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ORIGINAL ARTICLE

Dll4 in the DCs isolated from OVA-sensitized mice is involved in Th17 differentiation inhibition by 1,25-dihydroxyvitamin D3 *in vitro*

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Abstract

Introduction: T helper 17 cell (Th17) cells play an important role in neutrophilic asthma, and 1,25(OH)2D3 has been reported to modulate the proliferation and differentiation of T cells. In this study, we examined the effects of 1,25(OH)2D3 on the dendritic cell (DC)-mediated regulation of Th17 differentiation from OVA-sensitized mice. **Methods:** DCs were isolated from ovalbumin-sensitized mouse spleens. Lipopolysaccharide (LPS) was administered to stimulate the DCs for 24 h, and dexamethasone or 1,25(OH)2D3 was applied simultaneously. The expression of Notch ligand delta-like ligand 4 (Dll4) in the DCs was detected in each group. All the groups of treated DCs were co-cultured with T cells, and Dll4 was inhibited in these groups. After 24 h, Th17 and Treg cell differentiation and the IL-17A levels were measured. **Results:** Dll4 expression was increased in LPS-treated DCs compared with the control group ($p=0.05$), resulting in increased Th17 cell differentiation ($p=0.002$). Treatment with 1,25(OH)2D3 inhibited the Dll4 expression ($p=0.04$) and decreased Th17 cell differentiation ($p=0.001$) in DCs that was induced by LPS. Directly inhibiting Dll4 reduced Th17 cell differentiation, and Th17 cell differentiation was not further inhibited by 1,25(OH)2D3 once Dll4 was blocked. **Conclusions:** These results suggest that Dll4 in the DCs isolated from OVA-sensitized mice is involved in Th17 differentiation inhibition by 1,25(OH)2D3.

Keywords

1,25-dihydroxyvitamin D3, asthma treatment, delta-like ligand 4, dendritic cells, T helper 17 cell

History

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Introduction

Asthma is a very common disease that is considered as a primary T helper 2 (Th2)-mediated disease. Asthma is characterized by the production of interleukin (IL)-4, IL-5, and IL-13 and the infiltration of eosinophils. Recently, four distinct inflammatory subtypes of asthma have been identified: the eosinophilic, neutrophilic, paucigranulocytic, and mixed granulocytic inflammatory patterns [1]. Of these four phenotypes, neutrophilic asthma is induced by Th17 cells, which are characterized by the production of IL-17 [2]. In the IL-17 family, which comprises six members (IL-17A-F), IL-17A is considered the primary IL-17 cytokine and is thought to negatively affect asthma by promoting airway hyper-responsiveness (AHR), neutrophilia, airway remodeling, late-phase airway and systemic inflammation, and pro-inflammatory cytokine production [3–6]. Because Th17 cells play an important role in neutrophilic asthma, increasing numbers of researchers have focused on Th17 cell function in severe asthma as a mechanism underlying neutrophilic inflammation.

The direction of T cell differentiation is determined by the interaction between antigen-presenting cells (APCs) and

T cells. Dendritic cells (DCs) are considered the most important APCs for the initiation of the antigen-induced immune responses. Specific signals, including innate cytokine production, major histocompatibility complex (MHC) molecule expression and co-stimulatory molecule activation, rapidly promote the activation of immune responses [7–9]. The identified family of Notch ligands in DCs also alters the course of T cell activation [10,11]. Several studies have demonstrated that Notch is involved in Th1, Th2, Th17, and Treg cell differentiation, depending on the ligand, the immune environment, the genetic makeup, and the disease model. In a mouse model of multiple sclerosis, the inhibition of delta-like ligand 4 (Dll4) decreases Th1/Th17 cell responses [12], and in experimental autoimmune encephalomyelitis, Dll4 blockade alleviates the disease by promoting regulatory T cell development [13]. Dll4 up-regulates ROR γ expression in T cells, which further enhance the differentiation of Th17 cell populations [14]. These findings have raised the question of whether Dll4 is involved in Th17 cell differentiation in asthma.

1,25(OH)2D3 (VitD3), the active form of vitamin D, has been reported to act as a modulator of the proliferation and differentiation of cells, including immune system cells, especially CD4⁺ T cells. VitD3 affects naive CD4⁺ T cells directly and DCs indirectly to regulate the Th1/Th2 balance [15,16]. Recent studies have confirmed that vitamin D

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reduces the differentiation and expansion of Th17 cells in young asthmatic children [17] and inhibits the increase in Th17 cytokine levels in patients with moderate-to-severe asthma *in vitro*; an antagonist of the ectonucleotidase CD39 reversed 1,25(OH)2D3-mediated inhibition of the IL-17A response [18]. These results raise a question of whether other mechanisms underlie the effect of VitD3 on Th17 cell differentiation in asthma.

Our previous study demonstrated that VitD3 up-regulates Jagged2 and induces the production of CD4+CD25+Foxp3+ regulatory T cells to inhibit allergic inflammation in the airway [19]. Recent studies have reported that Dll4 promotes IL-17 production and ROR γ activation [14] and that VitD3 inhibits IL-17A production in T cells from rheumatoid arthritis patients [20]. We hypothesized that VitD3 decreases Dll4 expression in DCs, leading to a shift in the Th17/Treg balance, and that Dll4 blockade inhibits the Th17 response and Th17-mediated neutrophilic airway inflammation in asthma.

Materials and methods

Experimental animals

C57BL/6J mice (female, 6–8 weeks old, 18–22 g body weight) were obtained from Chongqing Experimental Animal Co. All the mice were bred under specific pathogen-free conditions. All the experimental protocols were approved by the Ethics Committee of Xinqiao Hospital (Third Military Medical University, Chongqing, China). The mice were sensitized with 0.02% ovalbumin (OVA) on the first and seventh days. Beginning on the 13th day, the mice were challenged through the respiratory tract with 1% aerosolized OVA for 30 min per day for 3 d to establish the asthma model.

Cell culture

The DCs were isolated from the sensitized mouse spleens using CD11c⁺ cell-binding magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions of the manufacturer. The purity of the DCs (>80%) was determined via flow cytometric detection of the fluorescently labeled anti-CD11c monoclonal antibody. The DCs were seeded at a density of 1×10^6 cells per well in six-well plates in the RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Life Technologies, Grand Island, NY). The DCs were divided into the following four groups: the control group was treated with 1 μ l/ml PBS (Life Technologies, Grand Island, NY) as an untreated negative control; the LPS group was treated with 1 μ g/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO); the LPS + Dex group was treated with 1 μ g/ml LPS and 10^{-8} mol/L dexamethasone (Dex; Sigma-Aldrich, St. Louis, MO); and the LPS + VitD3 group was treated with 1 μ g/ml LPS and 10^{-8} mol/l 1,25(OH)2D3 (Sigma-Aldrich, St. Louis, MO). All the cells were incubated at 37 °C in 5% CO₂ for 24 h. T cells were isolated from the sensitized mice using CD4⁺ T cell-binding magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to yield ~80% purity. After washing the cultured DCs with RPMI-1640 medium, the purified T cells were co-cultured with differently treated DCs at a ratio of

1×10^5 DCs per 1×10^6 T cells, and the cultures were incubated for an additional 24 h. For Dll4 inhibition, the co-cultures were treated with 5 μ l/ml Dll4-blocking antibody (eBioscience, San Diego, CA), and an anti-IgG antibody (eBioscience, San Diego, CA) was used as the isotype control.

Western blot

The treated DCs were collected and lysed in RIPA buffer (Thermo Scientific, Waltham, MA) containing a protease inhibitor cocktail for 30 min on ice. The lysates were centrifuged at 12 000 rpm for 20 min at 4 °C. The protein concentrations were determined, and the protein samples were denatured for 10 min at 95 °C. Subsequently, 60 μ g of total protein from each sample was loaded onto an SDS-PAGE gel for electrophoresis. The separated proteins were transferred from the gel to a BioTrace polyvinylidene difluoride (PVDF) transfer membrane. The membranes were blocked for 1 h with TBS-Tween containing 5% fat-free milk and probed overnight at 4 °C using the following primary Abs: mouse anti-Dll4 (Abcam, Hong Kong, China) and mouse anti-GAPDH mAb (loading control). The membranes were washed three times, probed for 1 h using secondary Abs and then visualized via Immobilon Western Chemiluminescent HRP Substrate.

Flow cytometric analysis of effector T cell (Th17 and Treg) subpopulations

Cells were collected, counted, and stained with the following fluorochrome-labeled antibodies: FITC-conjugated anti-CD4, PE-cy5-conjugated anti-CD25, and PE-conjugated anti-IL-17A and anti-Foxp3 (all from eBioscience, San Diego, CA). For intracellular cytokine staining, the cells were stimulated in culture medium with a 250 $\times$ PAM/ionomycin mixture and 250 $\times$ BFA/monensin mixture (Multiscience Biotech, Hangzhou, China) for 6 h at 37 °C in 5% CO₂ to facilitate colony formation. Then, the cells were washed and stained for 30 min with the surface cell markers anti-CD4 and anti-CD25, followed by fixation and permeabilization using FIX&PERM buffer (eBioscience, San Diego, CA). After permeabilization, intracellular antibodies against IL-17A and Foxp3 were applied for 30 min at 4 °C in flow cytometry staining buffer (eBioscience, San Diego, CA), and the flow cytometry results were analyzed using FlowJo software (eBioscience, San Diego, CA). Apoptosis was measured after co-culturing and staining with Annexin V-FITC and PI (Beyotime Biotechnology, Hangzhou, China).

Enzyme-linked immunosorbent assay (ELISA) quantification of cytokine production

The concentrations of secreted IL-17A in the supernatants of the DCs co-cultured with T cells were determined using ELISA kits (R&D Systems, Minneapolis, MN). The limit of sensitivity was 0.46–4.80 pg/ml.

Statistical analysis

The data are expressed as the mean $\pm$ standard deviation (SD). The data were analyzed via one-way ANOVA using SPSS version 19.0 software (SPSS Inc., Chicago, IL). $p < 0.05$ was considered statistically significant.

Results

1,25(OH)2D3 decreased the expression of Dll4 in LPS-treated spleen DCs

DCs were isolated from the sensitized mouse spleen and treated with LPS to mimic neutrophil induced T cell differentiation in asthma [4,21]. The purity of the DCs, as measured by flow cytometry, was greater than 80% (Figure 1A); therefore, these cells could be used for the following studies. After culturing the cells in LPS, LPS + Dex or LPS + VitD3 for 24 h, the DCs were harvested, and Dll4 protein expression in the DCs was confirmed via Western blot. As shown in Figure 2, LPS increased the expression of Dll4 compared with the control treatment ($p=0.05$; Figure 2B), and both VitD3 and Dex inhibited the LPS-induced up-regulation of Dll4 expression ($p=0.04$ and $p=0.15$, respectively; Figure 2B), although VitD3 induced a greater decrease in Dll4 expression compared with Dex.

VitD3-treated DCs exhibited suppressed Th17 cell differentiation and biological activity

The treated DCs were co-cultured with T cells for 24 h; the purity of these T cells was more than 80% (Figure 1C) as measured by flow cytometry. The LPS-treated DCs exhibited increased differentiation of both Th17 ($p=0.002$; Figure 3B and A) cells and Tregs ($p=0.024$; Figure 3F and E) compared with the control cells. The VitD3 + LPS-treated DCs in which Dll4 was down-regulated by VitD3 exhibited inhibited Th17 differentiation compared with the LPS-treated DCs ($p=0.001$; Figure 3D and B), whereas Treg differentiation was only marginally affected (Figure 3H and F). The Dex + LPS-treated DCs exhibited suppressed differentiation of both Th17 cells ($p=0.001$; Figure 3C and B) and Tregs ($p=0.008$; Figure 3G and F) compared with the LPS-treated DCs. The VitD3 + LPS-treated DCs exhibited a lower Th17/Treg ratio than the LPS-treated DCs ($p=0.026$; Figure 3S) because VitD3 decreased Th17 differentiation (Figure 3Q) more effectively than Treg differentiation (Figure 3R), whereas LPS induced the differentiation of both T cell types (Figure 3Q and R). The Dex + LPS-treated DCs did not exhibit a reduced Th17/Treg ratio (Figure 3S) because this treatment decreased both Th17 and Treg differentiation (Figure 3Q and R). The levels of IL-17A, the primary Th17 cytokine in the co-culture medium, were evaluated via ELISA. The level of IL-17A in the LPS group was higher

than that in the control group ($p<0.001$; Figure 4), and the level in both the Dex + LPS and VitD3 + LPS groups was less than that in the LPS group ($p<0.001$ for both). No significant difference in apoptosis was detected among the four groups (Figure 5); thus, apoptosis did not affect the T cell ratio.

1,25(OH)2D3 decreased Th17 cell differentiation by blocking Dll4

The expression of Dll4 in APCs has been associated with the development of Th17 responses. During the co-culture period, all groups were treated with 5 μ l/ml Dll4-blocking antibody. After culturing for 24 h, Th17 and Treg cell differentiation was measured via flow cytometry. The Dll4-blocked LPS-treated DCs exhibited similar Th17 differentiation as the Dll4-blocked control-treated DCs (Figure 3J and I) and Th17 differentiation was decreased compared with the non-Dll4-blocked LPS-treated DCs ($p=0.01$; Figure 3Q). Furthermore, with the Dll4 blocked, the LPS-treated group exhibited enhanced Treg differentiation compared with the control-treated group ($p=0.001$; Figure 3N and M). The Dll4-blocked VitD3 + LPS-treated DCs exhibited similar Th17 cell differentiation to both the Dll4-blocked LPS-treated and VitD3 + LPS-treated DCs (Figure 3). These findings revealed that Dll4 plays an important role in Th17 cell differentiation and Th17 cell differentiation was not further inhibited by VitD3 once Dll4 was blocked, indicating that Dll4 is involved in Th17 differentiation inhibition by VitD3. The Dll4-blocked LPS-treated DCs exhibited a lower Th17/Treg ratio than the non-Dll4-blocked LPS-treated DCs ($p=0.02$; Figure 3S) because the application of the Dll4-blocking antibody prevented Dll4 from inducing Th17 cell differentiation (Figure 3Q) but did not affect Treg cell differentiation (Figure 3R). These results indicate that Dll4 inhibition only affected Th17 cell differentiation. With Dll4 blocked, Dex + LPS-treated DCs exhibited suppressed Treg differentiation but not Th17 cell differentiation compared with the LPS-treated DCs. Dex inhibited the LPS-induced up-regulation of Dll4 expression, although to a lesser extent than VitD3. Furthermore, in the Dll4-blocked Dex + LPS-treated group, the level of IL-17A was decreased compared with the Dll4-blocked control and LPS-treated groups ($p=0.02$ and $p=0.03$, respectively; Figure 4). These results revealed that Dll4 was more strongly affected by VitD3 than by Dex and that Dex may regulate Th17 differentiation via alternative pathways.

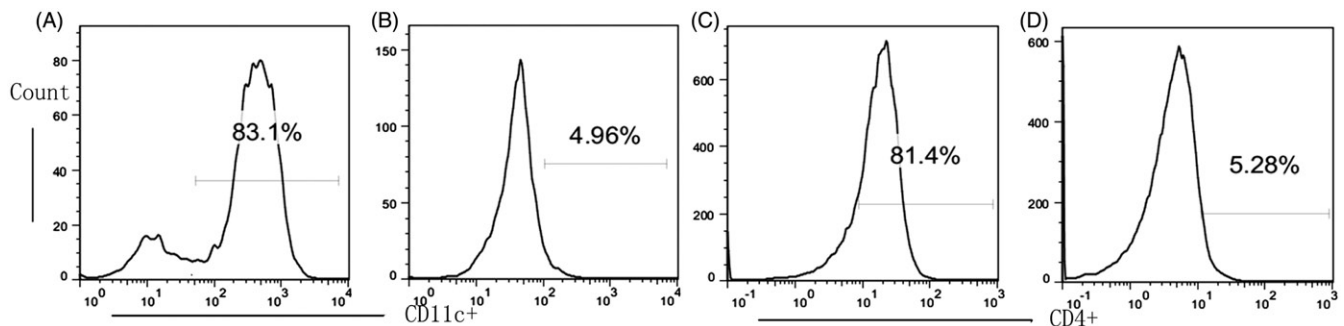
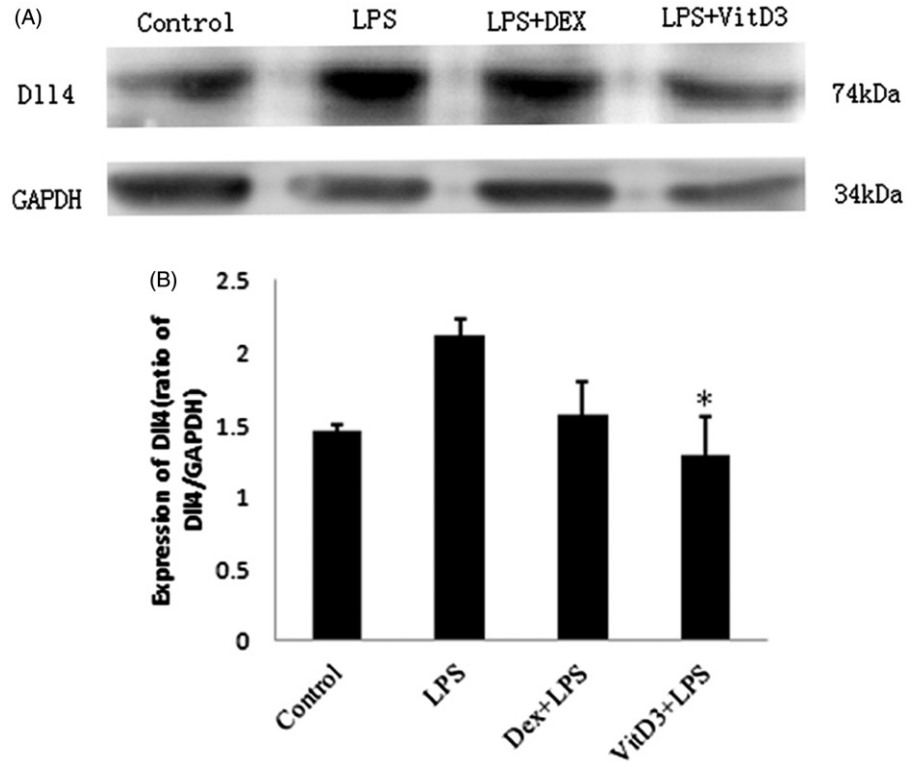


Figure 1. The purity of DCs and T cells was confirmed via flow cytometry with gating. (A) The purity of the CD11c+ DCs in the cultures was >80%; (B) the cells were incubated with an isotype control antibody. (C) The purity of CD4+ T cells was >80%; (D) the cells were incubated with an isotype control antibody.

Figure 2. The expression of Dll4 in mouse spleen DCs was detected via Western blot in the different groups. The spleen DCs were treated with LPS, Dex + LPS or VitD3 + LPS for 24 h. (A) Dll4 protein expression was confirmed, and the GAPDH expression level was used to normalize the protein loading among samples. (B) The expression of Dll4 in both the Dex + LPS and VitD3 + LPS groups was decreased compared with that in the LPS group, although the result was more significant in the VitD3 + LPS group. All results are representative of three independent experiments. * $p < 0.05$ versus the LPS group.



Discussion

At present, no effective preventive strategy or known cure for asthma is available [22,23]; the prevalence of asthma is increasing, and its societal burden remains high. Based on the additional information about asthma phenotypes, novel drugs may provide an opportunity to personalize asthma management and to directly target the mechanisms responsible for this disease. Vitamin D is used as an adjuvant drug for asthma, and serum 1,25(OH)2D3 is positively correlated with lung function and negatively correlated with the severity of asthma [24–26]. The mechanism by which VitD3 modulates Th17 cell differentiation in asthma is unclear. In our study, we found that 1,25(OH)2D3 inhibited LPS-induced Dll4 expression in DCs from OVA-sensitized mice, that Dll4 expression was associated with Th17 cell differentiation, and that treatment with anti-Dll4 ameliorated the increase in Th17 cell differentiation and prevent VitD3 decreasing the Th17 cell differentiation. Thus, VitD3 reduced Th17 cell differentiation by inhibiting Dll4 from OVA-sensitized mice.

T cell differentiation is regulated by multiple molecular pathways, including T cell receptor signaling, MHC molecule expression, stimulation with multiple molecules and cytokine production. Many recent studies have demonstrated that the Notch family plays a crucial role in T cell differentiation. Notch is a family of heterodimeric transmembrane receptors that regulates numerous developmental processes. There are four Notch receptors (N1–4) and five Notch ligands (delta-like 1, 3, and 4; Jagged 1 and 2). Dll4 has been reported to negatively regulate the neovasculature during airway remodeling by affecting Tregs [27]. In this study, the LPS-treated DCs exhibited enhanced Th17 differentiation and Dll4 expression compared with the control DCs. blocking Dll4 activity in the LPS-treated DCs ameliorated Th17 cell

differentiation, indicating that Dll4 promotes Th17 cell differentiation. This result is similar to the finding in mycobacteria-elicited pulmonary granulomatous immune responses [28]. A previous study demonstrated that the inhibition of Dll4 alleviates experimental autoimmune encephalomyelitis by promoting regulatory T cell development [13]. In our study, Dll4 blockade did not affect Treg differentiation. This discrepancy in the impact of Dll4 inhibition on Treg differentiation between these studies may be due to the use of different disease models.

Vitamin D3 is increasingly recognized as a complex immune-modulatory molecule that is positively correlated with lung function and negatively correlated with asthma severity. It has been revealed that VitD3 reduces the production of IL-17A via the modulation of the vitamin D receptor (VDR) pathway in a mouse model [29,30] and inhibits the differentiation and maintenance of Th17 cells by attenuating the production of IL-6 [31,32] and the transcription of Th17 cells [33,34]. A clinical study also indicated that vitamin D3 intake decreases IL-17 + CD4+ T helper cells [35]. Using *in vitro* studies, Nanze [18] reported that 1,25(OH)2D3 inhibits Th17 cytokines in patient with moderate-to-severe asthma, and an antagonist of the ectonucleotidase CD39 reversed the 1,25(OH)2D3-mediated inhibition of the IL-17A response in asthma. In this study, we provided evidence that VitD3 reduces Dll4 expression in LPS-treated DCs, thereby preventing Th17 differentiation and blocking Dll4 can prevent VitD3 decreasing Th17 differentiation. These findings indicate that VitD3 reduces the production of Th17 cells via the DC-mediated expression of the Notch ligand Dll4. We also found that VitD3 decreases the Th17/Treg ratio *in vitro*, as VitD3 more strongly decreased Th17 cell differentiation than Treg differentiation. Consistent with a prior study, VitD3 inhibited Th17 cytokine production

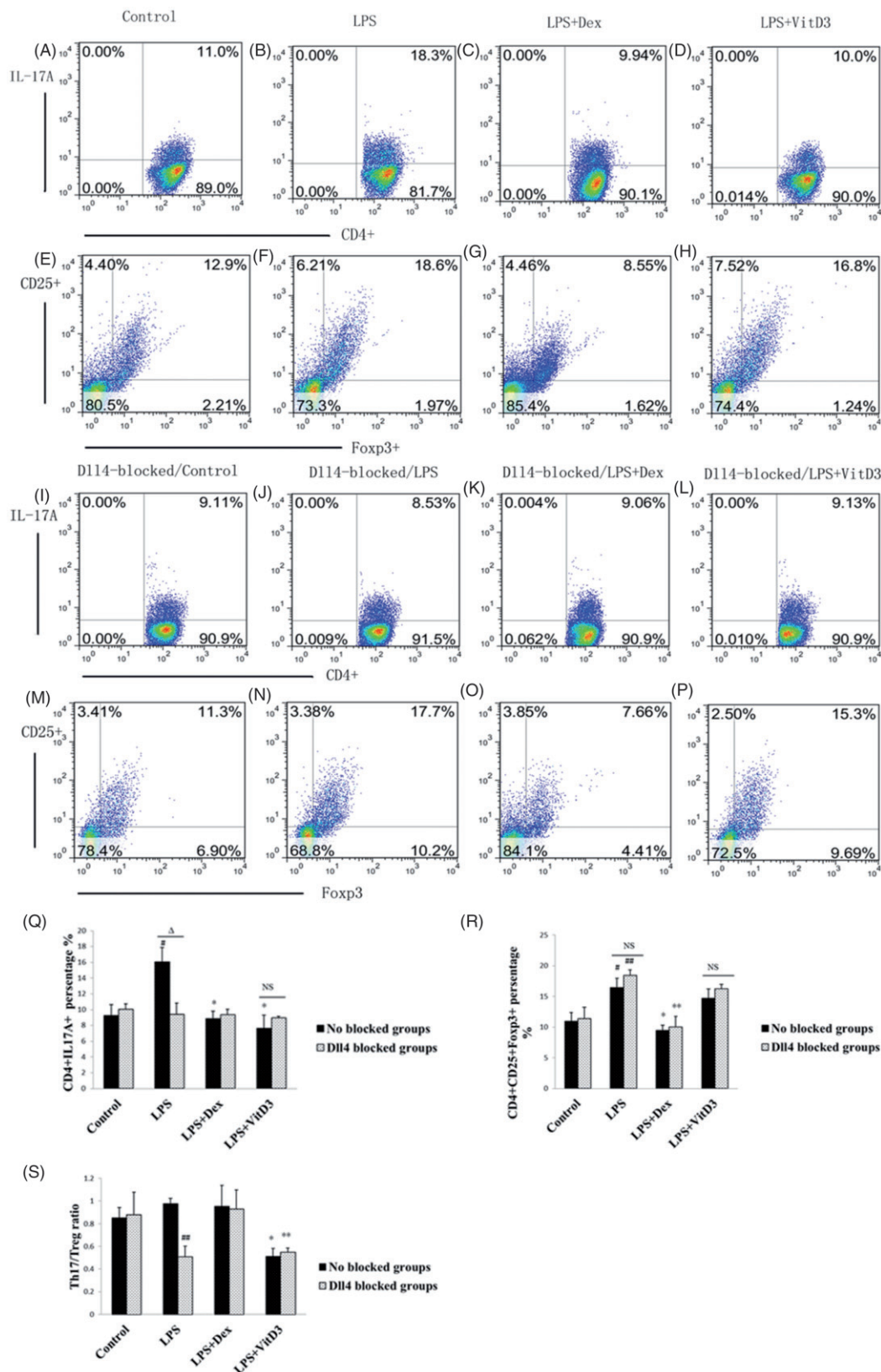


Figure 3. The differentiation of Th17 and Treg cells after co-culturing was determined via flow cytometry with or without Dll4-blocking antibody. All cells in the picture are CD4⁺ cells. The sensitized spleen DCs were treated with LPS, Dex + LPS or VitD3 + LPS for 24 h and then co-cultured with CD4⁺ T cells for another 24 h. (A, B, C, and D) Th17 differentiation was detected as CD4⁺IL-17A⁺ in the absence of the blocked Dll4 antibody. (E, F, G, and H) Treg differentiation was detected as CD4⁺CD25⁺Foxp3⁺ in the absence of the Dll4 antibody. The treated DCs were cultured with T cells for 24 h, and 5 μl/ml Dll4-blocking antibody was added to the culture medium in each group. (I, J, K, and L) Th17 cells (detected as CD4⁺IL-17A⁺) and (M, N, O, and P) Treg cells (detected as CD4⁺CD25⁺Foxp3⁺) in the presence of the Dll4 antibody; after Dll4 inhibition, Th17 differentiation did not differ significantly among the four groups. Statistical analysis of (Q) the percentage of differentiated Th17 cells, (R) the percentage of differentiated Tregs and (S) the Th17/Treg ratio. All results are representative of three independent experiments. #p < 0.05 versus the control group; *p < 0.05 versus the LPS group; ##p < 0.05 versus the Dll4-inhibited control group; **p < 0.05 versus the Dll4-inhibited LPS group; Δp < 0.05 versus the corresponding Dll4-inhibited group; NS, no significant difference.

in asthma [18], revealing that VitD3 inhibited the biological activity of Th17 cells. According to the Global Initiative for Asthma, comprehensive treatment for asthma has been proposed, and the roles of many of the currently available asthma drugs, have been well-defined in patients with mild to moderate disease. However, no effective preventive strategy or known cure for asthma is available. Vitamin D, when used as an adjuvant drug, is inversely correlated with various characteristics of asthma, such as airway remodeling, the IgE and eosinophil levels, and AHR [36–38]. In our study, we found that VitD3 might inhibit Th17 cell differentiation and biological activity by decreasing Dll4 expression. This finding revealed the effect of Dll4 on the regulation of Th17 cell differentiation and the role of VitD3 in decreasing Th17 differentiation. Additionally, recent studies reported that serum interleukin-17 has a negative correlation with vitamin D levels in young asthmatics and 1,25(OH)2D3 inhibits Th17 cytokines in moderate-to-severe asthma patient [18,39]. Clinically, because neutrophilic asthma is induced by Th17 cells, VitD3 may effectively alleviate neutrophilic asthma by suppressing Th17 cell production.

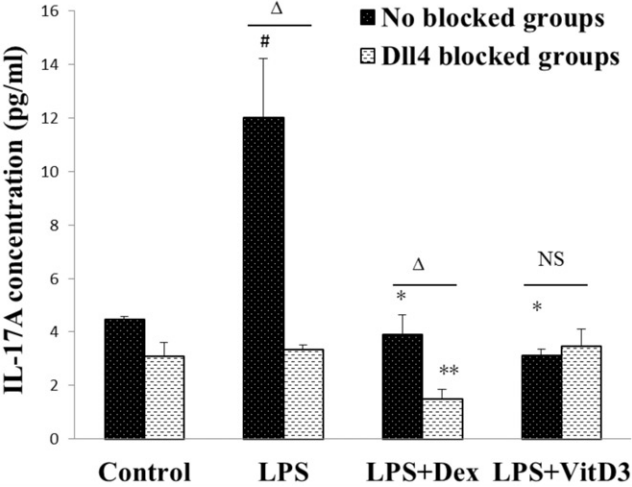


Figure 4. Dll4 inhibition impaired the biological activity of Th17 cells. The levels of IL-17A, the primary Th17 cytokine in the co-culture medium, were measured via ELISA in the non-Dll4-blocked and Dll4-blocked groups. All data are representative of three independent experiments. # $p < 0.05$ versus the control group; * $p < 0.05$ versus the LPS group; ** $p < 0.05$ versus the Dll4-inhibited LPS group; $\Delta p < 0.05$.

Recent studies have reported that 1,25(OH)2D3, but not glucocorticoids, inhibits increases in the levels of Th17 cytokines [18] and that Th17-predominant asthmatic patients develop resistance to glucocorticoids *in vitro* [40]. In our study, we also found that dexamethasone reduces both Th17 cell and Treg differentiation but does not decrease the Th17/Treg ratio.

Under our co-culture conditions, DCs are end-stage cells that do not differentiate. Because of the 1:10 cell ratio of DCs to T cells, the Th17/Treg ratio is rarely affected by T cell proliferation. This finding concerning the mechanism by which Vitamin D regulates Th17 cell differentiation provides an experimental basis for the use of Vitamin D and expands its potential value for the clinical treatment of asthma. However, these results must be confirmed by *in vivo* experiments. In conclusion, Dll4 in the DCs isolated from OVA-sensitized mice is involved in Th17 differentiation inhibition by 1,25(OH)2D3.

Declaration of interest

The authors report that they have no conflicts of interests. This work was supported by grants from the National Natural Science Foundation of China (Nos. 81270075) awarded to Changzheng Wang. The funders had no role in study design, data collection and analysis, the decision to publish, or manuscript preparation.

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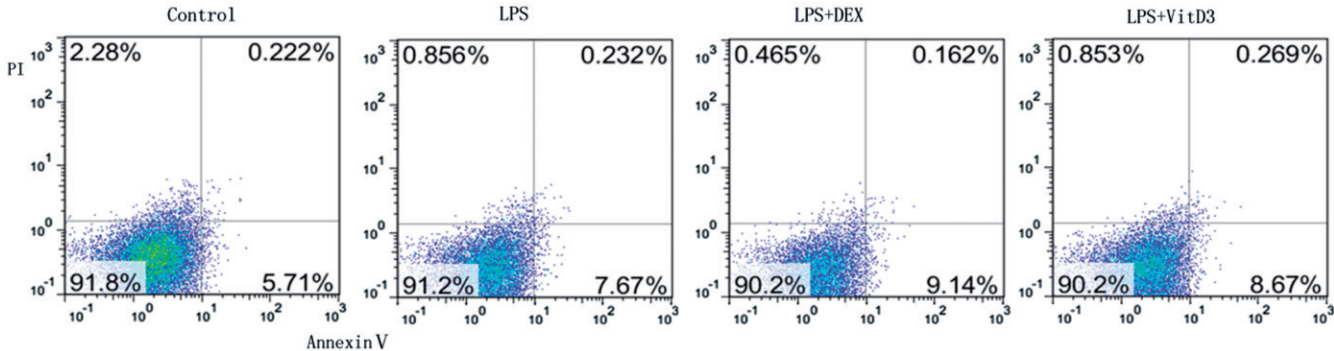


Figure 5. Apoptosis as determined via flow cytometric analysis of T cells stained with Annexin V-FITC after co-culturing. No difference was detected among the groups.

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