



Effect of miR-506-3p on Proliferation and Apoptosis of Airway Smooth Muscle Cells in Asthmatic Mice by Regulating CCL2 Gene Expression and Mediating TLR4/NF- κ B Signaling Pathway Activation

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Abstract

We aimed to investigate the effect of miR-506-3p on the proliferation and apoptosis of airway smooth muscle cells (ASMCS) in asthmatic mice by regulating the activation of TLR4/NF- κ B signaling pathway through targeted regulation of C–C Motif Chemokine Ligand 2 (CCL2) expression. Twenty-four BALB/c mice of specific pathogen-free grade were selected to establish asthmatic mouse model, which were randomly divided into normal control group and asthma model group ($n = 12$ for each group). HE and IHC staining, bioinformatics and dual luciferase reporter assay, RT-PCR MTT, flow cytometry and Western blot were used in this research. HE staining showed airway epithelium thickening, submucosal inflammatory cell infiltration and airway smooth muscle thickening, and the positive expression rate of CCL2 was significantly increased in asthma model group (all $P < 0.05$). CCL2 was the target gene of miR-506-3p. Moreover, the expression of miR-506-3p in asthma model group was significantly decreased, the mRNA and protein expression levels of CCL2, TLR4, NF- κ B (p65) and Bcl-2 were significantly increased, while those of Bax were decreased (all $P < 0.05$). In miR-506-3p mimic group or siRNA-CCL2 group, the expression of CCL2, TLR4, NF- κ B (p65) and Bcl-2 decreased obviously, while that of Bax increased, cell proliferation decreased, G1 phase prolonged, G2 & S phases shortened, and apoptosis rate increased significantly (all $P < 0.05$), whereas the opposite trends were found in miR-506-3p inhibitor group (all $P < 0.05$). However, there was no statistical difference in the above-mentioned indexes in miR-506-3p inhibitor + siRNA-CCL2 group (all $P > 0.05$). Overexpression of miR-506-3p can inhibit ASMCS proliferation and promote apoptosis via inhibiting CCL2 expression and suppressing the activation of TLR4/NF- κ B signaling pathway. Inhibited expression of miR-506-3p can reverse the positive role of CCL2 gene silencing. Our study is the first to prove the beneficial role of miR-506-3p-CCL2-TLR4/NF- κ B regulatory axis in the development of asthma.

Keywords miR-506-3p · CCL2 · TLR4/NF- κ B signaling pathway · Asthma · Airway smooth muscle cells · Proliferation · Apoptosis

Introduction

Bronchial asthma (asthma) is a common chronic respiratory disease [1]. According to the report of global initiative for asthma (GINA), there are about 300 million patients with asthma in the world, and it is expected to increase to 400 million by 2025 [2, 3]. Direct and indirect costs caused

by asthma have resulted in huge personal and social economic burden. It has become one of the chronic diseases that seriously threaten the health of all human beings [4]. In-depth study of its pathogenesis and exploration of effective intervention measures is the focus of academic attention. Traditionally, airway inflammation is the key link in the pathogenesis of asthma, and airway remodeling is the inevitable result of chronic development of airway inflammation [5, 6]. Under the stimulation of various inflammatory factors, the regulation mechanism of airway injury/repair is disordered, which leads to a series of pathological changes, such as epithelial metaplasia, hyperplasia of mucous glands, sub-epithelial basement membrane fibrosis, thickening of

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smooth muscle layer, deposition of extracellular matrix and neovascularization. [7–9].

It has been reported that airway remodeling and smooth muscle layer thickening are typical features of asthma [10]. Different from other chronic lung diseases, airway smooth muscle layer thickening is the most typical pathological change of asthma [11]. Furthermore, airway smooth muscle cells (ASMCS) are found to be passive cells in asthma, and can actively participate in the whole process of airway remodeling [12]. There is evidence that abnormal proliferation and hypertrophy of ASMCS may be one of the initiating factors of asthma [13]. Recent studies have gradually found that asthmatic ASMCS can also participate in immune regulation through the synthesis of interleukin, chemokines and cell adhesion factors, which further aggravates chronic inflammatory response of asthma [14–18]. Significantly, ASMCS play a key role in the pathogenesis of asthma [19–21], with the research on the mechanism and intervention of its dysfunction as the hot spot at present.

NF- κ B is an important nuclear factor widely expressed in various tissues and cells [22]. Its activation and nuclear transfer involve various complex signal transduction processes [23]. Via regulating the expression of multiple genes, NF- κ B participates in many biological activities, such as immune response, inflammatory response, apoptosis and tumorigenesis. [24]. Despite the absence of complete understanding of the role of NF- κ B signaling pathway in asthma, previous studies have found that NF- κ B is continuously activated in asthmatic patients or animal models [25, 26], suggesting that NF- κ B plays an important role in asthma. More importantly, Toll-like receptors (TLRs) recognize ligand conversion signals through leucine-rich repeats in extracellular domain and induce corresponding signal conversion cascade reactions, resulting in the activation of NF- κ B [27]. So far, the intervention of this signaling pathway is likely to provide a new intervention target for the treatment of asthma.

In addition, microRNA (miRNA) is a highly conserved, single stranded, non-coding small RNA, which mainly regulates gene expression by degrading mRNA or hindering its translation at the transcriptional level [28]. As an important regulator of life activities, miRNA plays a quite important role in the occurrence and development of tumors, cardiovascular diseases and various chronic diseases, and it is possibly to become a new target for clinical diagnosis and treatment in an early stage [29, 30]. There have been abundant studies concerning the role of miRNAs in human diseases. For example, miR-506-3p can act on PI3K-Akt signaling pathway and play a negative regulatory role by mediating the expression of PIK3CA [31]; it can inhibit the metastasis ability of colorectal cancer cells by regulating UHRF1, acting as a tumor suppressor gene in colorectal cancer [32]; and overexpression of miR-506-3p can inhibit the inflammatory

reaction and apoptosis of cardiomyocytes, which may be related to TRAF6 [33]. These studies suggest the critical role of miR-506-3p in various human diseases. In addition, C–C Motif Chemokine Ligand 2 (CCL2), also known as chemoattractant protein-1 (MCP-1), is one of the earliest and widely studied CC chemokine family member [34]. Chemokines are small molecule cytokines that can make cells chemotactic, which play important physiological and pathological roles and are involved in inflammation, tumors, autoimmune diseases, allergic diseases, etc. [35, 36]. In recent decades, the incidence of autoimmune diseases such as allergic asthma has increased significantly worldwide. Chronic asthma is characterized by inflammation and increased secretion of MCP-1/CCL2 [37]. CCL2 is secreted by a variety of inflammatory cells, such as mast cells, monocytes and epithelial cells. Therefore, the expression level of CCL2 in lung tissue can reflect the severity of asthma.

In view of the above interpretation, the present study was performed to explore the specific interaction or relationship of miR-506-3p, CCL2 and TLR4/NF- κ B signaling pathway. Through the exploration of the effect of miR-506-3p on the proliferation and apoptosis of ASMCS in asthmatic mice by regulating the activation of TLR4/NF- κ B signaling pathway via regulating CCL2 expression, this study is expected to clarify the pathogenesis of asthma and provide new theoretical basis and prevention targets for the prevention of asthma.

Materials and Methods

Modeling and Grouping

Twenty-four BALB/c mice of either gender, weighing 20–25 g and 7 weeks old, were used and divided into the normal control group and the asthma model group, with 12 mice in each group. For the establishment of asthma model, ovalbumin (OVA) sensitizing solution (10 μ g OVA emulsified with 2 mg AL(OH)₃) was injected intraperitoneally three times on days 0, 7 and 14 in the asthma model group. All mice were challenged with airway allergens for 3 consecutive times. To be specific, on days 15, 16 and 17, mice were placed in a self-prepared transparent plastic box (22*20*15 cm) and atomized with 2% OVA solution (2 g OVA dissolved in 100 mL distilled water) by ultrasonic atomizer. More than 90% of the particles were detected by laser turbidimetric method in the range of 1–2 μ m. The airway was stimulated once a day (40 min each) for 3 consecutive days. After that, the animal reaction was observed, and the positive manifestations were restlessness, prostration, cyanosis of lips, shortness of breath, abdominal muscle twitch and incontinence. The normal control group was given phosphate buffer saline (PBS) instead of OVA intraperitoneal injection and atomization inhalation, and the rest

operation steps were the same as those in the asthma model group. All animal experiments in this study were in strict accordance with the management and use principles of local Animal Research Center. Furthermore, 24 h after the last challenge, the lung tissue was harvested and fixed with 4% paraformaldehyde. The lung tissue slices prepared was about 5 μm in thickness. Hematoxylin–eosin (HE) staining used to observe the pathological changes of bronchi and lungs in each group under light microscope.

HE Staining for the Observation of Pathological Changes

In the process of HE staining, after dehydration and transparent processing, the tissue blocks were embedded and the tissue slices with a thickness of about 4 μm were prepared. The next steps were baking, dewaxing, dehydration, hematoxylin staining, hydrochloric ethanol differentiation, 0.25% ammonia re-bluing, eosin staining, transparent processing in xylene, etc. After staining, the slide was sealed with clean cover glass with neutral gum. The pathological changes were observed under the microscope.

Immunohistochemical Detection of CCL2 Positive Expression

The positive expression of CCL2 was detected according to the operation steps of two-step immunohistochemical detection kit. In the control test, the primary antibody was replaced by normal rabbit serum with the same dilution, with no difference in the remaining steps. Frozen tissue was sectioned (4 μm in thickness) and fixed with pre-cooled acetone, which were then placed in a wet box. After 3 times of PBS washing (2 min each), the frozen tissue sections were dripped with 3% hydrogen peroxide solution and incubated for 10 min to block endogenous peroxidase. After repeated washing with PBS washing, antigen retrieval was performed, followed by another PBS washing. Afterwards, the primary antibody of anti-CCL2 was added and incubated at 4 °C overnight. With PBS washing (3 times, 2 min each), the second antibody (HRP-labeled goat anti-rabbit IgG antibody) was added and incubated at room temperature for 20 min. With repeated PBS washing (3 times, 2 min each), DAB solution was prepared immediately, with the degree of development controlled under the microscope. Subsequently, after 30 s of hematoxylin staining, the sections were differentiated by hydrochloric ethanol, washed with distilled water, and stained again, followed by gradient alcohol dehydration (70%–80%–90%–95%—anhydrous ethanol), transparent processing using xylene (2 times, 2 min each), and sealing with neutral gum. Cells with brown nuclei were regarded as positive cells. Five high-power visual field was selected for each pathological section randomly, with a total of 500 cells

counted (positive cells < 10%, negative, positive cells $\geq$ 10% and < 50%, positive, positive cells > 50%, strong positive).

Isolation and Culture of ASMCS from Normal and Asthmatic Mice

The mice were killed by cervical dislocation. The skin of mice was disinfected with 75% alcohol. After the exposure of the chest by cutting off the skin and muscle, the left lung, right middle lung and right lower lobe were quickly removed and placed in pre-cooled D-Hank solution. The lung tissue, bronchial artery and vein, fat, residual connective tissue as well as internal and external membrane were scraped to obtain the remaining trachea and bronchus. Following the exposure of the trachea and bronchus longitudinally, and the outer membrane and inner membrane were scraped out with a curette until the tissue was transparent. After the bronchial smooth muscle was isolated, the tissue was cut into small pieces of 1 mm $\times$ 1 mm in size with ophthalmic iris scissors. The tissue blocks were digested in D-Hank solution containing collagenase, papain and trypsin, and digested in 5% CO₂ incubator at 37 °C. Single ASMCS were obtained by blowing and beating, filtration, centrifugation and suspension using DMEM medium, and subcultured to the 4th–8th generation. ASMCS showed a long spindle shape under the inverted phase contrast microscope, which could be connected or overlapped with each other and fluctuated ups and downs, showing a typical “peak valley” shape during fusion growth. After immunohistochemical SABC staining with anti- α -actin mAb, cells with light brown positive myofilaments around the nucleus stained with light blue were regarded as ASMCS.

Cell Grouping and Transfection

Cells of passage 6 were digested by trypsin and inoculated into 24-well plates. Cells grew into monolayer by culture. Cells were divided into seven groups according to different transfection protocols: Control group (ASMCS of normal control group), blank group (ASMCS of the model group without the transfection of any sequences), NC group (transfected with negative control sequence), miR-506-3p mimic group (transfected with miR-506-3p mimic sequence), miR-506-3p inhibitor group (transfected with miR-506-3p inhibitor sequence), siRNA-CCL2 group (transfected with siRNA-CCL2 sequence), miR-506-3p inhibitor + siRNA-CCL2 group (transfected with miR-506-3p inhibitor and siRNA-CCL2 sequences). The transfection sequence was constructed by Sangon Biotech (Shanghai) Co., Ltd. Corresponding siRNA sequences were (all 5′–3′): miR-506-3p mimic: UAAGGCACCCUUCUGAGUAGA; miR-506 inhibitor: UCUACUCAGAAGGGUGCCUUA; siRNA-CCL2: CTCGCGAGCTATAGAAGAA. The negative

control sequence was a random sequence that was also provided by Sangon Biotech. Furthermore, the control groups of miR-506-3p mimic group, miR-506-3p inhibitor group, siRNA-CCL2 group and miR-506-3p inhibitor + siRNA-CCL2 group were set to detect corresponding expression levels by RT-PCR, with the aim to identify the successful transfection for subsequent experiments, which were mimic NC group, inhibitor NC group, siRNA-NC group, as well as inhibitor NC + siRNA-NC group and miR-506-3p inhibitor + siRNA-NC group, respectively.

On the day before transfection, cells of appropriate amount were seeded on the 24-well plate, and medium without antibiotics was added into each well. The cell density was 30–50% at the time of transfection. For each sample, the lipofectamine 2000 mixture was prepared as follows: The plasmid with a concentration of 20 μM was diluted with 50 μL serum-free Opti-MEM medium (the final concentration of RNA added into cells was 100 nm), and the mixture was gently mixed; meanwhile, 1 μL lipofectamine 2000 was diluted with 50 μL serum-free Opti-MEM medium, and then gently mixed and incubated at room temperature for 5 min. Then, the above two mixtures were mixed gently and incubated at room temperature for 20 min. The diluted lipofectamine 2000 should be mixed with the diluted plasmid within 25 min to maintain the activity of the transfection reagent. In addition, when mixing reagents, pay attention not to blow or vibrate violently, just flick the tube wall with fingers. After that, the above-mentioned mixture was added into the culture well containing cells and culture medium, and was gently mixed. Next, the culture plate was placed in a 5% CO_2 incubator at 37 $^\circ\text{C}$, and the medium containing mixed solution in the well was removed after 4–6 h, and the fresh medium was replaced for further culture. After 48 h, cells were collected for further experiment.

Bioinformatics and Dual Luciferase Reporter Assay to Verify the Targeting Relationship Between miR-506-3p and CCL2

RNA22 tool (<https://cm.jefferson.edu/rna22/>), a biological prediction website for target gene prediction of miRNA, was used to carry out target gene analysis of miR-506-3p, followed by the verification of the target relationship between CCL2 and miR-506-3p. The 0.25 μg normal plasmid and plasmid containing binding site mutation were co-transferred into the experimental cells with 100 nm miR-506-3p mimics and 20 ng Ranilla luciferase plasmid (pRL-TK) by Lipofectamine 2000. Serum medium was added after 6 h of transfection. Then, after 48 h of transfection, cells were collected and the luciferase activity was measured according to the instructions of the kit. The kit used was Promega dual luciferase reporter assay gene detection kit (#E1910).

RT-PCR Detection of miR-506-3p, CCL2, TLR4 and NF- κB (p65) mRNA Expressions in Tissues and Cells

Twenty-four hours after the last challenge, mice were killed by cervical vertebra dislocation. The lung tissue samples were taken for RT-PCR detection of miR-506-3p, CCL2, TLR4, NF- κB (p65), Bcl-2 and Bax mRNA expressions in tissues, and detection in cells was the same as that in tissues. Total RNA was extracted from lung tissue and ASMCS in each transfection group with Trizol (Invitrogen, USA). The absorbance ratio of RNA samples was detected to be 1.9–2.0 by ultraviolet spectrophotometer at the wavelength of 260/280 nm. The SYBR Primescript™ RT-PCR reagent kit (TaKaRa, China) was used for detection using ABI Prism 7500 (Applied Biosystems, USA). Reaction system: SYBR® Premix Ex Taq™ II (2 $\times$) 25 μL , 2 μL PCR forward primer, 2 μL PCR reverse primer, 1 μL ROX Reference Dye (50 $\times$), 4 μL DNA template, and 16 μL ddH₂O. Real-time fluorescent quantitative PCR was performed by ABI7500 quantitative PCR (7500, ABI, USA), with the reaction conditions of pre-denaturation at 95 $^\circ\text{C}$ for 30 s, denaturation at 95 $^\circ\text{C}$ for 5 s, as well as annealing and extension at 60 $^\circ\text{C}$ for 30 s, with a total cycle of 40 times. Three parallel tubes were made for each sample to be tested and the relative standard substance of each gradient concentration. After PCR reaction, the data were analyzed by 7500 system SDS software, and the relative initial copy number of each sample was calculated. U6 was used as internal reference for miR-506-3p, and GAPDH as the internal reference for CCL2, TLR4, NF- κB (p65), Bcl-2 and Bax. The mRNA levels of miR-506-3p, CCL2, TLR4 and NF- κB (p65) were calculated by relative quantitative $2^{-\Delta\Delta\text{CT}}$ method. The test was repeated three times.

Western Blot Detection of CCL2, TLR4, NF- κB (p65), Bcl-2 and Bax Protein Expressions in Tissues and Cells

Similarly, mice were killed by cervical vertebra dislocation 24 h after the last challenge. The lung tissue samples were taken for Western blot detection of CCL2, TLR4, NF- κB (p65), Bcl-2 and Bax protein expressions in tissues, and detection in cells was the same as that in tissues. The RIPA lysate (Beyotime Biotechnology Co., Ltd., Beijing) was used to decompose and homogenize tissues/cells on ice, and the homogenate was centrifuged at 17,000g at low temperature for 1 h. The supernatant was taken and the protein content of the sample was determined by BCA protein content determination kit. The sample buffer solution at the ratio of 1:4 was added, boiled in boiling water for 5 min to realize complete denaturation of the protein, followed by centrifugation at 17,000g for 15 min, and the supernatant was collected. The equivalent samples were taken for 7.5% or 15%

polyacrylamide discontinuous gel electrophoresis, and then the proteins separated from the gel were transferred onto the cellulose nitrate membrane. After membrane transferring, the membrane was sealed with 15% skimmed milk powder (dissolved by TBST) at room temperature for 2 h. Then, the primary antibodies of CCL2 (1:10,000, ab200343), TLR4 (1:500, ab13556), NF- κ B (p65) (1:2000, ab76311), Bcl-2 (1:1000, ab32124) and Bax (1:5000, ab32503) were added to the membrane for reaction overnight at 4 °C. After TBST washing, corresponding secondary antibody of Goat Anti-Rabbit IgG H&L (HRP) (1:10,000, ab97051) was added to incubate at room temperature for 2 h. With another TBST washing, appropriate amount of liquid A and liquid B in ECL reagent box was collected to mix well. After drying of the membrane with clean filter paper, the mixed luminous liquid was dripped evenly on the membrane to react in the chemiluminescence imaging system for further collection of the images. Integrated density value was measured by the computer image analysis system. GAPDH was used as the internal reference. The test was repeated three times.

MTT Detection of Cell Proliferation After ASMCS Transfection According to Different Protocols

After 48 h of transfection, cells were digested and then blown into single cell suspension with 10% FBS/RPMI1640. With cell counting, the concentration of cells was adjusted into 3×10^3 cells/mL. Cells were then inoculated into the 96-well plate at a density of 200 μ L per well, with four replicates in each group. Cells were incubated for 0 h, 24 h, 48 h, 72 h and 96 h in the cell incubator. After that, an amount of 200 μ L serum-free medium containing 10% MTT was added into each well. After 4 h of incubation, the mixture of MTT was absorbed, and then 150 μ L DMSO was added into each well, and the crystal was fully dissolved by shaking at room temperature for 10 min. At the wavelength of 490 nm, the absorbance value of each well was measured on the enzyme-linked immunosorbent monitor, and the results was recorded. The cell growth curve was drawn with time as horizontal axis and the absorbance value as vertical axis.

Flow Cytometry Detection of Cell Cycle and Apoptosis After ASMCS Transfection According to Different Protocols

In the detection of cell cycle distribution, 48 h after transfection, cells were collected and washed with pre-cold PBS for three times, the supernatant was centrifuged and resuspended with PBS. The cell concentration was adjusted to about 1×10^5 /mL. With fixation using pre-cooled 75% ethanol for 1 h, cells were centrifuged and the ethanol discarded. After washing with PBS twice, 100 μ L RNase A was added for reaction in dark, followed by a 37 °C water

bath for 30 min. After the addition of 400 μ L PI for staining, cells were cultured in dark at 4 °C for 30 min. The red fluorescence light was recorded at the excitation wavelength of 488 nm detected by flow cytometry, and the cell cycle was detected.

After 48 h of transfection, cells and culture medium (including the floating cells in the upper layer) were digested and collected, centrifuged at 1500 rpm/min for 5 min, and the upper liquid was discarded, and then cells were re-suspended and precipitated with $1 \times$ PBS. The cell concentration in each flow cytometry tube was adjusted to 1×10^6 /mL. Annexin V and PI double staining was performed according to the instructions of the kit, and the apoptotic cells were detected by flow cytometry. In terms of result analysis, the early apoptosis rate was detected by flow cytometry, and the average value of each group was obtained for result analysis. Apoptotic cells were resistant to PI dye, but necrotic cells were not. The DNA of cells with membrane damage can be stained by PI to produce red fluorescence, while cells with intact cell membrane would not. Therefore, PI and not produce red fluorescence signal would not stain normal living cells and cells with early apoptosis. On the double-variable scatter plot, the lower left quadrant was (FITC−/PI−), representing living cells; the upper right quadrant was (FITC+/PI+), representing necrotic cells; and the lower right quadrant was (FITC+/PI−), representing apoptotic cells.

Statistical Analysis

SPSS 22.0 (SPSS, Chicago, Illinois, USA) was used for statistical analysis. The measurement data were expressed by mean $\pm$ standard deviation. The comparison between groups was conducted using independent sample *t* test. One-way analysis of variance was used for comparison among groups, and pairwise comparison was performed by LSD method. The count data were expressed by percentage and analyzed by chi-square test. Statistically significant difference was presented by $P < 0.05$.

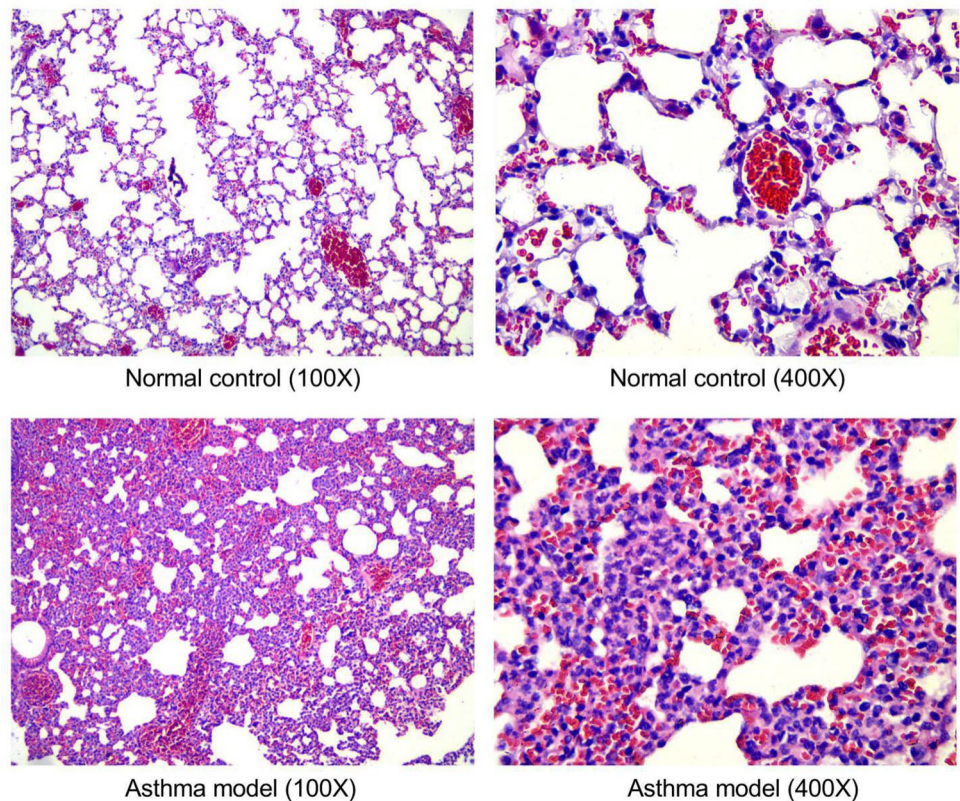
Results

Pathological Changes of Asthmatic Mice After Modeling

In the normal control group, the structure of bronchial lumen and alveoli was complete, and there was no obvious inflammatory cell infiltration in the bronchial wall, with order arrangement of the airway epithelial cells (Fig. 1).

In the asthma model group, there were bronchial lumen stenosis, wall thickening, disordered lung tissue structure, widened alveolar septum, wall rupture, inflammatory cell infiltration around the airway and lung interstitium, mainly

Fig. 1 HE staining results of lung tissue in normal control group and asthma model group ($\times 100$ and $\times 400$, respectively)



neutrophil infiltration; besides, blood vessels dilated and congested, lymphocytes and eosinophils infiltrated in peripheral blood vessels and alveoli, which indicated the successful establishment of asthma model in mice (Fig. 1).

Increased Positive Expression Rate of CCL2 Detected by Immunohistochemistry

CCL2 was weakly positive in normal control group. In the asthma model group, CCL2 was highly expressed, and the positive cells stained were brown and increased significantly (Fig. 2). Compared with the normal control group, the positive expression rate of CCL2 in asthma model group was significantly increased, with statistical difference ($P < 0.05$).

Detection Results of RT-PCR and Western Blot in Tissues

The expression of miR-506-3p in asthma model group was significantly decreased, while the mRNA expression levels of CCL2, TLR4 and NF- κ B (p65) were significantly increased (all $P < 0.05$) compared with the normal control group as well as the protein expression of Bax in asthma model group was significantly decreased, while those of CCL2, TLR4, NF- κ B (p65) and Bcl-2 were increased obviously (all $P < 0.05$) (Fig. 3).

CCL2 as the Target Gene of miR-506-3p

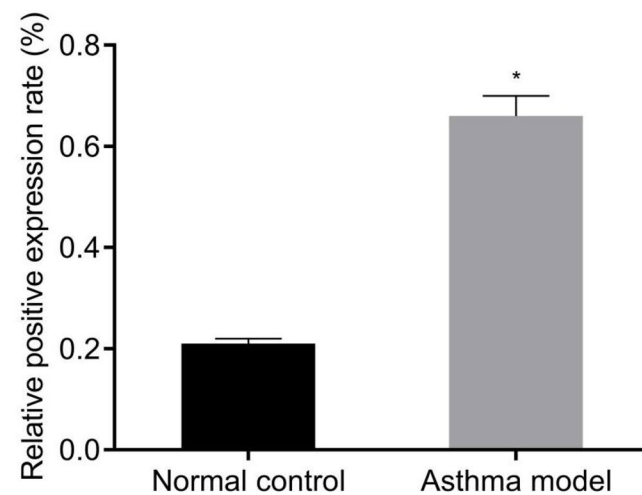
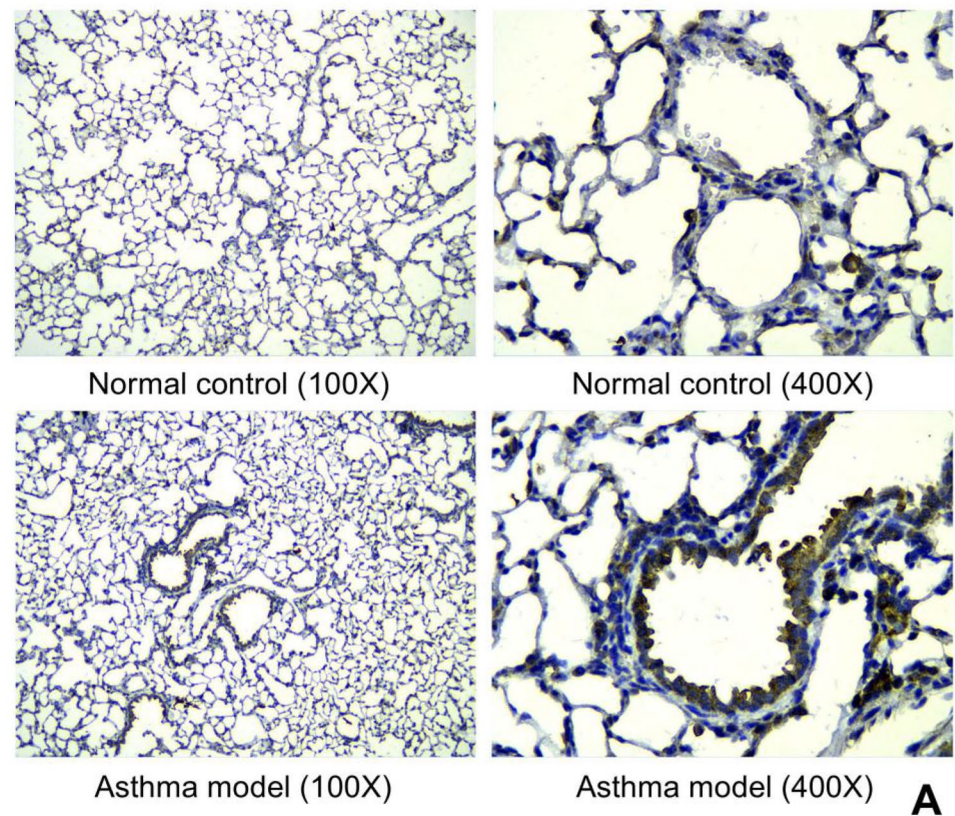
Through online analysis, there was a specific binding region between CCL2 gene and miR-506-3p sequence. CCL2 was the target gene of miR-506-3p (Fig. 4a). Furthermore, CCL2 was identified as the target gene of miR-506-3p by dual luciferase reporter assay (Fig. 4b).

It was found that compared with NC group, the luciferase signal of miR-506-3p mimic/Wt-CCL2 co-transfection decreased in miR-506-3p mimic transfection group ($P < 0.05$). However, there was no significant difference in luciferase activity of mutant 3'UTR ($P > 0.05$). These results indicate that miR-506-3p can specifically bind to CCL2 gene.

Identification of Successful Transfection

MiR-506-3p mimic group showed a highly increase in the expression of miR-506-3p compared with mimic NC group ($P < 0.05$), and a significant decrease was found in the expression of miR-506-3p in miR-506-3p inhibitor group than that in inhibitor NC group (Fig. 5) ($P < 0.05$). In addition, si-CCL2 group showed decreased expression of CCL2 than that in siRNA-NC group ($P < 0.05$). Meanwhile, compared with inhibitor NC + siRNA-NC group, miR-506-3p inhibitor + siRNA-NC group exhibited a decrease in the expression of miR-506-3p but increase in that of CCL2 (both $P < 0.05$);

Fig. 2 The positive expression rate of CCL2 in lung tissue of normal control group and asthma model group. *Note a* Immunohistochemical results of lung tissue ($\times 100$ and $\times 400$, respectively). *b* Histogram of CCL2 positive expression rate. $n=12$, independent sample t test was used for statistical analysis. *Compared with the normal control group, $P<0.05$



besides, compared with miR-506-3p inhibitor + siRNA-NC group, there was significant decrease in the expression of CCL2, and a slight increase in the expression of miR-506-3p in miR-506-3p inhibitor + siRNA-CCL2 group (both $P<0.05$) (Fig. 5).

MiR-506-3p Inhibited the Activation of TLR4/NF- κ B Pathway by Inhibiting CCL2 Gene Expression

The results of RT-PCR showed that the expression of miR-506-3p was significantly lower than control group, while

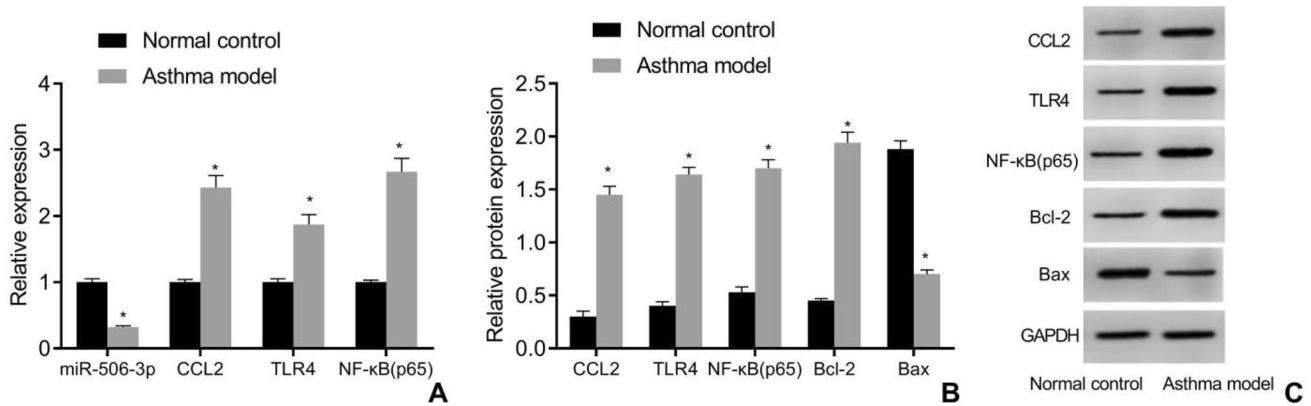


Fig. 3 Detection results of RT-PCR and Western blot in tissues. *Note* **a** Histogram of index mRNA expressions by RT-PCR. **b** Histogram of index protein expressions by Western blot. **c** Images of electro-

phoretic bands by Western blot. $n=12$, independent sample t test was used for statistical analysis. *Compared with the normal control group, $P<0.05$

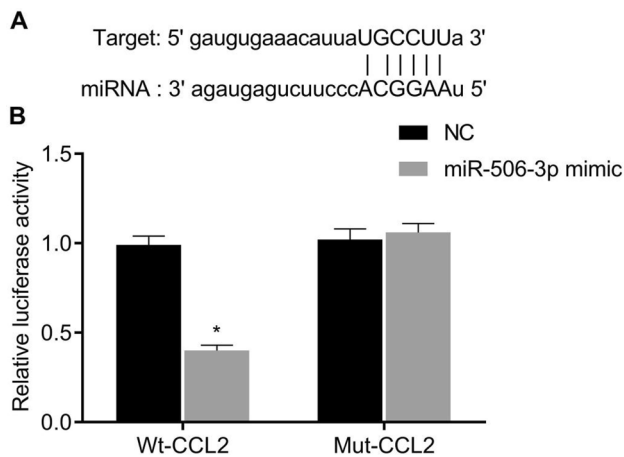


Fig. 4 Detection results of the target relationship between miR-506-3p and CCL2. *Note* **a** Target relationship diagram of CCL2 and miR-506-3p. **b** Results of dual luciferase reporter assay; $n=3$, independent sample t test was used for statistical analysis. *Compared with NC group, $P<0.05$

the mRNA and protein expression levels of CCL2, TLR4 and NF-κB (p65) in each group were significantly increased (all $P<0.05$). No obvious difference was found between Blank and NC groups (all $P>0.05$) (Fig. 6). Furthermore, compared with Blank and NC groups, miR-506-3p mimic group showed evidently increased expression of miR-506-3p ($P<0.05$), yet without significant difference in siRNA-CCL2 group ($P>0.05$), but the mRNA expression levels of CCL2, TLR4 and NF-κB (p65) were significantly decreased in miR-506-3p mimic and siRNA-CCL2 groups (all $P<0.05$). Furthermore, there was remarkable decrease in the expression of miR-506-3p in miR-506-3p inhibitor group, while the mRNA expression levels of CCL2, TLR4 and NF-κB (p65) were notably increased (all $P<0.05$). Meanwhile, there was

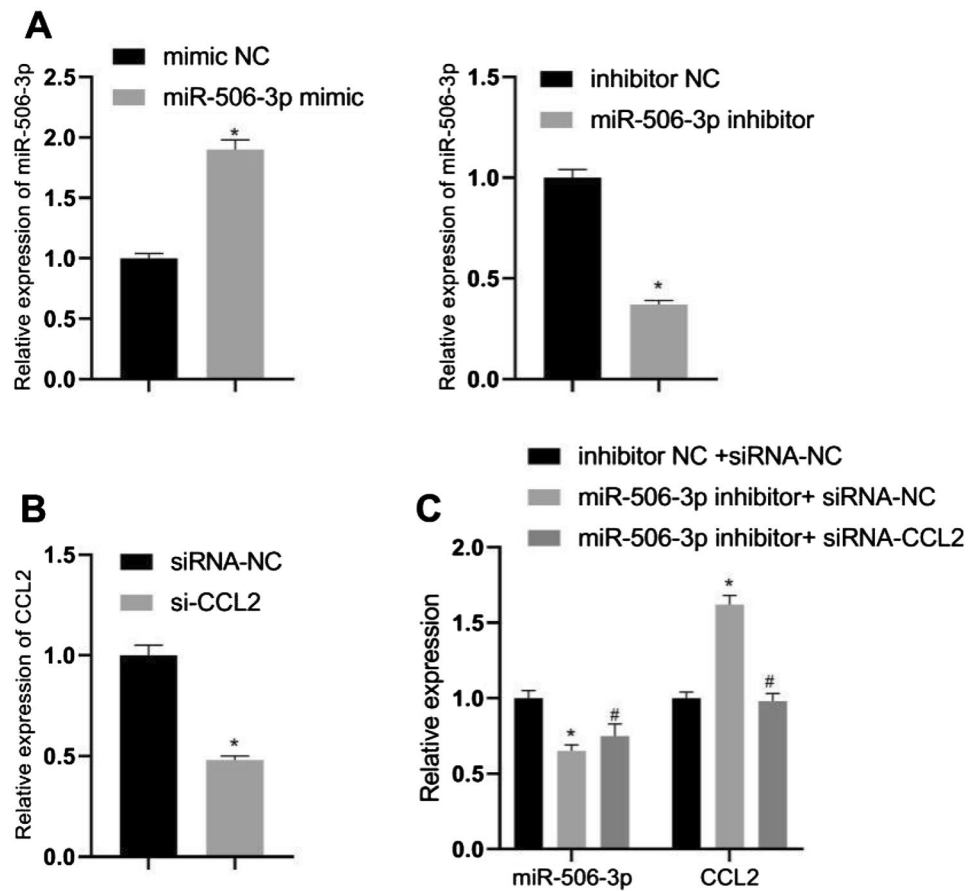
obvious decrease in the expression of miR-506-3p in miR-506-3p inhibitor+siRNA-CCL2 (all $P<0.05$). However, no significant difference was found in the mRNA expression of CCL2, TLR4 and NF-κB (p65) (all $P>0.05$) (Fig. 6).

Meanwhile, the results of Western blot showed that the protein expression of Bax was significantly lower than that of control group, and the protein expression levels of CCL2, TLR4, NF-κB (p65) and Bcl-2 in each group were significantly increased (all $P<0.05$). No obvious difference was found between Blank and NC groups (all $P>0.05$) (Fig. 6). Furthermore, compared with Blank and NC groups, miR-506-3p mimic group showed evidently increased protein expression of Bax ($P<0.05$), without difference in siRNA-CCL2 group ($P>0.05$), but the protein expression levels of CCL2, TLR4, NF-κB (p65) and Bcl-2 were significantly decreased in miR-506-3p mimic group and siRNA-CCL2 group (all $P<0.05$). Furthermore, there was remarkable decrease in the protein expression of Bax in miR-506-3p inhibitor group, while those of CCL2, TLR4, NF-κB (p65) and Bcl-2 were notably increased (all $P<0.05$). However, no significant difference was found in the protein expression levels of CCL2, TLR4, NF-κB (p65), Bcl-2 and Bax (all $P>0.05$) (Fig. 6).

Inhibitory Role of miR-506-3p Regulating CCL2 Gene Expression in Cell Proliferation of ASMCS

As shown in Fig. 7 in terms of the detection results of MTT assay, there was obvious increase in cell proliferation in other groups when compared with control group (all $P<0.05$). Furthermore, compared with Blank and NC groups, miR-506-3p mimic group and siRNA-CCL2 group revealed significantly decreased cell proliferation, while miR-506-3p inhibitor group had obviously increased cell proliferation (all $P<0.05$). However, no obvious change was found in miR-506-3p

Fig. 5 Cell plasmid transfection identification in each group with qRT-PCR. **Note** **a** Identification of successful transfection of miR-506-3p mimic and inhibitor, *compared with miR-506-3p mimic NC group and miR-506-3p inhibitor NC group, respectively, $P < 0.05$. **b** Identification of successful transfection of si-CCL2, *compared with siRNA-NC group, $P < 0.05$. **c** Identification of successful transfection of miR-506-3p inhibitor and siRNA-CCL2, *compared with inhibitor NC + siRNA-NC group, #compared with miR-506-3p inhibitor + siRNA-NC, $P < 0.05$



inhibitor + siRNA-CCL2 group when compared with Blank and NC groups (both $P > 0.05$).

Effect of miR-506-3p Regulating CCL2 Gene Expression on Cell Cycle Distribution of ASMCS

According to the detection results of flow cytometry for cell cycle distribution in Fig. 8, compared with Control group, G1 phase was shortened (cell proportion decreased) and G2 & S phases was prolonged (cell proportion increased) in other groups (all $P < 0.05$). Meanwhile, compared with Blank and NC groups, miR-506-3p mimic and siRNA-CCL2 groups showed prolonged G1 phase (cell proportion increased) and shortened G2 & S phases (cell proportion decreased); whereas miR-506-3p inhibitor group displayed shortened G1 phase (cell proportion decreased) and prolonged G2 & S phases (cell proportion increased) ($P < 0.05$). However, no obvious difference was found in miR-506-3p inhibitor + siRNA-CCL2 group ($P > 0.05$).

Promoting Role of miR-506-3p Regulating CCL2 Gene Expression in Cell Apoptosis of ASMCS

Based on the detection results of flow cytometry for cell apoptosis (Fig. 9), compared with Control group, there was significant decreased trend in the remaining groups (all $P < 0.05$). Meanwhile, compared with Blank and NC groups, miR-506-3p mimic and siRNA-CCL2 groups exhibited significantly increased cell apoptosis, while miR-506-3p inhibitor group had obviously decreased cell apoptosis (all $P < 0.05$). Nevertheless, there was no statistical significance in the cell apoptosis of miR-506-3p inhibitor + siRNA-CCL2 group when compared with Blank and NC groups ($P > 0.05$).

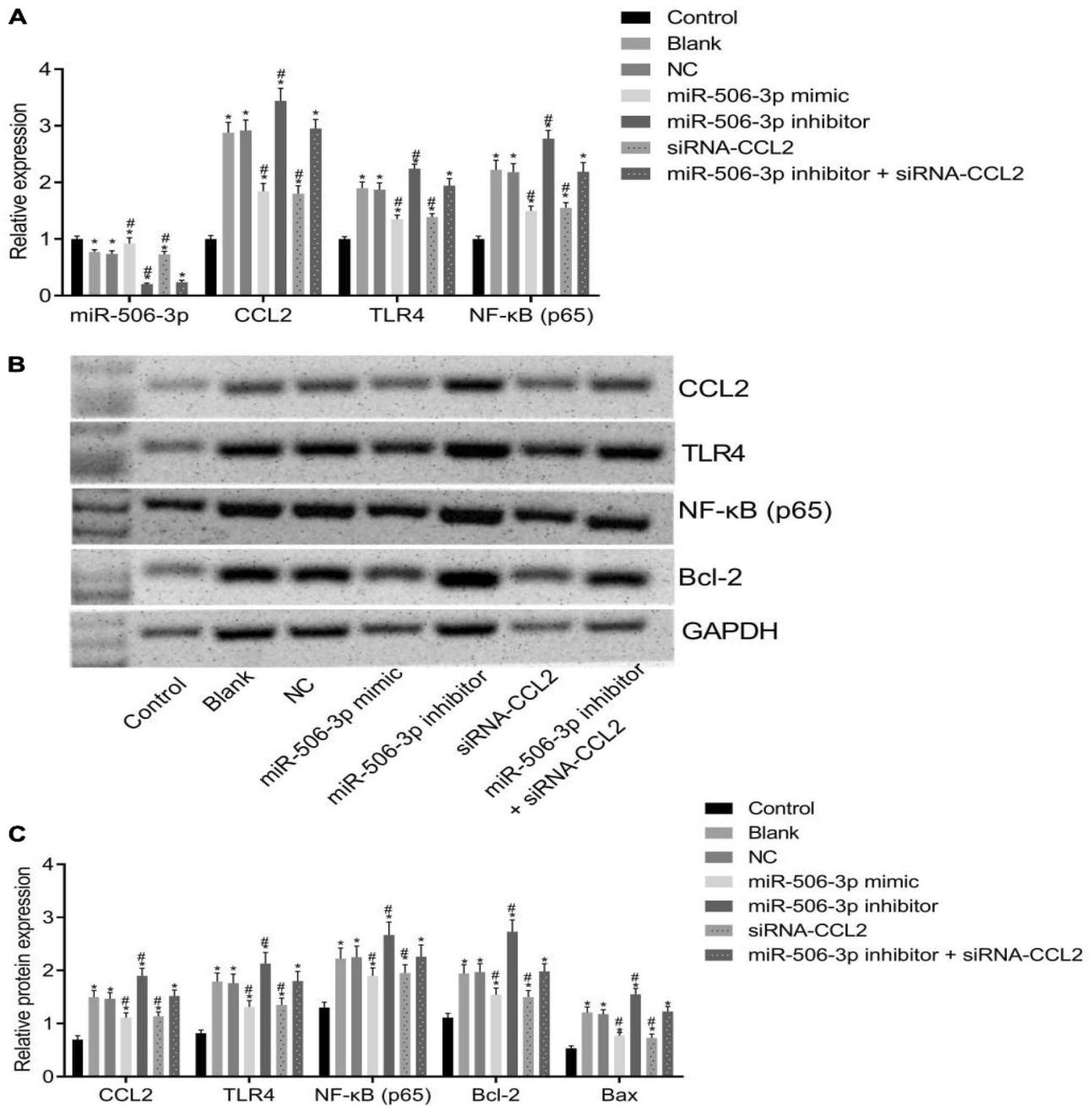


Fig. 6 Detection results using RT-PCR and Western blot in each group after cell transfection. *Note a* Detection results using RT-PCR in each group after cell transfection. *b* Electrophoretic bands detected using Western blot in each group after cell transfection. *c* Detection

results using Western blot in each group after cell transfection; $n=3$, independent sample t test was used for statistical analysis. *Compared with Control group, $P<0.05$. #Compared with Blank group and NC group, $P<0.05$

Discussion

Airway remodeling is a key link and independent factor in the pathogenesis of asthma [38]. Dysfunction of ASMCS is the core pathological basis of airway remodeling, which not only leads to irreversible changes of airway

wall thickening structure, but also is the main effector cell of airway hyperresponsiveness and chronic inflammation [17].

The imbalance of intracellular signal pathway regulatory network caused by various endogenous or exogenous stimuli is considered to be the key factor leading to structural and functional abnormalities of ASMCS [39]. NF-κB is a

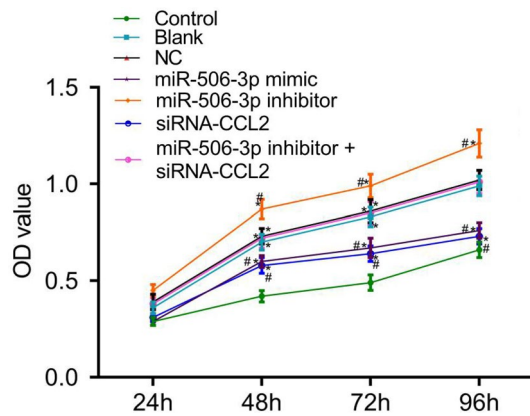


Fig. 7 The proliferation of ASMCS in each group after cell transfection by MTT assay. Note $n=3$, two-way ANOVA was used for comparison. *Compared with Control group, $P<0.05$. #Compared with Blank group and NC group, $P<0.05$

common pathway for many cytokines to regulate cell biological effects such as proliferation and secretion [40]. Besides, TLRs play a key role in immune regulation and inflammatory response, which is a bridge between innate immunity and adaptive immunity [41]; TLR4 can specifically recognize lipopolysaccharide, mediate inflammatory response and initiate immune response [42]. In view of the above interpretations, we speculate here that TLR4/NF- κ B signaling pathway may be the junction of cooperative molecular markers/cell structural changes and multiple cell dysfunction

in ASMCS. Previous studies have found the effect of TLR4/NF- κ B signaling pathway on the regulation of inflammatory response and immune response [43, 44]. In asthmatic mice, TLR4/NF- κ B signaling pathway may be continuously activated, which may lead to the persistent abnormal expression of inflammatory genes in the lung [45].

Increasingly more evidences have shown that miRNAs play an important role in the pathogenesis of human diseases [46, 47]. At the post-transcriptional level, miRNAs are modified by nuclease and inserted into RNA induced silencing complex to inhibit gene expression. The study of searching for related miRNAs and identifying their potential target genes have revealed the pathogenesis of many human diseases.

In our study, following pathological change observation of asthmatic mice after modeling, it confirmed a successful establishment of asthma model in mice. Furthermore, with a series exploration in tissues, there was increased positive expression of CCL2 in the modeling mice, down-regulation of miR-506-3p expression, and up-regulated expression of CCL2, TLR4 and NF- κ B (p65). The above-mentioned results may support that there exist high expression of CCL2 but low expression of miR-506-3p, as well as activated TLR4/NF- κ B signaling pathway in asthma, and also provide potential insights that overexpression of miR-506-3p may exert a beneficial role in preventing the development of asthma through inhibiting the activation of TLR4/NF- κ B signaling pathway by silencing the expression of CCL2. The hypothesis is thus explored in the subsequent study.

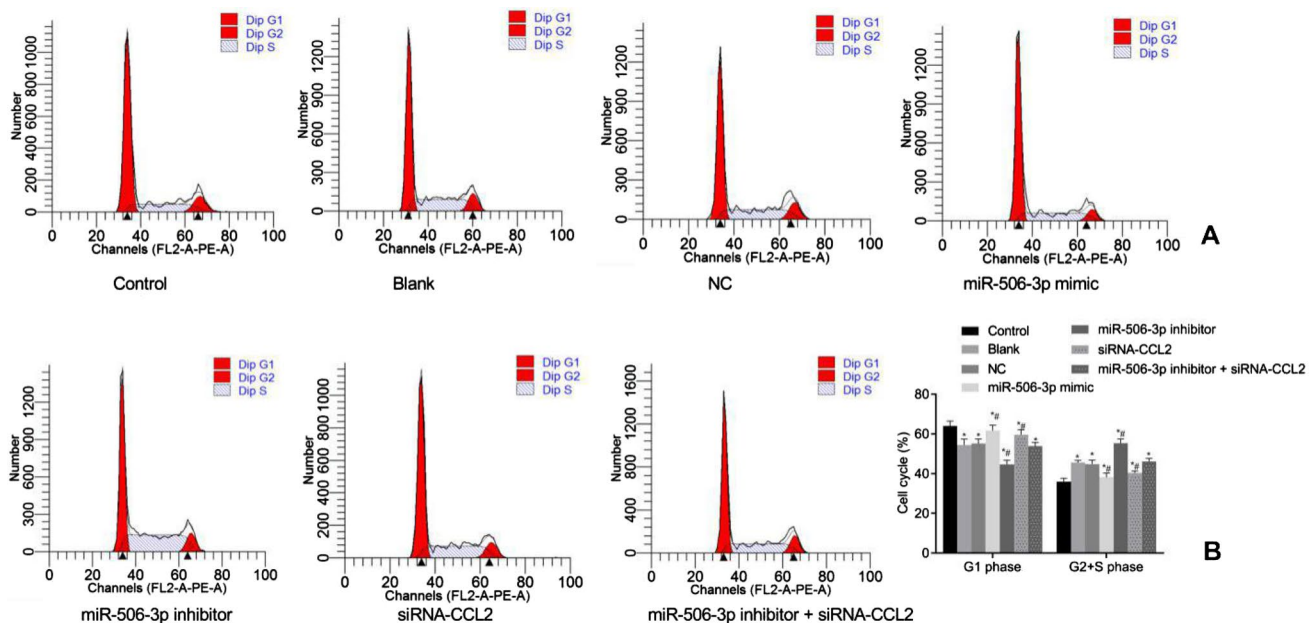


Fig. 8 The distribution of cell cycle in each group after cell transfection by flow cytometry. Note **a** Detection of cell cycle distribution by flow cytometry. **b** Histogram of each cell cycle in each group; $n=3$,

one-way ANOVA was used for comparison. *Compared with Control group, $P<0.05$. #Compared with Blank group and NC group, $P<0.05$

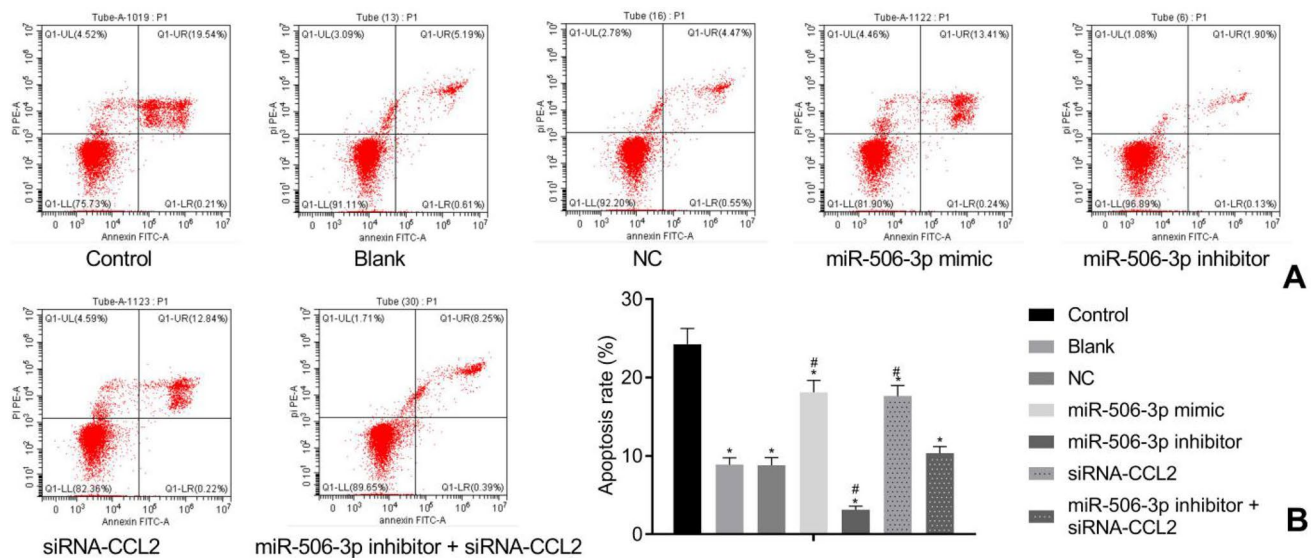


Fig. 9 The apoptosis of cells in each group after cell transfection by flow cytometry. *Note a* Detection of cell apoptosis by flow cytometry. *b* Histogram of each cell apoptosis in each group; $n = 3$, one-way

ANOVA was used for comparison. *Compared with Control group, $P < 0.05$. #Compared with Blank group and NC group, $P < 0.05$

Firstly, CCL2 was identified to be the target gene of miR-506-3p and corresponding plasmids were successfully transfected in each group after cell modeling according to different transfection protocols. Our study verified that miR-506-3p inhibited the activation of TLR4/NF- κ B pathway by inhibiting the expression of CCL2 gene using RT-PCR and Western blot. As evidenced by corresponding results in different transfection groups, overexpression of miR-506-3p could inhibit the expression of CCL2, TLR4 and NF- κ B (p65), similar results of decreased expression of TLR4 and NF- κ B (p65) was detected after silencing CCL2 expression. By contrast, inhibited expression of miR-506-3p resulted in increased expression of CCL2, TLR4 and NF- κ B (p65). More importantly, no significant difference was found in the mRNA and protein expression of CCL2, TLR4 and NF- κ B (p65) after combined treatment of inhibited expression of miR-506-3p and CCL2 when compared with the controls, which supported that down-regulated expression of miR-506-3p might reverse the positive role of CCL2 gene silencing in the inhibited activation of TLR4/NF- κ B pathway, providing potential basis for subsequent study.

For further verification of the aforementioned miR-506-3p-CCL2-TLR4/NF- κ B axis in the development of asthma, our study discovered that miR-506-3p inhibited cell proliferation, affected cell cycle distribution and promoted cell apoptosis of ASMCS through silencing the expression of CCL2 gene. To be specific, overexpression of miR-506-3p and silenced CCL2 gene expression could significantly decreased cell proliferation, prolonged G1 phase and shortened G2 & S phases, and promoted ASMCS apoptosis; whereas the opposite trends were found after inhibited

expression of miR-506-3p. Meanwhile, there was no statistical difference in cell proliferation, cell cycle distribution and cell apoptosis after combined treatment of inhibited expression of miR-506-3p and CCL2. It indicates that the combined treatment reversed the positive effect on CCL2 gene expression silencing, which also proves that miR-506-3p dose silences CCL2 gene expression to retard the progression of asthma. It may be related to the inhibited activation of TLR4/NF- κ B pathway, as described above. Meanwhile and significantly, the regulation mechanism of apoptosis is multifaceted, some genes and cytokines play important roles in the regulation of apoptosis, such as Bcl-2 family. The Bcl-2 gene family contains two types of genes with opposite functions, one is anti-apoptosis gene such as Bcl-2; the other is pro-apoptosis gene such as Bax. These two genes are considered to be the most important regulatory genes to inhibit and promote apoptosis. Specifically, Bcl-2 is a gene cloned from follicular lymphomas. It can inhibit the decrease of mitochondrial membrane potential, decrease the release of cytochrome and apoptosis-inducing factor and the activation of Caspase, thus inhibiting cell apoptosis. Besides, Bax has high homology with Bcl-2, which can activate Caspase and induce apoptosis. Bcl-2 can form a dimer with Bax, and the balance between Bax and Bcl-2 function significantly in regulating apoptosis. In our study, miR-506-3p can promote the apoptosis of ASMCS via targeted inhibition of CCL2 expression and suppressed activation of TLR4/NF- κ B signaling pathway, manifesting in decreased expression of Bcl-2 and increased expression of Bax.

As for the highlights of the present study, our study for the first time reveals the positive role of

miR-506-3p-CCL2-TLR4/NF- κ B regulatory axis in asthma in an established animal model of asthma and a cell model of isolated ASMCS, which is convincing to some extent. In addition, there is a rationality in our explored mechanism based on miR-506-3p-CCL2-TLR4/NF- κ B axis. For example, Jing et al. established a mouse model with chronic asthma and reported in their study that overexpression of miR-485 exerts an inhibitory effect on ASMCS proliferation and promotes apoptosis through the Smurf2-mediated TGF- β /Smads signaling pathway [48]. Zhang et al. also discovered a positive role of the lncRNA GAS5/miR-10a/BDNF regulatory axis in promoting ASMCS proliferation to aggravate asthma [49]. Corresponding reasoning mechanisms are similar to those explored in our study, which in turn identifies the reliable findings in our study. Meanwhile, there are few studies related to miR-506-3p, CCL2 or TLR4/NF- κ B signaling pathway in asthma, which may provide new thoughts for the molecular therapy of asthma.

To sum up, miR-506-3p can inhibit the proliferation and promote the apoptosis of ASMCS via inhibition of CCL2 gene expression and suppressed activation of TLR4/NF- κ B signaling pathway. Our study is critical to prove the beneficial role of miR-506-3p-CCL2-TLR4/NF- κ B regulatory axis in inhibiting the development of asthma. While asthma is a complicated process resulted from the regulation of various factors that interact with each other mutually. Although our study has confirmed the potential role of miR-506-3p-CCL2-TLR4/NF- κ B regulatory axis in asthma, we still wonders if there are additional signaling pathways involved in the process; how about the downstream targets of TLR4/NF- κ B; what is the upstream mechanism of miR-506-3p regulation; are there other target genes involved in the development of asthma besides TLR4 that are regulated by miR-506-3p; etc. These are the directions we will study continuously in the future. Simultaneously, our future study may continue to emphasize on the investigation of the intrinsic mechanism and application of the proposed regulatory axis in asthma and relevant autoimmune diseases.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary Figure 1: A schematic diagram summarizing the overall findings of the study with potential implications

