Canadian Respiratory Journal

# Challenges in the Current Management of Severe Asthma and Treatment Option Selection

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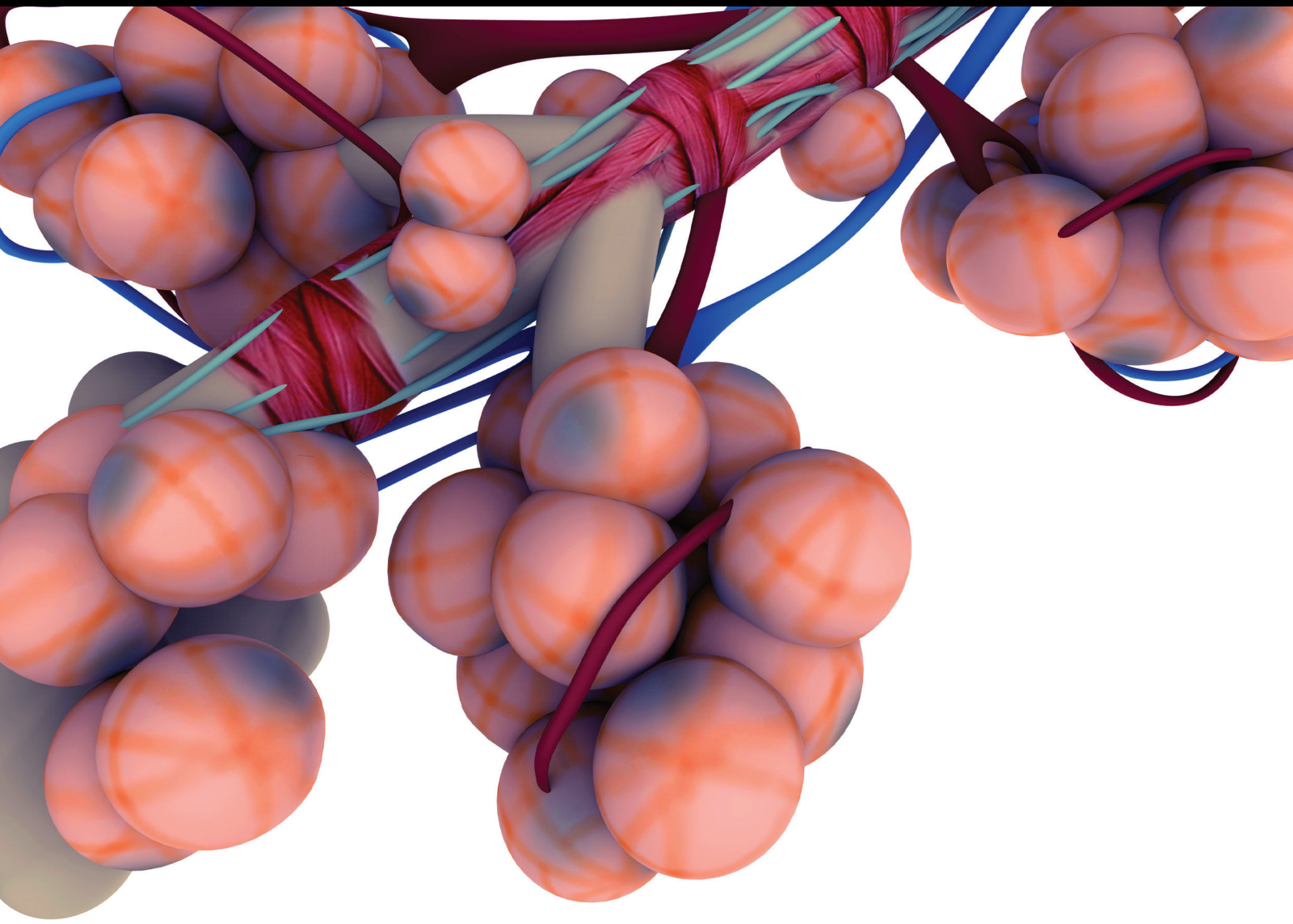

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*Research Article*

# Serum Levels of Epithelial-Derived Cytokines as Interleukin-25 and Thymic Stromal Lymphopoietin after a Single Dose of Mepolizumab in Patients with Severe Non-Allergic Eosinophilic Asthma: A Short Report

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The bronchial epithelium has continuous contact with environmental agents initiating and maintaining airway type 2 inflammation in asthma. However, there is a lack of data on whether reduced airway eosinophilic inflammation can affect the production of epithelial-derived mediators, such as interleukin-25 (IL-25) and thymic stromal lymphopoietin (TSLP). The aim of this study was to investigate the changes in serum levels of IL-25 and TSLP after a single dose of mepolizumab, a humanized monoclonal antibody to interleukin-5 (IL-5), in patients with severe non-allergic eosinophilic asthma (SNEA). We examined 9 SNEA patients before and four weeks after administration of 100 mg mepolizumab subcutaneously. The fractional exhaled nitric oxide (FeNO) level was analysed using an electrochemical assay (NIOX VERO®, Circassia, UK). Serum IL-25 and TSLP levels were measured by ELISA. Four weeks after the single dose of mepolizumab, blood eosinophil count significantly decreased from $0.55 \pm 0.20 \times 10^9$/l to $0.14 \pm 0.04 \times 10^9$/l ($p = 0.01$) and $FEV_1$ increased from $2.1 \pm 0.5$ l ($65.4 \pm 8.8\%$ of predicted) to $2.6 \pm 0.4$ l ($76.4 \pm 9.1\%$ of predicted) ($p = 0.04$), while FeNO level has not changed ($32.3 \pm 8.4$ *vs* $42.9 \pm 12.6$ ppb). Serum IL-25 level significantly decreased from $48.0 \pm 17.2$ pg/mL to $34.8 \pm 17.1$ pg/mL ($p = 0.02$) with same tendency in TSLP level: from $359.8 \pm 71.3$ pg/mL to $275.6 \pm 47.8$ pg/mL ($p = 0.02$). It has also been noticed a significant relation between changes in the blood eosinophil count and serum IL-25 level ($r = 0.81$, $p = 0.008$), as well as between changes in serum IL-25 and TSLP levels ($r = 0.93$, $p = 0.004$) after a single dose of mepolizumab. Thus, anti-IL-5 treatment with mepolizumab might diminish the production of bronchial epithelial-derived cytokines IL-25 and TSLP in patients with SNEA which is potentially related to reduced eosinophilic inflammation. This trial is registered in ClinicalTrial.gov with identifier NCT03388359.

## 1. Introduction

Asthma is a common, life-lasting airway disease, associated with a high social and economic burden. About 3–8% of all asthma patients have severe asthma, suffering from frequent symptoms and recurrent exacerbations despite the combined treatment with high-dose of inhaled steroids and long-acting bronchodilators, often supplemented with oral steroids [1, 2]. All this leads to a significant loss of life quality and labour productivity, increased mortality risk [3, 4]. The cost of severe asthma treatment represents a significant part of the total cost of all asthma cases [3, 4]. Therefore, severe asthma is the most research-intensive areas of respiratory medicine in the last decade.

Eosinophilic airway inflammation has a key position in the pathogenesis of severe eosinophilic asthma [5, 6]. After

activation, eosinophils synthesize a row of cytokines, chemokines, growth factors, and other eosinophil-derived proinflammatory products, and all of them contribute to the airway inflammation in asthma, including airway epithelial cell damage, airway dysfunction, and remodeling [7–9]. Interleukin- 5 (IL-5) is one of the main promoters of eosinophil production, maturation, and release from bone marrow. It also activates eosinophils and prolongs their survival in the circulation, as well as providing an essential signal for their migration into tissue [10]. However, the initial immune response to inhaled air pollutants or other external triggers occurs already in the bronchial epithelium [11–16]. Therefore, dysfunction of epithelial cells is becoming an increasingly important part of the pathogenesis of asthma. There are data that cytokines interleukin- 25 (IL-25) and thymic stromal lymphopoietin (TSLP) are some of the major airway type 2 inflammation regulators derived from the bronchial epithelium [14, 17]. These cytokines have been described as epithelial-derived alarmins that activate and potentiate the inner immune cascade, including airway eosinophilic inflammation, in the presence of actual damage [14, 16–18].

It is unknown whether anti-IL-5-directed treatment affects only eosinophilic inflammation or also other mediators which are involved in airway type 2 inflammation. In this study, we aimed at assessing the changes in serum levels of epithelial-derived mediators as IL-25 and TSLP on mepolizumab, a humanized monoclonal antibody to IL-5, treatment in patients with severe non-allergic eosinophilic asthma (SNEA). We designed to use a single dose of mepolizumab to avoid asthma exacerbations that could influence the intensity of type 2 inflammation, whereas positive drug effect on reduction in blood eosinophils and lung function improvement is observed already after the first dose [19, 20].

## 2. Materials and Methods

*2.1. Subjects.* The study was conducted with the permission of the Regional Biomedical Research Ethics Committee of the Lithuanian University of Health Sciences (BE-2-13) and after signing the informed consent forms. The study was registered in the U.S. National Institutes of Health trial registry ClinicalTrials.gov with identifier NCT03388359.

The study included patients with adult-onset SNEA (the inclusion criteria listed below). Non-allergic asthma was chosen to eliminate allergens as an uncontrollable factor which damage the epithelium and may significantly alter cytokine levels and affect airway type 2 inflammation activity.

The participants were men and women between the ages of 18 and 65 years, recruited from the Department of Pulmonology at Hospital of the Lithuanian University of Health Sciences Kaunas Clinics.

Inclusion criteria were as follows: asthma diagnosis for at least 12 months; non-allergic phenotype, confirmed by the absence of allergy-specific symptoms (watery runny nose or nasal obstruction, conjunctivitis, rashes, urticaria, without dietary restrictions, and any symptoms of digestion) and with negative skin prick tests; blood eosinophil count $\geq 0.15 \times 10^9$/l during the screening visit or documented $\geq 0.30 \times 10^9$/l blood eosinophil count in the 12-month period before the screening denying other possible common causes of eosinophilia (e.g., helminths, allergies); no other reasons that could lead to poor control of asthma symptoms; documented at least 12-month treatment of high doses of inhaled steroids combined with long-acting beta-agonist ± long-acting antimuscarinic agent ± episodic use of oral steroids prior to inclusion in the study; and in the 12 months before the screening visit ≥2 exacerbations of asthma that required treatment with systemic steroids.

The study was open for non-smokers only.

Exclusion criteria included asthma exacerbation, active airway infection, and use of oral steroids 1 month prior to the study; clinically significant permanent allergy symptoms; and treatment with targeted (biological) therapy (e.g., omalizumab, mepolizumab, and benralizumab) at the screening visit.

*2.2. Study Design.* At the screening visit, the inclusion/exclusion criteria were assessed, and informed consent was obtained. During the first study visit, FeNO level was analysed, $FEV_1$ was measured, and blood samples were collected for evaluation of blood eosinophil count and epithelial-derived mediators concentrations. After all the procedures, mepolizumab 100 mg was injected subcutaneously. The second study visit was scheduled for 4 weeks. Then, FeNO and $FEV_1$ were re-evaluated, and blood samples were re-taken. Only patients without asthma exacerbation during this 4 weeks period were re-evaluated. The study design scheme is presented in Figure 1.

*2.3. Pulmonary Function Testing.* The lung function was evaluated for all study subjects by measuring baseline $FEV_1$ using an ultrasonic spirometer (Ganshorn Medizin Electronic, Germany) and compared with the predicted value matched for age, body height, and sex according to the standard methodology. $FEV_1$ was measured three times and recorded only the highest of three reproducible measurements.

*2.4. FeNO Measurement.* All study subjects underwent FeNO analysis with an online method using a single-breath exhalation and an electrochemical assay (NIOX VERO®, Circassia, UK), according to ATS-ERS guidelines [21]. Patients made an inspiration of eNO-free air via a mouthpiece immediately followed by full exhalation at a constant rate (50 mL/sec) for at least 10 seconds. The mean of three readings at the end of the expiration (plateau phase) was taken as the representative value for each measurement.

*2.5. Skin Prick Test.* All study subjects were screened for allergies by the skin prick test at least 6 months prior to enrollment. Standardized allergen extracts (Stallergenes S.A., France) were used for the following allergens: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat and dog dander, mixture of pollen of 5 grasses, birch pollen, mugwort, *Alternaria*, *Aspergillus*, and *Cladosporium*. Negative control was performed with diluent (saline) and

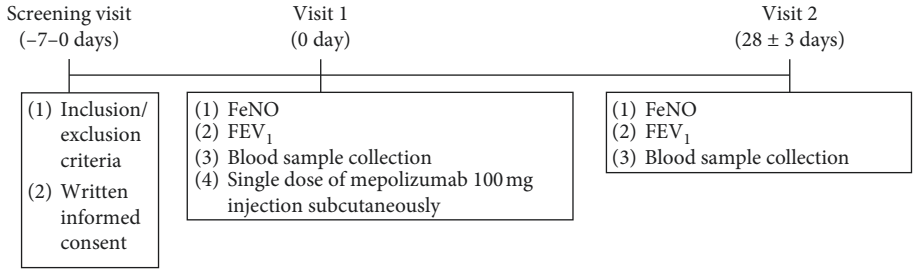


Figure 1: The study design scheme. $FEV_1$—forced expiratory volume in 1st second; FeNO—fractional exhaled nitric oxide.

positive control with histamine hydrochloride (10 mg/mL). Results of the test were evaluated 15 min after application. The skin prick test was estimated to be positive when the mean wheal diameter reaches ≥3 mm.

*2.6. Detection of Protein Level by Investigating Individuals' Blood Serum Samples.* Protein (IL-25 and TSLP) levels in blood serum samples were measured by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The following ELISA kits were used for experiments: IL-25 (R&D Systems, USA) lower limit of detection (LLD)—11.7 pg/mL and TSLP (R&D Systems, USA) LLD—7.8 pg/mL. 100 $\mu$l of serum samples was used for experiments. Blood was collected into BD Vacutainer® SST™ II Advance Blood Collection Tubes and allowed to clot for 30 min. After that, the tubes were centrifuged at 1300 ×g 10 min at room temperature to separate serum from clotted blood. Serum immediately was collected and divided into 1 mL cryogenic tubes that were frozen in −80°C for further proteins level analysis. ELISA measurements were performed after a sufficient amount of samples was collected. The results were expressed as protein concentration per 1 mL of serum.

*2.7. Statistical Analysis.* Statistical analysis was performed by using GraphPad Prism 6 for Windows (ver. 6.05, 2014; GraphPad Software Inc., San Diego, CA). Protein concentration data were represented as the mean ± standard error of the mean or with specific values for each subject, including the overall mean. Significant differences between two dependent groups were determined using the Wilcoxon matched-pairs signed-rank test. Spearman rank correlation coefficient was used to evaluate correlations. $p < 0.05$ was considered as statistically significant.

## 3. Results and Discussion

*3.1. Characteristics of Study Population.* In this study, we examined 9 patients with adult-onset SNEA at two time points: before and 4 weeks after 100 mg subcutaneous injection of mepolizumab. The subjects with SNEA were middle or elderly aged with a tendency to overweight, on the Global Initiative for Asthma (GINA) step 4-5 treatment, and had impaired lung function, increased blood eosinophil count, and elevated FeNO level (more detailed data are presented in Table 1). Any patient experienced asthma exacerbation up to a second visit. Four weeks after a single dose of mepolizumab, significant reduction of the blood eosinophilia and improvement in lung function were observed, while FeNO level remained stable (Table 1).

Table 1: Demographic and clinical characteristics of the study population.

| | SNEA subjects | |
|---|---|---|
| Number ($n$) | 9 | |
| Sex (M/F) | 5/4 | |
| Age (years) | 53 ± 5.2 | |
| BMI (kg/m$^2$) | 28.9 ± 1.6 | |
| High-dose iCS + LABA | 5 | |
| High-dose iCS + LABA + LAMA | 3 | |
| High-dose iCS + LABA + theophylline | 1 | |
| | At baseline visit | 4 weeks after a single dose of mepolizumab |
| $FEV_1$ (l) | 2.1 ± 0.5 | 2.6 ± 0.4* |
| $FEV_1$ (% of predicted) | 65.4 ± 8.8 | 76.4 ± 9.1* |
| Blood eosinophil count (×10$^9$/l) | 0.55 ± 0.20 | 0.14 ± 0.04* |
| FeNO (ppb) | 32.3 ± 8.4 | 42.9 ± 12.6 |

F: female; M: male; $FEV_1$: forced expiratory volume in 1st second; FeNO: fractional exhaled nitric oxide; iCS: inhaled glucocorticosteroids; LABA: long-acting $\beta$-adrenoceptor agonists; LAMA: long-acting muscarinic antagonists. Data are presented as the mean ± standard error of the mean. * $p < 0.05$ comparing with the baseline visit.

*3.2. Changes in Blood Eosinophil Count and Serum IL-25 and TSLP Levels after a Single Dose of Mepolizumab.* Four weeks after the single dose of mepolizumab, blood eosinophil count significantly decreased from $0.55 \pm 0.20 \times 10^9$/l to $0.14 \pm 0.04 \times 10^9$/l ($p = 0.01$, Figure 2(a)). In this study, we found that the serum level of IL-25 was significantly reduced from 48.0 ± 17.2 pg/mL to 34.8 ± 17.1 pg/mL already after a single dose of add-on treatment with mepolizumab ($p = 0.02$, Figure 2(b)). The change in serum TSLP level was similar: 359.8 ± 71.3 pg/mL, before mepolizumab administration, and 275.6 ± 47.8 pg/mL ($p = 0.02$), 4 weeks after a single dose of mepolizumab (Figure 2(c)).

Significant correlations were observed between changes in the blood eosinophil count and serum IL-25 level ($r = 0.81$, $p = 0.008$) (Figure 3), as well as between changes in serum

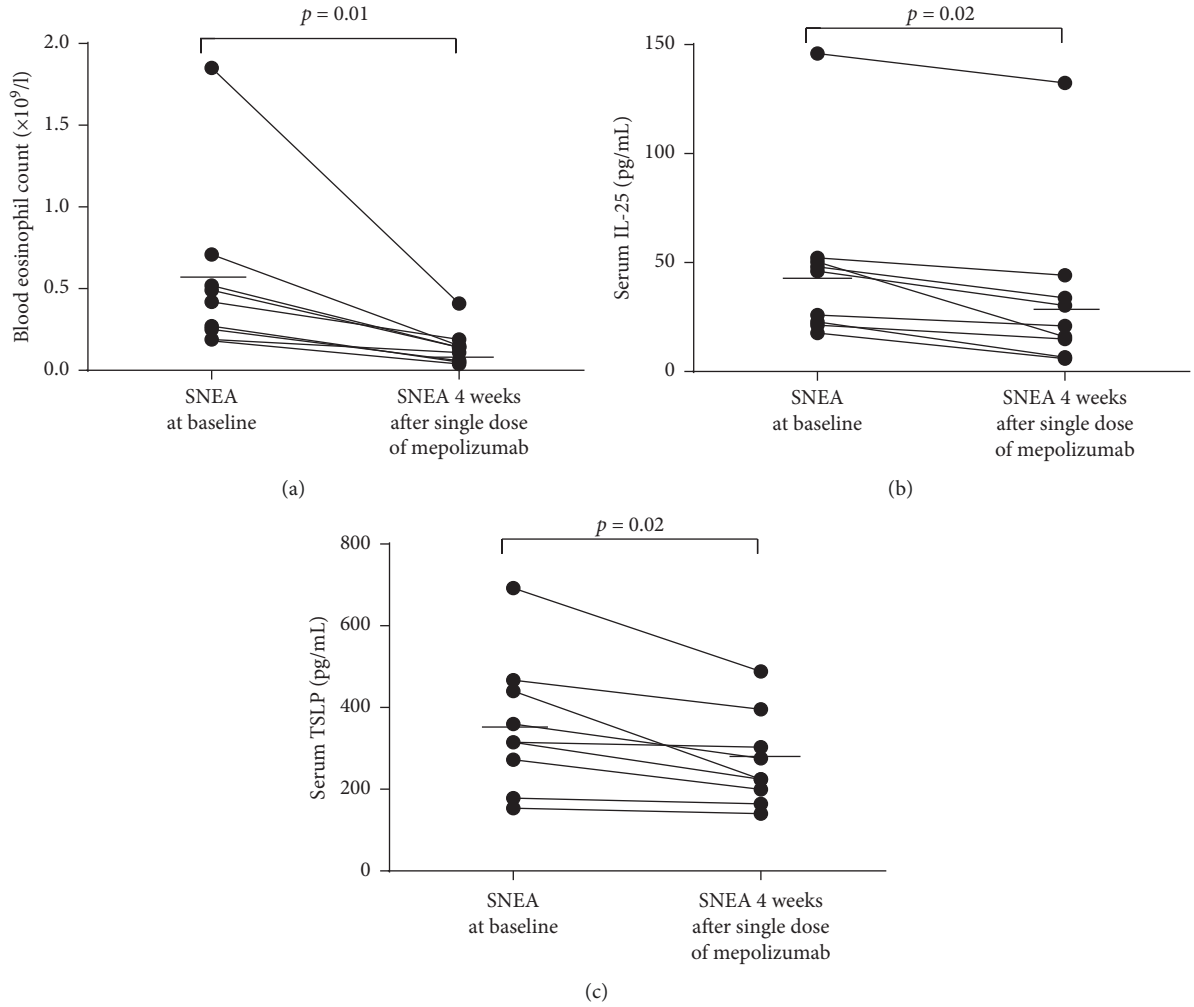


Figure 2: Changes in blood eosinophil count and serum interleukin-25 (IL-25) and thymic stromal lymphopoietin (TSLP) level in patients with severe non-allergic eosinophilic asthma (SNEA) 4 weeks after a single dose of mepolizumab ($n = 9$). (a) Blood eosinophil count. (b) IL-25. (c) TSLP. Statistical analysis—Wilcoxon matched-pairs signed-rank test. Data are presented with specific values for each subject, including the overall mean.

IL-25 and TSLP levels ($r = 0.93$, $p = 0.004$) (Figure 4). However, the correlation between changes in the blood eosinophil count and serum TSLP level was not significant ($r = 0.49$, $p > 0.05$).

## 4. Discussion

In this study, attention was drawn to the bronchial epithelium dysfunction and how the level of epithelial-derived cytokines changes during treatment with anti-IL-5 drug when eosinophilic inflammatory activity is reduced. It was found that a single dose of mepolizumab in patients with SNEA significantly decreased blood eosinophil count and improved lung function, whereas FeNO did not. We have also noticed that anti-IL-5 treatment reduced the serum level of epithelial-derived cytokines, such as IL-25 and TSLP.

Blood eosinophil depletion and lung function improvement (measured by $FEV_1$) during treatment with mepolizumab have been demonstrated in many studies to confirm the appropriateness of mepolizumab during severe eosinophilic asthma [19, 22–24]. Our study does not contradict this, suggesting that patients have been appropriately selected and that the given treatment effectively inhibits eosinophilic inflammation, assuming that the newly identified results—changes in IL-25 and TSLP—are associated with the action of mepolizumab.

IL-25 and TSLP are epithelial-derived cytokines that play an essential role in stimulating Th2 cytokine response and initiating airway type 2 inflammation in asthma [16–18]. By investigating eosinophilic asthma pathogenesis, it was noted that IL-25 significantly influences the production of type 2 cytokines, including IL-5, essential for eosinophilic inflammation [25–27]. Analogous results were obtained with the TSLP [28–31]. There is evidence that these cytokines are related to asthma severity. It is found that the amount of IL-25 in the sputum was correlated with the severity of asthma—the highest level of IL-25 in sputum was found in severe asthma [28]. The significant changes in TSLP protein

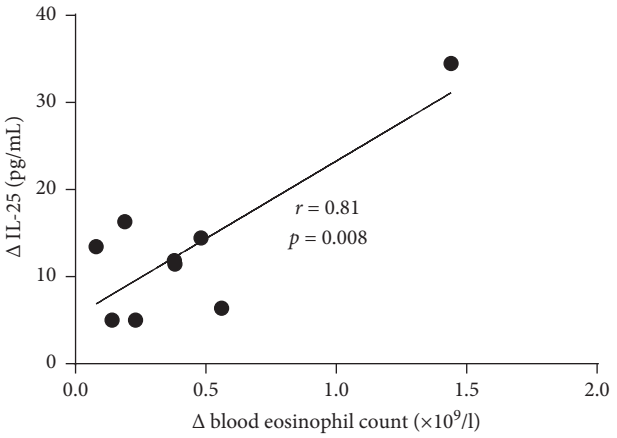


Figure 3: Correlation between changes in the blood eosinophil count and serum interleukin-25 (IL-25) level 4 weeks after a single dose of mepolizumab in patients with severe non-allergic eosinophilic asthma ($n = 9$). $r$—Spearman rank correlation coefficient.

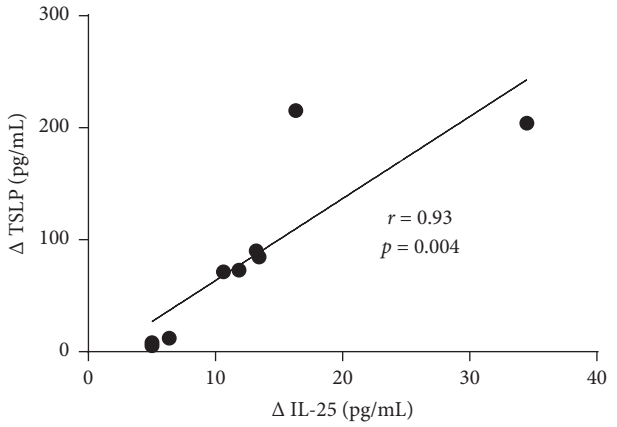


Figure 4: Correlation between changes in serum interleukin-25 (IL-25) and thymic stromal lymphopoietin (TSLP) level in patients 4 weeks after a single dose of mepolizumab with severe non-allergic eosinophilic asthma ($n = 9$). $r$—Spearman rank correlation coefficient.

expression were identified in lung tissue, and an inverse correlation with the severity of bronchial obstruction was observed [29]. The importance of IL-25 and TSLP in eosinophilic asthma is increasingly being explored. The change in their production is believed to play an essential role in the pathogenesis of asthma by initiating airway type 2 inflammation and promoting the production of type 2 cytokines [6, 17, 18, 30, 32]. However, no studies have yet been carried out to evaluate the effect of anti-IL-5 treatment on the bronchial epithelium-derived cytokines. We found that reduced activity of eosinophilic inflammation with mepolizumab affects the level of epithelial-derived cytokines IL-25 and TSLP and it was associated with changes in blood eosinophil count. How the reduction of eosinophilic inflammation is associated with epithelial cytokine level has only speculations. The bronchial epithelium has the naïve bronchial epithelial cells, which produce some levels of IL-5 [31]. Animal studies have shown that bronchial epithelial cells, isolated from mice with OVA-induced allergic airway disease, produced elevated levels of IL-5 mRNA and protein as compared to bronchial epithelial cells from naïve mice. Therefore, IL-5 produced by epithelial cells contributed to mucous metaplasia and airway eosinophilia and can impact the microenvironment of the lung, modifying pathologic and protective immune responses in the airways [31]. Thus, bronchial-epithelial eosinophilia leads to the production of an additional amount of IL-5, and it is an important element for supporting eosinophilic inflammation. In addition, eosinophils release granular proteins that can damage the bronchial epithelial cells [33] and thus lead to higher epithelial-derived cytokine production. In terms of direct effects of IL-5 on bronchial epithelial cells, there are data that differentiated human airway epithelial cells express functional IL-5 receptors and that this cytokine may promote epithelial cell growth and proliferation (at the same time also affect the production of cytokines) [34]. In response to exogenous stimuli, epithelial-derived IL-25 and TSLP together with other bioactive substances elicit innate lymphoid cell (ILC2) responses in the lungs [6, 17]. Activated ILC2s can subsequently promote IL-5-mediated eosinophil recruitment and produce large amounts of Th2 cytokines, including IL-5, which enhances eosinophil adhesion to bronchial epithelial cells. ILC2s also produce amphiregulin, which promotes the repair of the airway epithelium [10]. Collectively, all these findings suggest that IL-5 affects airway physiology in asthma in part through effects on airway epithelial cells [34]. Thus, the hypothesis is that by blocking IL-5, mepolizumab selectively inhibits both the inner cascade IL-5 and the epithelial-derived IL-5, aggravate eosinophil adhesion to bronchial epithelial cells, reduce its infiltration with eosinophils and their degranulation, and thus reduce the eosinophilic inflammation and subsequent epithelial damage, as well as epithelial cytokine production. This is reinforced by us obtaining a significant correlation between changes in the blood eosinophil count and serum IL-25 level and changes in serum IL-25 and TSLP levels after a single dose of anti-IL-5 treatment with mepolizumab in patients with SNEA.

According to the study data, blood levels of certain cytokines are unlikely to accurately reflect the processes in the lungs as we did not find a reliable correlation between changes in TSLP and blood eosinophil count. These insights are also observed in a study with atopic dermatitis, where serum TSLP level did not significantly correlate with disease severity, blood eosinophil counts, and serum total immunoglobulin E levels, suggesting that TSLP does not mainly enter the blood circulation [35] or related to how acute the disease is. There are data that the early exaggerated production of TSLP might be important for initiating immune processes but may not be through serum TSLP [36]. However, the importance of TSLP as a key cytokine for epithelial damage has been demonstrated by a significant correlation with IL-25.

Additionally, FeNO is attributed to eosinophilic inflammatory biomarkers, and the relationship with eosinophilia and FeNO level is established [23], but, according to studies, no significant changes were found in FeNO level on

eosinophilic inflammation-reducing treatment [37, 38]. The results of our study were analogous. However, FeNO is referred to as a validated eosinophilic biomarker and its stability, despite suppressed eosinophilic inflammation, is more difficult to understand. FeNO can be influenced by many factors, including inhaled corticosteroids and body mass index [39–42]. It is also associated with interleukin- 4 and interleukin- 13, but these cytokines are crucially important in allergic asthma cases [42, 43]. In our study, SNEA patients received inhaled steroids in high doses and had a slightly increased body mass index; however, they were all non-atopic. Therefore, in the case of severe asthma, the assessment of FeNO as a marker of eosinophilic inflammatory activity may not be valuable but is likely more sensitive to withdrawal of steroids [44].

The study is limited by the lack of a control group for comparison of the effect of mepolizumab on the levels of epithelial-derived cytokines. However, this was a brief observation study for 4 weeks to minimize the risk of exacerbations and course variability of asthma that could have an impact on TSLP and IL-25 production. Thus, only clinically stable severe asthma patients, free of systemic steroids at least 1 month before the study, were included and patients without asthma exacerbation during 4 weeks period after a single dose of mepolizumab were re-evaluated. Additionally, all study subjects were non-atopic and non-smokers, so it could be presumed that contact with inhaled non-specific air particles, which trigger the epithelium, is constant.

## 5. Conclusion

Our study results demonstrate that anti-IL-5 treatment with mepolizumab might diminish the production of bronchial epithelial-derived cytokines IL-25 and TSLP in patients with SNEA which is potentially related to reduced eosinophilic inflammation. These findings indicate that mepolizumab by modulation of bronchial epithelium function could provide a broader impact on the severe eosinophilic asthma pathophysiology. This may be important when making clinical decisions for patients non-responsive to other biologics for severe refractory asthma.

## Abbreviations

ELISA: Enzyme-linked immunosorbent assay
FeNO: Exhaled nitric oxide concentration
$FEV_1$: Forced expiratory volume in 1st second
GINA: Global Initiative for Asthma
ILC2: Type 2 innate lymphoid cells
IL-5: Interleukin-5
IL-25: Interleukin-25
LLD: Lower limit of detection
SNEA: Severe non-allergic eosinophilic asthma
TSLP: Thymic stromal lymphopoietin.

## Data Availability

The study data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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*Research Article*

# Effect of Tiotropium Bromide on Airway Inflammation and Programmed Cell Death 5 in a Mouse Model of Ovalbumin-Induced Allergic Asthma

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*Rationale*. We previously demonstrated increased expression of programmed cell death 5 (PDCD5) in asthmatic patients and ovalbumin-induced allergic asthma. International guidelines (GINA 2019) have included the use of tiotropium bromide for chronic treatment of the most severe and frequently exacerbated asthma in patients ≥6 years old, who do not have good response to inhaled corticosteroids. *Objective*. To explore the role of tiotropium and its effect on PDCD5 level in a mouse model of chronic asthma. *Methods*. We divided 12 female mice into 2 groups: untreated asthma ($n = 6$) and tiotropium-treated asthma ($n = 6$). The impact of tiotropium was assessed by histology of lung tissue and morphometry. Pulmonary function was tested by using pressure sensors. The number of cells in bronchoalveolar lavage fluid (BALF) was detected. Levels of PDCD5, active caspase-3, and muscarinic acetylcholine receptors M2 (ChRM2) and M3 (ChRM3) were examined. *Results*. Tiotropium treatment significantly reduced airway inflammation and remodeling in asthmatic mice and intensified the lung function. PDCD5 level was reduced with tiotropium ($p < 0.05$). Moreover, active caspase-3 level was decreased with tiotropium ($p < 0.001$), and ChRM3 level was increased. *Conclusions*. Tiotropium treatment may alleviate the pathological changes with asthma by regulating apoptosis.

## 1. Introduction

Allergic asthma is a major health concern worldwide, with chronic inflammatory disorder of the airways and airway remodeling. Airway remodeling can lead to irreversible airflow limitation and accelerate lung function decline [1]. Despite advances in the pathogenesis and therapeutics for asthma, for some patients, the disease remains uncontrolled without good response to inhaled corticosteroids. Novel treatment strategies need to be explored.

Tiotropium bromide (tiotropium), a selective long-acting, muscarinic acetylcholine receptor (mAChR) antagonist, is important for treating chronic obstructive pulmonary disease (COPD). Anticholinergic drugs relax airway smooth muscle by blocking mAChRs in the airway and are thus considered an alternative bronchodilator therapeutic option for asthma. Clinical evidence revealed that tiotropium can reduce severe asthma relapse [2]. In Europe and the United States, tiotropium is approved for patients ≥6 years old and with asthma uncontrolled by medium- to high-dose inhaled corticosteroids/long-acting $\beta$2-agonists according to the Global Initiative for Asthma (GINA) 2019 Steps 4 and 5 with a history of exacerbations [3, 4]. Evidence from mouse models show that tiotropium can suppress inflammation and airway remodeling in chronic asthma [5–8]. However, the underlying mechanism is still unclear.

Increasing evidence has shown that changes in programmed cell death or apoptosis mechanisms of resident and mobile cells of the airways may directly contribute to the development and clinical severity of asthma [9]. Apoptosis is also an important process in ameliorating inflammation.

Programmed cell death 5 (PDCD5) is a strong candidate of apoptosis-regulating proteins because of its known role in programmed cell death [10]. PDCD5 was reported to be associated with accelerated apoptosis in response to various stimuli [11]. Moreover, caspases are essential for apoptosis.

Caspase-3 is considered the key executioner caspase in apoptosis [12]. We previously reported increased serum PDCD5 level in asthmatic patients. In addition, we found PDCD5 upregulated in bronchoalveolar lavage fluid (BALF) and lung tissue of ovalbumin- (OVA-) challenged asthmatic mice as compared with controls [13, 14]. Finally, the expression of PDCD5 was correlated with asthma severity and active caspase-3 levels.

In the present study, we established a mouse model of allergic asthma to investigate the effect of tiotropium on the change in asthmatic pathology and the expression of PDCD5.

## 2. Methods

*2.1. Mice and Reagents.* The study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. The animal protocol was approved by the Committee on the Ethics of Animal Experiments of Peking University Health Science Center, Beijing (Permit No. LA2011-062). Twelve BALB/c mice (female, 6–8 weeks old) were obtained from the Department of Laboratory Animal Science (Peking University Health Science Center). They were kept under pathogen-free conditions and had free access to food and water during experiments. Mice were randomly divided into 2 groups ($n = 6$ each): OVA-challenged group (untreated asthma group) and OVA-challenged tiotropium-treated group (tiotropium-treated asthma group).

Chicken egg OVA and aluminum hydroxide powder were from Sigma-Aldrich (St Louis, MO, USA). Tiotropium powder for inhalation (Spirva) was a gift from Boehringer Ingelheim (Ingelheim, Germany). Periodic acid-Schiff staining (PAS) and Masson's trichrome staining (Masson) kits were from Shanghai Yuanye Bio-Technology. The home-made mouse anti-PDCD5 monoclonal antibody and the PDCD5 ELISA kit were gifts from Prof. Yingyu Chen (Center for Human Disease Genomics, Peking University, Beijing). The antiactive caspase-3 antibody was from Abcam (Cambridge, MA, USA). Mouse anti-mAChR M2 (ChRM2) and anti-mAChR M3 (ChRM3) antibodies were from Shanghai Gongshuo Bio-Technology.

*2.2. Induction of Allergic Asthma Mouse Model and Treatment Protocols.* The modified OVA inhalation method was used to generate the allergic asthma mouse model as described in [14]. In brief, OVA sensitization involved an intraperitoneal injection of 20 $\mu$g OVA absorbed with 2.25 mg aluminum hydroxide gel on days 1 and 14. On day 21, mice were placed in an acrylic box ($40 \times 30 \times 15$ cm) connected to an ultrasonic nebulizer (model YC-Y800, Yadu, Beijing). The untreated asthma group was challenged by repeated inhalation with 30 ml OVA (2.5% weight/volume diluted in physiological saline) for 30 min/day on 3 consecutive days/week for up to 8 weeks. For tiotropium treatment, mice were sensitized and challenged as for the untreated asthma group and then from day 21 were treated with tiotropium (36 $\mu$g dissolved in 3 ml sterile physiological saline) with a nebulizer for 5 min before each OVA challenge. Mice were killed 24 h after the last exposure.

*2.3. Pulmonary Function Measurement.* Mice were anesthetized with 10% urethane injected intraperitoneally and were intubated endotracheally by using trocars. Pulmonary function was assessed by using an animal ventilator (ADInstruments, Australia) connected to a pressure sensor. The peak expiratory flow (PEF), peak inspiratory flow (PIF), intra-airway pressure (IP), and maximum rising slope of IP (IP slope) were detected, and data were analyzed by using Chart 4.1 (ADInstruments, Australia).

*2.4. Bronchoalveolar Lavage Fluid (BALF) Cytology.* The mouse lungs were successively lavaged three times with 0.5 ml physiological saline. Recovered BALF was pooled and centrifuged (2000 rpm, 5 min). BALF cells were pelleted and stained with Wright–Giemsa. A differential count of 200 cells was performed. The supernatant was stored at −80°C.

*2.5. Histopathology Study of the Lungs.* The lavaged lungs were inflated with 10% formalin and immersed in 10% formalin fixation solution. Fixed lung tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) for inflammatory cell infiltration and proliferation of smooth muscle, Masson's trichrome for airway fibrosis and collagen deposition, and periodic acid-Schiff (PAS) for goblet cells. An expert respiratory pathologist blinded to treatment groups graded the extent of inflammation in the lungs according to a semiquantitative scoring system [14].

*2.6. ELISA.* To measure the content of PDCD5 in supernatants of BALF, supernatants (100 $\mu$l/well) were added to 96-well ELISA plates and incubated for 60 min at 37°C. After washing, 1 $\mu$g/ml anti-PDCD5 antibody (100 $\mu$l/well) was added and incubated for 60 min at 37°C. After three washes, TMB solution (100 $\mu$l/well) was added for incubation in the dark at room temperature for 15 min. Color development was stopped by adding 2 M $H_2SO_4$ (50 $\mu$l), and absorbance was measured at OD 450 nm ($OD_{450}$).

*2.7. Immunohistochemistry (IHC).* IHC was used to detect the expression of PDCD5, active caspase-3, ChRM2, and ChRM3 in the lung. Paraffin sections of 4 $\mu$m lung tissue were stained with antibodies for PDCD5 (1 : 300), active caspase-3 (1 : 100), and ChRM2 and ChRM3 (1 : 100) by incubating overnight at 4°C. Secondary staining with a goat antimouse antibody involved using an ABC kit and DAB (DAKO, Carpinteria, CA, USA).

*2.8. Statistical Analysis.* Data are presented as mean ± SD. One-way ANOVA was used to compare multiple samples and the Student's independent $t$ test to compare two groups.

Pearson correlation coefficient was used for correlation analysis. $p < 0.05$ was considered statistically significant. Data were analyzed by using SPSS 13.0 and GraphPad Prism 5.0.

## 3. Results

*3.1. Tiotropium Treatment Enhanced Pulmonary Function.* Tiotropium treatment enhanced PIF from $2.26 \pm 0.03$ L/s in the untreated asthma group to $2.29 \pm 0.01$ L/s in the tiotropium-treated asthma group and PEF from $4.66 \pm 0.04$ L/s to $4.80 \pm 0.12$ L/s ($p < 0.05$). The IP slope decreased from $96.02 \pm 3.69$ mmHg/s in untreated asthmatic mice to $74.90 \pm 4.90$ mmHg/s in tiotropium-treated asthmatic mice ($p < 0.001$) (Table 1).

*3.2. Tiotropium Treatment Attenuated Chronic Airway Inflammation and Airway Remodeling.* After tiotropium treatment, the total number of inflammatory cells in BALF of asthmatic mice ($35.92 \pm 9.05 \times 10^4$) showed a decreasing trend without statistical significance ($27.0 \pm 3.97 \times 10^4$) (Table 1).

Histological staining revealed decreased inflammatory cell infiltration in airways and pulmonary vasculature, goblet cell hyperplasia, smooth muscle cell proliferation, peribronchial fibrosis, and collagen in tiotropium-treated asthmatic mice ($p < 0.05$; Table 1).

*3.3. Tiotropium Treatment Reduced PDCD5 Level.* To determine whether PDCD5 was affected by treatment, we tested PDCD5 in mouse BALF and lung tissue. The PDCD5 protein level was higher in BALF of untreated than tiotropium-treated asthmatic mice ($39.89 \pm 7.74$ vs. $29.58 \pm 7.49$ $\mu$g/L, $p < 0.05$) (Table 2) (Figure 1(a)). On IHC, PDCD5 protein staining was reduced in airway epithelial and inflammatory cells after tiotropium treatment ($5.90 \pm 0.58$ vs. $4.47 \pm 0.50$, $p < 0.01$) (Figures 1(b) and 1(c)). In lung tissue, PDCD5 staining intensity was positively correlated with scores for inflammatory cell infiltration, goblet cell metaplasia, and collagen deposition ($p < 0.01$) (Table 3).

*3.4. Tiotropium Treatment Reduced Active Caspase-3 Level.* IHC revealed decreased active caspase-3 level after tiotropium treatment ($5.83 \pm 0.41$ vs. $3.00 \pm 1.10$, $p < 0.001$) (Figures 1(b) and 1(c)). Moreover, active caspase-3 protein level was positively correlated with scores for inflammatory cell infiltration, goblet cell metaplasia, collagen deposition, and total cell number in BALF ($p < 0.05$) (Table 3). PDCD5 and active caspase-3 levels were positively correlated ($r = 0.862$, $p < 0.001$).

*3.5. Elevated ChRM3 Level in Lung Tissues with Tiotropium Treatment.* On IHC, ChRM2 and ChRM3 were expressed mainly in the airway mucosa (including airway epithelial cells and goblet cells), smooth muscle layer, and inflammatory cells around the airway. ChRM3 level was higher with tiotropium treatment than without tiotropium treatment ($5.88 \pm 0.35$ vs. $3.10 \pm 1.07$, $p < 0.001$), with no difference in ChRM2 level with and without tiotropium treatment ($2.33 \pm 0.52$ vs. $2.17 \pm 0.41$, $p > 0.05$) (Figures 1(b) and 1(c)).

## 4. Discussion

Our previous studies reported increased serum PDCD5 level in asthmatic patients and upregulated PDCD5 in BALF and lung tissue of untreated asthmatic mice versus controls, which was correlated with asthma severity [13, 14]. In the present study, we established a mouse model of chronic allergic asthma and diminished the severity of asthma by treatment with tiotropium. Tiotropium treatment improved the lung function of asthmatic mice with relief of airway inflammation and remodeling, accompanied by downregulated PDCD5 and active caspase-3. Tiotropium may be beneficial therapeutically as a bronchodilator and an anti-inflammatory agent by regulating apoptosis.

Although tiotropium is an anticholinergic bronchodilator commonly used to treat COPD, novel pharmacological strategies suggest its benefit as an anti-inflammatory drug [15, 16]. Clinical research proposed the use of tiotropium as an alternative treatment for asthmatic patients ≥6 years old with uncontrolled asthma because its effects appeared equivalent to salmeterol [17, 18]. However, only a few publications exist on the therapeutic effect of tiotropium in animal models of asthma, suggesting its anti-inflammatory effects on allergic airway inflammation besides bronchodilation [5–8, 19].

OVA-sensitized mice can show pathological and clinical features similar to those observed in human chronic asthma. In accordance with previous studies, we found that tiotropium might attenuate airway inflammation and airway remodeling, thus diminishing the severity of asthma. The inflammatory cell number was decreased in airways and pulmonary vasculature with tiotropium treatment, and goblet cell hyperplasia, smooth muscle cell proliferation, peribronchial fibrosis, and collagen deposition were decreased. Nevertheless, tiotropium has been found to inhibit airway resistance and compliance in asthmatic mice [6]. In agreement with this result, PIF and PEF were enhanced after tiotropium treatment in our study, which implies that tiotropium can enhance the pulmonary function of asthmatic mice. However, in disagreement with most studies and our histopathological results, we found no significant difference in BALF cytology, although total cell count and eosinophil percentage were decreased. Similarly, in a guinea pig model of chronic asthma, hematoxylin and eosin results showed that only airway eosinophilia in the submucosa of cartilaginous airways was reduced by tiotropium, with no difference in airway eosinophilia in adventitia of cartilaginous airways after tiotropium treatment or in the submucosa and adventitia of noncartilaginous airways [20]. The possible reason is that being less invasive, BALF may not be enough to reflect differences in this study.

Much evidence has shown that dysregulated cell apoptosis may play a central role in the development of airway

Table 1: Characteristics of untreated asthma mice and tiotropium-treated asthma mice.

| Characteristics | Untreated asthma mice | Tiotropium-treated asthma mice | $p$ value |
|---|---|---|---|
| *Pulmonary function* | | | |
| Peak inspiratory flow (L/s) | 2.26 ± 0.03 | 2.29 ± 0.01 | 0.030 |
| Peak expiratory flow (L/s) | 4.66 ± 0.04 | 4.80 ± 0.12 | 0.034 |
| Intra-airway pressure (IP, mmHg) | 2.38 ± 0.06 | 2.33 ± 0.06 | 0.182 |
| Maximum rising slope of IP (mmHg/s) | 96.02 ± 3.69 | 74.90 ± 4.90 | <0.001 |
| *BALF cytology* | | | |
| Total cell count ($\times 10^4$) | 35.92 ± 9.05 | 27.00 ± 3.97 | 0.064 |
| Macrophages (%) | 67.00 ± 4.86 | 66.50 ± 3.73 | 0.846 |
| Eosinophils (%) | 11.50 ± 3.02 | 10.50 ± 1.87 | 0.509 |
| Neutrophils (%) | 10.67 ± 2.50 | 11.5 ± 1.87 | 0.530 |
| Lymphocytes (%) | 10.83 ± 1.47 | 11.50 ± 2.17 | 0.549 |
| *Pathological scores* | | | |
| Inflammatory cell infiltration | 2.50 ± 0.84 | 1.25 ± 0.42 | 0.013 |
| Goblet cell hyperplasia | 1.92 ± 0.58 | 1.17 ± 0.41 | 0.030 |
| Collagen deposition | 1.42 ± 0.38 | 0.67 ± 0.26 | 0.003 |

Data are mean ± SD.

Table 2: Programmed cell death 5 (PDCD5) level in BALF and lung tissues in mouse groups.

| PDCD5 | Untreated asthma mice | Tiotropium-treated asthma mice |
|---|---|---|
| BALF ($\mu$g/L) | 39.89 ± 7.74 | 29.58 ± 7.49* |
| Lung tissue | 5.90 ± 0.58 | 4.47 ± 0.50** |

Data are mean ± SD. * $p < 0.05$ and ** $p < 0.01$ compared with untreated asthma mice.

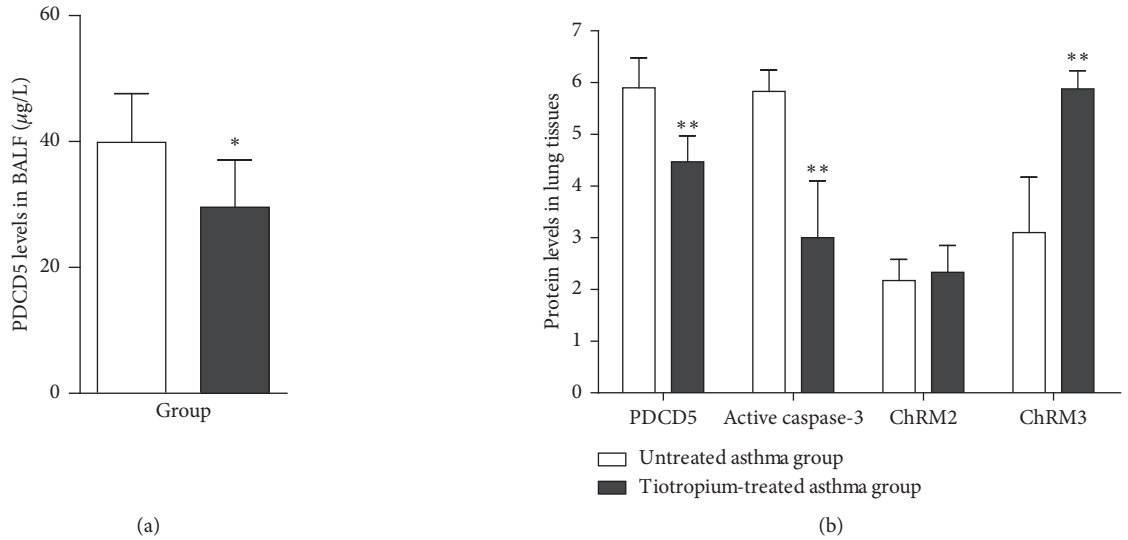


Figure 1: Continued.

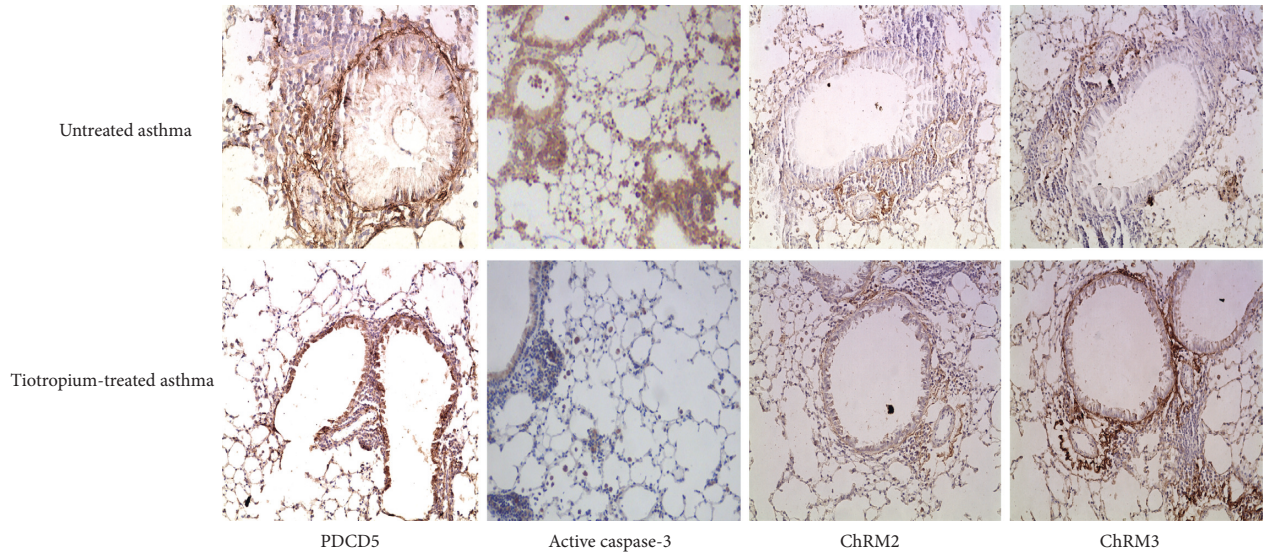


Figure 1: Protein expression. (a) PDCD5 protein level in BALF. (b) Protein levels in lung tissue. (c) Representative immunohistochemical staining of PDCD5, active caspase-3, and muscarinic acetylcholine receptors M2 (ChRM2) and M3 (ChRM3). Data are mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with untreated asthma mice.

Table 3: Correlation between PDCD5/active caspase-3 level and various clinicopathologic indexes ($n = 12$).

| Protein level in lung tissue | | Inflammatory cell infiltration | Goblet cell metaplasia | Collagen deposition | Total cell number in BALF |
|---|---|---|---|---|---|
| PDCD5 | $r$ | 0.904 | 0.814 | 0.773 | 0.258 |
| | $p$ value | <0.001 | 0.001 | 0.003 | 0.418 |
| Active caspase-3 | $r$ | 0.754 | 0.681 | 0.739 | 0.625 |
| | $p$ value | 0.005 | 0.015 | 0.006 | 0.030 |

BALF: bronchoalveolar lavage fluid.

inflammation in asthma [21]. The process of apoptosis is important because it allows for rapid clearance of senescent or damaged cells, for limited tissue injury. PDCD5 was upregulated in cells undergoing apoptosis. In this study, PDCD5 expression in asthmatic airway epithelium and inflammatory cells was decreased when tiotropium relieved airway inflammation and remodeling. In agreement with our previous results, the level of PDCD5 was correlated with the lung function, inflammatory cell infiltration, goblet cell metaplasia, and collagen deposition. The consistent correlation between PDCD5 expression and severity of asthma indicated not only the potential role of PDCD5 in monitoring asthmatic severity but also a correlation between tiotropium and apoptosis. Tiotropium was previously suggested to affect apoptosis. In COPD patients, tiotropium was found to reduce CD4+ and increase CD8+ peripheral blood T-cell apoptosis via caspase-3 and caspase-8 activity and IκB-mediated mechanisms [22]. In a subacute cigarette exposure mouse model, tiotropium significantly decreased the number of macrophages and caspase-3-labeled cells of the lungs [23]. In this study, we suggest that tiotropium might relieve airway inflammation by regulating apoptosis of airway epithelium and inflammatory cells. PDCD5 could evoke apoptosis of cells [24, 25]. The initiation of apoptosis serves to terminate the inflammatory process by reducing the number of inflammatory cells, but the persistence of inflammation may be due to abnormalities in the regulation of cell apoptosis, leading to a chronic or everlasting inflammatory cell survival and accumulation. Thus, with resolution of airway inflammation and clearance of inflammatory cells with tiotropium, PDCD5 expression was reduced. However, further study is needed to explore the effect of tiotropium on apoptosis.

Active caspase-3 is the key executioner of caspase and an early marker of apoptosis [12, 26]; therefore, we measured the level of active caspase-3 to confirm the correlation between tiotropium and apoptosis. Similar to PDCD5, activated caspase-3 was reduced with tiotropium treatment, and such downregulation was positively correlated with PDCD5 level ($p < 0.001$). Downregulated active caspase-3 indicates decreased cell apoptosis during the resolution of airway inflammation. We further identified the correlation between tiotropium and apoptosis.

Tiotropium is a selective ChRM3 antagonist that dissociates more slowly from M3 than M2 or M1 muscarinic receptors [27]. ChRM1 receptors are mainly distributed in the peripheral lung tissue and in the alveolar walls [28] and regulate cholinergic transmission [29]. ChRM2 receptors are on smooth muscle (SM) cells and fibroblasts [28]. Together with ChRM2, ChRM3 receptors are the most represented in

human airways; they are predominantly expressed in SM cells and mediate SM ACh-induced contraction [28, 29]. ChRM3 is a G-protein-mediated receptor and may play an important role in the pharmacological effects of tiotropium [30, 31]. Some studies demonstrated that ChRM2 and ChRM3 receptors mediate proliferation of lung fibroblasts or SM cells, and tiotropium can inhibit such proliferation [32–34]. Considering the possible role of ChRM2 and ChRM3 receptors in tiotropium regulating apoptosis, we examined their levels in lung tissues. In lipopolysaccharide-induced lung inflammation, blockage of mAChR exerts anti-inflammatory properties, with ChRM3 receptors playing an important role by mediating NF-$\kappa$B signaling [35]. In murine models of COPD and asthma, ChRM3 expression was inhibited by the administration of tiotropium [36, 37]. Holownia et al. [38] found that tiotropium could increase cytosolic ChRM3 protein level in induced sputum cells of COPD patients. Similarly, ipratropium bromide, another common acetylcholine receptor antagonist, could upregulate ChRM3 expression in bronchial walls of an asthmatic murine model [39]. The results of existing studies are inconsistent. In our study, ChRM3 level was elevated after tiotropium treatment. Upregulated ChRM3 might be a compensatory result after continuous application of antagonists. No difference in ChRM2 level between the two groups may suggest that tiotropium did not affect ChRM2 level, perhaps because tiotropium dissociated faster from ChRM2 than ChRM3. These results suggest that anti-inflammatory effects rather than a cholinergic action in airway muscle may be the main role of tiotropium.

There were several limitations to this study. First, our asthmatic mice model is a chronic asthma model and not treated with steroids; thus, pathogenesis and pathological changes may not be similar to clinical refractory asthma. Recent published data suggested no significant difference in the response to mometasone or tiotropium as compared with placebo in mild persistent asthma with low eosinophil level [40]. Thus, exploring the mechanisms of tiotropium in asthma is relevant for all disease severity levels (so patient therapy should be individual and probably more effective in low eosinophilic asthma). Second, we did not perform in-depth research of the pathogenesis of severe asthma. Third, the molecular mechanism of the effect of tiotropium on apoptosis was not studied. Treating airway inflammatory cells with tiotropium may help explore the underlying mechanism.

## 5. Conclusions

Taken together, we found that tiotropium could improve the lung function and reduce PDCD5 level in OVA-induced asthmatic mice. Tiotropium may relieve clinical pathological changes by regulating apoptosis in asthma. Future studies are needed to determine the role of tiotropium in asthma.

## Data Availability

The data used to support the findings of this study are included within the article.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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