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microRNA-22-3p plays a protecting role in a murine asthma model through the inhibition of the NLRP3/Caspase-1/IL-1 β axis

Running title: miR-22-3p is protective in asthma

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New Findings

What is the central question of this study?

The relevance of miR-22-3p has been indicated in asthma, while how its protective role exerted in asthma has not been previously reported.

What is the main finding and its importance?

Upregulation miR-223 hampered the airway inflammation and release of inflammatory cytokines through blocking the activation of NLRP3/Caspase-1/IL-1 β signaling pathway in asthma.

Abstract

Asthma, a great public health burden, is triggered by inflammatory responses in the airways that are not addressed appropriately by current therapies. This study aims to investigate the regulatory mechanism of microRNA-22-3p (miR-22-3p) on the proliferation of bronchial epithelial cells exposed to lipopolysaccharide (LPS) and expressions of pro-inflammatory cytokines in a murine asthma model challenged by ovalbumin (OVA). We first confirmed the overexpression of miR-22-3p in the murine asthma model and bronchial epithelial cells.

miR-22-3p remarkably reversed the decline in bronchial epithelial cell viability, enhancement in apoptosis rate, and release of inflammatory factors induced by LPS. miR-22-3p targeted and conversely regulated NACHT, LRR and PYD domains-containing protein 3 (NLRP3). Overexpression of NLRP3 counteracted the inhibitory effect of miR-22-3p on inflammatory damage in bronchial epithelial cells through activation of caspase-1/IL-1 β . In an *in vivo* model, overexpression of miR-22-3p significantly attenuated airway obstruction and tissue damage in mice. In summary, our study underscores that miR-22-3p serves both as a negative regulator of the NLRP3/caspase-1/IL-1 β axis and a protective factor against the inflammatory response, thus standing out a future therapeutic modality for asthma.

Keywords: microRNA-22-3p; NLRP3; Caspase-1; IL-1 β ; Asthma

1 Introduction

MicroRNAs (miRNAs), short 18-22 nt single-stranded RNAs that play important parts in modulating protein expression and regulating cellular signaling, have been implicated in the development of chronic airway diseases, including asthma (Tan et al., 2020). These symptoms of asthma result from chronic airway inflammatory response to a trigger, most frequently inhaled allergen(s), that induces airway hyperresponsiveness (AHR), airway remodeling as well as mucus hypersecretion (Frey et al., 2020). Repeated injury, repair, and remodeling of the airway epithelium following stimuli exposure contributes to histological changes and functional aberrations in the airway mucosal epithelium, which were closely related to the pathophysiology of asthma (Gon & Hashimoto, 2018). Circulating miR-125b, miR-16, miR-299-5p, miR-206, along with miR-133b have been identified as possible predictive biomarkers of allergic and asthmatic status (Panganiban et al., 2016). In addition, miR-22-3p was found to be significantly downregulated in 62 patients with asthma compared with healthy controls, indicating it may have impact on the mediation of the immune response and inflammatory cytokine release by interacting with its putative targets (Dong et al., 2016). miR-22-3p was shown to act as an anti-inflammatory factor in spinal cord injury and retinal pigment epithelial cells (Hu et al., 2019; Li et al., 2020; Fang et al., 2021). As chronic inflammation is a major cause and feature of asthma (Xu et al., 2020a), it is of great importance to study the effect of miR-22-3p on the inflammation caused by asthma.

Inflammasomes, major molecular contributors to inflammation, are comprised of a central protein, an adaptor protein apoptosis speck-like protein and a caspase-1 protein (Yerramothu et al., 2018). NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome was established to be upregulated in neutrophilic asthma and might modulate the inflammatory responses observed in asthma by generating interleukin (IL)-1 β (Simpson et al., 2014). The NLRP3/Caspase-1/IL-1 β axis was also found to be activated in human lumbar cartilaginous endplate degeneration (Tang et al., 2016). More specifically, blocking the NLRP3/caspase-1 axis efficiently prevented the development of toluene diisocyanate-induced asthma, thus could be applied as a therapeutic target for asthmatics (Chen et al., 2019). Interestingly, the correlation between miR-22 and NLRP3 has been highlighted in high glucose-induced human endothelial cells (Song et al., 2019). In the present work, we induced a murine asthma model *in vivo* with ovalbumin (OVA) and an *in-vitro* model with lipopolysaccharide (LPS) in human bronchial epithelial cells BEAS-2B cells. Therefore, this

study intended to identify the influence of miR-22-3p on the cell viability and apoptosis of BEAS-2B cells and cytokines in asthma and shed light on asthma treatment.

2 Materials and Methods

2.1 Ethical approval

All in vivo manipulations were approved by the Animal Ethical Committee of Liaocheng People's Hospital (Approval number: NO. 2020013) and strictly performed in accordance with the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals (8th edition, 2011; (Council, 2011). Efforts were made to avoid all unnecessary distress to the animals.

2.2 Mice

Forty female BALB/c mice (6 weeks old, 18-22 g, Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were bred in an animal facility with free access to food and water under a 12-h light/dark cycle. The mice were allowed to acclimatize to laboratory conditions for 7 days prior to the experiments.

The mice were randomly divided into four groups (10 mice in each): the control group, the model group, the lentivirus (LV)-negative control (NC) group, and the LV-mimic group. Mice were sensitized with asthma through two intraperitoneal injections of 20 µg OVA (Grade V, Sigma-Aldrich Chemical Company, St Louis, MO, USA) absorbed onto 2 mg Inject Alum Adjuvant (Thermo Fisher Scientific Inc., Waltham, MA, USA) in 200 µL sterilized 0.9% saline at week 1 and 2 (once a week). Mice were then inhaled with 5% aerosolized OVA in saline for 30 min from week 3 to week 10 (three times a week). Control mice injected and inhaled the same volume of 0.9% saline with the same frequency as the murine asthma model. Mice in the LV-NC and LV-mimic groups were injected with the corresponding lentivirus (20 µL) via the tail vein from week 7 to week 10 three times a week after each OVA challenge. Lentiviral-coated miR-22-3p overexpression plasmid and empty lentiviral plasmid were from GenePharma (Shanghai, China) with a viral titer of 1×10^8 TU/mL.

2.3 Airway resistance test

At 8 a.m. the day after the last challenge or lentivirus injection, mice were subjected to an anesthetization with a mixture of ketamine (90 mg/kg) and xylazine (18 mg/kg) using intramuscular injection to insert a tracheotomy tube. A volume-cycled ventilator (Flexivent, SCIREQ Scientific, Montreal, QC, Canada) with a positive-end expiratory pressure of 2 cm H₂O was utilized for ventilation. Mice were then subjected to increasing doses of aerosolized methacholine (0-30 mg/mL) to measure AHR. The airway resistance was analyzed with the Flexivent software and Pulmodyn data-acquisition software (Hugo Sachs Electronic). The forced oscillation technique was used by measuring the piston pushing volume and the pressure in the cylinder, and the mechanical load provided by the whole lung and bronchi of the subject animal can be calculated (resistance of the whole respiratory system).

2.4 Broncho alveolar lavage fluid (BALF) and cell counts

Mice were euthanized by an intraperitoneal injection of sodium pentobarbital (150 mg/kg) after the airway resistance test. The trachea was exposed for an intravenous retention with a 1 mL sterile syringe. BAL was recuperated by instillation of 0.5 mL saline for three times. The lavage fractions were pooled (no less than 1.2 mL). The BALF samples were then centrifuged at 1,200 rpm at 4°C for 10 min. Cell precipitate was suspended in saline, placed on slides, and stained with Wright-Giemsa staining solution (Sigma-Aldrich). Total cell count and eosinophil count in BALF were counted by using an optical microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

2.5 Lung histopathology

The left lungs of mice were collected and fixed in 4% paraformaldehyde for 24 h before being dehydrated and paraffin-embedded. Then, 4-μm embedded lung tissue sections were mounted on slides and stained with hematoxylin and eosin (HE, Sigma-Aldrich) to identify tissue inflammation. Mucus production was identified by a periodic acid-Schiff (PAS) staining kit (Beijing Solarbio Life Sciences Co., Ltd., Beijing, China), and collagen deposition was assessed by a Masson staining kit (Solarbio). The tissue sections were viewed under a microscope (Olympus).

2.6 Quantitative RT-PCR

Total RNA was extracted from mouse right lung tissue homogenates and cells using Trizol (Invitrogen Inc., Carlsbad, CA, USA). RNA was reversely transcribed to complementary DNA (cDNA) using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Reverse transcription of miRNAs was performed using the All-in-One™ miRNA RT-qPCR assay kit (Genecopoeia, Rockville, MD, USA). RT-qPCR was performed using a 7300 real-time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). U6 served as an endogenous control for miRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acted as an endogenous control for mRNA. The $2^{-\Delta\Delta CT}$ method was used to analyze expression. The mRNA and miRNA primers are listed in Table 1.

2.7 Cell culture and treatment

BEAS-2B cells (CRL-9609, American Type Culture Collection, Manassas, VA, USA) were grown in Roswell Park Memorial Institute-1640 medium (Gibco, Carlsbad, CA, USA) plus 10% FBS (Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C with 5% CO₂. Bronchial epithelial cells in a pro-inflammatory immune microenvironment were simulated by treating BEAS-2B cells with LPS (10 µg/mL, 25 µg/mL, 50 µg/mL, Sigma Aldrich, St. Louis, MO, USA) for 24 h, using phosphate-buffered saline as a control. To study the relationship between miR-22-3p expression and LPS stimulation time, we treated BEAS-2B cells with 25 ng/mL LPS for 0, 6, 12, and 24 h, respectively. The miR-22-3p mimic and NC mimic used for cell transfection were purchased from GenePharma Co., Ltd. (Shanghai, China). GenePharma was also commissioned to clone the NLRP3 sequence and insert it into the pcDNA 3.1 vector to construct the NLRP3 overexpression plasmids (pcDNA-NLRP3), with the empty vector serving as a control (pcDNA). All transfections were performed with Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

2.8 Methyl thiazolyl tetrazolium (MTT) assay

MTT cell proliferation kit (Beyotime Biotechnology Co., Ltd., Shanghai, China) was used to detect the viability of cells. Briefly, 10 µL MTT solution was added to 3×10^3 3000 cells in each well of the 96-well plates for another 4-h incubation. Each well was then incubated with 100 µL Formazan dissolving solution to dissolve the Formazan precipitate. After 3 to 4 h, the purple Formazan crystals were dissolved, and the optical density (OD)

value at 570 nm was measured using a spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China) to calculate the cell viability.

2.9 5-Ethynyl-2'-deoxyuridine (EdU) staining

BEAS-2B cells (1×10^5) were stained with 50 μ M EdU solution (Beyotime) at 37°C for 2 h in a 5% CO₂ incubator, fixed with 4% formaldehyde and treated with 1× click reaction buffer (Beyotime) for 30 min in darkness. Afterwards, the nuclear was stained with 4',6-diamidino-2-phenylindole (DAPI). Images were observed using an EVOS M5000 microscope (Thermo Fisher Scientific). The EdU positive ratio was determined as a ratio of the number of EdU-positive cells (in red) to the total number of DAPI-positive cells (in blue).

2.10 Flow cytometry

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kits (BioVision, Inc., Exton, PA, USA) were applied for cell apoptosis determination. In short, the transfected cells (3×10^4) were detached with trypsin (Gibco) and resuspended in 1× binding buffer. The cells were double stained with Annexin V-FITC and PI for 20 min. A FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (Tree Star Inc., Ashland, OR, USA) were used to quantify the apoptosis rate.

2.11 Enzyme-linked immunosorbent assay (ELISA)

The concentration of inflammatory factors in cell culture supernatants was determined using the human IL-6 ELISA kit (ab178013, Abcam Inc., Cambridge, UK) and the human IL-8 ELISA kit (ab46032, Abcam) according to the manufacturer's protocols.

2.12 Western blot

Cells were lysed in ice-cold radio immunoprecipitation assay lysis buffer (Beyotime Biotechnology Co., Ltd., Shanghai, China) to collect total protein. The proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After sealing with 5% bovine serum albumin, the membranes were subsequently incubated with primary antibodies against NLRP3 (1:1000, ab263899, Abcam), Caspase-1 (1:1000, #3866, Cell Signaling Technologies, Beverly, MA, USA), IL-1 β (1:5000, ab33774, Abcam) overnight at 4°C, and GAPDH (1:2000, ab128915, Abcam) was used as an internal reference gene. The membranes

were then incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody to IgG (1:10,000, ab205718, Abcam) for 2 h at 37°C. The target protein signal was detected using Pierce enhanced chemiluminescence Western Blotting substrate (Thermo Fisher Scientific), and the protein levels were standardized relative to GAPDH using Image Pro Plus 6.0 software.

2.13 Dual-luciferase reporter assay

The potential binding sites between miR-22-3p and NLRP3 were obtained from miRDB (<http://mirdb.org/>), and the 3'-untranslated region (3'UTR) fragment of NLRP3 containing the putative binding sites or mutant sequence of miR-22-3p was cloned into the pmirGlo vector (GenePharma) to construct the NLRP3-wild type (WT)/NLRP3-mutant (MT) luciferase reporter vectors. BEAS-2B cells were co-transfected with the above vectors and miR-22-3p mimic for 48 h, and luciferase activity was measured with a dual-luciferase assay kit (Promega).

2.14 RNA immunoprecipitation (RIP)

RIP analysis was performed in BEAS-2B cells using the Imprint[®] RNA Immunoprecipitation Kit (Sigma-Aldrich). Briefly, cells were collected, lysed in RIP lysis buffer, and incubated with 40 µL of Protein A magnetic beads coupled to anti-IgG or anti-Ago2 at 4°C overnight. Total RNA was isolated using the GenElute[™] Total RNA Purification Kit (Sigma-Aldrich). Enrichment of miR-22-3p and NLRP3 was quantified using RT-qPCR.

2.15 Statistical analysis

All statistical analyses were performed using Prism version 8.0 (GraphPad software, La Jolla, CA, USA). Data analyses were implemented with unpaired *t*-test or one-way or two-way analysis of variance (ANOVA), followed by the Tukey's method for pairwise comparison between two items. Data were presented as means ± standard deviation (SD). Two-tailed $p < 0.05$ was regarded as statistical significance. Each *in vitro* reaction was run in triplicate.

3 Results

3.1 miR-22-3p expression was significantly reduced in asthma

We constructed the murine asthma model by sensitization to OVA, and the control mice were treated with saline (Figure 1A). We found that OVA induced a significant enhancement in airway resistance to elevated concentrations of Methacholine in mice (Figure 1B). The cells in BALF were counted, and it was found that sensitization to OVA resulted in an increase in the number of total cells and eosinophils (Figure 1C). In addition, we also performed histological examination of airway tissues from mice (Figure 1D). Using HE staining, we observed more inflammatory infiltrates in the airway tissues of the murine asthma model, while PAS staining suggested that sensitization to OVA resulted in mucus secretion. Masson's staining implied that sensitization to OVA contributed to thicker airway collagen deposition. We measured the expression of miR-22-3p in mouse lung tissue homogenates by RT-qPCR and observed that its expression was much lower (Figure 1E).

In addition, we simulated the in a pro-inflammatory immune microenvironment *in vitro* by treating human bronchial epithelial cells BEAS-2B with different concentrations of LPS for 24 h. By MTT experiments, we found that the viability of BEAS-2B cells gradually decreased with increasing LPS concentrations (Figure 1F). At an LPS concentration of 25 ng/mL, the viability of the cells had been reduced by more than half, so an LPS concentration of 25 ng/mL was chosen for subsequent experiments. The levels of inflammatory factors in the supernatant of BEAS-2B cells after 25 ng/mL LPS treatment for 24 h were examined using ELISA. LPS treatment significantly promoted the release of pro-inflammatory factors IL-6 and IL-8 (Figure 1G) from the cells. EdU experiments were then performed to examine the DNA synthesis activity of the cells, which demonstrated that LPS significantly repressed the synthesis of cellular DNA (Figure 1H). Whereas flow cytometry analysis revealed that LPS significantly induced apoptosis (Figure 1I). We then treated BEAS-2B cells with 25 ng/mL LPS and detected miR-22-3p expression at different time points by RT-qPCR. LPS treatment inhibited miR-22-3p expression in a time-dependent manner (Figure 1J).

3.2 Overexpression of miR-22-3p inhibits LPS-induced bronchial epithelial cell injury

To investigate the effects of miR-22-3p on BEAS-2B cells, we transfected NC mimic or miR-22-3p mimic into BEAS-2B cells. We determined the effective transfection using RT-qPCR (Figure 2A). Cells were then treated with 25 ng/mL LPS for 24 h, and cell viability

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was examined by MTT experiments, where we observed that overexpression of miR-22-3p reversed the repressive effect of LPS on cell viability (Figure 2B). EdU experiments on cellular DNA synthesis activity displayed that miR-22-3p mimic significantly restored the cellular DNA synthesis impaired by LPS (Figure 2C). Flow cytometric analysis showed that miR-22-3p appreciably attenuated LPS-induced apoptosis (Figure 2D). Finally, the levels of pro-inflammatory factors in cell supernatants were examined by ELISA. Overexpression of miR-22-3p was found to significantly inhibit the release of IL-6 and IL-8 from BEAS-2B cells and to reduce the inflammatory damage of the cells (Figure 2E).

3.3 miR-22-3p targets NLRP3

To find downstream targets by which miR-22-3p exerts a protective role against inflammation in BEAS-2B cells, prediction websites, including miRDB, miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>), mirDIP (<http://ophid.utoronto.ca/mirDIP/>), TargetScan (<http://www.targetscan.org/>), StarBase (<http://starbase.sysu.edu.cn/>), and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>) were used (Figure 3A). There were seven common intersections, ESR1, RCOR1, NLRP3, RAB5B, ARPC5, CCNT2, and SP1.

We examined the expression of seven potential targets in LPS-treated BEAS-2B cells transfected with NC mimic or miR-22-3p mimic. RT-qPCR revealed that only NLRP3 expression was differentially expressed before and after transfection (Figure 3B). The potential binding sites of miR-22-3p to NLRP3 (Figure 3C) were downloaded from miRDB. Luciferase reporter plasmids of NLRP3-WT with a potential binding site with miR-22-3p and NLRP3-MT with the mutated binding site were constructed, and co-transfected with NC mimic or miR-22-3p mimic, respectively, into BEAS-2B cells and assayed for luciferase activity after 48 h (Figure 3D). miR-22-3p considerably inhibited the luciferase activity of NLRP3-WT, while had no significant effect on the luciferase activity of NLRP3-MT. RIP experiments were then performed to test the binding ability of miR-22-3p to NLRP3 via Ago2 (Figure 3E). We noted that anti-Ago2 was significantly enriched for miR-22-3p and NLRP3 compared to anti-IgG, while miR-22-3p mimic promoted the enrichment levels of miR-22-3p and NLRP3 by anti-Ago2. Finally, we performed western blot to detect NLRP3 expression in LPS-treated BEAS-2B cells transfected with NC mimic and miR-22-3p mimic. As expected, miR-22-3p mimic also significantly inhibited NLRP3 protein expression (Figure 3F).

3.4 NLRP3 attenuates the protective effects of miR-22-3p on bronchial epithelial cells by inducing the caspase-1/IL-1 β axis

NLRP3 mediates inflammation through activation of the caspase-1/IL-1 β axis in liver diseases (Szabo & Petrasek, 2015). We first detected NLRP3 expression by RT-qPCR in BEAS-2B cells treated with LPS (25 ng/mL) at different time points and found that LPS promoted NLRP3 expression in a time-dependent manner (Figure 4A). We then transfected the NLRP3 overexpression plasmid pcDNA-NLRP3 into BEAS-2B cells overexpressing miR-22-3p and measured effective transfection by RT-qPCR (Figure 4B). Cells were treated with LPS (25 ng/mL) for 24 h, and protein expression of NLRP3, Caspase-1 and IL-1 β was detected by western blot (Figure 4C). We found that the activation of the caspase-1/IL-1 β pathway was significantly promoted by overexpression of NLRP3.

Moreover, we noted through MTT assay and EdU staining that the promoting effects of miR-22-3p mimic on viability and DNA synthesis were significantly reversed by overexpression of NLRP3 (Figure 4D, E). Flow cytometry results also exhibited that overexpression of NLRP3 facilitated apoptosis (Figure 4F). Finally, it was showed that NLRP3 upregulation significantly promoted the release of pro-inflammatory factors via ELISA (Figure 4G).

3.5 Overexpression of miR-22-3p ameliorates lung injury in OVA-induced murine asthma model

We investigated the effects of miR-22-3p on asthma in *in vivo* experiments. We injected lentiviral vector containing miR-22-3p mimic, termed as LV-mimic, after OVA challenge at weeks 7-10, with the empty lentiviral vector LV-NC as a control. In the airway resistance test, LV-mimic significantly reduced airway resistance to elevate concentrations of Methacholine in mice (Figure 5A). The cells in BALF were counted, and LV-mimic was found to significantly reduce the number of total cells and eosinophils (Figure 5B). In the histological examination, we observed that although there was still some damage in the lungs, the inflammatory infiltration in the airway tissues of mice in the LV-mimic group was significantly reduced, the secretion of airway mucus was significantly alleviated, and airway collagen deposition was also improved compared with mice in the LV-NC group (Figure 5C). We performed RT-qPCR in mouse right lung tissue homogenates and found that LV-mimic significantly promoted miR-22-3p expression and decreased NLRP3 expression (Figure 5D).

4 Discussion

The airway epithelium is believed to be crucial to both the initiation, progression as well as exacerbations of asthma (Holgate et al., 2009). Therefore, an improved understanding in the molecular mechanisms behind inflammation of airway epithelium is of great importance to provide novel insights for the development of therapeutic interventions for asthma. In the present investigation, we found that miR-22-3p was significantly downregulated lung tissues of the murine asthma model and LPS-induced human bronchial epithelial cells BEAS-2B. miR-22-3p drastically hampered the inflammation and cell apoptosis both *in vivo* and *in vitro*, via the NLRP3/caspase-1/IL-1 β pathway. Our findings illuminated that miR-22-3p exerted a protective role against asthma.

Eosinophils are main effector cells against parasites, bacteria, and viruses, and involved in local and global inflammation, which are essential to asthma (Ferrari et al., 2020). Therefore, our induced murine asthma model had elevated eosinophil and total cell counts along with excess mucus secretion and thickening of airway collagen deposition, indicating the success of animal modeling. In addition, LPS was also found to promote the levels of IL-6 and IL-8, suggesting the success of cell modeling. miR-22 lies in chromosome 17p13.3 between markers D17S1866 and D17S1574, and resides in the exon 2 of the non-coding C17orf91 gene (Fan et al., 2017). miR-22-3p has been shown to be downregulated in synovial tissues of patients with rheumatoid arthritis (Lin et al., 2014). Also, its downregulation was found in rat spinal cord tissues after ischemia/reperfusion injury (Fang *et al.*, 2021). Consistently, we identified that miR-22-3p was depleted in both cell and animal models of asthma. Later, we performed gain-of-function assays on BEAS-2B cells to examine the effects of miR-22-3p mimic *in vitro*, which revealed that miR-22-3p accelerated BEAS-2B cell viability, while prevented apoptosis and repressed inflammation under the condition of 25 ng/mL LPS. Moreover, we found the upregulation of miR-22-3p alleviated airway inflammation in the OVA-induced murine asthma model. In line with our observation, miR-22-3p notably curbed the inflammation and apoptosis by lowering the levels of IL-1 β and IL-6 in rats with sepsis-induced acute kidney injury and in HK-2 cells exposed to LPS (Wang et al., 2020b). Therefore, these findings showed that miR-22-3p may be participated in the pathogenesis of asthma.

In this study, we observed that NLRP3 was one of the seven targets of miR-22-3p filtered by six bioinformatic tools. Subsequent dual-luciferase and RIP assays substantiated their targeting relationship. Afterwards, western blot results corroborated that NLRP3 activated the downstream Caspase-1/IL-1 β axis. Previously, overexpression of miR-22-3p significantly reduced, whereas inhibition of miR-22-3p increased the expression patterns of NLRP3, Caspase-1, and mature IL-1 β in retinal pigment epithelial cells (Hu *et al.*, 2019). Our rescue experiments showed that NLRP3 upregulation abrogated the protective role of miR-22-3p in BEAS-2B cells against LPS-induced injuries by repressing cell viability and amplifying inflammation and apoptosis. Likewise, upregulation of NLRP3 reversed the protecting effects of miR-223 on oxidized low-density lipoprotein-treated human vascular endothelial cells (Wang *et al.*, 2020a). More specifically, miR-223 played protective roles in mice with neutrophilic asthma through the suppression of NLRP3 inflammasome (Xu *et al.*, 2020b). Furthermore, a NLRP3 selective inhibitor MCC950 has been reported to inhibit LPS-induced inflammation and pyroptosis of H9C2 cells (Li *et al.*, 2021). IL-1 β , generated by activated Caspase-1, stimulates positive regulation for pro-inflammatory cytokines, thereby increasing inflammation (Provoost *et al.*, 2011; Szabo & Petrasek, 2015). Moreover, blockade of IL-1 β activity with an IL-1 receptor antagonist eradicated obesity-induced AHR (Kim *et al.*, 2014). In addition, Caspase-1 are necessary for the Th17 response in nitrogen dioxide-induced AHR (Martin *et al.*, 2013). Overall, Kim *et al.* raised the probability of targeting the NLRP3 inflammasome and/or IL-1 β as a therapeutic option in severe, steroid-insensitive asthma (Kim *et al.*, 2015). Whether inhibition of L-1 β and Caspase-1 regulated the viability and inflammatory status in BEAS-2B cells is not present in this study, and it will require further experiments.

5 Conclusion

In summary, the present work has exhibited the interaction between miR-22-3p and NLRP3 in airway inflammation. Upregulation miR-223 has the potency to hamper the airway inflammation and release of inflammatory cytokines through blocking the activation of NLRP3/Caspase-1/IL-1 β signaling pathway in mice treated with OVA and human bronchial epithelial cells BEAS-2B treated with LPS, which is involved in the pathogenesis of asthma. Collectively, this study might provide a prospective target for the treatment regimens of asthma in the near future.

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Authors' contributions

SFG and RC is the guarantor of integrity of the entire study and contributed to the concepts; LNZ and MQW contributed to the design and definition of intellectual content of this study; YYW, WJD and YJ contributed to the experimental studies, data acquisition and statistical analysis; XMK contributed to the source, validation and methodology. All authors contributed to the manuscript preparation, read and approved the final manuscript. XMK is the person responsible for “research governance”, that is regulations, principles and standards of good practice in research carried out at the institution.

Conflicts of interest

The authors declare no potential conflicts of interest.

Availability of data and materials

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

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Figure legend

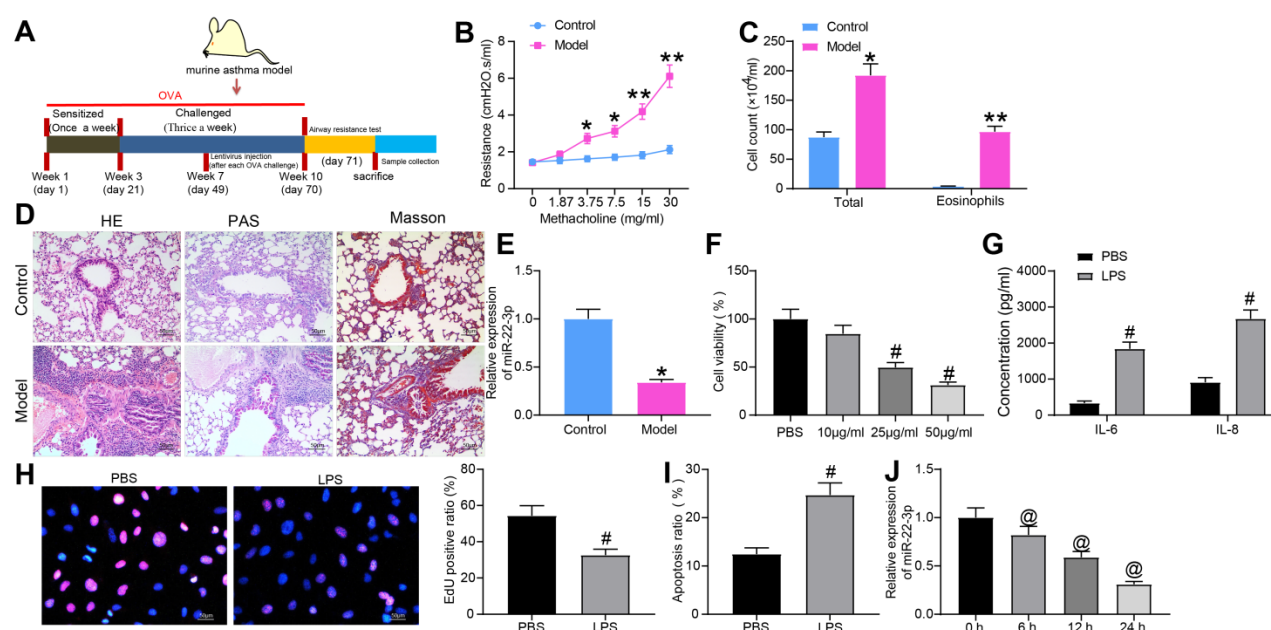


Figure 1 miR-22-3p expresses poorly in lung tissues of the murine asthma model and LPS-induced cells. A, the schematic timeline showed that BALB/c mice were sensitized with OVA; B, airway resistance in mice assessed using airway resistance test; C, number of total cells and eosinophils in BALF; D, histologic changes of OVA-induced damage to mouse airway tissues; E, miR-22-3p expression in OVA-induced mouse lung tissue examined by RT-qPCR; F, the viability of BEAS-2B cells measured using MTT assay; G, the release of pro-inflammatory factors in BEAS-2B cells evaluated using ELISA assay; H, the DNA synthesis activity of BEAS-2B cells tested using EdU staining; I, the apoptosis rate of BEAS-2B cells tested by flow cytometry analysis; J, the expression of miR-22-3p in response to different LPS treatment time examined using RT-qPCR. Results are shown as mean $\pm$ SD ($n = 10$). *p* values were obtained using an unpaired *t* test (panels E, H and I), one-way (panels F and J) or two-way ANOVA (row factor, panels B, C and G). Each in vitro reaction was run in triplicate. **p* < 0.05, ***p* < 0.01 vs. control mice; #*p* < 0.05 vs. BEAS-2B cells treated with PBS; @*p* < 0.05 vs. BEAS-2B cells treated 25 ng/mL LPS at the baseline.

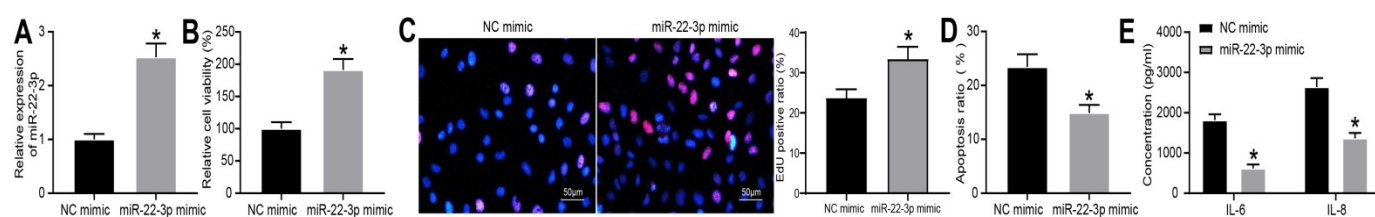


Figure 2 Inflammation is ameliorated by miR-22-3p overexpression in BEAS-2B cells.

BEAS-2B cells were treated with miR-22-3p mimic or NC mimic. A, transfection efficiency of miR-22-3p mimic in BEAS-2B detected by RT-qPCR; B, the viability of BEAS-2B cells measured using MTT assay; C, the DNA synthesis activity of BEAS-2B cells tested using EdU staining; D, the apoptosis rate of BEAS-2B cells tested by flow cytometry analysis; E, release of pro-inflammatory factors in BEAS-2B cells evaluated using ELISA assay. Results are shown as mean $\pm$ SD. *p* values were obtained using an unpaired *t* test (panels A, B, C and D) or two-way ANOVA (row factor, panel E). Each in vitro reaction was run in triplicate. **p* < 0.05 vs. BEAS-2B cells treated with NC mimic.

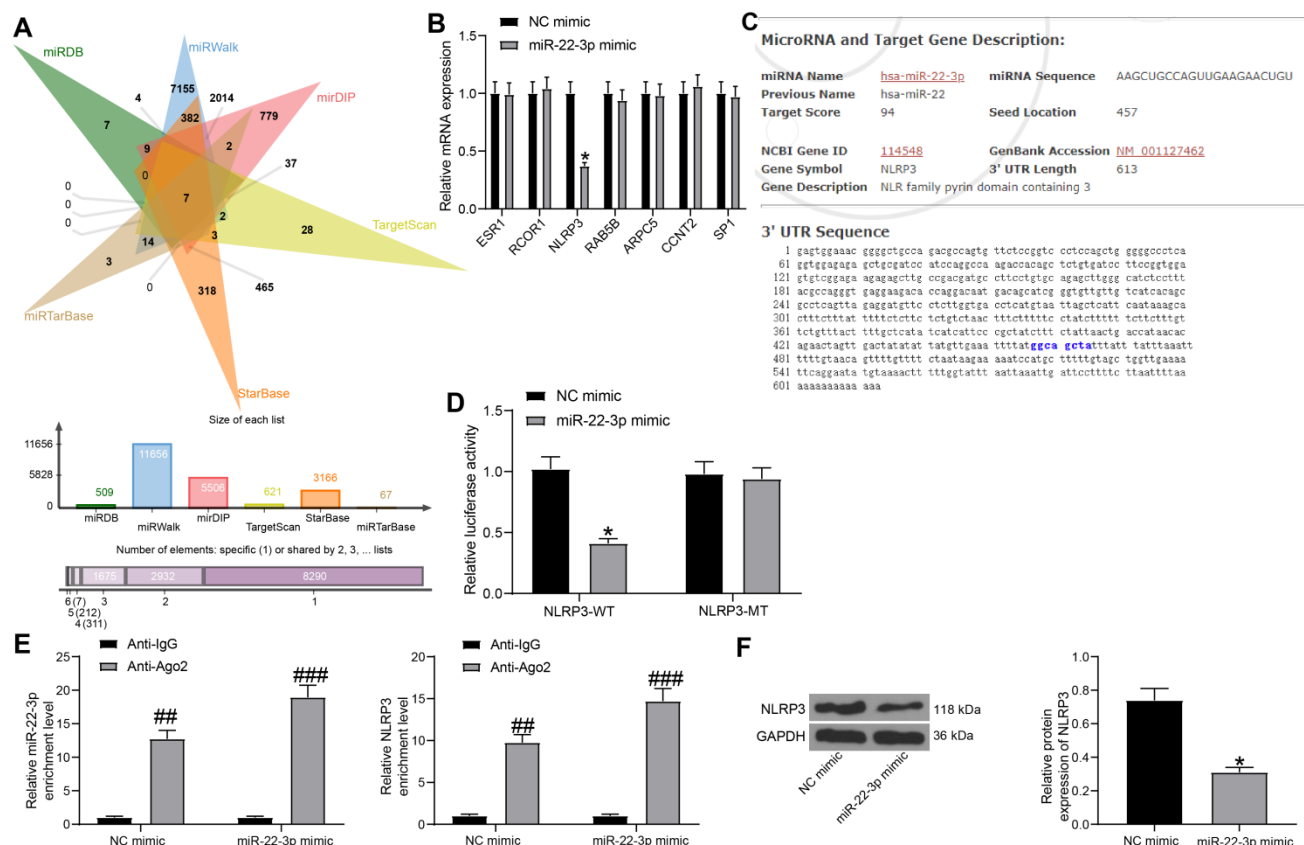


Figure 3 miR-22-3p targets NLRP3 mRNA in BEAS-2B cells. A, potential target genes of miR-22-3p predicted by bioinformatic websites; B, the mRNA expression of potential target genes in response to miR-22-3p mimic detected by RT-qPCR; C, potential binding sites of miR-22-3p to NLRP3; D, NLRP3-WT/NLRP3-MT luciferase activity examined using dual-luciferase assay; E, the binding of miR-22-3p to NLRP3 measured using RIP experiments; F, the NLRP3 protein expression in response to miR-22-3p mimic detected by western blot. Results are shown as mean $\pm$ SD. p values were obtained using an unpaired t test (panel F), one-way (panel B) or two-way ANOVA (row factor, panels D and E). Each in vitro reaction was run in triplicate. * $p < 0.05$ vs. BEAS-2B cells transfected with NC mimic; ## $p < 0.01$, ### $p < 0.001$ vs. Anti-IgG transfection.

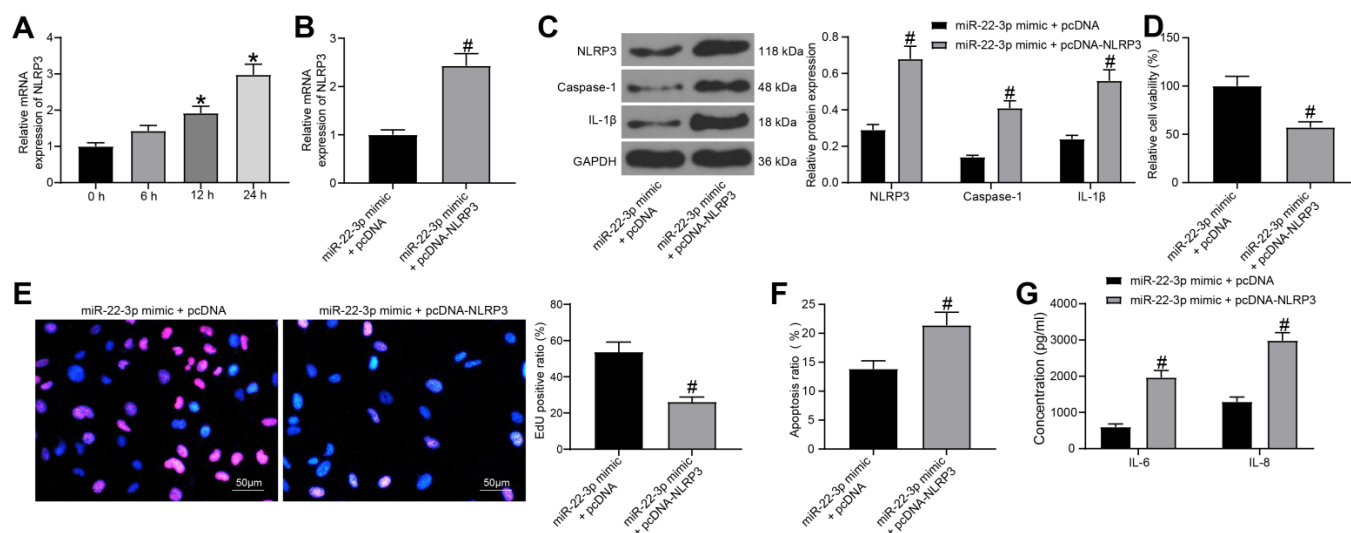


Figure 4 miR-22-3p modulates inflammation by altering NLRP3 expression in BEAS-2B cells. BEAS-2B cells were co-transfected with miR-22-3p mimic and pcDNA-NLRP3 or pcDNA. A, NLRP3 mRNA expression at different time points of LPS treatment measured using RT-qPCR; B, the transfection efficiency of pcDNA-NLRP3 in BEAS-2B transfected with miR-22-3p mimic measured using RT-qPCR; C, the protein expression of caspase-1 and IL-1 β in response to pcDNA-NLRP3 measured using western blot; D, the viability of BEAS-2B cells measured using MTT assay; E, the DNA synthesis activity of BEAS-2B cells tested using tested using EdU staining; F, the apoptosis rate of BEAS-2B cells measured using flow cytometry analysis; G, the release of pro-inflammatory factors in BEAS-2B cells measured using ELISA assay. Results are shown as mean $\pm$ SD. *p* values were obtained using an unpaired *t* test (panels B, D, E and F), one-way (panel A) or two-way ANOVA (row factor, panel C and G). Each in vitro reaction was run in triplicate. **p* < 0.05 vs. BEAS-2B cells treated 25 ng/mL LPS at the baseline; #*p* < 0.05 vs. BEAS-2B cells transfected with miR-22-3p mimic + pcDNA.

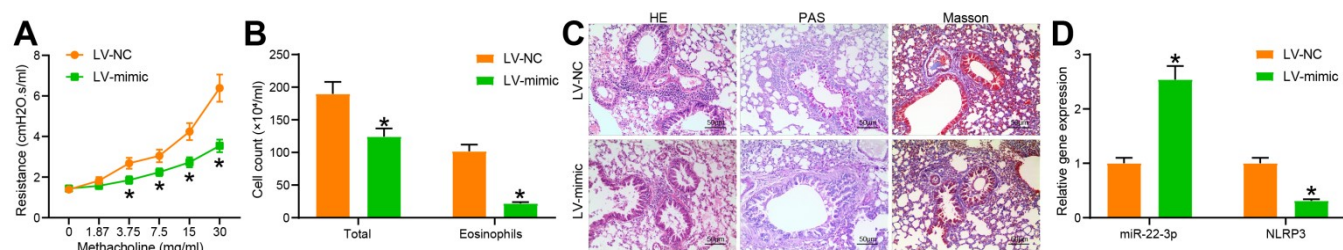


Figure 5 miR-22-3p exerts therapeutic effects on OVA-induced mice. OVA-induced mice were injected with LV-mimic or LV-NC. A, airway resistance in mice assessed using airway resistance test; B, total cell and eosinophil counts in BALF; C, histologic changes of OVA-induced lung airway injury in mice; D, expression of miR-22-3p and NLRP3 in mouse lung tissues by RT-qPCR. Results are shown as mean $\pm$ SD ($n = 10$). p values were obtained using a two-way ANOVA (row factor, panels A, B and D). $*p < 0.05$ vs. mice injected with LV-NC.

Table 1 Primer sequences

Targets	Forward primer (5'-3')	Reverse primer (5'-3')
hsa-miR-22-3p	GTTCTTCAGTGGCAAGC	GAACATGTCTGCGTATCTC
mmu-miR-22-3p	GTTCTTCAGTGGCAAGCTT	GAACATGTCTGCGTATCTC
hsa-NLRP3	GGACTGAAGCACCTGTTGTGCA	TCCTGAGTCTCCCAAGGCATTC
mmu-NLRP3	TCACAACTCGCCCAAGGAGGAA	AAGAGACCACGGCAGAAGCTAG
hsa-ESR1	GCTTACTGACCAACCTGGCAGA	GGATCTCTAGCCAGGCACATTC
hsa-RCOR1	CCAGTAACCAGAAGCCTGTGAAG	AAGCCACCAGTTTCTCAGGAGG
hsa-RAB5B	GGAGACTTCAGCCAAGACAGCT	ACACTGGCTCTTGTTCTGCTGG
hsa-ARPC5	GAGAACAAGTTCGTGGACGAAGA	CTTCACTGCCTGACTCTTGGTG
hsa-CCNT2	GGCTGCAAAAGTGGAAGAACAGG	CCAGTTCTTGAGTCTGTTGAAGG
hsa-SP1	ACGCTTCACACGTTTCGGATGAG	TGACAGGTGGTCACTCCTCATG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
U6	CTCGCTTCGGCAGCACAT	TTTGCGTGTGCATCCTTGCG

Note: hsa, homo sapiens; mmu, mus musculus; miR-22-3p, microRNA-22-3p; NLRP3, NACHT, LRR and PYD domains-containing protein 3; ESR1, estrogen receptor alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.