Am J Transl Res 2022;14(9):6187-6195
www.ajtr.org /ISSN:1943-8141/AJTR0142900

Original Article

Blockade of interleukin-6 receptor attenuates apoptosis and modulates the inflammatory response in Mycoplasma pneumoniae infected A549 cells

Wei Fang, Jingyu Huang, Jun Wang, Tengyi Huang, Dajia Lin, Jun Yin

Department of Clinical Laboratory Medicine, Second Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, People's Republic of China

Received March 15, 2022; Accepted July 23, 2022; Epub September 15, 2022; Published September 30, 2022

Abstract: Objective: To investigate the effect of silencing the interleukin (IL)-6 gene on the induction of inflammation, oxidative stress and apoptosis in Mycoplasma pneumoniae (MP) infected A549 cells and its mechanism of action. Methods: IL-6 small interfering RNA (siRNA) was synthesized and transfected into A549 cells, which were divided into a blank control group, a negative control group, and an IL-6 siRNA group. The mRNA and protein expression of IL-6 and the protein expression of CyclinD1, Cleaved caspase-3, Bax, B-cell lymphoma 2 (Bcl-2), signal transducer and activator of transcription 3 (STAT3), phosphorylated STAT3 (p-STAT3), matrix metalloproteinase (MMP)-2 and MMP-9 were measured. Besides, cell viability and apoptosis were determined. Additionally, the levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), IL-1β, IL-8 and tumor necrosis factor (TNF)-α were measured. Results: The mRNA and protein levels of IL-6 in the IL-6 siRNA group were lower than those in the blank and negative control groups ($P < 0.05$). The IL-6 siRNA group had higher viability but lower apoptosis rate of A549 cells at 24 h, 48 h and 72 h than the blank and negative control groups ($P < 0.05$). The IL-6 siRNA group had lower protein expression levels of Cleaved caspase-3 and Bax, but higher protein expression levels of CyclinD1 and Bcl-2 than the blank and negative control groups ($P < 0.05$). The IL-6 siRNA group had lower levels of IL-6, IL-8, TNF-α and MDA, but higher levels of SOD and GSH-PX than the blank and negative control groups ($P < 0.05$). Conclusion: Silencing the IL-6 gene can reduce the MP-induced inflammatory response and oxidative stress of A549 cells, enhance cell viability and inhibit apoptosis. Meanwhile, it was also found that STAT3 expression was inhibited after silencing IL-6 gene expression. Therefore, it is speculated that IL-6 may play a role by regulating STAT3, but its exact molecular biological mechanism still needs to be further explored.

Keywords: Mycoplasma pneumoniae, interleukin-6, A549 cells, apoptosis, inflammatory response

Introduction

Mycoplasma pneumoniae (MP) is a common agent of acute respiratory infections with a cell size between viruses and bacteria, exacerbating chronic obstructive pulmonary disease, asthma and other extrapulmonary systemic complications [1, 2]. The pathogenic mode of MP includes intracellular colonization, adhesion to host cells, disruption of cell membranes and cytotoxic effects, which can cause alveolar damage and structural disruption of epithelial cells, leading to impairment of lung function [3-5]. Currently, the pathogenesis of MP infection is not yet fully elucidated and is mostly thought to be related to an imbalance in the immune inflammatory response after infection. Studies have confirmed that MP infection induces the secretion of several cytokines and inflammatory mediators, such as interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-10, and some of them recruit neutrophils in the early stages of inflammation, thus connecting the mechanisms of intrinsic and adaptive immunity that mediate the airway mucosa, and participate in defense against chronic inflammation and other key pathophysiological processes of pathogenic bacterial infection [6, 7].

IL-6 is a cytokine involved in a variety of biological activities, secreted by activated mononuclear macrophages, epithelial cells and lympho-

cytes, and it plays an important role in anti-infection and hematopoiesis [8]. It was found that serum IL-6 level was elevated in children with MP infection, and the level was related to the change of Th1/Th2 balance, which was helpful to guide clinical medication and evaluate the severity of disease [9]. The above studies all suggest that IL-6 may be an important and key target for the development of MP infection, and regulating the expression of IL-6 may have specific significance against MP infection. For this reason, in this study, the IL-6 gene was silenced by small interfering RNA (siRNA) technology and transfected into A549 cells to observe the effect and mechanism of silencing IL-6 on the inflammation, oxidative stress and apoptosis of MP-induced A549 cells, thus providing new biological targets and protocols for treatment.

Materials and methods

Materials

Cell lines and strains: A549 cell lines were purchased from Shanghai Enzyme Research Biotechnology Co. MP stain ATCC15531 was purchased from American Type Culture Collection Inc. (Chicago, IL, USA).

Methods

MP culture: The MP culture was inoculated in mycoplasma broth and incubated in an incubator at 37°C with saturated humidity and 5% CO_2. When the medium color changed from red to yellow, serial passage was carried out, and the third passage was taken. The concentration of MP was measured by color change unit (CCU), and 1×10^4 CCU/mL bacterial solution was retained for use.

Cell treatment: A549 cells were cultured in high sugar medium [containing 100 U/mL penicillin, 10% fetal bovine serum and 100 U/mL streptomycin (Gibco, USA)] at 37°C in 5% CO_2, and the cells were passaged every 2-3 days. Thereafter, the cells were digested and passaged with trypsin (Gibco, USA), and the cells in logarithmic growth phase were taken for further experiments. A549 cells were inoculated into 24-well plates at a density of 1×10^5/mL, and the experiment was started after the cells grew into monolayers. Each well was added with 750 μL of MP bacterial solution at a concentration of 1×10^4 CCU/mL, and four replicate wells were set up and incubated at 37°C in 5% CO_2 for 4 h to establish a model of MP infection in A549 cells.

Cell transfection: The IL-6 siRNA was synthesized by Shanghai GenePharma Co., Ltd. A total of 3 sequences were screened, and the sequence with the strongest interference effect was screened by pre-experiments for subsequent experiments. The sequences finally used for the trial were IL-6 siRNA sequence: 5'-AGAGTCCTTCAGAGAGATA-3', and negative control group (NC-RNAi) sequence: 5'-TTCTCCGAACGTGTCACGT-3'. A549 cells were divided into a blank control group, a negative control group and an IL-6 siRNA group. A549 cells in good growth condition were seeded into 6-well plates at 5×10^8/L. Serum-free medium was mixed with IL-6 siRNA, siRNA NC and lipo-2000 (Thermo Fisher Scientific, USA), respectively, and incubated for 20 min at room temperature. When the cells reached 80% confluence, the above mixture was added, the medium was changed 6 h after transfection, and the culture was continued for 48 h to extract RNA and protein.

qRT-PCR assay: Total RNA of the cells was extracted by Trizol method [RNA extraction kit (Shanghai Haifang Biotechnology Co., Ltd., China)]. The residual genomic DNA in the total RNA was removed by RNA Reverse Transcription Kit (Beijing Taize Jiaye Technology Development Co., Ltd., China), and cDNA was constructed by reverse transcription. Subsequently, SYBR® Premix Ex Taq was used to perform real-time fluorescence quantitative polymerase chain reaction (PCR) (7500 fluorescence real-time PCR instrument, ABI, USA) at the following conditions: 95°C for 10 min, 95°C for 15 s, 60°C for 15 s, 72°C for 30 s for 40 cycles, reaction system 20 μL. The primers were designed and synthesized by Shanghai Biotechnology, China. The relative expression of IL-6 mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal reference. The primer sequences were as follows: IL-6: F: 5'-GGTACATCCTCGACGGCATCT-3', R: 5'-GTGCCTCTTTIGCTGCTTTCAC-3'; GAPDH: F: 5'-GGGAAGCTCACTGGCATGGCCTTCC-3', R: 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The experiment was repeated three times.

Western blot assay: The culture medium was discarded, and PBS was added and washed 3

times. Cellular proteins were extracted with Total Protein Extraction Kit (Taraka Corporation, Japan). The protein was quantified by BCA method. 1× loading buffer was added, followed by SDS-PAGE electrophoresis, 0.45 μm polyvinylidene fluoride (PVDF) membrane transfer and 5% skim milk blocking. All primary antibodies were supplied by Cell Signaling Technology, Inc. Primary antibodies, including CyclinD1 (1:1000, No. 2978), Cleaved caspase-3 (1:1000, No. 9661), Bax (1:1000, No. 2772T), Bcl-2 (1:1000, No. 2876), STAT3 (1:1000, No. 9132L), p-STAT3 (1:1000, No. 9131), MMP-2 (1:1000, No. 4022S), MMP-9 (1:1000, No. 3852S) and GAPDH (1:1000, No. 2118L) were added, incubated overnight at 4°C, and washed 3 times with TBST. Afterwards, secondary antibody, goat anti-rabbit IgG/HRP (1:10000, No. bs-0295G-HRP, Beijing Solarbio Science & Technology Co., Ltd., China) was added and incubated for 1 h at room temperature, exposed to ECL color development, and quantified using Image J software. The experiment was repeated three times.

MTT assay: A549 cells in good growth condition were inoculated 1×10^4 cells per well into 96-well culture plates. Then, 200 μL of culture medium was added, and after 24 h, 48 h and 72 h of continuous incubation, 10 μL of MTT reagent (Shanghai Hengfei Biotechnology Co., Ltd., China) was added to each well, and the incubation was continued for 1 h in a 37°C incubator. The absorbance value (A) of each sample was measured by a microplate reader at 450 nm wavelength, and the cell viability was calculated. The experiment was repeated three times.

Enzyme-linked immunosorbent assay (ELISA): A549 cells in good growth condition were placed in a centrifuge tube and mixed with complete medium to obtain a cell suspension. The levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), IL-1β, IL-8 and TNF-α were determined according to instructions of the ELISA kits for MDA, SOD, GSH-PX, IL-1β, IL-8 and TNF-α (Beyotime Biotechnology, Shanghai, China). The experiment was repeated three times.

Flow cytometry: A549 cells (3×10^5 cells) in good growth condition were seeded into 6-well plates, cultured for 24 h to reach 90% confluence, digested with EDTA-free trypsin for 2 min, and fully pipetted to obtain single cells. Then, 5 μL of fluorescently labeled Annexin V (Annexin V-FITC) and propidium iodide (PI) reagents (Shanghai Hengfei Biotechnology Co., Ltd., China) were added to each suspension, and incubated in dark for 15 min. The apoptosis rate was detected by flow cytometry (flow cytometer, Backman Corporation, USA). The experiment was repeated three times.

Statistical analysis

Data in this study were analyzed with SPSS 24.0. The measurement data conforming to a normal distribution were described as mean ± standard deviation. The one-way analysis of variance (ANOVA) was used for comparison among multiple groups, and least significant difference (LSD)-t test was used for pairwise comparison. $P < 0.05$ was considered statistically significant.

Results

mRNA and protein levels of IL-6 and the effect on cell proliferation

The mRNA and protein levels of IL-6 in the IL-6 siRNA group were lower than those in the blank and negative control groups ($P < 0.05$). There was no statistical difference in the mRNA and protein levels of IL-6 between the blank and negative control groups ($P > 0.05$). The viability of A549 cells in the IL-6 siRNA group was higher than that in the blank and negative control groups at 24 h, 48 h and 72 h ($P < 0.05$). There was no statistical difference in the viability of A549 cells at 24 h, 48 h and 72 h between the blank and negative control groups ($P > 0.05$) (**Figure 1**).

Effect of silencing IL-6 on apoptosis and expression of apoptosis-related proteins of A549 cells

The apoptosis rate in the IL-6 siRNA group was lower than that in the blank and negative control groups ($P < 0.05$). There was no statistical difference in the apoptosis rate between the blank and negative control groups ($P > 0.05$) (**Figure 2**). The protein levels of Cleaved caspase-3 and Bax in the IL-6 siRNA group were lower than those in the blank and negative control groups ($P < 0.05$), while the protein levels of

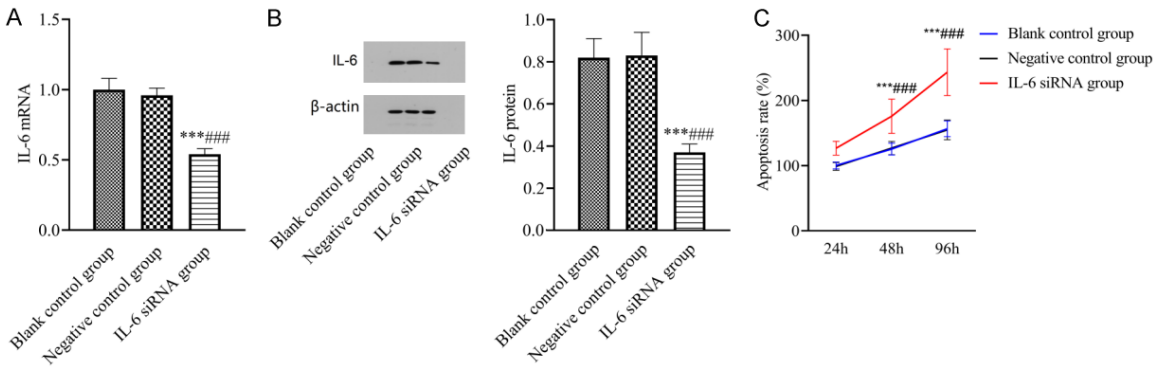

Figure 1. Expression of IL-6 and effect on cell on proliferation. A: IL-6 mRNA; B: IL-6 protein; C: Cell proliferation rate. Note: IL-6, interleukin-6; siRNA, small interfering RNA. Compared with the blank control group, $^{***}P < 0.001$, compared the negative control group, $^{###}P < 0.001$.

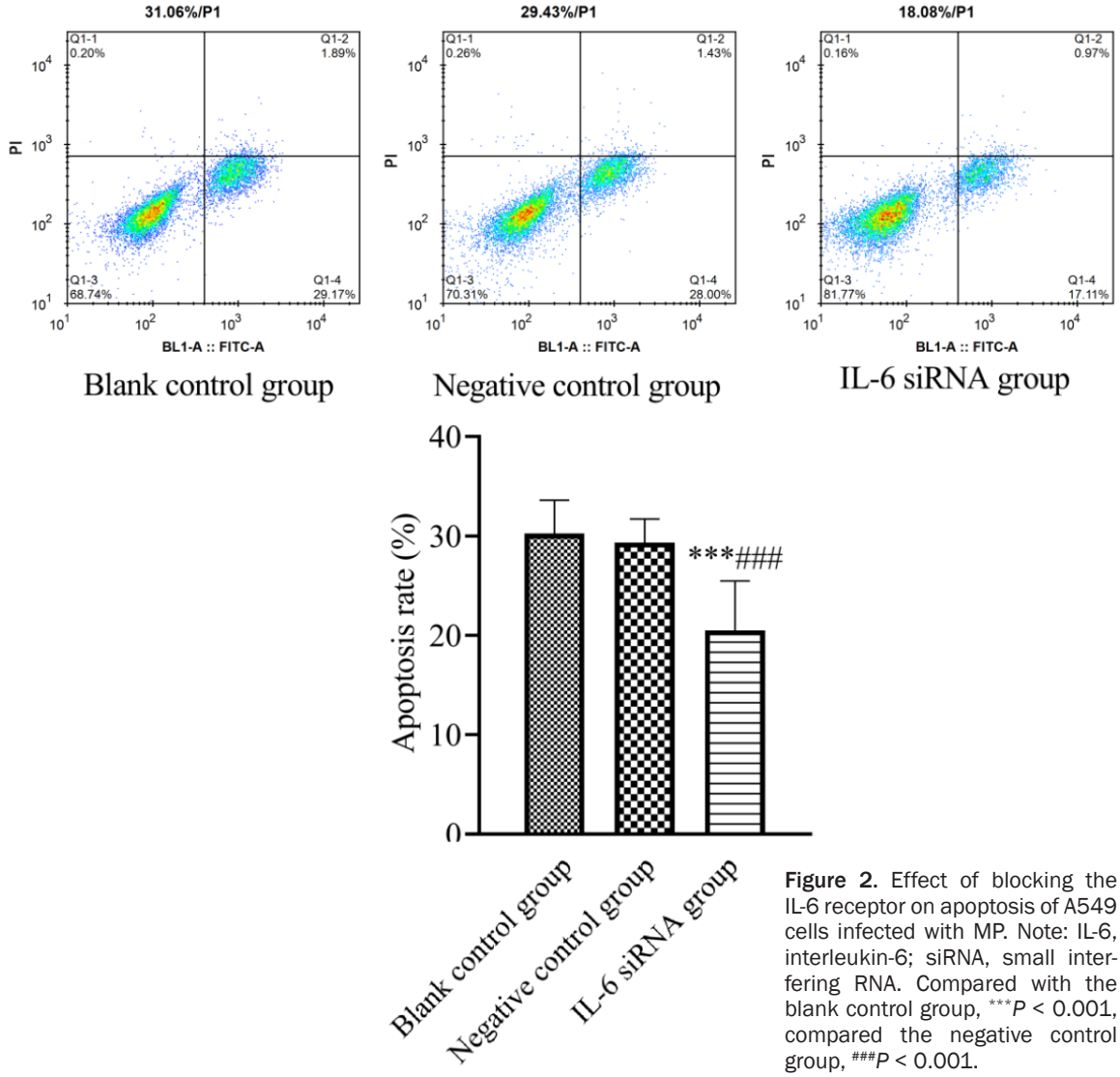

Figure 2. Effect of blocking the IL-6 receptor on apoptosis of A549 cells infected with MP. Note: IL-6, interleukin-6; siRNA, small interfering RNA. Compared with the blank control group, $^{***}P < 0.001$, compared the negative control group, $^{###}P < 0.001$.

CyclinD1 and Bcl-2 in the IL-6 siRNA group were higher than those in the blank and negative control groups ($P < 0.05$). There was no significant difference in the protein expression of

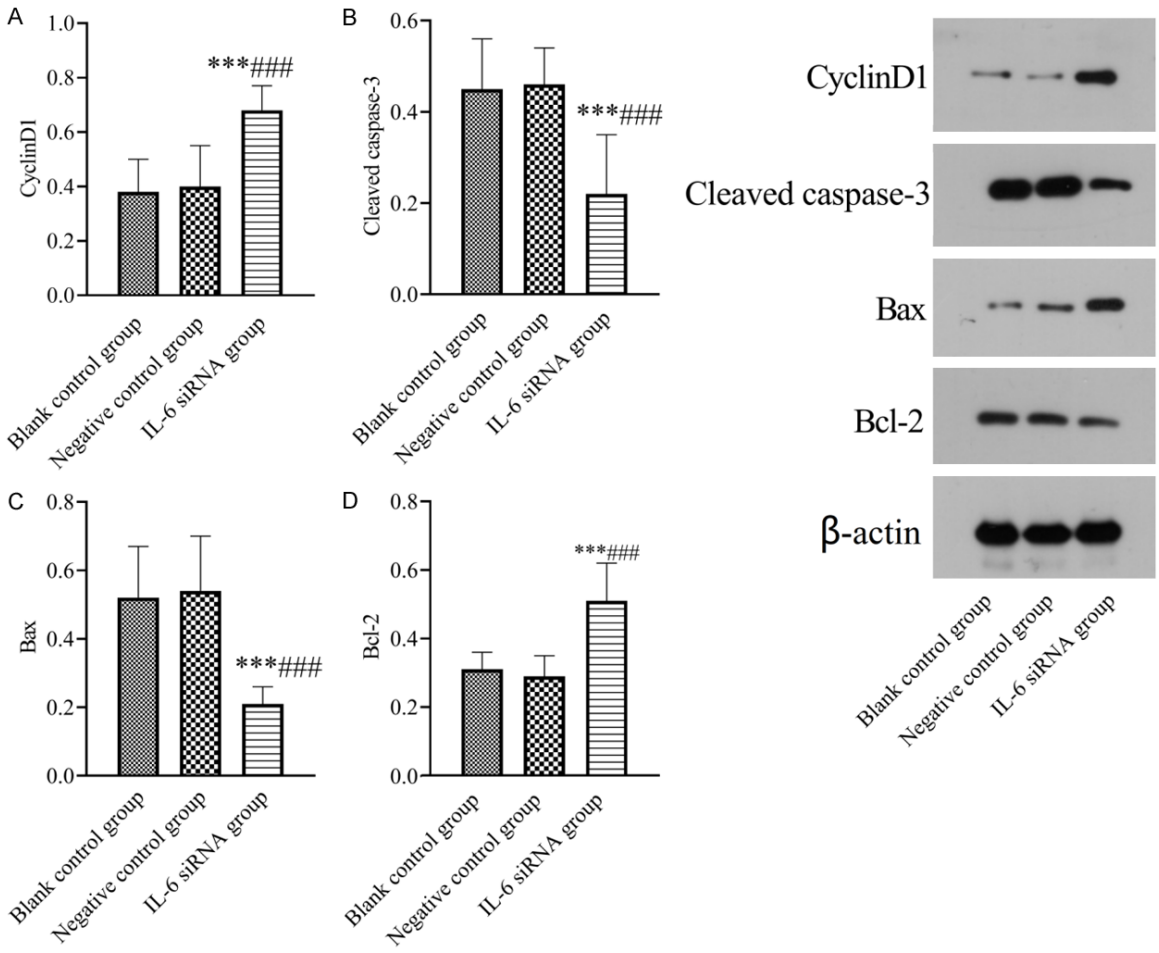

Figure 3. Effect of blocking the IL-6 receptor on expression of apoptosis-related proteins of A549 cells infected with MP. A: CyclinD1; B: Cleaved caspase-3; C: Bax; D: Bcl-2. Note: IL-6, interleukin-6; siRNA, small interfering RNA. Compared with the blank control group, $^{***}P < 0.001$, compared the negative control group, $^{###}P < 0.001$.

CyclinD1, Bcl-2, Cleaved caspase-3 and Bax between the blank and negative control groups ($P > 0.05$) (**Figure 3**). This suggested that silencing IL-6 inhibited apoptosis of A549 cells.

Effect of silencing IL-6 on STAT3, p-STAT3, MMP-2 and MMP-9 protein expression

The protein expression levels of p-STAT3, MMP-2 and MMP-9 in the IL-6 siRNA group were lower than those in the blank and negative control groups ($P < 0.05$). There was no statistical difference in the protein expression levels of STAT3, p-STAT3, MMP-2 and MMP-9 between the blank and negative control groups ($P > 0.05$). This suggested that silencing IL-6 reduced STAT3 phosphorylation level and down-regulated the activity of STAT3 signaling pathway (**Figure 4**).

Effect of silencing IL-6 on inflammatory response

The levels of IL-6, IL-8 and TNF-α in the IL-6 siRNA group were lower than those in the blank and negative control groups ($P < 0.05$). There was no statistical difference in the levels of IL-6, IL-8 and TNF-α between the blank and negative control groups ($P > 0.05$). This indicated that silencing IL-6 inhibited the secretion of inflammatory factors and reduced the inflammatory response (**Figure 5**).

Effect of silencing IL-6 on oxidative factors

The MDA level in the IL-6 siRNA group was lower than that in the blank and negative control groups, while the SOD and GSH-PX levels in the IL-6 siRNA group were higher than those in the

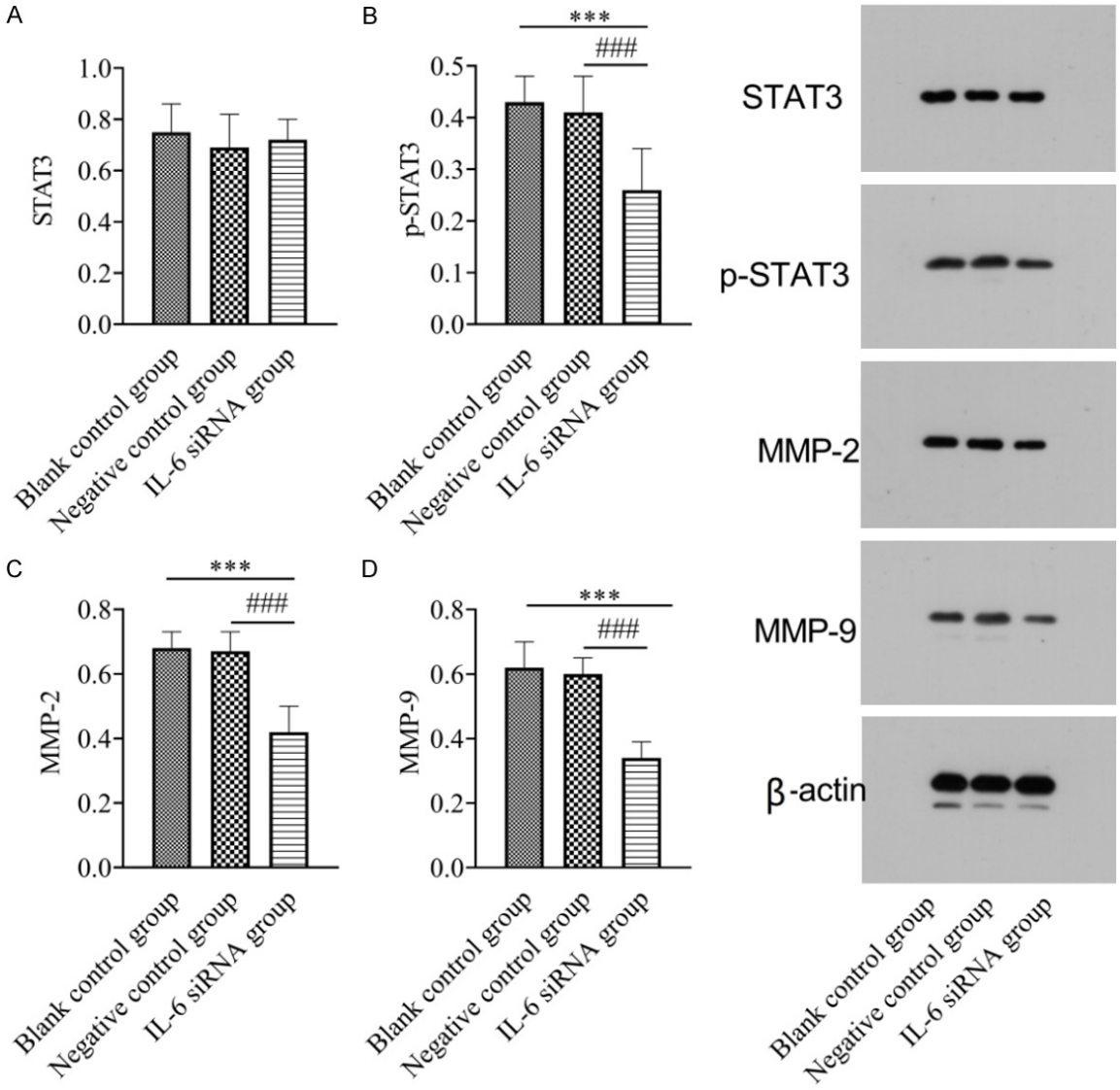

Figure 4. Effect of blocking the IL-6 receptor on STAT3, p-STAT3, MMP-2 and MMP-9 protein expression of A549 cells infected with MP. A: STAT3; B: p-STAT3; C: MMP-2; D: MMP-9. Note: IL-6, interleukin-6; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; p-STAT3, phosphorylated STAT3; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9. Compared with blank control group, $^{***}P < 0.001$, compared with negative control group, $^{###}P < 0.001$.

blank and negative control groups ($P < 0.05$). There was no statistical difference in the MDA, SOD and GSH-PX levels between the blank and negative control groups ($P > 0.05$). This indicated that silencing IL-6 reduced the oxidative stress response (**Figure 6**).

Discussion

MP can induce chronic airway inflammation and hyper-responsiveness by proliferating, aggregating and releasing inflammatory factors and chemotactic mediators in the airway epithelium and by participating in the airway epithelial inflammatory response and immune response process [10, 11]. IL-6, mainly expressed on human chromosome 7, is an important non-specific inflammatory factor in MP infection and is involved in the inflammatory pathological changes of pneumonia [12]. It was found that serum IL-6 was highly expressed in patients with MP infection, and its level was closely related to TNF-α and Gal-3 levels, which could effectively predict the therapeutic out-

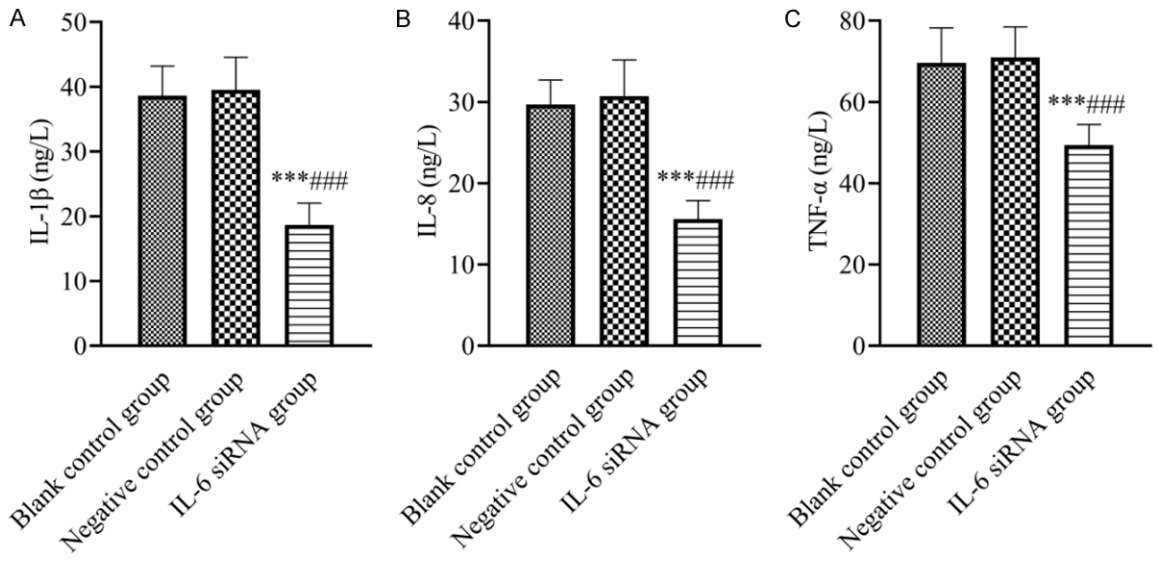

Figure 5. Comparison of inflammatory responses. A: IL-1β; B: IL-8; C: TNF-α. Note: IL-6, interleukin-6; siRNA, small interfering RNA; IL-1β, interleukin-1β; IL-8, interleukin-8; TNF-α, tumor necrosis factor-α. Compared with the blank control group, $^{***}P < 0.001$, compared with the negative control group, $^{###}P < 0.001$.

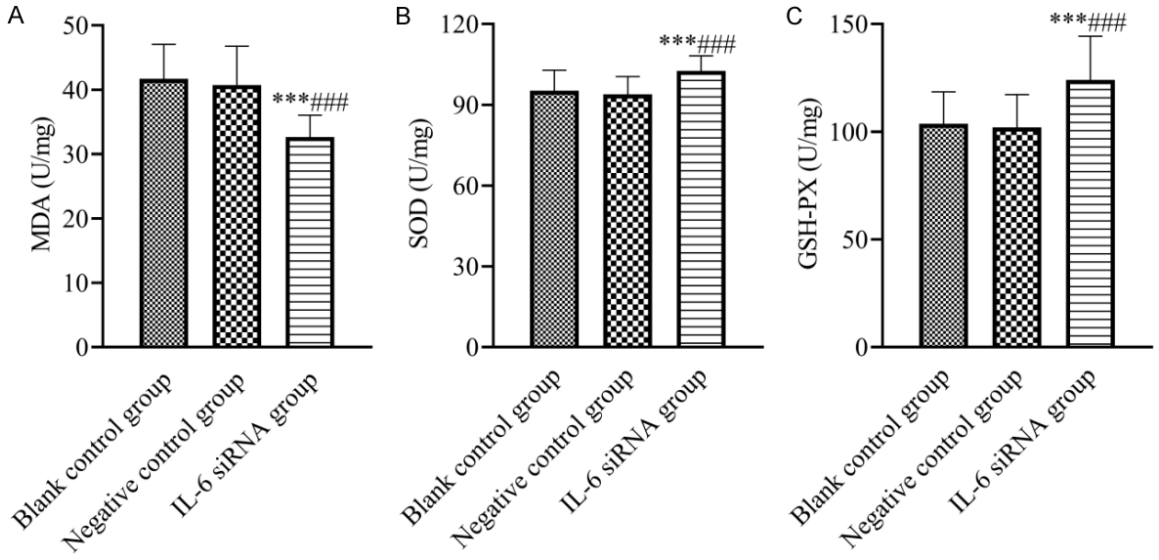

Figure 6. Comparison of oxidation factors. A: MDA; B: SOD; C: GSH-PX. Note: IL-6, interleukin-6; siRNA, small interfering RNA; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase. Compared with the blank control group, $^{***}P < 0.001$, compared with the negative control group, $^{###}P < 0.001$.

comes [13]. The results of this study showed that down-regulation of IL-6 gene expression enhanced the cell viability of MP-induced A549 cells, suggesting that IL-6 may be an important factor in the development of MP. Huang et al. [14] found that with the aggravation of MP in children, the levels of TLR4 in peripheral blood monocytes and IL-6 and TNF-α in serum also increased significantly, which is similar to our findings.

The Bcl-2 gene family and caspase-3 can regulate the apoptosis process, among which Bcl-2 inhibits Bax cytotoxicity and caspase-3 activation by combining with apoptosis stimulating factors, thus exerting anti-apoptotic effects; Bax activates the pro-apoptotic protein caspase-9 and releases cytochrome C by changing mitochondrial permeability; caspase-3 participates in the regulation and initiation of the entire apoptosis process by activating apopto-

sis stimulating factors [15]. In this study, apoptosis-related protein expression was measured by Western blot, and the results showed that silencing IL-6 could enhance the function of anti-apoptotic genes, promote the inactivation of pro-apoptotic genes, and inhibit apoptosis.

STAT3 is a key molecule mediating the inflammatory response and is activated by phosphorylation shuttling between the cytoplasm and nucleus, promoting the release of a series of inflammation-related proteins and exacerbating the inflammatory response [16-18]. It was found that STAT3 tyrosine phosphorylation and activation of STAT signaling pathway can induce the expression of downstream genes such as chemokines, adhesion factors and cytokines such as IL-6 and IL-1β upon stimulation by inflammation and infection, leading to acute lung tissue injury [19]. It has been reported that STAT3 may be associated with asthma-induced bronchial epithelial cell injury, and down-regulation of STAT3 expression can inhibit the activation of the JAK/STAT3 signaling pathway, attenuate airway injury, impede epithelial cell secretion of inflammatory factors and reduce the airway inflammatory response [20]. Therefore, inhibition of the activation of the JAK/STAT3 signaling pathway is particularly critical to attenuate airway inflammatory response. IL-6 can induce the differentiation of CD4+ T cells to Th17 cells by activating the specific transfer factor RORγt in Th17 cells through STAT3 pathway and signaling; moreover, IL-6 can induce the production of MMP factors such as MMP-2 and MMP-9 in tumor cells and induce the degradation of tumor cell extracellular matrix [21, 22]. In contrast, activation of STAT3 directly binds to the gene promoter, thus regulating MMP-2 expression. It can be speculated that increased phosphorylation level of STAT3 may play an important role in IL-6 regulating MMP-2 and MMP-9 proteins, thus affecting biological behaviors such as cell migration and invasion [23-25]. The results of this study showed that after the down-regulation of IL-6 expression, the STAT3 phosphorylation of MP-induced A549 cells was also significantly reduced, and the activity of STAT3 signaling pathway was down-regulated. Meanwhile, secretion of MMP-2, MMP-9, inflammatory factors such as IL-8 and TNF-α, as well as oxidative factors such as SOD and GSH-PX were also decreased.

However, this study has the following shortcomings. First, due to the limitation of experimental funds and the defect of experimental design, a STAT3 knockdown group was not set up. In the next study, the STAT3 blocking group will be set up to observe the therapeutic effect of IL-6 on STAT3. Second, the mechanism of MP infection is complex, but this study only discussed from the aspects of STAT3 signaling pathways and inflammatory reaction, and the mechanism was relatively single. The reliability and significance of the results of this study still need to be verified by more clinical studies or animal experiments in the future.

In summary, silencing of the IL-6 gene can alleviate MP-induced inflammation and oxidative stress in A549 cells, enhance cell viability and inhibit apoptosis. At the same time, IL-6 may play its role by regulating STAT3.

Acknowledgements

This research did not receive any specific funding from agencies in the public, commercial, or not-for-profit sectors.

Disclosure of conflict of interest

None.

Address correspondence to: Jun Yin, Department of Clinical Laboratory Medicine, Second Affiliated Hospital of Shantou University Medical College, Dongxia North Road, Shantou 515041, Guangdong Province, People's Republic of China. Tel: +86-0754-88915950; E-mail: Nobelalex@163.com

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