

Original Article

Mechanisms of the anti-asthma effect of nebulized anti-NGF sustained-release microsphere inhalation

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Abstract: Nebulized anti-nerve growth factor microspheres (referred to as NANMs) may exert an anti-asthma effect that can modulate immunological-pathological-related changes in asthmatic rats. However, research into the evidence and mechanism of the inhibition of asthma exacerbation by NANMs is lacking. The mechanism of action and the therapeutic effects of inhibition of NANMs were analysed in ovalbumin (OVA)-induced asthma rats in this study. Rats were divided into control, asthma (OVA-induced group), anti-NGF, and NANM groups according to a random number method. We evaluated the reticular basement membrane thickness, airway wall thickness, and collagen thickness. We investigated the pulmonary pathological changes and used immunohistochemical methods to detect and compare the expression of NGF, TGF- β 1, and Smad-3. Our results revealed that NANMs reduced the symptoms and the average pulmonary resistance values of lung function, decreased the thickness of the reticular basement membrane and collagen deposition, and attenuated the expression of NGF protein and mRNA levels. Moreover, TGF- β 1 and Smad-3 were significantly reduced by NANMs in lung tissues. NANMs ameliorated OVA-induced murine asthma by regulating airway remodelling in asthmatic conditions and improving lung function and pathology. TGF- β 1, Smad-3 and NGF were inhibited in the lung tissues, and thus, NANMs have anti-asthmatic effects.

Keywords: Anti-NGF, mechanisms, anti-asthmatic, nerve growth factor, asthma

Introduction

NGF is related to allergic and inflammatory diseases and plays an important role in the process of asthma [1]. Few previous reports have examined the relationship between anti-NGF and pulmonary pathological changes in asthma, such as changes in collagen thickness and NGF mRNA expression [2, 3]. However, there are still some difficulties in applying anti-NGF in patients with asthma. For example, the specific mechanism of the clinical application of anti-NGF and its pharmacological action are not clear. Additionally, the short intravenous half-life of anti-NGF, its quick dilution and metabolism after administration, and its neutralization by NGF affect its efficacy. Microsphere formulations feature long-term drug release, an alternative route of administration, diversification, long-lasting efficacy, and safety [4]. Therefore,

we speculated that the joint advantages of both anti-NGF and microspheres may be useful in treating asthma exacerbation. There is existing evidence of the usefulness of anti-NGF for treating asthma [5]. However, nebulized anti-nerve growth factor microsphere (NANM) inhalation its mechanism and therapeutic effects are not yet clear. Whether animal behaviour and pulmonary pathology, pulmonary function, and total TGF- β 1 in bronchoalveolar lavage fluid (BALF) can be affected by NANMs remains unclear. TGF- β 1 is the main regulator of the immune response leading to airway remodelling by collagen deposition [6]. It initiates pathways that exert a variety of biological effects [7]. Smads are also important proteins, and previous studies have shown that TGF- β 1 and Smad-3 are both related to airway remodelling [8]. In the present study, which includes the most recent data, to explore the mechanisms

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of how NANMs might suppress airway remodeling in an asthma model, we used a well-established and well-characterized model of chronic allergic asthma characterized by pathological airway remodelling.

Materials and methods

Preparation of NANMs

Anti-NGF microspheres were produced via the polymer alloy method [9] with some modifications. Briefly, 300 µg of anti-rat NGF antibody solution (Sinobioway Bio-Medicine Co., Ltd.; Xiamen, China) was mixed with 0.5 ml of bovine serum albumin (BSA) solution containing 10 mg of BSA (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China). A freeze-drying treatment was performed with the following conditions: a pre-freezing temperature of -50°C with a 20°C/min rate of temperature reduction, followed by incubation at -20°C for 3 h and at 20°C for 12 h. After the freeze-drying treatment, the freeze-dried anti-NGF powder was evenly dispersed in a BSA matrix. Approximately 300 mg of polylactide (PLA)/polylactic coglycolic acid (PLGA) (W/W = 1:3) was added to a tube, and the prepared freeze-dried anti-NGF powder was transferred. The polymer was then dissolved in 2 ml of dichloromethane to form the oil phase, and 10 ml of 2% polyvinyl alcohol served as the water phase. The water phase was added to the oil phase and homogenated at 1700 r/min for 30 s to yield a solid-in-oil-in-water (S/O/W) emulsion. The emulsion was then transferred to 400 ml of 10% deionized water sodium chloride solution. Then, magnetic stirring was performed at room temperature at 1700 r/min for 30 s. A total of 256.7 mg of anti-NGF microsphere was produced and stored at 4°C for the subsequent experiments.

Animal modelling and grouping

Forty female Sprague-Dawley rats (Laboratory Animal Center of Guilin Medical University, Guilin, China), weighting 125 ± 5 g and aged 7 to 8 weeks, were divided into control, asthma, anti-NGF, and NANM groups according to a random number method with ten animals in each group. All animals were fed an ovalbumin (OVA)-free diet, given free access to water and maintained in a clean, quiet and photophygous environment. On days 0 and 7, all rats, including the

control group rats, were sensitized with an intraperitoneal injection of 1 mg OVA (Sigma-Aldrich, St. Louis, MO, USA) and 200 µg of aluminium hydroxide (Aldrich, Milwaukee, WI, USA) in 0.5 mL of phosphate-buffered saline (PBS). The asthma group rats were exposed to 1% aerosolized OVA (1 g OVA in 100 ml of sterile PBS in a nebulizer) for 30 min every two days from days 14 to 72. The anti-NGF group rats were challenged with an aerosolized OVA solution followed by 2 ml of intravenously injected anti-NGF antibody of (Wuhan Boster Biological Technology, Ltd.; Wuhan, China) every two days from days 14 to 72. The NANM group rats were challenged with an aerosolized OVA solution followed by NANM atomization inhalation therapy every two days from days 14 to 72, and the control group underwent the same treatment except with sterile PBS. To confirm the establishment of the model, spirometry and haematoxylin/eosin (HE) staining of the lung tissues and lung function tests were performed. BALF and inflammatory cell counts were also measured.

Pulmonary function testing

Transpulmonary pressure, flow rate, and tidal volume were recorded at baseline using a whole-body plethysmograph system (Buxco Electronics Inc.; Troy, NY, USA) at an inhaled histamine concentration of 0, and alterations in the transpulmonary pressure, flow rate, and tidal volume were recorded before and after the atomization of 0.64 mg/mL of histamine (Sinopharm Chemical Reagent Beijing Co., Ltd.; Beijing, China) for 20 s. Airway responsiveness was presented as pulmonary airway resistance.

Measurement of total TGF-β1

The concentration of TGF-β1 was measured by ELISA kits in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). After incubating the wells with the receptor and washing thoroughly, the specific antibody for each measurement was added to each test well. TGF-β1 was detected by a horseradish peroxidase-based colorimetric assay.

Histological examination

For the histological examination of the bronchial mucosa reticular basement membrane thick-

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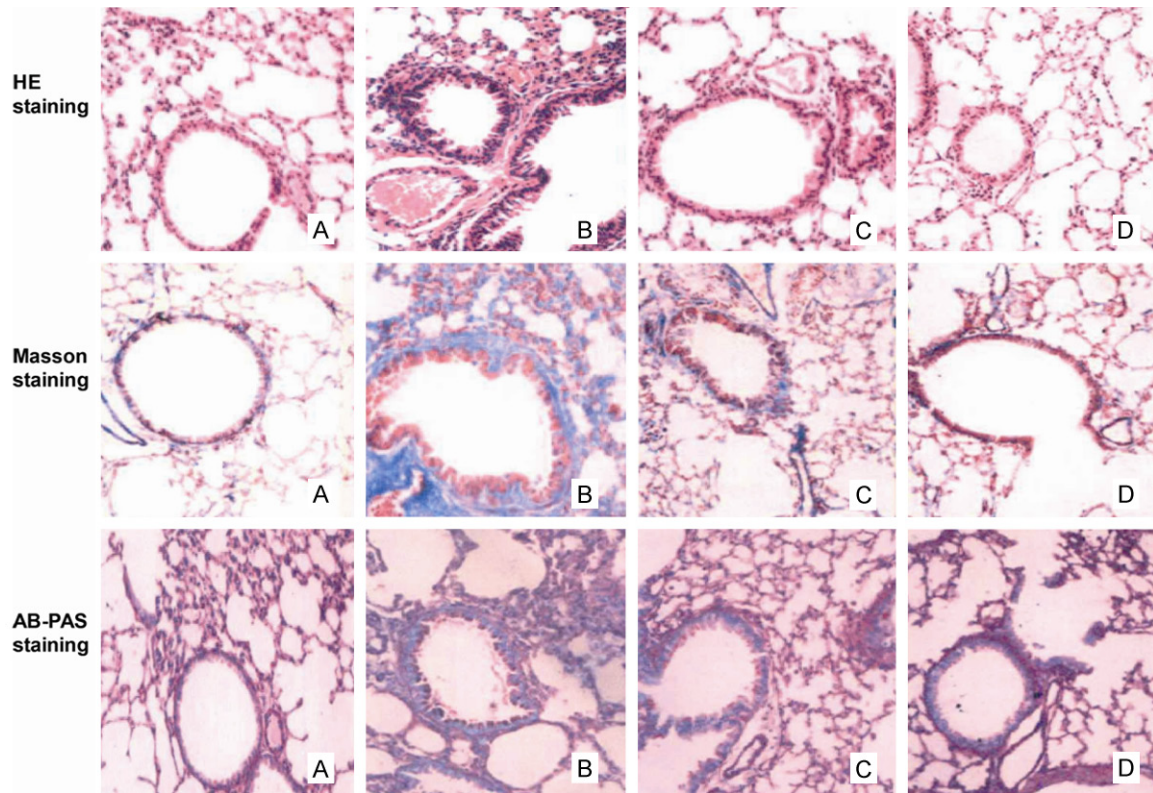


Figure 1. The pathology of lung tissues in each rat group (HE $\times 200$) (Masson $\times 200$) (AB-PAS $\times 200$). Groups: A: Control; B: Asthma; C: Anti-NGF; D: NANM.

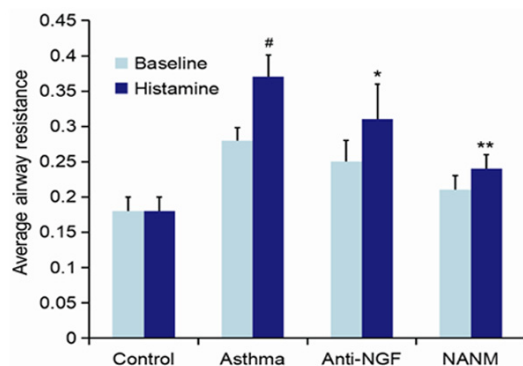


Figure 2. The average airway resistance of the rats before and after histamine excitation ($\bar{x} \pm S$) (cm-H₂O). [#] $P < 0.05$ compared with the control group, ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with the asthma group.

ness, samples were acquired from two locations separated by a distance of 200 μm . On the sampled slices, three sites at intervals of 100 μm were used to record the bronchial mucosa reticular basement membrane thickness. The three values were averaged to obtain the reticular basement membrane thickness.

The thickness was measured with a micrometre at a magnification of $\times 1000$ from the base under the bronchial subepithelium to the outer edge of the reticular layer. Collagen was measured according to hydroxyproline content.

Immunohistochemical staining

Three specimens were sampled from the opening of the right main bronchus and each bronchus at the middle and lower lobes for biopsy. Biopsy tissues were excised according to a standardized protocol and evaluated by immunohistochemical staining, including HE staining, Alcian blue-periodic acid Schiff (AB-PAS) staining and Masson staining. For HE staining, each slice was assessed for the degree of inflammatory cell infiltration of the left main bronchus and the surrounding three large vessels, and the mean values were obtained. The basement membrane perimeter (BM) and smooth muscle area (AM) were measured, and muscle thickness was calculated as AM/BM (μm). Five fields from each immunohistochemically stained section were evaluated by a

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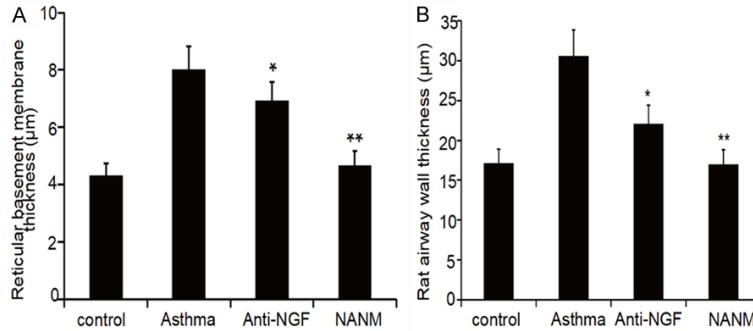


Figure 3. Comparison of reticular basement membrane thickness and airway wall thickness in rats ($\bar{x} \pm S$). * $P < 0.05$ compared with the asthma group, ** $P < 0.01$ compared with the asthma group. The following figures show the same comparisons.

single observer to determine the grey value of the NGF-positive area at a higher magnification. The mean grey value (A) was calculated. The antibody was diluted 1:150, and PBS was used as a negative control.

Real-time PCR (RT-PCR) assay

Total RNA was isolated from the biopsy tissues and reverse transcribed into cDNA. NGF mRNA expression was quantified using the RT-PCR assay, and GAPDH served as an internal control.

Western blot analysis

After electrophoresis and transfer, the membranes were incubated with the monoclonal mouse anti-TGF- β 1 (1:1000, dilution, Santa Cruz, CA, USA), anti-P-Smad3 (1:1000, dilution; Cell Signalling Technology, Danvers, MA, USA), and anti- β -actin (1:1000, dilution) antibodies overnight at 4°C. A goat anti-rat antibody (1:1000, dilution, Santa Cruz, CA, USA) was used as the secondary antibody. The membranes were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody. Membranes were photographed, and for quantitative analyses, densitometric band values were detected by using the western blotting luminol reagent (ELIPIS Biotech., Inc, Daejeon, Korea).

Statistical analysis

The statistical analysis was performed using SPSS 21.0 software (SPSS, Inc.; Chicago, IL, USA). All data are shown as the mean \pm stan-

dard error (SEM). Differences in the means across the groups were tested for statistical significance using one-way analysis of variance (ANOVA), followed by a multiple comparison tests with the Bonferroni adjustment. A P value of < 0.05 was considered to indicate statistical significance.

Results

Effect of NANMs on animal behaviour and pulmonary pathology

To investigate the development of airway inflammation in the lung tissue, we evaluated the pathology of the lesions. Lung lesions were evaluated with HE staining, Masson staining and AB-PAS staining. Mild symptoms appeared in the NANM group when the animals were in their home environment. We observed significant infiltration of inflammatory cells into the lung tissues in the asthma group compared with the NANM group. The symptoms were reduced to some degree in the NANM group relative to the other groups, and the tracheal epithelium was nearly complete, with no significant inflammatory exudate cavity phenomenon. AB-PAS staining and Masson staining revealed similar findings. The severity of pathology decreased in the order of asthma group $>$ anti-NGF group $>$ NANM group. The NANM group exhibited markedly attenuated infiltration of inflammatory cells compared with the asthma group (**Figure 1**).

Effect of NANMs on pulmonary function

The average pulmonary resistance value after excitation followed the order of asthma group $>$ anti-NGF group $>$ NANM group. The average pulmonary resistance value difference between the asthma group and NANM group was statistically significant ($P < 0.05$) (**Figure 2**).

Effect of NANMs on the thickness of the airway wall and the reticular basement membrane

To investigate the change in pathology, we examined the thickness of the airway wall and the reticular basement membrane in the rats of each group (**Figure 3**). The thickness param-

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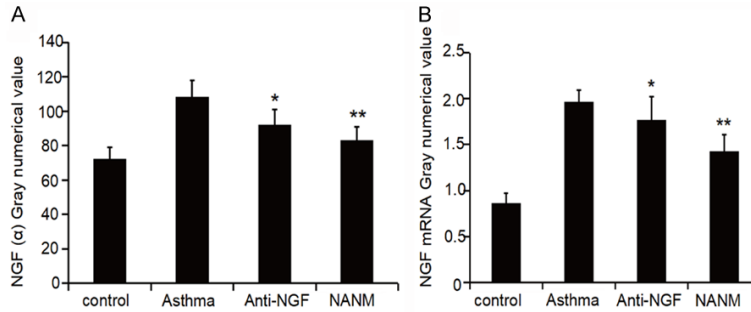


Figure 4. Comparison of NGF protein expression and NGF mRNA in the lungs of rats in the asthma, control, anti-NGF and NANM groups ($\bar{x} \pm s$).

Effect of NANMs on the expression of the NGF in lung tissue

To determine whether NANMs influenced the expression of NGF protein and mRNA in the lung tissue, we detected the expression levels of NGF protein and mRNA by their grey numerical values. NGF protein and mRNA production were obviously higher in the asthma group than in the control group. However, the NANM group had an obviously lower production of NGF protein and lower mRNA grey numerical values than both the asthma group and the anti-NGF group (**Figure 4**).

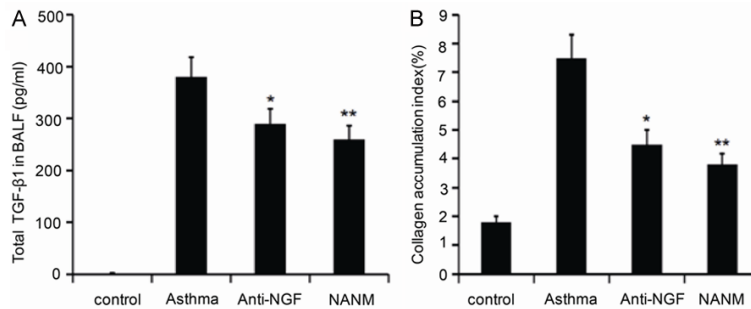


Figure 5. Effects of NANMs on collagen deposition in BALF and total TGF-β1 production in lung tissues.

Effect of NANMs on the expression of collagen deposition and total TGF-β1 in BALF

To further clarify the relationship between collagen synthesis and total TGF-β1 activation, we detected the expression of collagen deposition and the total TGF-β1. As shown in **Figure 5A**, **5B**, the expression of collagen deposition and total TGF-β1 were obviously higher in the asthma group rats than in the control group rats, which was similar to what was detected in the anti-NGF group. However, the NANM group rats had an obviously lower expression levels of collagen and total TGF-β1 than the asthma group rats.

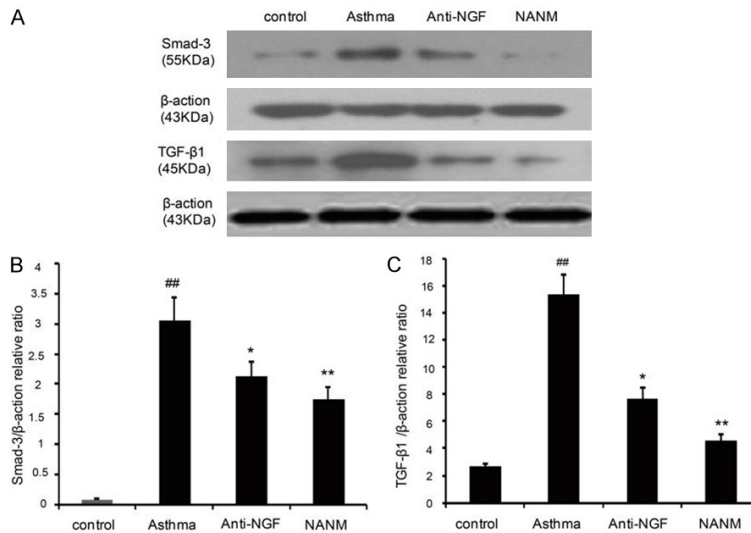


Figure 6. Effects of NANMs on the expression of Smad-3 and TGF-β1.

Effect of NANMs on TGF-β1 and Smad-3 expression in lung tissue

ters of the NANM group were statistically different than those of the asthma group ($P < 0.05$), suggesting that NANMs can improve the thicknesses of the reticular basement membrane and the airway wall in asthmatic airway inflammation.

To analyse whether NANMs affected the expression of the active factors of the TGF-β1 signaling pathway on asthma, TGF-β1 expression and the intracellular effector of Smad-3 were evaluated. As shown in **Figure 6**, the expression levels of Smad-3 and TGF-β1 were increased in

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the asthma group compared with the control group. The expression levels of Smad-3 and TGF- β 1 were obviously reduced in the NANM group. Compared with the asthma group, the NANM group showed an obviously decreased in the relative ratio of Smad-3/ and TGF- β 1/ β -actin ($P < 0.01$, **Figure 6B, 6C**).

Discussion

The purpose of this study was to investigate the effects of NANM treatment for improving airway remodelling and the immunological-pathological-related changes associated with asthma in an asthma model. The results of our study indicated that NANMs could improve lung function and pathology, inhibit NGF expression, and suppress airway remodelling. Moreover, NANM treatment decreased collagen deposition and the expression of Smad-3 and TGF- β 1. As far as we know, this is the first study to report accurate data that NANMs have obvious anti-asthma effects. Asthma can induce NGF overexpression in the lungs [10], and the excessive expression of NGF components can synergistically cooperate with other pathogenic factors that participate in asthma pathogenesis to induce critical damage [2]. NGF plays a “bonding” role in asthma [11], and it was therefore hypothesized that anti-NGF regulation may effectively reduce lung inflammation and airway remodelling [2], thereby delaying or even reversing the process of asthma. Some recent experimental studies have shown that anti-NGF can antagonize airway inflammation induced by asthma [3, 5, 11]. Therefore, the key to effective intervention strategies for asthma is to rationally identify anti-NGF regulation strategies with high value for clinical applications. NANM treatment via sustained-release microspheres as a carrier can improve drug release and drug bioavailability, increase stability, and reduce or even circumvent adverse reactions. However, the mechanism of the effect of inhaled NANMs in asthma remain unclear, and there are no currently available reports on this topic. In this study, we propose that inhaled NANMs adhere to the endothelial-exposed nerve endings in the damaged airway, causing a long-lasting inhibition of the proliferation of nerve endings and neurotransmitter release to control airway inflammation in asthma.

This study concludes that treatment with NANMs improved behavioural and pathological changes associated with asthmatic lungs in the

NANM group, suggesting that this administration route may be superior to intravenous injection. Therefore, inhalation may increase the local blood concentration of the drug above the threshold concentration. Additionally, microspheres are effective controlled-release formulations with improved stability and good biological tolerance that can reduce the extent of damage in the body and concentrate the drug in the target area. These features may improve the delivery of drugs with good therapeutic indices and increase their efficacy.

The average pulmonary resistance value indicated that NANM treatment could more obviously reduce the average airway resistance. Additionally, the differences between the NANM and anti-NGF groups were statistically significant, indicating that local inhalation of anti-NGF microspheres is an optimal route of administration.

These parameters were obviously different between the NANM and asthma groups, suggesting that NANMs can decrease the reticular basement membrane thickness, collagen deposition, airway wall thickness, and NGF expression. In addition, treatment with NANMs more effectively reduced NGF expression compared with intravenous anti-NGF therapy. Due to its accurate targeting, the drug dosage can be reduced, and the drug is predominantly concentrated in the target tissue for slow release, resulting in an effective treatment option for patients with asthma. Consistent with a previous study [12], our data indicate that neurogenic-mediated immunoinflammatory mechanisms play key roles in animal behaviour and pulmonary pathology. The results of our study suggest that treatment with NANMs significantly attenuates infiltration of inflammatory cells in pulmonary pathology. Therefore, the results of our study confirm that NANMs may possibly act as an effective anti-asthma treatment drug to treat asthma.

Furthermore, with NANM treatment, regardless of the decrease in the average pulmonary resistance, the NANM treatment group exhibited simultaneous decreases in the deposition of collagen and basement membrane thickness in the airway wall of asthmatic rats. In the lung tissues, we speculate that these changes after NANM treatment may have been due to an increase in TGF- β 1 activity, as the expression of total TGF- β 1 in BALF was significantly increased.

TGF- β 1 regulates collagen deposition through the Smad pathway via binding and activation of its special type I serine/threonine kinase receptors and type II serine/threonine kinase receptors [13]. Recent scientific studies have indicated the Smad-3 signalling pathway as a master pathway of airway remodelling in order to address in a wide range of inflammatory mediators [14]. Moreover, it is crucial for synthesis of most of the extracellular matrix (ECM) components, including TGF- β 1 and collagen deposition [15]. Thus, the TGF- β /Smad signalling pathway plays an irreplaceable and important role in the pathogenesis of asthma [16]. Previous research proved that the effect of inhibition of airway remodelling is influenced by regulation of the TGF- β 1/Smad-3 signalling pathway in asthma [17, 18]. In this study, the asthma group rats showed more lung inflammation and airway remodelling, which led to excessive collagen deposition and expression of Smad-3 and TGF- β 1. Regarding the potential effects of NANM treatment, the NANMs obviously blocked lung inflammation and airway remodelling through inhibiting the release of inflammatory mediators, such as TGF- β 1.

In conclusion, our results revealed that NANMs obviously improved lung function and pathology, inhibited NGF expression, suppressed airway remodelling, and modulated the TGF- β /Smad-3 signalling pathway in asthmatic conditions. Those biological effects were associated with the inhibition TGF- β /Smad-3 signalling (Figure 6). The results of our study collectively showed that NANMs improve immunological and pathological-related changes in asthma and may potentially be used as an effective therapeutic agent for asthma.

Acknowledgements

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Disclosure of conflict of interest

None.

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