One mechanism of glucocorticoid action in asthma may involve the inhibition of IL-25 expression

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Abstract. While the mechanism of action of classic cytokines in asthma has received increased attention from researchers, certain non-classical cytokines, such as IL-25, also participate in this mechanism. The present study was performed to investigate the changes in IL-25 (IL-17E) mRNA and protein in bronchial asthma and to further characterize the mechanism underlying the action of glucocorticoids in asthma. A total of 96 specific pathogen-free BALB/c male mice were randomly divided into three normal groups (after the first allergization, after the second allergization and after excitation), three asthma groups (with the same three subgroups), a dexamethasone group and a budesonide group (n=12/group). An asthma model was established via the ovalbumin-sensitized excitation method. Mice in the dexamethasone group received intraperitoneal injections of dexamethasone 1 h prior to each excitation, the budesonide group received a budesonide suspension via inhalation 2 h before and after each provocation, and the normal group was sensitized and challenged with isotonic saline. IL-25 protein expression levels in the bronchoalveolar lavage fluid were measured by ELISA, and the relative IL-25 mRNA content in lung tissue was determined by reverse transcription-quantitative polymerase chain reaction. Compared with the normal groups, both the protein and mRNA levels of IL-25 were significantly increased ($P<0.05$) in the asthma groups. Dexamethasone and budesonide groups exhibited significant protein and mRNA reductions in IL-25, as compared with the asthma group after excitation ($P<0.05$), whereas these two groups significantly increased levels compared with the normal group after excitation ($P<0.05$). No significant differences in IL-25 mRNA expression levels were detected in the dexamethasone and budesonide groups when compared with the normal group after excitation. Therefore, we conclude that IL-25 is involved throughout the process of inflammation and inflammatory immune pathogenesis in asthma. One of the mechanisms of glucocorticoid action in asthma may involve inhibition of IL-25 expression.

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Introduction

The pathogenesis of asthma is yet to be fully elucidated. Immune and endocrine mechanisms, as well as genetic background, are considered to have an important role in asthma (1-3). Among these, the imbalance of Th1/Th2 cells has been demonstrated to be the most important among this class of immune factors, due to imbalances in their quantity, activation and function. Therefore, the Th2-type immune response is one of the predominant characteristics of airway inflammation due to asthma and is associated with the formation and maintenence of factors during the pathophysiological process of asthma (1-3). Th2 cytokines have a promoting effect in this process; however, the majority of research in this field has been predominantly focused on interleukin (IL)-4, IL-5, IL-10 and IL-13 (1,2), certain non-classical cytokines, such as IL-25 (IL-17E), also have an important role in asthma (3). Various studies using exogenous IL-25 or transgenic animals have demonstrated that IL-25 is able to promote the Th2-type immune response (4-7).

To date, there remains no cure for asthma in clinical practice, and anti-inflammatory therapy remains the primary strategy for treating asthma. Glucocorticoids are the preferred therapeutic strategy for treating asthma due to their powerful and comprehensive anti-inflammatory function. These compounds correct the Th1/Th2 imabalance, although the effects of glucocorticoids on the expression of the IL-25 gene remain unclear (8). There are two main types of hormone dosage protocols for glucocorticoids, systemic and local use. Dexamethasone is a common systemic-use hormone, and budesonide is a local-use hormone, which is inhaled (1). However, it remains unclear whether these two delivery routes have the same impact on IL-25.

Therefore, the present study established an asthma model with dexamethasone and budesonide groups and detected the expression levels of IL-25 mRNA at various time points (following the first allergization, following the second allergization and post-excitation) and different sections [lung tissue and bronchoalveolar lavage fluid (BALF)] to investigate the

Table I. Forward and reverse primers for interleukin (IL)-25 and β-actin mRNA.

Gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
IL-25	NM_080729.2	CTCAACAGCAGGGCCATCTC	GTCTGTAGGCTGACGCAGTGTG
β-actin	NM_007393.3	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

alterations in IL-25 mRNA and protein expression levels in bronchial asthma, to compare the treatment effect of systemic- and local-use glucocorticoids, and to further characterize the mechanism underlying the action of glucocorticoids in asthma.

Materials and methods

Animals and materials. A total of 96 specific pathogen-free male BALB/c mice (Shanghai Laboratory Animal Center of the Chinese Academy of Sciences, Shanghai, China); ultrasonic nebulizer (Pari Company, Starnberg, Germany); iQ5 multi-color real-time PCR detection system (BioRad Laboratories, Inc., Hercules, CA, USA); mastercycler gradient PCR cycler (Eppendorf AG, Hamburg, Germany); TU1810 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China); ovalbumin (OVA, V-grade) and aluminum hydroxide gel (Sigma-Aldrich, St. Louis, MO, USA); budesonide suspension (AstraZeneca, London, UK); 0.9% sodium chloride solution (isotonic saline) and dexamethasone injection (Sichuan Kelun Pharmaceutical Co., Ltd., Sichuan, China); mouse IL-25 ELISA kit (060728; Shenzhen Xin Bo Sheng Biological Technology Co., Ltd., Shenzhen, China); and RNA extraction, reverse transcription and RNA purification kits (Takara Biotechnology Co., Ltd., Dalian, China).

Groups. A total of 96 mice, aged 6-8 weeks and weighing 18±2 g, were randomly divided into three normal groups (after the first allergization, after the second allergization and after excitation), three asthma groups (after the first allergization, after the second allergization and after excitation), a dexamethasone group and a budesonide group. Each group contained 12 mice. Mice were maintained at a density of five mice/cage at 25±2˚C (60-70% humidity) with a 12-h light/dark cycle and *ad libitum* access to food and water. The present study was approved by the Medical Ethics Committee of Zun Yi Medical College.

Model establishment. Mice in the asthma, dexamethasone and budesonide groups received intraperitoneal injections with 0.1 ml solution containing 25 μg OVA mixed with 2 mg aluminum hydroxide gel at day 0 and day 10, and were subsequently excited with a 1% OVA solution through ultrasonic atomization for 30 min from day 14 to day 20 (9). Additionally, mice in the dexamethasone group received an intraperitoneal injection of 2 mg/kg dexamethasone 1 h prior to each excitation (10), and the budesonide group was administered 0.5 mg budesonide suspension mixed with 5 ml isotonic saline via inhalation for 15 min 2 h before and after each provocation (11). Mice in the normal group were sensitized and challenged with isotonic saline.

Sample collection. Mice in the normal and asthma groups that only received the first sensitization were anesthetized via an intraperitoneal injection of 10% chloral hydrate (0.075 ml/mouse; Jiang-lai Biological Research Reagent Professional Supplier, Shanghai, China) and sacrificed on day 9 by exposing their abdominal aorta, whereas mice in the normal and asthma groups that received a second sensitization were sacrificed on day 13. Clinical manifestations of the mice were assessed in the normal and asthma groups after excitation, as were the dexamethasone and budesonide groups during excitation, and these mice were sacrificed within 24 h of the last excitation. The left lungs of the mice and their BALF were harvested, as were the right lungs of the mice in the normal and asthma groups after excitation, and the dexamethasone and budesonide groups, to analyze pathological sections for hematoxylin and eosin staining. Briefly, a small amount of BALF was dropped onto the counting board and the leukocytes were counted under low magnification from one side of the four big boxes, respectively. The total number of cells was then calculated according to the following formula: C (a/ml) = n/4 x 10^4, where C is the total number of cells and n is the number of leukocytes from one side of the four big boxes.

The remaining BALF was then centrifuged at 300 x *g* for 10 min at 4˚C, and the sedimentation was used to create slides with Wright-Giemsa compound stain. The total number of eosinophils (EOS) in the BALF samples were recorded under high magnification from ≥200 white cells by a single individual who was blinded to the protocol, and the percentages of EOS among the leukocytes were calculated.

ELISA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). IL-25 levels were determined via ELISA, according to the manufacturer's protocol. For RT-qPCR, primer sequences (Table I) were extracted from GenBank, designed with Primer 3.0 software (primer3.ut.ee/) and synthesized by the Shanghai Biological Engineering Technology Services Company (Shanghai, China). Total RNA from 50-100 mg of left lung tissue was homogenized in TRIzol, extracted using chloroform and purified via precipitation with equal amounts of isopropanol and 75% alcohol. RNA quality was determined by the 260/280 ratios. Purified RNA was reverse transcribed with Oligo-dT primers and MuLV reverse transcriptase (Takara Biotechnology Co., Ltd.). Reverse transcription was performed in a 10-μl reaction mixture containing 2 μl PrimeScript Buffer (5X), 0.5 μl PrimeScript RT Enzyme mix, 0.5 μl Oligo dT Primer, 0.5 μl random hexamers, 6.5 μl total RNA. Thermal cycling conditions were as follows: 37˚C for 15 min, 8˚C for 5 sec and holding at 4˚C. The Power SYBR Green Master Mix (Takara Biotechnology Co., Ltd.) was used for qPCR analysis in a 20-μl reaction volume containing 10 μl SYBR *Premix Ex Taq* II, 1.6 μl PCR Primer, 2 μl cDNA and 6.4 μl dH2O. PCR thermal cycling

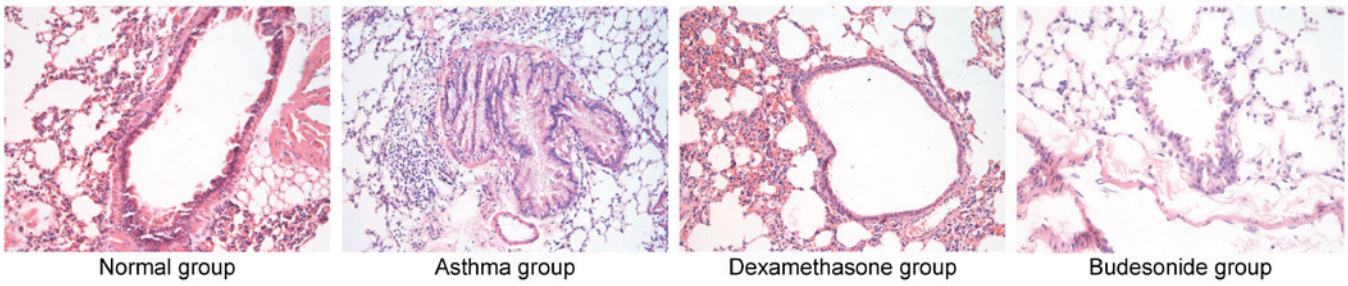

Figure 1. Pneumonic pathology of the four groups following excitation, as detected by hematoxylin and eosin staining (magnification, x400).

was performed at 95˚C for 3 min (pre-denaturation), 95˚C for 10 sec (denaturation) and 60˚C for 45 sec (annealing) for 40 cycles. Relative expression of IL-25 mRNA was calculated according to the $2^{-\Delta\Delta Cq}$ method (12) and were normalized to the housekeeping gene, β-actin. Procedures were repeated three times for each sample.

Statistical analysis. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. Data with a normal distribution were expressed as the mean ± standard deviation. Comparisons between two groups were performed using the independent samples *t*-test, and comparisons among multiple groups were performed using one-way analysis of variance or the pair-wise least significant difference *t*-test when the variance was homogeneous. A non-parametric test was employed when the variance was heterogeneous after calibration. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical manifestations in the mice. Following excitation, mice in the asthma group exhibited irritability, increased nasal secretions, scratching of the nose, shortness of breath, respiratory distress, and unresponsive and limp limbs. These characteristics were markedly reduced in the budesonide and dexamethasone groups, suggesting that budesonide and dexamethasone treatment may reduce the allergic clinical manifestations of asthma.

Pneumonic pathology. Pathological sections from the asthma group following excitation exhibited luminal narrowing in the bronchi, bronchioles and alveoli, a widened alveolar septum, inflammatory cells (predominantly eosinophils), infiltration of the tube wall and the pulmonary interstitial, exudates in the bronchioles and alveoli, and partial epithelial necrosis of the bronchi and bronchioles with goblet cell hyperplasia. In contrast, the inflammatory responses in the sections harvested from mice in the budesonide and dexamethasone groups were markedly reduced (Fig. 1), suggesting that budesonide and dexamethasone treatment may reduce the allergic clinical manifestations of asthma.

Cell counting. Cell counts obtained from the BALF are shown in Figs. 2-4. These results demonstrated that the total white cell count significantly increased in a time-dependent manner over the period that the asthma model was established

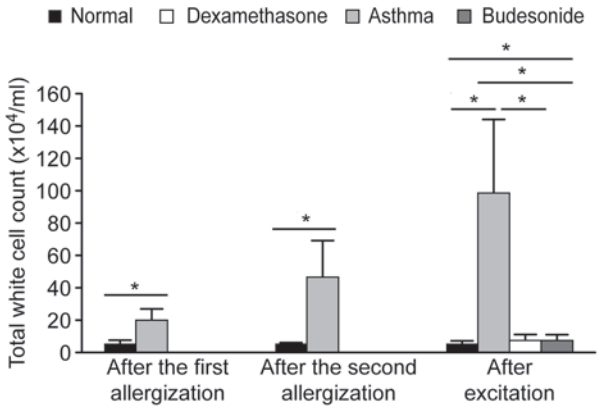

Figure 2. Total white cell count in the bronchoalveolar lavage fluid. Total white cell count was significantly lower in the asthma group after the first allergization, as compared with after second allergization and excitation. Total white cell count was significantly lower in the asthma group after the second allergization, as compared with after excitation. *P<0.05.

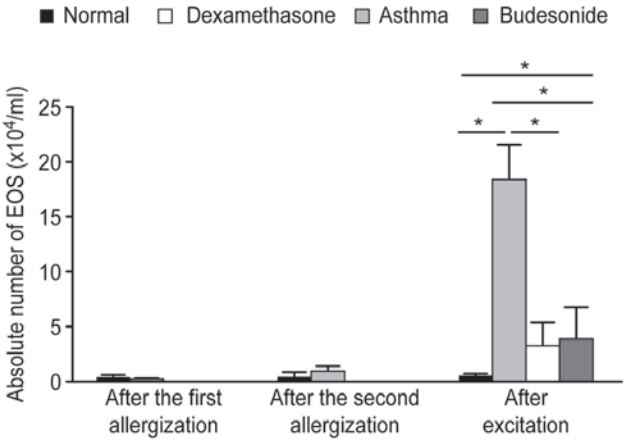

Figure 3. Absolute number of EOS in the bronchoalveolar lavage fluid. Absolute number of EOS was higher in the asthma group after excitation, as compared with after the first and second allergizations (*P<0.05). EOS, eosinophils.

(P<0.05). Furthermore, the number and percentage of EOS significantly increased until the model was successfully established (P<0.05). In contrast, dexamethasone and budesonide administration significantly decreased the number of white cells and EOS to normal levels (P<0.05). These findings suggest that budesonide and dexamethasone treatment may reduce the inflammatory responses in asthma by reducing the total white cell count and the number and percentage of EOS.

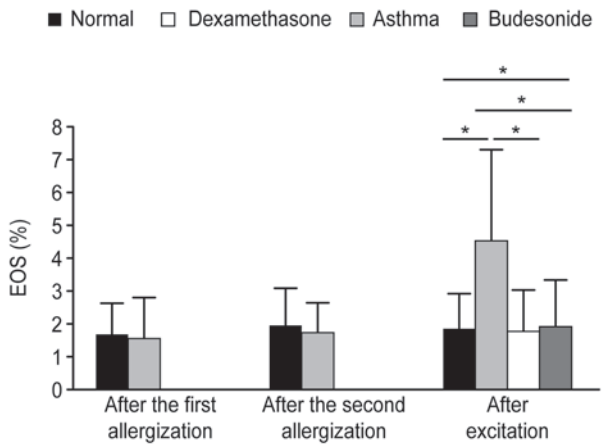

Figure 4. Percentage of EOS among all white cells. Percentage of EOS was significantly higher in the asthma group after excitation, as compared with after the first and second allergizations (*P<0.05). EOS, eosinophils.

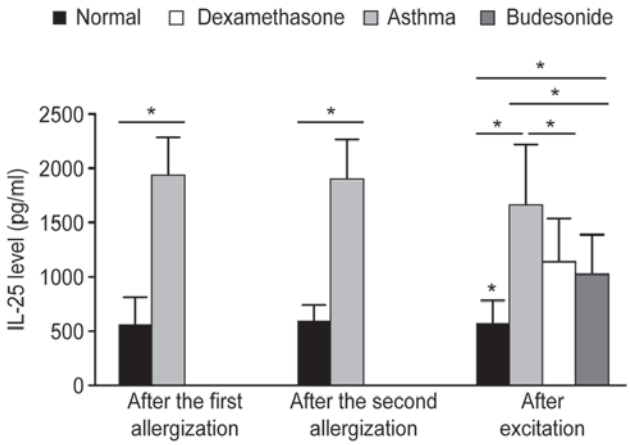

Figure 5. Concentration of IL-25 in the bronchoalveolar lavage fluid. IL-25 levels were lower in the normal group after excitation, as compared with the dexamethasone and budesonide groups (*P<0.05). IL, interleukin.

IL-25 protein and mRNA expression levels. The results of ELISA analysis demonstrated that IL-25 protein levels were significantly increased in the asthma group, as compared with the normal group after each sensitization and challenge (P<0.05). In contrast, the protein expression levels of IL-25 were significantly inhibited in the dexamethasone and budesonide groups, as compared with the asthma group (P<0.05); although IL-25 levels were significantly elevated in the dexamethasone and budesonide groups when compared with the normal group (P<0.05; Fig. 5).

The results of RT-qPCR analysis demonstrated that the relative levels of IL-25 mRNA were significantly increased in the asthma group, as compared with the normal group after each sensitization and challenge (P<0.05). In contrast, dexamethasone and budesonide significantly inhibited the expression of IL-25 mRNA (P<0.05), and there was no significant difference in IL-25 mRNA expression when the dexamethasone and budesonide groups were compared with the asthma group (Fig. 6). These findings suggest that budesonide and dexamethasone may reduce the inflammatory responses in asthma by inhibiting the expression of IL-25 mRNA.

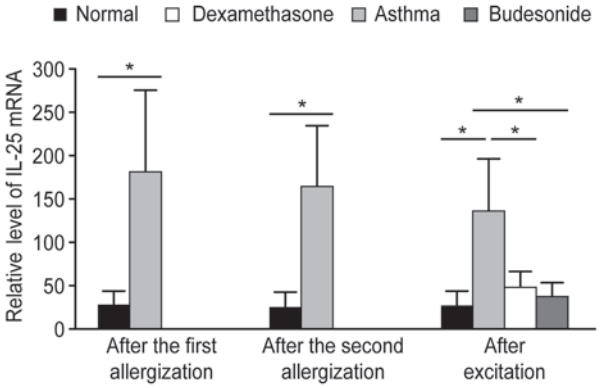

Figure 6. Relative transcript levels of interleukin (IL)-25 mRNA in the lung tissue. *P<0.05.

Discussion

IL-25, which belongs to the IL-17 family, is predominantly derived from Th2 cells, although a small quantity is also derived from the mast cells, macrophages, eosinophils and basophils. The receptor for IL-25 is IL-17RB. IL-25 predominantly targets memory Th2 cells and the subsidiary cells of the non-T and non-B cells, such as marrow-derived mast cells and alveolar macrophages (6,7,13). Previous studies have shown that IL-25 can induce inflammation and the inflammatory immune reaction of asthma; increase eosinophils, Th2 cells, IL-4, IL-5 and IL-13 in the lungs; increase IgE, IgA and IgG1 levels in serum; and induce airway hyperreactivity, hyperplasia of the lung epithelial cells, mucus hypersecretion and airway remodeling (4-7,14,15). Therefore, the present finding that IL-25 mRNA and protein expression levels were increased after each allergization and excitation verified the involvement of IL-25 throughout the inflammation and inflammatory immune process of asthma.

We hypothesize that the mechanism responsible for the increase in IL-25 may be associated with increases in IL-25-secreting cells, such as Th2 cells, eosinophils and basophils, and the induction of leukocytes in the BALF by various initiating factors, which may enhance the expression of the IL-25 gene. The increased IL-25 mRNA expression levels demonstrated in the present study may also promote the expression of IL-25 and further elevate IL-25 levels in the BALF through the secretion of pneumonic cells.

Wang *et al* (16) indicated that the mechanisms by which IL-25 participates in the inflammation associated with asthma included the following: i) During the early activation of T cells, IL-25 may activate the T-cell nuclear factor c1 and the JunB transcription factor, thus increasing the generation of IL-4 and the expression of GATA-3 and promoting the differentiation of Th2 cells; and ii) IL-25 may promote the proliferation of central memory Th2 cells to generate Th2-type cytokines.

Meanwhile, the mechanisms by which IL-25 induces the inflammatory immune response of asthma include the following: i) IL-25 induces target cells to express IL-4, IL-5 and IL-13 (4); ii) synergistically with anti-CD3 and anti-CD28, IL-25 stimulates Th2 cells, particularly memory Th2 cells, to express IL-4, IL-5, IL-6, IL-10, CXCL9, CXCL10 and CCL5 (17); iii) IL-25 also stimulates Th2 cells to express intercellular adhesion molecule-1, MCP-1 (monocyte chemoattractant protein-1, monocyte

inflammatory protein-1α, IL-8 and IL-6 (17); and iv) IL-25 mediates airway inflammation through the signal transduction protein activator of transcription-6 signaling pathway (7), promotes the aggregation of neutrophils (18) and induces the inflammatory cascade, which increases allergic airway inflammation (14).

In the present experiment, dexamethasone inhibited the expression of IL-25 mRNA and protein, indicating that glucocorticoids may reduce the severity of asthma by inhibiting the transcription and protein expression of the IL-25 gene. We hypothesize that the underlying mechanisms may be that dexamethasone suppressed the transcription and protein expression of the IL-25 gene, which decreased the production of IL-25 mRNA, while also suppressing the synthesis and release of IL-25, thus reducing the stability of IL-25 and downregulating the function of the target cells of IL-25 and their associated signaling pathways.

Since, in the dexamethasone group, IL-25 levels did not completely decrease to normal levels, IL-25 may be involved in the mechanism of glucocorticoid resistance. This phenomenon may be explained by the long duration of time that is required for the clearance of IL-25, and that fact that certain pro-inflammatory factors increase the stability of IL-25 and decrease its degradation. Previous studies have indicated that soluble IL-25R and an IL-25 monoclonal antibody (mAb) can alleviate lung inflammation in mice, and the IL-25 mAb can prevent the binding of IL-25 and IL-25R in the human body (6,14,19,20). Therefore, IL-25 may be considered as an adjunctive treatment for asthma.

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