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Asthma Clinical Improvement and Reduction in The Number of $CD4^+CD25^+foxp3^+$Treg and $CD4^+IL\text{-}10^+$ Cells After Administration of Immunotherapy *House Dust Mite* and Adjuvant Probiotics and/ or *Nigella Sativa* Powder in Mild Asthmatic Children

Nieken Susanti[1], Wisnu Barlianto[2], Handono Kalim[3], HMS Candra Kusuma[4]
[1](Pediatric Department, Brawijaya University, Indonesia)
[2](Allergy-Immunology Division-Pediatric Department, Brawijaya University, Indonesia)
[3](Rheumatology Division- Derpartment of Internal medicine, Brawijaya University, Indonesia)
[4](Pulmonology Division-Pediatric Department, Brawijaya University, Indonesia)

Abstract: *Recent studies suggest that regulatory T cells (Treg cells) and immunosuppressive cytokines such as interleukine-10 (IL-10) have a role in pathogenesis of asthma and efficacy of allergen specific immunotherapy (SIT). Since immunotherapy had limitations, several adjuvants proposed to improve its efficacy such as probiotic and Nigella sativa. The aim of this study is to evaluate the effect of combination of immunotherapy house dust mite and probiotic or Nigella sativa either on the induction of $CD4^+CD25^+foxp3^+$Treg and $CD4^+IL\text{-}10^+$ or the control of asthma symptoms in mild asthmatic children. Thirty one mild asthmatic children were evaluated and then randomized to receive immunotherapy plus placebo, immunotherapy plus probiotic, immunotherapy plus Nigella sativa, or immunotherapy plus probiotic plus Nigella sativa for 14 weeks. The number of $CD4^+CD25^+foxp3^+$Treg, $CD4^+IL\text{-}10^+$ and Asthma Control Test (ACT) score were measured before and after treatment. $CD4^+CD25^+foxp3^+$Treg cell number has unsignificant decreased on all treatment groups. There was an unsignificant increase of $CD4^+IL\text{-}10^+$ number in immunotherapy plus placebo group whereas unsignificant decrease was found in the other three groups. All of groups showed a significant increase of ACT score except immunotherapy and placebo group. Adjuvant probiotic or Nigella sativa in immunotherapy can improve asthma symptoms in mild asthmatic children.*

Keywords – *Asthma control test, $CD4^+CD25^+foxp3^+$Treg, $CD4^+IL\text{-}10^+$, immunotherapy, Nigella sativa, probiotic*

I. Introduction

Asthma is chronic respiratory allergic disease which is often found in children[1, 2]. It prevalence increases globally by 50% every decade in developed and developing countries[3].

Current treatments for asthma are mainly based on pharmacological interventions, such as treatment with glucocorticoids or β_2-agonist. Although these treatments are highly effective for controlling disease in most individuals, many patients must take these drugs for life. Moreover, up to 30% of patients do not achieve optimal disease control with these drugs. These issues all highlight the need for new strategies that have specific and long-lasting effects for the treatment of asthmatic disease[4].

Based on recent asthma guidelines, like the Global Initiative for Asthma (GINA) guidelines, asthma treatment focuses on achieving and maintaining asthma control rather than managing asthma attacks. Several tools have been developed to determine the level of control and to guide treatment such as the *Asthma Control Test* (ACT), which useful in the detection of poorly controlled asthma in adults and children [5, 6]. Latest asthma treatment also focuses on enhancing the role of Treg cells to protect the progression of asthma [7]. Treg cells play important roles in the immunological dysregulation underlying allergic diseases[8]. Several studies showed a significantly decreasing of $CD4^+CD25^+foxp3^+$Treg and IL-10 in peripheral blood and *broncho alveolar lavage fluid* (BALF) asthmatic patients[9].

Some allergic disease therapies are known to increase Treg cells and allergen immunotherapy is the only treatment to date that can affect the natural course asthma and has long-lasting effects [7]. Many double-blind, placebo-controlled randomized clinical trials demonstrate immunotherapy is effective for the treatment of asthma. An allergen immunotherapy has the ability to form Treg are capable of secreting cytokines such as IL-10[10]. Since immunotherapy is specific for the antigen used, all relevant antigens need to be included in the treatment extract because any omission may decrease its effectiveness [11]. In recent years, adjuvant therapies have been investigated in order to increase the efficacy of SIT in the therapy of allergic diseases. *Mycobacterium vaccae* , lipopolysaccharide, *Lactobacillus* spp., oral bacterial extracts and *Nigella sativa* have been examined for their adjuvant effects [12].

Few clinical studies evaluate probiotic and *Nigella sativa* effect on asthma [13]. Previous research suggests that probiotics may enhance the production of Treg and regulatory cytokines (IL-10) in vitro in humans

[14]. Preliminary studies in mice showed ethanol extract of *Nigella sativa* can increase the number of $CD4^+CD25^+foxp3^+$Treg lymphocytes in the lungs of mice models of asthma [15]. In vivo administration of *Nigella sativa* and specific immunotherapy effective in reducing the symptoms of allergic rhinitis and asthma [12, 16].

The aim of this study is to evaluate the effect of combination of immunotherapy *house dust mite* (HDM) and probiotic or *Nigella sativa* either on the induction of $CD4^+CD25^+foxp3^+$Treg and $CD4^+IL\text{-}10^+$ or the control of asthma symptoms in mild asthmatic children using ACT.

II. Methods

2.1 Study design

This clinical trial was single-blind, randomized, pre and post control group study conducted at the outpatient allergy and immunology of Department of Pediatrics at the Saiful Anwar General Hospital Malang, Indonesia. The patients were randomized into immunotherapy and placebo, immunotherapy and *Nigella sativa*, immunotherapy and probiotic, or immunotherapy and *Nigella sativa* and probiotic group using randomization table. An evaluation of $CD4^+CD25^+foxp3^+$Treg, $CD4^+IL\text{-}10^+$, and ACT score were conducted before and after treatment for 14 weeks.

2.2 Patients

This study enrolled 31 asthmatic children (age, 4-14 years old) who were positive sensitized house dust mite allergens by skin prict test, diagnosed with intermittent or mild persistent asthma by GINA criteria, and the elderly subjects signed informed consent. Patients with comorbid asthma (sinusitis, otitis media, tuberculosis, pneumonia, nasal polyps, gastro-esophageal reflux, or other anatomical abnormalities), suffer from immunodeficiency, autoimmune disease, or have a cardiovascular disorder, have a history of severe allergic (anaphylactic shock and asthma severe life-threatening attacks), taking therapeutic doses of corticosteroids for 1-2 weeks; vitamin D3 doses of more than 2000 IU / day for 3-4 months; β blockers, ACE inhibitors, antibiotics, leukotriene antagonists, teofillin, anti-cholinergic, cromolyn and ketotifen in the 2 weeks before and during the study and had a history of respiratory failure and intubation in the last 6 months were excluded from the study.

2.3 Administration of immunotherapy and adjuvant *Nigella sativa* or probiotic

Children were given subcutaneous immunotherapy house dust mite (1:100) according to immunotherapy protocol of RS. dr. Saiful Anwar Malang (Fig. 1). We used probiotics produced by PROBI of Medifarma containing $2x10^9$ cfu/g of a mixture of live bacteria *Lactobacillus acidophilus* La-5™ and *Bifidobacterium lactis* Bb-12™, taken daily at a dose of 1 sachet per day. Each capsule of *Nigella sativa* consist of black cumin powder and administered at a dose of 15 mg/kg/day, while the placebo capsule contained only starch powder made from rice.

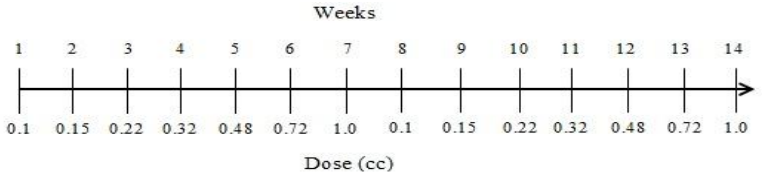

Figure 1. Build up phase of immunotherapy protocol RS. dr. Saiful Anwar Malang

2.4 Asthma Control Test

We used Bahasa Indonesia version of childhood-asthma control test (C-ACT) for children age 4-11 years old and Bahasa Indonesia version of asthma control test (ACT) for children 12-14 years old. The cultural linguistic validation process consist of three steps: forward translation, backward translation and patient testing. The C-ACT consists of seven items, addresses the previous 4 weeks and is divided into two parts. One part is filled in by the child and consists of four questions on perception of asthma control, limitation of activities, coughing and awakenings at night. Each question has four response options. The second part is filled in by the parent or caregiver and consists of three questions (daytime complaints, daytime wheezing and awakenings at night) with six response options. The sum of all scores yields the C-ACT score, ranging from 0 (poorest asthma control) to 27 (optimal asthma control). A cut-off point ≤19 indicates uncontrolled asthma. The ACT is a patient-completed questionnaire and consists of five items evaluating the preceding 4 weeks (limitation of activities, shortness of breath, awakenings at night, use of reliever medication and patient's perception of asthma control). Each question has five response options, resulting in scores of 1–5. The sum of all scores yields the total ACT score, ranging from 5 (poorest asthma control) to 25 (optimal asthma control). It has been validated from the age of 12 years and a score ≤19 indicates poorly controlled asthma. In this study we used Indonesian version that has been validated.

2.5 $CD4^+CD25^+foxp3^+$Treg and $CD4^+IL\text{-}10^+$ cells analysis

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA blood by Ficoll-Hypaque Premium centrifugation gradient within 4 h of collection. To determine the percentage of $CD4^+CD25^+foxp3^+$Treg and $CD4^+IL\text{-}10^+$, PBMC was analyzed using flow cytometry. Cells were stained with human Treg cell-staining kit according to the manufacturer's protocols. Briefly, cells were incubated with a coctail of FITC anti human CD4 (BioLegend) and PE/Cy-5 anti human CD25 (BioLegend) in the dark at 4^0C for 15 minutes. Cells were then fixed and permeabilized with fixation/permeabilization buffer in the dark at 4^0C for 20 minutes, washed once with permiabilization buffer and then resuspended in 1 mL of fixation/permeabilization buffer and incubated in the dark at 4^0C for 15 minutes. They were then incubated with coctail of PE anti-human Foxp3 (BioLegend) in the dark at room temperature for 30 minutes, and then washed once with permeabilization buffer. $CD4^+IL\text{-}10^+$ were prepared using the same procedure without added PE/Cy-5 anti human CD25. All the labeled cells were resuspended in 0,5 mL cell staining buffer and analyzed by flow citometry using a FACSCalibur instrument (Becton Dickinson) and the Cell Quest software package (Becton Dickinson).

2.6 Statistical analysis

Statistical analysis was performed with the SPSS 21.0 software. The internal consistency of C-ACT and ACT were measured by Cronbach's Alpha Coefficient. Value of Cronbach's Alpha Coefficient was 0.912 ($\alpha \geq 0.70$) which mean ACT acceptable to used. Data from flow cytometry and ACT score were analyzed using Paired t-test for paired data, independent t-test for unpaired data and Anova One way for data between treatment groups. To determine correlation between $CD4^+CD25^+foxp3^+$Treg and $CD4^+IL\text{-}10^+$ or between $CD4^+CD25^+foxp3^+$Treg or $CD4^+IL\text{-}10^+$ and ACT score used by Pearson product moment test. The data are presented as means ± standard deviations.

III. Result

3.1 Sample characteristics

Randomization results show the distribution of samples to the immunotherapy and placebo group (n = 7), immunotherapy and probiotics group (n = 8), and Nigella sativa immunotherapy group (n = 8), immunotherapy and probiotics group and Nigella sativa (n = 8).

Most of the samples in this study is 20 (20/31) had a history of atopic disease, including history of parental asthma 11 (11/20), rhinitis 8 (8/20) and urticaria 1 (1/20). Skin test prict showed 14 (14/31) samples had positive results against house dust mite allergen, and the other (17/31) had positive results against some allergens. Most samples (4/7) in the immunotherapy group had intermittent asthma, whereas samples in the immunotherapy and *Nigella sativa*, immunotherapy and probiotics, and the immunotherapy and *Nigella sativa* and probiotics group mostly had mild persistent asthma that is 6 (6/8), 7 (7/8), and 5 (5/8). No side effects of administration of immunotherapy, *Nigella sativa* and probiotics was found in this study. Characteristics of each sample can be seen in TABLE 1.

Table 1 Sample Characteristics

Sample Characteristic	Group 1 (n=7)	Group 2 (n=8)	Group 3 (n=8)	Group 4 (n=8)
a. Age (year)				
< 5 years old	1	3	2	1
≥ 5 years old	6	5	6	7
b. Gender				
Boy	4	4	4	5
Girl	3	4	4	3
c. History of parenteral atopic disease				
Negative	3	2	3	3
Positive	4	6	5	5
d. *Skin prict test* result				
HMD	3	3	5	3
Multiple allergen:	4	5	3	5
HMD + food allergen	4	3	1	4
HMD + pet allergen	0	1	0	0
HMD + food + pet allergen	0	1	2	1
e. Nutritional status				
Good nutrition	7	8	8	8
Malnutrition	0	0	0	0
f. Symptom				
Cough	3	1	1	2
Dyspneu	1	1	0	0
Cough + rhinitis	0	0	0	2
Cough + dyspneu	3	6	3	1
Cough + rhinitis + dyspneu	0	0	4	3
g. Diagnosis				
Intermittent asthma	5	2	1	3
Intermittent asthma	4	1	1	0
Intermitten asthma + mild intermittent rhinitis	1	0	0	1
Intermitten asthma + moderate-severe intermittent rhinitis	0	1	0	2
Mild persistent asthma	2	6	7	5
Mild persistent asthma	1	6	4	3
Mild persistent asthma + mild intermittent rhinitis	0	0	3	1
Mild persistent asthma + moderate-severe intermittent rhinitis	1	0	0	1

Note: Group 1 = immunotherapy and placebo; group 2 = immunotherapy and *Nigella sativa*; Group 3 = immunotherapy and probiotic; Group 4 = immunotherapy and *Nigella sativa* and probiotic

In this study, we found a significant increase of ACT scores in the immunotherapy and *Nigella sativa* (p-value = 0.001 <α), immunotherapy and probiotics (p-value = 0.004 <α), and immunotherapy and *Nigella sativa* and probiotics (p-value = = 0.000 <α) groups. The increase in ACT scores are also obtained in the immunotherapy and placebo group, although the results were not statistically significant (p-value = 0.062> α) (Fig. 2).

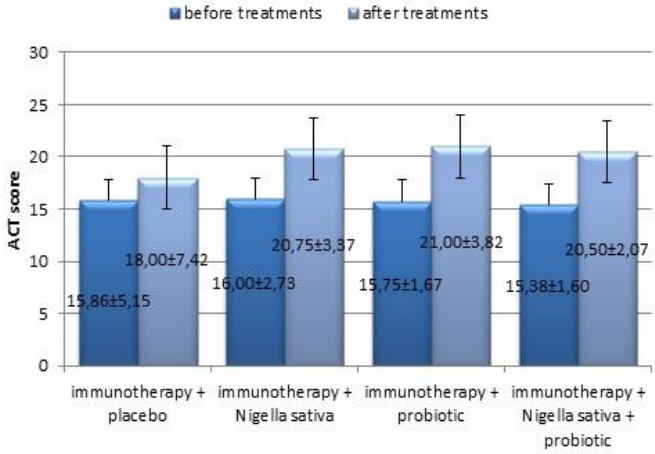

Figure 2 There is a significant improvement of ACT scores in the three treatment groups and no significant increase in the immunotherapy and placebo. ACT scores obtained no differences between treatment groups.

Decrease of mean number of CD4⁺CD25⁺Foxp3⁺Treg in PBMC after treatments

Flow cytometry results showed unsignificant decrease of mean number of CD4⁺CD25⁺Foxp3⁺Treg in all treatment groups. CD4⁺CD25⁺Foxp3⁺Treg among all treatment groups were not significantly different. The mean number of CD4⁺CD25⁺Foxp3⁺Treg obtained the highest in the group receiving adjuvant immunotherapy HDM and *Nigella sativa* and probiotics (0.15 ± 0.11%), but before getting the treatment the mean number of CD4⁺CD25⁺Foxp3⁺Treg in the group was also the highest (0.15 ± 0.11). Three other groups have the same average number of CD4⁺CD25⁺Foxp3⁺Treg (HDM immunotherapy and placebo = 0.1 ± 0.01%; HDM and adjuvant immunotherapy *Nigella sativa* = 0.11 ± 0.05%; adjuvant immunotherapy and probiotics HDM = 0.11 ± 0.08%) (Fig. 3).

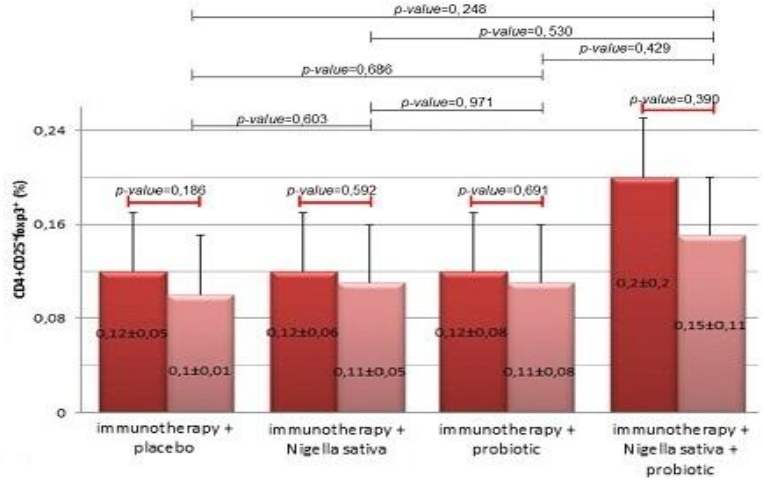

Figure 3 There was no significant difference in the number of CD4⁺CD25⁺Foxp3⁺Treg among all treatment groups, and between before and after treatment in each group although the obtained decrease in the number of CD4⁺CD25⁺Foxp3⁺Treg.

Increase in the number of CD4⁺IL-10⁺ in the immunotherapy group and placebo, and a decrease in the other three groups

In this study the average number of CD4⁺IL-10⁺ increased in the immunotherapy and placebo group, whereas the average number of CD4⁺IL-10⁺ decreased in the other three treatment groups although the increase or decrease is not significant. The number CD4⁺IL-10⁺ between treatment groups (placebo group and

immunotherapy, immunotherapy and *Nigella Sativa*, immunotherapy and probiotics, and immunotherapy and and *Nigella Sativa* and probiotics) before and after treatment did not differ significantly (Fig. 4).

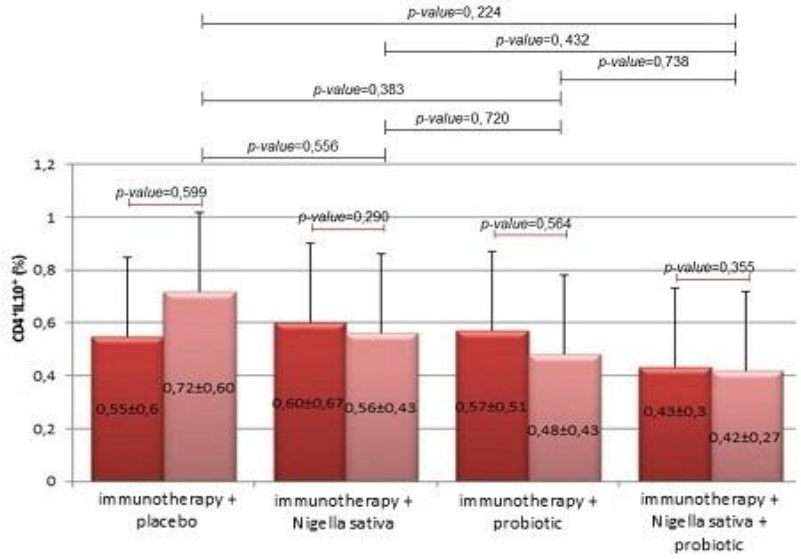

Figure 4 There is an increase in the number of $CD4^+IL\text{-}10^+$ cells following administration of immunotherapy and placebo, and a decrease in the number of $CD4^+IL\text{-}10^+$ after administration of three other treatments, although not significantly.

There are differences in the number of $CD4^+IL\text{-}10^+$ between samples with positive test results prict skin against HDM and multiple allergens, as well as between intermittent and mild persistent asthma.

Statistical analysis showed that there was significant difference in the number of $CD4^+IL\text{-}10^+$ between samples with a diagnosis of intermittent asthma and mild persistent asthma (p-value = 0.001 <α) and the samples were sensitive to HDM with multiple allergens (p-value = 0.032 <α). The number of $CD4^+IL\text{-}10^+$ in the samples with a diagnosis of intermittent asthma (0.86 ± 0.46) was higher than mild persistent asthma (0.36 ± 0.31) and were sensitive to HDM (0.74 ± 0.57) is higher rather than with multiple allergens (0.40 ± 0.24). There was no significant difference between samples with parental history of atopy and without parental history of atopy (p-value = 0.394> α) (TABLE 2).

ACT scores on group characteristics of history of parental atopy and asthma diagnosis was significantly different

There was a significant difference in ACT scores between groups with and without a history of parental atopic disease, and this difference take place before and after treatment. Significant differences of ACT scores were also obtained between intermittent asthma and mild persistent asthma group but this difference was not obtained after treatment administration. ACT scores in the mild persistent asthma group was lower than in the group with ACT scores in the intermittent asthma group (TABLE 2).

Table 2 Comparison of the number of $CD4^+CD25^+Foxp3^+$Treg, $CD4^+IL\text{-}10^+$, and asthma control test scores between groups on each of the characteristics of the sample

Characteristics	n	CD4+CD25+foxp3+Treg		$CD4^+IL\text{-}10^+$		ACT scores			
						Pre		Post	
		mean±SD	p-value	mean±SD	p-value	mean±SD	p-value	mean±SD	p-value
History of parental atopic disease									
Negative	20	0,13±0,08	0,521	0,64±0,48	0,394	16,65±2,00	0,015*	21,35±2,91	0,036*
Positive	11	0,11±0,07		0,47±0,37		14,09±3,56		17,91±5,82	
Skin Prick Test									
Single	13	0,13±0,09	0,205	0,74±0,57	0,032*	16,42±2,79	0,089	20,5±3,52	0,350
Multiple	18	0,12±0,06		0,40±0,24		15,17±2,92		19,82±5,11	
Diagnosis									
Intermittent asthma	11	0,12±0,09	0,966	0,86±0,46	0,001*	17,81±2,13	0,02*	21,63±2,65	0,154
Mild persistent asthma	20	0,12±0,07		0,36±0,31		14,60±2,62		19,30±4,99	

Obtained a weak correlation between the number of $CD4^+CD25^+Foxp3^+$Treg dan $CD4^+IL\text{-}10^+$ in the immunotherapy group

TABLE 3 shows the relationship between the number of $CD4^+CD25^+Foxp3^+$Treg and $CD4^+IL\text{-}10^+$ is significant (p-value = 0.011 <α) with a correlation coefficient of 0.086. Positive value means that if there is an increase in $CD4^+CD25^+Foxp3^+$Treg there will be an increase in $CD4^+IL\text{-}10^+$ in patients with mild asthma child, or vice versa. However, no significant association between the number of $CD4^+CD25^+Foxp3^+$Treg cells and $CD4^+IL\text{-}10^+$ cells in the other three treatment groups.

Relationship between ACT scores and the number of $CD4^+CD25^+Foxp3^+$Treg as well as the relationship between ACT scores and the number of $CD4^+IL\text{-}10^+$. In all treatment groups in this study found a positive relationship, which means that when it obtained an increase of the number of $CD4^+CD25^+Foxp3^+$Treg or $CD4^+IL\text{-}10^+$mit is also associated with an increase of ACT scores, although statistically it turns out that relationship was not significant (TABLE 3).

Table 3 Correlation between the number of $CD4^+CD25^+Foxp3^+$Treg and $CD4^+IL\text{-}10^+$, between ACT scores and the number of $CD4^+CD25^+Foxp3^+$Treg or $CD4^+IL\text{-}10^+$

GROUP	Correlation between the number of $CD4^+CD25^+foxp3^+$Treg and $CD4^+IL10^+$		Correlation between the number of$CD4^+CD25^+foxp3^+$Treg and ACT score		Correlation between the number of $CD4^+IL\text{-}10^+$ and ACT score	
	Correlation coefficient (r)	*p value*	Correlation coefficient (r)	*p value*	Correlation coefficient (r)	*p value*
Immunotherapy	0,086	0,011*	0,522	0,229	0,512	0,241
Immunotherapy + *Nigella sativa*	0,124	0,077	0,108	0,798	0,090	0,833
Immunotherapy + probiotic	0,445	0,269	0,503	0,203	0,259	0,535
Immunotherapy + *Nigella sativa*+ probiotic	0,063	0,882	0,621	0,101	0,166	0,695

IV. Discussion

In this study, the value of ACT in all treatment groups experienced an increase. Immunotherapy in the treatment group obtained an increase in the value of ACT is not significant. Immunotherapy in the treatment group 4/7 samples had sensitization to HDM and food. HDM immunotherapy alone allegedly giving underprivileged suppress clinical manifestations caused by sensitization than HDM. This is consistent with the research of Anderson *et al.* which showed suppression of $CD4^+CD25^+$ cells in peripheral blood are stimulated by grass pollen allergens can be reduced during the pollen season, and otherwise not found in $CD4^+CD25^+$ cells in peripheral blood are stimulated by HDM allergens [17].

An increase in the real value of ACT in the other three treatment groups showed clinically that the administration of adjuvant probiotics and / or *Nigella sativa* is able to cover the shortfall of immunotherapy treatment. The number of $CD4^+CD25^+Foxp3^+$Treg and $CD4^+IL\text{-}10^+$ is not associated with the clinical success of immunotherapy. This is consistent with research that shows Ajduk *et al*. granting HDM immunotherapy for 1.5-2 years improve asthma clinical variables (symptom scores, medication needs, FEV_1, and PEF) despite no change in the number of cells obtained $CD3^+CD4^+CD25^+Foxp3^+$Treg and decreased IL-10 [18]. Administration of *Nigella sativa* improve clinical symptoms of allergic diseases (asthma, allergic rhinitis and atopic dermatitis) significantly compared with placebo [19]. *Nigella sativa* has the effect of respiratory muscle bronchodilation and anti-inflammatory effects. *Nigella sativa* have antagonistic effects on muscarinic receptors and histamine, tehadap inhibitory effect of calcium channel and the opening of potassium channels, and the effect of β-adrenoceptor stimulation, all it causes bronchodilation airway muscles. The main content of *Nigella sativa* is *Timoquinone* (TQ) has anti-inflammatory effects by decreasing Th2 cytokines (IL-4, IL-5 and IL-13), serum IgE, 5-lipooksigenase and cyclooxygenase (COX) in the metabolism of arachidonic acid, thromboxane and leukotriene synthesis and inhibits $Ca2^+$ influk so as to prevent mast cell degranulation and decreased TNF [12, 20, 21]. Ability of probiotic immune regulation depends on the ratio of IL-10 and IL-12 [22].

After administering the treatment for 14 weeks, the number of $CD4^+CD25^+Foxp3^+$Treg had unsignificant decrease in the HDM immunotherapy group. Similar to our results, the results of flow cytometry analysis of peripheral blood HDM allergic asthmatic children by Wei et al showed no significant difference in the number of $CD4^+CD25^+Foxp3^+$Treg cells between the group that received specific immunotherapy for 1.5-2 years and asthma group [23]. Adjuk *et al*. studied the number and function of $CD4^+$Treg cells ($CD4^+CD45RO^+CTLA\text{-}4^+$ and $CD3^+CD4^+CD25^+Foxp3^+$) in the peripheral blood of 16 children HDM allergic

asthma who received subcutaneous specific immunotherapy. In these studies, the number of $CD3^+CD4^+CD25^+Foxp3^+$Treg cells did not change after the administration of immunotherapy for 1 year despite the improvement obtained clinical variables including symptom scores, medication needs, forced expiratory volume in 1 second (FEV_1), the mean peak expiratory flow (PEF) [18]. Specific immunotherapy in asthmatic mice Balb / c increased the number of $CD4^+CD25^+Foxp3^+$Treg significantly in the spleen and blood on the first day after injection, and the increase can't be found in the blood after day fourth [24]. Krestan *et al*. reported in the early maintenance phase that is one month after administration of wasp venom immunotherapy longitudinally significant decline of $CD4^+CD25^+Foxp3^+$Treg temporary which accompanied by significant increase of expression of CCR7 and CD62L on memory cells ($CD4^+CD25^+Foxp3^+$ $CD45RO^+$) Treg [25]. CCR7 and CD62L is a marker of lymphocyte migration to limfonodi. The results of the above studies indicate that the number of $CD4^+CD25^+Foxp3^+$Treg in peripheral blood of allergic patients who have received specific immunotherapy is inconsistent, where differences in the results of these studies can be caused by patient demographic characteristics, including the degree of disease severity and atopic status were different, and the difference in specific immunotherapy protocol and blood sampling time after specific immunotherapy. Another possibility is the difference in the distribution of Treg cells (peripheral blood versus effector organs) caused by homing of $CD4^+CD25^+Foxp3^+$Treg cell both to the lungs and lymph nodes into secondary organs. $CCR7^+CD62L^+$Tregs cells and $CD4^+CD25^+Foxp3^+$Treg in the spleen was increase after specific immunotherapy showed homing $CD4^+CD25^+Foxp3^+$Treg cells to secondary lymphoid organs in asthma, and suggested the homing of $CD4^+CD25^+Foxp3^+$Treg -mediated lung to CCR4. Research homing in vivo showed that CCR4 is important in the migration of $CD4^+CD25^+Foxp3^+$Treg. Homing of $CD4^+CD25^+Foxp3^+$Treg to the lung impaired without CCR4 [26].

In group of immunotherapy, the number of $CD4^+IL\text{-}10^+$ cell increased although not significantly. Wei *et al*. showed HDM immunotherapy administration increased the number of $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ in PBMCs were significantly [23], $CD4^+CD25^+Foxp3^-IL\text{-}10^+$ and $CD4^+IL\text{-}10^+$ in allergic asthma children [27]. In contrast to our study, that study provides HDM immunotherapy longer than our study is up with the maintenance phase (1.5-2 years), and evaluate the number of $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ and $CD4^+CD25^+Foxp3^-IL\text{-}10^+$. Administration of HDM immunotherapy in patients with allergic asthma showed increased $CD4^+IL\text{-}10^+$ significant compared with group asma. Study conducted by Jutel *et al*. use a semi-rush protocol for 3 months in adult asthma patients shows the number of $CD4^+CD25^+Foxp3^+$Treg are increased [28]. If the effector and regulatory cells was induced in the skin, IL-10 had an important role in the regulation of the lung [29, 30]. Treg cells ($Foxp3^+$Treg cells or $Foxp3^-$Tr1) induced by antigen immunization and adjuvant through the skin to secrete IL-10 that can prevent worsening of the inflammatory process [29, 31]. We conclude that administration of HDM immunotherapy increased the number of $CD4^+IL\text{-}10^+$. In this study we also get a weak correlation between the number of $CD4^+CD25^+Foxp3^+$Treg by the number of $CD4^+IL\text{-}10^+$. IL-10 is not only produced by $CD4^+CD25^+Foxp3^+$Treg. IL-10 is a cytokine that has the ability to suppress T cell proliferation, which is produced by $Foxp3^+$iTreg, Tr1, Th1, Th2, B cells, monocytes-macrophages, dendritic cells, eosinophils and mast cells [32].

In this study the number of $CD4^+CD25^+Foxp3^+$Treg cells at specific HDM immunotherapy treatment group and probiotics decreased but not significantly and the number of $CD4^+CD25^+Foxp3^+$Treg cells in this group was not significantly different to the immunotherapy group. Experimental studies using a combination of the two strains of *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* Bb-12, as in this study have not been studied so that the role of cytokines produced by this probiotic (mixed life *Lactobacillus acidophilus* La-5^{TM} and *Bifidobacterium lactis* Bb-12^{TM}) against the number of $CD4^+CD25^+Foxp3^+$Treg cells in this study is not known with certainty. Immunological effects of probiotics on the number of $CD4^+CD25^+Foxp3^+$Treg cells still vary depending on the time of administration of probiotics, strains of bacteria used, and the specific allergens [33]. Administration of *Bifidobacterium bifidum* Bb-12 in Balb / c asthma started at birth until age 8 weeks increased significantly the expression of Foxp3 in spleen and peribronchial lymph nodes. Probiotic treatment before sensitization phase showed a non-significant effect. This is consistent with the hypothesis "window of oppurtinity" the intervention of probiotics will be successful if given early in the colonization process. This hypothesis may explain why the use of probiotics in allergic disease that has been occurred failed to show significant effects on immunological and clinical parameters [34]. Strain probiotics affect the success of therapy, this is evidenced in studies on proximal small bowel biopsies of healthy volunteers who consumed *Lactobacillus* strains with different species showed after 6 hours consumption of *Lactobacillus* strains induce the expression of different cytokines [35].

L. acidophilus La-5 increases the secretion of IL-2 and TNFα [36]. IL-2 and TGF-β is a cytokine that plays an important role in the generation of $CD4^+CD25^+Foxp3^+$iTreg [37]. IL-2 is required in the activation and function optimization of $Foxp3^+$ nTreg and iTreg [38]. TNF decreased the expression and function of $Foxp3^+$nTreg in children HDM allergic asthma. Combination IL-2 and TNF significantly decreased $CD4^+CD25^{high}Foxp3^+$ in children of non-atopi [39]. Six days after the colonization of *Bifidobacterium lactis*

Bb-12 increased IL-6 in rat intestinal epithelial cells [40]. Increased soluble IL-6 receptor antibody and administration of anti-IL-6 increases the number and immunosuppression function of $CD4^+CD25^+Foxp3^+$Treg in lung allergic asthma patients [41]. IL-6 altered Foxp3 Treg into Th17 cells [42]. IL-6 will alter Foxp3 nTreg not iTreg into cells that produce IL-17 [43]. Another possibility is that $CD4^+CD25^+Foxp3^+$Treg cells induced by probiotic migration to local lymph tissue. T lymphocytes which are activated following the flow of lymph toward mesenterial lymph nodes into the blood then circulates throughout the body, and homing to the intestinal mucosa [44, 45]. The migration process is evidenced by a study conducted by Kwon *et al*. ie probiotics IRT5 5 x 108 cfu/day after 20 days of sensitization phase increases the production of $CD4^+Foxp3^+$Treg cells in mesenteric lymph nodes and via chemokines (CCL1 and CCL22) and receptors (CCR4 and CCR8) migration to sites of inflammation in infammatory bowel disease and rheumatoid arthritis [46].

The number of $CD4^+IL\text{-}10^+$ in group of specific immunotherapy and probiotics was decrease although not statistically significant. *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* Bb-12 is not a potent inducer of IL-10, and *L. acidophillus* is a potent inducer of IL-12. Francis et al showed early immune response to allergens can be suppressed by IL-10, but subsequent reactivation of allergen specifics are influenced by the local cytokine environment. For example, although IL-10 can suppress the immune response, reactivation of allergen in the presence of IL-12 produced after vaccination of high-dose grass pollen deviation will induce an immune response to a Th1 [47]. We suspect though giving immunotherapy can increase $CD4^+IL\text{-}10^+$, but reactivation of allergen specific cell by HDM immunotherapy in the presence of IL-12 induced by probiotics will deviate immune response to Th1. In the treatment group of immunotherapy and adjuvant probiotics, there were 7 of 8 samples were diagnosed with mild persistent asthma, and 3 of them with comorbid mild intermittent rhinitis. This is consistent with the previous discussion that the number of $CD4^+IL\text{-}10^+$ is inversely proportional to the severity of asthma. Our results are supported by Charoenying *et al*. that showed the number of IL-10 in moderate persistent asthma is lower than the mild persistent asthma in HDM allergic asthma children [48].

Only one preliminary study examined the effects of *Nigella sativa* on the number of $CD4^+CD25^+Foxp3^+$Treg cells in vivo. That study using murine asthma which given ethanol extract of *Nigella sativa* with several doses (1.2, 2.4 and 4.8 mg/kg/day) for 5 days after sensitization and with a dose of 4.8 mg / kg / day along with sensitization. The results showed an increase in the number of $CD4^+CD25^+Foxp3^+$Treg cells in a dose groups 2.4 and 4.8 mg/kg/day. Barlianto *et al*. concluded that the increase in the number of $CD4^+CD25^+Foxp3^+$Treg cells can be caused by chronic administration of ovalbumin and the role of TGF-β [15]. There was no increase in the number of $CD4^+CD25^+Foxp3^+$Treg cells in our study because the study subjects, the degree of severity, and different research methods.

Results of this study showed that the number of $CD4^+IL\text{-}10^+$ cell in the group of Nigella sativa and immunotherapy treatment was not significantly decreased after treatment. Mostly sample in this group (6 of the 8) had mild persistent asthma. Similar results were also obtained in the study of Barlianto *et al*. ie there was no significant difference of mean IL-10 in all dose groups Nigella sativa, and showed a significant increase of TGF-β in all dose groups [15]. TGF-β plays a leading role in oral tolerance, not IL-10 [49]. Oral tolerance studies showed $Foxp3^+$Treg can inhibit airway inflammation depend on TGF-β not IL-10 [29, 49]. TGF-β is secreted by $Foxp3^+$Treg mediate airway tolerance when induced $Foxp3^+$Treg cells in mucosal tissues and lymph nodes of lung and colon [29, 31].

V. Conclusion

Adjuvant probiotics (mixed live bacteria *Lactobacillus acidophilus* La-5™ and *Bifidobacterium lactis* Bb-12 ™) and / or Nigella sativa may increase the effectiveness of specific immunotherapy HDM in improving the clinical manifestations of mild asthma, although not able to increase the number of $CD4^+CD25^+Foxp3^+$Treg cell and $CD4^+IL\text{-}10^+$ cell in PBMC. The weakness in this study is not enrolled a negative control. This study also enrolled asthmatic children who had positive sensitization of multiple allergen, as well as child who had mild asthma accompanied by allergic rhinitis which can be confounding our results. Further research is needed to assess the function of $CD4^+CD25^+Foxp3^+$Treg and $CD4^+IL\text{-}10^+$, knowing the mechanism of therapeutic effects of probiotics and Nigella sativa and research that measures the number and function of $CD4^+CD25^+Foxp3^+$Treg and $CD4^+IL\text{-}10^+$ in broncho alveolar lavage (BAL).

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