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Original Article

1,25-dihydroxyvitamin D3 reduces mouse airway inflammation of neutrophilic asthma by transcriptional modulation of interleukin-17A

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Abstract: Corticosteroid resistance and severe airflow obstruction have been proved to participate in the neutrophilic inflammation of airway in uncontrollable asthmatics. IL-17 is one of the pro-inflammatory cytokines produced by Th17 cells, and it plays an important role in the neutrophilic inflammation of airway in steroid-resistant asthmatics. Recent data have proved that $1,25(OH)_2D_3$ represses IL-17A in inflammation and Th17-mediated autoimmunity through vitamin D receptors(VDR) at the level of transcription. Our study validated that $1,25\text{-}(OH)_2D_3$ can modulate IL-17A on the transcriptional level by using Runx1, thus reducing inflammation in the airway of mice with neutrophilic asthma. $1,25(OH)_2D_3$ may be promising for the therapeutic applications of neutrophilic asthma.

Keywords: $1,25(OH)_2D_3$, neutrophilic asthma, IL-17, airway inflammation

Introduction

Asthma is a serious health problem affecting people of all ages and causing severe limits in daily life when uncontrolled [1]. It has been defined as a chronic airway disorder which includes airway inflammation and hyper-responsiveness. There are 2 types of airway inflammation: eosinophilic inflammation and non-eosinophilic inflammation. Most asthmatic patients with chronic eosinophilic inflammation are sensitive to inhaled corticosteroids [2]. However, some with neutrophilic inflammation are poorly responsive to steroid therapy and therefore are at risk of developing severe asthma [3-5]. The precise pathology is unclear, but some studies suggest IL-17 is closely related to steroid-resistant neutrophilic airway inflammation [6]. IL-17 is a pro-inflammatory cytokine produced by Th17 cells with IL-17A being its main form. IL-17 was up-regulated in the airways of OVA-sensitized mice. The change after inhalation of allergen is associated with allergen-induced T cell activation and neutrophilic aggregation [7]. Steroid treatment in asthmatics could not attenuate airway neutrophilia and IL-17A expression [8].

$1,25(OH)_2D_3$ is the active form of vitamin D. It takes part in the construction of normal skeletal architecture and metabolism of calcium and phosphorus [8]. $1,25(OH)_2D_3$ achieves its biological effects mainly via binding to the vitamin D receptors(VDR) and Retinoid X receptor-á (RXR-á). Previous studies have shown that $1,25(OH)_2D_3$ have a suppressive effect on inflammation and Th17-mediated autoimmunity [7, 9]. Nanzer et al. reported that the IL-17A level in the peripheral blood of asthmatics couldn't be inhibited by glucocorticoids, but could be inhibited by $1,25(OH)_2D_3$ [10]. Recent data have indicated that $1,25(OH)_2D_3$ could curb IL-17A at the transcription level, which is mediated by VDR. The mechanism includes nuclear factor for activated T cells (NFAT) blocking, histone deacetylase (HDAC) recruitment, sequestration of Runx1 and direct induction of Foxp3 [11-14].

However, little is known about the role of $1,25(OH)_2D_3$ on inhibiting airway inflammation

of neutrophilic asthma by blocking the IL-17A pathway and its regulatory mechanism. In this article, we revealed the positive effect of $1,25(OH)_2D_3$ on airway inflammation and hyperresponsiveness of neutrophilic asthma mouse models, as well as its negative effect on IL-17A. The negative effect of $1,25(OH)_2D_3$ on IL-17A could be relieved by Runx1. Our results suggest that transcriptional modulation of IL-17A through Runx1 by $1,25(OH)_2D_3$/VDR was involved in the the suppression of the inflammation of mouse airway in neutrophilic asthma.

Methods

Mice

Female BALB/c mice (6-8 weeks) weighing 18-22 g were obtained from Changzhou Cavens Lab Animal Co. Ltd (Jiangsu, China, permit number SYXK 2011-0035). The mice were fed according to standard laboratory diet. They were kept in a room in the animal center of the Lung Transplant Lab of Wuxi People's Hospital. The room was temperature- and humidity-controlled, and provided 12 h of light and 12 h of darkness every day. This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health* and was conducted with the approval of the Ethics Committee of Nanjing Medical University. All surgeries were performed under sodium pentobarbital anesthesia to minimize suffering.

Protocols and pharmacologic treatments

We induced a mouse model of neutrophilic asthma in this study. Mice were intraperitoneally injected with 200 ug ovalbumin (OVA, Sigma-Aldrich, USA) emulsified in 2 mg aluminum hydroxide (Alum, Sigma-Aldrich, USA) in 200 ul normal saline on days 0 and 7. The model mice received intranasal sensitization of 100 ug OVA plus 10 ug lipopolysaccharide (LPS, Sigma-Aldrich, USA) in 50 ul normal saline on days 0, 7, 21 and 35. Additionally, the model mice were injected intraperitoneally with different volumes of $1,25(OH)_2D_3$ diluted in ethanol (Sigma-Aldrich, USA) 1 hour before an airway challenge with aerosolized OVA (from 1% to 11%, wt/vol, in normal saline, 60 minutes per day, 5 consecutive days per week) through an ultrasonic nebulization from day 14 to 53. The animals were euthanized and studied on day 54.

In this study, the mice were randomly divided into six groups (n=8 per group): (i) mice sensitized with normal saline (control group, NC group). (ii) mice sensitized with OVA plus LPS and challenged with OVA (neutrophilic asthma group, NA group). (iii) mice treated with ethanol 1 hour before the same challenge with OVA (CH group). (iv) mice treated with 0.2 ml $1,25(OH)_2D_3$ 1 hour before the same challenge with OVA (low dose group, L group). (v): mice treated with 0.5 ml $1,25(OH)_2D_3$ 1 hour before the same challenge with OVA (middle dose group, M group). (vi) mice treated with 1 ml $1,25(OH)_2D_3$ 1 hour before the same challenge with OVA (high dose group, H group).

Measurement of airway hyperresponsiveness

24 hours after the final OVA challenge, we assessed airway hyper-responsiveness in conscious and unrestrained mice through whole-body plethysmography (Buxco Electronics Inc., NY, USA). Briefly, each mouse was placed in a plastic chamber and exposed to methacholine aerosols ranging from 16.25 to 50 mg/mL in PBS for 3 min. The Penh values were recorded continuously from 5 s to 3 min after each methacholine challenge.

Bronchoalveolar lavage fluid collection and cell count

The mice were euthanized by intraperitoneal injection of an overdose of pentobarbital 48 h after the last OVA challenge, followed with a tracheostomy. For the bronchoalveolar lavage fluid (BALF) collection, 0.4 mL ice-cold PBS was infused for three times through a 20 G plastic catheter. The recovery rate of BALF was above 80%. The reclaimed BALF was immediately centrifuged at 1500 rpm for 10 min at 4°C. The supernatant remains was stored in an -80°C freezer for further cytokine analysis. The precipitate was then resuspended by 50 μl PBS for the total cell count. Wright-Giemsa-stained smears were used for the cell differentiation. Trypan blue staining was used for exclusion of dead cells.

Lung histopathology and morphometry

After the mice were killed, lower lobes of their right lungs were fixed in 10% formaldehyde for 24 h and then embedded in paraffin. Tissues were cut into 4 μm sections and stained with hematoxylin and eosin (HE). We performed

Periodic Acid-Schiff (PAS) staining for a better inflammatory cell differentiation of lung tissues. The sections were observed with a microscope at ×400 magnification. We analyzed five randomly selected airway sections in each group. The numbers of eosinophils, neutrophils and total cells were counted by Image-Pro Plus. This process was performed in a routine histology lab.

Measurement of the levels of IL-17A in BALF and culture supernatants

The levels of IL-17A in BALF and culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (eBioscience, CA, USA). The absorbance at 450 nm was measured using a microplate reader (Beckman Coulter, USA). Absolute concentrations were obtained by running standard curves on the same ELISA plates.

Th17 differentiation

$CD4^+$ T cells were isolated from BALB/c mice spleens by a MACS column (CD4+ T cell isolation kit II, Miltenyi Biotec) according to the manufacturer's protocol. Purified $CD4^+$ T cells were cultured in complete RMPI 1640 and treated with either vehicle or different doses of $1,25(OH)_2D_3$ (0.1 nM, 1 nM, 10 nM) for 3 days under the condition (which will be called the "Th17 condition" in the following passage) in which we mimicked the presence of Th17 by using 1 ug/ml anti-CD3, 1 ug/ml anti-CD28, 50 ug/ml anti-IFN-γ, 10 ug/ml anti-IL-4, 20 ng/ml IL-6, 3 ng/ml TGF-β, 20 ng/ml IL-23. Cells were cultured for 4 to 6 days and then rested for an additional 4 to 6 days (1 stimulation cycle).

Transient-transfection and luciferase assay

The 2-kb mouse IL-17A (mIL-17A) promoter construct and CMV-RunX1 was obtained from Genechem company. HEK293T cells were seeded in a 24-well culture dish 24 h prior to transfection. Cells were transfected with lipofectamine 2000 according to the manufacturer's instructions. 24 h after transfection, cells were treated with the following: (i) vehicle, (ii) $1,25(OH)_2D_3$ (0.1 nM to 10 nM), (iii) activation with PMA (107 M), and (iv) $1,25(OH)_2D_3$ plus PMA.

RNA-mediated interference

After purification as described above, $CD4^+$ T cells were directly transfected by nucleofection with RunX1 siRNA or control siRNA for knockdown of Runx1. 2×10^5 cells were transfected with 80 pmols of either RunX1 siRNA or control siRNA. After transfection, cells were incubated for 4 h at 37°C and activated with 1 ug/ml anti-CD28 and anti-CD3. Then they were cultured under Th17 condition as described above. After 48 h of activation, we collected part of the transfected cells and extracted total RNA for Real-time PCR analyzation. The remaining cells were collected after 'rest' for another 48 h and re-stimulated with PMA and ionomycin. Total RNA was extracted for Real-time PCR analyzation. IL-17A production in culture supernatants was measured by ELISA.

Real time PCR

Total RNA was isolated from lung samples and Th17 cells using RNAiso Plus Reagent (Takara, Japan). Then a PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan) was used to perform reverse transcription. The IL-17 mRNA was quantified by real-time PCR analysis, which was carried out using a SYBR Premix Ex TaqTMII (Takara, Japan). Primers used for mouse IL-17A were 5'-GAAGGCCCTCAGACTACCTCAA-3' and 5'-TCATGTGGTGGTCCAGCTTTC-3' (40 cycles). Primers used for RunX1 were 5'-TTTCAGGAGTGGTGACGCCT-3' and 5'-AGCAGGTACCACTGGTCTTC-3'. All data was measured for the expression of GAPDH mRNA for the sample (GAPDH primers, 5'-GAAGGCCCTCAGACTACCTCAA-3' and 5'-TCATGTGGTGGTCCAGCTTTC-3'). All runs were performed at least twice. Primer sequences were designed by Primer 5 according to the corresponding structures of mouse genes.

Western bolt analysis

Nuclear and cytoplasmic protein fractions were extracted from differentiated T cells using the RIPA lysates (CWBIO, China). The protein concentrations were assessed using the BCA protein assay kit (CWBIO, China). Protein samples were stored in a -80°C freezer until being used for immunoblotting. 30 ug samples were run on 12% SDS/PAGE and transferred to a PVDF membrane (Millipore, USA), which was blocked with 5% fat-free milk in TBST (0.1% Tween-20 in TBS) for 2 h at room temperature. Blocked

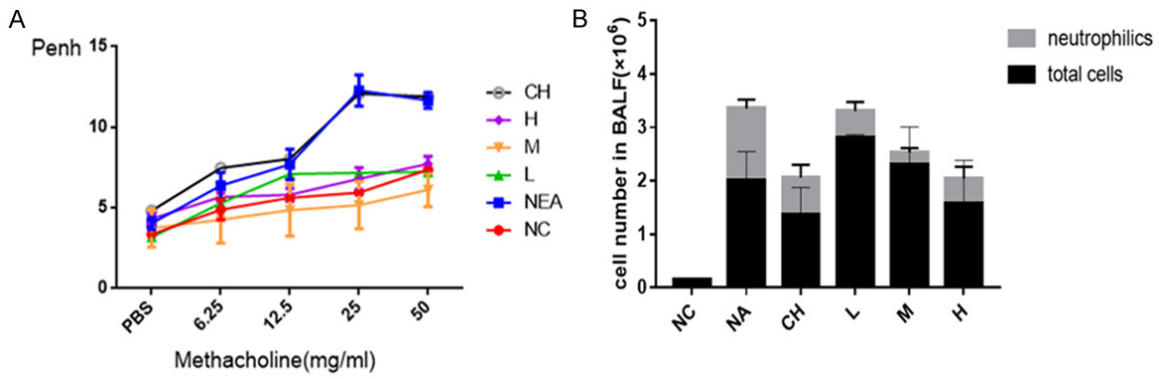

Figure 1. The effect of $1,25(OH)_2D_3$ on the murine neutrophilic asthma model. $1,25(OH)_2D_3$ suppresses the elevated methacholine responsiveness induced by OVA challenge. Methacholine responsiveness was assessed 24 h after the last OVA-challenge (A). $1,25(OH)_2D_3$ reduces the recruitment of inflammatory cells in the BALF (B). (n=8, $P < 0.05$).

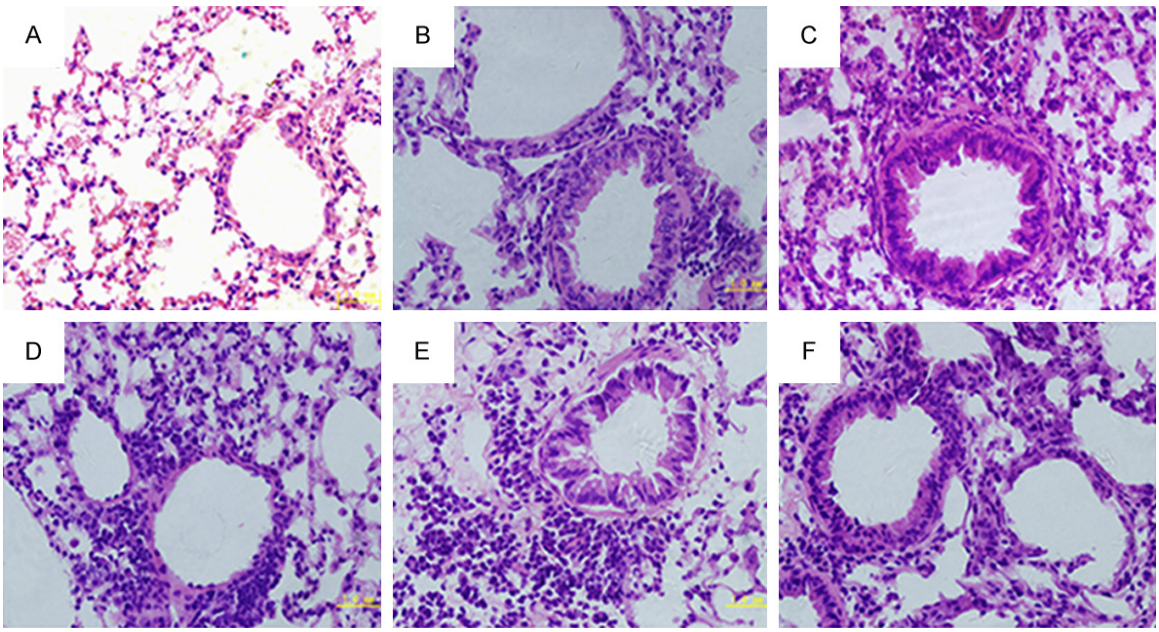

Figure 2. H&E staining of the lung tissues. Representative results of lung pathology (original magnification, 400×) in mice from the NA (A), NC (B), CH (C) and low (D), middle (E), high (F) dose of $1,25(OH)_2D_3$ treated groups were analyzed. The larger number of inflammatory cells, including neutrophils, in the asthma group in comparison to the control could be decreased by $1,25(OH)_2D_3$.

membranes were incubated with primary antibodies, including anti-Runx1 (Santa Cruz) or anti-VDR (Biotechnology inc) at a dilution of 1:2000 in 1% Tris-buffered saline overnight. Membranes were then rinsed and incubated with goat anti-rabbit/mouse IgG-horseradish peroxidase (HRP) antibodies (CWBIO, China) at 1:2000 dilution in 1% TBST for 2 h at room temperature. After being thoroughly washed in TBST, the quantification of the proteins on membranes was detected by fluorography using ECL (enhanced chemiluminescence) reagents (Millipore, USA) according to its instructions and analyzed in image J.

Chromatin immunoprecipitation (ChIP) assay

Mouse primary $CD4^+$ T cells were isolated and polarized under Th17 condition as described. The chromatin immunoprecipitation (ChIP) assay was performed according the CHIP kit instructions. (Thermo scienitific, USA) Immun-

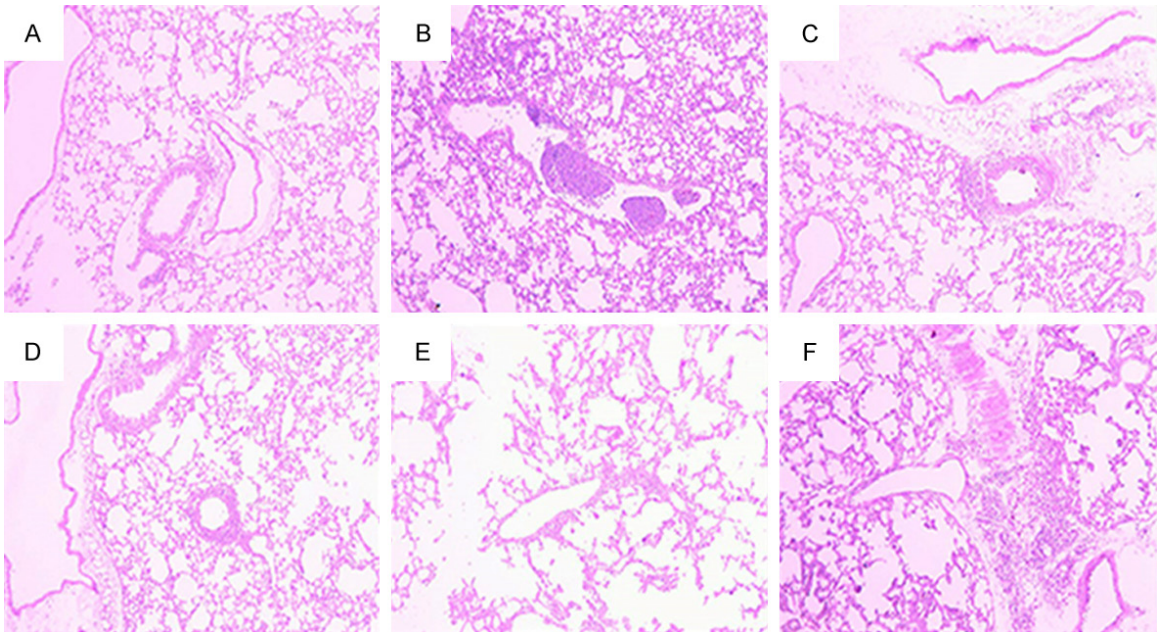

Figure 3. PAS staining of the lung tissues. Representative results of lung pathology (original magnification, 200×) in mice from the NA (A), NC (B), CH (C) and low (D), middle (E), high (F) dose of $1,25(OH)_2D_3$ treated groups were analyzed. The larger number of inflammatory cells, including neutrophils, in the asthma group in comparison to the control could be decreased by $1,25(OH)_2D_3$.

Table 1. The numbers of eosinophils, neutrophils and total cells in lung tissues analyzed from PAS staining

	Eosinophils	Neutrophils	Total cells
C	2.3±0.57	2.0±1.2	62.8±20.5
CH	29.0±2.65*	52.3±9.07*	214.8±27.84*
NA	34.7±10.79*	53.4±11.93*	226.6±11.85*
L	22.0±2.65*	33.0±4.58*,Δ	220.4±35.03*
M	18.4±2.52*,Δ	25.3±4.04*,Δ	202.5±27.47*
H	11.0±3.61Δ	21.7±5.69*,Δ	172.3±22.9*,Δ

The values expressed as mean ± SD (n=5). *P < 0.05, compared with control group. ΔP < 0.05, compared with NA group.

oprecipitations were performed with anti-Runx1. DNA precipitates were isolated and subjected to PCR, and the primers we used are murine IL-17A promoter fragments with Runx1 binding sites: 5'-AAACCACATGGTGTTGTGGTAT-3' and 5'-TGACCGATAGAATAACTATTTGA-3'. DNA acquired prior to precipitation was collected and used as the input. A total of 10% of input was used for PCR evaluation. PCRs using the primers designed to amplify the upstream region of respective promoters were used as a negative control to exclude nonspecific binding.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6.01 (GraphPad Software, San Diego, CA). Data were expressed as mean ± SD. Differences between groups were analyzed using ANOVA tests or unpaired, two-tailed, parametric Student's t-test. $P < 0.05$ were considered statistically significant.

Results

$1,25(OH)_2D_3$ reduces methacholine responsiveness induced by OVA challenge

The NA and CH groups exhibited significantly increased AHR as methacholine concentrations increased compared with the NC, L, M and H groups ($P < 0.01$, **Figure 1A**). There was no significant difference between the NA and CH groups. However, the methacholine responsiveness induced by OVA challenge could be decreased by the $1,25(OH)_2D_3$ treatment. Moreover, the inhibition of methacholine responsiveness was enhanced with the elevation of the concentration of $1,25(OH)_2D_3$ ($P < 0.05$, **Figure 1A**). We also found that the M group showed the lowest Penh values.

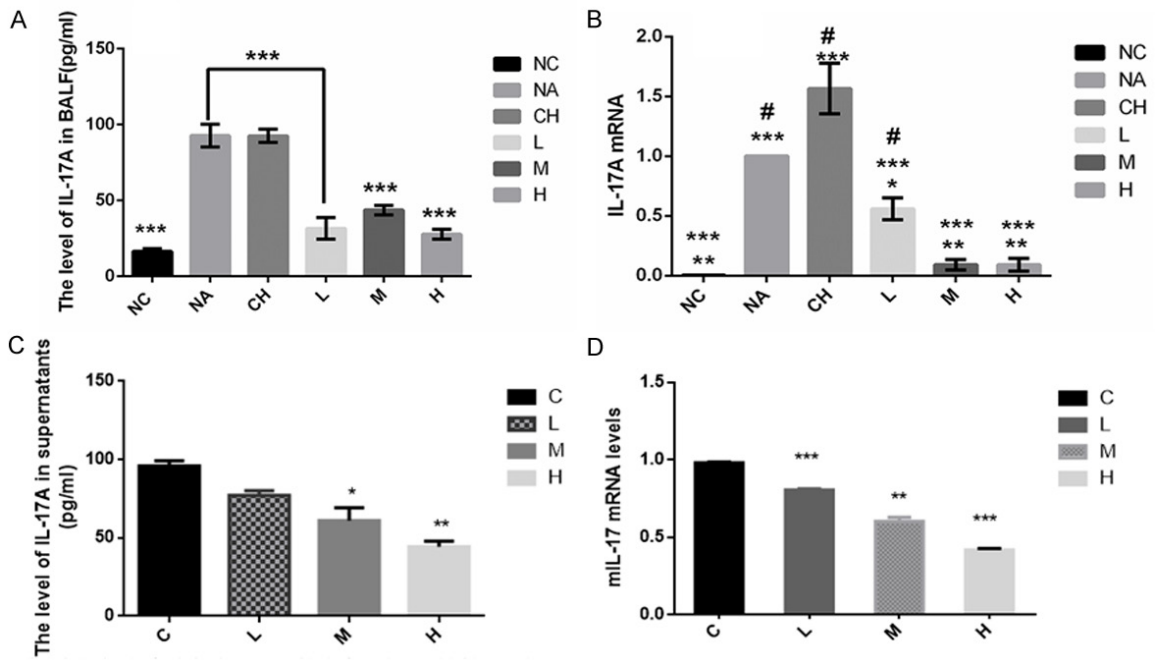

Figure 4. $1,25(OH)_2D_3$ suppresses IL-17A expression. IL-17A level measured by ELISA was increased in BALF from OVA-challenged mice and $1,25(OH)_2D_3$ significantly reduced this increment (A). IL-17A mRNA levels were significantly elevated in lung tissues from OVA-challenged mice and decreased after $1,25(OH)_2D_3$ treatment (B). T cells isolated from splenocytes were activated under Th 17 condition in the presence or absence of $1,25(OH)_2D_3$ (0.1 to 10 nM). 48 h later, supernatants were used to measure the level of IL-17A by ELISA. IL-17A was significantly reduced by $1,25(OH)_2D_3$ in M and H groups compared with control group (C). After 3 days of differentiation of the mouse primary $CD4^+$ T cells under Th17 condition, we observed how IL-17A mRNA was inhibited by different concentrations of $1,25(OH)_2D_3$ (D). Compared with the NA group, **P < 0.01; *P < 0.05, compared with the NC group; #P < 0.01, and the CH group; ***P < 0.01. Results are presented as mean ± SD (n=5).

$1,25(OH)_2D_3$ prevents the neutrophilic airway inflammation

To investigate the effect of $1,25(OH)_2D_3$ on neutrophilic asthma, the murine neutrophilic asthma model received intraperitoneally injection of $1,25(OH)_2D_3$. The OVA-challenged group had significantly more total cells and neutrophils in the BALF than the NC group (P < 0.01). The administration of $1,25(OH)_2D_3$ markedly decreased inflammatory cells, especially neutrophils in BALF (P < 0.01, **Figure 1B**).

H&E stained lung sections showed a large number of inflammatory cells including neutrophils in the parabronchial and perivascular regions of the asthma group. Markedly, a reduction in this pulmonary inflammation and neutrophil infiltration was observed in mice in the $1,25(OH)_2D_3$ treated group compared to NA group, especially in the high dose group (**Figure 2**). PAS stained lung sections showed similar results. (**Figure 3**, **Table 1**).

$1,25(OH)_2D_3$ suppresses IL-17A in mouse models of neutrophilc asthma

We examined the effect of $1,25(OH)_2D_3$ on IL-17A in mouse BALF and lung tissues. Compared with the NC group, the IL-17A levels in BALF of the NA and CH groups were significantly higher in Alum/OVA-sensitized/challenged mice compared with the NC group (P < 0.001, **Figure 4A**). IL-17A levels were statistically lower in $1,25(OH)_2D_3$ treated groups. However, we found no difference between the L, M and H groups (P < 0.001, **Figure 4A**). IL-17A mRNA levels were significantly elevated in lung tissues from OVA-challenged mice, and these levels decreased after 1,25-dihydroxyvitamin D3 treatment (P < 0.05, **Figure 4B**).

$1,25(OH)_2D_3$ suppresses the secretion of IL-17A in mouse $CD4^+$ T cells

We treated T cells isolated from mouse spleens under Th17 condition with vehicle or different doses of $1,25(OH)_2D_3$. A significant reduction in

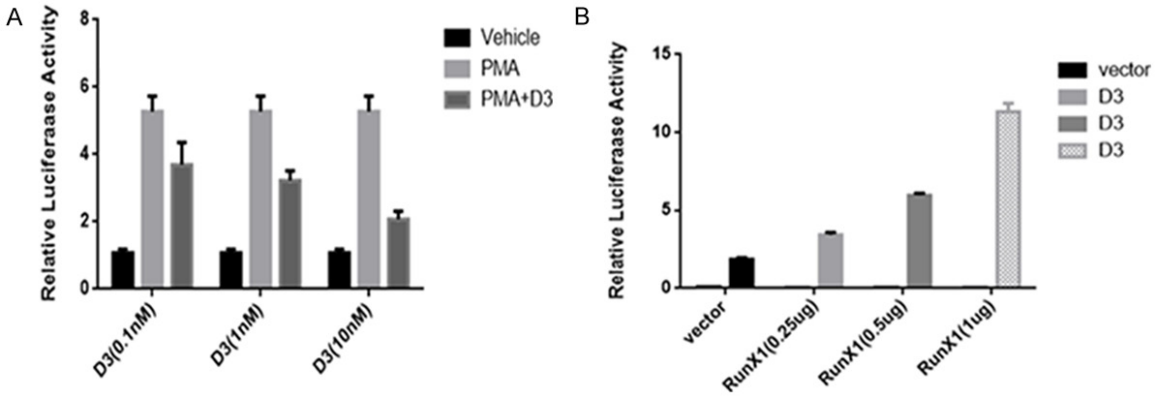

Figure 5. IL-17A transcription is repressed by $1,25(OH)_2D_3$ and relieved by Runx1. HEK293T cells transfected with the mIL-17A promoter were activated 4 h with PMA in the presence of increasing concentrations of $1,25(OH)_2D_3$ (A). RunX1 relieves the repression of $1,25\text{-}(OH)_2D_3$ on mIL-17A transcription and enhances activation. HEK293T cells were co-transfected with the 2-kb mIL17A promoter and increasing concentrations of Runx1 expression vector or empty control vector. Cells were activated for 4 h with PMA in the presence or absence of 10 nM $1,25(OH)_2D_3$ (B). ($P < 0.05$, n=5).

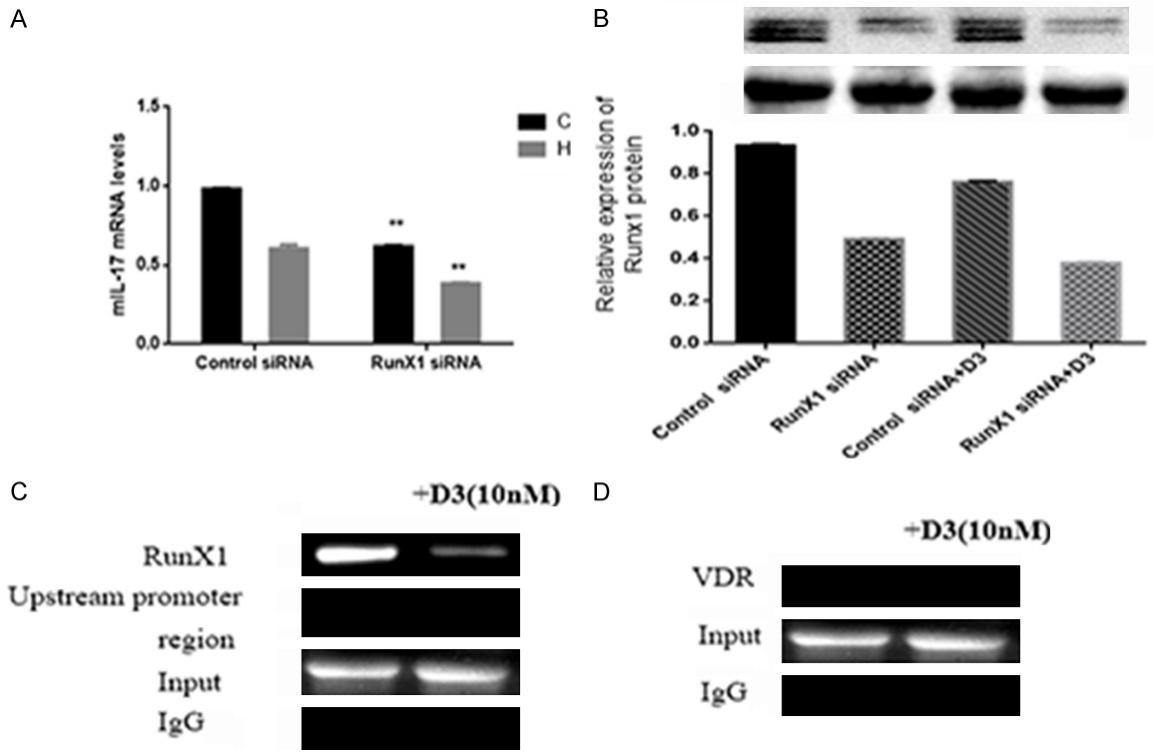

Figure 6. RunX1 is required for IL-17A expression in mouse $CD4^+$ T cells. Inhibition of mIL-17A mRNA levels by Runx1-specific siRNA was observed (A). Western blot analysis of Runx1 protein levels was done to confirm knockdown of Runx1 by Runx1-specific siRNA (B). ChIP assays were performed in mouse $CD4^+$ T cells polarized under Th17 condition. RunX1 was recruited to the RunX1 sites in the IL-17A promoter, but this recruitment was markedly decreased in the presence of $1,25(OH)_2D_3$ (C). VDR recruitment to Runx sites was not observed with or without $1,25(OH)_2D_3$ (D). ($P < 0.05$, n=5).

IL-17A secretion was observed in the presence of middle and high doses of $1,25(OH)_2D_3$ ($P < 0.05$), while there was no significant difference between the low dose group and the control group (**Figure 4C**). In addition, we found that in the presence of $1,25(OH)_2D_3$, the expression of IL-17A mRNA was inhibited in mouse $CD4^+$ T cells under Th17 condition (**Figure 4D**).

IL-17A transcription is directly repressed by $1,25(OH)_2D_3$, which could be relieved by Runx1

It remains poorly understood whether IL-17A transcription is involved in the mechanism of inhibition of mouse IL-17A secretion and mRNA levels. We transfected HEK293T cells with a mouse 2-kb IL-17A promoter and CMV-RunX1 for 24 h and found that IL-17A transcription was significantly repressed in response to $1,25(OH)_2D_3$ ($P < 0.05$, **Figure 5A**).

Additionally, we assessed the ability of RunX1 to rescue $1,25(OH)_2D_3$-mediated inhibition of mIL-17A transcription. We transfected several kinds of plasmids into HEK293T cells that were treated with 1,25-dihydroxyvitamin D3 (10 nM), one was the RunX1 empty plasmid, the others were plasmids mixed with the IL-17A promoter and different concentrations of RunX1. The activity of IL-17A luciferase increased along with the increase of the RunX1 plasmid concentration, which indicated that Runx1 offsets the inhibition by 1,25-dihydroxyvitamin D3 on L-17A transcription, enhancing its activity ($P < 0.05$, **Figure 5B**).

Runx1 is required for IL-17A expression in mouse $CD4^+$ T cells

To investigate the effect of RunX1 on the IL-17A expression, $CD4^+$ T cells were transfected with RunX1 siRNA (**Figure 6A**, **6B**). The expression of IL-17A mRNA levels was statistically reduced after transfection, which indicates that RunX1 is required for mouse IL-17A expression ($P < 0.05$, **Figure 6A**). Western bolt of RunX1 protein levels was done to confirm the transfection effect of RunX1 siRNA (**Figure 6B**).

$1,25(OH)_2D_3$ decreases recruitment of Runx1 to IL-17A promoter

$CD4^+$ T cells were cultured under Th17 condition and activated in the presence or absence of 10 nM $1,25(OH)_2D_3$ for 3 days. We found that Runx1 was recruited to the Runx1 sites in the IL-17A promoter. However, VDR was not observed to be recruited to the Runx1 sites. Also, the recruitment of Runx1 to the mIL-17A promoter could be decreased by $1,25(OH)_2D_3$ (**Figure 6C**, **6D**).

Discussion

Asthma is a heterogeneous disease characterized by chronic airway inflammation, bronchial hyper-responsiveness and airway structural remodeling [15, 16]. Currently, most asthmatics are sensitive to inhaled corticosteroids (ICS) [17, 18]. However, ICS seems to be useless for neutrophilic asthmatics and corticosteroid resistance resulted in prolonged hospitalization and high rates of mortality [19]. IL-17 is one of the pro-inflammatory cytokines produced by Th17 cells and IL-17A is its main existing form [20]. IL-17A has been reported to be a negative regulator of established allergic asthma and correlate with the severity of asthma [21]. $1,25(OH)_2D_3$ have been reported to have a suppressive effect on inflammation and Th17-mediated autoimmunity. Tang et al. demonstrated that $1,25(OH)_2D_3$ could prevent and partially reverse experimental autoimmune uveitis (EAU) by reducing IL-17 production [22]. Additionally, $1,25(OH)_2D_3$ was proved to be able to suppress IL-17A induction in mouse models of 2,4,6-trinitrobenzene sulfuric acid (TNBS) colitis and early rheumatoid arthritis [23, 24], as well as Candida albicans-stimulated peripheral blood mononuclear cells (PBMC) [25]. Nanzer et al. studied PBMC from asthmatic patients in vitro. They revealed that patients with severe asthma showed much higher IL-17A levels and $1,25(OH)_2D_3$ could inhibit this increase in a glucocorticoid-independent fashion [10]. Researchers also indicated that $1,25(OH)_2D_3$ could repress IL-17A at the transcriptional level by VDR through mechanisms including nuclear factor for activated T cells (NFAT) inhibition, histone deacetylase (HDAC) recruitment, sequestration of Runx1 and direct induction of Foxp3 [21, 26-28].

However, little is known about the role of $1,25(OH)_2D_3$ in neutrophilic asthma. It is still unclear whether $1,25(OH)_2D_3$ could inhibit airway inflammation by blocking the IL-17A pathway and its regulation mechanism. In previous studies, increased neutrophils and almost no eosinophils in BALF was observed in neutrophilic asthma mice induced by OVA-LPS. Besides, H&E stained lung tissues around the bronchi and vessels also showed more inflammatory cells, including neutrophils [28, 29]. In this study, we established a mouse model of neutrophilic asthma through sensitizing OVA with LPS and aluminum hydroxide. The neutrophilic asthma group exhibited significant neutrophil infiltration in the BALF and lung tissue. Airway hyper-responsiveness was increased in NA group. Moreover, IL-17A was up-regulated in OVA-challenged mice. The positive connection

between sputum IL-17 and neutrophils in acute asthmatics has been studied [26]. Hellings et al. reported up-regulation of IL-17 in OVA-sensitized mice airways. In addition, IL-17 was closely connected with allergen-induced T cell activation and neutrophilic influx into airways after allergen inhalation [26, 30]. IL-17 mRNA expression was found to be increased in BAL cells of horses affected by heaves, indirectly leading to recruitment of neutrophils into the airways [27]. A recent study by Murcia et al. indicated that IL-17 could directly activate and possibly recruit equine neutrophils, which was not attenuated by DEX [28]. Similarly, IL-17 was proved to play an important role in neutrophil recruitment in the airways of rats and mice, as well as humans.

In this study, we treated OVA-sensitized mice with different doses of 1,25-$(OH)_2D_3$. Neutrophilic cells in BALF, production of IL-17A and airway responsiveness all decreased with the elevation of the dose of 1,25-$(OH)_2D_3$. Likewise, we have found that 1,25-$(OH)_2D_3$ reduced IL-17A levels in mouse $CD4^+$ T cells under Th17 condition.

$1,25(OH)_2D_3$ achieves its biological effect mainly via binding to the vitamin D receptors (VDR) and Retinoid X receptor-á (RXR-á) in the nucleus of various cells [31]. The VDR is a member of the superfamily of nuclear hormone receptors and exists in plenty of immune cells including stimulated macrophages, dendritic cells, monocytes and activated B and T cells. The VDR is considered to be a ligand-activated transcription factor that binds specific DNA sequence elements [32]. For high-affinity DNA binding to cognate vitamin D response elements (VDREs) located in the regulatory regions of 1,25D target genes, the ligand-bound VDR is required to heterodimerize with RXRs, followed by the activation of transcription. In this study, IL-17A transcription could be significantly repressed by $1,25(OH)_2D_3$ in HEK293T cells after being transfected with a 2-kb IL-17 transcription motor construct. It revealed that the $1,25(OH)_2D_3$ could negatively regulate airway inflammation in neutrophilic asthmatic mice by inhibiting the production and transcription of IL-17A.

Moreover, we have proved that the suppressive effect of $1,25(OH)_2D_3$ on IL-17A transcription could be attenuated by Runx1. The RunX family is constituted of 3 highly homologous proteins, which are all expressed in Th17 cells [11-13]. Several researches have shown that IL-17 transcription and expression was dependent on Runx1 [14, 33]. Runx1 transcription factor interplays with distinct molecules leading to various aspects of T cell differentiation. In Th cell differentiation, Runx1 interacts with Gata3 to suppress IL-4 secretion and induce IFN-g production [34]. There are sequences present in the 2-kb promoter fragment in the upstream of IL-17 transcription start site, including upstream of the RORγt-/Runx1-binding site to promote transcription. As reported previously, IL-17 expression could be strongly suppressed by the silence of Runx1. Moreover, Runx1 could not have effects on IL-17 transcription without RORγt. However, the binding of Foxp3 or T-bet to Runx1 inhibited RORγt- and Runx1-induced IL-17 expression [33, 35]. Our study has found that overexpression of Runx1 could rescue $1,25(OH)_2D_3$-mediated inhibition of IL-17A transcription and enhance activation of IL-17A transcription. Under the same condition, the IL-17 expression level increased with the Runx1 dose in vitro. The expression level of IL-17 in mouse $CD4^+$ T cells transfected with Runx1-specific siRNA was inhibited, which revealed that RunX1 is required for IL-17A expression in vivo.

Likewise, our study showed that the negative effect of $1,25(OH)_2D_3$ can involve isolation of Runx1 by VDR. Previous studies have indicated that in osteoblastic cells, Runx2 and VDR could cooperate in the $1,25(OH)_2D_3$ stimulation of osteopontin and osteocalcin [36, 37]. Joshi et al. showed that VDR could suppress IL-17A through interaction with Runx1, leading to prevention of Runx1 activation and Runx1 sequestration in mouse models of MS [9]. Through CHIP studies, we found that Runx1 was recruited to the Runx1 sites in the IL-17A promoter. However, $1,25(OH)_2D_3$ could markedly decrease this recruitment. Thus, we can conclude that Runx1 sequestration by $1,25(OH)_2D_3$/VDR may be a common mechanism for down-regulation of IL-17, which is positively regulated by Runx1. These results may provide us with new insights for therapeutic targets for the control of neutrophilic asthma, as well as corticosteroid-resistant asthma.

Disclosure of conflict of interest

None.

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