

ORIGINAL ARTICLE

Anti-Dll4 Antibody Inhibits the Differentiation of Th17 Cells in Asthmatic Mice

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Abstract— T helper 17 (Th17) cells play an important role in allergic asthma, and the Notch ligand Delta-like ligand (Dll)4 has been reported to direct the differentiation of Th17 cells. In this study, experimental animals were divided into five groups (control group, asthma group, physiological saline group, anti-Dll4 antibody group, and immunoglobulin G group). The study aimed to explore the effect of anti-Dll4 antibody on the differentiation of Th17 cell in asthmatic mice. Dll4 protein expressions were performed by immunohistochemical imaging. The proportion of Th17 cells in mouse spleen-isolated CD4⁺ T cells were measured by flow cytometry. The protein expression of Th17 transcription factor retinoid-related orphan nuclear receptor (ROR γ t) was detected by Western blotting. Interleukin (IL)-17 levels in serum were measured by enzyme-linked immunosorbent assay (ELISA). The study found that the expression of Dll4 in lung tissue from the asthma group significantly increased compared with the anti-Dll4 antibody group. The ratio of Th17 cells in CD4⁺ T cells was significantly downregulated, and the protein expression of ROR γ t in spleen significantly reduced in the anti-Dll4 antibody group compared with the asthma group. Moreover, the IL-17 level in serum from the anti-Dll4 antibody group significantly reduced compared with the asthma group. These results suggested that anti-Dll4 antibody could inhibit the differentiation of Th17 cells in asthmatic mice.

KEY WORDS: asthma; delta-like ligand 4; interleukin-17; notch; T helper 17.

INTRODUCTION

Asthma is a chronic airway inflammation that involves many inflammatory cells such as lymphocytes, eosinophils, and mast cells, of which T lymphocytes play an important role in the pathogenesis of asthma. T helper

17 (Th17) cells and the cytokine interleukin (IL)-17 could aggravate asthma airway inflammation [1, 2].

Radtke et al. [3] found four Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five Notch ligands (Jagged1, Jagged2, Delta-like ligand (Dll)1, Dll3, and Dll4) in mammals. The present findings showed that the levels of Notch1 and Notch2 receptors were upregulated in the asthma group [4]. Huang et al. [5] indicated that T-regulatory (Treg) cells could improve airway remodeling through the Dll4-Notch signaling pathway. Dll4 in the dendritic cells (DC) isolated from ovalbumin (OVA)-sensitized mice is involved in inhibition of Th17 differentiation by 1,25-dihydroxyvitamin D3 in vitro [6]. Dll4 defines the involvement of human DC in regulating Th1 and Th17

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differentiation [7]. Mukherjee et al. [8] thought that Dll4 upregulates retinoid-related orphan nuclear receptor (ROR γ t) expression in T cells and that both ROR γ t and IL-17 gene promoters are direct transcriptional Notch targets that augment the differentiation of Th17 cell populations.

Recent research suggested that the Notch signaling pathway played an important role in the differentiation and activation of T cells, and it is closely related to the pathogenesis of asthma [9]. Therefore, it was needed to further explore whether anti-Dll4 antibody could regulate the differentiation of Th17 cells in a mouse model of asthma through in vitro intervention of anti-Dll4 antibody.

MATERIALS AND METHODS

Animal Model of Asthma Setup

Male BALB/C mice (4- to 6-week-old), weighing 18–22 g, were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and kept in a pathogen-free environment in the animal center of Wenzhou Medical University. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University (Permit Number: wyd2014–0119). An ovalbumin (OVA)-induced asthma model was established as in previous studies [4, 10]. Experimental animals were divided into five groups: control group ($n = 6$), asthma group ($n = 6$), physiological saline group ($n = 6$), anti-Dll4 antibody group ($n = 6$), and immunoglobulin G (IgG) group ($n = 6$). BALB/c mice were sensitized by intraperitoneal injection of 10 μ g OVA, emulsified in 20 mg Al (OH) $_3$ gel in 0.1 mL of normal saline (NS) on days 1 and 13, and were exposed to OVA (1 mg/mL) aerosol challenges for 30 min once a day for eight consecutive days from day 25 to day 32. Anti-Dll4 antibody (eBioscience, CA, USA) was administered intraperitoneally for 30 min before each OVA challenge to block the Dll4-Notch signaling pathway with a dose of 200 μ g in a volume of 100 μ l per mouse per injection. The IgG group mice were sensitized and challenged with OVA and injected in the same time as well as the anti-Dll4 antibody group with IgG (eBioscience, CA, USA) with a dose of 200 μ g in a volume of 100 μ l per mouse per injection as control. The physiological saline group mice were sensitized and challenged with OVA and treated with physiological saline as the control group. The experimental animals were sacrificed within 24 h after the last allergen challenge.

Histopathological Examination

The mouse lungs were first fixed with 4% paraformaldehyde for 4 h. They were then dehydrated in ethyl alcohol, embedded with paraffin, cut into 3- μ m sections, and stained with hematoxylin and eosin (HE) staining. Lung tissue slices were evaluated through a light microscope (Nikon, Japan) to observe infiltration of inflammatory cells and airway morphology.

Immunohistochemistry

Lung tissues were immunohistochemically analyzed with Dll4 antibody (Santa Cruz Biotechnology, USA). The sections were counterstained using hematoxylin (Thermo Shandon, PA, USA). The expression of Dll4 was measured using Image-Pro Plus 6.0 via optical density analysis.

T Lymphocyte Isolation and Purification

The mouse spleens were fragmented into small pieces, pressed with the plunger of a disposable syringe, and washed in phosphate-buffered saline through a nylon mesh. Mononuclear cells were isolated by mouse lymphocyte separation medium.

Flow Cytometry Analysis

Fluorescein isothiocyanate-labeled anti-mouse CD4 $^+$ and phycoerythrin (PE)-labeled anti-mouse IL-17A were used to detect Th17 cells. Matching IgG was used as the isotype control. All antibodies were purchased from BD Bioscience, USA. For intracellular cytokine staining, the cells were counted and stimulated with 100 ng/mL phorbol myristate acetate and 1 μ g/mL ionomycin in the presence of 1.6 μ g/mL monensin (Beyotime Biotechnology, Hangzhou, China) for 5 h at 37 $^{\circ}$ C in 5% CO $_2$. The cells were collected, washed, surface-stained with FITC-labeled anti-mouse CD4 $^+$ antibody, and resuspended in flow cytometry staining buffer (eBioscience, CA, USA). Then, they were stained with PE-labeled anti-mouse IL-17A antibody. After washing, the cells were resuspended and subjected to FACScalibur flow cytometer (BD Accui C6, USA). Analysis of flow cytometry results was performed using FlowJo software (eBioscience, CA, USA).

Western Blotting

The protein expressions of Th17 transcription factor retinoid-related orphan nuclear receptor (ROR γ t) were detected by Western blotting. T lymphocytes isolated from mouse spleen were collected and lysed in Radio-

Immunoprecipitation Assay (RIPA) buffer with protease inhibitor mixture (Beyotime Biotechnology, Hangzhou, China) on ice. Total protein (30 μ g) was resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto BioTrace polyvinylidene difluoride membranes. The membranes were blocked for 1 h with Tris-buffered saline and Tween 20 containing 5% powdered skimmed milk and then probed overnight at 4 °C with primary antibodies. Secondary antibodies were diluted in blocking buffer and incubated with the membranes for 1 h at room temperature. Immunostained proteins were detected by enhanced chemiluminescence (ECL).

Enzyme-Linked Immunosorbent Assay

Interleukin (IL)-17 levels in serum were measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience, CA, USA).

Statistical Analysis

All results were expressed as means $\pm$ standard deviation (SD) and analyzed by one-way analysis of variance, using SPSS 21.0 (SPSS, IL, USA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Effect of Anti-Dll4 Antibody on Asthma Airway Inflammation

The control group showed less inflammatory cell infiltration in surrounding small blood vessels in HE-stained lung tissues. Compared with the control group, the asthma group, physiological saline group, and IgG group showed significant infiltration of inflammatory cells in the airways and small perivascular spaces. The anti-Dll4 antibody group showed significantly reduced inflammatory cell infiltration (Fig. 1).

Effect of Anti-Dll4 Antibody on Dll4 Protein Expression in Lung Tissues

Dll4 protein expression was assessed by immunohistochemical imaging and semi-quantified using Image-Pro Plus software after hematoxylin staining. The expression of Dll4 in lung tissues from the asthma group (0.27 ± 0.03), physiological saline group (0.27 ± 0.04), and IgG group (0.25 ± 0.03) significantly increased compared with that from the control group (0.18 ± 0.03 , $P < 0.01$). The expression of Dll4 protein in lung tissue from the asthma group significantly increased compared with that from the anti-Dll4 antibody group (0.13 ± 0.03 , $P < 0.01$) (Fig. 2).

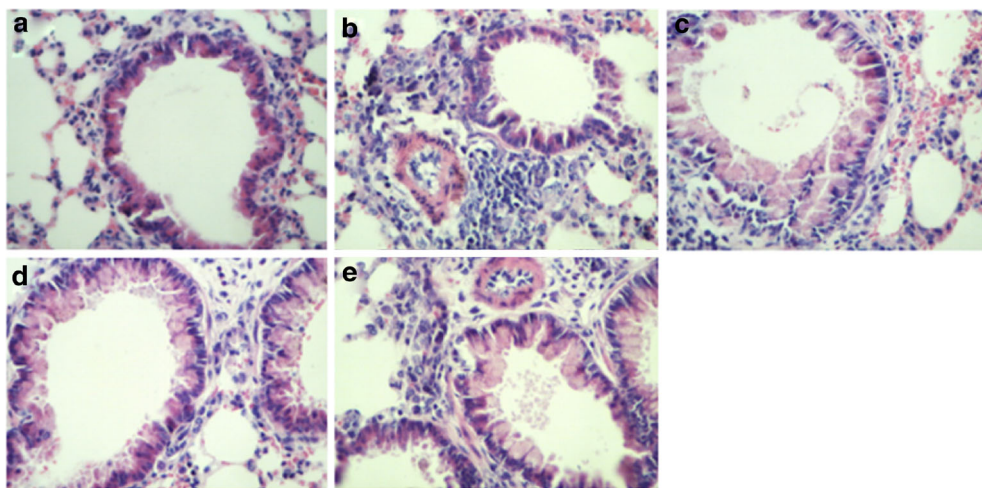


Fig. 1. Anti-Dll4 antibody reduced airway inflammation in asthmatic mice. HE-stained lung tissues (light microscope). **a** Control group. **b** Asthma group. **c** Physiological saline group. **d** Anti-Dll4 antibody group. **e** IgG group.

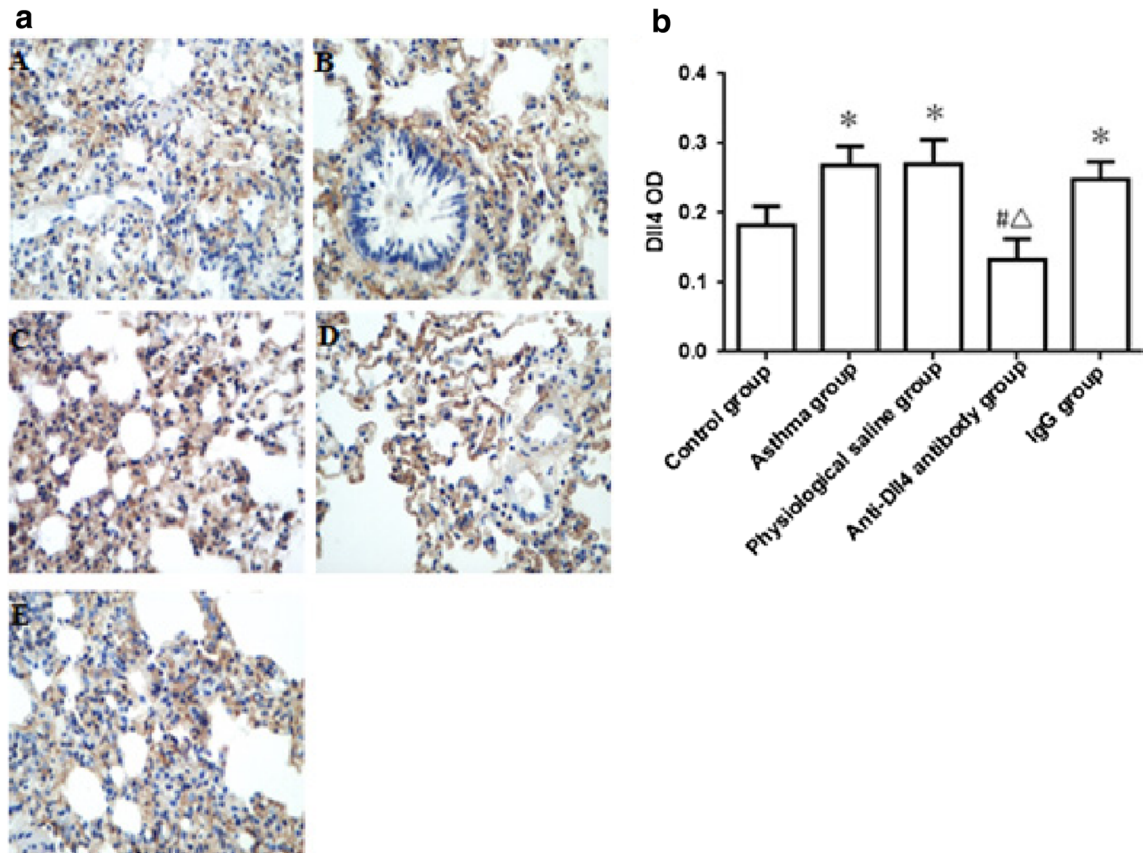


Fig. 2. Anti-Dll4 antibody downregulated Dll4 protein expression. **a** Dll4 protein expression was detected by immunohistochemical imaging. *A* Control group. *B* Asthma group. *C* Physiological saline group. *D* Anti-Dll4 antibody group. *E* IgG group. **b** The protein of Dll4 was semi-quantified using Image-Pro Plus software. Data were expressed as means $\pm$ SD. * $P < 0.01$ compared with control groups; # $P < 0.01$ compared with asthma group; $\Delta P < 0.01$ compared with IgG group.

Anti-Dll4 Antibody Decreased the Frequency of Th17 Cells in the Spleen

The proportion of Th17 cells in $CD4^+$ T cells was determined by flow cytometry. Significantly upregulated proportions of Th17 cells in $CD4^+$ T cells of mice spleen from the asthma group ($1.63 \pm 0.52\%$), physiological saline group ($1.65 \pm 0.77\%$), and IgG group ($1.72 \pm 0.47\%$) were observed compared with the control group ($0.58 \pm 0.31\%$, $P < 0.05$). However, the proportions of Th17 cells in $CD4^+$ T cells were significantly downregulated in the anti-Dll4 antibody group compared with those in the asthma group (0.67 ± 0.20 versus $1.63 \pm 0.52\%$, $P < 0.05$) (Fig. 3).

Anti-Dll4 Antibody Regulated ROR γ t Expression in the Spleen of Asthmatic Mice

Significantly elevated protein expression of ROR γ t was observed in lung tissue from the asthma group

(0.24 ± 0.05), physiological saline group (0.24 ± 0.05), and IgG group (0.22 ± 0.03) compared with the control group (0.08 ± 0.01 , $P < 0.01$). However, protein expression of ROR γ t in lymphocytes isolated from mouse spleen from the anti-Dll4 antibody group significantly reduced compared with that from the asthma group (0.11 ± 0.02 versus 0.24 ± 0.05 , $P < 0.01$) (Fig. 4).

Anti-Dll4 Antibody Reduced the Production of IL-17 in Asthmatic Mice

IL-17 level was evaluated by ELISA. Significantly increased IL-17 levels in serum from the asthma group (149.78 ± 16.82 pg/ml) and IgG group (136.74 ± 8.48 pg/ml) were noted compared with those in the control group (107.44 ± 17.83 pg/ml, $P < 0.01$). However, IL-17 levels in serum from the anti-Dll4 antibody group significantly reduced compared with those from the

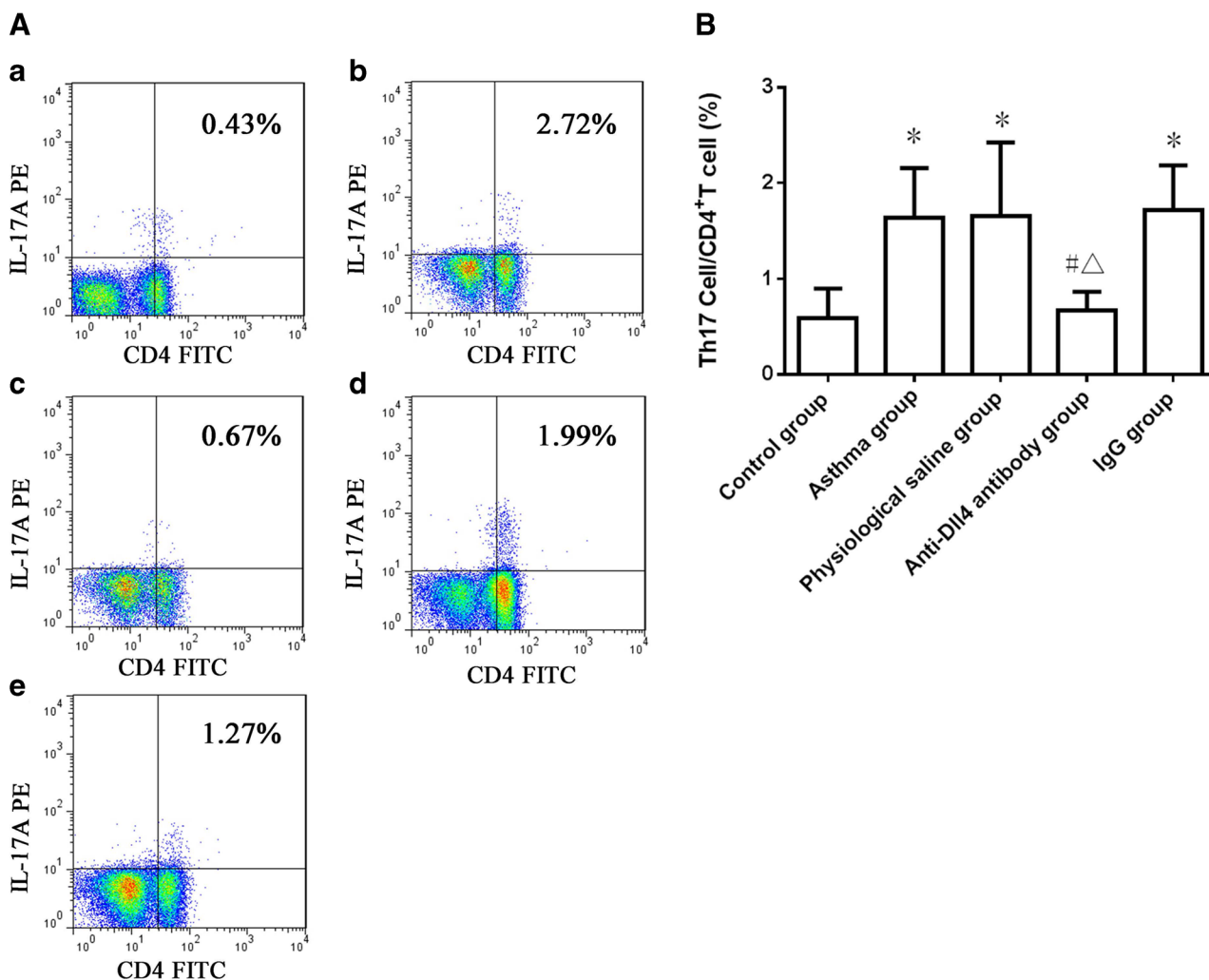


Fig. 3. Anti-Dll4 antibody decreased the differentiation of Th17 cells. **a** Proportion of Th17 cells in CD4⁺ T cells was determined by flow cytometry. **A** Control group. **B** Asthma group. **C** Physiological saline group. **D** Anti-Dll4 antibody group. **E** IgG group. **b** Data were expressed as means $\pm$ SD. * P < 0.05 compared with control groups; [#] P < 0.05 compared with asthma group; ^Δ P < 0.05 compared with IgG group.

asthma group (117.66 ± 5.54 versus 149.78 ± 16.82 pg/ml, P < 0.01) (Fig. 5).

DISCUSSION

Th17 cells are characterized by their expression of ROR γ t and production of IL-17. They play a critical role in the development of inflammatory diseases such as asthma [11]. The concentration of IL-17A in culture supernatant was significantly higher in asthmatics compared with normal controls, and IL-17A levels significantly increased 24 h after allergen challenge [12]. Many studies have found

that inhibiting the differentiation of Th17 cells could improve the development of asthma [13–15]. The present study proved that anti-Dll4 antibody could inhibit the differentiation of Th17 cells in asthmatic mice.

It was reported that Th17 cells were active players in the airway inflammation of allergic asthma. A marked increase in IL-17A levels was observed in the sputum of patients with severe asthma [16]. Excess IL-17-secreting cells were also observed in the lung tissue of these patients [17]. The increase in Th17 cells and IL-17A levels in atopic asthma after allergen inhalation challenge suggests a possible role for Th17 in allergen-induced airway responses [12]. Significantly upregulated proportion of Th17 cells in CD4⁺ T cells of mice spleen

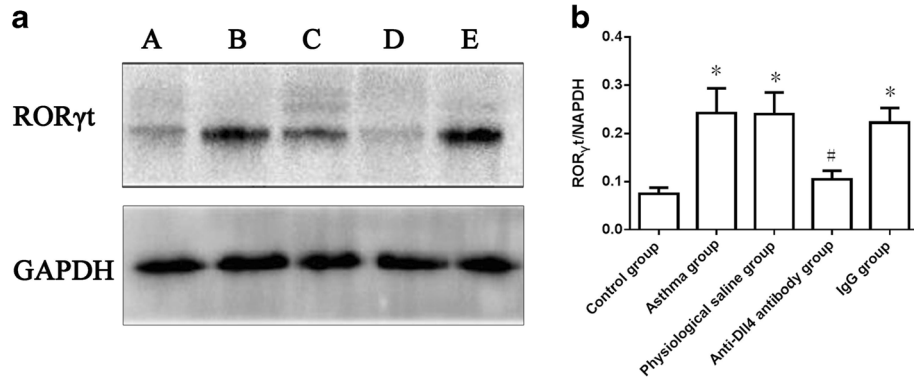


Fig. 4. **a** Western blotting analysis of the protein expression of ROR γ t. *A* Control group. *B* Asthma group. *C* Physiological saline group. *D* Anti-Dll4 antibody group. *E* IgG group. **b** Statistical analysis of the protein expression of ROR γ t. Data were expressed as means $\pm$ SD. * $P < 0.01$ compared with control groups; # $P < 0.01$ compared with asthma group.

and significantly increased IL-17 levels in serum from the asthma group were observed compared with those from the control group in this study.

The Notch signaling pathway plays a critical role in the differentiation and activation of T cells [9]. The roles of Notch in adaptive immunity have expanded to include the regulation of T cell differentiation and function [18]. It has been shown that γ -secretase inhibitor (GSI) directly regulates Th17 responses through a Notch signaling-dependent pathway in the mouse model of allergic asthma [19]. The ligand in the Notch signaling pathway has a specific regulatory effect on some inflammation-associated diseases. The present study showed that the expression of Dll4 protein in the lung tissues

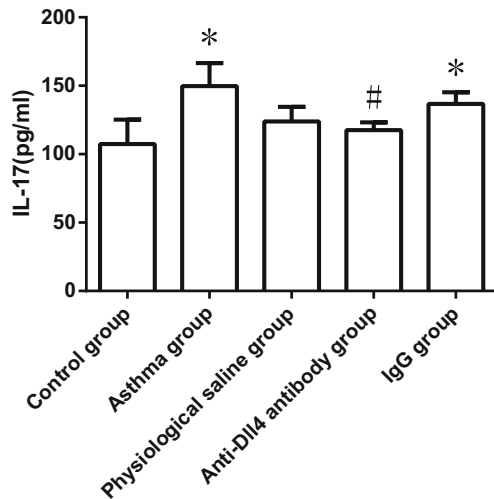


Fig. 5. Level of IL-17 was evaluated by ELISA. Data were expressed as means $\pm$ SD. * $P < 0.01$ compared with control groups; # $P < 0.01$ compared with asthma group.

of asthmatic mice was remarkably higher than that in the control group. Meng et al. [7] found that Dll4 was a unique functional molecule of human circulating DCs critical for directing Th1 and Th17 differentiation. Piggott et al. [20] showed that Jagged1-Fc could competitively inhibit the Notch signaling pathway and inhibit the response of Th1 and Th17, suggesting that the interaction between Notch and its ligand was involved in the regulatory effects on the maintenance and survival of T cells. Eixarch et al. [21] verified that Dll4 directly participated in regulating the production of Th1 and Th17 by the antigen-specific T cells. Dll4 upregulates ROR γ t expression in T cells and both ROR γ t and IL-17 gene promoters are direct transcriptional Notch targets that further enhance the differentiation of Th17 cell populations [8]. Blockade of Dll-like receptor signaling impaired Th17 function, as manifested by a significant reduction in Th17 cells in the spleen and bronchoalveolar lavage fluid [22]. The expression of Dll4 in lung tissue from the asthma group significantly increased compared with that from the anti-Dll4 antibody group. The proportion of Th17 cells in CD4⁺ T cells was significantly downregulated, and the protein expression of ROR γ t in spleen significantly reduced in the anti-Dll4 antibody group compared with that in the asthma group. Also, IL-17 levels in serum from the anti-Dll4 antibody group significantly reduced compared with the asthma group.

In summary, this study suggested that blocking the Notch signaling pathway using anti-Dll4 antibody could inhibit the differentiation of Th17 cells in asthmatic mice (Fig. 6).

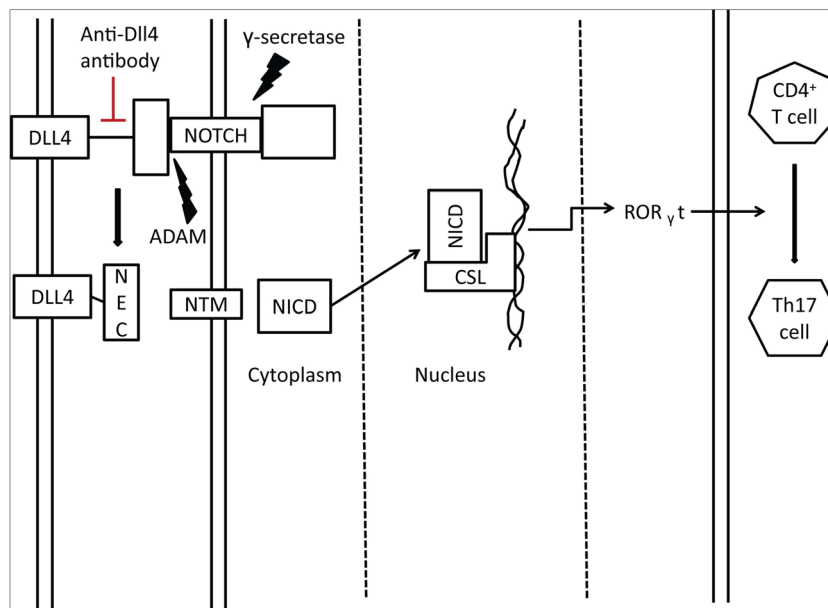


Fig. 6. Notch receptors are engaged with a Notch ligand (Dll4) and induces sequential cleavages by A-Disintegrin-and-metalloprotease (ADEM) and γ -secretase in Notch releasing the Notch intracellular domain (NICD) from the membrane NICD translocates to the nucleus where it directly interacts with the CSL (CBF1,Su (H),LAG1) transcription factor and recruits coactivators to induce target gene expression. Target gene upregulates the protein expression of ROR γ t and promotes the differentiation of Th17 cells. Anti-Dll4 antibody used in these studies is shown in the figure.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare no conflicts of interest.

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