Long non-coding RNA GAS5 protects against *Mycoplasma pneumoniae* pneumonia by regulating the microRNA-222-3p/TIMP3 axis

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Abstract. *Mycoplasma pneumoniae* pneumonia (MPP) is a type of pneumonia induced by *M. pneumoniae* (MP) infection. The present study investigated the effect of long non-coding RNA growth arrest-specific 5 (GAS5) in MPP and the underlying molecular mechanism of this. The expression of GAS5, microRNA-222-3p, (miR-222-3p) and tissue inhibitor of metalloproteinases-3 (TIMP3) in MPP was investigated using reverse transcription-quantitative PCR. Lipid-associated membrane protein (LAMP)-induced THP-1 cells were used to model MPP. The viability of LAMP-induced THP-1 cells was analyzed using an MTT assay. Expression levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) pro-inflammatory cytokines, and the anti-inflammatory cytokine heme oxygenase-1 (HO-1) in LAMP-induced THP-1 cells were measured by ELISA. A dual-luciferase reporter assay assessed the associations among GAS5, miR-222-3p and TIMP3. The expression of GAS5 and TIMP3 was downregulated in MPP. Expression of miR-222-3p was upregulated. GAS5-overexpression increased the viability of LAMP-induced THP-1 cells. GAS5 upregulation decreased the levels of IL-1β, IL-6, TNF-α and HO-1 levels in LAMP-induced THP-1 cells. GAS5 directly interacted with miR-222-3p. TIMP3 was a target of miR-222-3p. miR-222-3p upregulation or TIMP3-knockdown reversed the promotion effect on cell viability as well as the inhibitory effect on inflammation caused by GAS5-overexpression in LAMP-induced THP-1 cells. GAS5-overexpression increased the viability and decreased the inflammation of LAMP-induced THP-1 cells by regulating the miR-222-3p/TIMP3 axis. These results demonstrated a potential therapeutic target for MPP treatment.

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Introduction

Mycoplasma pneumoniae pneumonia (MPP) is a type of pneumonia induced by *M. pneumoniae* infection (1). MPP frequently occurs in children and infants. The clinical presentation is low-grade fever, cough and asthma-like symptoms (2). Despite the advancement in medical science, the incidence of severe or fatal MPP continues to rise (3). Severe MPP is characterized by pulmonary fibrosis, obstructive bronchiolitis and copious pleural effusion (PE), and may be life-threatening (4). Macrolide antibiotics are the first-line therapy for MPP (5). However, macrolide resistance develops frequently in children with MPP, with a rate of 87.2% reported in one previous study (6). Alternative and effective therapeutic strategies in children with MPP are required.

Long non-coding RNAs (lncRNAs) are essential for the regulation of respiratory diseases, including idiopathic pulmonary fibrosis (IPF) (7), acute lung injury (ALI) (8), and pneumonia (9). The growth arrest-specific 5 (GAS5) lncRNA is crucial in numerous inflammatory diseases. GAS5 upregulation alleviates renal fibrosis and inflammatory reactions in rats with diabetic nephropathy (10). GAS5-silencing decreases the viability and aggravates the inflammatory injury of lipopolysaccharide (LPS)-induced chondrocytes (11). Notably, GAS5 is poorly expressed in ALI, and its overexpression attenuates inflammation in ALI mice (12). However, the precise role of GAS5 in the progression of MPP remains unclear.

As biological molecules, microRNAs (miRNAs) participate in the progression of pneumonia. miR-217 contributes toward lung injury and inflammation in interstitial pneumonia (13). miR-21 is upregulated in and promotes the development of ventilator-associated pneumonia (14). miR-155 was reported to induce impaired bacterial clearance and increase mortality in patients suffering from pneumonia that developed following influenza (15). Importantly, miR-222-3p expression is enhanced in children with MPP (16). miR-222-3p is a target of GAS5 in papillary thyroid carcinoma (17). However, the specific regulatory association between GAS5 and miR-222-3p in MPP remains to be elucidated.

The regulatory functions of miRNAs involve the targeting of mRNAs through complementary sequences (18). Tissue inhibitor of metalloproteinase 3 (TIMP3), a member of the TIMP family, participates in various inflammatory diseases, including liver ischemia/reperfusion injury (19), osteoarthritis (20) and IPF (21). Notably, miR-222-3p may directly target TIMP3 in osteosarcoma (22). However, the potential regulatory mechanism of GAS5 associated with the miR-222-3p/TIMP3 axis in MPP remains unknown.

The present study investigated the expression of GAS5, miR-222-3p and TIMP3 in patients with MPP. Lipid-associated membrane proteins (LAMPs) were induced in THP-1 cells to model MPP. The associations among GAS5, miR-222-3p and TIMP3 were confirmed. Subsequently, whether GAS5 controlled the viability and inflammation of LAMP-induced THP-1 cells by regulating the miR-222-3p/TIMP3 axis was investigated. The findings indicated a potential novel therapeutic target for MPP.

Materials and methods

Patients and samples. A total of 25 children with MPP from the Pediatric Intensive Care Unit of the Liaocheng Second People's Hospital (Linqing, China) were enrolled between July 2017 and October 2018. Exclusion criteria included premature delivery, immunodeficiency, recurrent pneumonia and recent use of immunomodulators and immunosuppressive agents. There were 16 children with PE and nine children without PE. Additionally, 25 healthy children comprised the control group. The healthy children had no history of MPP, immune system diseases, or other acute and chronic infectious diseases. Peripheral blood samples were collected from children in the MPP and control groups. A portion of the peripheral blood samples were used for the measurement of inflammatory cytokines and another portion was used to extract peripheral blood mononuclear cells (PBMCs). Next, the PBMCs were isolated by Ficoll-Plaque density gradient centrifugation (GE Healthcare) from all blood samples at 1,000 x g for 30 min at 20˚C. The PBMC layer was extracted and washed by adding three volumes of PBS, centrifuged at 250 x g for 10 min at 20˚C. The PBMCs were maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.), containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere of 5% CO_2. Logarithmic growth phase cells were used for further assays. The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Liaocheng Second People's Hospital (approval no. 2017-013). Written informed consent was obtained from each child and their guardian.

Extraction of LAMPs from MP. MP M129 [29342; American Type Culture Collection (ATCC)] was used as the standard MP strain. The MP was cultured in PPLO broth medium (BioLife) at 37˚C for 5-7 days and harvested when the red pH indicator turned orange. Following centrifugation at 10,000 x g for 20 min at 4˚C, MP pellets were resuspended in 10 ml Tris-buffered saline (TBS; 50 mM Tris pH 8.0, 0.15 M NaCl), containing 1 mM EDTA (TBSE). MP was then lysed for 1 h at 4˚C by adding 2% (v/v) Triton X-114 into the suspension. To isolate LAMPs, the lysate was incubated for 10 min at 37˚C to allow phase separation. The upper aqueous phase was removed and replaced with the same volume of TBSE. The phase separation procedure was repeated twice. The final phase was resuspended in TBSE to the original volume, and 2.5 volumes of ethanol were added to precipitate LAMPs at -20˚C overnight. After the supernatant was discarded, the isolated LAMPs were resuspended in PBS, treated with low temperature ultrasound and stored at -70˚C. The concentration of LAMPs was determined using an enhanced BCA Protein assay kit (Beyotime Institute of Biotechnology).

Cell culture and treatment. THP-1 cells ($1x10^5$ cells/ml), obtained from ATCC, were cultured in RPMI-1640 medium containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere of 5% CO_2. THP-1 cells were harvested and centrifuged after reaching exponential growth. The resulting cell pellet was suspended in serum-free RPMI-1640 medium, $1x10^6$ cells were distributed across a 24-well plate. The cells were co-cultured with different levels of LAMPs (2, 4 and 6 μg/ml) for 16 h. Cells in the control group were treated with PBS. THP-1 cells co-cultured with 6 μg/ml LAMPs were considered as LAMP-induced THP-1 cells and were examined in subsequent experiments.

Cell transfection. Short hairpin (sh)-GAS5, sh-negative control (NC) and sh-TIMP3 were synthesized by Shanghai GenePharma Co., Ltd., and then inserted into the pGLVU6/Puro vector (Shanghai GenePharma Co., Ltd.). The pcDNA3.1-NC (pcDNA3.1), pcDNA3.1-GAS5 (oe-GAS5), miR-222-3p mimics (sense, 5'-AGCUACAUCUGGCUACUG GGU-3' and antisense, 5'-CCAGUAGCCAGAUGUAGC UUU-3'), NC mimics (sense, 5'-UUCUCCGAACGUGUC ACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGA ATT-3'), miR-222-3p inhibitor (5'-ACCCAGUAGCCAGAU GUAGCU-3'), and NC inhibitor (5'-CAGUACUUUUGUGUA GUACAA-3') were synthesized by Shanghai GenePharma Co., Ltd. LAMP-induced THP-1 cells ($1x10^5$ cells/well) were seeded onto a 24-well plate and cultured until growth was 80% confluent. The lipid complex was pre-prepared by mixing 25 μl Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) diluted in serum-free RPMI-1640 medium and 25 μl specific nucleic acids (sh-GAS5/sh-TIMP3/sh-NC/oe-GAS/pcDNA3.1 and/or miR-222-3p mimics or mimics NC/miR-222-3p inhibitor/inhibitor NC; shRNA, 200 ng; miRNA, 50 nM) diluted in serum-free medium for 10 min. Cells were then incubated with 50 μl lipid complex for 48 h at 37˚C. After 48 h transfection, the transfected cells were used for subsequent assays.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA samples were obtained through reverse transcription (37˚C for 15 min; 85˚C for 5 sec) using the PrimeScript RT Reagent kit (Takara Bio, Inc.). miScript SYBR Green PCR kit (Qiagen, Inc.) was used to conduct a qPCR analysis. RT-qPCR was performed using a 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95˚C for 3 min and 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec, and a final extension step at 72˚C for 10 min. Relative expression

was calculated using the $2^{-\Delta\Delta Cq}$ method (23). GAPDH, U6 and β-actin were used for the normalization of GAS5, miR-222-3p and TIMP3, respectively. The primer sequences are presented in Table I.

Western blot analysis. A Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology) was used to extract nuclear and cytoplasmic proteins. PBMCs were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) on ice to extract the total protein. Protein concentration was evaluated by bicinchoninic acid assay (Beyotime Institute of Biotechnology). Equal amounts (30 μg) of protein samples were separated by 10% SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride membranes, blocked with 5% skimmed milk for 1 h at 37˚C, and incubated at 4˚C overnight with primary antibodies against anti-TIMP3 (1:1,000; cat. no. SAB4502973; Sigma-Aldrich; Merck KGaA) or anti-Tubulin (1:200; cat. no. T3526; Sigma-Aldrich; Merck KGaA). The membranes were then incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG (1:5,000; cat. no. 12-348; Sigma-Aldrich; Merck KGaA) secondary antibody for 1 h at 25˚C. Finally, the bands were visualized using an enhanced chemiluminescence kit (Invitrogen; Thermo Fisher Scientific, Inc.). Protein bands were semi-quantified by densitometric analysis using ImageJ software (version 1.51, National Institutes of Health).

Viability assay. The viability of LAMP-induced THP-1 cells was measured using an MTT cell proliferation assay kit (Sigma-Aldrich; Merck KGaA). In brief, the LAMP-induced THP-1 cells with different transfections were seeded onto 96-well plates ($2x10^3$ cells/well) and cultured with 5% CO_2 at 37˚C. When the cell reached 80% confluence, they were incubated with 20 μg MTT reagents for 2 h at 37˚C in a humidified culture chamber supplied with 5% CO_2. Following incubation, supernatants were removed and 150 μl DMSO was added to dissolve the formazan crystals. The optical density values were measured at 450 nm using a plate reader and were used to evaluate cell viability.

ELISA. Peripheral blood samples from each group were transferred to a serum separating tube and centrifuged at 1,000 x g at 4˚C for 10 min. The serum was harvested. Additionally, LAMP-induced THP-1 cells from each group were centrifuged at 1,000 x g at 4˚C for 10 min. Each supernatant was collected. The levels of interleukin (IL)-6 and interferon (IFN)-γ in serum were measured using a Human IL-6 ELISA kit (cat. no. ab178013; Abcam) and Human IFN-γ ELISA kit (cat. no. ab46025; Abcam). The levels of IL-1β, IL-6, tumor necrosis factor-α (TNF-α) and heme oxygenase-1 (HO-1) in LAMP-induced THP-1 cells were assessed using Human IL-1β ELISA kit (cat. no. ab214025; Abcam), Human IL-6 ELISA kit (cat. no. ab178013; Abcam), Human TNF-α ELISA kit (cat. no. ab181421; Abcam) and Human HO-1 ELISA kit (cat. no. ab207621; Abcam), respectively.

Dual-luciferase reporter assay. The potential binding sites of GAS5 and miR-222-3p or miR-222-3p and TIMP3 were predicted by starBase (http://starbase.sysu.edu.cn/starbase2/) or TargetScan (http://www.targetscan.org/vert_72/). Wild-type (WT) fragments of the 3'-UTR of GAS5/TIMP3 with putative binding sites of miR-222-3p were purchased from Shanghai GenePharma Co., Ltd. and cloned into a psiCHECK-2 Dual-Luciferase miRNA Target Expression Vector (Promega Corporation). GAS5/TIMP3-3'-UTR-Mut reporter containing mutant miR-222-3p binding sites was used and generated using a Quikchange Multi Site-directed Mutagenesis kit (Stratagene; Agilent Technologies, Inc.). Subsequently, the recombinant vectors were co-transfected with miR-NC or miR-222-3p mimics into THP-1 cells. Luciferase activity was evaluated 48 h post-transfection by Dual Luciferase Reporter assay system (Promega Corporation), and firefly luciferase activity was normalized to that of *Renilla* luciferase.

Table I. Primer sequences.

Name of primer	Sequence (5'-3')
GAS5-F	CTTCTGGGCTCAAGTGATCCT
GAS5-R	TTGTGCCATGAGACTCCATCAG
GAPDH-F	CGACTTATACATGGCCTTA
GAPDH-R	TTCCGATCACTGTTGGAAT
miR-222-3p-F	AGCTACATCTGGCTACTGGGT
miR-222-3p-R	GCGAGCACAGAATTAATACGAC
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
TIMP3-F	ACCGAGGCTTCACCAAGATG
TIMP3-R	CATCATAGACGCGACCTGTCA
β-actin-F	TGGAATCCTGTGGCATCCATGAAAC
β-actin-R	ACGCAGCTCAGTAACAGTCCG

F, forward; R, reverse; GAS5, growth arrest-specific 5; TIMP3, tissue inhibitor of metalloproteinases-3.

Statistical analyses. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation. The differences between two groups or among multiple groups were assessed using Student's t-test or one-way analysis of variance followed by Tukey's post-hoc test. The significance of the correlations was determined by Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Decreased GAS5 expression in PBMCs of patients with MPP. The demographic and clinical characteristics of the children with MPP and control groups are presented in Table II. Age, sex and white blood cell counts were not significantly different between the MPP and control groups. The levels of neutrophils and C-reactive protein in children with MPP were significantly higher than those of the control group ($P<0.001$). RT-qPCR was performed to confirm whether GAS5 was differentially expressed in the PBMCs of patients with MPP. GAS5 expression was significantly decreased in PBMCs of the MPP group compared with the control group ($P<0.001$; Fig. 1A). Additionally, GAS5 expression was significantly decreased in MPP cases with PE compared with those

Table II. Demographic and clinical characteristics of children with MPP and controls.

Parameter	MPP (n=25)	Control (n=25)	P-value
Age, mean ± SD, years	6.5±2.8	6.7±2.8	0.786
Sex, male/female, n	13/12	14/11	0.365
White blood cells, mean ± SD, $x10^9$/l	8.2±3.7	7.6±1.9	0.126
Neutrophils mean ± SD, $x10^9$/l	7.9±2.6	1.9±0.7	<0.001
C-reactive protein, 25th-75th percentile, mg/l	17.1 (9.8-43.5)	0.16 (0.09-0.7)	<0.001
Lactate dehydrogenase, mean ± SD, U/l	456.4±150.5	-	-
Lymphocytes subgroups, mean ± SD, %			
$CD3^+$	67.9±8.6	-	-
$CD3^+CD4^+$	36.7±7.1	-	-
$CD3^+CD8^+$	27.6±6.9	-	-
$CD3^-CD19^+$	18.5±5.1	-	-
$CD3^-CD$ (16^+ 56^+)	12.8±6.5	-	-
$CD19^+CD23^+$	9.1±4.4	-	-

MPP, *Mycoplasma pneumoniae* pneumonia; SD, standard deviation.

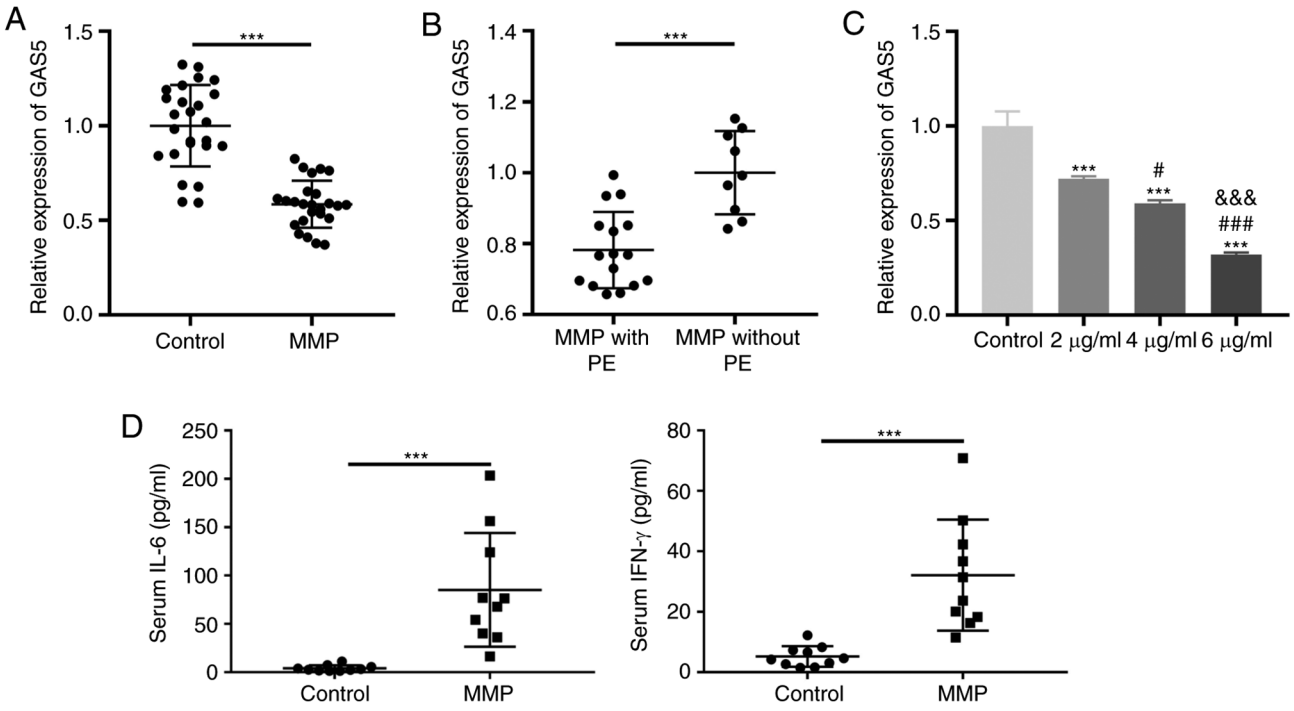

Figure 1. GAS5 expression is decreased in the PBMCs of patients with MPP. (A) The expression of GAS5 in PBMCs of patients with MPP and the control group was detected by RT-qPCR. ***P<0.001 vs. Control. (B) The expression of GAS5 in PBMCs of patients with MPP with and without PE was assessed by RT-qPCR. ***P<0.001 vs. MPP without PE. (C) The expression of GAS5 in different concentrations of LAMPs (2, 4 and 6 μg/ml) treated THP-1 cells was measured by RT-qPCR. ***P<0.001 vs. Control; #P<0.05, ###P<0.001 vs. 2 μg/ml; &&&P<0.001 vs. 4 μg/ml. (D) The levels of IL-6 and IFN-γ in serum in the control and MMP groups were measured by ELISA. ***P<0.001 vs. Control. PBMCs, peripheral blood mononuclear cells; MPP, *Mycoplasma pneumoniae* pneumonia; RT-qPCR, reverse transcription-quantitative PCR; PE, pleural effusion; LAMPs, lipid-associated membrane proteins; IL, interleukin; IFN, interferon; GAS5, growth arrest-specific 5.

without PE (P<0.001; Fig. 1B). The level of GAS5 expressed by THP-1 cells significantly decreased in a dose-dependent manner following induction with LAMPs (P<0.001; Fig. 1C). Ten peripheral blood samples were randomly selected from control and MMP groups, respectively. Next, the levels of IL-6 and IFN-γ in serum were measured by ELISA. The results demonstrated that the levels of IL-6 and IFN-γ in serum in the MMP group were higher than those in the control group (P<0.001; Fig. 1D).

GAS5 enhances the viability and inhibits the inflammation of LAMP-induced THP-1 cells. To investigate the effect of GAS5 on MPP *in vitro*, LAMP-induced THP-1 cells were used as the MPP model at the cellular level. As demonstrated

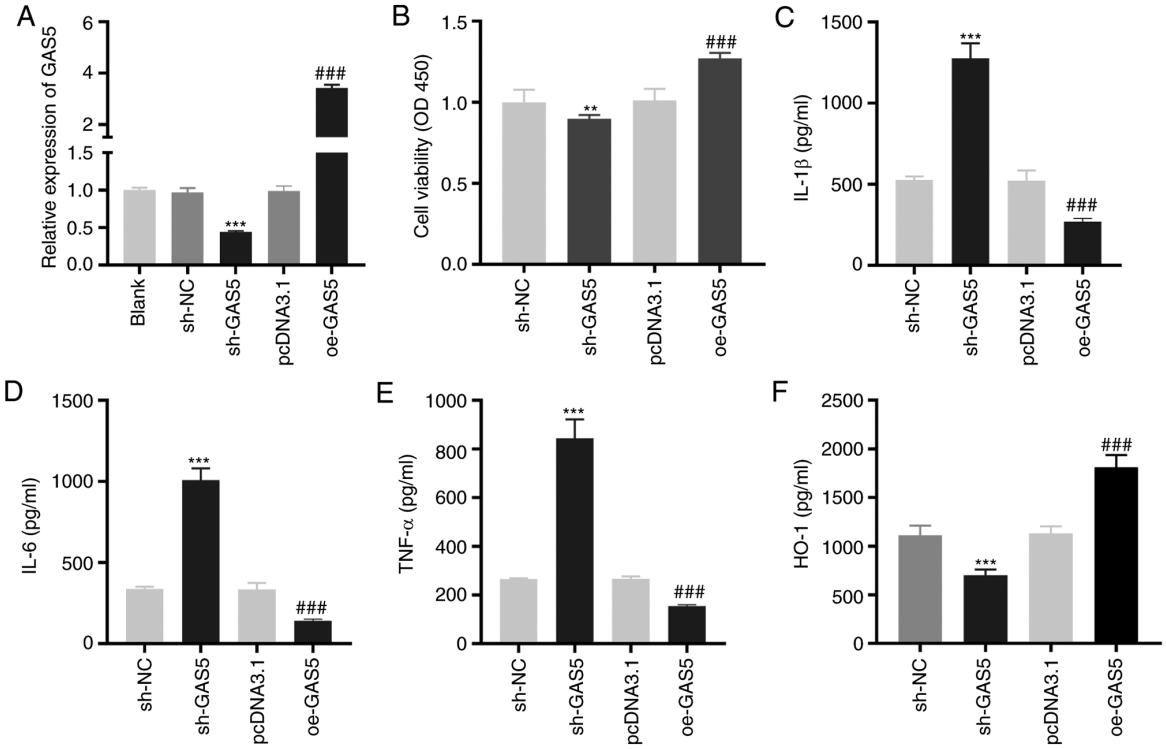

Figure 2. GAS5 increases the viability and inhibits the inflammation of LAMP-induced THP-1 cells. (A) The transfection efficiency of sh-NC, sh-GAS5, pcDNA3.1 and oe-GAS5 in LAMP-induced THP-1 cells was investigated by reverse transcription-quantitative PCR. ***P<0.001 vs. sh-NC; ###P<0.001 vs. pcDNA3.1. (B) The viability of LAMP-induced THP-1 cells was detected by MTT assay. **P<0.01 vs. sh-NC; ###P<0.001 vs. pcDNA3.1. (C-F) ELISA was performed to confirm the level of IL-1β, IL-6, TNF-α and HO-1 in LAMP-induced THP-1 cells. ***P<0.001 vs. sh-NC; ###P<0.001 vs. pcDNA3.1. LAMPs, lipid-associated membrane proteins; sh, short-hairpin RNA; NC, negative control; IL, interleukin; TNF-α, tumor necrosis factor-α; HO-1, heme oxygenase-1; GAS5, growth arrest-specific 5.

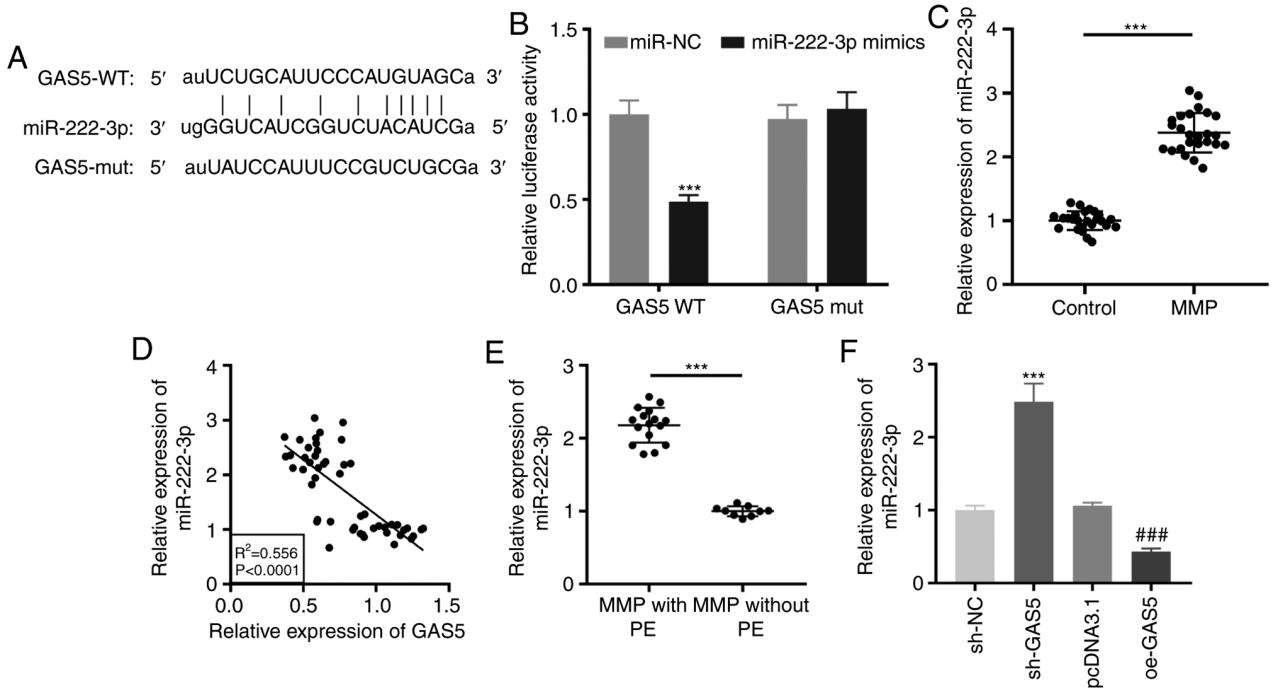

Figure 3. miR-222-3p serves as a target of GAS5. (A) The putative binding site of miR-222-3p in GAS5 was predicted by starBase. (B) Relative luciferase activity in LAMP-induced THP-1 cells was measured by dual-luciferase reporter assay. ***P<0.001 vs. miR-NC. (C) RT-qPCR was performed to measure the expression of miR-222-3p in PBMCs of patients with MPP and the control group. ***P<0.001 vs. Control. (D) The correlation between the expression of GAS5 and miR-222-3p was analyzed in the PBMCs of all participants. (E) The expression of miR-222-3p in PBMCs of MPP patients with and without PE was detected by RT-qPCR. ***P<0.001 vs. MPP without PE. (F) The effect of GAS5 upregulation and downregulation on the miR-222-3p expression in LAMP-induced THP-1 cells was evaluated by RT-qPCR. ***P<0.001 vs. sh-NC; ###P<0.001 vs. pcDNA3.1. LAMP, lipid-associated membrane protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PBMCs, peripheral blood mononuclear cells; MPP, *Mycoplasma pneumoniae* pneumonia; PE, pleural effusion; sh, short hairpin RNA; NC, negative control; GAS5, growth arrest-specific 5; WT, wild-type; mut, mutant.

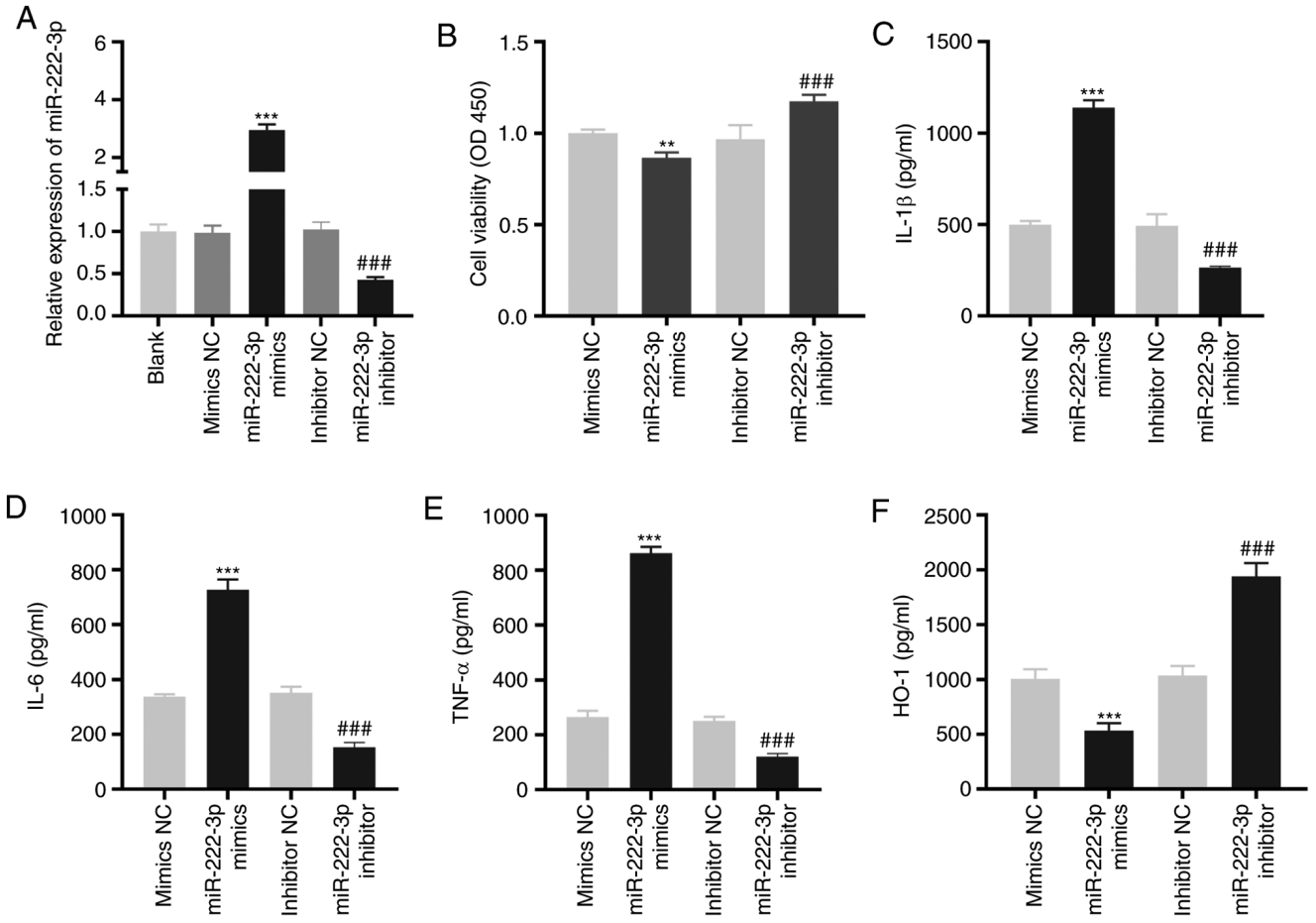

Figure 4. miR-222-3p decreases the viability and increases the inflammation of LAMP-induced THP-1 cells. (A) The transfection efficiency of NC mimics, miR-222-3p mimics, NC inhibitors and miR-222-3p inhibitors in LAMP-induced THP-1 cells was assessed by reverse transcription-quantitative PCR. ***P<0.001 vs. NC mimics; $^{###}$P<0.001 vs. NC inhibitors. (B) MTT assay was performed to measure the viability of LAMP-induced THP-1 cells. **P<0.01 vs. NC mimics; $^{###}$P<0.001 vs. NC inhibitors. (C-F) The concentrations of IL-1β, IL-6, TNF-α and HO-1 in LAMP-induced THP-1 cells were detected by ELISA. ***P<0.001 vs. NC mimics; $^{###}$P<0.001 vs. NC inhibitors. LAMP, lipid-associated membrane protein; NC, negative control; IL, interleukin; TNF-α, tumor necrosis factor-α; HO-1, heme oxygenase-1.

in Fig. 2A, GAS5 expression was significantly inhibited or enhanced by the transfection of sh-GAS5 or oe-GAS5 into LAMP-induced THP-1 cells (P<0.001). The MTT assay revealed that GAS5-silencing significantly decreased the viability (P<0.01), while GAS5-overexpression significantly increased the viability of LAMP-induced THP-1 cells (P<0.001; Fig. 2B). The levels of IL-1β, IL-6 and TNF-α in LAMP-induced THP-1 cells were significantly increased by GAS5 silencing and decreased when GAS5 was overexpressed (P<0.001; Fig. 2C-E). Transfection of sh-GAS5 or oe-GAS5 significantly downregulated or upregulated the level of the anti-inflammatory cytokine HO-1 in LAMP-induced THP-1 cells (P<0.001; Fig. 2F).

miR-222-3p as a target of GAS5. Using the starBase online software, it was found that GAS5 contained the target site of miR-222-3p (Fig. 3A). A dual-luciferase reporter assay confirmed that miR-222-3p mimics reintroduction clearly decreased the luciferase activity in THP-1 cells transfected with GAS5 WT compared with the activity observed in miR-NC (P<0.001; Fig. 3B). RT-qPCR revealed that miR-222-3p expression was significantly enhanced in PBMCs of patients with MPP (P<0.001; Fig. 3C). As demonstrated in Fig. 3D, a negative correlation between GAS5 and miR-222-3p expression was observed in PBMCs of all participants (R^2=0.556, P<0.0001). miR-222-3p was highly expressed in patients with MPP with PE (P<0.001; Fig. 3E). Notably, downregulation or upregulation of GAS5 significantly increased or decreased, respectively, miR-222-3p expression in LAMP-induced THP-1 cells (P<0.001; Fig. 3F).

miR-222-3p decreases the viability and increases the inflammation of LAMP-induced THP-1 cells. As demonstrated in Fig. 4A, miR-222-3p was enhanced or inhibited by the transfection of miR-222-3p mimics or miR-222-3p inhibitors, respectively, into LAMP-induced THP-1 cells (P<0.001). The overexpression of miR-222-3p significantly decreased viability (P<0.01), while its inhibition significantly increased the viability of LAMP-induced THP-1 cells (P<0.001; Fig. 4B). miR-222-3p-overexpression or inhibition increased or decreased, respectively, the levels of IL-1β, IL-6 and TNF-α in LAMP-induced THP-1 cells (P<0.001; Fig. 4C-E). Furthermore, the level of HO-1 in LAMP-induced THP-1 cells was significantly decreased or increased by the upregulation or downregulation, respectively, of miR-222-3p (P<0.001; Fig. 4F).

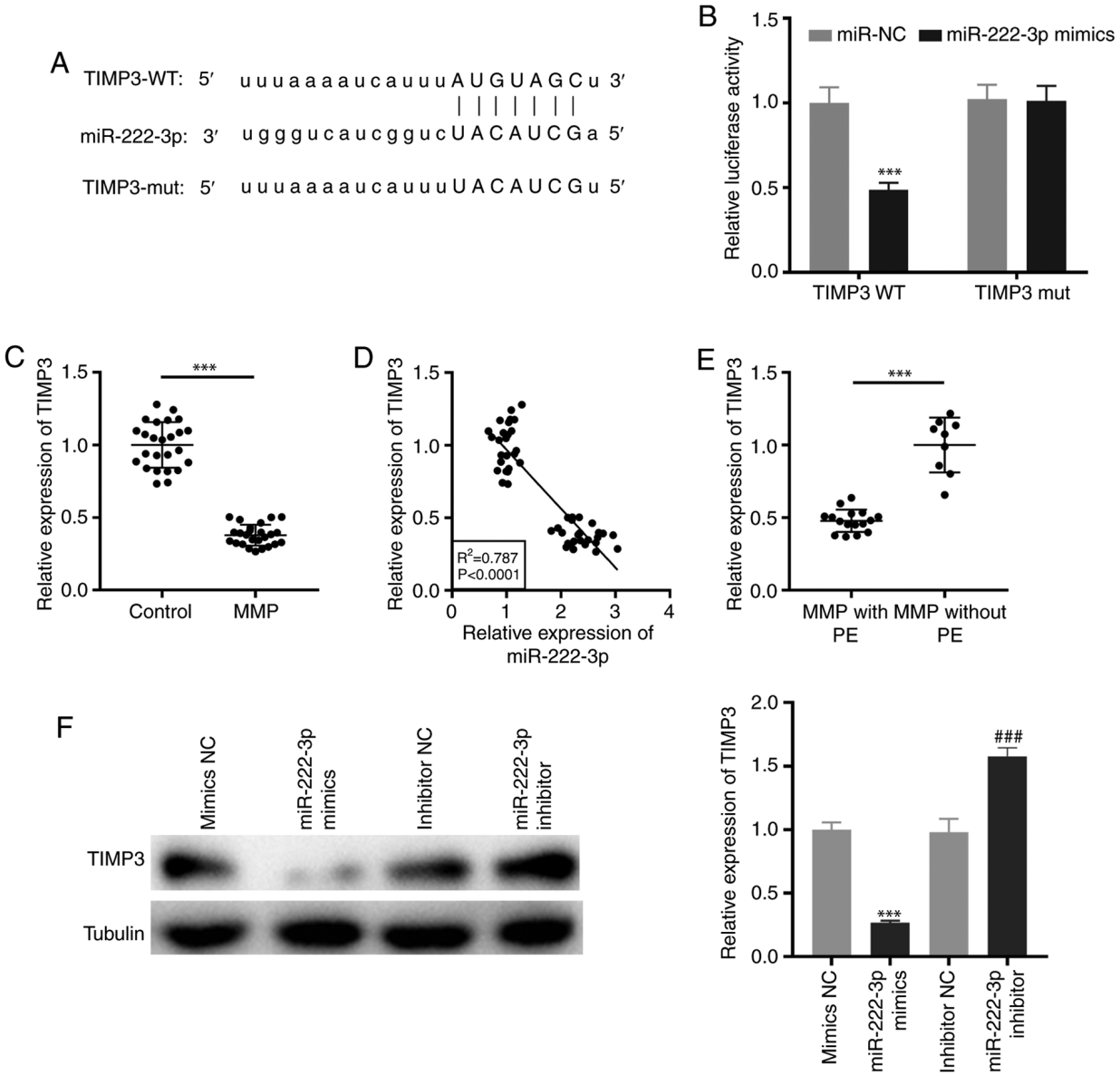

Figure 5. TIMP3 is targeted by miR-222-3p. (A) The binding site for miR-222-3p on the 3'-UTR of TIMP3 was predicted by TargetScan. (B) Dual-luciferase reporter assay was performed to measure the relative luciferase activity in LAMP-induced THP-1 cells. ***P<0.001 vs. miR-NC. (C) The TIMP3 expression in PBMCs of patients with MPP and the control group was detected by RT-qPCR. ***P<0.001 vs. Control. (D) The correlation between the expression of TIMP3 and miR-222-3p was analyzed in the PBMCs of all participants. (E) The expression of TIMP3 in PBMCs of patients with MPP with and without PE was measured by RT-qPCR. ***P<0.001 vs. MPP without PE. (F) The protein expression of TIMP3 in LAMP-induced THP-1 cells was measured by western blot analysis. ***P<0.001 vs. NC mimics; ###P<0.001 vs. NC inhibitors. TIMP3, tissue inhibitor of metalloproteinases-3; 3'-UTR, 3'-untranslated region; LAMP, lipid-associated membrane protein; NC, negative control; PBMCs, peripheral blood mononuclear cells; MPP, *Mycoplasma pneumoniae* pneumonia; RT-qPCR, reverse transcription-quantitative PCR; PE, pleural effusion; WT, wild-type; mut, mutant.

Targeting of TIMP3 by miR-222-3p. TargetScan was used to predict the binding site for miR-222-3p on the 3'-UTR of TIMP3 (Fig. 5A). miR-222-3p mimics notably decreased the luciferase activity of the WT TIMP3 reporter vector in THP-1 cells (P<0.001; Fig. 5B). Additionally, RT-qPCR demonstrated that TIMP3 expression was significantly downregulated in PBMCs of patients with MPP (P<0.001; Fig. 5C). As illustrated in Fig. 5D, the expression of TIMP3 was negatively correlated with miR-222-3p expression in PBMCs of all participants (R^2=0.787, P<0.0001). Notably, TIMP3 expression was significantly suppressed in patients with MPP with PE (P<0.001; Fig. 5E). Furthermore, western blot analysis revealed that transfection of miR-222-3p mimics or miR-222-3p inhibitor may suppress or promote, respectively, the protein expression of TIMP3 in LAMP-induced THP-1 cells (P<0.001; Fig. 5F).

GAS5 increases the viability and attenuates the inflammation of LAMP-induced THP-1 cells by regulating the miR-222-3p/TIMP3 axis. As demonstrated in Fig. 6A, the expression of TIMP3 was significantly inhibited by transfecting with sh-TIMP3 in LAMP-induced THP-1 cells (P<0.001). To investigate whether GAS5 regulated the miR-222-3p/TIMP3 axis in MPP, feedback experiments were performed in LAMP-induced THP-1 cells. GAS5-overexpression notably increased TIMP3 protein expression, and miR-222-3p-overexpression or TIMP3-knockdown rescued the increased

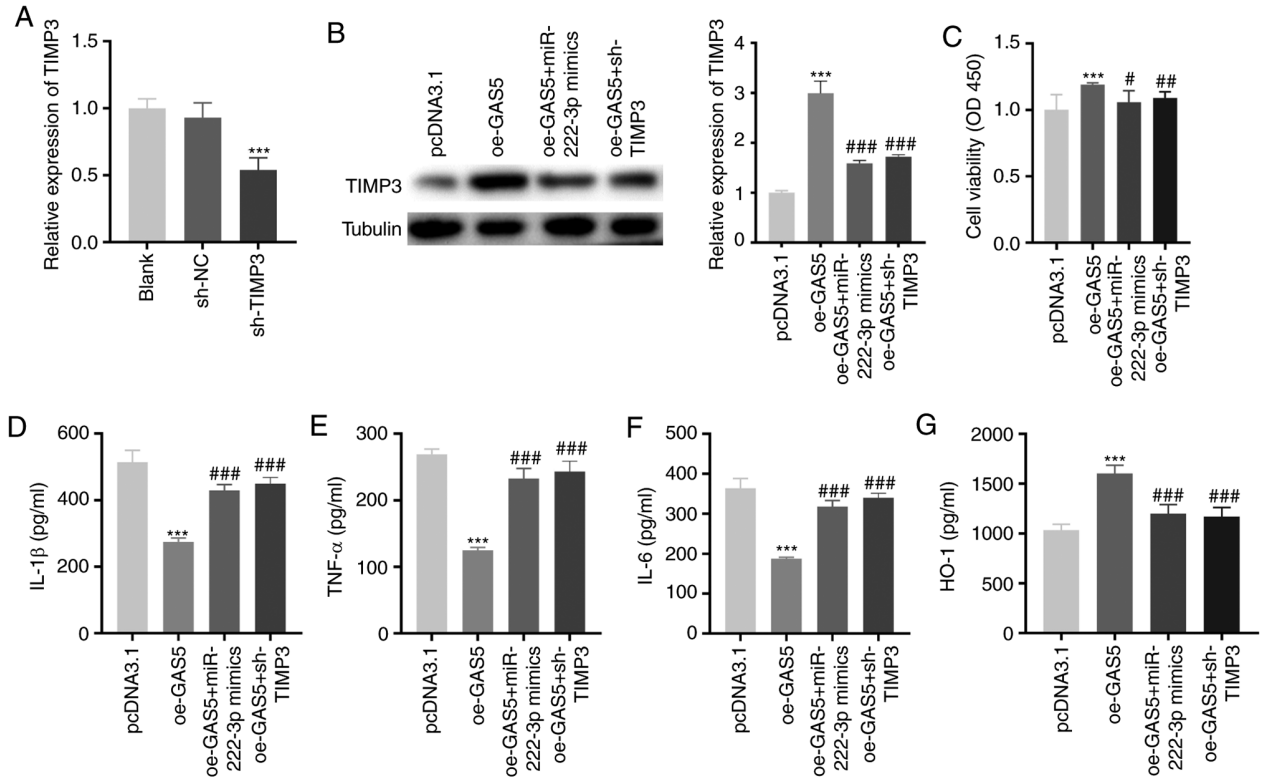

Figure 6. GAS5 increases the viability and attenuates the inflammation of LAMP-induced THP-1 cells via regulating the miR-222-3p/TIMP3 axis. (A) The transfection efficiency of sh-NC and sh-TIMP3 in LAMP-induced THP-1 cells was measured by reverse transcription-quantitative PCR. ***P<0.001 vs. sh-NC. (B) The promoting effect of GAS5-overexpression on TIMP3 protein expression in LAMP-induced THP-1 cells was mitigated by miR-222-3p-overexpression or TIMP3-knockdown. ***P<0.001 vs. pcDNA3.1; $^{###}$P<0.001 vs. oe-GAS5. (C) Overexpression of miR-222-3p or inhibition of TIMP3 reversed the enhancing effect of GAS5-overexpression on the viability of LAMP-induced THP-1 cells. ***P<0.001 vs. pcDNA3.1; $^{#}$P<0.05, $^{##}$P<0.01 vs. oe-GAS5. (D-G) miR-222-3p-overexpression or TIMP3 inhibition not only reversed the decrease in IL-1β, IL-6 and TNF-α, but also weakened the promoting effect on the level of HO-1 caused by GAS5-overexpression in LAMP-induced THP-1 cells. ***P<0.001 vs. pcDNA3.1; $^{###}$P<0.001 vs. oe-GAS5. LAMP, lipid-associated membrane protein; TIMP3, tissue inhibitor of metalloproteinases-3; sh, short-hairpin RNA; NC, negative control; IL, interleukin; TNF-α, tumor necrosis factor-α; HO-1, heme oxygenase-1; GAS5, growth arrest-specific 5.

TIMP3 protein expression caused by GAS5-overexpression in LAMP-induced THP-1 cells (P<0.001; Fig. 6B). GAS5-overexpression increased cell viability (P<0.001). The upregulation of miR-222-3p or downregulation of TIMP3 reversed the promotion effect of GAS5-overexpression on the viability of LAMP-induced THP-1 cells (P<0.05; Fig. 6C). GAS5-overexpression significantly decreased the levels of IL-1β, IL-6 and TNF-α, and increased the level of HO-1 in LAMP-induced THP-1 cells (P<0.001; Fig. 6D-G). Notably, miR-222-3p-overexpression or TIMP-knockdown mitigated the inhibitory effect of GAS5-overexpression on the levels of the pro-inflammatory cytokines and weakened the promotion effect of GAS5-overexpression on the level of anti-inflammatory cytokines in LAMP-induced THP-1 cells (P<0.001).

Discussion

Downregulation of lncRNAs is associated with the pathogenesis of respiratory diseases. GAS5 expression is downregulated in diverse respiratory diseases, including acute respiratory distress syndrome (24), non-small cell lung cancer (25) and ALI (12). In the present study, GAS5 expression was markedly decreased in PBMCs of patients with MPP and in LAMP-induced THP-1 cells. Furthermore, the results of the present study demonstrated that GAS5 may be associated with the development of MPP. LncRNAs serve as critical factors to modulate cell viability in certain inflammatory diseases. LncRNA CASC9 may mitigate the effect of LPS on the viability of human small airway epithelial cells (26). LncRNA TUG1 suppressed MRC-5 cell viability in a pneumonia model (27). Notably, GAS5 increases the viability of endothelial progenitor cells, attenuating the development of atherosclerosis (28). In the present study, GAS5-overexpression increased the viability of LAMP-induced THP-1 cells, suggesting that GAS5 may increase cell viability in MPP. Certain lncRNAs are involved in the regulation of inflammation in lung diseases. In sepsis-induced ALI, lncRNA TUG1 ameliorates inflammation by inhibiting miR-34b-5p (29). MALAT1-silencing increases IL-6 and TNF-α levels in ALI rats (30). Notably, GAS5 has an anti-inflammatory role in numerous diseases. GAS5 regulates miR-429 to increase DUSP1 expression, attenuating the inflammation of alveolar epithelial cells in ALI (12). GAS5 inhibits endoplasmic reticulum stress-induced inflammation by targeting SERCA2b in diabetic retinopathy (31). In the present study, GAS5-overexpression suppressed the levels of IL-1β, IL-6 and TNF-α, and enhanced the HO-1 level in LAMP-induced THP-1 cells. These results indicated that GAS5 may attenuate inflammation in MPP. By contrast, GAS5 has a pro-inflammatory role in certain diseases. For instance,

GAS5 accelerates microglial inflammatory responses in Parkinson's disease (32). Silencing of GAS5 decreases the levels of TNF-α and IL-6 in the supernatant of osteoarthritic chondrocytes (33). GAS5 may exert different regulatory effects on inflammation in different diseases. The regulatory role of GAS5 in inflammation is controversial and further research on the detailed regulatory mechanism of GAS5 on the inflammation of MPP is required.

Previous studies have demonstrated that GAS5 may interact with miRNAs to modulate the development of inflammatory diseases (34,35). For instance, GAS5 attenuates inflammation in systemic lupus erythematosus by suppressing miR-92a-3p expression (36). GAS5 decreases TNF-α and IL-6 levels in diabetic nephropathy by inhibiting miR-452-5p expression (37). In the present study, miR-222-3p was determined to be a target of GAS5. miR-222-3p expression was upregulated in patients with MMP compared with the children in the control group. Notably, miR-222-3p expression was negatively correlated with GAS5 expression in patients with MPP. We hypothesized that GAS5 may influence MPP by regulating miR-222-3p. miR-222 is upregulated and promotes the pathogenesis of inflammatory diseases. For instance, miR-222 accelerates endothelial cell inflammation and dysfunction in atherosclerosis (38). miR-222 is highly expressed and induces the inflammatory responses of microglia cells in intracerebral hemorrhage (39). Notably, miR-222-silencing attenuates staphylococcal enterotoxin B-induced inflammation in ALI (40). In the present study, miR-222-3p decreased the viability and accelerated the inflammation of LAMP-induced THP-1 cells. Furthermore, miR-222-3p markedly reversed the effects of GAS5-overexpression on LAMP-induced THP-1 cells. These results suggested that GAS5 may alleviate MPP by inhibiting miR-222-3p expression *in vitro*.

TIMP3 expression is usually downregulated in diverse inflammatory diseases, including heart failure (41), interstitial lung disease (42) and cardiac ischemia/reperfusion injury (43). Similarly, TIMP3 expression was clearly decreased in MPP and LAMP-induced THP-1 cells in the present study. TIMP3 generally exerts its role in certain diseases by interacting with miRNAs. For instance, miR-712 facilitates endothelial inflammation and atherosclerosis by inhibiting TIMP3 expression (44). miR-34b-5p-silencing hampers bleomycin-induced pulmonary fibrosis by increasing TIMP3 expression (21). Notably, miR-222 inhibition enhances TIMP3 expression to alleviate the development of pulmonary artery hypertension (45). In the present study, TIMP3 was a target of miR-222-3p. The expression of TIMP3 was lower in patients with MMP than in the children in control group. Notably, there was a negative correlation between the expression of TIMP3 and miR-222-3p in patients with MPP. We hypothesized that miR-222-3p may be involved in MPP development by modulating TIMP3. Considering the interaction between GAS5 and miR-222-3p, we hypothesized that silencing of GAS5 may downregulate TIMP3 expression by increasing miR-222-3p expression in MMP. The feedback data demonstrated that TIMP3-knockdown attenuated the enhancing effect on cell viability and weakened the anti-inflammatory effect caused by GAS5-overexpression in LAMP-induced THP-1 cells. These findings suggested that GAS5 may exert its protective effect by regulating the miR-222-3p/TIMP3 axis in MPP.

There were two limitations to the present study. To begin with, the detailed regulatory mechanism of GAS5 on the inflammation of MPP is not fully understood. Furthermore, the effect of GAS5 on MPP was investigated at the cellular level. Establishment of an MPP animal model is required to confirm the role of GAS5 *in vivo*.

In conclusion, the expression of GAS5 was downregulated in patients with MPP and LAMP-induced THP-1 cells. GAS5-overexpression increased the viability and attenuated the inflammation of LAMP-induced THP-1 cells by regulating the miR-222-3p/TIMP3 axis. These results indicated that GAS5 may be a promising therapeutic target for MPP.

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Availability of data and materials

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

Authors' contributions

LY and XL were responsible for conceptualization and design of the study, and performed the methods and software analysis, as well as having roles in the supervision of the entire study. XZ was responsible for data acquisition, original draft preparation and project management. XZ and XL were responsible for reviewing and editing the manuscript. LY, XZ and XL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The Second People's Hospital of Liaocheng (Linqing, China) and written informed consent was obtained from each child and their guardian.

Patient consent for publication

Written informed consent for publication was obtained from each child and their guardian.

Competing interests

The authors declare that they have no competing interests.

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