Iranian Journal of Basic Medical Sciences
ijbms.mums.ac.ir

1,25-Dihydroxyvitamin D3 ameliorates neutrophilic asthma through modulation of ERS-NLRP3 inflammasome axis in a mouse model

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ARTICLE INFO

Article type:
Original

Article history:
Received: May 8, 2025
Accepted: Nov 17, 2025

Keywords:
$1,25\text{-}(OH)_2D_3$
Airway inflammation
Endoplasmic reticulum - stress
Neutrophilic asthma
NLRP3 inflammasome

ABSTRACT

Objective(s): Asthma is a heterogeneous disease. Neutrophilic asthma is associated with severe asthma and poor response to inhaled corticosteroids. We investigated whether 1,25-dihydroxyvitamin D3 can ameliorate neutrophilic airway inflammation, inhibit endoplasmic reticulum stress (ERS), and ERS-induced NLRP3 inflammasome activation in a murine model of neutrophilic asthma.

Materials and Methods: BALB/c mice were established as an experimental model of neutrophilic asthma. $1,25\text{-}(OH)_2D_3$ was administered for intervention to investigate the effect of Vitamin D3 on neutrophilic airway inflammation, ERS-induced NLRP3 activation. Inflammatory cells were counted in BALF, and lung tissue was histologically examined. Concentrations of IL-17, IL-1β, and IL-18 in BALF were measured. Immunohistochemical analysis of lung Ly-6G expression and AHR was tested. Furthermore, the percentage of Th17 cells was determined by flow cytometry. CHOP, GRP78, and NLRP3 protein expression was also examined by western blot.

Results: We observed that the infiltration of inflammatory cells and goblet cell hyperplasia in lung and airway tissue were reduced by $1,25\text{-}(OH)_2D_3$ treatment. The total number of inflammatory cells and the percentage of neutrophils in BALF were significantly less in the treatment groups. $1,25\text{-}(OH)_2D_3$ obviously suppressed neutrophils infiltration and Ly-6G expression in neutrophilic asthma. $1,25\text{-}(OH)_2D_3$ reduced Th17 cell percentage and IL-17 levels. The expression levels of GRP78 and CHOP were lower in the $1,25\text{-}(OH)_2D_3$ treatment group than those in the NA model group. And, $1,25\text{-}(OH)_2D_3$ decreased NLRP3 protein expression levels in the lungs of NA mice.

Conclusion: Our results suggest that 1,25-dihydroxyvitamin D3 ameliorates neutrophilic asthma in mice by a mechanism linked to reduced ERS and NLRP3 inflammasome activation, supporting further investigation of its potential as a therapeutic candidate.

▶ Please cite this article as:
Zhang JH, Guo Q, Wei LY, Chen XY. 1,25-Dihydroxyvitamin D3 ameliorates neutrophilic asthma through modulation of ERS-NLRP3 inflammasome axis in a mouse model. Iran J Basic Med Sci 2026; 29: 438-445. doi: https://dx.doi.org/10.22038/ijbms.2026.87901.19026

Introduction

Asthma is a chronic airway inflammatory disease displaying high heterogeneity. Its heterogeneity is reflected in the existing different clinical and molecular phenotypes of the disease. Asthma is characterized by multiple immunological mechanisms (endotypes) determining variable clinical presentations (phenotypes). According to sputum granulocyte levels, asthma can be classified into four phenotypes: eosinophilic (EA), neutrophilic (NA), paucigranulocytic (MA), and mixed granulocytic asthma (1). Neutrophilic asthma deserves considerable interest among those different phenotypes for NA accounts for more than half of non-eosinophilic asthma, and neutrophilic airway inflammation is highly associated with severe asthma, poor response to inhaled corticosteroids (ICS), as well as more severe airflow obstruction, and more frequent exacerbations. In addition, the pathological mechanisms underlying neutrophilic asthma remain incompletely elucidated. As current conventional treatments, such as steroids, fail to prevent neutrophilic asthma, there is an urgent need for effective therapeutic approaches to prevent or treat this type of asthma.

Neutrophilic asthma is characterized by increased involvement of Th1 (IFN-γ) and Th17 (IL-17A) cytokines. In particular, IL-17A has been found to be elevated in severe asthma and can initiate the release of various cytokines/chemokines, thereby driving neutrophilic airway inflammation. T helper (Th) 17 cells play an essential role in the pathological mechanisms of neutrophilic asthma through the release of IL-17 cytokines. In recent years, many studies have shown that NLRP3 (Nod-like receptor protein 3) inflammasome activation increases in the neutrophilic asthmatic airways. Inflammasomes form the critical component of the innate immune system. The NLRP3 inflammasome is an intracellular multiprotein complex that serves as a crucial regulator in the pathogenesis of diverse inflammatory diseases, causing the release of

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proinflammatory cytokines such as interleukin-1β and IL-18 by a caspase-1-dependent process. Simpson *et al.* reported upregulation of the NLRP3 inflammasome, particularly in neutrophilic asthma (2). Kim *et al.* also proved NLRP3 inflammasome responses drive experimental severe, steroid-resistant asthma and are potential therapeutic targets in this disease (3). In addition, endoplasmic reticulum stress (ERS) participates in many immune and inflammatory responses. The endoplasmic reticulum is an intracellular organelle that is responsible for protein folding and trafficking. Cellular stresses, including hypoxia, Ca^{2+} overload, I/R, and ROS, can disrupt ER homeostasis, resulting in the production of numerous unfolded or misfolded proteins and initiating the unfolded protein response (UPR) and activation of ERS. ERS can activate the NLRP3 inflammasome to induce inflammatory responses via oxidative stress, calcium homeostasis, and NF-κB activation (4). The interactions between ERS and the NLRP3 inflammasome lead to pathological processes in various inflammatory diseases. Recent work by Pathinayake and colleagues demonstrated that increased ER stress and UPR pathways are related to clinical severity and inflammatory phenotypes in asthma; ER stress genes displayed a significant correlation with classic Th2 genes, Th17 genes, and NLRP3 in sputum from asthmatic participants (5).

Vitamin D is well known for its functions in calcium and bone homeostasis. More recently, immunomodulatory and anti-inflammatory functions of vitamin D have been employed successfully to treat different autoimmune diseases and inflammatory diseases. Epidemiological evidence suggests an association between vitamin D insufficiency and poorer asthma control. Many studies have shown that vitamin D administration alleviates airway remodeling and airway inflammation in asthma. (6, 7). Recently, Vitamin D has been shown to inhibit the NLRP3 inflammasome signaling (8). Furthermore, Vitamin D played critical roles in modulating inflammation and reactive oxygen species (ROS) by suppressing ERS (9, 10). Taking into account ERS proved as an essential factor for the activation of the NLRP3 inflammasomes and the anti-inflammation properties of vitamin D, we here investigated whether 1,25-dihydroxyvitamin D3(1,25-$(OH)_2D_3$), the physiologically active form of vitamin D, can ameliorate neutrophilic airway inflammation, inhibit ERS and ERS-induced NLRP3 inflammasome activation in a murine model of neutrophilic asthma.

Materials and Methods

Animals and care

Female BALB/c mice, aged 6 weeks and weighing 20±2 g, were purchased from the Laboratory Animal Center of Guangxi Medical University (Nanning, Guangxi, China) and allowed to acclimate to their environment for 7 days. Mice were raised under specific pathogen-free (SPF) conditions. Animal care and experimental procedures were carried out strictly in accordance with established institutional guidelines and with approval from the Research Animal Care Committee at Guangxi Medical University for Animal Experimentation. All mice were provided with standard food and water *ad libitum*, on a 12:12 hr light: dark cycle, in an air-conditioned room at 23 °C and 45% relative humidity.

Materials

Ovalbumin (OVA), Grade V, was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). LPS, 1,25-$(OH)_2D_3$, and methacholine were also obtained from Sigma. The monoclonal antibodies FITC anti-mouse CD4, anti-mouse IL-17 PE were from eBioscience. Fixation Medium was obtained from Invitrogen (Camarillo, CA, USA). Monensin, Ionomycin, and Phorbol ester were purchased from Sigma-Aldrich. Monoclonal antibody-based mouse IL-17 and IL-1β, and IL-18 ELISA kits were from R&D (R&D Systems, Minneapolis, MN, USA). Primary antibodies against CHOP, GRP78, and NLRP3 were supplied by Cell Signaling Technology (CST, MA, USA). Monoclonal antibody for immunohistochemistry anti-mouse Ly-6G (Invitrogen, Carlsbad, CA, USA).

Experimental groups and animal model of neutrophilic asthma

All the mice were randomly separated into three groups consisting of eight animals each: (1) normal control group (group A), (2) NA model group (group B), (3) Vit D3 treatment group (group C). Mice in groups B and C were administered OVA/LPS to induce an experimental model of neutrophilic asthma. The mice of group C accepted 1,25-$(OH)_2D_3$ treatment. A neutrophilic asthma model was established in accordance with a previously published experimental protocol, with minor modification (11). Briefly, BALB/c mice were sensitized intranasally with 25 μg OVA (grade V, Sigma-Aldrich, St. Louis, MO, USA) and 1 μg LPS in saline on days 1 to 3 and on day 14. After the initial sensitization, the mice were challenged with aerosols for 1 hour, consisting of 3% OVA daily from day 21 to 25, by an ultrasonic nebulizer (WH-2000, Guangzhou, China) in a closed chamber. The normal control group was administered the same volume of saline only and nebulized with saline (instead of OVA) for comparison. Mice in group C were treated with a 0.1% 1,25-$(OH_)_2D_3$ solution (4 μg/kg, based on body weight) via intraperitoneal injection daily, 1 hr before airway challenge with OVA (on days 21-25). Groups A and B were sham-injected with saline intraperitoneally.

Airway hyperresponsiveness (AHR) determination

AHR was measured in mice 12 hr after the last aerosol challenge by non-invasive whole-body plethysmography. We use a double-chamber plethysmography device, BUXCO TBL3999 (Buxco Electronics Inc., Troy, NY, USA), to assess airway function. Data were expressed as the increase in sRaw (specific airway resistance), calculated as follows: sRaw rise = sRaw(methacholine) - sRaw(PBS). We placed the mice in a chamber, allowed them to acclimate for 5-10 min, and then exposed them to nebulized PBS for 3 min to establish baseline sRaw values. We then exposed the mice to increased concentrations of aerosolized methacholine. AHR of each mouse was assessed by delivering increasing doses of inhaled methacholine (0, 6.25, 12.5, and 25 mg/ml). Recordings were obtained for 3 min in each nebulization cycle. The sRaw values measured during each 3-min sequence were averaged.

Sample collections

All animals were sacrificed 24 hr after the last aerosol challenge. Mice were intraperitoneally injected with an overdose of pentobarbital and then sacrificed. BALF samples were harvested, and lung tissue specimens were collected for further study. The right upper lobe of lung tissue was fixed in 10% formalin for histological and immunohistochemical

staining. The other lung tissues were stored at -80 °C for WB and Flow Cytometric analysis.

Differential cell counting in BALF

Bronchoalveolar lavage (BAL) was performed, and BAL fluid (BALF) was obtained using a previously described method. BALF cells and differentials were counted. BALF was centrifuged at 500 × g for 5 min. The supernatant from BALF was stored at -20 °C until assayed for cytokine measurement. The cell pellet was resuspended in 200 µl of PBS, and the cells were fixed and stained with Diff-Quick (Baxter-Dale, Dudingen, Germany) staining. Total cell count was performed using an automatic cell counter. Cell differential counts and percentages were determined under a light microscope in a blinded manner. A minimum of 300 cells was counted and classified as eosinophils, neutrophils, and lymphocytes on the basis of morphologic criteria and staining characteristics in randomly selected fields of the slide.

Histological examination of lung tissue

The lung was fixed in 4% paraformaldehyde in PBS and embedded in paraffin, cut into 5-µm sections according to general histochemical procedure. HE staining of the lung sections was conducted to assess inflammation by the degree of peribronchiolar and perivascular inflammation. The AB-PAS stains of the lung are performed to identify goblet cell hyperplasia in the epithelium and submucosal gland hypertrophy.

Immunohistochemical analysis of lung Ly-6G expression

Slides were stained with anti-mouse Ly-6G to determine neutrophil infiltration in the lung tissues of mice by immunohistochemistry. The lung sections (5 µm) were dewaxed, rehydrated, and incubated in 3% hydrogen peroxidase to block endogenous peroxidase activity. Tissue slides were also incubated in 5% normal goat serum to block nonspecific binding. The lung sections were then incubated overnight at 4 °C with the primary Ab diluted in 5% normal goat serum or normal goat serum as a negative control. The revelation was performed using an appropriate secondary antibody for 30 min at room temperature, according to the supplier's instructions. Immunoreactivity was visualized by a treatment with diaminobenzidine (Sigma-Aldrich). Images were obtained under a light microscope (Olympus Corporation, Tokyo, Japan).

Cytokine concentrations analysis

Concentrations of IL-17, IL-1β, and IL-18 in BALF were measured by enzyme-linked immunosorbent assay (ELISA) using commercial mouse ELISA kits according to the manufacturer's protocols. The absorbance at 450 nm wavelength was measured using a microplate ELISA Reader, and the content of each group was calculated according to the standard curve.

Western blotting analysis

Tissues were cut into pieces and crushed in a cryotube by shaking with a sterile steel ball. RIPA lysis buffer was added to extract total protein from lung tissues. The protein content was determined by the BCA method. Ten microliters of protein samples were used for western blot analysis, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was removed, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore) of appropriate size, which was then soaked in methanol. The membrane was then sealed with 5% blocking buffer (5% nonfat milk in 20 mM Tris–HCl, pH 7.5), 137 mM NaCl, and 0.1% Tween 20 at room temperature for 1 hr. The membranes were incubated with primary antibody overnight at 4 °C. The next day, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000 dilution) at room temperature for 2 hr, and then detected with ECL (Amersham Pharmacia Biotech). GAPDH was used as an internal reference. The gray value of the band was detected and analyzed using ImageJ Software.

Flow cytometry

Single-cell suspensions from lung tissues were prepared by mechanical disruption followed by enzymatic digestion. The percentages of Th17 from the lung tissue were analyzed by flow cytometry. Cells were stained with fluorescein isothiocyanate-conjugated antibodies, and staining with isotype control antibodies was performed in all experiments. The expression markers were determined using the FITC anti-mouse CD4 Antibody, PE anti-mouse IL-17A Antibody, purchased from BioLegend (USA). Prepare 100 µl tubes for each specimen. For each sample to be analyzed, 1×10^6 cells were added to an appropriate FITC anti-mouse CD4 antibody according to the manufacturer's instructions or the appropriate isotype controls. Cells were incubated for 15 min at room temperature in the dark. And then cells were fixed/permeabilized in 100 µl Perm/Fix buffer (BD Bioscience). After once washing in PBS +0.1%NaN3 + 5%FBS, cells were stained with the recommended volume of anti-mouse IL-17 PE according to the manufacturer's instructions or the corresponding isotype controls. Cells were incubated for 20 min in the dark at room temperature. After washing twice with Perm/Wash buffer, the cells were resuspended in staining buffer. Samples were analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed with FlowJo software (TreeStar, Version 10.6.1).

Statistical analysis

Statistical analyses were performed using SPSS (version 23.0; SPSS, Inc., Chicago, USA). The data are presented as means ± SEM (standard error of the mean). Multiple groups were analyzed using a one-way analysis of variance (ANOVA), followed by *post hoc* comparisons using Tukey's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of 1,25-$(OH)_2D_3$ on AHR

AHR of mice in response to methacholine (Mch) measured using the non-invasive whole-body plethysmography was significantly increased in the NA model group mice. 1,25-$(OH)_2D_3$ treatment significantly reduced sRaw. sRaw detected when mice were stimulated by Mch (12.5, 25.0mg/ml) in the NA group was increased significantly compared to the control group ($P<0.05$). The sRaw increase in the 1,25-$(OH)_2D_3$ treated group was markedly less than the NA model group ($P<0.05$) (Figure 1).

Effect of 1,25-$(OH)_2D_3$ on inflammatory cells in BALF

Total inflammatory cells and differential counts in BALF

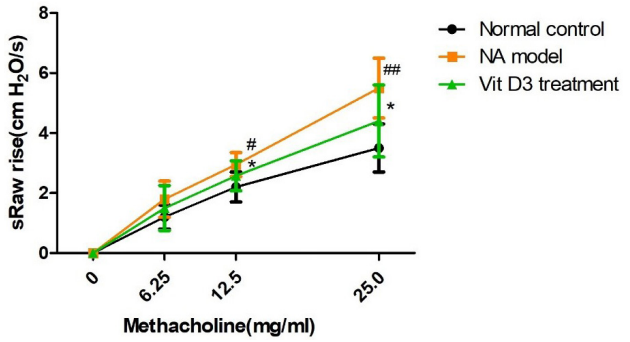

Figure 1. Change in the mouse airway resistance in response to increasing concentrations of methacholine
Data were expressed as the increase in sRaw (specific airway resistance). #*P*<0.05, ## *P*<0.01, compared with the normal control group; * *P*<0.05, compared with the model group. n=8 per group. NA: Neutrophilic asthma

were investigated to analyze the effect of 1,25-$(OH)_2D_3$ on airway inflammation. The total number of cells in BALF showed a marked increase in the NA model group in comparison to that in the control group. Both the percentage of eosinophils and neutrophils in the NA model group showed a significant increase compared to the control group. 1,25-$(OH)_2D_3$ treatment led to a significant reduction of neutrophil and eosinophil percentage as well as a decline in total inflammatory cells in comparison to the NA model group (Figure 2A, B).

Effect of 1,25-$(OH)_2D_3$ on histological change of lung tissue

OVA/LPS challenge induced airway inflammation in the NA model group, while HE-stained slides of lung tissue showed apparent inflammatory cell infiltration versus control model mice. And AB/PAS staining of lung tissue showed significant goblet cell hyperplasia and mucus hypersecretion within the bronchi in the NA model group. After 1,25-$(OH)_2D_3$ treatment, airway inflammation decreased compared to the NA group. As shown by HE and AB/PAS staining of lung tissue, the infiltration of inflammatory cells and goblet cell hyperplasia were reduced (Figure 3A, B).

Effect of 1,25-$(OH)_2D_3$ on neutrophils infiltration in lung tissue

Immunostaining for Ly-6G expression in lung tissue, a granulocytic marker of cell maturity, revealed that Ly-6G expression was elevated in the lungs of mice in the NA model group compared with the normal control group. Ly-6G+ neutrophils infiltrated predominantly within the airway and alveolar walls. Treatment with 1,25-$(OH)_2D_3$

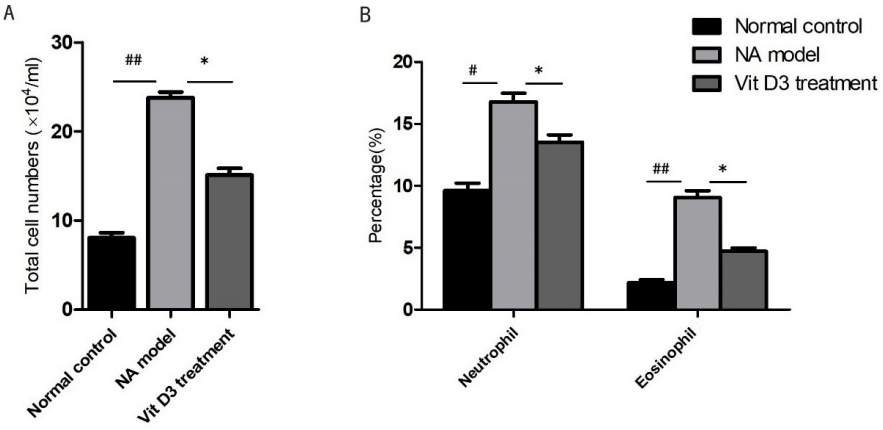

Figure 2. Total number of mouse inflammatory cells, neutrophil, and eosinophil percentages calculated in BALF
(A) The number of total inflammatory cells in the BALF. (B) The mean percentage of neutrophils and eosinophils in the BALF. Data expressed as mean± SEM (n=8/group). ## *P*<0.01, # *P*<0.05, compared with the normal control group; * *P*<0.05, compared with the model group. BALF: Bronchoalveolar lavage fluid

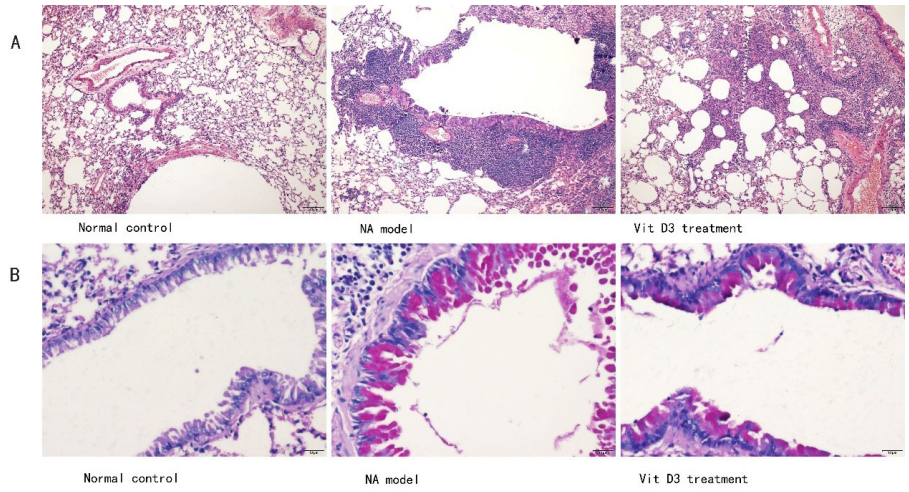

Figure 3. Histological change of mouse lung tissue (A) HE staining; (B) AB-PAS staining
Original magnification: ×200.

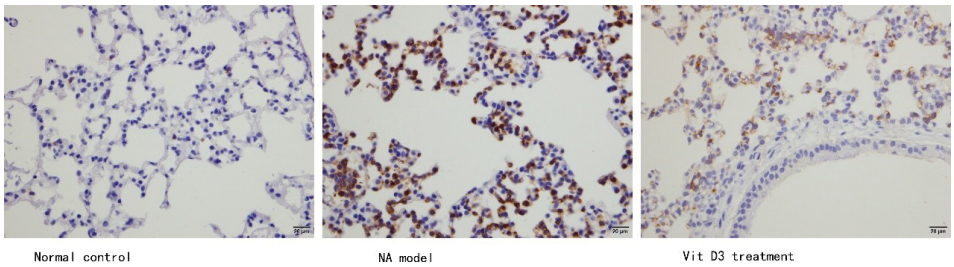

Figure 4. Immunostaining for Ly-6G expression in lung tissue
Magnification: ×400.

obviously suppressed neutrophil infiltration and Ly-6G expression (Figure 4).

3.5 Effect of 1,25-(OH)$_2$D$_3$ on inflammatory cytokine levels

In the mice of the NA model group, OVA/LPS challenge resulted in the elevation of concentrations of IL-17, IL-1β, and IL-18 in BALF compared to normal control mice, as measured by ELISA. 1,25-(OH)$_2$D$_3$ significantly reduced the concentrations of IL-17 and IL-1β, IL-18 versus the model group (Figure 5).

Protein expression of CHOP, GRP78, and NLRP3 in lung tissue

The expressions of CHOP, GRP78 protein, and NLRP3 protein were both significantly increased in the NA model group compared with the normal group (P<0.05). 1,25-(OH)$_2$D$_3$ treatment reduced the levels of CHOP, GRP78 and NLRP3 proteins (P<0.05) (Figure 6).

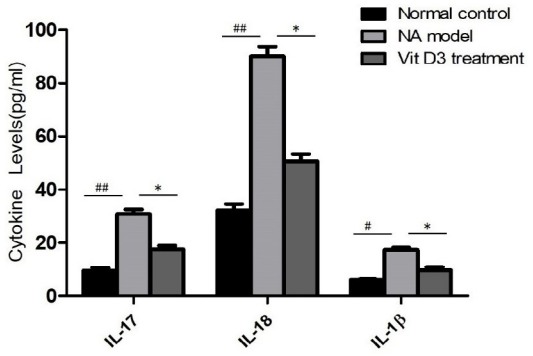

Figure 5. Cytokine Levels in BALF were Quantified by Enzyme-Linked Immunosorbent Assay (ELISA)
Data expressed as mean± SEM (n=8/group). ## P<0.01, # P<0.05, compared with the normal control group; * P<0.05, compared with the model group. NA: Neutrophilic asthma

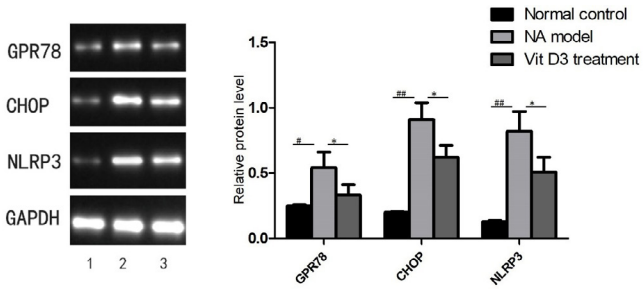

Figure 6. Expression of CHOP, GRP78, and NLRP3 in the mouse lung tissue by western blot. 1. Normal control group; 2. NA model group; 3. Vit D3 treatment group. Data expressed as mean± SEM (n=8/group). ## P<0.01, # P<0.05, compared with the normal control group; * P<0.05, compared with the model group. NA: Neutrophilic asthma

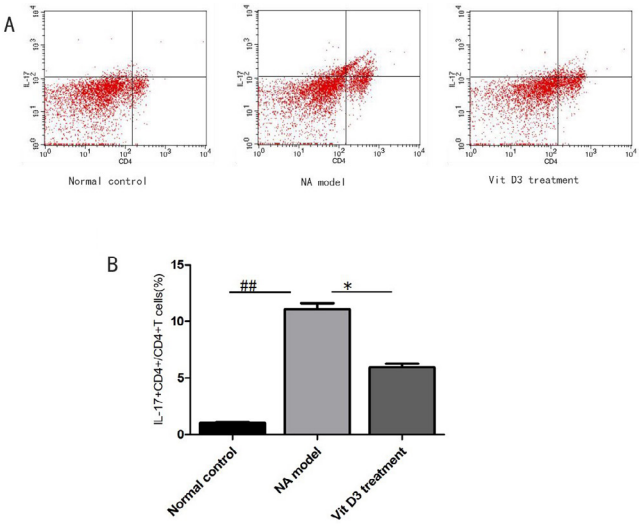

Figure 7. The percentage of mouse Th17 cells analyzed by FCM
(A) Representative results of Th17 cells for flow cytometry. (B) Comparative analysis of Th17 percentage in three groups. ## P<0.01, compared with the normal control group; * P<0.05, compared with the model group. FCM: Flow cytometer

Effect of 1,25-(OH)$_2$D$_3$ on proportion of Th17 cells

The percentage of Th17 cells in CD4+T cells of the lung was examined by flow cytometry. We could find that the percentage of Th17 cells in the NA group was higher than the normal control group (P<0.05). 1,25-(OH)$_2$D$_3$ decreased the percentages of Th17 cells versus the NA model group (P<0.05) (Figure 7).

Discussion

ERS maintains cellular protein homeostasis under multiple stimuli, serving as a fundamental cellular stress response. ER stress involves the accumulation of unfolded or misfolded proteins due to the disruption of homeostasis in the ER. Unresolved ERS contributes to the pathogenesis of many human diseases, including autoimmune and inflammatory diseases. Inflammasomes are integral parts of the innate immune response that mediate the activation of potent inflammatory mediators. They can promote the expression, maturation, and release of a multitude of proinflammatory cytokines upon activation and lead to a cascade of inflammatory responses. In recent years, emerging evidence suggests that ERS and inflammasomes interact with each other in inflammatory signaling cascades. A growing body of evidence has confirmed that ERS is involved in exacerbating inflammasome-induced inflammation cascades, and ERS-induced activation of the NLRP3 inflammasome is the pathological basis of various inflammatory diseases. Emerging research findings provide an opportunity for therapeutic targeting in inflammatory

diseases.

In recent years, many studies have reported the contributions of ERS to the pathogenesis of various pulmonary diseases, such as ARDS, pulmonary fibrosis, and asthma (12-15). ER stress may perform important regulatory functions in airway inflammation, apoptosis, mucus secretion, AHR, and airway remodeling of asthma. More recently, emerging evidence has revealed the therapeutic effects in asthma and various diseases through different methods of intervention, inhibiting ERS (16-19).

Vitamin D3 is an important hormone acting as a regulator of calcium homeostasis. Meanwhile, vitamin D3 has immunomodulatory function and has an inhibitory effect on various inflammatory diseases. Vitamin D3 deficiency is associated with asthma severity and control (20). Furthermore, experimental studies have shown that 1,25-dihydroxyvitamin D, an immunomodulator, exerts a protective role in asthma through the modulation of immune function. Taher *et al.* demonstrate that 1,25-$(OH)_2D_3$ potentiates the efficacy of immunotherapy and that the regulatory cytokines IL-10 and TGF-beta play a crucial role in the effector phase of this mouse model. (21) Zhou *et al.* found that 1,25-$(OH)_2D_3$ lowered many symptoms of inflammatory responses and decreased the expression of iNOS in OVA-induced experimental asthma. (22) In a study of Sprague-Dawley rats, Huang *et al.* revealed that administration of vitamin D alleviated the airway remodeling in asthma by down-regulating the activity of the Wnt/β-catenin signaling pathway (6). Previous studies suggest that vitamin D3 exerts its beneficial effect in asthma. However, the precise molecular mechanism underlying its immunomodulating properties remains to be fully elucidated.

In a study, Cao *et al.* found that 1,25-dihydroxyvitamin D3 was able to abolish NLRP3 inflammasome activation and subsequently inhibit caspase-1 activation and IL-1β secretion via the vitamin D receptor (VDR). VitD3 specifically prevented NLRP3-mediated apoptosis-associated speck-like protein with a caspase-recruitment domain (ASC) oligomerization. In addition to this, NLRP3 binding to NIMA-related kinase 7 (NEK7) was also inhibited. (23) Other studies observed that vitamin D3 ameliorated inflammation by inactivating the NLRP3 inflammasome (24, 25). Furthermore, Wen G and coworkers' findings showed that 1,25-$(OH)_2D_3$ is effective in attenuating ER stress and the NF-κB-driven inflammatory response in MCF-7 cells. (26) Research results by Yuan *et al.* suggest that vitamin D may ameliorate impaired wound healing in diabetic mice by suppressing ER stress.(9) Neutrophilic asthma is a severe airway inflammatory disease. So, could neutrophilic airway inflammation be attenuated by 1,25-$(OH)_2D_3$ through the inhibition of ERS and ERS-induced NLRP3 inflammasome activation?

In the present study, we found that 1,25-$(OH)_2D_3$ treatment reduced infiltration of inflammatory cells and goblet cell hyperplasia in lung and airway tissue, thus reducing inflammation in asthma. The total number of inflammatory cells and the percentage of neutrophils in BALF were significantly lower in the treatment groups compared with the NA model group. Importantly, in this study, we observed the effect of 1,25-$(OH)_2D_3$ on neutrophil infiltration in lung tissue through immunohistochemical staining for Ly-6G expression. Treatment with 1,25-$(OH)_2D_3$ obviously suppressed neutrophil infiltration and Ly-6G expression in neutrophilic asthma. Th17 cells and their signature cytokine, IL-17, have been implicated in the development of severe asthma (27). We investigated the production of IL-17 in the BALF by ELISA; the results revealed that treatment with 1,25-$(OH)_2D_3$ decreased IL-17 levels in the lung. Besides, we detected the percentage of Th17 cells in CD4+T cells in the pulmonary cell suspension by flow cytometry. In the NA group, their percentage was higher than that of the control group. 1,25-$(OH)_2D_3$ reduced the percentage of Th17 cells as compared with the NA model group. The NLRP3 inflammasome leads to the activation of caspase-1, which promotes the maturation and release of the inflammatory cytokine interleukin-1β (IL-1β) and IL-18. We observed that, in the treatment group, IL-1β and IL-18 production were decreased compared with the NA model group. This result shows that the protective role of 1,25-$(OH)_2D_3$ in neutrophilic asthma may be related to its inhibitory effects on NLRP3 activation. Then, whether 1,25-$(OH)_2D_3$ can ameliorate neutrophilic airway inflammation by inhibiting ERS-induced NLRP3 inflammasome activation in neutrophilic asthma? GRP78 and CHOP, two major components of ERS, were determined by Western blotting. Results showed that the expression levels of GRP78 and CHOP were lower in the 1,25-$(OH)_2D_3$ treatment group than those in the NA model group. In addition, NLRP3 protein expression was also detected by Western blotting. Also, treatment with 1,25-$(OH)_2D_3$ decreased NLRP3 protein expression levels in the lungs of NA mice. These findings indicate that administration of 1,25-$(OH)_2D_3$ was associated with suppressed ERS-mediated NLRP3 inflammasome activation, along with a corresponding decrease in neutrophil infiltration, Th17 cell proportion, and inflammatory cytokine levels in lung tissue. These observed changes coincided with attenuated airway inflammation, reduced mucus production, and improved airway hyperresponsiveness.

This study has several limitations. While this study provides evidence supporting the potential role of 1,25-$(OH)_2D_3$ in modulating neutrophilic airway inflammation through the ERS-NLRP3 pathway, the limitations should be acknowledged. First, the absence of a positive control group utilizing a specific inhibitor of ERS or NLRP3 inflammasome prevents direct benchmarking of the intervention's efficacy and fully validating the proposed mechanism. Second, the conclusions are drawn from a single animal model of neutrophilic asthma, and their generalizability to other models or human pathophysiology requires further investigation. Finally, although our data show a compelling association, the precise causal relationship and detailed molecular mechanisms remain to be fully elucidated. Future studies employing genetic approaches, such as cell-specific knockout models, and clinical trials in human populations are warranted to confirm these findings and translate them into therapeutic applications.

Conclusion

In summary, our study demonstrates that 1,25-Dihydroxyvitamin D3 treatment is associated with reduced airway inflammation and decreased levels of inflammatory cytokines, including IL-1β, IL-18, and IL-17, in a murine model of neutrophilic asthma. These changes were accompanied by suppression of ERS markers and NLRP3 inflammasome activation. The collective findings support a potential role for 1,25-$(OH)_2D_3$ in ameliorating

neutrophilic inflammation, possibly by modulating ERS-induced NLRP3 inflammasome activation.

While these results highlight a promising therapeutic candidate for neutrophilic asthma, several limitations should be considered. The observational nature of the data precludes definitive causal conclusions, and the use of a single animal model necessitates further validation in other experimental systems. Additionally, the absence of a positive control group limits direct mechanistic benchmarking. Future studies incorporating genetic approaches and pathway-specific inhibitors are warranted to elucidate the precise mechanism of action. Future studies will also include a dose-response analysis to determine the optimal therapeutic dosage. Ultimately, well-designed clinical trials will be essential to evaluate the translational potential of 1,25-$(OH)_2D_3$ in patients with neutrophilic asthma.

Acknowledgment

This work was supported by the Guangxi Natural Science Foundation, China (No. 2024GXNSFAA010409).

Authors' Contributions

JH Z supervised, directed, and managed the study. Q G and LY W conducted experiments, gathered data, and performed analysis; XY C prepared the manuscript. All authors reviewed and approved the final version.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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