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Decrease in IL-4 Expression after Immunotherapy with Local House Dust Mite Allergen in Atopic Dermatitis in BALB/c Mice

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Abstract

The efficacy of immunotherapy in atopic dermatitis (AD) patients has been debated in years. Study of immunotherapy in AD mouse model can help finding objective information in the mechanism of immunotherapy, especially interleukin (IL)-4 that widely known has important role in AD pathogenesis. Thirty-three male BALB/c mice aged 6-8 weeks were randomly assigned to 3 different groups: immunotherapy, AD model, and control group. The mice in immunotherapy and AD model group were sensitized with house dust mites (HDM) Dermatophagoides pteronyssinus (Der-p) allergen extract, patched for the first 7 days and nebulized continuously from day 1-93. Subcutaneous Der-p immunotherapy injection was given every 3 days in increasing doses. The skin samples were taken on day 93 and was assessed for IL-4 with immunohistochemistry method. The data were analyzed with comparative analysis. The mice in immunotherapy group had lower IL-4 expression compared to AD model group which received placebo injections, and slightly higher than the control group. The mean IL-4 expression comparison between groups of mice was not significant ($p = 0.098$). This study found a decrease of IL-4 after immunotherapy with local HDM allergen in AD BALB/c mice compared to placebo group.

KEYWORDS
Atopic dermatitis, BALB/c mice, House dust mites, IL-4, Tropical disease

INTRODUCTION

Atopic dermatitis (AD) is a chronic and multifactorial inflammatory skin disease with the main complaint of itching. Despite not being a life-threatening disorder, AD poses a considerable social, psychological, and financial burden (Sastre *et al*., 2020). The prevalence of AD in Europe is 11.4-16.9%. Southeast Asia and Latin America is identified as emerging areas with an increasing prevalence of AD, including in Surabaya, Indonesia (Sihaloho and Indramaya, 2015; Simon *et al*., 2019). This disease is a precondition of other atopic diseases such as allergic asthma and allergic rhinitis known as atopic march. Both genetic and environmental factors contribute to the atopic march, which results in type 2 immunological reactions and possibly high Immunoglobulin E (IgE) levels (Aw *et al*., 2020). Environmental factors can trigger AD by causing skin inflammation and the skin barrier disruption. It has been shown that cytokines of the T-helper 2 (Th2) type worsen the epidermal barrier. As a result, allergens and irritants may enter the epidermis and prolong the inflammation (Simon *et al*., 2019).

The most frequent aeroallergens associated with atopic disorders are house dust mites (HDM) (Tham *et al*., 2016). As many as 63.3% of AD patients reported of having positive Skin Prick Test (SPT) results for HDM in the Dermatology and Venerology Outpatient Clinic Dr. Soetomo General Academic Hospital Surabaya, Indonesia, between 2017 and 2019 (Nugroho *et al*., 2022). The most common species that can cause allergic responses in Asia are Dermatophagoides pteronyssinus and Dermatophagoides farinae. Storage mites such as Blomia tropicalis and Tyrophagus putrescentiae are also frequently found in Asia (Tham *et al*., 2016). At relative humidity levels more than 50%, house dust mites will survive and grow, at relative humidity levels below this, they may get dry and stop growing. Dust mites and the allergies they create are a significant issue for those who live in humid, temperate, and tropical environments (Sarwar, 2020).

Various modalities of AD therapy have been attempted, but generally only symptomatic. Probiotics have also been suggested as an additional therapy since they were found to be effective in relieving clinical symptoms (Prakoeswa *et al*., 2022). Exposure to unavoidable allergens often leads to relapse. Long-term use of topical drugs can cause side effects (Katoh *et al*., 2020). Immunotherapy is the only causative therapy for allergic diseases by increasing tolerance to allergens (Alvaro-Lozano *et al*., 2020). Immunotherapy plays a role in increasing tolerance to allergens, allergen administration is done in increasing doses until hyposensitization is achieved (Caminiti *et al*., 2020). The therapeutic advantages and cost saving of allergic rhinitis treatment for children are well supported by studies on immunotherapy conducted in Indonesia (Endaryanto and Nugraha, 2021). Currently, there is no biological marker that is considered valid in predict-

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ing the success of immunotherapy. Regulatory T (Treg) cells and their cytokines such as interleukin (IL)-10, transforming growth factor beta (TGF-β), and IL-2 which have been shown to reduce Th2 immune responses (Tang, 2020). Recent studies in immunotherapy on animal models with different allergen and method also showed a decrease in Th2 and Th17 cytokines, as well as an increase in Th1 cytokines, demonstrate efforts to rebalance immunological dysregulation in AD (Shershakova *et al*., 2015; Shin *et al*., 2018). This study aimed to find objective information of the IL-4 role as well-known marker of Th2 immune responses in AD pathogenesis.

MATERIALS AND METHODS

Mice preparation

Thirty-three male BALB/c mice aged 6-8 weeks were purchased from the Faculty of Veterinarian Medicine at University of Airlangga, Surabaya, Indonesia. The mice were kept in a pathogen-free environment and were put in individual cages throughout the experiment. This study had received ethical clearance from the Ethical Committee of Faculty of Veterinarian Medicine at University of Airlangga, Surabaya, Indonesia (No.2.KE.11.09.2021). The mice were randomly assigned to 3 different groups, 11 mice each. The groups were control, AD model, and immunotherapy group. The individual cages for the mice were made of acrylic plastics with 12 x 8 x 8 in size. The mice were numbered, and the cages were labeled to prevent error in applying procedures to each group. Before the start of immunotherapy, the mice in immunotherapy and AD model group were sensitized with HDM by patch and nebulized Dermatophagoides pteronyssinus (Der-p) allergen extract in phosphate-buffered saline (PBS) for 7 days continuously. The mice were shaved with electric razor before patch application. The dose for patch was 100 µg and 10-6 µg for nebulization. Meanwhile, the control group received NaCl 0.9% as the placebo. The Der-p allergen extract was produced by Teaching Industry Allergen by Dr.Soetomo Hospital-Universitas Airlangga, Surabaya, Indonesia (Putera *et al*., 2021).

Immunotherapy protocol

The Der-p immunotherapy was given to the mice in immunotherapy group with escalating doses of 0.1, 1, 10, and 100 µg in 100 µL PBS. Subcutaneous injection was given to the neck of each mouse. The AD model and control group were given PBS injection instead of Der-p as the placebo. The injections were carried out every 3 days from day 15 to 60. The first dose of immunotherapy (0.1 µg) was given on day 15, 18, 21, and 24; the second dose (1 µg) on day 27, 30, 33, and 36; the third dose (10 µg) on day 39, 42, 45, and 48; the fourth dose (100 µg) on day 51, 52, 59, and 62. After immunotherapy, the mice had 2 exposures to Der-p patch for 1 week, separated by 2 weeks interval in between the exposure. The Der-p nebulization was given daily since the sensitization phase until the end of treatment on day 93. The Der-p allergen doses for patch and nebulization exposure as well as the placebo were the same to that on sensitization phase.

Th2 cytokine expression (IL-4)

The skin tissue from the back of the mouse was fixed with 10% paraformaldehyde, immersed in paraffin, then cut for 4 µm thick. Immunostaining was carried out to the skin tissue to measure Th2 cytokine expression with immunohistochemistry (IHC) method. For the evaluation of IL-4 expression, semiquantitative immunoreactive score (IRS) scale was applied. Semiquantitative IRS scale was counted by multiplying the score of positive cells percentage and the score of color reaction intensity. The score of positive cells percentage was divided into 0 (no positive cell reaction), 1 (1-10% positive cell reaction), 2 (11-50% positive cell reaction), 3 (51-80% positive cell reaction), and 4 (>80% positive cell reaction). Meanwhile, the score of color reaction intensity was divided into 0 (no color reaction), 1 (low color reaction), 2 (moderate color reaction), and 3 (intense color reaction). The IRS were evaluated on 10 fields of view and counted for mean values for each sample.

Statistical analysis

The mean IRS for each mouse was analyzed with IBM SPSS Software ver. 26 (IBM, USA) and assessed for the normality of the data with Shapiro-Wilks normality test. Non-parametric Kruskal-Wallis H test was used for comparative analysis because the data did not show normal distribution. Data visualization was made with GraphPad Prism software ver. 8 (GraphPad, USA).

RESULTS

Eleven mice from each group were evaluated for IL-4 at day 93. The mice in immunotherapy group had lower IL-4 expression compared to AD model group which received placebo injections. The mean±SD of IL-4 expression for control group was 2.755±0.723, AD model group was 3.000±0.343, and the immunotherapy group was 2.764±0.932. The comparison for each group was illustrated in Figs. 1 and 2. The mean IL-4 expression comparison between groups of mice was not significant (Kruskal–Wallis H test, p = 0.098).

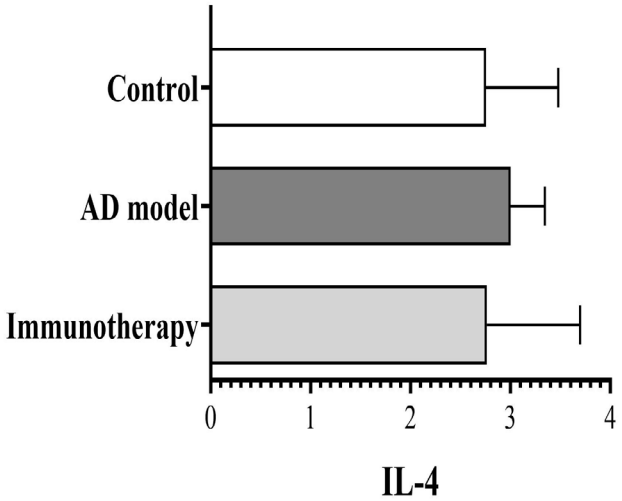

Fig. 1. The data visualization of IL-4 expression from each group. The AD model had the highest IL-4 counts compared to immunotherapy and control group. However, the statistical analysis showed no significant difference between groups. The data are shown in mean±SD.

DISCUSSION

According to latest studies, AD is caused by a complex combination of genetics, skin barrier disruption, immunological dysfunction, and environmental factors (Dubin *et al*., 2021). Elevated serum immunoglobulin E (IgE) levels, allergen sensitivity, a predominance of Th2 cytokines, an increase in T cells that express cutaneous lymphocyte associated antigen, and other markers are all part of the pathogenesis associated with immunological dys-

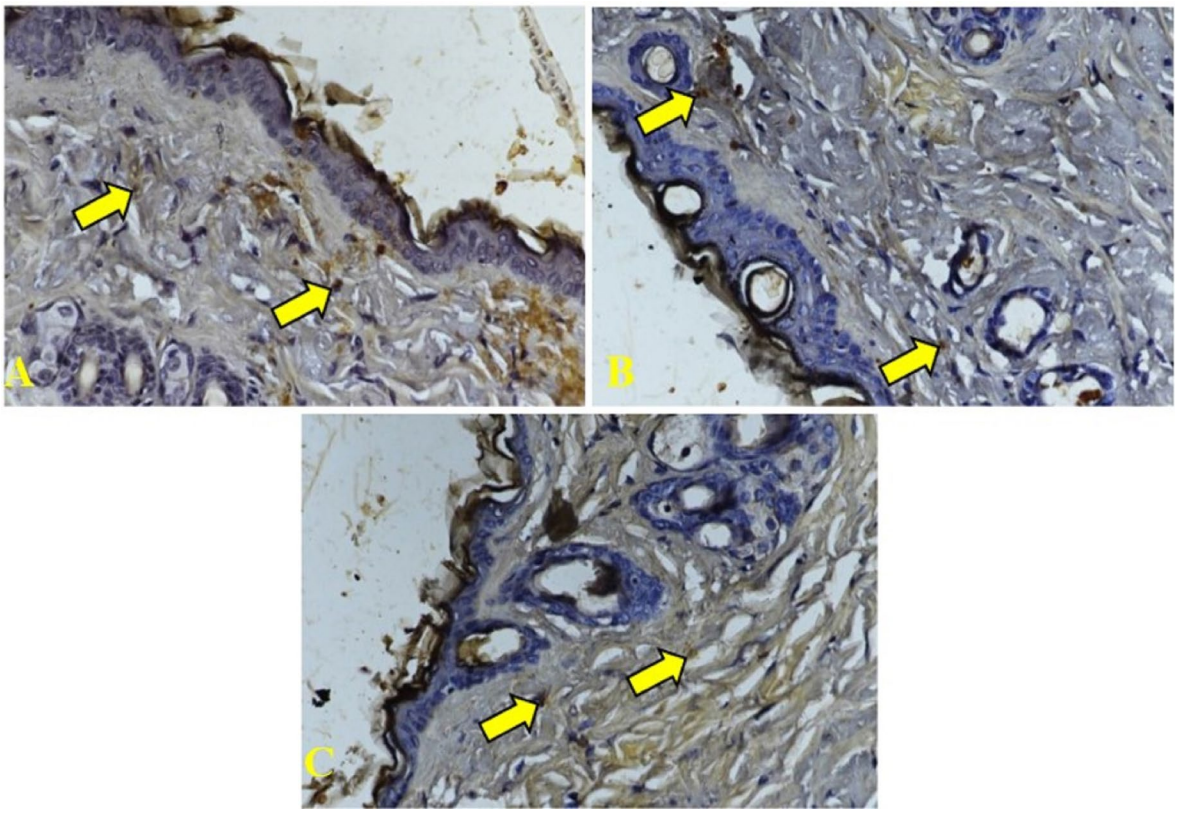

Fig. 2. Comparison of IL-4 expression in the skin of (a) control group, (b) AD model group, and (c) immunotherapy group. The arrow indicates the presence of IL-4 expression in the dermis which is indicated by the presence of brown chromogen (Immunohistochemistry, 400x magnification).

function in AD. Genetic and environmental factors cause damage to the skin barrier that triggers Langerhans cells and inflammatory dendritic epidermal cells to produce Th2 cytokines (IL-4, IL-5, IL-13, IL-31) (Yang *et al*., 2020).

It has been demonstrated that Th2 cytokines contribute to the barrier disruption in AD. IL-4 and IL-13 act on epidermal keratinocytes, inducing hyperplasia and also reduced differentiation, inhibiting full maturation (Dubin *et al*., 2021). An increase in the level of cytokines IL-4 and IL-13 causes a drop in filaggrin, ceramide, and skin lipids as well as induces B cells to produce IgE, whilst IL-5 induces eosinophils production (Yang *et al*., 2020). Elevated IgE levels in AD skin and serum results in allergic inflammation and pruritis symptoms (Dubin *et al*., 2021). Furthermore, it has been suspected that IL-31 has a special role in the onset of pruritus related with AD (Cho and Chu, 2019). The increase in Th2 cytokines is dominant in all types of AD lesions, especially in pediatric patients (Renert-Yuval and Guttman-Yassky, 2020). It is well known that IL-4 induces the transformation of naive T cells into Th2 cells. Th2 cells produce various inflammatory cytokines including IL-4, IL-13, IL-5, and IL-9 that are essential for recruiting eosinophils and mast cells, as well as increasing B cell activity. IL-4 and IL-13 are essential for the long-term existence of Th2 cells and the production of important Th2 cytokines (Dubin *et al*., 2021). Thus, IL-4 and IL-13 play an important role in the Th2 immune response in AD.

The histology of AD skin lesions, bronchial biopsies, and bronchoalveolar lavages, as well as the cellular infiltration in upper airways, suggest that allergic rhinitis, atopic asthma, and dermatitis have similar immunological processes (Jacquet, 2011). Based on histology, the presence of peripheral and lung eosinophilic and Th2 cellular infiltrates has shown that IL-4 and IL-13 may play a role in the pathogenesis of allergic asthma. The smooth muscle contraction, thickening of the basement membrane, goblet cell hyperplasia, and mucus production that cause airway blockage may also be mediated by these cytokines (Gandhi *et al*., 2017). It is also well known that the release of Th2 cytokines, particularly IL-4 and IL-13, plays a significant role in the development of allergic rhinitis. Through a reduction in the production of tight junction proteins in epithelial cells, Th2 cytokines also affect the integrity of the nasal epithelial barrier in people with allergic rhinitis and asthma (Husna *et al*., 2022).

In this study, HDM allergen extract was used to induce AD-like reaction in BALB/c mice. Enzymatically active natural Der-p 1 has been shown to directly activate the adaptive immune system. As a result, it promotes Th2 sensitization by, at the very least, cleavage of CD23 from B cells to increase the production of IgE and CD25 from T cells to decrease the production of the Th1 cytokine interferon-γ (IFN-γ). Numerous proteins and macromolecules produced by HDMs also have the potential to stimulate innate immunity. While several studies have shown that HDM allergen's biological activities increase their allergenicity, recent research has shown that contaminating microbial elements in HDM play a crucial role as adjuvant factors to induce Th2-biased allergy reactions (Jacquet, 2011).

Immunotherapy prevents both early and late allergic reactions. Immunotherapy causes an increase in allergen-specific IgG, notably the IgG4-blocking antibody, which suppresses both IgE-dependent histamine release from basophils and IgE-mediated antigen presentation to T cells. Immunotherapy alters the cytokine profile of the peripheral and mucosal system from Th2 to Th1 by acting on T cells. Th2 immune responses are suppressed, and allergic symptoms are under control by Treg cells and their cytokines, particularly IL-10, TGF-β, IL-2, and IL-2R (Tang, 2020). Th2 cells and their associated cytokines, such as IL-4, IL-5, and IL-13, along with T-cell proliferation are inhibited as a result of the development of allergen-specific tolerance after immunotherapy (Alvaro-Lozano *et al*., 2020).

The lower IL-4 expression in immunotherapy group compared to the AD model group revealed that the Th2 immune response was suppressed after the administration of immunotherapy. The IL-4 expression was evaluated by cultivating the skin lesion formed on the back of the mouse and processing the tissue with IHC method. However, the results did not show statistically significant difference. Similar research of immunotherapy in AD mouse model using Dermatophagoides farinae (Der-f) found that immunotherapy considerably increased mRNA expression of the Treg cytokine IL-10 while significantly suppressing the Th2 cytokines IL-4 and IL-13 compared to placebo group. From the same study, the IL-4 expression in the serum and splenocytes also showed decrease level but not statistically significant. Addi-

tionally, the 24-hour incubation of lymph node cells with 100 g/ml Der-f extract led to the detection of the Der-f-induced synthesis of IL-4, IL-10, and TGF-1 (Shin *et al*., 2018). Another research of immunotherapy in AD mouse model using ovalbumin (OVA) allergen reported that the levels of IL-4 and IL-5 in supernatants of OVA-stimulated splenocytes from sensitized mice were found to be greater than in controls. The IL-4 expression decreased significantly during OVA immunotherapy with both modified and unmodified allergens (Shershakova *et al*., 2015).

CONCLUSION

This study concluded that IL-4 decreases after immunotherapy with local house dust mite allergen in atopic dermatitis of BALB/c mice.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding this publication.

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