

Combination of azithromycin and methylprednisolone alleviates *Mycoplasma pneumoniae* induced pneumonia by regulating miR-499a-5p/STAT3 axis

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Abstract. *Mycoplasma pneumoniae* (*M. pneumoniae*) is a contributing factor to community-acquired pneumonia in children. The present study sought to explain the underlying mechanism of azithromycin (AZM) combined with methylprednisolone (MP) in the treatment of *M. pneumoniae* infection. Peripheral blood samples were obtained from patients with *M. pneumoniae* and healthy volunteers for analysis. A549 cells were infected with *M. pneumoniae* to construct an *in vitro* cell model with *M. pneumoniae*, followed by treatment with AZM and MP. Cell Counting Kit-8 and TUNEL assays were conducted to detect cell viability and apoptosis. RT-qPCR was employed to measure the expression levels of microRNA (miR)-499a-5p and STAT3. Western blotting was performed to measure the expression of STAT3 and apoptosis-related proteins. Luciferase report assay was performed to verify the binding site between miR-499a-5p and STAT3. The production of inflammatory cytokines was determined using ELISA kits. The results exhibited the downregulated miR-499a-5p and dysregulated inflammatory cytokines in peripheral blood of patients and *M. pneumoniae*-infected A549 cells. AZM and MP treatment alone or combined significantly inhibited inflammatory response, cell viability loss and promoted apoptosis in A549 cells infected with *M. pneumoniae*, which was partly reversed by inhibition of miR-499a-5p. Furthermore, miR-499a-5p could negatively regulate its direct target STAT3. In addition, STAT3 is also regulated by AZM and MP. Collectively, the present results suggested that combination treatment of AZM and MP could inhibit *M. pneumoniae* infection-induced inflammation, cell

viability loss and promoted apoptosis partly by regulating miR-499a-5p/STAT3 axis.

Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is globally recognized as an important pathogen leading to upper and lower respiratory tract infection in human, particularly in children (1). ~40% of children over five years of age suffering from community-acquired pneumonia (CAP) are caused by *M. pneumoniae* (2). *M. pneumoniae*-induced pneumonia (MPP) accounts for 30% of pneumonia (3). As many as 18% of pediatric pneumonia patients need hospitalization (4). Furthermore, it has been reported that *M. pneumoniae* infection in children aged 5~15 years is closely linked with lobar pneumonia, featuring as acute, severe and prone to complications such as pleural effusion, atelectasis and extrapulmonary system involvement (5). Currently, macrolides, such as azithromycin (AZM), are recommended as the first-line therapy for *M. pneumoniae* infection. However, the therapeutic effect is limited due to the drug resistance.

The activation of systemic inflammatory response is a critical characteristic for severe pneumonia, and the dysregulation of inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin (IL)-6 and IL-10, contributes to the development of this disease (6). Methylprednisolone (MP) is a synthetic glucocorticoid preparation which inhibits immune and inflammatory response (7). A combined treatment of MP and AZM provided improved results compared with AZM alone in terms of faster improvement in clinical manifestations, lower clinical symptom score, and attenuated laboratory and radiographic items in refractory MPP of children, indicating that the combination of MP and AZM may be a promising therapeutic strategy for children with MPP (8).

At present, RNAomics, including microRNA (miRNA or miR), piRNA, mRNA and lncRNA, have emerged as a research focus in the post-genome era. miRNAs are a class of single-stranded non-coding RNA with regulatory functions (9). It was demonstrated that dysregulated miRNA expression is associated with lung-related diseases, and miRNA is involved in the regulation of various biological behaviors (10).

Existing studies have revealed a downregulated expression of miR-499a-5p in clinical patients suffering from hepatitis B virus or autism, and in *in vivo* models including gliomas, lung

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adenocarcinoma and myocardial injury (11-15). In particular, miR-499a-5p was declined in sepsis-induced lung injury, and restoration of miR-499-5p protected the lung from injury by inhibiting inflammation (16). Therefore, the involvement of miR-499a-5p in inflammation- and infection-related diseases was hypothesized (15,17). However, whether miR-499a-5p is also downregulated in MPP remains unclear.

In the present study, *M. pneumoniae* strain was utilized to infect A549 cells to mimic MPP *in vitro*, and the role of miR-499a-5p in MPP was investigated for the first time. In addition, the potential mechanism underlying combination of MP and AZM in MPP was also explored.

Materials and methods

Clinical specimen collection. This study included hospitalized children aged 5-10 years who were diagnosed with MPP at Wuhan Fourth Hospital (Wuhan, China) from January to December 2019. Children with compromised immunity, or suffering from chronic lung diseases or asthma were excluded from the present study. Peripheral blood samples were obtained from 30 fasting patients with MPP (18 females and 12 males) and 30 fasting healthy volunteers (17 females and 13 males) in the morning. All patients were in the acute stage of MPP and did not take any medication prior to sample collection. The present study was approved (approval no. 2018-KY-08) by the Ethics Committee of Wuhan Fourth Hospital (Wuhan, China). Patients were enrolled in the study after written informed consent was obtained from their parents or guardians.

Cell culture and treatment. *M. pneumoniae* standard strain ATCC15531 [American Type Culture Collection (ATCC)] was cultured in PPLO-yeast-extract-glucose-penicillin media containing 20% fetal bovine serum. A549 cells were obtained from ATCC and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cell culture conditions comprised humidified air, 5% CO₂ and 37°C.

To establish MPP model *in vitro*, 1x10⁶ ccu *M. pneumoniae* suspensions were used to infect 1x10⁵ A549 cells for 4 h (18,19). Then, the medium was refreshed to remove the unbound *M. pneumoniae*. For treatment, A549 cells were treated with 20 µg/ml AZM (Pfizer Pharmaceuticals) alone or in combination with 400 µg/ml MP (MP sodium succinate; Pfizer, Inc.) 1 h prior to *M. pneumoniae* induction (20,21).

Cell counting kit (CCK)-8 assay. Cell proliferation was assessed by CCK-8 assay. After the A549 cells seeded in a 96-well plate at a density 5x10³ cells/well were infected with *M. pneumoniae* or treated with AZM and MP for 24, 48 and 72 h, respectively, 10 µl of CCK-8 reagent (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well for another 4 h incubation at 37°C. Absorbance at 450 nm was determined using a microplate reader (ELx808; Omega Bio-Tek, Inc.).

Cytokines analysis. The production of C-reactive protein (CRP) and inflammatory cytokines, including TNF-α (cat. no. DTA00D), IL-6 (cat. no. D6050), IL-10 (cat. no. D1000B)

and CRP (cat. no. DCRP00) in the cell culture medium were detected using their corresponding ELISA assay kits (R&D Systems, Inc.) following the manufacturer's protocol.

TUNEL assay. Cell apoptosis was determined using TUNEL assay (cat. no. C1098, Beyotime Institute of Biotechnology). After indicated treatment, A549 cells in a 24-well plate (2x10⁵ cells/well) were fixed in 4% paraformaldehyde for 25 min at room temperature and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate), followed by incubation with 0.3% H₂O₂ in methanol at room temperature for 20 min. Then, TUNEL detection solution was added to cells for another 1 h of incubation at 37°C, followed by incubation with stop solution for 10 min at 37°C. Finally, cells were incubated with DAB solution at room temperature for 10 min in the dark and counterstained with hematoxylin to stain the nuclei. After mounting with neutral resin, cells in five fields of view were randomly selected for observation under a light microscope (Olympus Corporation).

Cell transfection. miR-499a-5p mimic (5'-UUAAGACUU GCAGUGAUGUUU-3') and miR-499a-5p inhibitor (5'-AAA CATCTCTGCAAGTCTTAA-3'), as well as their negative controls (mimic control (5'-ACUACUGAGUGACAGUAG A-3') and inhibitor control (5'-CAGUACUUUUGUGUAGUA CAA-3')), were synthesized by Guangzhou RiboBio Co., Ltd. A549 cells at the logarithmic phase were seeded into six-well plates (1.5x10⁶ cells/well) and cultured overnight. When 80-90% confluence was achieved, A549 cells were transfected with miR-499a-5p mimic (50 nM), mimic control (50 nM), miR-499a-5p inhibitor (50 nM) and inhibitor control (50 nM) using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) in line with the manufacturer's protocol, respectively. After 48 h of transfection at 37°C, A549 cells were harvested for further experiments.

Western blotting. The total proteins were extracted from A549 cells using RIPA buffer with protease inhibitors (Beyotime Institute of Biotechnology). After determining the protein concentration with BCA protein assay kit (Beyotime Institute of Biotechnology), the proteins (30 µg) were electrophoresed via 10% SDS-PAGE and transferred onto PVDF membrane (MilliporeSigma). After blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with primary antibodies against Bcl-2 (1:1,000; cat. no. ab196495), Bax (1:1,000; cat. no. ab182733), cleaved caspase3 (1:500; cat. no. ab32042), caspase3 (1:500; cat. no. ab13847), STAT3 (1:1,000; cat. no. ab68153) and GAPDH (1:1,000; cat. no. ab181603) at 4°C overnight. The aforementioned antibodies were all purchased from Abcam. Then, these membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Enhanced chemiluminescence (MilliporeSigma) was used to visualize reactive protein bands on X-ray film. The protein bands were quantitatively analyzed by ImageJ v1.8.0 software (National Institutes of Health).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from clinical samples or

Table I. Characteristics of the subjects in the present study.

Characteristics	Patients with MPP (n=30)	Healthy volunteers (n=30)
Sex		
Female	18 (60%)	17 (57%)
Male	12 (40%)	13 (43%)
Age, years		
5-7	20 (67%)	19 (58%)
8-10	10 (33%)	11 (42%)
Mean age, years	7.03±1.67	7.17±1.74
CRP (mg/l)	6.00±1.25	23.62±2.58

MPP, *Mycoplasma pneumoniae*-induced pneumonia; CRP, C-reactive protein.

A549 cells was extracted using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After measuring the purity at a wavelength of 260 nm and concentration of RNA using NanoDrop 2000 (Thermo Fisher Scientific, Inc.), the extracted RNA was reversely transcribed into cDNAs using PrimerScript reverse transcriptase kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol, followed by qPCR using SYBR Green kit (Qiagen GmbH) on an ABI7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 10 min initial denaturation at 94°C, 15 sec denaturation at 94°C and 30 sec of annealing at 55°C (40 cycles) and final extension for 1 min at 72°C. The values were calculated using the $2^{-\Delta\Delta C_q}$ method (22) and normalized to U6 or GAPDH expression. The sequences of the primers used were as followed: miR-499a-5p forward, 5'-GCCGAG TTAAGACTTGCAGTGA-3' and reverse, 5'-CTCAACTGG TGTCGTGGA-3'; STAT3 forward, 5'-CATCCTGAAGCT GACCCAGG-3' and reverse, 5'-TCCTCACATGGGGGA GGTAAG-3'; GAPDH forward, 5'-AATGGGCAGCCGTTA GGAAA-3' and reverse, 5'-CGCGCCCAATACGACCAA ATC-3'; and U6 forward, 5'-AACGCTTCACGAATTTGC GT-3' and reverse, 5'-CTCGCTTCGGCAGCACA-3'.

Bioinformatics. The potential binding site between STAT3 and miR-499a-5p was predicted by Starbase v2.0 (<https://starbase.sysu.edu.cn/>).

Luciferase reporter assay. The STAT3 sequence including the putative binding sites of miR-499a-5p was sub-cloned and inserted into the pmirGLO vector (Promega Corporation) to form wild-type STAT3 (STAT3-WT) and mutant STAT3 (STAT3-MUT) vectors. Then, A549 cells were co-transfected with STAT3-WT or STAT3-MUT vector, as well as miR-499a-5p mimic and mimic control using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.). After 48 h of transfection, relative luciferase activity was detected using a dual-luciferase reporter assay kit (Promega Corporation) and normalized to that of *Renilla*.

Statistical analysis. All data were analyzed using SPSS software (version 22.0; IBM Corp.). Differences between two groups were determined using unpaired Student's

t-test, and comparison among groups was analyzed using one-way ANOVA analysis followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of miR-499a-5p and inflammatory cytokines between patients and volunteers with or without MPP treatment. A total of 30 children with MPP and 30 healthy volunteers were recruited in the present study (Table I). First, the peripheral blood from children with MPP and healthy volunteers was collected for analysis. As revealed in Fig. 1A-D, the levels of TNF- α , IL-6 and CRP were significantly increased while the level of IL-10 was significantly decreased in patients compared with those in the healthy volunteers. In addition, a downregulated expression of miR-499a-5p was detected in peripheral blood of patients compared with the healthy volunteers (Fig. 1E). The aforementioned findings suggested that miR-499a-5p may be associated with the development of MPP.

***M. pneumoniae* infection induces inflammation and apoptosis of A549 cells.** To establish MPP model *in vitro*, 1×10^6 ccu *M. pneumoniae* suspensions were used to infect A549 cells for 4 h. It was revealed that the levels of TNF- α , IL-6 and CRP were significantly elevated while the level of IL-10 was significantly decreased in *M. pneumoniae*-infected A549 cells (Fig. 2A-D), which were consistent with the aforementioned results from clinical samples. Furthermore, after *M. pneumoniae* infection, cell viability was also significantly decreased (Fig. 2E). TUNEL assay also revealed the remarkably increased apoptosis of *M. pneumoniae*-infected cells (Fig. 2F). Accordingly, the decreased expression of Bcl-2, increased expression of Bax and cleaved caspase-3 as well as unchanged level of caspase 3 were observed (Fig. 2G).

Treatment with MP and AZM attenuates inflammation and apoptosis in *M. pneumoniae*-infected A549 cells. After successful establishment of MPP model *in vitro*, MP and AZM were used for treatment. Compared with the model group, AZM or MP treatment reduced levels of inflammatory factors

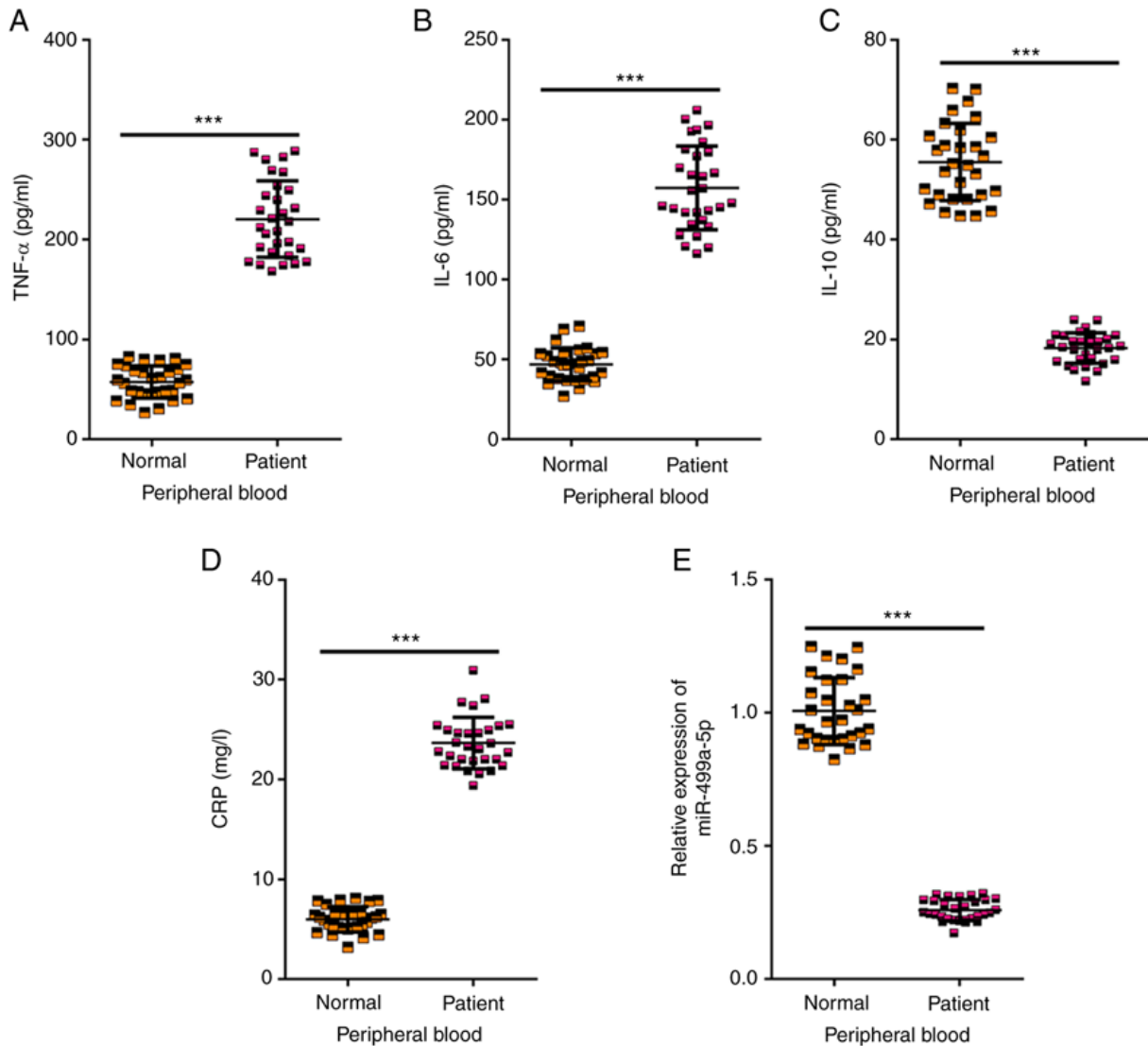


Figure 1. Comparison of miR-499a-5p and inflammatory cytokines between children with or without MPP. (A-D) After the peripheral blood from children with MPP and healthy volunteers was collected, the production of inflammatory cytokines, including (A) TNF- α , (B) IL-6, (C) IL-10 and (D) CRP was determined using corresponding ELISA kits. (E) Expression level of miR-499a-5p was assessed using reverse transcription-quantitative PCR. ***P<0.001. miR, microRNA; MPP, *Mycoplasma pneumoniae*-induced pneumonia; CRP, C-reactive protein.

in *M. pneumoniae*-infected A549 cells. Particularly, the combination of AZM and MP exhibited a more pronounced inhibition on inflammation (Fig. 3A-D). Additionally, CCK-8 and TUNEL assays showed that AZM alone or the combination of MP promoted cell viability and decreased cell apoptosis in *M. pneumoniae*-infected A549 cells (Fig. 3E and F). Western blot analysis also detected the upregulated expression of Bcl-2 and the downregulated expression of Bax and cleaved caspase-3 (Fig. 3G).

Effects of miR-449a-5p during MPP treatment. To identify the explicit role of miR-499a-5p in MPP, the expression level of miR-499a-5p in MPP model was also detected *in vitro*. As revealed in Fig. 4A, there was a decrease in the expression of miR-499a-5p in the model group and an increase in the level of miR-499a-5p in the AZM + MP group. Thus, miR-499a-5p may be involved in the progression of MPP. To verify our hypothesis, A549 cells were transfected with miR-499a-5p inhibitor to suppress the expression of miR-499a-5p (Fig. 4B).

In addition, the transfected and untransfected A549 cells were infected with *M. pneumoniae* and treated with AZM and MP, and miR-499a-5p inhibitor greatly reversed the elevated miR-499a-5p expression caused by AZM and MP treatment (Fig. 4C). Inhibition of miR-499a-5p significantly reversed the promotive effect of combination treatment of AZM and MP on cell viability (Fig. 4D). Furthermore, the inhibitory effects of AZM and MP combination treatment on inflammatory response and cell apoptosis were also retarded by miR-499a-5p inhibitor (Fig. 4E-J).

miR-499a-5p targets and negatively regulates STAT3. To further discover the functional mechanism of miR-499a-5p, a potential binding site between STAT3 and miR-499a-5p was identified through Starbase (Fig. 5A). Subsequently, this binding connection between STAT3 and miR-499a-5p was verified by luciferase reporter assay (Fig. 5B and C). In addition, RT-qPCR and western blotting assays revealed that both the mRNA and protein expression of

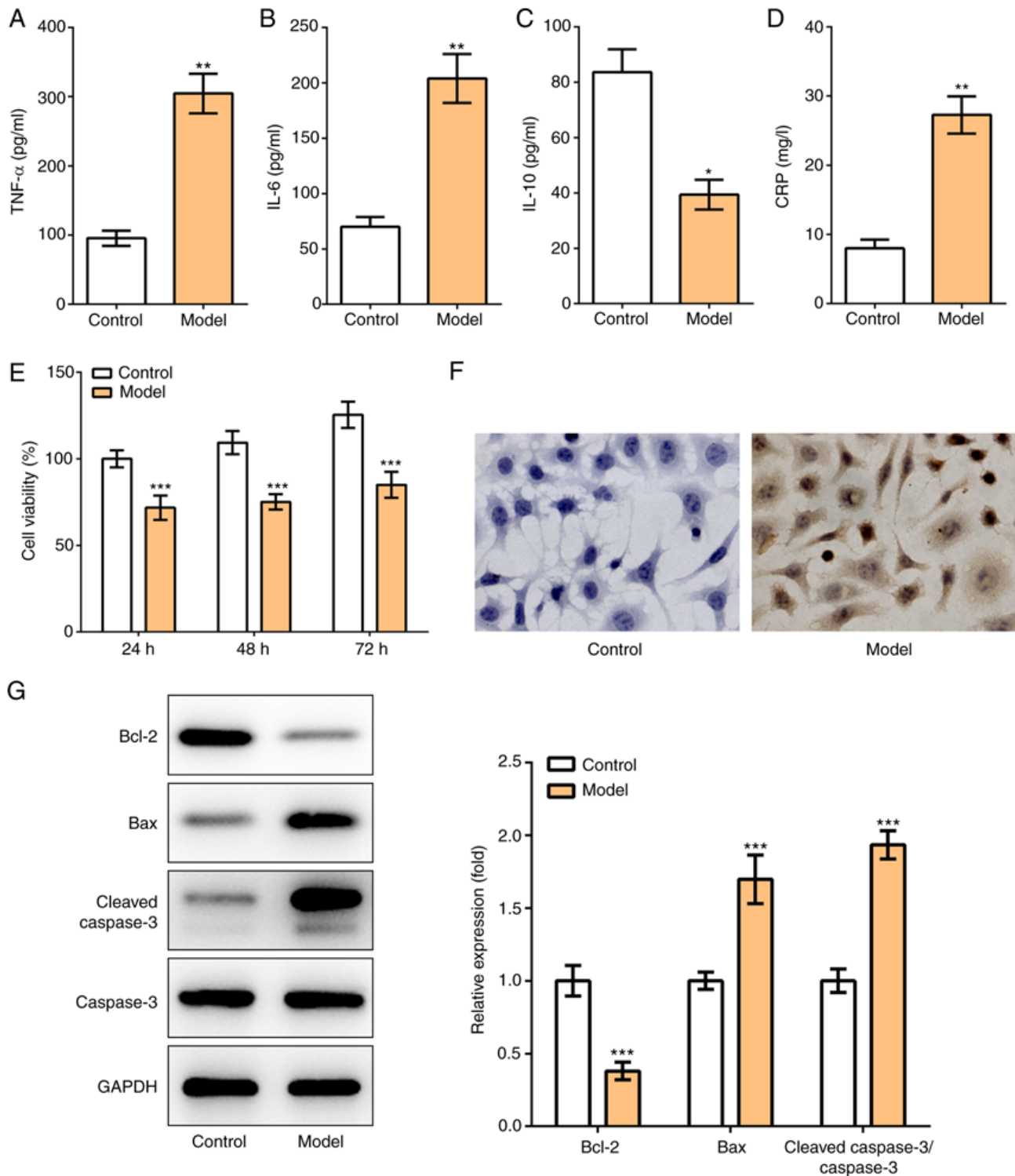


Figure 2. *M. pneumoniae* infection induces inflammation and apoptosis of A549 cells. (A-D) A549 cells were infected with *M. pneumoniae* to induce MPP model *in vitro*, and the production of inflammatory cytokines, including (A) TNF- α , (B) IL-6, (C) IL-10 and (D) CRP was determined using corresponding ELISA kits. (E) Cell Counting Kit-8 assay was applied to detect cell viability. (F) TUNEL assay was conducted to detect cell apoptosis (magnification, x200). (G) The protein expression levels of Bcl-2, Bax, cleaved caspase-3 and caspase-3 were detected using western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. *M. pneumoniae*, *Mycoplasma pneumoniae*; MPP, *Mycoplasma pneumoniae*-induced pneumonia; CRP, C-reactive protein.

STAT3 were significantly decreased upon miR-499a-5p overexpression but increased following miR-499a-5p inhibition (Fig. 5D and E), indicating that miR-499a-5p could target and negatively regulate STAT3. Furthermore, the mRNA and protein expression of STAT3 were also elevated in *M. pneumoniae*-infected A549 cells; however,

the combination treatment of AZM and MP inhibited the elevated STAT3, and this inhibitory effect was then weakened by miR-499a-5p inhibitor (Fig. 5F and G). These findings suggested that miR-499a-5p targets and negatively regulates STAT3, which is involved in the pathogenic mechanism and treatment of MPP.

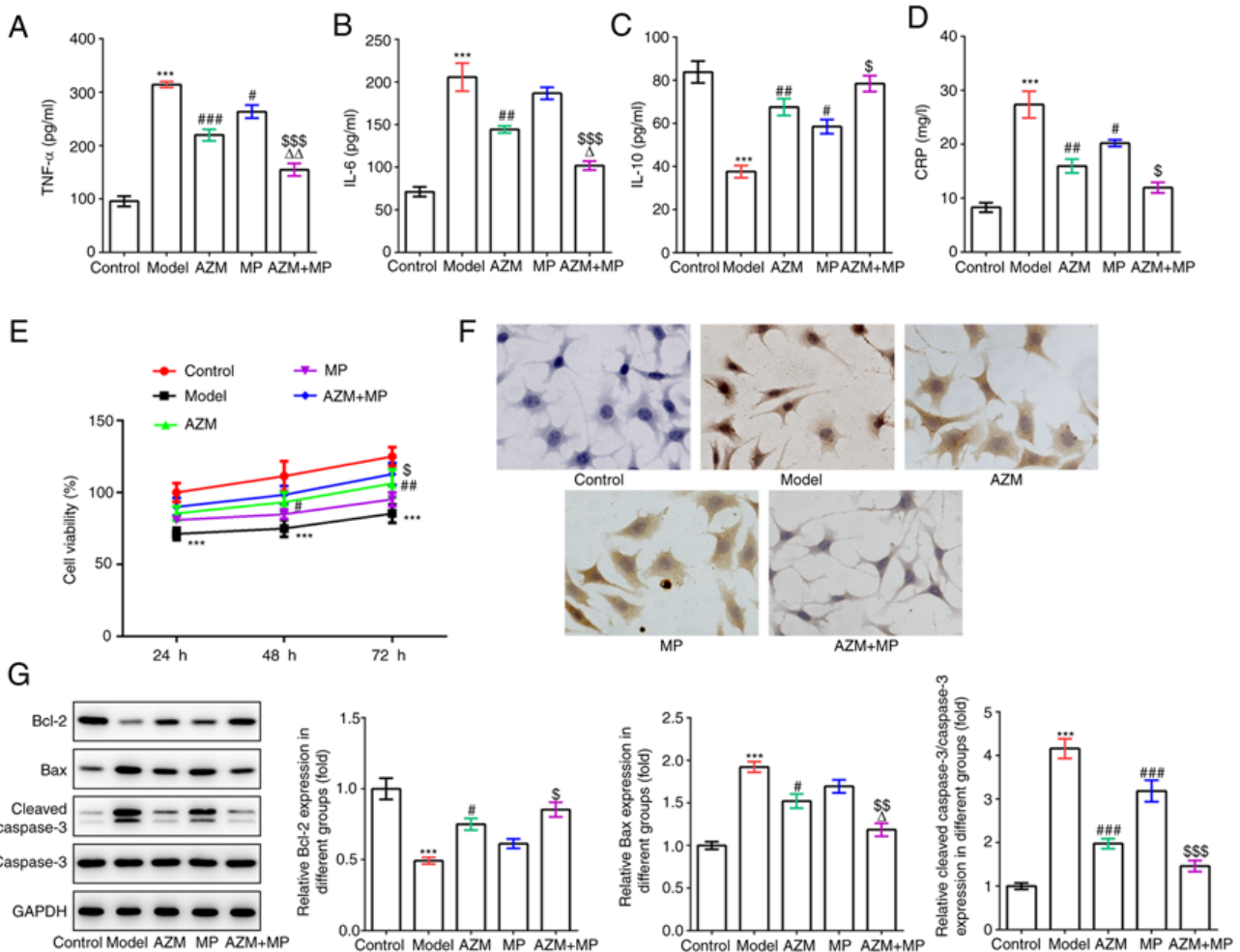


Figure 3. Treatment with MP and AZM attenuates inflammation and apoptosis in *M. pneumoniae*-infected A549 cells. (A-D) The production of inflammatory cytokines, including (A) TNF- α , (B) IL-6, (C) IL-10 and (D) CRP was determined using corresponding ELISA kits in *M. pneumoniae*-induced pneumonia model with or without AZM and MP treatment. (E) Cell Counting Kit-8 assay was applied to detect cell viability. (F) TUNEL assay was conducted to detect cell apoptosis (magnification, $\times 200$). (G) The protein expression levels of Bcl-2, Bax, cleaved caspase-3 and caspase-3 were detected using western blotting. *** $P < 0.001$ vs. control; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. model; \$ $P < 0.05$, \$\$ $P < 0.01$ and \$\$\$ $P < 0.001$ vs. MP; $\Delta P < 0.05$ and $\Delta\Delta P < 0.01$ vs. AZM. MP, methylprednisolone; AZM, azithromycin; *M. pneumoniae*, *Mycoplasma pneumoniae*; CRP, C-reactive protein.

Discussion

M. pneumoniae is one of the important pathogenic microorganisms responsible for respiratory tract infection in humans, particularly in children. Attention has been markedly paid to uncover the mechanism of AZM and MP in the prevention and treatment of MPP. In the present study, MPP cell models were established to study the effect of AZM and MP during *M. pneumoniae* infection and to uncover the underlying mechanism. It was identified that combination treatment with AZM and MP was more effective against *M. pneumoniae*-induced inflammatory, cell viability loss and apoptosis than treatment with AZM or MP alone. Furthermore, the role of miR-499a-5p in *M. pneumoniae*-infected A549 cells was characterized and it was revealed that combination treatment with AZM and MP may exert its function in *M. pneumoniae*-induced A549 cells by regulating miR-499a-5p/STAT3 axis.

M. pneumoniae infection shows various clinical manifestations, ranging from asymptomatic infection to fatal pneumonia. In pneumonia pathogenesis, the imbalance between pro-inflammatory cytokines such as TNF- α and IL-6

and anti-inflammatory cytokines such as IL-10 is crucial in triggering inflammatory response (23). A previous study has demonstrated that *M. pneumoniae* infection induced inflammation and innate immune cell activation in the lung by activation of NLRP3 (NLR-family, leucine-rich repeat protein 3) inflammasome complex (24). Additionally, CRP is a non-specific inflammatory marker, and higher CRP level is usually found in more severe patients with MPP (25). In the present study, an imbalance of inflammatory cytokines was also observed, as evidenced by elevated TNF- α , IL-6 and CRP expression levels and reduced IL-10 level upon *M. pneumoniae* infection, leading to inflammatory response in *M. pneumoniae*-infected A549 cells.

Cell apoptosis is another essential factor in the pneumonia pathogenesis that contributes to the loss of defense function of alveolar epithelial cells (26). It has been previously demonstrated that *M. pneumoniae* contributes to DNA damage in host mucosal epithelial cells, leading to cell apoptosis and necrosis through the release of toxic metabolites (27). Therefore, in the present study, it was also hypothesized that *M. pneumoniae* infection results in cell apoptosis of A549 cells, which was

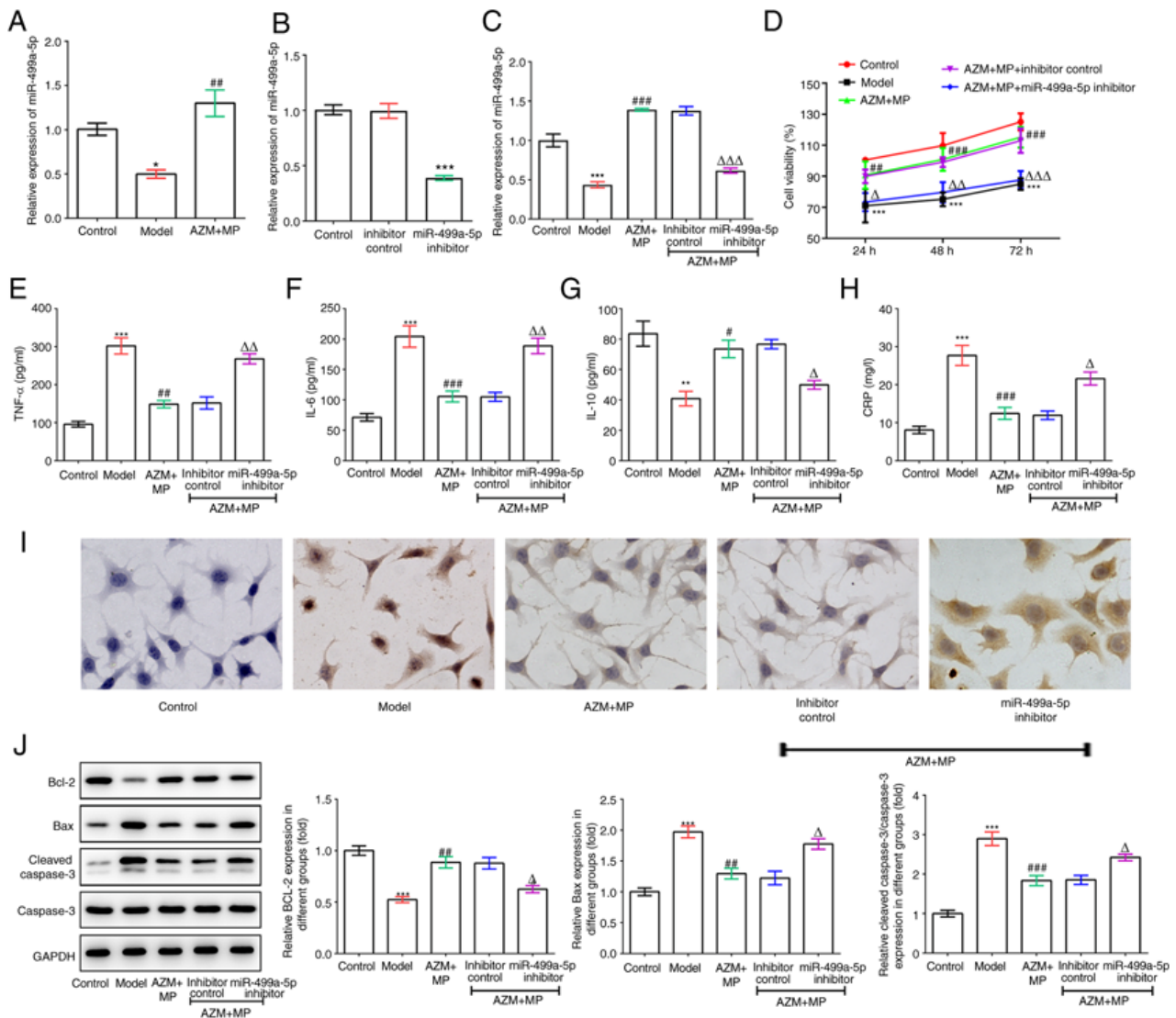


Figure 4. Effects of miR-499a-5p during MPP treatment. (A) The expression level of miR-499a-5p was detected using RT-qPCR in MPP model with AZM and MP treatment. (B) A549 cells were transfected with miR-499a-5p inhibitor or inhibitory control and the expression level of miR-499a-5p was detected using RT-qPCR. (C) The transfected and untransfected A549 cells were infected with *M. pneumoniae* and treated with AZM and MP and the expression level of miR-499a-5p was detected using RT-qPCR. (D) Cell Counting Kit-8 assay was applied to detect cell viability. (E-H) The production of inflammatory cytokines, including (E) TNF- α , (F) IL-6, (G) IL-10 and (H) CRP was determined using corresponding ELISA kits. (I) TUNEL assay was conducted to detect cell apoptosis (magnification, $\times 200$). (J) The protein expression levels of Bcl-2, Bax, cleaved caspase-3 and caspase-3 were detected using western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. model; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ and $\Delta\Delta\Delta P < 0.001$ vs. inhibitor control. miR, microRNA; MPP, *M. pneumoniae*-induced pneumonia; RT-qPCR, reverse transcription-quantitative PCR; AZM, azithromycin; MP, methylprednisolone; *M. pneumoniae*, *Mycoplasma pneumoniae*; CRP, C-reactive protein.

then verified by elevated apoptotic rate, decreased expression of Bcl-2 and increased expression of Bax and cleaved caspase-3.

During the treatment of MPP, though macrolides, such as AZM, are recommended as the first-line therapy, the therapeutic effect is limited due to drug resistance. AZM alone is more likely to cause refractory mycoplasma pneumonia. Furthermore, the children with low immunity can also develop refractory mycoplasma pneumonia. Therefore, based on the treatment of AZM, MP, as a potent glucocorticoid, can effectively suppress the immune response of the body, reduce alveolar edema, inhibit leukocyte phagocytosis and infiltration, improve bronchial obstruction and block the disease progress. In the present study, decreased levels of TNF- α , IL-6 and CRP, and increased level of IL-10, as well as reduced level

of cell apoptosis, were found in the combination treatment of AZM and MP, indicating that the combination therapy had a more effective influence on blocking inflammatory response and suppressing apoptosis in MPP.

Notably, it was also revealed that miR-499a-5p was not only closely connected with the progression of MPP, but also affected the effects of AZM and MP combination therapy. miR-499a-5p inhibitor partly weakened the protection efficacy of the combination treatment against cell viability loss, inflammation and apoptosis. Just as aforementioned, miR-499a-5p was involved in inflammation- and infection-related diseases. In addition, miR-499a-5p exhibited a protective role against the progression of multiple diseases by regulating its different targeted genes. For instance, Gu *et al* (28) uncovered that miR-499a-5p could inhibit the proliferation, and enhance

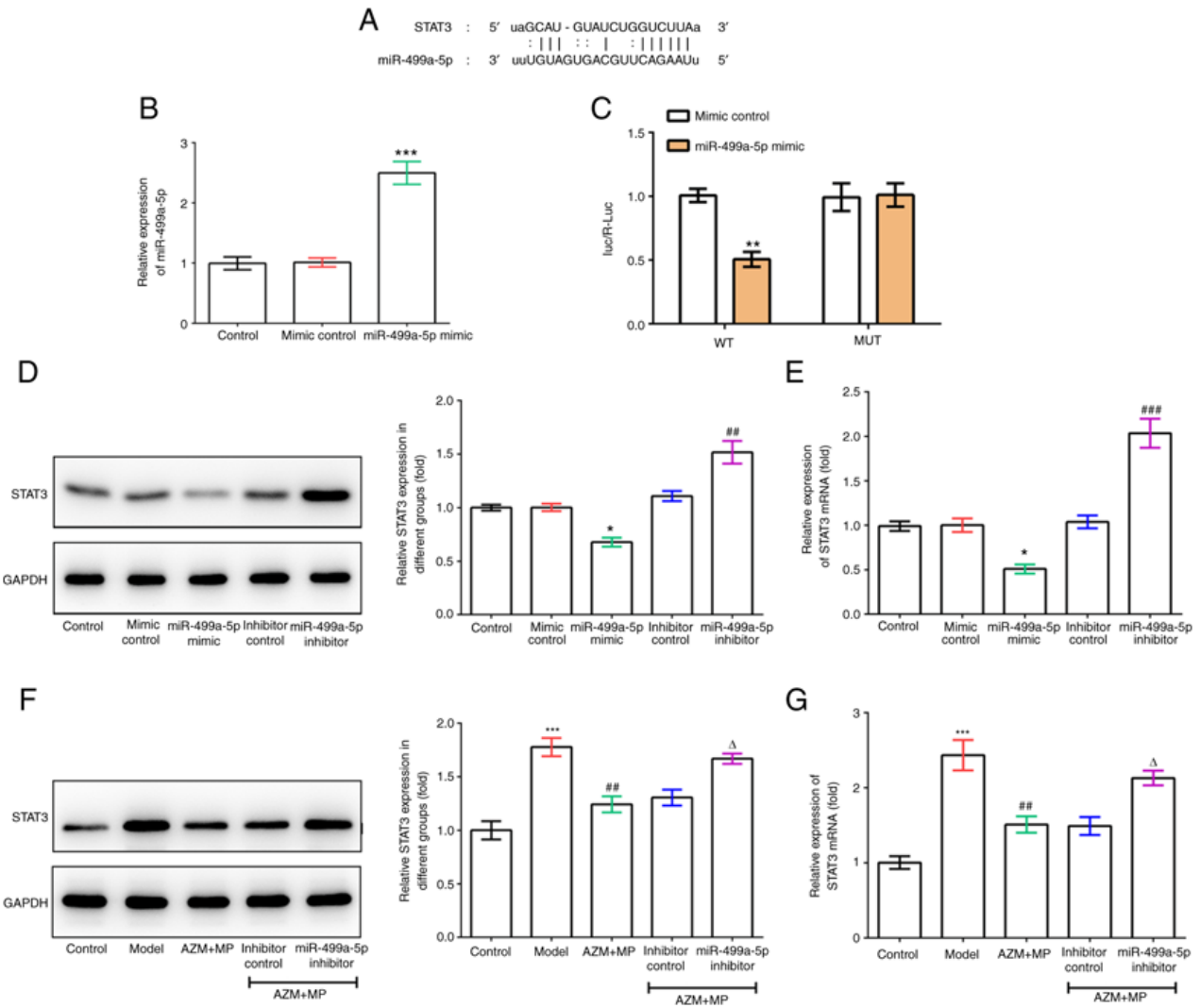


Figure 5. miR-499a-5p targets and negatively regulates STAT3. (A) The potential binding site between miR-499a-5p and STAT3 was predicted by StarBase. (B) A549 cells were transfected with miR-499a-5p mimic and mimic control, then the expression level of miR-499a-5p was detected by RT-qPCR. (C) Luciferase reporter assay was conducted to identify the relationship of miR-499a-5p and STAT3. (D and E) A549 cells were transfected with miR-499a-5p mimic, miR-499a-5p inhibitor and their controls, then the mRNA level and protein expression of STAT3 in each group were detected using RT-qPCR and western blotting, respectively. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. mimic control; ## $P < 0.01$ and ### $P < 0.001$ vs. inhibitor control. (F and G) Combination treatment of AZM and MP was used in *M. pneumoniae*-infected A549 cells with or without miR-499a-5p inhibition and the mRNA and protein expression of STAT3 in each group were detected using RT-qPCR and western blotting, respectively. *** $P < 0.001$ vs. control; ## $P < 0.01$ vs. model; $\Delta P < 0.05$ vs. AZM + MP + inhibitor control. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; AZM, azithromycin; MP, methylprednisolone; *M. pneumoniae*, *Mycoplasma pneumoniae*.

apoptosis of cervical cancer cells by targeting eIF4E; Zhao *et al* (11) found a decreased level of miR-499a-5p in damaged cardiomyocyte induced by hypoxia-reoxygenation, and overexpression of miR-499a-5p could effectively mitigate cardiomyocyte injury by reducing cell apoptosis via directly targeting CD38. In the present study, STAT3 was demonstrated to be a direct target of miR-499a-5p. STAT3 is a key signaling protein to facilitate multiple cellular processes, including growth, differentiation and apoptosis (29). STAT3 is responsible for autosomal dominant hyperimmunoglobulin E syndrome which is associated with various infections such as lung and skin infection (30). It has been reported that *M. pneumoniae* can induce airway mucus hypersecretion by regulating STAT3-mediated signaling pathways (31). STAT3 activation was also observed in A549 cells stimulated by lipid-associated membrane proteins from *M. pneumoniae*, and was closely associated with inflammation (32). In the present

study, it was revealed that *M. pneumoniae* infection induced a higher expression of STAT3, which was then attenuated upon the combination treatment of AZM and MP. Considering the relationship between miR-499a-5p and STAT3, these results indicated that miR-499a-5p may be an important regulator for MPP formation and treatment.

However, there are certain limitations to the present study. First of all, the effect of combined AZM and MP was only investigated in an *in vitro* cell model, and an *in vivo* experiment is needed to validate our conclusion. In addition, miR-499a-5p/STAT3 axis is one of the potential regulatory mechanisms involved in the protective effects of AZM and MP in MPP, and more mechanisms of action need deep exploration.

In conclusion, all the results of the present study support the idea that combination treatment of AZM and MP could inhibit *M. pneumoniae* infection-induced inflammation, cell viability loss and apoptosis partly by regulating miR-499a-5p/STAT3

axis. Consequently, combination treatment of AZM and MP will be an alternative ideal therapeutic strategy for children with MPP in clinical practice.

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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

YC and CH designed the experiments. YC, SD, LT, HC and JC conducted the experiments. YC, SD, LT and CH analyzed and interpreted the data. YC drafted the manuscript. CH revised the manuscript. All authors read and approved the final manuscript. YC and SD confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. 2018-KY-08) by the Ethics Committee of Wuhan Fourth Hospital (Wuhan, China). Patients were enrolled in the study after written informed consent was obtained from their parents or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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