ORIGINAL ARTICLE

Effect of 1,25-Dihydroxy Vitamin D_3 on the Expression of Interleukin-17 and -21 in an Asthma Mice Model

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ABSTRACT

Objective: *To explore the effect of 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) on the expression of interleukin (IL)-17 and IL-21 in asthma by establishing an asthma mice model.*

Methods: *Forty female Kunming mice were randomly divided into normal control group (group A, n = 10), asthma group (group B, n = 10), $1,25(OH)_2D_3$ intervention group (group C, n = 10) and dexamethasone treatment group (group D, n = 10). Asthma mice model was established and subsequently treated with different intervention. The airway lesions in mice were observed and the serum IL-17, IL-21 and immunoglobulin E (IgE) levels were determined using the enzyme-linked immunosorbent assay (ELISA) method. Immunohistochemical method was used to detect the expression of IL-17 and IL-21 in lung tissues.*

Results: *Serum IL-17 and IgE levels in groups B, C and D were remarkably higher than those in group A, especially in group B ($F = 31.276, 18.677$, $p < 0.01$). However, serum IL-21 level in group A was the highest, significantly higher than the other three groups ($F = 22.406$, $p < 0.01$). Immunohistochemical test revealed that the expression of IL-17 in group B was significantly higher than that in groups A, C and D, while IL-21 was strongly expressed in groups A and D but weakly expressed in group B.*

Conclusion: *In the pathogenesis of asthma, $1,25(OH)_2D_3$ intervention can reduce the expression of IL-17 but elevate the expression of IL-21, suggesting it has a certain effect on asthma.*

Keywords: 1,25-dihydroxy vitamin D_3, asthma, interleukin 17, interleukin 21, mice

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INTRODUCTION

Bronchial asthma is a common respiratory disease. It is estimated that there are about 300 million people worldwide suffering from asthma. In some countries such as Britain, Australia and New Zealand, the prevalence rate of asthma is as high as 20% (1). T cells, especially $CD4^+$ T cells, mainly including Th1, Th2, Th17 and Treg subsets, have been known to play an important immunoregulatory role in the pathogenesis of asthma. Th17 cell is a new subset of T helper cells found in recent years, named for its production of interleukin (IL)-17. It is widely involved in the immune response (2). Interleukin-21 is a newly discovered cytokine, which can also be produced by Th17 cells. It has a better inhibitory role than IL-6 in the pathogenic inflammatory response (3).

1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) is the active form of vitamin D, which is formed from vitamin D3 catalysed by 25-hydroxylase and 1α-hydroxylase. 1,25-dihydroxy vitamin D_3 binds to its vitamin D receptor on the target organs and subsequently activates its downstream molecules, playing a corresponding biological effect (4, 5). It has been reported that $1,25(OH)_2D_3$ has immunoregulatory effects on all subsets of $CD4^+$ T cells in the pathogenesis of bronchial asthma. For instance, in the classical mechanism of Th1/Th2 cellular immune imbalance in asthma, $1,25(OH)_2D_3$ can not only inhibit the proliferation of Th1 cells and reduce the levels of interferon gamma (IFN-γ), IL-2 and IL-5 (6, 7), but also promote the expression of Th2 cytokines IL-4 and IL-13, leading to Th2-dominant imbalance of Th1/Th2 cells (8). In addition, $1,25(OH)_2D_3$ can promote the differentiation of Treg cells and increase serum IL-10 and transforming growth factor beta 1 (TGF-β1) levels. In the allergic airway

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inflammation in mice, $1,25(OH)_2D_3$ can improve the effect of allergen immunotherapy (9).

The discovery of Th17 cells breaks through the limitations of Th1/Th2 model in the pathogenesis of asthma. Interleukin-17, mainly produced by Th17 cells, can act on various inflammatory cells in the pathogenesis of asthma and cause the production of proinflammatory cytokines and chemokines, promoting neutrophil infiltration and airway mucosa cell metaplasia. Besides, it is involved in the airway remodelling (10). Interleukin-21 is also an important cytokine produced by Th17 cells, which has been regarded as a potential immunotherapy cytokine due to its inhibitory effects on airway hyper-responsiveness and allergy (11). In the animal models of Th17 cell-mediated autoimmune uveitis and colitis, $1,25(OH)_2D_3$ has been confirmed to have inhibitory effects on the production of IL-17 and being able to prevent the pathological development of the disease (12), suggesting that $1,25(OH)_2D_3$ has regulatory effect on IL-17. However, the regulatory effect of $1,25(OH)_2D_3$ on the expression of cytokines IL-17 and IL-21 produced by Th17 cells in the pathogenesis of asthma has been reported less. In this study, an asthma mice model was established to explore the effect of $1,25(OH)_2D_3$ on the expression of cytokines IL-17 and IL-21 produced by Th17 cells.

SUBJECTS AND METHODS

Establishment of asthma model and treatment

Forty healthy female Kunming mice, special pathogen free (SPF), aged four to six weeks and weighing 20 ± 2 g comprised the study subjects. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao University, from the Experimental Animal and Animal Experiment Centre of the Affiliated Hospital of the Medical College of Qingdao University (qualified number: SCXK (Lu), 20090007). Mice were randomly divided into normal control group (group A, n = 10), asthma group (group B, n = 10), $1,25(OH)_2D_3$ intervention group (group C, n = 10) and dexamethasone treatment group (group D, n = 10).

Asthma model was established following the method reported previously (13) with some modifications. Briefly, mice in groups B, C and D received intraperitoneal injection of 0.2 mL freshly prepared chicken ovalbumin (OVA, Sigma-Aldrich, USA) mix suspension (containing 2 mg OVA and 5 mg aluminum hydroxide) at one, eight and 15 days for sensitization. Meanwhile, mice in group A received only intraperitoneal injection of 0.2 mL physiological saline at each time point. At 22 days, mice in each group were put in a homemade atomization box with a size of 20 × 20 × 30 cm^3 to inhale 2% OVA-physiological saline atomizing solution. Each inhalation lasted for 40 minutes, once daily for seven days.

During the atomizing inhalation, the appearance of symptoms such as shortness of breath, proneness and motionless, abdominal muscle spasm and excremental and urinary incontinence in mice was regarded as the asthma model being successfully established. Mice in group C received intraperitoneal injection of 0.2 mL freshly prepared $1,25(OH)_2D_3$ (2.5 mg/kg, Sigma-Aldrich, USA) solution 30 minutes before each atomizing inhalation, while mice in group D received intraperitoneal injection of 0.2 mL freshly prepared dexamethasone solution (0.5 mg/kg) 30 minutes before each atomizing inhalation. Mice in groups A and B received equal volumes of physiological saline by intra-peritoneal injection and atomizing inhalation. Model preparation took a total of 28 days.

Within 24 hours after the last atomizing inhalation, mice were anaesthetized with 10% chloral hydrate (0.5 mL/kg) by intraperitoneal injection. Blood was collected by enucleating the eyeball and centrifuged at 3000 r/min for 10 minutes for isolating the serum. Serum of each blood sample was collected and stored at - 20 °C before use. The trachea of each mouse was exposed with a transverse incision, through which a modified remaining needle was inserted and fixed by suture. Then the heart and lungs were exposed. After ligating the root of the left lung, right pulmonary bronchial alveolar wash was performed with 0.3 mL freshly prepared phosphate buffered saline (PBS) for three times and all the lavage fluid was collected at 30 seconds after the washing (recovery > 80%). The lavage fluid was centrifuged at 3000 r/min for five minutes and the supernatant was stored at -20 °C before use. The cell sediment was re-suspended with 50 μl PBS. The total cell number of the cell sediment was counted on a cell count sheet. The left lung was cut and fixed in 4% paraformaldehyde for 48 hours and made into paraffin-embedding block. Then the lung specimens were cut into 4–5 μm thick slices and stained by haematoxylin and eosin for pathological examination.

Serum IL-17, IL-21 and immunoglobulin E (IgE) levels were determined using enzyme-linked immunosorbent assay (ELISA) kit (R&D Company, USA) according to the manufacturer's instruction. Lung tissue slides were used to detect the expression of IL-17 and IL-21 in lung tissues. Primary anti-bodies (rabbit anti-mouse polyclonal antibody) for IL-17 and IL-21 were from Beijing ZSGB Company (China) and used in a dilution of 1:200 and 1:400, respectively. Goat anti-rabbit secondary antibody (PV9001 kit, Beijing ZSGB Company, China) was used to detect the immunoreactivity of the cells. Immunohistochemical method was performed according to the instructions of the PV9001 kit.

Statistical analysis

Data are shown as mean ± standard deviation (SD) and processed using SPSS software v 17.0 (Chicago, IL, USA). Normal distribution test and homogeneity test of variance were performed on all of the data before the data were

analysed. One-factor analysis of variance was used to compare the differences among the groups when the data fit normal distribution and homogeneity of variance. Difference was statistically significant when $p < 0.05$.

RESULTS

Asthma mice model

During the atomizing excitation, all mice in groups B, C and D presented symptoms such as forelimbs scratching nose, mouth and torso, shortness of breath, forelimbs lifted, cyanosis of the oral area and limb endings, abdominal muscle twitching, excremental and urinary incontinence, dysphoria or even proneness and motionless in some severe cases. But all of the symptoms could be gradually stopped in a few minutes after the atomization ended. Continuous excitation could cause loss of body weight, less hair lustre and decreased food intake in the mice in groups B, C and D. However, mice in group A acted freely without any symptoms described above.

Total count of cells and eosinophil count in bronchoalveolar lavage fluid of group B were significantly higher than those in the other three groups ($p < 0.01$). Except for no statistically significant difference between groups C and D ($p > 0.05$), comparisons between any other two groups showed statistical significance ($p < 0.01$; Table 1).

Serum IL-17, IL-21 and IgE levels in asthma mice

Both serum IL-17 and IgE levels were higher in groups B, C and D than in group A, especially in group B, while serum IL-21 level showed the reverse, being higher in group A than in the other three groups ($p < 0.01$). As for serum IL-17, IL-21 and IgE levels, no statistically significant difference was found between groups C and D ($p > 0.05$). Serum IL-21 level also showed no significant difference between groups A and D ($p > 0.05$). Otherwise, serum IL-17, IL-21 and IgE levels showed marked differences among the other comparisons ($p < 0.01$; Table 2).

Table 1: Total count of cells and eosinophil count in bronchoalveolar lavage fluid

Groups	n	Total count of cells ($\times 10^4$/mL)	Eosinophil count ($\times 10^4$/mL)
Control (group A)	10	6.36 ± 2.13	0.19 ± 0.11
Asthma (group B)	10	21.00 ± 6.69[a]	5.14 ± 2.03[a]
Vitamin D (group C)	10	12.46 ± 2.79[a,b]	2.8 ± 0.63[a,b]
Hexadecadrol (group D)	10	9.19 ± 1.54[a,b,c]	2.13 ± 0.72[a,b,c]
F-value		27.05	32.96
p-value		< 0.001	< 0.001

Data are shown as mean ± SD. [a]$p < 0.01$, *vs* group A; [b]$p < 0.01$, *vs* group B; [c]$p > 0.05$, *vs* group C

Pathological changes in lung tissues of mice

Observed under a light microscope, lung tissues in group A showed normal bronchial and alveolar structure, complete airway epithelium and neat cilia arrangement. No inflammatory cell infiltration was found in the lung tissues of group A (Fig. 1A). However, in groups B, C and D, bronchial epithelial damage, structure disorder, thickening bronchial wall, luminal stenosis, different amounts of inflammatory cells infiltration around the bronchi and blood vessels and in pulmonary mesenchyme could be observed in the lung tissues (Fig. 1B–D). In addition, mucus could be seen in the bronchial lumen and the alveolar structure was destroyed. The alveolar septum had been broken and some alveolar fused. The lungs in group B revealed the most serious inflammation, followed by the lungs in group C and then group D (Fig. 1B–D).

Expression of IL-17 and IL-21 in lung tissues

Punctate cellular brown granules in cytoplasm were considered to be positive for IL-17 and IL-21. The expression of IL-17 was the strongest in the cytoplasm of bronchial epithelium cells in the asthma group (Fig. 2B) but was the weakest in the control group (Fig. 2A). Interleukin-17 was also positive in lung tissues in the $1,25(OH)_2D_3$ intervention group and dexamethasone treatment group, but it was lower than that in group B (Fig. 2C, D). Interleukin-21 was weakly expressed in group B (Fig. 3B), but strongly expressed in groups A, C and D, especially in groups A and D (Fig. 3A, C, D).

Table 2: Serum interleukin-17, -21 and IgE levels in the four groups

Groups	n	IL-17 (pg/mL)	IL-21 (pg/mL)	IgE (μg/mL)
Control (group A)	10	14.03 ± 1.66	21.62 ± 4.67	16.19 ± 4.24
Asthma (group B)	10	28.62 ± 3.98[a]	9.95 ± 1.93[a]	31.34 ± 6.53[a]
Vitamin D (group C)	10	21.91 ± 3.13[a,b]	15.27 ± 2.53[a,b]	26.07 ± 3.80[a,b]
Hexadecadrol (group D)	10	18.98 ± 4.37[a,b,c]	19.15 ± 3.77[b,c]	24.25 ± 3.08[a,b,c]
F-value		31.276	22.406	18.677
p-value		< 0.001	< 0.001	< 0.001

Data are shown as mean ± SD. [a]$p < 0.01$, *vs* group A; [b]$p < 0.01$, *vs* group B; [c]$p > 0.05$, *vs* group C.

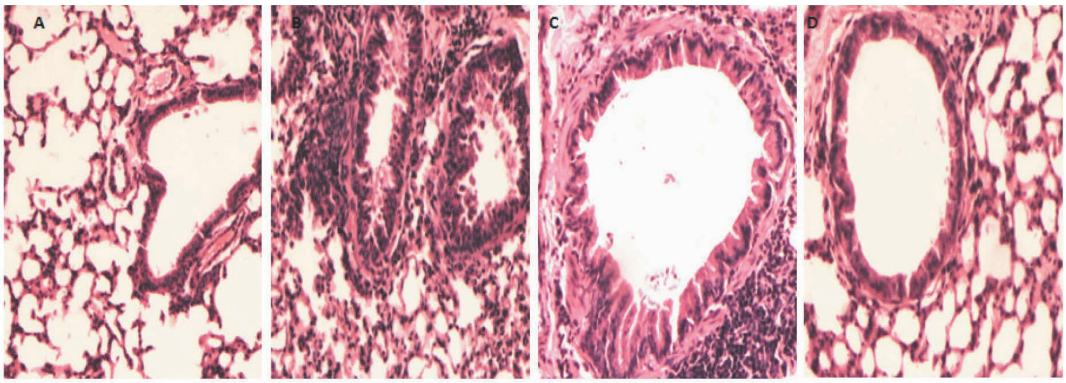

Fig. 1. Pathological examination of mice lung tissues in the four groups stained by haematoxylin and eosin (original magnification, 200×).
A) control group; B) asthma group; C) 1,25-dihydroxy vitamin D3 intervention group; D) dexamethasone treatment group. As compared with group A, group D showed the mildest inflammatory response. Mild damage in bronchial epithelium, mild hyperplasia of epithelial and smooth muscle cells and a few infiltrating inflammatory cells around the bronchi could be observed in the lung tissues of group D. Obvious airway smooth muscle spasm, thickening wall and luminal stenosis, cavity with mucus, damaged alveolar structure and a large number of inflammatory cells infiltration around the bronchi could be seen in the lung tissues of group B. Lung tissues of group C revealed a moderate inflammatory response.

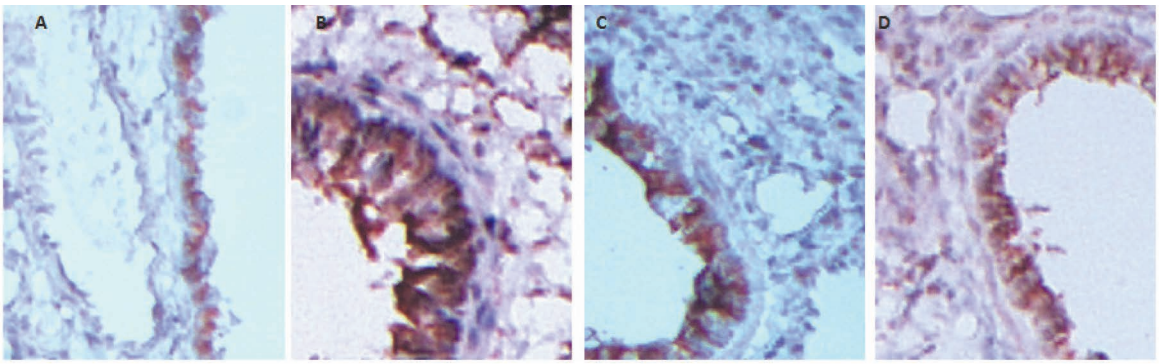

Fig. 2. The expression of interleukin (IL)-17 in the lung tissues of mice in the four groups determined by immunohistochemical staining (original magnification, 400×).
A) control group; B) asthma group; C) 1,25-dihydroxy vitamin D3 intervention group; D) dexamethasone treatment group. Interleukin-17 (brown) was highly expressed in the cytoplasm of bronchial epithelial cells in group B but weakly expressed in group A. Groups C and D revealed a moderate expression of IL-17.

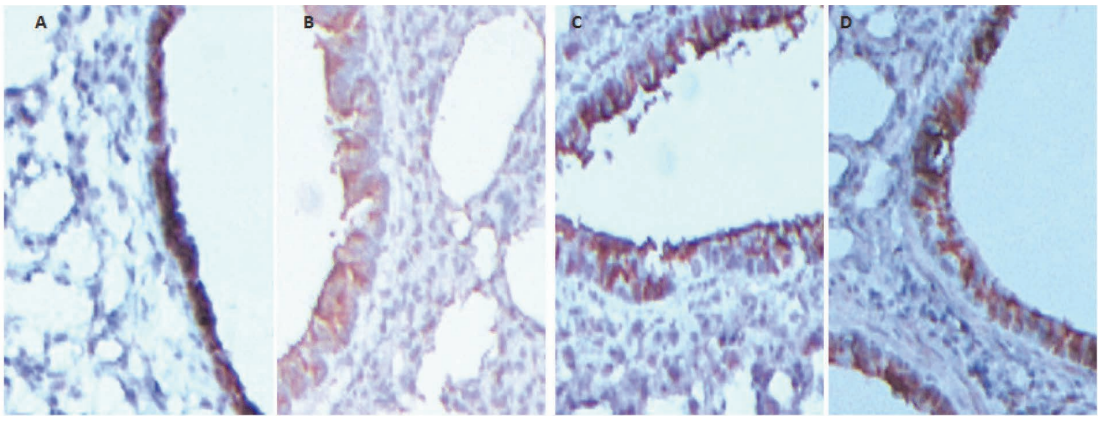

Fig. 3. The expression of interleukin (IL)-21 in the lung tissues of mice in the four groups determined by immunohistochemical staining (original magnification, 400×).
A) control group; B) asthma group; C) 1,25-dihydroxy vitamin D3 intervention group; D) dexamethasone treatment group. The expression of IL-21 was the highest in groups A and D but was the lowest in group B.

DISCUSSION

It is well known that bronchial asthma is a kind of allergic disease caused by immunologic abnormality, characterized by persistent airway inflammation, airway hyper-responsiveness (AHR) and airway remodelling. Persistent airway inflammation, AHR and airway remodelling interact and influence each other and are all associated with lymphocytes. T helper lymphocytes play a key role in the pathogenesis of asthma (14). For instance, imbalance of Th1/Th2 cells contributes to the pathogenesis of asthma. With the stimulation of allergen, Th2 cells present increasing immune function, while Th1 cells are relatively inhibited, leading to the pathological process of Th2 cytokines and increased IgE level (15). Studies have reported that the number and function of Treg cells changed also contributes to the onset of asthma (16).

Th17 cells are a subset of T helper cells, newly discovered in recent years. It has an important role in the regulation of immune response. Interleukin-17A, also known as IL-17, is the main production of Th17 cells. It has been reported that IL-17 level increases in the lung tissue, sputum and bronchoalveolar lavage fluid of bronchial asthma patients, suggesting that IL-17 is involved in the pathogenesis of asthma and Th17 cells play an inducing role to neutrophils in lung inflammation (17, 18). Interleukin-17 also plays an important role in the occurrence of airway hyper-respon-siveness. Kudo *et al* (19) proved in an *in vitro* study that IL-17 can directly affect the airway smooth muscle of mice and human to strengthen its contractile response, resulting in the emergence of airway hyper-responsiveness. In addition, $1,25(OH)_2D_3$ has been proven to have an inhibitory effect on the expression of IL-13 and IL-17 in $CD4^+$ T cells of cord blood induced by lipopolysaccharide (20), suggesting that $1,25(OH)_2D_3$ may play a protective role in the early stage of allergen sensitization.

Interleukin-21 is also a newly discovered cytokine, mainly produced by Th17 cells. It can interact with a variety of cells such as T cells, B cells, NK cells and dendritic cells, playing regulatory roles in cellular immunity and humoral immunity (21). Studies have shown that IL-21 is closely related to IgE level *in vivo*. It promotes the proliferation and differentiation of B cells, as well as induces the expression of the modifier gene Bcl-2 and differentiation inhibitory factor Id2 in B cells, and thereby downregulates the production of IgE (22). In allergic diseases, the increasing amount of eosinophils can lead to airway hyper-responsiveness. Suto *et al* (23) reported that IL-21 intervention for ovalbumin inhalation-induced asthma mice model can significantly reduce the production of antigen specific IgE and the aggregation of eosinophils in the respiratory tract. All the above results indicated that IL-21 can reduce type IV allergy and serves as a protective factor in the pathogenesis of asthma. However, due to scant research on the role of IL-21 in the pathogenesis of asthma, it is still unclear how IL-21 acts and whether it has protective effects.

Our results showed that IL-17 and IL-21 were involved in the pathogenesis of asthma. The similar increasing trend of serum IL-17 and IgE levels in asthma mice indicated that IL-17 may induce or exacerbate asthma by promoting the synthesis and release of IgE. However, IL-21 may down-regulate the IgE level.

1,25-dihydroxy vitamin D3 has become a research hotspot in recent years on the pathogenesis of asthma. In this study, we only explored the effect of $1,25(OH)_2D_3$ on Th17 cells in the development of asthma. Currently, the immuno-regulatory effect of Th17 cells on the pathogenesis of asthma has also drawn much attention. However, the effect of $1,25(OH)_2D_3$ on Th17 cells in the onset of asthma is seldom reported. An *in vitro* study from Joshi *et al* (24) showed that $1,25(OH)_2D_3$ can inhibit the expression of IL-17A. Palmer *et al* (25) also found that $1,25(OH)_2D_3$ can elevate the expression of IL-21 but inhibit the expression of IL-2, T-bet, STAT1 and STAT4. In this study, we confirmed that $1,25(OH)_2D_3$ intervention can reduce the level of IL-17 but increase the expression of IL-21, which is consistent with the above results. In addition, the interventional effect of $1,25(OH)_2D_3$ is similar to that of dexamethasone, indicating $1,25(OH)_2D_3$ can regulate the levels of IL-17 and IL-21 and may have a similar effect on controlling asthma with dexamethasone treatment.

In conclusion, $1,25(OH)_2D_3$ intervention can reduce serum IL-17 level and increase serum IL-21 level, suggesting that it can have certain effect on asthma control. However, research on the therapeutic effect of $1,25(OH)_2D_3$ on asthma is still in the stage of animal experiments; the right time and suitable doses for the clinical application of vitamin D still cannot be defined. Thus, its clinical application still has many limitations and needs further research.

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AUTHORS' NOTE

All of the authors declare that they have no conflicts of interest regarding this paper.

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