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Mycobacterium vaccae nebulization protects Balb/c mice against bronchial asthma through neural mechanisms

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ABSTRACT

Objectives: Bronchial asthma can be effectively controlled but not be cured, its etiology and pathogenesis are still unclear, and there are no effective preventive measures. The key characteristic of asthma is chronic airway inflammation, and recent research has found that airway neurogenic inflammation plays an important role in asthma. We previously found that *Mycobacterium vaccae* nebulization protects against asthma. Therefore, this objective of this study is to explore the effect of *M. vaccae* nebulization on asthmatic neural mechanisms.

Methods: A total 18 of female Balb/c mice were randomized into normal, asthma control, and *M. vaccae* nebulization (Neb.group) groups, and mice in the Neb.group were nebulized with *M. vaccae* one month before the asthmatic model was established. Then, 1 month later, the mice were sensitized and challenged with ovalbumin. Twenty-four hours after the last challenge, mouse airway responsiveness; pulmonary brain-derived neurotrophic factor (BDNF), neurofilament-medium length (NF-M, using NF09 antibody), and acetylcholine expression; and nerve growth factor (NGF) mRNA level were determined.

Results: We found that the BDNF, NF09, acetylcholine expression, and NGF mRNA level were decreased in the Neb.group compared with levels in the asthma control group.

Conclusion: *M. vaccae* nebulization may protected in Balb/c mice against bronchial asthma through neural mechanisms.

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Introduction

Asthma is a heterogeneous disease characterized by chronic airway inflammation. It is defined by a history of respiratory symptoms such as wheezing, shortness of breath, chest tightness, and cough that vary over time and in intensity, accompanied by variable expiratory airflow limitation. The key characteristic of asthma is chronic airway inflammation, and recent studies have found that airway neurogenic inflammation plays an important role in the inflammatory mechanism of asthma.

Research studies have demonstrated that NGF may aggravate airway inflammation, hyper-responsiveness, and remodeling (1) and, therefore, could be a target in asthma therapy (2). In addition, BDNF is an important neuroprotective factor that can be produced by lung immunoinflammatory and airway parietal cells. Studies have shown increases in BDNF levels in asthma patients and animal models (3,4), and the level

of plasma BDNF is related to the severity of asthma (5,6).

The NGF and BDNF expression levels increase in the airway epithelial cells in allergic inflammation, which increases eosinophils in tissues and aggravates airway inflammation (7). Previous studies have found that neurotrophic factors such as NGF and BDNF are survival and activation factors of eosinophils in patients with allergic asthma, which play an important role in eosinophilic inflammation in allergic asthma (8). A recent research study demonstrated that nebulization with antinerve growth factor microspheres inhibited airway remodeling in an OVA-induced asthmatic rat model (9).

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophic factor 3 (NT3) are the main members of the neurotrophic factor family and they all play an important regulatory role in airway neurogenic inflammation. Neurofilament (NF) is one of the structural proteins of airway nerve endings

and its number and distribution density can reflect the neurosynaptic density indirectly. Thus NF reflects the state of the nerve network, including the structural and functional changes in the airway nerve network; therefore, changes in airway nerve plasticity may be related to the airway hyper-responsiveness.

The etiology and pathogenesis of asthma are still unclear, and this condition can only be controlled, but not cured. The hygiene theory suggests that early infection is negatively correlated with bronchial asthma (10,11) and the tuberculin reaction is negatively correlated with the occurrence of allergic diseases (12–14). Studies have confirmed that *Bacillus Calmette-Guerin* (BCG) can protect against allergic bronchial asthma (15). *Mycobacterium vaccae*, which is mainly composed of *Mycobacterium* proteins, is similar to BCG vaccine and is often used as an adjuvant treatment for tuberculosis (TB). *M. vaccae* immunotherapy may be effective in the treatment of pulmonary TB (16). Recent research studies have found that intranasal immunotherapy in combination with a mycobacterial adjuvant induces regulatory T cell differentiation and attenuates allergic responses *in vivo* (17).

Our previous study demonstrated that *M. vaccae* reduced airway hyper-responsiveness and protected against bronchial asthma in mice (18). We subsequently wondered what effects *M. vaccae* has on neural mechanisms since there are no relevant research studies, presently. Therefore, in this research study, we simulated the BCG vaccination immunization course in the human body (immunity was produced 4–6 weeks after BCG vaccination), nebulized mice with *M. vaccae* for 4 weeks before establishing an asthmatic model, and then investigated the effect of *M. vaccae* on the neural mechanism of asthma using immunohistochemistry and fluorescence quantitative polymerase chain reaction (PCR). These investigations were aimed at providing a theoretical basis for *M. vaccae* use in asthma prevention.

Methods

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of

the National Institutes of Health (NIH), and was approved by the Guangxi Medical University Animal Care and Use Committee (Protocol number: 20131002). All surgeries were performed under Nembutal anesthesia and every effort was made to minimize the suffering of the experimental animals.

Animal model establishment

A total of 18 female Balb/c mice (20–25 g) were randomized into the normal, asthma control, and *M. vaccae* nebulization (Neb.group) groups. Mice in the Neb.group were nebulized with 22.5 μ g *M. vaccae* (Anhui Longkema Biological Pharmaceutical Co., Anhui, China) mixed with 10 ml phosphate-buffered saline (PBS) once daily for 5 consecutive days, while mice in normal and asthma control groups were nebulized with PBS. The asthmatic model was a modification of that used in our previous study (18).

Briefly, each mouse was injected intraperitoneally with a mixture of 20 μ g ovalbumin (OVA, Grade V, Sigma-Aldrich, St. Louis, MO), 1 mg aluminum hydroxide gel (Pierce, Waltham, MA), and 200 μ L PBS once weekly for 3 consecutive weeks and 4 weeks later, they were nebulized with 1% OVA/PBS suspension (1 g OVA in 100 ml PBS) 30 min daily for 5 consecutive days (Figure 1).

Airway responsiveness measurement

The airway responsiveness of the mice was measured 24 h after the last challenge using a noninvasive lung function machine (FinePointe™ NAM system TBL4500, Buxco, Wilmington, NC), according to our previous method (18). After calibration and a 5 min adaptation, the mice were nebulized with 20 μ L PBS and methacholine (Mch; Sigma-Aldrich) at concentrations of 6.25, 12.5, and 25 mg/mL for 30 s. Data were recorded for 3 min and the mice were allowed to recover for 4 min. The results were analyzed automatically after the experiment ended. The airway responsiveness is presented as the expiratory flow at 50% tidal volume (EF₅₀).

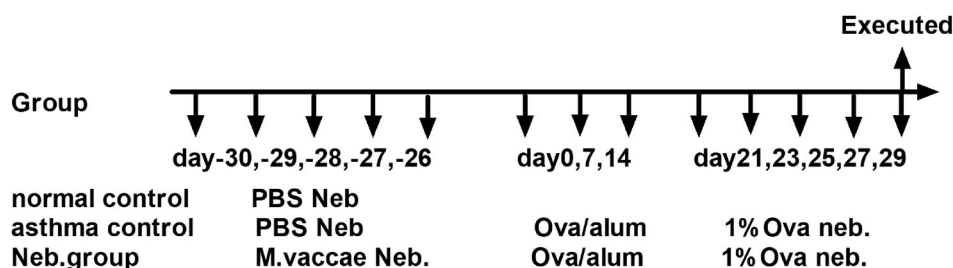


Figure 1. Experimental protocol. *M. vaccae*: *Mycobacterium vaccae*; Neb.: nebulization.

Specimen collection

The mice were anesthetized using an intraperitoneal injection of 1% Nembutal (50 mg/kg body weight), exsanguinated through the eyeballs, and fixed on their backs. The lungs were lavaged using 500 μ L iced PBS thrice, and the bronchoalveolar lavage fluid (BALF) was collected in Eppendorf tubes on ice. The recovery of BALF was >80%. The bronchopulmonary tissues were carefully separated using mosquito forceps and ophthalmic scissors. The left upper lung tissues were fixed in formalin for histological analysis. The left lower lobes were cleaned with PBS, cut into small pieces, frozen immediately in liquid nitrogen for 2–3 h, and then stored in a refrigerator at -80°C for fluorescence quantitative PCR.

Bronchoalveolar lavage fluid cell count and cell classification

The BALF was centrifuged for 10 min at 4°C and 1500 r/min, the supernatant was discarded, and the sediment resuspended and mixed in 100 μ L PBS. Of this, 30 μ L was used for cell counts in the cell counting chamber, 30 μ L was smeared for Wright's staining, and 200 inflammatory cells were counted under an oil microscope. Cells were classified based on morphological and staining characteristics.

Histology

The formalin (4%)-fixed lung tissues were paraffin-embedded and sliced into 3 mm-thick sections, which were then stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) for morphological evaluation.

Immunohistochemistry

The pulmonary tissues were fixed in formalin, routinely processed, and embedded in paraffin. Pulmonary BDNF, NF09, and acetylcholine expression levels were assessed according to immunohistochemical methods. The value of integrated optical density (IOD) was measured by image analysis software (Image-Pro Plus 6.0), three fields of vision were randomly observed in each section, and the average value was taken.

NGF mRNA expression

The NGF mRNA levels were measured using immunofluorescence PCR. The PCR primers for NGF were designed as follows: NGF forward: TGCCAA

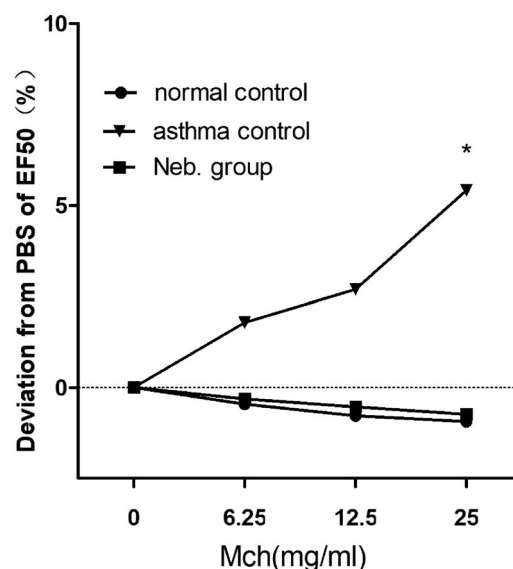


Figure 2. Airway hyper-responsiveness. * $p < 0.05$, Neb.group vs. asthma control group.

Table 1. Total cell count and cell classification count.

Group	Total cells ($\times 10^7/\text{L}$)	Percentages in total cells (%)	
		Eosinophils	Lymphocytes
Normal control	2.813 ± 0.5939	1.063 ± 0.3204	10.81 ± 1.487
Asthma control	$21.94 \pm 2.337^*$	$12.06 \pm 1.450^*$	$20.38 \pm 2.532^*$
Neb.group	$11.00 \pm 1.389^{**}$	$6.00 \pm 1.389^{**}$	$12.38 \pm 1.356^{**}$

* $p < 0.01$, compared with Normal control.

** $p < 0.01$, compared with Asthma control group.

GGACGCAGCTTTC and reverse: TGAAGTTTA GTCCAGTGGCTTCAG and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) forward: TGTGT CCGTCGTGGATCTGA and reverse: TTGCTGTTG AAGTCGCAGGAG (TaKaRa Biotechnology, Japan).

Statistical analysis

The statistical package for the social sciences (SPSS) 21.0 was used to calculate the standard deviations between experimental samples when each experimental group contained an equal number of data sets. Where the data set numbers differed between groups, the standard error of the mean was used. Normally distributed data and data with two independent variables were analyzed using a one-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis. The relationships of lung functions with BDNF, NF09, and Acetylcholine levels were performed using Spearman's rank correlation test. In all other instances, statistical differences between groups were calculated using the Student's t -test, and $p < 0.05$ was considered significant. GraphPad Prism 8.0 was used to construct the graphs.

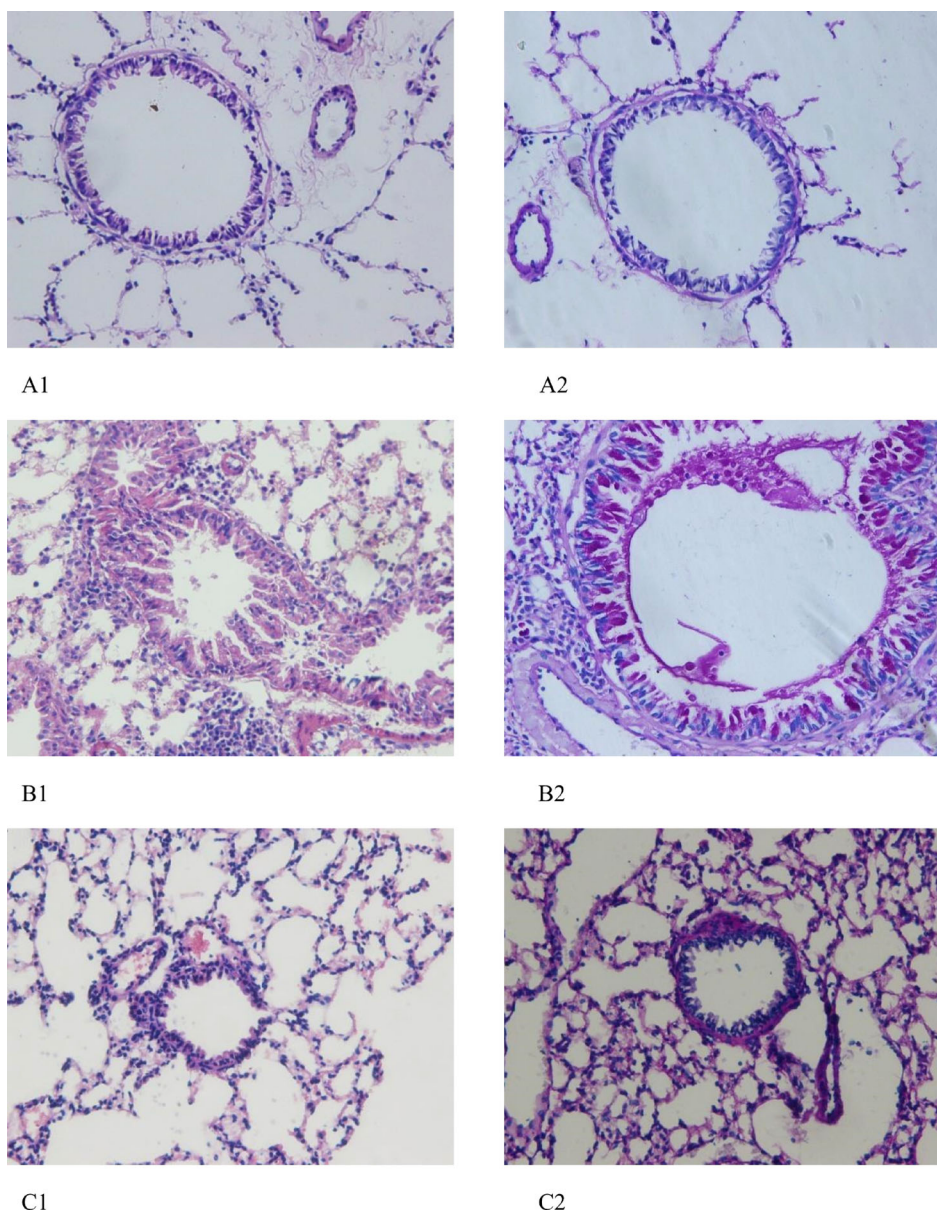


Figure 3. Lung histopathology. The lungs in the normal control group mice showed complete airway epithelium mucosa, the cilia were ordered, the basilar membranes and smooth muscle were thin, the lung small vascular endothelium was smooth and glossy, there was no inflammatory cell infiltration around the vessels and airways, there were few goblet cells, and no mucus secretion (Figure 3A1 and 3A2). By contrast, in the asthma control group, there was cellular swelling of the airway epithelia, the plicae mucosae were increased, cilia were disordered, the bronchial mucous membranes were broken, many inflammatory cells infiltrated the bronchioles, the vessels had an alveolar space and interalveolar septum, the alveolar wall was thickened showing hyperemia and dropsy, the basilar membrane was thickened, the smooth muscle showed hyperplasia, and PAS staining showed airway epithelia and goblet cell proliferation and hypertrophy, generous mucus and mucus plugs, and desquamation of the epithelia (Figure 3B1 and 3B2). In the *M. vaccae* group, the airway lumen was unobstructed and the epithelia lined up in order; PAS staining showed no goblet cells or mucus secretion (Figure 3C1 and 3C2).

Result

Asthmatic symptoms and manifestations

No animals died before the mice were euthanized. During nebulization, mice in the asthma control group showed the following symptoms: agitation, scratching their ears and cheeks, forelimb crispation, shortness of breath, cyanosis of the lips and forelimbs, and urinary

and fecal incontinence. Mice in the *M. vaccae* group showed none of the above manifestations.

Airway responsiveness

The airway responsiveness of the asthma control group was significantly higher than that of normal

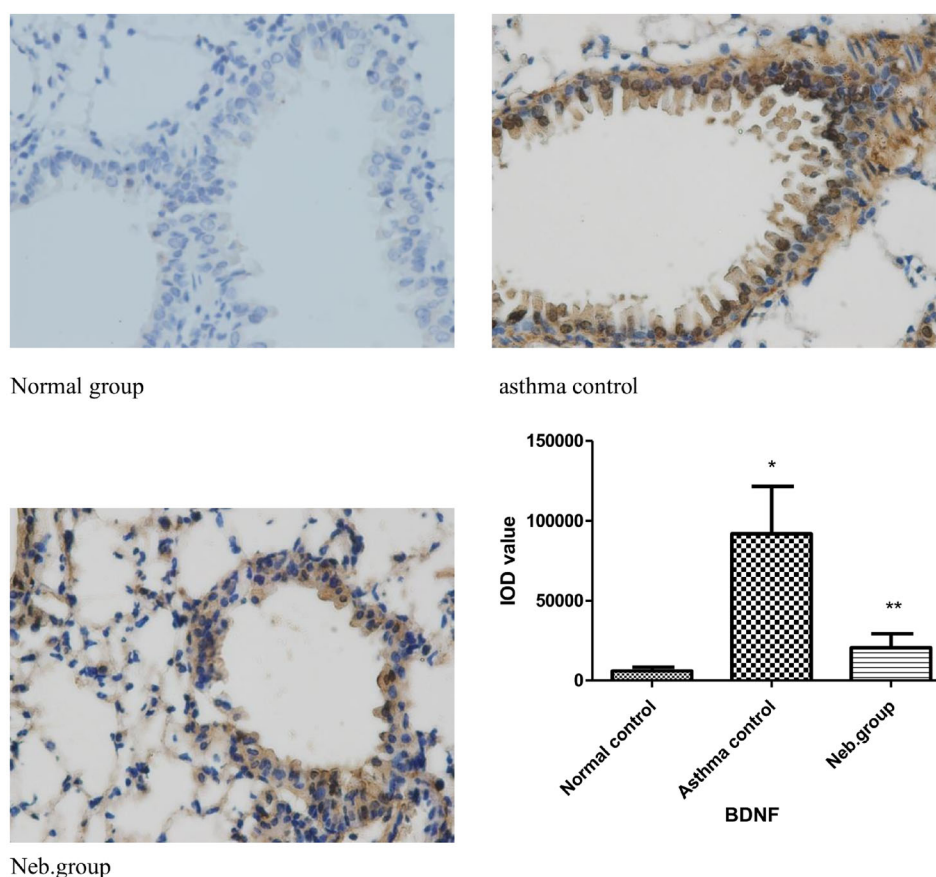


Figure 4. Expression of BDNF in pulmonary (400 $\times$) by immunohistochemical staining. The IOD (integral optical density) value result showed that pulmonary BDNF expression in Neb.group decreased sharply compared with Asthma control group. * $p < 0.0001$, compared with normal control; ** $p < 0.001$, compared with asthma control group.

control group ($p < 0.05$). The airway responsiveness of the *M. vaccae* group was significantly lower than that of the asthma control group ($p < 0.05$, Figure 2).

Bronchoalveolar lavage fluid total cell count and cell classification

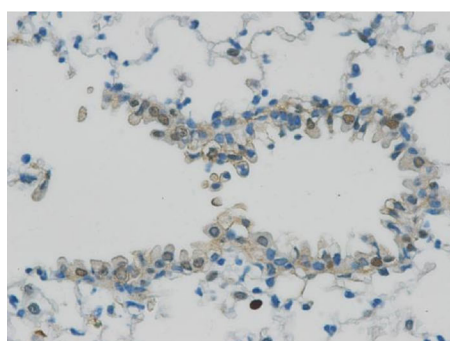
There was a significant difference in the total number of cells, and the percentages of eosinophils and lymphocytes in the BALF among the 3 groups. The total cell count and the eosinophil/lymphocyte ratio in the asthma control group were higher than that in the normal group ($p < 0.01$). The total cell count and the eosinophil/lymphocyte ratio in the Neb. group were lower than that in the asthma group ($p < 0.01$). The results are shown in Table 1.

Histology

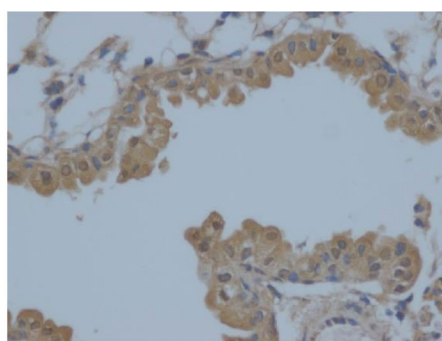
The lungs of the mice in the normal control group showed complete airway epithelium mucosa. Specifically, the cilia were ordered, the basilar

membranes and smooth muscle were thin, and the small vascular endothelium of the lung was smooth and glossy. Furthermore, there was no inflammatory cell infiltration around the vessels and airways, few goblet cells, and no mucus secretion (Figure 3A1 and 3A2). In contrast, the asthma control group showed cellular swelling of the airway epithelia, increased plicae mucosae, disordered cilia, broken bronchial mucous membranes, and considerable inflammatory cells infiltration in the bronchioles.

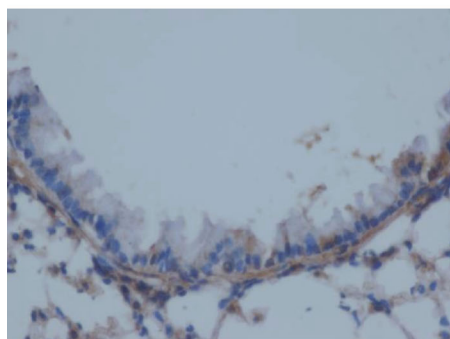
In addition, the vessels had an alveolar space and interalveolar septum, the alveolar wall was thickened with hyperemia and dropsy, the basilar membrane was thickened, the smooth muscle showed hyperplasia, and PAS staining showed airway epithelia and goblet cell proliferation and hypertrophy with generous mucus, mucus plugs, and epithelial desquamation (Figure 3B1 and 3B2). In the *M. vaccae* group, the airway lumen was unobstructed and the epithelia lined up in order while PAS staining showed no goblet cells or mucus secretion (Figure 3C1 and 3C2).



Normal control



Asthma control



Neb.group

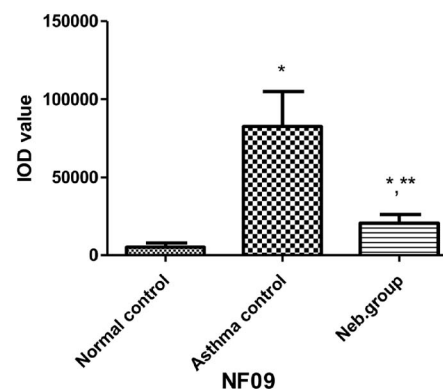


Figure 5. Expression of NF09 in pulmonary (400 $\times$) by immunohistochemical staining. The IOD value result showed that pulmonary NF09 expression in Neb.group decreased sharply compared with Asthma control group. * $p < 0.0001$, compared with normal control; ** $p < 0.001$, compared with asthma control group.

Pulmonary BDNF, NF09, and acetylcholine expression

Pulmonary BDNF, NF09, and acetylcholine expression in the *M. vaccae* group was significantly lower than that of asthma control group was (Figures 4–6).

Pulmonary NGF mRNA expression

The pulmonary NGF mRNA expression in the asthma control group was significantly higher than that of the normal group ($p < 0.0001$). Furthermore, the expression of NGF mRNA in the *M. vaccae* group was significantly lower than that in asthma control group was ($p < 0.5$, Figure 7).

Association between lung function and NF09, acetylcholine and BDNF level

There was a significant positive correlation between the Normal control lung function and lung BDNF (Spearman $r = 1.00$, $p = 0.01$). However, no significant correlation was found between the Normal control lung function and lung NF09 and Acetylcholine. There

was also no significant correlation between the lung function and NF09, Acetylcholine and BDNF level both in Asthma control group and Neb.group. The results are shown in Table 2.

Discussion

As hypothesized, we found *M. vaccae* nebulization can decreased airway hyper-responsiveness, alleviate the airway inflammation in asthmatic mice. We also found it can lowered pulmonary BDNF, NF09, and acetylcholine expression in asthmatic mice. The research demonstrated that *M. vaccae* nebulization may protect Balb/c mice against bronchial asthma through neural mechanisms.

Bronchial asthma can be effectively controlled but not be cured and long-term recurrent attacks can lead to airway remodeling that could subsequently develop into chronic obstructive pulmonary disease. The etiology and pathogenesis of asthma are still not very clear, and there are no effective preventive measures. Studies have shown that *Mycobacterium* infection is negatively correlated with the onset of asthma. BCG is

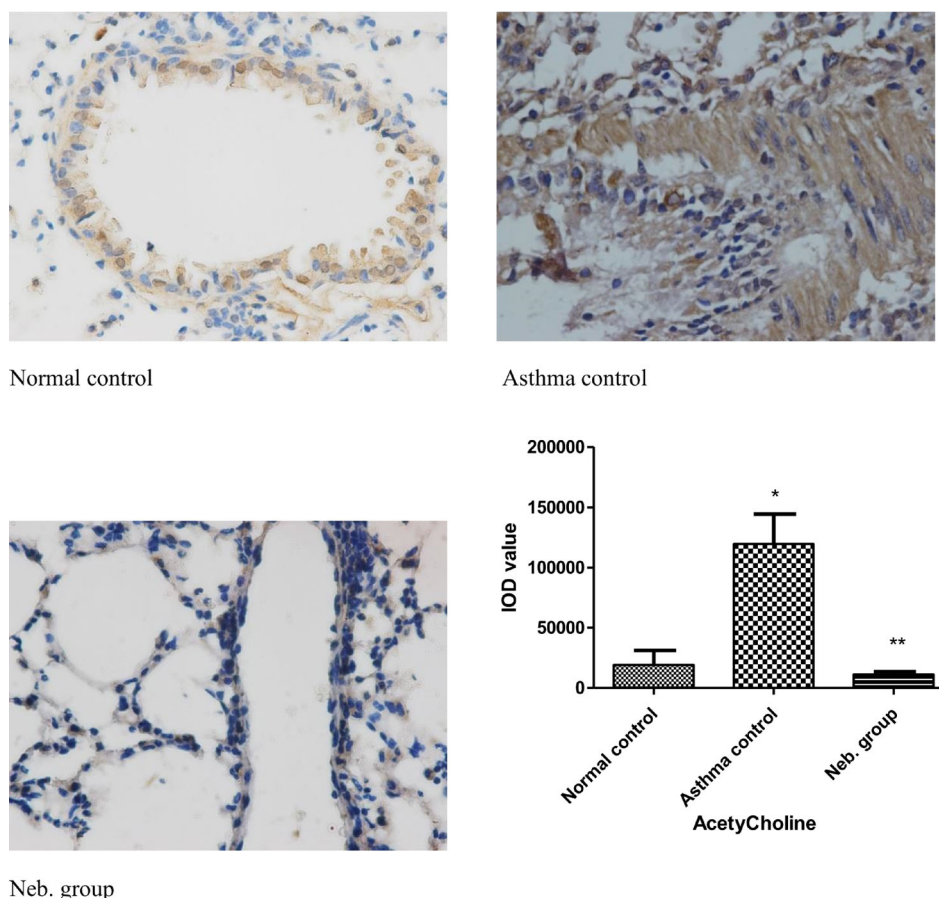


Figure 6. Expression of Acetylcholine in pulmonary (400 $\times$) by immunohistochemical staining. The IOD value result showed that pulmonary BDNF expression in Neb.group decreased sharply compared with Asthma control group. * $p < 0.0001$, compared with normal control; ** $p < 0.0001$, compared with asthma control group.

a live attenuated vaccine prepared from *Mycobacterium bovis*. Clinical studies have found that BCG has an unstable effect in preventing asthma (19). Animal experiments confirmed that BCG could protect against asthma and inhibit airway remodeling in bronchial asthma (20). *M. vaccae* is a nontoxic mycobacterium, which is often used as an adjuvant treatment for tuberculosis (TB). Nebulization therapy is simple and feasible with few side effects, and an important method for the treatment of respiratory diseases. In this study, mice were nebulized with *M. vaccae* for 1 month before the asthmatic model was established to simulate BCG vaccine inoculation (immunity was produced 4 to 6 weeks after BCG vaccination). The aim of this study was to observe the preventive effects of *M. vaccae* and its neurological mechanism in asthma.

Neurotrophic factors are expressed not only in the nervous system, but also in peripheral tissues including the lungs (21). Recent studies have shown that airway neurogenic inflammation plays an important role in the inflammatory mechanism of asthma. NGF and BDNF are main members of the neurotrophic factor

family that play an important regulatory role in airway neurogenic inflammation. Anti-NGF therapy may be beneficial in preventing and treating asthma (22). NF is one of the structural proteins of airway nerve endings and an important component of the nerve axon skeleton. Its quantity and distribution density can indirectly reflect the synaptic density of the nerve, thus, reflecting the state of the nerve network.

The immunohistochemical analysis showed that the expression of BDNF and NF09 in the lung tissue of the asthma control group was significantly higher than that of normal control group was, which was consistent with previously reported results in the literature. The expression levels of BDNF and NF09 in the *M. vaccae* group were significantly lower than those in the asthma control group were. The results suggest that levels of NGF, BDNF, and NF09 in the lung can be significantly decreased by *M. vaccae* nebulization, which may be an important prevention mechanism in asthma.

Our results showed that after *M. vaccae* nebulization of the asthmatic mice, airway inflammation, lung eosinophils, mucus secretion, and airway

responsiveness were reduced, indicating that this treatment could prevent bronchial asthma. Neurological mechanisms have an important role in the pathogenesis of bronchial asthma. The

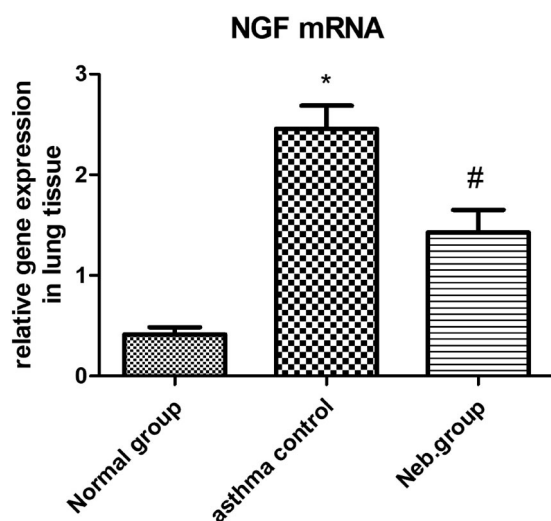


Figure 7-1. Results of NGF mRNA expression. * $p < 0.0001$, compared with Normal group; # $p < 0.05$, compared with asthma control group.

immunohistochemical and quantitative fluorescence PCR results showed that *M. vaccae* nebulization significantly reduce the levels of NGF, BDNF, and NF09, which could be useful for the prevention of asthma in Balb/c mice.

Our results also showed that there was a positive correlation between BDNF level and lung function in the normal control group, suggesting that BDNF plays an important role in the mechanism of airway hyper-responsiveness. But, we also found that there was no significant correlation between lung function and NF09, acetylcholine level in normal control group, and there was no significant correlation between lung function and BDNF, NF09 and acetylcholine in both asthma group and Neb. group either. However, our research demonstrated that the airway reactivity and BDNF, NF09 and acetylcholine level of asthmatic mice decreased significantly after *M. vaccae* nebulization, suggesting that the airway hyper-responsiveness mechanism in asthmatic mice is extremely complex.

M. vaccae is mainly composed of protein and short-term *M. vaccae* nebulization may have an “antigenic” effect that may lead to other types of

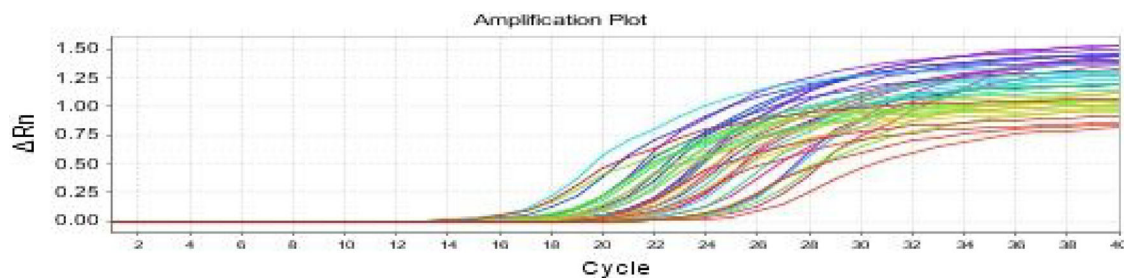


Figure 7-2. Pulmonary NGF mRNA amplification plot.

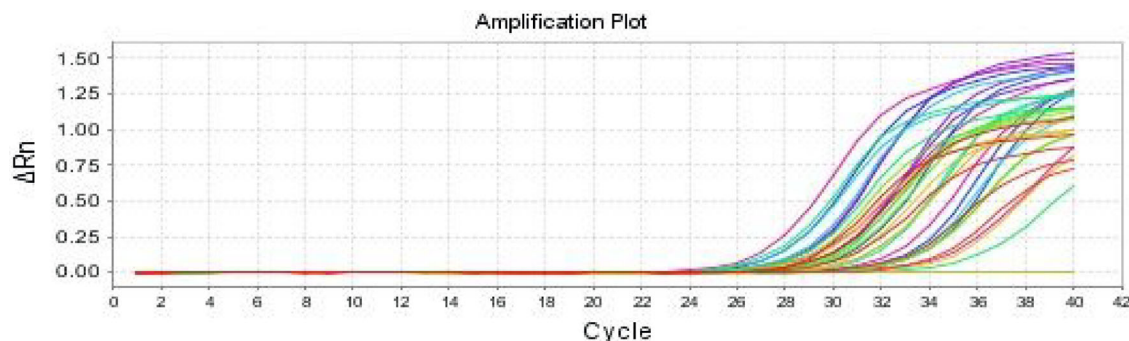


Figure 7-3. Gapdh mRNA amplification plot.

Table 2. Spearman correlation between lung function and NF09, Acetylcholine and BDNF level.

group	NF09 level		Acetylcholine level		BDNF level	
	r	p	r	p	r	p
Normal control Lung function	0.086	0.872	−0.600	0.285	1.000	0.01
Asthma control Lung function	0.486	0.329	−0.600	0.285	−0.429	0.289
Neb.group Lung function	0.200	0.800	0.700	0.188	−0.214	0.645

airway inflammation. However, *M. vaccae* nebulization significantly inhibited neuroinflammation and decreased the airway responsiveness in asthma. Furthermore, this nebulization treatment is simple and easy to administer with few side effects, any associated discomfort can be treated immediately, and patients can administer the treatment at home.

This study also has several limitations. First, we only used immunohistochemical method to detect the pulmonary NF09, BDNF and *acetylcholine* expression and immunofluorescence PCR to detect NGF expression, additional experiments such as ELISAs and/or Western blots would be performed to support and complement the immune-histological images in the future research. Second, the research only performed in animal.

Conclusions

Our study suggests that *M. vaccae* nebulization reduced pulmonary NGF, NF09, and BDNF expression significantly, and decreased the pulmonary *acetylcholine* levels. *M. vaccae* played an important role in regulating the nervous mechanism and anticholinergic function in the mouse model of asthma. In the future, we plan to use *M. vaccae* nebulization in asthmatic patients to further study its asthmatic prevention effect.

Declaration of interest

The authors declare no conflict of the interest.

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