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Dexamethasone alleviate allergic airway inflammation in mice by inhibiting the activation of NLRP3 inflammasome

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ABSTRACT

Dexamethasone (DEX) is the mainstay treatment for asthma, which is a common chronic airway inflammation disease. However, the mechanism of DEX resolute symptoms of asthma is not completely clear. Here, we aimed to analyze the effect of DEX on airway inflammation in OVA-induced mice and whether this effect is related to the inhibition of the activation of NLRP3 inflammasome. Female (C57BL/6) mice were used to establish the allergic airway inflammation model by inhalation OVA. The number of inflammatory cells in the bronchi alveolar lavage fluid (BALF) was counted by Swiss-Giemsa staining, and the contents of IL-1 β , IL-18, IL-5 and IL-17 were detected by ELISA. The degree of inflammatory cells infiltration and mucous cells proliferation in lung tissue were separately observed by H&E and PAS staining. The proteins expression of NLRP3, pro-caspase-1, caspase-1, IL-1 β , IL-6 and IL-17 in lung tissue were detected by Western blotting. We found that DEX significantly inhibited OVA-induced inflammatory cells infiltration, airway mucus secretion and goblet cell proliferation in mice. The total and classified numbers of inflammatory cells and the levels of IL-1 β , IL-18, IL-5 and IL-17 in the BALF of the experimental group were significantly lower than those of the model group after DEX treatment. DEX also significantly inhibited the activity of NLRP3 inflammasome and reduced the protein contents of Pro-Caspase-1, Caspase-1, Caspase-1/Pro-Caspase-1, IL-1 β , IL-6 and IL-17 in lung tissues. Our study suggested that DEX alleviates allergic airway inflammation by inhibiting the activity of NLRP3 inflammasome and the levels of IL-1 β and IL-18.

1. Introduction

Bronchial asthma is a common chronic allergic airway inflammation, disease involving a variety of inflammatory cells. Asthma affects more than 20% of the population in the world [1], especially children in industrialized countries [2]. The incidence of allergic asthma has continued to rise over the past few decades [3]. However, T helper2 (Th2) cells and allergic inflammation play an important role in the symptoms and pathophysiology of asthma [4,5].

Interestingly, inflammasome is a variety of protein signal complexes, including NLRP1, NLRP3, IPAF and AIM2, of which NLRP3 inflammasome is one of the extensive features of inflammatory diseases. Recent studies have shown that lung epithelial cells expressed the

activated NLRP3 inflammasome and caspase-1 [6]. More study demonstrated NLRP3 plays a key role in respiratory epithelial-mediated immune response [7]. Previous studies showed that the expression of NLRP3 is significantly increased in the lung tissue of asthmatic mice, and the expression of IL-1 β and IL-18 is also increased in the peripheral blood of patients with asthma [8]. It has been reported that the activation of NLRP3 inflammasome can activate Caspase-1 and increase the production of IL-1 β and IL-18 [9]. At the same time, IL-1 β regulated by NLRP3 inflammasome may lead to a vicious circle of asthma [10]. Therefore, NLRP3 inflammasome plays an important role in host defense response [11].

At present, glucocorticoid therapy, represented by DEX, is still the mainstay treatment for asthma [12]. DEX is an immunosuppressant

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with anti-inflammatory effect [13,14]. It can alleviate the inflammation and pathology of asthma. However, the mechanism of DEX regulating immune response needs to be further studied. In this study, the asthma model of C57 mice was established, and the relationship between the efficacy of DEX and the activation of NLRP3 inflammasome was determined.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice (five weeks old) were acquired from the Shanghai Laboratory Animal Center Co., Ltd. (SLAC, Shanghai, China). The mice were raised in an SPF laboratory with accessing food and water freely. They were allowed to adapt themselves for one week before the experiment. This study was approved by the Animal Ethic Committee of Anhui Medical University and complied with the regulations on the Management of Experimental Animals in the whole process of the experiment.

2.2. Drug and reagents

DEX and OVA were purchased from sigma Co., Ltd. (Louis, MO, USA). Antibodies of NLRP3 and Caspase-1 were purchased from AdipoGen (San Diego, USA). Antibodies of TNF- α and IL-6 were brought from Cell Singling Technology (Cell Signaling Technology, Danvers, MA). IL-17 antibody was gotten from Abcam (Abcam, Cambridge, MA). ELISA kits of IL-1 β , IL-5 and IL-17 were purchased from Wuhan Huamei Co., Ltd. (Shanghai, China). ELISA kits of IL-18 were brought from Wuhan USCN Business Co., Ltd. (Shanghai, China). HRP-conjugated anti-rabbit IgG secondary antibody brought from Agilent (California, USA). Anti-GAPDH was acquired from KANGCHEN Biotech (Shanghai, China).

2.3. OVA-induced asthma and therapeutic interventions

Mice were randomly divided into three groups: control group, model group (OVA) and experimental (OVA + DEX) group. Each group contains six mice. The mice in the Model group and OVA + DEX group were intraperitoneally injected with 0.5 ml saline containing 10 μ g OVA and 1 mg aluminum potassium sulfate on day 0 and 7. Normal group were intraperitoneally injected with the same amount of saline containing 1 mg aluminum potassium sulfate. From the 14th day, the normal group began to inhale saline, while the other two groups inhaled 1% OVA solution for 30 min for seven consecutive days. One hour before the challenge, the normal group and the model group were intraperitoneally injected with saline of 0.4 ml, while the DEX group was intraperitoneally injected with 0.4 ml DEX solution (2 mg/kg/d) (Fig. 1). At 24 h after final challenge, the mice were sacrificed.

2.4. Samples collection

Within 24 h after the last challenge, the blood samples were

collected and then centrifuged for 10 min at 4 °C, 3000 rpm/min. The supernatant was collected and stored at -80 °C. Immediately after sacrifice, the BALF from the left lung of mice was harvested by endotracheal intubation, and then centrifuged for 10 min at 4 °C, 3000 rpm/min. The supernatants were stored at -80 °C and the pellets used for cell counting. A part of the right lung tissues were fixed in 4% paraformaldehyde for histology analysis, and the other parts were stored at -80 °C for Western blotting.

2.5. BALF cell count

After BALF centrifugation, the cell pellets were re-suspended with 200 μ l PBS. BALF cells were counted as previously described [15,16]. Briefly, Wright's-Giemsa staining one of the staining methods for distinguishing blood cells was used to count the number of lymphocytes, monocytes, neutrophils, and eosinophils. Under the microscope, leukocytes were counted and classified by counting at least 200 cells for each mouse. However, total cells were counted by hemocytometer.

2.6. Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's instructions of ELISA kits, the levels of IL-1 β , IL-18, IL-5 and IL-13 in BALF supernatant were measured. The concentration was calculated by using the standard curve generated by the standard sample.

2.7. Lung histological analysis

The fixed lung tissues of each mouse were embedded in paraffin and then cut into the paraffin-embedded tissue sections (4 μ m), which were stained with hematoxylin/eosin (H&E) or periodic acid-Schiff (PAS) to observe the histological changes. Image pro plus 6.0 was used to measure the total area of the bronchial wall (Wat) and bronchial basement membrane perimeter (Pbm). The ratio of Wat to Pbm (Wat/Pbm) was used for evaluating the thickness of the bronchial wall. Three images were analyzed per section at 200 $\times$ magnification [17,18]. The methods for evaluating the score of per bronchial inflammatory infiltration are as follows: 0, normal; 1, a few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep and 4, a ring of inflammatory cells > 4 cells deep. In addition, the evaluation methods of goblet cell proliferation around the airway are based on follows: 0, normal; 1, less than 25% of the epithelium; 2, 25–50% of the epithelium; 3, 50–75% of the epithelium; and 4, > 75% of the epithelium [19].

2.8. Western blotting

The contents of NLRP3, Caspase-1, IL-1 β , IL-6, IL-17 and TNF- α in mice were detected by western blotting. The lung tissues of each mouse were homogenized in RIPA buffer with protease inhibitor cocktail and 1 mM EDTA. The same amount of protein (25 μ g/lane) was separately electrophoresed in 12% SDS-PAGE gel, and transferred to PVDF at a low temperature. The membranes were blocked for one hour in 5%

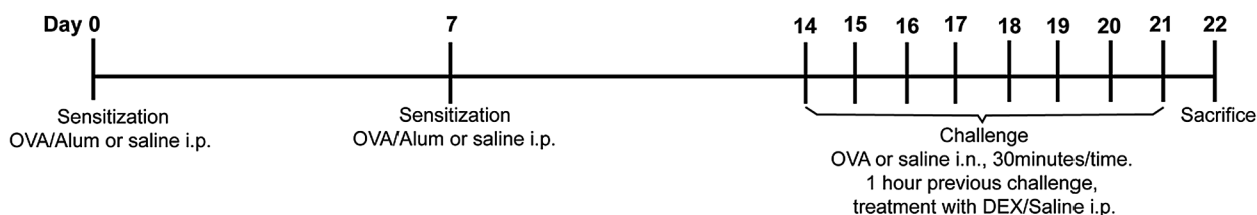


Fig. 1. The timeline of the acute asthma model in mice. Mice were sensitized by intraperitoneal injection of 0.5 ml OVA /Alum or saline on day 0 and 7. Then from the 14th day, the normal group began to inhale saline, while the other two groups inhaled 1% OVA solution for 30 min for seven days. One hour before OVA challenge, the mice in the administration group were injected DEX on day 14–21. The normal group and model group mice were given the same amount of saline. All mice were sacrificed on day 22.

skimmed milk powder (The TBST solution contains 5% skimmed milk powder and 0.1% Tween 20). After that, the membranes were incubated with primary antibody (NLRP3, Caspase1, IL-1 β , IL-6, IL-17 and TNF- α 1:1000) overnight at 4 °C and incubated the secondary antibody (1:4000) at room temperature for 1 h. The protein bands were scanned by ChemiScope 3000mini Chemiluminescence imaging system (Qing Xiang, Shanghai, China), and analyzed by Image J 1.38x software (NIH, Bethesda, MD, USA).

2.9. Statistical analysis

SPSS version 19.0 software (SPSS Inc., Chicago, IL, United States) was applied for statistical analysis. All data were expressed as the mean $\pm$ SEM and analyzed using one-way analysis of variance followed by Bonferroni test. A P value less than 0.05 were considered statistically significant.

3. Results

3.1. DEX reduced the number inflammatory cell in BALF of allergic mice

In order to detect the effects of DEX on airway inflammation in allergic mice, the total number and classification of inflammatory cells in BALF were observed by hemocytometer and Wright's-Giemsa. As shown in Fig. 2, the number of total leukocytes, eosinophils, monocytes, neutrophils and lymphocytes in BALF of the OVA group was significantly higher than that in the normal group. While, compared with OVA group, the number of inflammatory cells in BALF distinctly decreased in the treatment group.

3.2. DEX inhibited the production of inflammatory cytokines in BALF

To analyze the effect of DEX on inflammatory factor secretions, ELISA was used to detect the levels of IL-1 β , IL-18, IL-5 and IL-17 in

BALF of OVA-induced mice. Compared with the normal group, IL-1 β , IL-18, IL-5 and IL-17 in the BALF were found to be increased in the asthma model group. In contrast, treatment with DEX can reduce the content of inflammatory cytokines in BALF of asthma mice (Fig. 3).

3.3. DEX alleviated inflammatory cell infiltration and airway thickening in lung tissue of allergic mice

To further evaluate the inflammatory infiltration in the lung tissue of mice, we stained the lung tissue of mice with H&E. As can be seen from Fig. 4A, after OVA challenge, the inflammatory cell infiltrations in the lung tissue of the OVA group were serious compared with those in the control group. However, DEX dramatically attenuated these effects in the treatment group. The one notable result showed that the thickening airway (Wat/Pbm) of OVA group enhanced than the normal group. DEX alleviated these serious effects in OVA + DEX group (Fig. 4C).

3.4. DEX is a negative regulator of goblet cells hyperplasia in OVA-induced asthma

We detected the number of goblet cells and mucus cells in the airway of OVA-induced mice. We found the number of goblet cells and mucus cells in OVA group significantly enhanced than the normal group. The content of goblet cells and mucus cells was meaningfully lower than that in the OVA group, when treated with dexamethasone (Fig. 4B).

3.5. DEX inhibited the expression of NLRP3, caspase-1, IL-1 β , IL-6, IL-17 and TNF- α in the lung of allergic mice

In order to further clarify the mechanism of DEX on allergic mice. Western blot analysis was used to determine the protein expression of NLRP3, IL-1 β , IL-6, IL-17 and TNF- α , which reflect the activation of

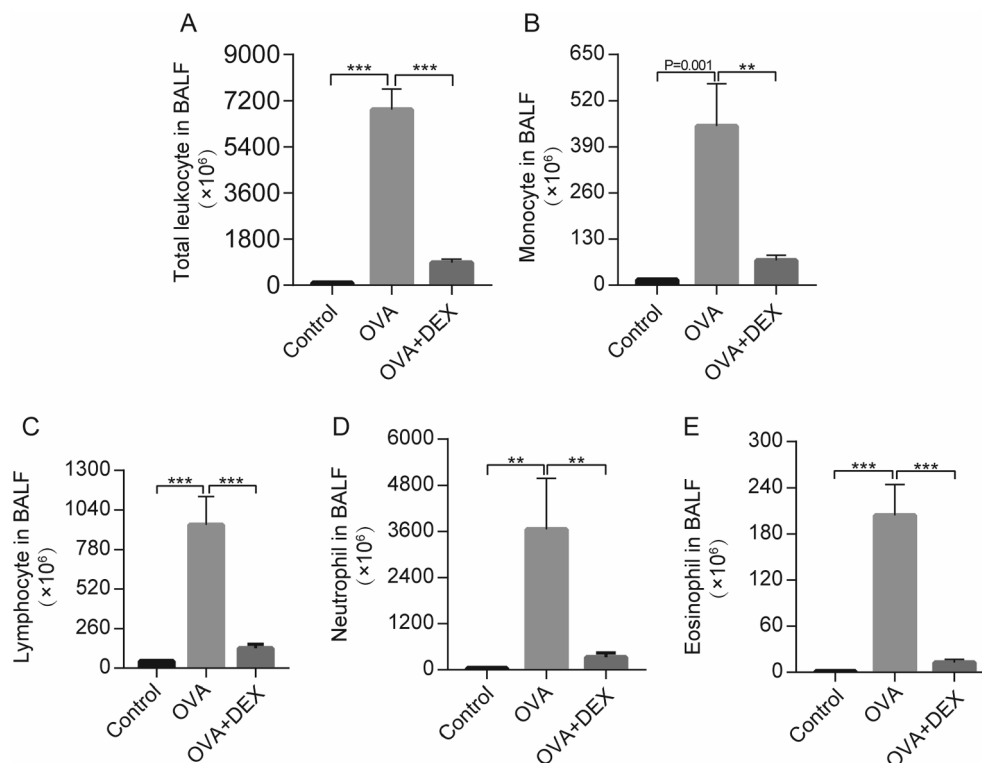


Fig. 2. Effects of DEX on inflammatory cells in mouse BALF induced by OVA. The data showed the changes of the total number of cells (A), monocytes (B), lymphocytes (C), neutrophils (D) and eosinophils (E) in BALF. There were six mice in each group. Data are the mean $\pm$ SEM. *P < 0.05, ** P < 0.01, ***P < 0.001.

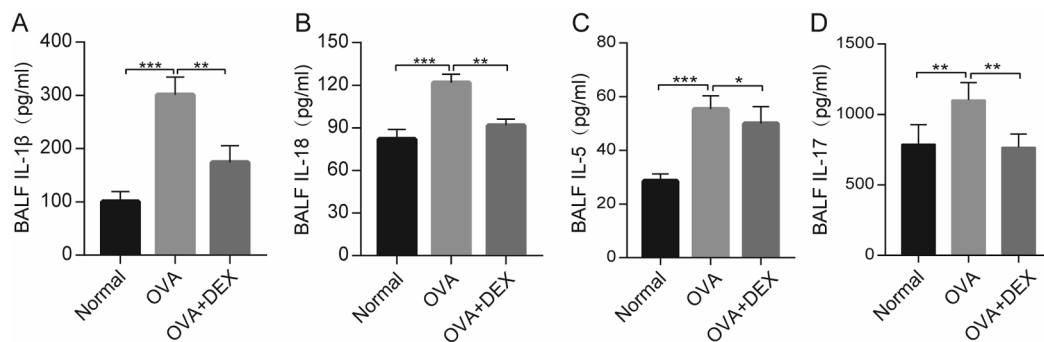


Fig. 3. DEX reduced the levels of pro-inflammatory cytokines in BALF of asthma mice. The BALF supernatants were collected for ELISA detection. The levels of IL-1 β (A), IL-18 (B), IL-5(C) and IL-17(D) were shown as above. Data were expressed as the mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001, n = 6 mice in each group.

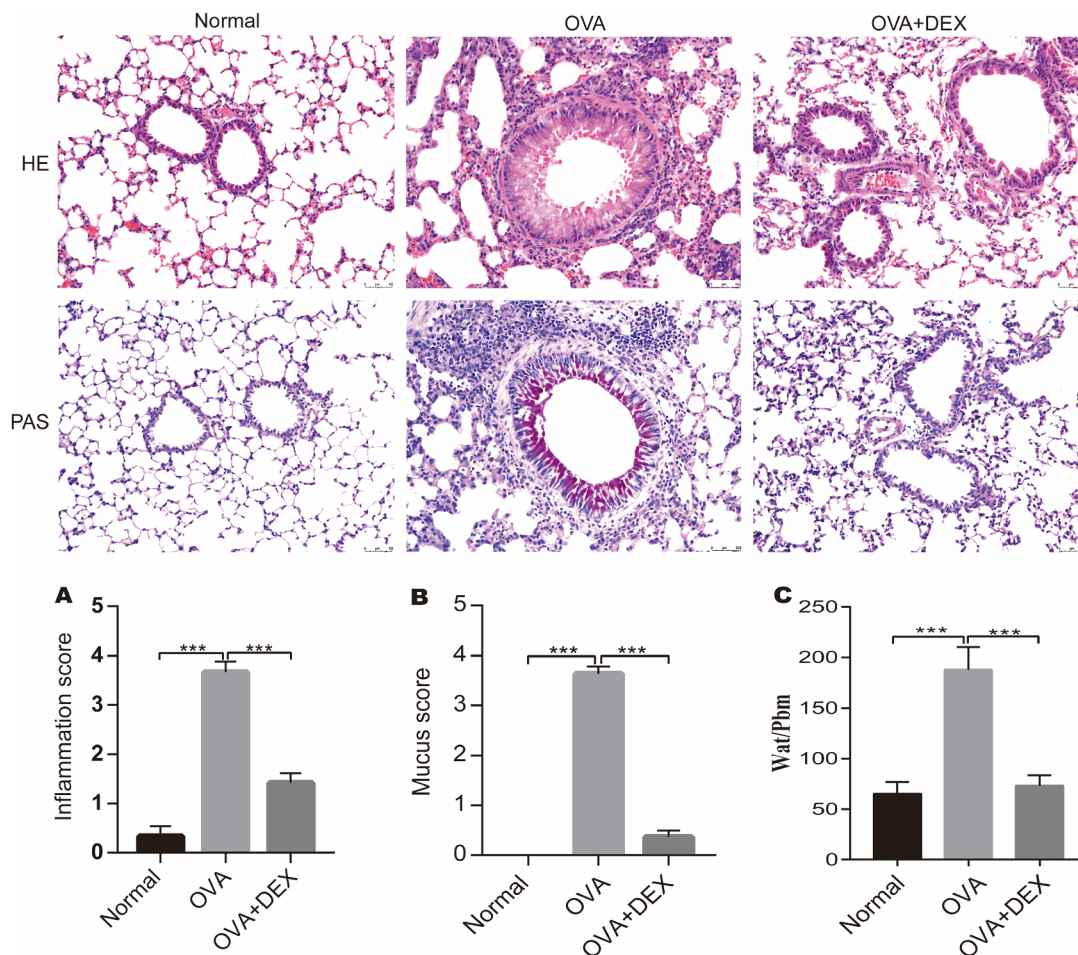


Fig. 4. Effects of DEX on pathologic features of OVA-induced allergic mice. Histological sections of lungs were stained with H&E staining for observing the lung injury. The goblet cells in the bronchus were observed by PAS staining. Pictures show representative samples from the different group. Scale bar = 50 μ m (magnification $\times$ 200). Inflammation scores (A), mucus secretion score (B) and Wat/Pbm (C) of every group was shown. Data were expressed as the mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

NLRP3 inflammasome and the inflammation degree in lung tissues of allergic mice. Besides NLRP3, Caspase-1 as another NLRP3 pathway active molecule, it can be cleaved from pro-Caspase-1. We determined the protein expression of pro-Caspase-1 and Caspase-1/pro-Caspase-1 by Western blot. As shown in Fig. 5 C-J, OVA challenging increased the protein levels of NLRP3, pro-Caspase-1, Caspase-1, Caspase-1/pro-Caspase-1, IL-1 β , IL-6, IL-17 and TNF- α , as compared with normal mice. Meanwhile, DEX treatment could reduce the levels of these proteins induced by the OVA.

4. Discussion

In this study, we demonstrated that treatment of DEX, one of the glucocorticoid, significantly inhibited OVA-induced infiltration of inflammatory cells into per bronchial regions, and reduced the number of inflammatory cells in BALF. Concomitantly, DEX suppressed the generation of pro-inflammatory cytokines and alleviated hyperplasia of airway goblet cells. Most notably, DEX inhibited increased activity of the NLRP3 inflammasome which is induced by OVA in lung tissues.

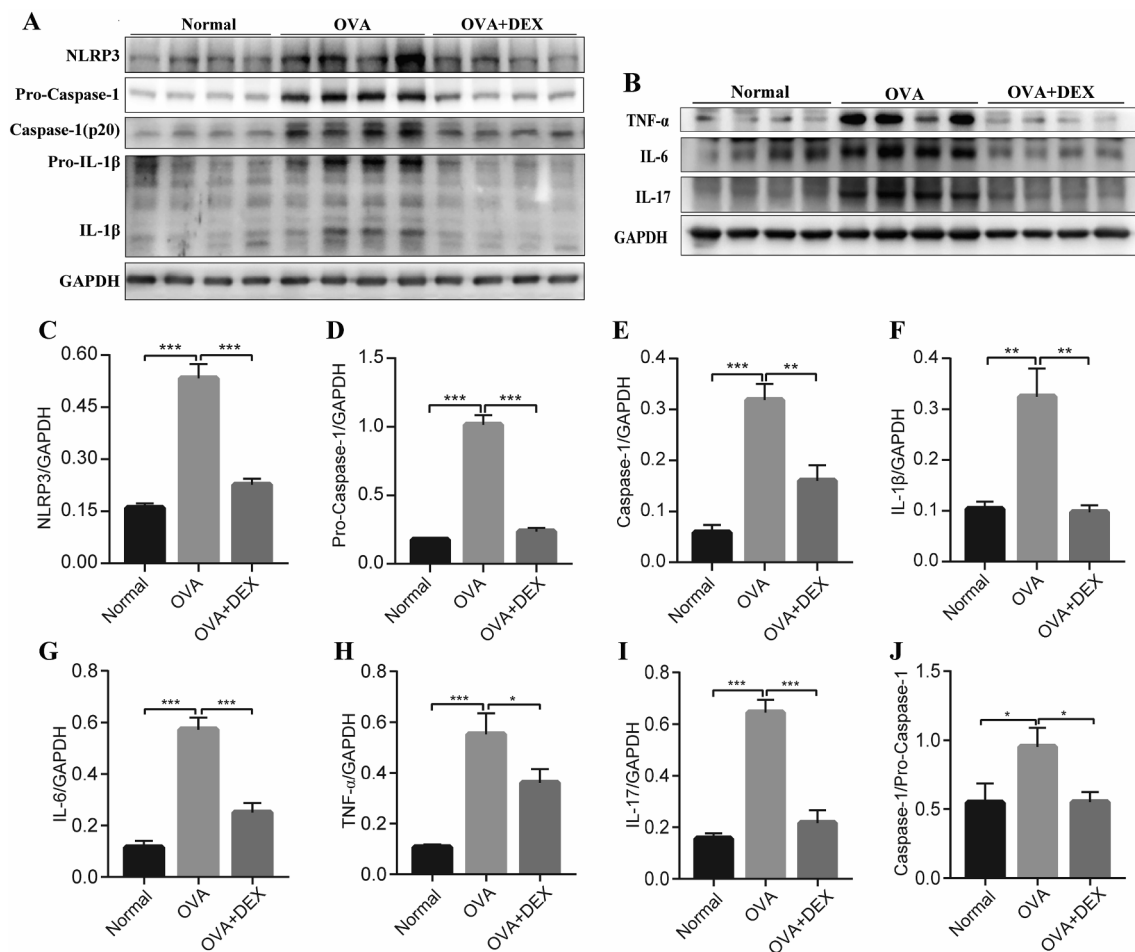


Fig. 5. DEX inhibited the expression of NLRP3, Caspase-1 and IL-1 β in OVA-induced allergic mice. NLRP3 inflammasome, Caspase-1, pro-caspase-1 and IL-1 β expression level were detected by western blotting. Relative levels of NLRP3 (C), pro-caspase-1 (D), Caspase-1 (E), IL-1 β (F), IL-6 (G), TNF- α (H), IL-17 (I) and Caspase-1/Pro-Caspase-1 (J) were shown. Data are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 6$ mice in each group.

DEX plays a key role in the medical field as an anti-inflammatory and immunosuppressive agent, especially in the treatment of asthma, and it remains the main agent for treatment. The processes of inflammation in airway's disease are complex and heterogeneous. So DEX plays an anti-inflammatory role in different ways. Previous studies have suggested that DEX reduces the number of eosinophils in human peripheral blood and the content of IgE in serum to alleviate airway inflammation in asthma [20,21]. Similarly, Wang ZR et al. demonstrated that DEX decreased EOS count of airway wall [22]. Other studies have shown that DEX not only reduced the number of eosinophils, inflammatory cells, and thickening of the airway epithelium but inhibited goblet cells hyperplasia [23]. In line with these findings, our study showed that DEX significantly decreased the number of eosinophils, neutrophils, monocytes and lymphocytes in BALF. It also suppressed thickening of the airway epithelium, infiltration of inflammatory cells into peribronchial regions and goblet cell hyperplasia/metaplasia. Our results support the role of DEX in anti-inflammation in vivo.

Although it was quite clear that DEX alleviated allergic airway inflammation, the mechanism of such effect is not fully understood. Many inflammatory cells and immune cells are involved in the occurrence and development of asthma [2]. However, lung epithelial tissues are the first defense barrier to respond to injuries and pathogens [24]. Previous study reported that NLRP3 inflammasome have distinct expression profiles in lung epithelial tissues [25,26]. Some study found pathogen-associated molecular patterns (PAMP) stimulate lung epithelial cells to express activated NLRP3 inflammasome [27]. In addition, the deficiency of NLRP3 inflammasome will impact the differentiation process

of Th2 [28], and NLRP3 inflammasome is involved in promoting asthma and activating the polarization of alveolar M2 macrophages [29]. Moreover, clinical and experimental studies demonstrated that the expression of NLRP3 inflammasome is significantly increased in patients with asthma and OVA-induced asthma mice [10,30,31]. It is suggested that NLRP3 inflammasome play a key factor in the pathology of asthma [32,33]. What's worse, the active NLRP3 leads to the activation of caspase-1, which further induces maturation of IL-1 β and IL-18[34]. This process will further aggravate the inflammatory response. Our current study demonstrated that treatment with DEX significantly lowered the protein expression of NLRP3, pro-Caspase-1, cleaved Caspase-1 and Caspase-1/pro-Caspase-1 in lung tissue. At the same time, Th2 cytokines in lung tissue and BALF were also decreased. This revealed that DEX might alleviate inflammation by inhibiting NLRP3 activation. Unlike our study, a previous study showed that DEX enhanced the expression and function of NLRP3 in human monocytes (THP-1) and monocyte-derived macrophages (THP-M) [35]. These different results may be caused by the different protocol and experimental object.

As mentioned above, NLRP3 inflammasome activation could lead to the activation of caspase-1, which induces the increased release of mature IL-1 β and IL-18 [10,36]. A similar observation was demonstrated that, active IL-1 β /IL-18 affects adaptive immune cells such as Th1 and Th17 during the infection [37,38]. Concomitantly, the level of IL-1 β in BALF [39] and serum [40] of patients with asthma are higher than those of normal controls. Similarly, the contents of IL-18 in lung tissue and serum of patients with asthma were higher than that of

normal subjects [41]. So IL-1 β /IL-18 might be one of the key factors results severe asthma and increasing IL-1 β could also be seen as a biomarker of asthma [42,43]. Likewise, our works suggested that the activation of NLRP3 inflammasome was induced by OVA. In addition, the expression of protein level in NLRP3, pro-caspase-1, caspase-1, IL-1 β , IL-6 and IL-17, including cytokines IL-1 β , IL-18 which are associated with activation of NLRP3 were both elevated in OVA-induced mice. In contrast, DEX markedly suppressed airway inflammation and the activation of NLRP3 inflammasome in OVA-induced mice. To our knowledge, little study proposes the relationship between DEX and NLRP3 inflammasome alone in the treatment of allergic asthma. What's more, there are few studies on DEX inhibiting IL-17 production to alleviate allergic asthma by inhibiting the activity of NLRP3 inflammasome.

In conclusion, our data indicated that DEX alleviated allergic airway inflammation by inhibiting the activation of NLRP3 inflammasome and reducing the levels of IL-1 β and IL-18. Our study enriched the theoretical basis of dexamethasone in the treatment of allergic diseases such as asthma. However, it's not so clear that the mechanism of dexamethasone regulating NLRP3 inflammasome, so further studies are required to explain the mechanism by which DEX regulates the activation of NLRP3.

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Author contributions

Minglong Guan and Xiaoyun Fan designed the study; Minglong Guan and Hengli Ma conducted most of the study, analyzed the data, and prepared the manuscript. Xu Chen and Manli Miao helped conduct the experiments. Huimei Wu helped design the experiment and edit the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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