p and TLR2.

miR-344b-1-3p significantly suppresses the autophagy induced by A. fumigatus spores. Following transfection with the NC mimic, inhibitor NC, mimics or inhibitor, the cells were exposed to *A. fumigatus* for 60 min to observe the effect of miR-344b-1-3p on spore-induced autophagy. The 60-min exposure time was selected to allow time for autophagy to initiate and also avoid the saturation response. To demonstrate the transfection effects of miR-344b-1-3p inhibitor or mimics, the expression of miR-344b-1-3p was measured by RT-qPCR assay. The results showed that the expression of miR-344b-1-3p was significantly increased in the miR-344b-1-3p mimics group, and was significantly decreased in the miR-344b-1-3p inhibitor group, compared with that in the NC groups, which indicates high transfection efficiency (Fig. 3A). In addition, the expression levels of ATG5, Beclin-1, LC3-1 and LC3-II were determined by western blotting. As shown in Fig. 3B, the expression levels significantly decreased following mimics treatment and significantly increased following inhibitor treatment. Similarly, the density of GFP-LC3 puncta markedly decreased and increased following treatment with mimics and inhibitor, respectively (Fig. 3C). These results indicate that miR-344b-1-3p mimics treatment significantly decreased spore-induced autophagy and that the inhibition of miR-344b-1-3p significantly increased autophagy. Consequently, miR-344b-1-3p may inhibit *A. fumigatus* autophagy by regulating the levels of ATG5, Beclin-1 and LC3.

Additionally, the levels of TLR2 were determined by western blot analysis; the levels were markedly decreased following mimics exposure and markedly increased following inhibitor exposure (Fig. 4A and B), indicating that TLR2 is a target of miR-344b-1-3p.

miR-344b-1-3p inhibits the autophagy induced by A. fumigatus spores by regulating TLR2. To investigate the roles of miR-344b-1-3p and TLR2 in the autophagy induced by *A. fumigatus* spores, cells were transfected with one of the following: Control, mimics NC, mimics, mimics + TLR2 NC, mimics NC + TLR2 and mimics + TLR2. The results from the western blot assay revealed that the expression levels of TLR2, Beclin-1, ATG5, LC3-I and LC3-II were significantly decreased in cells transfected with miR-344b-1-3p mimics compared with those in the NC group. The expression levels of TLR2, Beclin-1, ATG5, LC3-I and LC3-II were significantly increased in cells transfected with mimics NC + TLR2 compared with those in cells in the mimics NC group. The expression levels of TLR2, Beclin-1, ATG5, LC3-I and LC3-II were markedly increased in the cells transfected with mimics + TLR2 compared with those in the mimics group (Fig. 4A-F). Similarly, the number of GFP-LC3 puncta in the cells was significantly decreased following miR-344b-1-3p

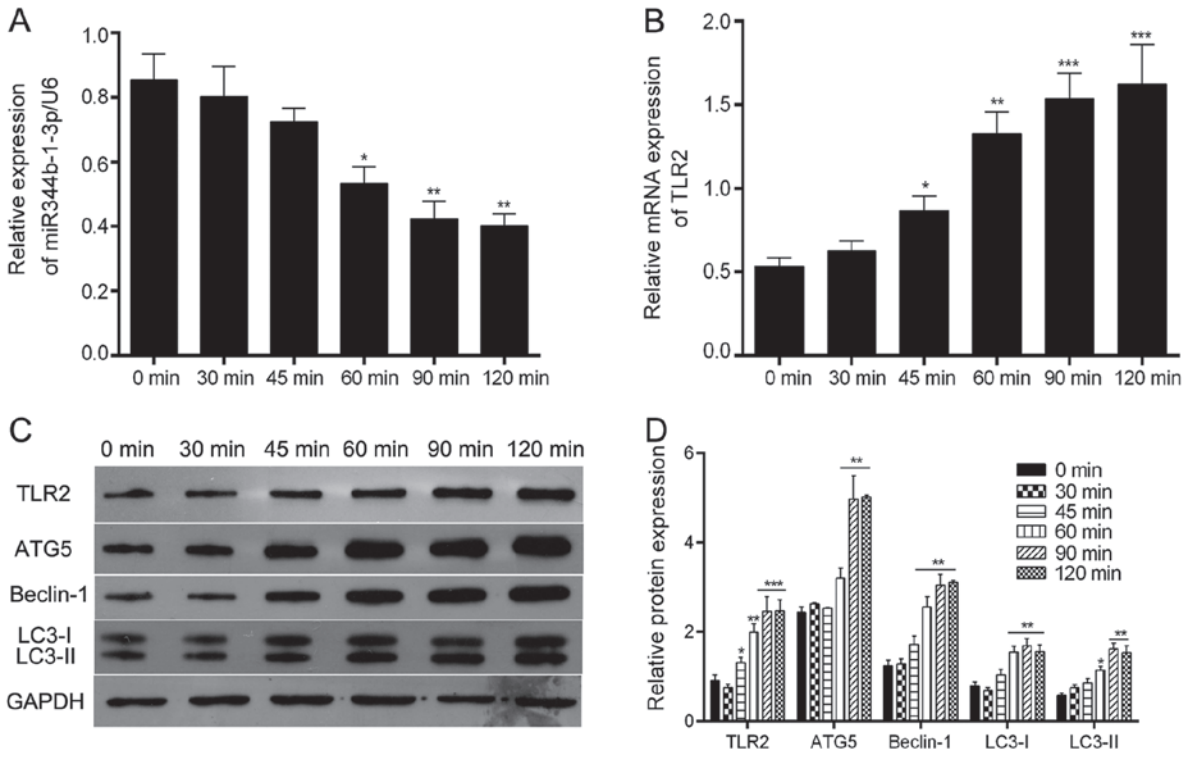

Figure 1. Effect of *A. fumigatus* infection on the expression of miR-344a-1-3p and TLR2. Following exposure to *A. fumigatus* for the indicated times, NR8383 cells were harvested. Total RNA was extracted, and (A) miR-344p-1-3a and (B) Tlr2 mRNA were detected by reverse transcription-quantitative polymerase chain reaction analysis. (C) Total protein was isolated, and TLR2, ATG5, Beclin-1, LC3-I and LC-II were analyzed by western blot analysis and normalized to GAPDH. (D) Quantification of protein expression. *P<0.05, **P<0.01 and ***P<0.001 compared with the uninfected group. *A. fumigatus, Aspergillus fumigatus*. miR, microRNA; TLR2, toll-like receptor 2; ATG5, autophagy-related protein 5; LC3, light chain 3.

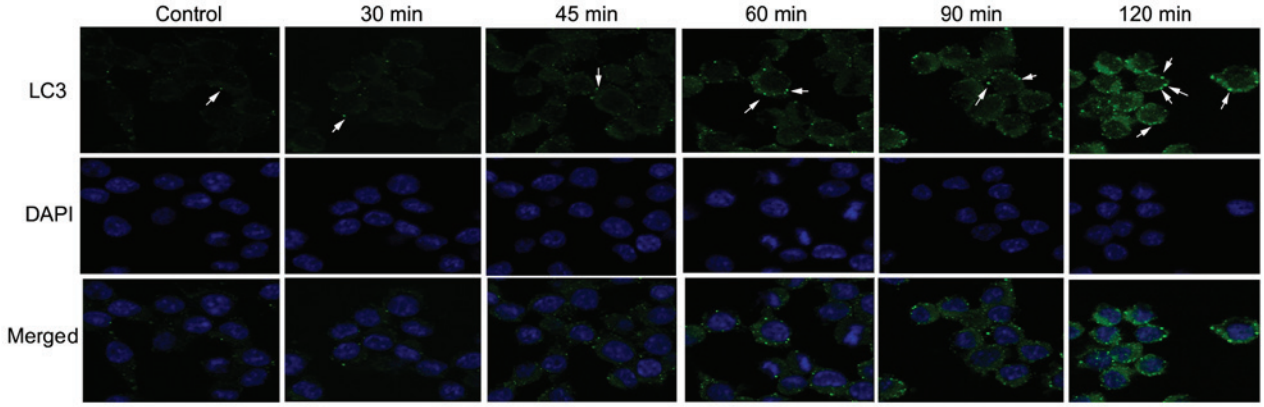

Figure 2. Effect of *Aspergillus fumigatus* infection on autophagy of NR8383 cells. Cells were infected for the indicated times and evaluated for autophagy by the detection of GFP-LC3 (green). Nuclei were stained with DAPI (blue). Cells were analyzed by confocal microscopy (magnification, x400; arrows indicate GFP-LC3 puncta). LC3, light chain 3.

mimics transfection, whereas the overexpression of TLR2 markedly attenuated the inhibitory effect on autophagy by miR-344b-1-3p (Fig. 4G). Therefore, these data indicated that miR-344b-1-3p may serve an important role in *Aspergillus* spore-induced autophagy by regulating TLR2.

Discussion

miRNAs are critical to multiple facets of immune system function, including pathogen recognition, inflammation activation and resolution. TLR2, an important pathogen recognition receptor, is targeted and regulated by miR-344b-1-3p, however, its role in regulation of immune function remains to be elucidated. To the best of our knowledge, miR-344b-1-3p was first associated with *A. fumigatus*-induced autophagy in macrophages, and may act by mediating recognition of the fungus.

Accumulating evidence suggests that autophagy is closely associated with immunity. *A. fumigatus* infection can elicit autophagy in several types of inflammatory cell, including monocytes and macrophages, and regulate their physiological activity (22,23). Studies have shown that autophagy directly affects the production of certain cytokines by mediating

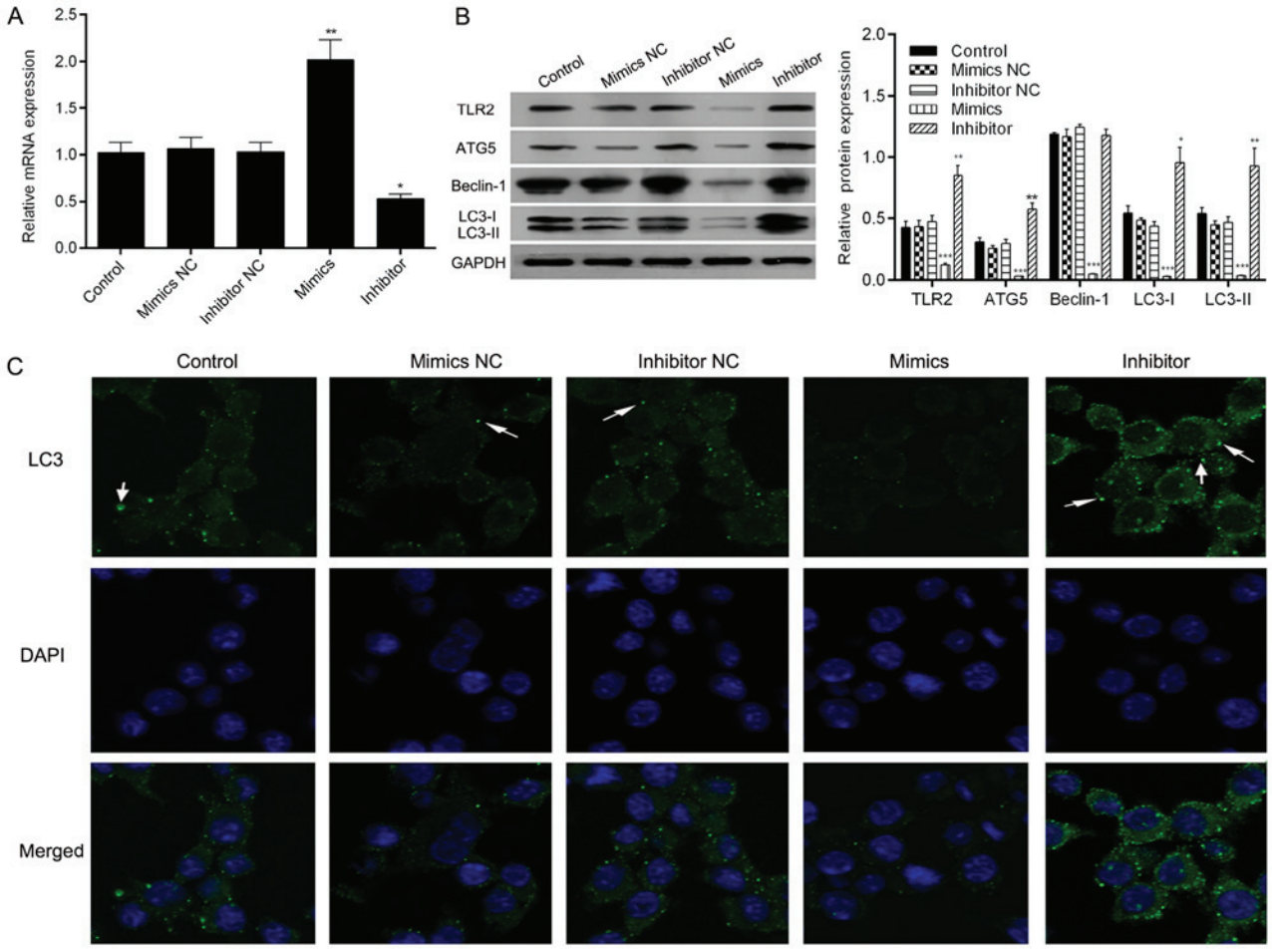

Figure 3. Effect of miR-344p-1-3p knockdown on the autophagy of NR8383 cells induced by *A. fumigatus* infection. Cells were transfected with miR-344p-1-3a mimics NC, inhibitor NC, mimics, or inhibitor, and then infected with *A. fumigatus* for 60 min. (A) Expression of miR-344p-1-3p was evaluated via reverse transcription-quantitative polymerase chain reaction assay in each group. (B) Levels of ATG5, Beclin-1, LC3-I, LC-II and TLR2 were analyzed by western blot analysis and normalized to those of GAPDH. *P<0.05, **P<0.01 and ***P<0.001 compared with the uninfected group. (C) Cells were visualized by detecting GFP-LC3 (green). Nuclei were stained with DAPI (blue) and analyzed by confocal microscopy. The control group received the same *A. fumigatus* treatment as the other groups (magnification, x400; arrows indicate GFP-LC3 puncta). *A. fumigatus, Aspergillus fumigatus*; miR, microRNA; NC, negative control; TLR2, toll-like receptor 2; ATG5, autophagy-related protein 5; LC3, light chain 3.

transcription, processing and secretion (24). These cytokines, which include interleukin-1β (IL-1β), IFN-γ, and tumor necrosis factor (TNF)-α, in turn dampen pro-inflammatory responses (25,26). Autophagy also accelerates macrophage aging, resulting in various functional changes, including impaired maturation, decreased antigen presentation capacity and reduced innate responses, alongside increased basal production of inflammatory cytokines (27). Additionally, autophagy triggers the death of activated macrophages through a caspase-independent pathway (28). Autophagy exerts dual functionality in macrophages, pro-inflammation and anti-inflammation, to achieve host protection and intracellular pathogen elimination.

Autophagy is both provoked and regulated. In its recognition of *A. fumigatus*, TLR2 serves a critical role in autophagy-induction in infected macrophages (14). In RAW264.7 cells, this induction is reached by activating multiple signaling pathways, including the JNK, ERK, and PI3K signaling pathways (29-31). Although the fungus used in these studies was not *A. fumigatus*, the same pathways were suggested to contribute to the autophagy in macrophages induced by *A. fumigatus*. Among the multiple functions of autophagy-induction, increased levels of pro-inflammatory cytokines have been reported. The cytokines, including IL-1β and TNF-α, can in turn upregulate autophagy, forming a positive feedback loop (24).

The ability of TLR2 to detect *Aspergillus* spores and induce autophagy is associated with its expression levels. Wu *et al* reported that induced expression of TLR2 by the fungus was noted in human corneal epithelial cells and mice macrophages (32,33). Consistent with these findings, in the present study, *A. fumigatus* exposure significantly increased the expression of TLR2 in rat alveolar cells, which was accompanied by the induction of autophagy and a decrease in the levels of miR-344b-1-3p. In addition, treatment with miRNA mimics in the present study attenuated the induction of TLR2 and autophagy. miR-344b-1-3p has been demonstrated to directly target TLR2 (20), suggesting a mechanism by which the miRNA may mediate autophagy. Additionally, targeting TLR2 with miR-344b-1-3p inhibited the expression of downstream genes, including TNF-α and IL-1β (20). In addition to the regulatory role of cytokines in autophagy,

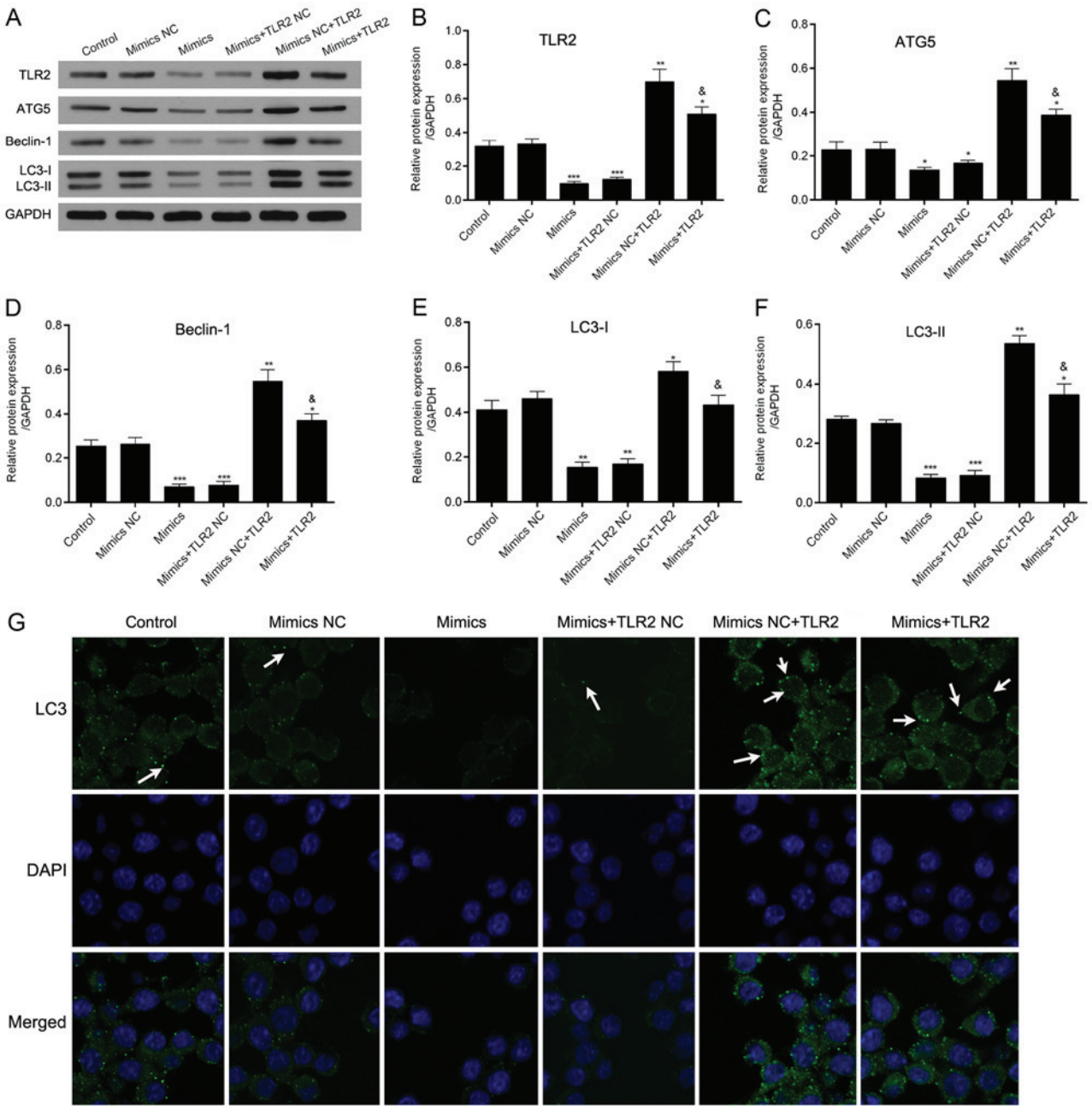

Figure 4. Effect of the overexpression of miR-344b-1-3p on the autophagy of *A. fumigatus*-infected NR8383 cells by TLR2. Cells were transfected with miR-344b-1-3p mimics and/or TLR2. The cells were then infected with *A. fumigatus* for 60 min. (A) Levels of TLR2, ATG5, Beclin-1, LC3-I and LC-II were analyzed by western blot analysis, GAPDH was used as loading control. Relative protein expression levels of (B) TLR2, (C) ATG5, (D) Beclin-1, (E) LC3-I (F) and LC-II were analyzed based on the protein gray values. *P<0.05, **P<0.01 or ***P<0.001 compared with the control group; $^{\&}$P<0.05 compared with the mimics NC + TLR2 group. (G) Cells were evaluated for autophagy by the detection of GFP-LC3 (green). Nuclei were stained with DAPI (blue) and analyzed by immunofluorescence microscopy (magnification, x400; arrows indicate GFP-LC3 puncta). *A. fumigatus, Aspergillus fumigatus*; miR, microRNA; NC, negative control; TLR2, toll-like receptor 2; ATG5, autophagy-related protein 5; LC3, light chain 3.

miR-344b-1-3p may also regulate autophagy in macrophages exposed to fungi by inhibiting cytokines. In addition to miR-344b-1-3p, miR-19 and miR-105 have been demonstrated to regulate the expression of TLR2 (34,35), indicating they may be associated with the activation of autophagy in macrophages. However, this requires confirmation by further investigations.

Taken together, the results of the present study demonstrate that the levels of miR-344b-1-3p decreased following challenge by *A. fumigatus*, which enhanced the activation of autophagy in infected macrophages by targeting TLR2 to protect against infection by intracellular mycobacteria. The present study reveals the importance of an miRNA in the regulation of autophagy and elimination of *A. fumigatus*. At present, miR-344b-1-3p has only been examined in rats. There is evidence to show that miR-344b-1-3p is an effective modulator of the TLR2 gene in COPD rats (1). It was also found that miR-344b-1-3p cannot be detected in *Homo sapiens*. In the future, there may be potential therapeutic interventions for saprophytic aspergillosis, allergic aspergillosis and invasive aspergillosis by artificially synthesizing miR-344b-1-3p and the targeted drugs.

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Availability of data and materials

The analyzed datasets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

All authors designed the study. YW, HX, YL, DH performed the experiments. YW, HX, YL, LC, YH and LL collected and analyzed the data. YW wrote the manuscript. DZ and WH provided advice on the experimental design and critically revised the manuscript. All authors read and approved the final version of the 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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