

# Oligo-fucoidan improved unbalance the Th1/Th2 and Treg/Th17 ratios in asthmatic patients: An *ex vivo* study

CHAO-HUEI YANG<sup>1\*</sup>, JING-JING TIAN<sup>2\*</sup>, WANG-SHENG KO<sup>1,2</sup>, CHIA-JU SHIH<sup>2</sup> and YA-LING CHIOU<sup>2</sup>

<sup>1</sup>Department of Internal Medicine, Kuang-Tien General Hospital, Taichung 43302; <sup>2</sup>Department of Nutrition, Master Program of Biomedical Nutrition, Hungkuang University, Taichung 43302, Taiwan R.O.C.

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**Abstract.** An imbalance in the helper T cells (Th)1/Th2 and regulatory T cells (Tregs)/Th17 ratios is believed to play a key role in asthmatic inflammatory responses. Fucoidan reportedly reduces the production of inflammatory factors. Nutritional intervention is an important tool in decreasing the severity of asthmatic disease. This study aimed to investigate the beneficial roles of oligo-fucoidan in balancing the T cell subtype ratios and reducing airway inflammation *ex vivo*. Peripheral blood mononuclear cells (PBMCs) were collected from 30 asthmatic subjects and 15 healthy subjects. Harvested PBMCs were stimulated and treated with or without oligo-fucoidan (100 or 500  $\mu$ g/ml) for 48 h. Cell surface and intracellular cytokine markers were examined by flow cytometry. The pro-inflammatory factors in plasma and culture supernatants were measured using ELISA kits. We found that oligo-fucoidan increases the proportion of Th1 and Treg cells, but did not affect the proportion of Th2 and Th17 cells. Oligo-fucoidan also increased the levels of interferon- $\gamma$  and interleukin-10. Thus, we concluded that oligo-fucoidan might improve the imbalance in Th1/Th2 and Treg/Th17 ratios to reduce airway inflammation, which could be a potential adjuvant therapy for allergic asthma.

## Introduction

Asthma is a chronic disease characterized by airway hyperresponsiveness, inflammation, and remodeling (1). The global prevalence of asthma in adults is estimated to vary widely from 0.8 to 13.4% (2). In 2000, the morbidity rate of asthma in Taiwan was approximately 7.57%; by 2011, this value had

risen to approximately 11.53%. In particular, the incidence of asthma in individuals aged 18-30 years old rose from 2.84% in 2000 to 11.85% in 2011 (3), a 4-fold increase in just 11 years. This demonstrates that asthma has become a common chronic disease and that attention must be given to its burden on health care and quality of life in Taiwan.

Asthma is marked by chronic airway inflammation. The respiratory submucosa of asthmatic patients has been found to be infiltrated by many inflammatory cells such as eosinophils, T cells, mast cell, neutrophils and dendritic cells during both asymptomatic periods and asthma episodes, resulting in the chronic and persistent inflammation of the patient's airway (1,4-7). These inflammatory cells release a variety of cytokines, including interleukin (IL)-4, IL-5, IL-13, and chemokines. They also induce the expression of airway tissue adhesion molecules such as intracellular adhesion molecule-1, vascular cell molecule-1, and E-selectin. This induction contributes to the chronic and persistent inflammation. In turn, repeated inflammation of the airway causes epithelial cell injury, smooth muscle proliferation or hypertrophy, and secretory cell proliferation resulting in increased mucous production toward airway remodeling and cause airway hypersensitivity (8); this eventually results in asthmatic pathogenesis (9,10). The immune mechanism of asthma is predominantly mediated by helper T (Th)2 cells. The activated Th2 cells into the airways and increase in the levels of Th2-type cytokines (IL-4, IL-5, IL-13) and involve in the isotype switching of antibodies produced by B cells from IgM to IgE to induce airway inflammation (11). Th1 cells could inhibit the development of Th2 cells to have an inflammation inhibitory role in asthma (12). In recent years, some studies have pointed out that a Th17/regulatory T cells (Treg) imbalance can also play a role in chronic airway inflammation (9,13-15). Evidence suggests that an imbalance in the Th1/Th2 and Th17/Treg cell ratios establishes the asthmatic inflammatory response.

The cause of airway inflammation is complex and involves many cells and inflammatory factors; consequently, drugs that control airway inflammation target several different pathways that produce inflammatory factors. Drug-based strategies to control airway inflammation include the use of a corticosteroid or the combined use of an inhaled corticosteroid and a long-acting  $\beta$ 2 agonist (16). Some patients are concerned about the adverse effects of these drugs, and seek alternative treatment. Cell and animal experiments focused

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*Correspondence to:* Miss Ya-Ling Chiou, Department of Nutrition, Master Program of Biomedical Nutrition, Hungkuang University, No. 1018 Sec. 6 Taiwan Boulevard, Shalu, Taichung 43302, Taiwan R.O.C.  
E-mail: chiouyl@sunrise.hk.edu.tw

\*Contributed equally

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on nutritional adjunctive therapy, an alternative treatment, have shown that vitamins A, E, and C, folate, and fish oil can relieve the symptoms of asthma (17). Another nutritional adjunctive therapy option is fucoidan. Fucoidan is a polysaccharide derived from brown seaweed extracts. It is structurally similar to a heparin molecule; it consists of repeating units of disaccharides containing an  $\alpha$ -1, 3-linked fucose and an  $\alpha$ -1, 4-linked fucose, forming an  $\alpha$ -1, 3-backbone with branches attached at C2 positions (18,19). Fucoidan is reported to possess antiviral, antioxidant, antimicrobial, anticoagulant, anticancer/antitumor, antiproliferative, and anti-inflammatory properties (19,20). Treatment of atopic allergic reactions with various fucoidan extracts improves allergic responses and prevents or reduces the symptoms of allergic disease by regulating the immune response, including an alteration in the Th1/Th2 balance, an inhibition of IgE production, and a suppression of mast cells degranulation (21,22). In our previous study, we found that oligo-fucoidan might reduce the proliferation of airway smooth muscle (ASM) cells (23). However, the effects of oligo-fucoidan on T cells during allergic disease remains unclear. In this study, peripheral blood mononuclear cells (PBMCs) from allergic asthmatic and non-asthmatic subjects were cultured and T cells were activated. We investigated the effects of oligo-fucoidan on the profiles of the T cells and the levels of cytokines produced during allergic inflammation.

## Materials and methods

**Study subjects.** The subjects were selected from outpatients at Kuang-Tien General Hospital (Taichung, Taiwan R.O.C.). The inclusion criteria were (1) presence of allergic asthma, (2) absence of complicated underlying disease, (3) lack of history of upper or lower airway disease during the month before the study, and (4) not having received immunotherapy or intravenous steroids during the four weeks before the study (inhaled steroids were allowed). The exclusion criteria were malignancy, infection, pregnancy, or other systematic immune disease. These subjects were collected from March 2016 to June 2016. Thirty patients with allergic asthma (13 women and 17 men) and 15 healthy subjects without allergic asthma (7 women and 8 men) were included in this study. Pulmonary function was assessed by spirometry according to the standards of the American Thoracic Society. All subjects were fully aware of the purpose and nature of the study, which was approved by Institutional Review Board (IRB) of Kuang-Tien General Hospital.

**Asthma control test (ACT).** The ACT is a simple test suitable for asthma patients (aged 12 years old and over) that helps patients determine their level of disease control in the past 4 weeks. The test contains five questions; the lowest score is 1 and the highest score is 5 for each item, A the lower score represents worse asthma control. The sum of the scores from the five questions is calculated, with 25 as the maximum. A total score >19 (excluding 19) indicates asthma control is good; a total score is <19 indicates asthma control is poor (24).

**Analysis of basic data and biochemical data of subjects.** We collected anthropometric data, including body weight and height, and blood pressure from subjects using standardized

techniques (IRB approved). Heparinized blood was collected to measure levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (Cre) in the hospital's inspection department. The total plasma IgE level was determined using the Two-site Sandwich Immunoassay Automated Chemiluminescence System (Centaur XP Immunoassay System, Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The remaining blood was separated into plasma and cells. Plasma was stored as aliquots in liquid nitrogen until analysis was performed.

**PBMCs stimulation assay.** PBMCs were isolated by Ficoll-Hypaque within 1 h of blood collection. Harvested PBMCs were washed twice with 1X phosphate-buffered saline (PBS) and resuspended at  $2 \times 10^6$  cells/ml in RPMI-1640 medium supplemented with 10% fetal bovine plasma, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mmol/l L-glutamine. Cells were stimulated with Con A (5  $\mu$ g/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and cultured with or without oligo-fucoidan (100 or 500  $\mu$ g/ml; Hi-Q oligo-fucoidan® as gift from Hi-Q Marine Biotech International Ltd., New Taipei City, Taiwan (R.O.C.)).

**Taiwan) for 48 h.** After incubation, the cells were centrifuged at 1,200 rpm for 10 min. The cell pellets were harvested for flow cytometry analysis (Beckman Coulter Epics xL; Beckman Coulter, Inc., Brea, CA, USA). The supernatants from the PBMCs stimulation assay were harvested and frozen at  $-20^\circ\text{C}$  until analysis was performed. Oligo-fucoidan was prepared by enzymatic hydrolysis of the original fucoidan from the brown algae *Sargassum hemiphyllum* to obtain molecules of approximately 0.5–0.8 kDa (92.1%). The oligo-fucoidan was dissolved in double distilled water (weight/volume) and filtered with a 0.45  $\mu$ m sterile filter (Merck Millipore, Darmstadt, Germany).

**Analysis of T cell subtypes.** PBMCs were re-stimulated with 50 ng/ml phorbol myristate acetate (PMA; Alexis Biochemicals, San Diego, CA, USA) and 1  $\mu$ M ionomycin (Sigma-Aldrich; Merck KGaA) in the presence of 1  $\mu$ l/ml monensin (BD Biosciences, Franklin Lakes, NJ, USA) for 4 h after Con A stimulation (modified form) (25). Half of the total re-stimulated cells were incubated with phycoerythrin (PE)-conjugated anti-human CD3 antibodies for 20 min at  $4^\circ\text{C}$  in the dark and were separated to stain with fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, anti-human CD8, or anti-human CD25 antibodies. The remaining re-stimulated cells were stained with FITC-conjugated anti-human CD4 and then suspended in fixation and permeabilization solution, to stain with PE-conjugated anti-human IFN- $\gamma$ , anti-human IL-4, anti-human IL-17A, or anti-human Foxp3 antibodies. PE-conjugated mouse IgG1 antibodies were used as isotype controls. These antibodies were purchased from BD Biosciences.

**Analysis of the levels of cytokines.** The supernatants from the PBMCs stimulation assay and plasma were used to analyze the levels of cytokines including, IFN- $\gamma$ , IL-4, IL-17A and IL-10 using ELISA kits (eBioscience, San Diego, CA, USA). All samples were analyzed in triple.

Table I. Patient characteristics.

Characteristic	Asthma group (n=30)	Non-asthma group (n=15)
Age	59.7±10.9	59.8±7.0
Sex (M/F)	17/13	8/7
BMI	24.8±4.2	25.5±2.9
AST (U/l)	21.4±6.38	29.7±10.7 <sup>b</sup>
ALT (U/l)	19.8±10.5	32.1±26.6
BUN (mg/dl)	16.1±7.21	17.5±5.7
Creatinine (mg/dl)	1.1±1.0	0.9±0.3
TG (mg/dl)	140±78.8	125.5±61.2
Cholesterol (mg/dl)	203.7±33.7	181.3±42.7
WBC (10 <sup>3</sup> /ul)	6.8±1.4	5.7±1.6
Eosinophil (%)	3.8±3.6	3.6±2.1
Total IgE (kUA/l)	337±441	43.8±32.0 <sup>b</sup>
ACT score	22.4±1.7	N.D.
FVC (L)	2.6±0.8	N.D.
FEV (L)	1.6±0.5	N.D.
FEV1/FVC pr	62.7±11.5	N.D.
Asthma severity classification <sup>a</sup> before treatment, n (%)		
Mild persistent	6 (20)	
Moderate persistent	21 (70)	
Severe persistent	3 (10)	
Stepwise pharmacologic therapy (%)		
Step 1	50	
Step 2	50	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; TG, Triglyceride; WBC, white blood cell; ACT, asthma control test; N.D., not determined; pr, predicted; <sup>a</sup>using GINA guide classification system; <sup>b</sup>significant different compared to asthma subject (P<0.05). The results are expressed as the mean ± SD unless otherwise indicated.

**Statistical analysis.** The results were expressed as the mean ± SD. SPSS for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. Statistical significance was determined by the unpaired *t*-test for comparisons between the Asthma and Non-asthma groups and was determined by one-way ANOVA with the least significant difference post hoc test for comparisons between different treatment groups. Two-tailed statistical tests were used, and P<0.05 was considered to indicate a statistically significant difference.

## Results

**Patient characteristics.** Thirty-two asthmatic patients were recruited; however, complete data were not available for two patients. Thus, 30 asthmatic patients (Asthma group) and 15 healthy volunteers (Non-asthma group) were included in the study. The asthma severity of the patients using GINA guide classification system and Stepwise pharmacologic therapy. Basic demographic characteristics for the two groups

were shown in Table I. There was no significant difference between the two groups for most components, except AST and total IgE levels (P<0.05). The subjects in the Asthma group had lung function below the normal range (FEV1/FVC predicted (pr), forced expiratory flow (FEF) (25-75%) pr, FEV1 po, and FEF (25-75%) po). The average ACT score was 22.4±1.7. There were 29 asthmatic patients over 19, indicating that the asthma symptoms in these patients were well-controlled.

**Oligo-fucoidan increased the Th1/Th2 ratio.** PBMCs were treated with different concentrations of oligo-fucoidan (OF100, 100 µg/ml and OF500: 500 µg/ml) for 48 h in stimulated (treated Con A) condition. Oligo-fucoidan treatment increased the proportion of CD3<sup>+</sup> CD4<sup>+</sup> T cells in the Asthma group more than it did in the Non-asthma group, but the difference was not significant (Table II).

Following T cell stimulation, oligo-fucoidan treatment increased the proportion of CD4<sup>+</sup> IFN-γ<sup>+</sup> cells in the Asthma group (control, 12.4±9.6%; OF100, 16.7±15.0%; and OF500, 16.6±12.5%) more so than in the Non-asthma group (P<0.05). In Fig. 1 showed T cells of one asthmatic and one non-asthmatic subject post-stimulation. Oligo-fucoidan further increased proportion of CD4<sup>+</sup> IFN-γ<sup>+</sup> cells in OF 100 group in asthma subject. On the other hand, the oligo-fucoidan treatment did not affect the proportion of CD4<sup>+</sup> IL-4<sup>+</sup> cells in either of these two groups. However, there was no significant difference between the Asthma group and Non-asthma group in the magnitude of the increase in the proportion of CD4<sup>+</sup> IFN-γ<sup>+</sup> cells following T cell stimulation; this increase was calculated by subtracting the proportion of CD4<sup>+</sup> IFN-γ<sup>+</sup> cells observed under unstimulated conditions from the proportion observed under stimulated conditions (Fig. 2).

While oligo-fucoidan treatment increased the ratio of Th1/Th2 (IFN-γ<sup>+</sup>/IL-4<sup>+</sup>) cells in the Asthma group, the increase was not significant (Table II). However, the oligo-fucoidan treatment, especially oligo-fucoidan treatment with 100 µg/ml, did significantly increase the ratio of Th1/Th2 (IFN-γ<sup>+</sup>/IL-4<sup>+</sup>) cells following T cell stimulation in the Asthma group (P<0.05), but not in the Non-asthma group (Fig. 3).

**Concentration of Th1 and Th2 cell cytokines in plasma and culture medium.** Heparinized peripheral blood (20 ml) was collected from each subject in both groups and separated into plasma and PBMCs. Plasma levels of IFN-γ (a Th1 cytokine) and IL-4 (a Th2 cytokine) were assayed using ELISA kits (Table III). There were no differences between the cytokine levels in either of the two groups.

IFN-γ and IL-4 levels in the culture medium of stimulated and unstimulated PBMCs from the patients were also assayed. Treatment with oligo-fucoidan increased the level of IFN-γ (P<0.05), but did not affect the level of IL-4 in the Asthma group (Table III). These results mirror the proportions of Th1 and Th2 cells (Table II). In addition, treatment with oligo-fucoidan more reduced the level of IL-4 and increased the level of IFN-γ more notably in the Non-asthma group than in the Asthma group (P<0.05).

**Oligo-fucoidan increased the Treg/Th17 ratio.** The proportion of CD4<sup>+</sup> IL-17<sup>+</sup> T cells was higher in the Asthma group than in

Table II. Changes in Th1 cell subtypes after different OF treated-PBMCs from two groups of subjects.

Subtype	Proportions of cells (%)					
	Asthma group (n=30)			Non-asthma group (n=15)		
	Control	OF 100	OF 500	Control	OF 100	OF 500
CD3 <sup>+</sup> CD4 <sup>+</sup>	23.9±13.4	26.1±12.8	25.6±13.0	22.2±13.4	26.8±13.6	21.5±12.5
CD4 <sup>+</sup> IL-4 <sup>+</sup>	0.3±0.34	0.5±1.4	0.3±0.4	0.5±0.4	0.7±0.6	0.6±0.5
CD4 <sup>+</sup> IFN- $\gamma$ <sup>+</sup>	12.4±9.6	16.7±15.0 <sup>a</sup>	16.6±12.5 <sup>a</sup>	14.5±7.0	21.8±11.2 <sup>a</sup>	19.3±10.0
IFN- $\gamma$ <sup>+</sup> /IL-4 <sup>+</sup>	52.0±48.4	113.4±136.8	80.4±88.2	37.4±29.1	54.2±45.2	60.9±59.1

OF, oligo fucoidan; Control, not treated with OF; OF 100, OF 100  $\mu$ g/ml; OF 500, OF 500  $\mu$ g/ml. <sup>a</sup>Significant different compared with control group among groups. IL, interleukin; PBMCs, peripheral blood mononuclear cells; OF, oligo fucoidan.

Table III. Concentration of Th1 and Th2 cell cytokines in plasma and culture medium.

Concentration (pg/ml)	Asthma group (n=30)		Non-asthma group (n=15)	
	Control	OF 100	Control	OF 100
Plasma				
IL-4	0.15±0.36		0.18±0.32	
IFN- $\gamma$	0.1±0.09		0.11±0.08	
Culture medium				
IL-4	0.14±0.05	0.12±0.03	0.20±0.05	0.14±0.03 <sup>a,b</sup>
IFN- $\gamma$	2.56±0.20	2.71±0.34 <sup>a</sup>	2.62±0.09	2.85±0.06 <sup>a,b</sup>

<sup>a</sup>Significant different compared with control group among groups. <sup>b</sup>Significant different compared with different group. Th, helper T cell; IL, interleukin; OF, oligo fucoidan.

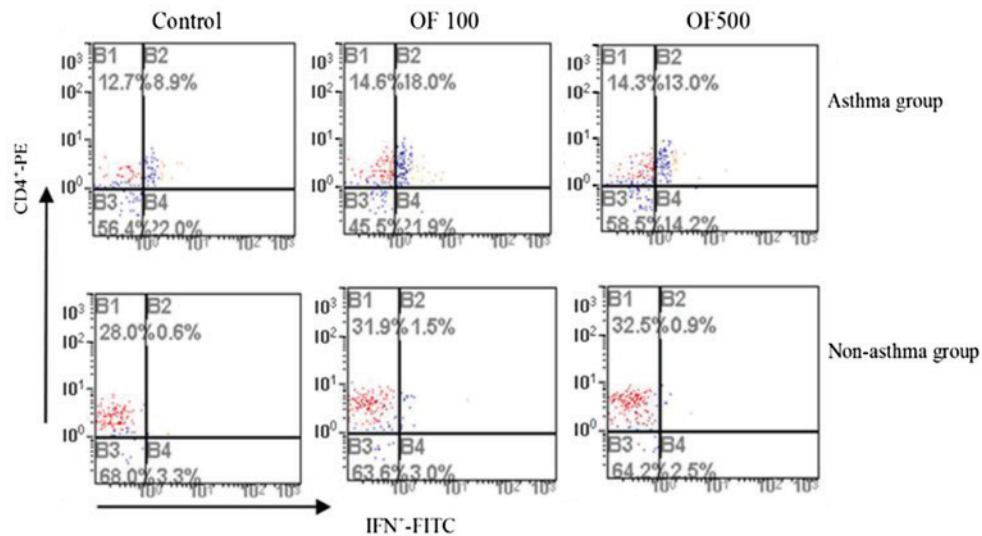


Figure 1. The level of CD4<sup>+</sup> IFN<sup>+</sup> cells after OF treatment of PBMCs. PBMCs from asthmatic and non-asthmatic subjects were stimulated and treated with or without OF (100 or 500  $\mu$ g/ml) for 48 h. The populations of CD4<sup>+</sup> IFN<sup>+</sup> cells were assayed by flow cytometry. Control, PBMCs not treated with OF; OF 100, PBMCs were treated with 100  $\mu$ g/ml OF for 48 h; OF 500, PBMCs were treated with 500  $\mu$ g/ml OF for 48 h. PBMCs, peripheral blood mononuclear cells; IL, interleukin; OF, oligo fucoidan.

the Non-asthma group (7.3±12.1 vs. 0.3±0.3;  $P<0.05$ ) (Table IV). Treatment with oligo-fucoidan decreased the proportions of CD4<sup>+</sup> IL-17<sup>+</sup> cells in both groups, but the difference were not

significant. There were also no significant differences between the two groups in the magnitude of the decrease in the proportion of CD4<sup>+</sup> IL-17<sup>+</sup> cells following stimulation (data not shown).



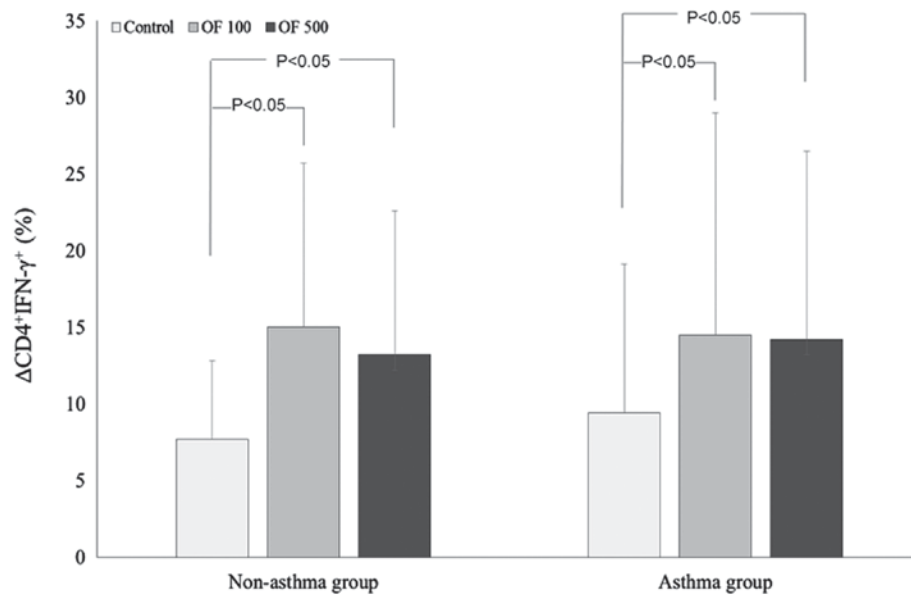


Figure 2. The degree of increasing of CD4<sup>+</sup> IFN<sup>+</sup> cells after OF treatment of PBMCs. PBMCs from asthmatic and non-asthmatic subjects were separated into unstimulated and stimulated groups. These cells were treated with or without OF (100 or 500  $\mu$ g/ml) for 48 h. The populations of CD4<sup>+</sup> IFN<sup>+</sup> cells were assayed by flow cytometry. Control, PBMCs not treated with OF; OF 100, PBMCs were treated with 100  $\mu$ g/ml OF for 48 h; OF 500, PBMCs were treated with 500  $\mu$ g/ml OF for 48 h.  $\Delta$ CD4<sup>+</sup> IFN<sup>+</sup>=the level of CD4<sup>+</sup> IFN<sup>+</sup> cells from stimulated cells minus the level of CD4<sup>+</sup> IFN<sup>+</sup> cells from unstimulated cells. OF, oligo fucoidan; PBMCs, peripheral blood mononuclear cells.

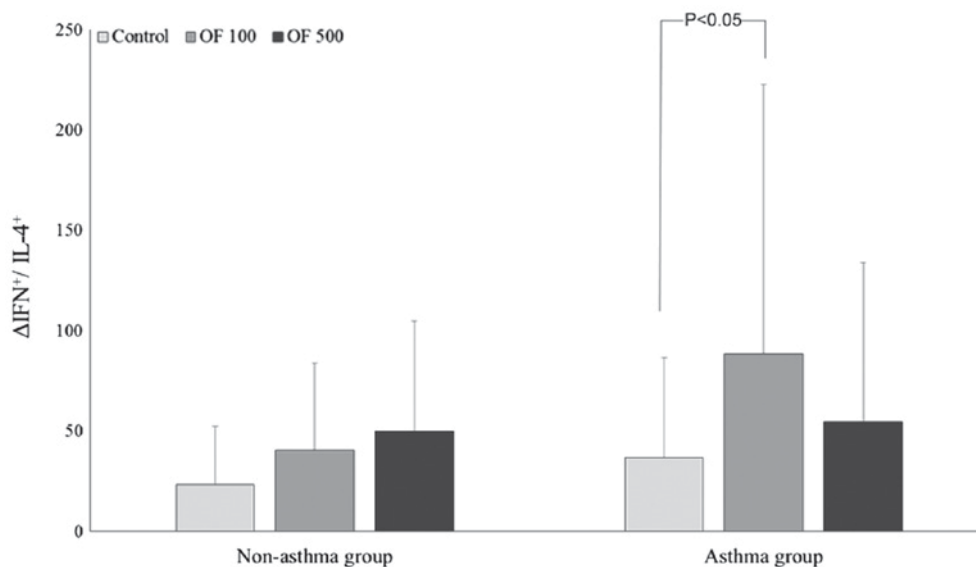


Figure 3. The degree of increasing of IFN<sup>+</sup>/IL-4<sup>+</sup> ratio after OF treatment of PBMCs. PBMCs from asthmatic and non-asthmatic subjects were separated into unstimulated and stimulated groups. These cells were treated with or without OF (100 or 500  $\mu$ g/ml) for 48 h. The populations of CD4<sup>+</sup> IFN<sup>+</sup> cells and CD4<sup>+</sup> IL-4<sup>+</sup> cells were assayed by flow cytometry. Control, PBMCs not treated with OF; OF 100, PBMCs were treated with 100  $\mu$ g/ml OF for 48 h; OF 500, PBMCs were treated with 500  $\mu$ g/ml OF for 48 h.  $\Delta$ IFN<sup>+</sup>/IL-4<sup>+</sup> ratio=the level of IFN<sup>+</sup>/IL-4<sup>+</sup> form stimulated cells minus the level of IFN<sup>+</sup>/IL-4<sup>+</sup> form unstimulated cells; OF, oligo fucoidan; PBMCs, peripheral blood mononuclear cells.

In asthmatic subjects, oligo-fucoidan treatment increased the proportion of Treg cells (CD4<sup>+</sup>Foxp3<sup>+</sup> cells) significantly (control: 41.1 $\pm$ 8.57%, OF100: 47.6 $\pm$ 7.4%, and OF500: 49.4 $\pm$ 7.4 (P<0.05) (Table IV).

Oligo-fucoidan treatment increased the ratio of Treg/Th17 (FOXP3<sup>+</sup>/IL-17<sup>+</sup>) in both the Asthma and Non-asthma groups (Table IV). However, there was no significant difference between the two regarding the degree of the Treg/Th17 ratio change following stimulation (data not shown).

*Concentration of Treg and Th17 cell cytokines in plasma and culture medium.* Plasma levels of IL-10 (a Treg cytokine) and IL-17 (a Th17 cytokine) were assayed using ELISA kits (Table V). There were no differences between the cytokine levels in the Asthma and Non-asthma groups. The levels of IL-10 and IL-17 from the culture medium of PBMCs from the patients were also assayed. The levels of IL-10 in Asthma group were higher than those in the Non-asthma group, but the difference was not significant. In addition, oligo-fucoidan increased

Table IV. The changes of Treg cell subtypes after different OF treated-PBMCs from two groups of subjects.

	Asthma group (n=30)			Non-asthma group (n=15)		
	Control <sup>1</sup>	OF 100 <sup>2</sup>	OF 500 <sup>3</sup>	Control	OF 100	OF 500
Proportions of cells (%)						
CD4 <sup>+</sup> IL-17 <sup>+</sup>	7.3±12.1	6.8±11.6	6.7±12.4	0.3±0.3 <sup>b</sup>	0.4±0.4 <sup>b</sup>	0.3±0.2 <sup>b</sup>
CD4 <sup>+</sup> FOXP3 <sup>+</sup>	41.13±8.7	47.6±7.4 <sup>a</sup>	49.4±7.4 <sup>a</sup>	38.4±15.4	45.6±13.8	47.0±14.4 <sup>a</sup>
FOXP3 <sup>+</sup> /IL-17 <sup>+</sup>	165.9±120.5	224.3±164.1	219.8±169.1	196.6±139.2	197.7±130.8	208.8±161.8

<sup>1</sup>Control, no treated OF; <sup>2</sup>OF 100, OF 100  $\mu$ g/ml; <sup>3</sup>OF 500, OF 500  $\mu$ g/ml. <sup>a</sup>Significant different compared with control group among groups.

<sup>b</sup>Significant different compared with different group. Tregs, regulatory T cells; OF, oligo fucoidan; PBMCs, peripheral blood mononuclear cells; IL, interleukin.

Table V. Concentration of Treg and Th17 cell cytokines in plasma and culture medium.

Concentration pg/ml	Asthma group (n=30)		Non-asthma group (n=15)	
	Control	OF 100	Control	OF 100
Plasma				
IL-10	0.11±0.11	3.17±0.12 <sup>a</sup>	0.18±0.30	2.92±0.07 <sup>a,b</sup>
IL-17A	0.15±0.31	3.01±0.21	0.30±0.60	3.04±0.12
Culture medium				
IL-10	2.04±0.57		1.89±0.53	
IL-17A	2.50±0.62		2.81±0.32 <sup>b</sup>	

<sup>a</sup>Significant different compared with control group among groups. <sup>b</sup>Significant different compared with different group. OF, oligo fucoidan; IL, interleukin; Tregs, regulatory T cells; Th, helper T cells.

the level of IL-10 in both the Asthma and Non-asthma groups ( $P<0.05$ ) (Table V). The levels of IL-17 in Asthma group were lower than those in Non-asthma group ( $P<0.05$ ). In addition, oligo-fucoidan more increased the level of IL-17 than control group in the Asthma and Non-asthma groups, but the difference was not significant.

## Discussion

The asthmatic immune response favors the production of Th2 cells over Th1 cells. Th1 cells may inhibit Th2 cell differentiation, effectively reducing the Th2-induced asthmatic response (26). However, excessive IFN- $\gamma$  release by Th1 cells can also cause airway inflammation (27). Therefore, an increase in the proportion of Th1 cells during allergic asthma is not necessarily a good phenomenon. In this study, we found that the proportion of Th1 cells and the level of IFN- $\gamma$  were significantly lower in asthmatic subjects than in non-asthmatic subjects (Tables II and III), and that treatment with oligo-fucoidan caused an increase in the proportion of Th1 cells and in the level of IFN- $\gamma$  in asthmatic subjects. However, there was no significant difference in the degree to which stimulating the cells increased in the proportion of Th1 cells and IFN- $\gamma$  levels between the Asthma and Non-asthma groups (Fig. 2). This suggests that oligo-fucoidan treatment might not overmuch increase the Th1 immune response, which

causes airway inflammation. Oligo-fucoidan treatment also increased the Th1/Th2 ratio, suggesting that it might reduce airway inflammation.

Because Th2 cells mediate airway inflammatory responses in allergic asthma, some drugs that reduce the number of Th2 cells, such as corticosteroids, are used to control airway inflammation (13,28). In this study, the asthmatic subjects all use corticosteroids to successfully control their asthmatic symptoms (Table I). There were no differences in the proportion of Th2 cells or IL-4 levels between asthmatic patients and healthy subjects that might be caused by these drugs. However, it is worth noting that oligo-fucoidan reduced the proportion of Th2 cells in unstimulated T cells. When the T cells of asthma patients were stimulated, oligo-fucoidan treatment did not effectively reduce the proportion of Th2 cells; however, the proportion of Th2 cells did not increase. This, may be due to the increased proportion of Th1 cells, which may reduce the Th2 cells-mediated airway inflammation. Although the inflammatory response in allergic asthma is predominantly Th2-mediated, Th17 cells also cause airway inflammation through IL-17A/F and IL-22 production and play an important role in the pathology of asthma (29). Th17 cells and the cytokines produced by Th17 cells increase airway inflammation through neutrophil and eosinophil activation (30-33). In this study, we found a larger population of Th17 cells in asthmatic subjects than in non-asthmatic subjects (Table IV) ( $P<0.05$ ). In addition, oligo-fucoidan treatment had

a tendency to decrease the proportion of Th17 cells in both groups.

Human Treg cells not only express CD25 on their cell surface, but also express the forkhead box P3 (Foxp3) transcription factor and are important in the establishment and maintenance of an immunosuppressive response (34-36). Treg cells can suppress other T cell subtypes by producing TGF- $\beta$  and IL-10 and function of Treg cells may be reduced in asthma (37). In this study, we found that oligo-fucoidan treatment significantly increased the proportion of Treg cells and the IL-10 levels (Tables IV and V) in asthmatic subjects. It is now known that Treg cells reduce T cell-induced inflammation by producing IL-10. However, the detailed mechanism of inflammation inhibition in Treg cell-mediated allergic asthma remain unclear.

Recent studies indicate that fucoidan can improve allergic responses by regulating the immune response, including alterations in the Th1/Th2 balance, inhibiting the production of IgE, suppressing the degranulation of mast cells, and inhibiting the proliferation of ASM (23). At present, little has been done to explore on the use of fucoidan in allergic diseases. This study was the first to characterize the immunomodulatory effects of oligo-fucoidan in the cells of asthmatic patients and emphasized its potential mechanism as a pharmaceutical and nutraceutical for the adjuvant therapy of allergic asthma. In this study, we compared the CD4<sup>+</sup> T cell profiles of asthmatic and non-asthmatic subjects and assessed the effects of oligo-fucoidan treatment on these profiles. Our results showed that oligo-fucoidan treatment could increase the ratios of Th1/Th2 and Treg/Th17 cells. Oligo-fucoidan might act on one or more of membrane receptors found on the immune cells include macrophages, dendritic cells to activate T cells toward Th 1 differentiation and producing IFN or toward Treg differentiation and producing IL-10. The mechanism which Oligo-fucoidan affects the differentiation of T cells is still unclear. Oligo-fucoidan might, like  $\beta$ -glucan, act on one or more of membrane receptors found on the immune cells including macrophages, dendritic cells to activate T cells and induce Th 1 differentiation and producing IFN or influence Treg differentiation and producing IL-10.

There are some studies which indicate that non oligo-fucoidan significantly reduces IgE production by B cells in PBMCs *in vitro* and *in vivo* (38-40). In this present study, we found that oligo-fucoidan significantly increased IFN- $\gamma$  production in PBMCs of asthmatic patients but did not affect IL-4 production. Our results suggest that oligo-fucoidan treatment has beneficial effects for allergic airway inflammation.

CD44 is a cell surface glycoprotein and the two main ligands for CD44 are hyaluronan (HA) and osteopontin (OPN) (41,42). On surface of the T cells, there is more expression of CD44 on activated T cells than naïve T cells (43). In allergic asthma, Th2 cells infiltrate the ASM layer and may cause ASMCs proliferation and inflammation. *In vitro* studies have shown that T cells adhere to ASMCs via CD44 to induce ASMCs proliferation (44). However, this study did not indicate what subtype of T cells to cause this phenomenon. In the other study indicated CD44 can inhibit cell death regulated by Fas (45). However, other study also indicated CD44 only regulates the survival of Th1 cells, but has no effect on Th2 or Th17 cells. Treatment of Th1 cells with CD44 agonist antibodies has been shown to increase cell survival by activating downstream PI3K-Akt pathways (46). In our study also found treatment with oligo-fucoidan caused an

increase in the proportion of Th1 cells and in the level of IFN- $\gamma$  in asthmatic subjects, but had no effect on Th2 or Th17 cells. In our previous study, we also found that oligo-fucoidan might reduce the proliferation of ASM cells. CD44 also expressed on the surface of smooth muscle cells. The role of oligo-fucoidan in CD44 of coculture Th1 cells and ASMCs remains to be clarified and this is also one of the focuses of future research. Some data do not show statistically significant differences due to the small sample size of the study. This *in vitro* experiment is preliminary to confirm the possibility of oligo-fucoidan for adjuvant treatment of asthma, and future research will be directed toward the role of oligo-fucoidan in conventional asthma therapy.

Oligo-fucoidan treatment can increase the ratio of Th1/Th2 and Treg/Th17 cells to ameliorate the imbalance in the T cell profile and could be a potential adjuvant therapy for allergic asthma. These results will provide the basis for future animal experiments and human trials.

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### Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author.

### Authors' contributions

All authors contributed extensively to the work presented in this paper. W-SK, C-HY and Y-LC designed the experiment. J-JT and C-JS performed the experiments. W-SK, C-HY, Y-LC and J-JT analyzed the data and prepared the manuscript.

### Ethics approval and consent to participate

Written informed consent was obtained from all patients for the present study, which was approved by IRB of Kuang-Tien General Hospital (approval no. 10457).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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