

ORIGINAL ARTICLE

The Natural Course of Atopic Dermatitis and the Association with Asthma

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Abstract—In this paper, we aimed to explore the potential mechanism underlying atopic dermatitis (AD) and its association with asthma. The BALB/c mice were randomly assigned to three groups, including the vehicle control (VD) group, the AD group, and the treatment (TR) group. The AD mice model was successfully constructed in the AD and TR group. The dermatitis severity scores and skin lesions were significantly increased in AD mice after DNCB application. Airway responsiveness in the AD group was significantly higher than in the TR group. The number of inflammatory cells was increased in skin lesions and bronchoalveolar lavage fluid (BALF) of AD mice. The levels of IL-4, IL-5, IFN- γ , and OVA-IgE in BALF supernatants of mice in the AD group were higher than those in the VC group. All the changes in AD mice were decreased by tacrolimus. These results indicate that AD may be a significant risk factor for atopic asthma development.

KEY WORDS: asthma; atopic dermatitis; airway inflammation; airway responsiveness; tacrolimus.

INTRODUCTION

Asthma is a chronic inflammation condition derived from the airways, which is characterized by increased airway responsiveness and inflammation. There is an increasing trend of asthma incidence since the 1970s [1]. It is estimated that asthma will affect 235 to 300 million people and result in 250,000 deaths in 2013 all over the world [2]. Asthma is caused by

complicated factors such as genes and environments [3]. Asthma can be classified into atopic (extrinsic) or non-atopic (intrinsic) disease [4]. Atopic (extrinsic) asthma can be prevented by avoiding allergens.

Atopic dermatitis (AD), one of the most common allergic skin inflammatory diseases, has a particularly high prevalence among infants and children [5, 6]. It has been suggested that AD is an “entry point” for subsequent allergic disease [7]. It is also reported that more than 50% of young children with severe AD will ultimately develop asthma [8]. The risk for developing asthma is higher in children with a heredity of allergic rhinitis [9]. Although great efforts have been made to explore the association between AD and asthma [10, 11], the underlying molecular mechanism involved in the progression from AD to asthma remains elusive.

Tacrolimus (FK506), a topical calcineurin inhibitor, is isolated from a fungus-like bacterium (*Streptomyces tsukubaensis*) [12]. Tacrolimus has showed efficacy in prevention of allergic dermatitis. Currently, tacrolimus is specifically recognized as a steroid-sparing alternative agent for AD management. In the current work, the AD-like immunologic lesions and airway sensitization were

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induced by 2, 4-dinitrochlorobenzene (DNCB) and ovalbumin (OVA) in BALB/c mice. The inflammation changes in AD mice were observed by treating with tacrolimus. In this study, we attempted to explore the association between asthma and AD in molecular level.

MATERIAL AND METHODS

Animals

Female specific pathogen-free (SPF) BALB/c mice (weighing from 16 to 19 g, 5 to 6 weeks old) were supplied by Shanghai Laboratory Animal Center. All the animals were maintained at a room temperature of 23 °C and 40% relative humidity in a 12/12 light–dark cycle with standard laboratory rat chow and water *ad libitum*. The experimental protocol was approved by the Medicine Ethical Committee for Laboratory Animals of Shanghai Jiao tong University.

Groups

BALB/c mice were given a 1-week acclimatization period and then randomly assigned to 3 groups ($n = 10$ for each group), including vehicle control (VC), AD, and treatment (TR) groups. To induce AD-like immunologic and skin lesions, the surface of the ventral and dorsal skin of mice was administered with DNCB. After complete removal of ventral hairs within an area approximately 2 cm × 2 cm, 25 µl of 7% DNCB solution (dissolved in acetone) was applied onto the ventral skin for sensitization on days 1 and 2. From days 6 to 18, 40 µl 1% DNCB solution and 40 µl OVA-PBS solution (40 µg OVA) were applied on the bare dorsal skin for every 4 days (Fig. 1). Mice in the VC group were treated with acetone in the same way as above.

Three days after sensitization, all the mice were challenged *via* the airways with aerosolized 1% OVA for 30 min every day from days 21 to 27. After the AD model construction, tacrolimus ointment was topically applied to the dorsal skin lesion of the mice in the treatment group 1 h after the aerosol inhalation each time. At 72 h after the last inhalation, airway hyper-responsiveness (AHR) was assessed, and tissues and cells were collected for further assays.

Measurement of Airway Hyper-Responsiveness

Airway hyper-responsiveness (AHR) was assessed by the change of airway function after challenged with aerosolized MCH (methacholine, Sigma-Aldrich, St. Louis,

USA) using barometric plethysmography (Buxco Electronics Inc., Troy, NY) as previously described [13, 14]. Briefly, pressure differences were measured between the main chamber of the plethysmograph, which contained conscious, spontaneously breathing animals, and the reference chamber (box pressure signal). Mice were challenged with aerosolized saline for baseline measurements or with MCH (3.125–50 mg/ml) for 3 min, and readings were taken and averaged for 3 min after each nebulization. Data were expressed using the dimensionless parameter, enhanced pause (Penh).

Sample Preparation

Mice were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital. Blood samples were collected by eyeball removal exsanguinations and centrifuged at 3000 rpm for 10 min at 4 °C. Then, the serum was aliquoted and stored at –80 °C until use.

The right lung was lavaged *via* the tracheal cannula with 0.5 mL phosphate-buffered saline (PBS; pH 7.2) for three times. Lavage fluid was recovered by gentle aspiration with a syringe. The collected bronchoalveolar lavage fluid (BALF) was then centrifuged at 2000 rpm/min for 10 min at 4 °C. The supernatant from the first wash was collected and stored at –80 °C for cytokine determination. The skin lesions and left lung were harvested, inflated with 4% paraformaldehyde, fixed in formalin for 24 and 6 h, respectively, and paraffin-embedded for histological analyses.

Quantification of Leukocytes in the Bronchoalveolar Lavage

After supernatant removal, cells in the lavage fluid were suspended in 0.5 ml of phosphate-buffered saline (pH 7.4). Cells were counted on a hemocytometer, and $(1-5) \times 10^3$ cells were spun onto glass microscope slides. The cell slides were air dried for 24–36 h, fixed, and stained with Wright's stain. The number of eosinophil (Eos), lymphocyte (Lym), macrophage (Mac), and neutrophil (Neu) recovered of 200 cells in duplicate slides were counted in a blinded fashion according to morphological criteria, and these data were expressed as absolute cell numbers.

Evaluation of Skin Dermatitis Severity

The severity of skin dermatitis in the dorsal and ventral skin was scored twice a week. The development of erythema/hemorrhage (1), scarring/dryness (2), edema (3), and excoriation/erosion (4) was scored as 0 (none), 1

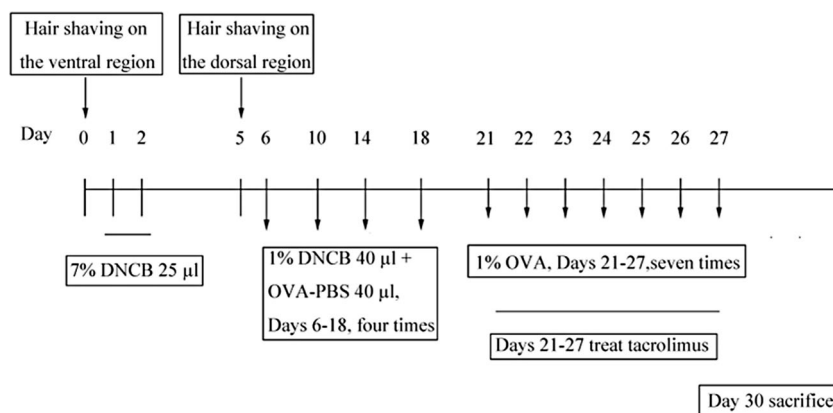


Fig. 1. Schematic diagram of the experimental protocol in mice. Mice were divided into three groups ($n = 10$ per group). To induce AD-like immunologic and skin lesions, DNCB was applied to the ventral and dorsal skin. After complete removal of ventral hairs within an area approximately 2 cm^2 , $25 \mu\text{l}$ of 7% DNCB was applied on the ventral skin for two consecutive days for sensitization. Four days after sensitization, the dorsal skin was challenged with $40 \mu\text{l}$ 1% DNCB solution and $40 \mu\text{l}$ OVA-PBS solution for every other 3 days. The vehicle control mice were treated in the same fashion with acetone. After inducing AD, all the mice were challenged *via* the airways with aerosolized 1% OVA for 30 min every day from days 21 to 27. Tacrolimus ointment was topically applied to the dorsal skin lesions of the treatment group 1 h after the aerosol inhalation each time. AD and vehicle control mice were applied to acetone in the same fashion. Finally, mice were sacrificed on day 30 and the skin or cells were processed.

(mild), 2 (moderate), or 3 (severe). Dermatitis score was calculated by summing up the scores of each item [15].

Histopathological Measurement

Sections ($5 \mu\text{m}$ in thickness) of left lung tissues were subjected to hematoxylin and eosin (H&E) staining for detecting inflammation changes. An arbitrary scope was given to each microscopic field viewed at a magnification of $\times 200$ by light microscopy.

Cytokine Assays

Interleukin (IL)-4, IL-5, OVA-specific IgE, and interferon (IFN)- γ were measured in the bronchoalveolar lavage (BAL) fluid and the serum samples using commercially available (enzyme-linked immunosorbent assay) ELISA kits (BD Biosciences, San Jose, CA, USA) as previously described [24, 25]. BAL fluid and serum were kept frozen at -80°C until cytokine analysis.

Statistical Analysis

All the data were expressed as mean $\pm$ SD (standard deviation) and analyzed by the SPSS software. The significant differences were assessed by one-way ANOVA and the Student-Newman-Keuls test. p value < 0.05 was considered significant.

RESULTS

AD-Like Skin Severity in BALB/c Mice

The skin conditions were investigated using the dermatitis severity scoring system. Repeated application of DNCB significantly increased dermatitis severity scores and induced the development of hemorrhage, edema, scarring, dryness, and erosion in BALB/c mice. After tacrolimus treatment, AD symptoms induced by DNCB were relieved (Fig. 2 and Table 1).

AD-Like Histopathological Changes in BALB/c Mice

Administration of DNCB significantly increased skin lesions in mice compared with the control group (Fig. 3). Furthermore, in comparison with the control group, skin lesions in rats of the AD group showed hyperkeratosis and dermis thickening, as well as infiltration of inflammatory cells such as neutrophils, lymphocytes, mast cells, and eosinophils (Fig. 3). These skin lesions were all decreased after tacrolimus treatment.

Airway Responsiveness

Airway responsiveness was tested on day 30. There was a significant difference in baseline measurement among VC, AD, and TR mice ($p < 0.05$) (Fig. 4). After aerosolizing MCH, the average of Penh increased gradually with the increasing concentrations of MCH (3.125, 6.25,

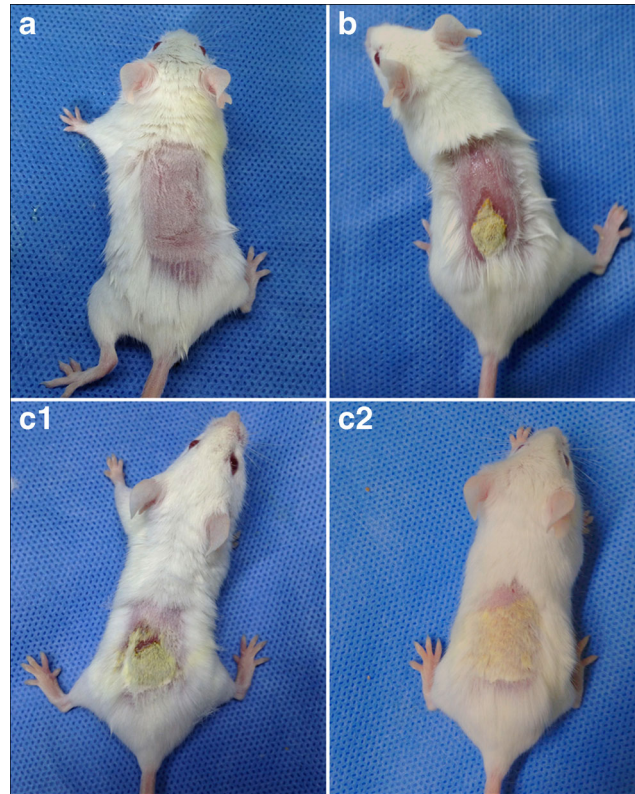


Fig. 2. Effects of tacrolimus on DNCB-induced AD skin severity in BALB/c mice. Representative photographs captured after treatment completion showing comparison of AD-like skin lesions. Results shown are representatives of three observations: vehicle control (VC) (a), AD (b), and treatment (TR) (c1 and c2; c1 before tacrolimus treatment, c2 after tacrolimus treatment).

12.5, 25, 50 mg/ml). The AD mice had significantly increased Penh at 3.125, 6.25, 12.5, 25, and 50 mg/ml MCH compared to the VC group of mice, and the Penh of the AD group was significantly higher than the Penh of the TR group at 12.5, 25 mg/ml MCH ($p < 0.05$) (Fig. 4).

Table 1. The Severity of AD was Macroscopically Assessed by SCORAD

Group	No. of mice	SCORAD
Vehicle control (VC)	10	0
AD	10	$16 \pm 1.633^*$
Treatment (TR)	10	$8.2 \pm 1.751^{**}$

The dermatitis score was defined as the sum of scores for seven clinical criteria: erythema, hemorrhage, edema, excoriation, erosion, scarring, and dryness. The degree of each symptom was scored as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe). The total score from the seven symptoms of each mouse was taken as the score for that mouse. Results are presented as mean $\pm$ SD. ($n = 5$). $^*p < 0.05$ vs. vehicle control, significantly different from the DNCB-treated group (AD); $^{**}p < 0.05$ vs. AD, significantly different from the control group (VC)

Histological Analysis of Lung Tissues

We then further confirmed airway inflammation changes in lung tissues by H&E staining (Fig. 5). H&E staining showed that, in comparison with VC mice, the number of inflammatory cells of AD mice was larger in both the peri-bronchiolar areas and alveolar septum. Infiltrated inflammatory cells in the peri-bronchiolar areas and alveolar septum were decreased in TR mice compared to AD mice.

The Number of Inflammatory Cells in BALF

In order to further analyze inflammatory changes in BALB/c mice, we counted the inflammatory cells in BALF. As shown in Fig. 6, the total number of leukocytes in BALF of AD mice was significantly higher than that in VC mice. After tacrolimus treatment, the number of leukocytes in BALF was significantly decreased in DNCB-treated mice ($p < 0.05$) (Fig. 6a). The number of neutrophils, lymphocyte, monocytes, and eosinophils in BALF from AD mice was all significantly higher than those in VC

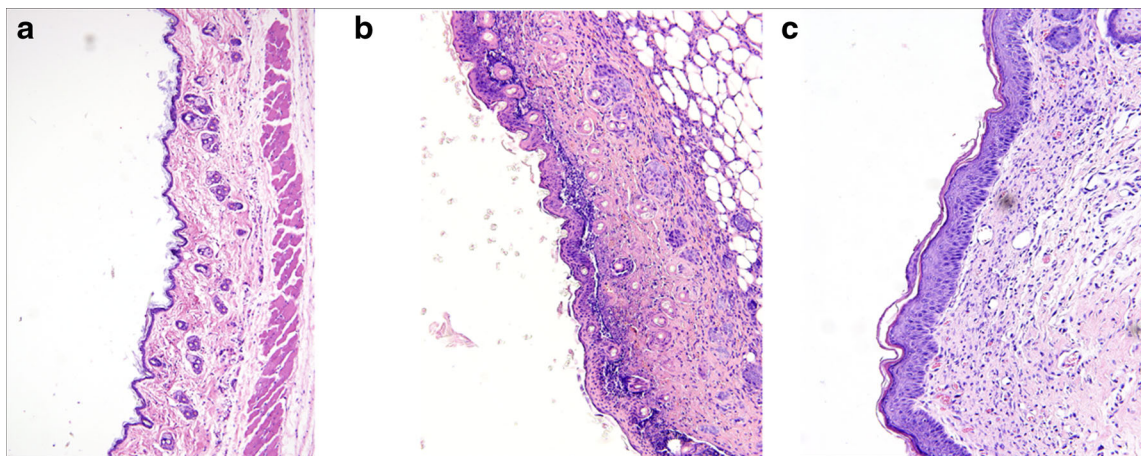


Fig. 3. Histopathological features of AD-like skin lesions in BALB/c mice. Sections were cut and stained with hematoxylin and eosin. Results shown are representatives of three observations in lung tissue from the three groups: vehicle control (VC) (a), AD (b), and treatment (TR) (c).

mice. Treatment with tacrolimus significantly decreased the number of neutrophils, lymphocyte, monocytes, and eosinophils in BALF of AD mice ($p < 0.05$) (Fig. 6b).

IL-4, IL-5, IFN- γ , and OVA-IgE Levels in BALF and Serum

The levels of IL-4, IL-5, IFN- γ , and OVA-IgE in BALF supernatants of mice in the AD group were higher than those in the VC group ($p < 0.05$) (Fig. 7). Comparing to the mice in the AD group, the levels of IL-4, IL-5, IFN- γ , and OVA-IgE were significantly decreased in TR mice ($p < 0.05$) (Fig. 7). Our results showed that repeated application of DNCB significantly increased the level of IL-4, IL-5, IFN- γ , and OVA-IgE in BALF supernatants of mice ($p < 0.05$). Tacrolimus inhibited the increased

levels of IL-4, IL-5, IFN- γ , and OVA-IgE induced by DNCB in BALF ($p < 0.05$) (Fig. 7).

In the mice of the AD group, the increased levels of IL-4, IL-5, and OVA-IgE in serum were reduced by the tacrolimus treatment ($p < 0.05$) (Fig. 8). The IFN- γ level in the serum was not changed in mice of all the groups.

DISCUSSION

AD can frequently induce the onset of asthma in early childhood. Although several studies have indicated that skin sensitization precedes airway sensitization [16–18], the mechanism underlying the progression from AD to asthma remains elusive. Understanding the pathogenesis

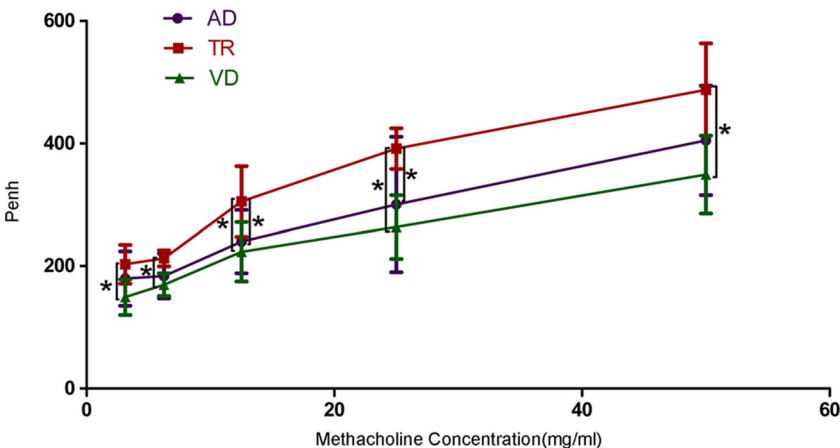


Fig. 4. Evaluation of AHR by MCH challenge in BALB/c mice. Penh values were measured in the three groups, vehicle control (VC), AD, and treatment (TR). Values are means $\pm$ SD. * $p < 0.05$.

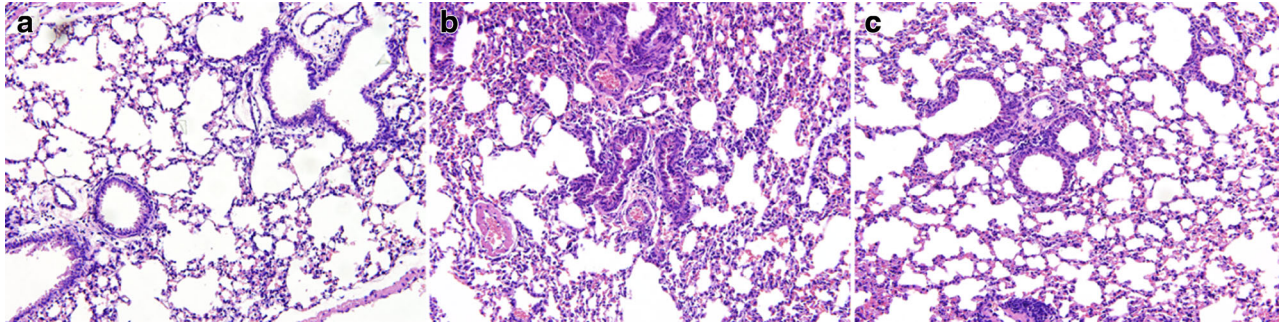


Fig. 5. Histopathological features of cellular infiltrations in the lung in BALB/c mice. Typical pictures of histological sections stained in hematoxylin and eosin ($\times 200$ magnification). All sections of lung tissues were stained with hematoxylin and eosin (H&E) and observed under light microscope ($\times 200$ magnification). Representative images from mice of vehicle control (VC) (a), AD (b), and treatment (TR) (c) were shown.

may help to develop more effective treatment for AD and prevention management for progressive diseases.

In a previous study, one of the pathophysiology of AD is the disrupted skin barrier [6]. In the present study, we successfully constructed an AD mice model with repeated application of DNCB. After AD model construction, the experimental animals presented with increasing dermatitis severity and skin lesions such as hyperkeratosis, dermis thickening, and inflammatory cell infiltration.

In order to explore the potential molecular association between asthma and AD, we investigated the changes in airway responsiveness, lung tissues histology, and inflammatory cells in BALF. It is reported that the main pathophysiology in asthma is the changes of airway inflammation and bronchial hyper-responsiveness [19, 20]. A previous evidence has showed that the eosinophilic airway inflammation is prevalent in asthmatic patients [21]. The airway inflammation is also found in other airway diseases

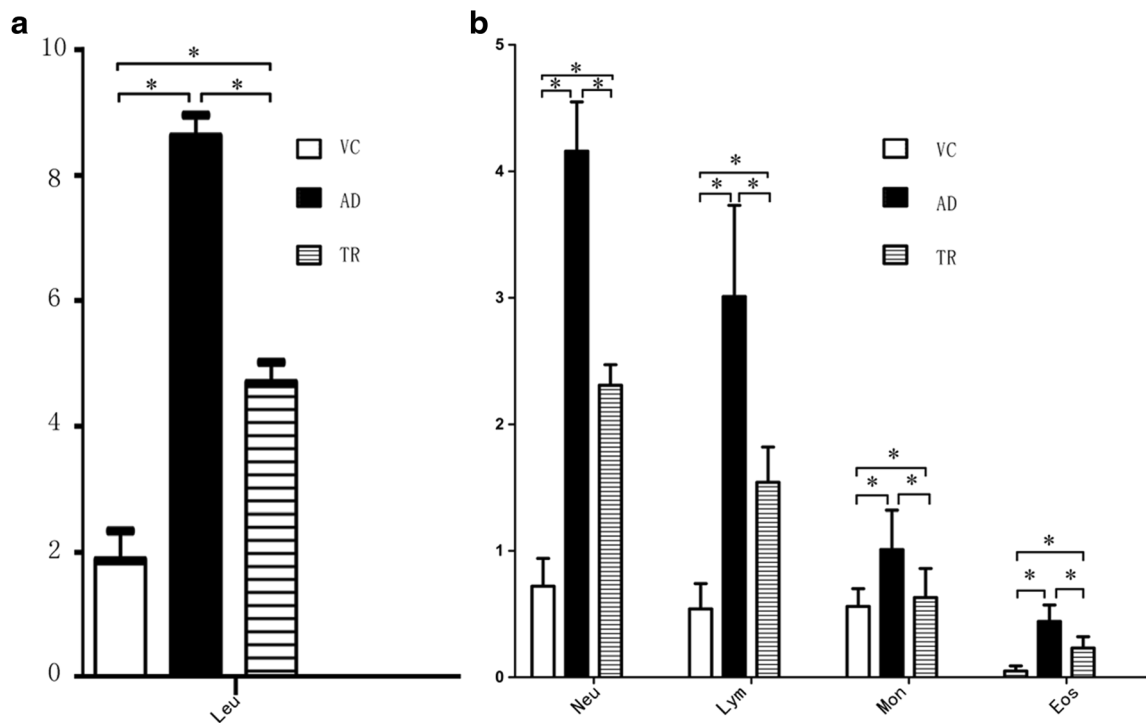


Fig. 6. The number of leukocytes in bronchoalveolar lavage (BAL) fluid in BALB/c mice. **a** The total leukocyte cells (Leu) were counted with cell counter. **b** The classified cells were performed with Wright staining. *Eos* eosinophil, *Lym* lymphocyte, *Mon* monocytes, *Neu* neutrophil. Data were expressed as mean $\pm$ SD, and the significant difference between two groups was analyzed by SNK tests, $*p < 0.05$.

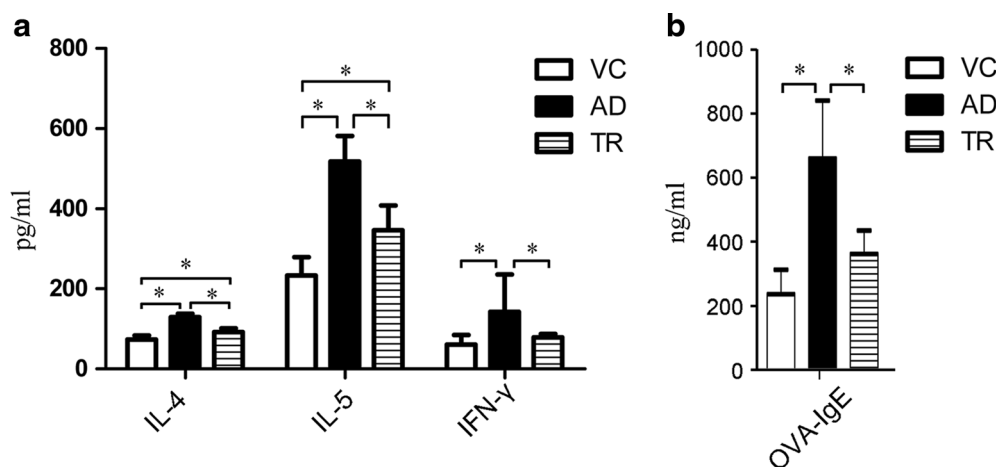


Fig. 7. Cytokines in bronchoalveolar lavage fluid in BALB/c mice. IL-4, IL-5, IFN-γ (a), and OVA-IgE (b) levels were analyzed by ELISA. Results are shown as mean ± SD * $p < 0.05$.

such as allergic rhinitis and chronic obstructive pulmonary disease [22]. Our data showed that the infiltration of inflammatory cells such as neutrophils, lymphocytes, mast cells, and eosinophils was observed in skin lesion. The number of infiltrated inflammatory cells in the lung tissue and BALF was significantly higher in AD mice than in VC mice. Tacrolimus treatment decreased inflammatory cells in the lung tissue and BALF of AD mice and prevented the risk for asthma development. It can be therefore hypothesized that airway inflammation may be a key mechanism involved in the progression from AD to asthma, and tacrolimus could prevent this progression *via* involving in inflammation.

In addition, AD has an immunological basis that primary T cell immunodeficiency disorders frequently

increase the concentrations of serum IgE and aggravate eczematous skin lesions [23]. AD does not occur in animals with the absence of T cells [24]. Previous studies have provided evidence that T cells are essential for inflammation and airway sensitivity [25, 26]. Th2-type cytokines, such as IL-4 and IL-5, contribute to the induction and maintenance of airway inflammation and asthmatic response. IL-5 is known to play a pivotal role in the maturation and differentiation of eosinophils [27]. One hypothesis suggests that cutaneous antigen-presenting cells play a key role in stimulating the Th2 response and promoting the AM. The epidermis of patients with AD contains an increased number of IgE-bearing Langerhans cells and inflammatory dendritic epidermal cells expressing the high-affinity receptor for IgE [28, 29]. These high-affinity

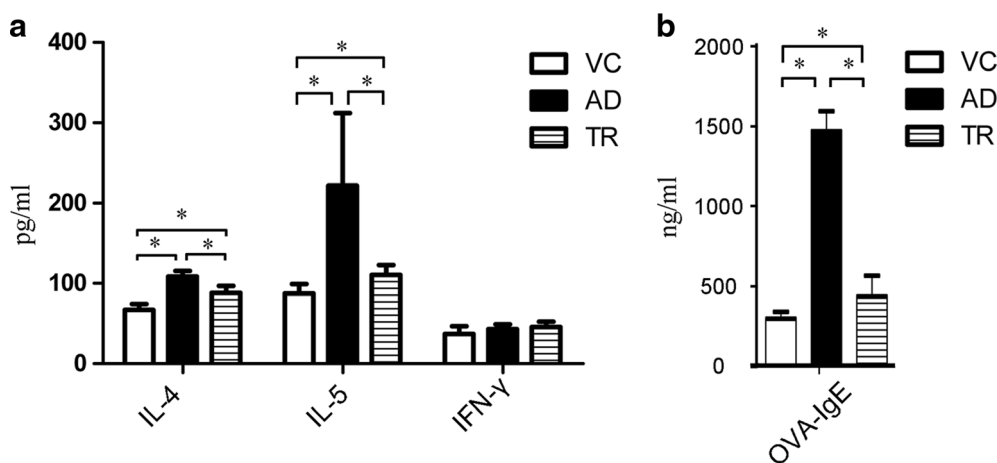


Fig. 8. Cytokines in the blood serum in BALB/c mice. IL-4, IL-5, IFN-γ (a), and OVA-IgE (b) levels were analyzed by ELISA. Results are shown as mean ± SD * $p < 0.05$.

receptors (FcεRI) for IgE contribute to the capture and internalization of allergens before their processing and antigen presentation to T cells in atopic skin. Langerhans cells positive for these receptors migrate to the lymph nodes and stimulate naive T cells, thus contributing to the expansion of the pool of Th2 cells and a systemic Th2 environment [30]. Subsequently, memory Th2 cells migrate through the circulatory system to various sites including the nasal and lung mucosa and bone marrow. In our paper, we also found that the level of OVA-specific serum IgE, IL-4, and IL-5 was increased in the AD mice model. Our data prompt us to hypothesize that Th2 cytokine IL-4, IL-5, and IgE are positively correlated with airway inflammation, and the Th2-type cytokines and IgE may play an important role in triggering the progression from AD to asthma.

Besides, tacrolimus binds to macrophilin, and further binds to calcineurin to dephosphorylate the activated T cell protein, thus to inhibit T cells [31]. Binding of calcineurin prevents translocation of the activated T cell protein into the cell nucleus and inhibits the activation of T cell cytokines. Tacrolimus also showed inhibitive effect on T-helper 1 and 2. In the present study, we observed that the pretreatment with a topical tacrolimus significantly relieved aerosolized OVA-induced airway inflammation and bronchial hyper-responsiveness. The levels of Th2-type cytokines IL-4, IL-5, and OVA-IgE were all significantly decreased in BALF and the serum of AD mice during tacrolimus treatment. Tacrolimus inhibited the molecular progression from AD to asthma. These data suggest that tacrolimus could effectively prevent the progression from AD to asthma *via* regulating Th2-type cytokines.

In conclusion, Th2-type inflammation and IgE production may be involved in the progression from AD to asthma. Tacrolimus could effectively prevent the progression from AD to asthma. AD is the most significant factor for atopic asthma development. Our work may help to understand the progression from AD to airway inflammatory diseases and the molecular association between AD and asthma. However, the findings of our study were not confirmed by using an *in vivo* model to AD. Further studies are still needed to verify our observation.

COMPLIANCE WITH ETHICAL STANDARDS

The experimental protocol was approved by the Medicine Ethical Committee for Laboratory Animals of Shanghai Jiao tong University.

Conflict of Interest. The authors declare that they have no conflict of interest.

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