

Eucheuma cottonii Sulfated Oligosaccharides Decrease Food Allergic Responses in Animal Models by Up-regulating Regulatory T (Treg) Cells

Sha-Sha Xu,^{†,||} Qing-Mei Liu,^{†,||} An-Feng Xiao,[†] Soheila J. Maleki,[§] Marcos Alcocer,[#] Yuan-Yuan Gao,[†] Min-Jie Cao,[†] and Guang-Ming Liu^{*,†,||}

[†]College of Food and Biological Engineering, Xiamen Key Laboratory of Marine Functional Food, Fujian Provincial Engineering Technology Research Center of Marine Functional Food, Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Jimei University, 43 Yindou Road, Xiamen 361021, Fujian, People's Republic of China

[§]Southern Regional Research Center, U.S. Department of Agriculture, Agriculture Research Service, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70124, United States;

[#]School of Biosciences, The University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom

ABSTRACT: In the present study, the anti-food allergy activity of *Eucheuma cottonii* sulfated oligosaccharide (ESO) was investigated. ESO was obtained by enzymatic degradation and purified by column chromatography. RBL-2H3 cells and BALB/c mouse model were used to test the anti-food allergy activity of ESO. The effects of ESO on the regulatory T (Treg) cells and bone marrow-derived mast cells (BMMCs) were investigated by flow cytometry. The results of in vivo assay showed that ESO decreased the levels of mast cell protease-1 and histamine and inhibited the levels of specific IgE by 77.7%. In addition, the production of interleukin (IL)-4 and IL-13 was diminished in the ESO groups compared to the non-ESO-treated group. Furthermore, ESO could up-regulate Treg cells by 22.2–97.1%. In conclusion, ESO decreased the allergy response in mice by reducing basophil degranulation, up-regulating Treg cells via Forkhead box protein 3 (Foxp3), and releasing IL-10. ESO may have preventive and therapeutic potential in allergic disease.

KEYWORDS: *Eucheuma cottonii* sulfated oligosaccharide (ESO), food allergy, murine model, mast cells, Treg cells

INTRODUCTION

Food allergy is a common, serious, and growing problem that affects nearly 5% of adults and 8% of children worldwide.¹ It has long been known that particular foods, namely, peanuts, milk, shellfish, wheat, and tree nuts, are able to trigger food allergies.² In China, 16.7% of the rural population is sensitized to shellfish or its derivatives.³ Tropomyosin (TM) was reported as the major sensitizing allergen in shellfish^{4,5} and has been widely chosen as the allergen to establish a food allergy model involving Chinese human studies.^{6–8}

Food allergy is an immunoglobulin E (IgE)-dependent type I hypersensitivity reaction due to the imbalance of Th1/Th2.⁹ Interleukin (IL)-4 and IL-13 are responsible for class switching in B cells, which results in the production of allergen-specific IgE antibodies.¹⁰ Allergic reactions to foods are due to the interaction of allergen-specific IgE antibody with its high-affinity receptor (FcεRI) on mast cells. When a specific antigen binds to the IgE linked to the FcεRI, it establishes receptor cross-linking and consequent release of mediators.¹¹

Mast cells, basophils, and Treg cells are important immune cells in the food allergy reaction.⁹ Recently it was reported that apoptosis of mast cells could alleviate food allergy symptoms.¹² In addition, Treg cells play an important role in the inhibition of immune responses by releasing IL-10 and transforming growth factor (TGF)-β that could contribute to the suppression.¹³ Improved preventive and therapeutic strategies to food allergy currently under study include some foods that

possibly have immunomodulatory effects and naturally bioactive substances.^{14,15}

Marine algae have recently received much attention for their valuable diverse biological activities, among them antioxidant, immunoregulatory, and antiallergic activities.^{16,17} Our previous study reported that sulfated polysaccharides from *Porphyra haitanensis* and *Gracilaria lemaneiformis* attenuated allergic symptoms in TM-allergic mice.^{7,8} Yoshida et al. showed that alginic acid oligosaccharide suppresses the development of Th2 and production of IgE. Furthermore, T cells responding to β-lactoglobulin were reduced by conjugation with acidic oligosaccharides.^{18,19} Uno et al. confirmed that oral administration of alginic acid oligosaccharide suppresses production of IgE.²⁰

The Rhodophyta *Eucheuma cottonii* is a major source of carrageenan, which exists as a sulfated polysaccharide containing galactose.²¹ It has shown a similar monosaccharide composition to sulfated polysaccharides from *P. haitanensis* and *G. lemaneiformis*, which were reported to have anti-food allergy activities.^{7,8} Furthermore, the extraction of *E. cottonii* was reported to have several biological activities, including anti-tumor, antiviral, and immunomodulatory activities.^{22,23} Polysaccharides obtained from *E. cottonii*, according to the methods

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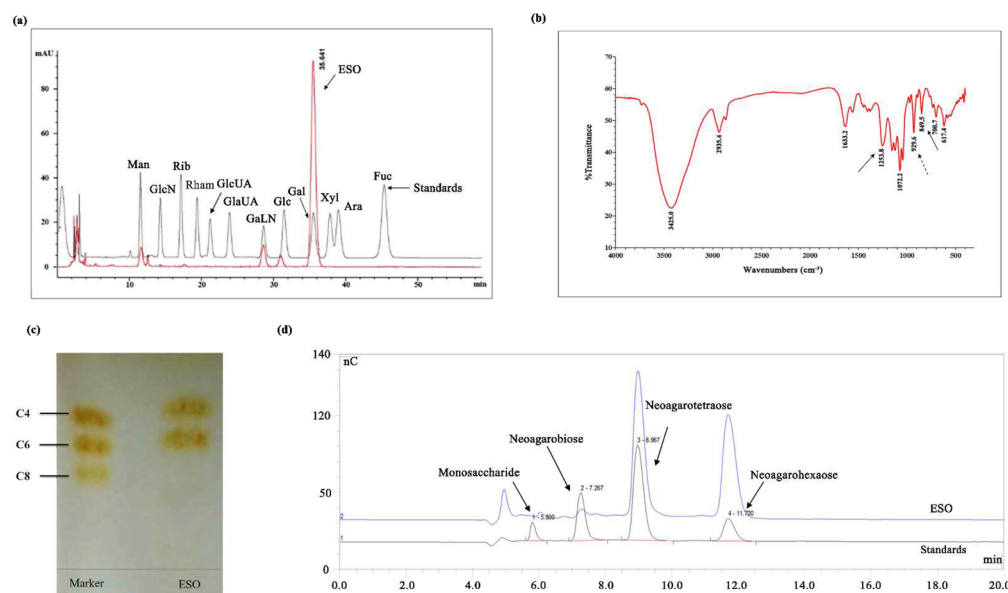


Figure 1. Structural analysis of the purified ESO by IC, TLC, and FTIR. (a) Monosaccharide composition of ESO by IC analysis (red line). The gray line represents the sugar standards, from left to right: mannose (Man), glucosamine (GlcN), ribose (Rib), rhamnose (Rham), glucuronic acid (GlcUA), galacturonic acid (GlaUA), galactosamine (GalN), glucose (Glc), galactose (Gal), xylose (Xyl), arabinose (Ara), and fucoidan (Fuc). (b) FTIR spectra of ESO between the wavelengths of 4000 and 500 cm^{-1} . The solid arrow represents the absorption bands at 1253 and 849 cm^{-1} of the sulfate group, and the dashed arrows show the absorption bands at 929 cm^{-1} of 3,6-anhydrogalactose. (c) TLC of ESO. The standards were neoagarotetraose (C4), neoagarohexaose (C6), and neoagaroctaoose (C8). (d) ESO composition, mainly neoagarotetraose and neoagarohexaose, determined by IC. The standards were monosaccharide, neoagarobiose, neoagarotetraose, and neoagarohexaose.

of Shi and Liu,^{7,8} were unable to evaluate the antifood allergy activity due to the poor solubility. Mou et al. reported that κ -carrageenan oligosaccharide derived from *E. cottonii* and prepared by enzymatic degradation had antioxidation capability and was beneficial for immunological regulation.²³ κ -Carrageenase was isolated in our laboratory and described in preliminary work.²⁴ In this work, the anti-food allergic activity of ESO obtained by κ -carrageenase enzymatic degradation was monitored using the rat basophilic cell line RBL-2H3 and mouse model of crab-TM allergy. Furthermore, the effects of ESO on CD4⁺Foxp3⁺ Treg cells in mice and bone marrow-derived mast cells (BMMCs) were evaluated.

MATERIALS AND METHODS

Reagents. Inject alum (Thermo Fisher Scientific Inc., Waltham, MA, USA); RPMI 1640, minimum essential medium with Eagle's salts (EMEM), and fetal bovine serum (FBS) (Hyclone, Logan, UT, USA); penicillin, streptomycin solution (Invitrogen, Grand Island, NY, USA); anti-dinitrophenyl (DNP)-IgE (Sigma, St. Louis, MO, USA); DNP-BSA (Biosearch, Petaluma, CA, USA); goat anti-mouse IgE, IgG1, IgG2a antibodies (Abcam Cambridge, UK); ELISA kit of histamine (IBL, Hamburg, Germany); and ELISA kits of IL-4, IL-13, IL-10, interferon (IFN)- γ and mouse mast cell proteinase (mMCP)-1 (eBioscience, San Diego, CA, USA) were obtained from the suppliers noted.

Purification and Chemical Analysis of Oligosaccharides from *E. cottonii*. Seaweed *E. cottonii* powder was kindly provided by Greenfresh (Fujian) Foodstuff Co. Ltd. The powder was treated with κ -carrageenase, at 40 $^{\circ}\text{C}$ for 4 h, which was produced by fermenting the marine bacterium *Pseudoalteromonas* sp. ASYS according to the method of Xiao.²⁴ Then the solution was centrifuged (7500g, 15 min) and the supernatant collected. The crude oligosaccharide was obtained after freeze-drying of the supernatant, which was further dissolved in distilled water and applied to an ultrafiltration membrane (5 kDa cut off), a DEAE-cellulose S2 column (3.5 $\times$ 50 cm) (Whatman, New York, USA), and Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, IL, USA) according to

the method of Shi with some modification.⁷ The lipopolysaccharide content of ESO was measured by using the limulus amoebocyte lysate assay (Associates of Cape Cod, New York, USA). The contents of carbohydrate, 3,6-anhydrogalactose (3,6-AG), and sulfate were determined as described by Shi.⁷ The monosaccharide composition of ESO was determined, after acid hydrolysis of the oligosaccharides, by ion chromatography (IC) analysis (ICS-3000, Dionex, Sunnyvale, CA, USA) via a CarboPac PA20 column (3 $\times$ 150 mm^2), using 0.25 M NaOH as the eluent at a flow rate of 0.5 mL/min. The infrared spectrum of the oligosaccharide fraction was measured by using a Fourier transform infrared spectrometer (FTIR) (VECTOR-22, BRUKER, Ettlingen, Germany) in the wavenumber range of 4000–500 cm^{-1} using the KBr disk method.

TLC analysis of the ESO was performed including several basic steps, as follows: (1) standards were the mixture of tetraose, hexaose, octasaccharide (1:1:1, w/w/w), which along with ESO were dissolved in pure water at final concentrations of 10 mg/mL; (2) solutions of the standards mixture and ESO (0.5 μL of each) were loaded onto a precoated silica gel plate (Ding Kang silica gel, Qingdao, China) and developed with a solvent system consisting of *n*-butanol/acetic acid/water (2:2:1, v/v/v); (3) the developed plate was stained by dipping in anhydrous sulfuric acid/ethanol (1:10 to 1:15, v/v) reagent for 5 s and heating at 150 $^{\circ}\text{C}$ for 10 s.²⁵

To further confirm the composition of ESO, the standards (mixture of monosaccharide, neoagarobiose, neoagarotetraose, and neoagarohexaose) were dissolved in ultrapure water, and the concentration was 20 $\mu\text{g}/\text{mL}$. The standards and ESO were analyzed by ion chromatography (IC) analysis (ICS-3000) via a Dionex CarboPac PA 100 anion-exchange column (4 $\times$ 250 mm^2). The eluent were 0.1 mol NaOH and 0.15 mol/L NaAc with a flow rate of 0.25 mL/min.

Protocol of the Food Allergy Mouse Model. Specific pathogen-free (SPF) female BALB/c mice (4–6 weeks of age) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Mice were housed in an SPF environment maintained at 22 ± 1 $^{\circ}\text{C}$ with a relative humidity of $55 \pm 10\%$. All experiments were performed under SPF conditions. The experimental protocols were approved by the local ethics committee

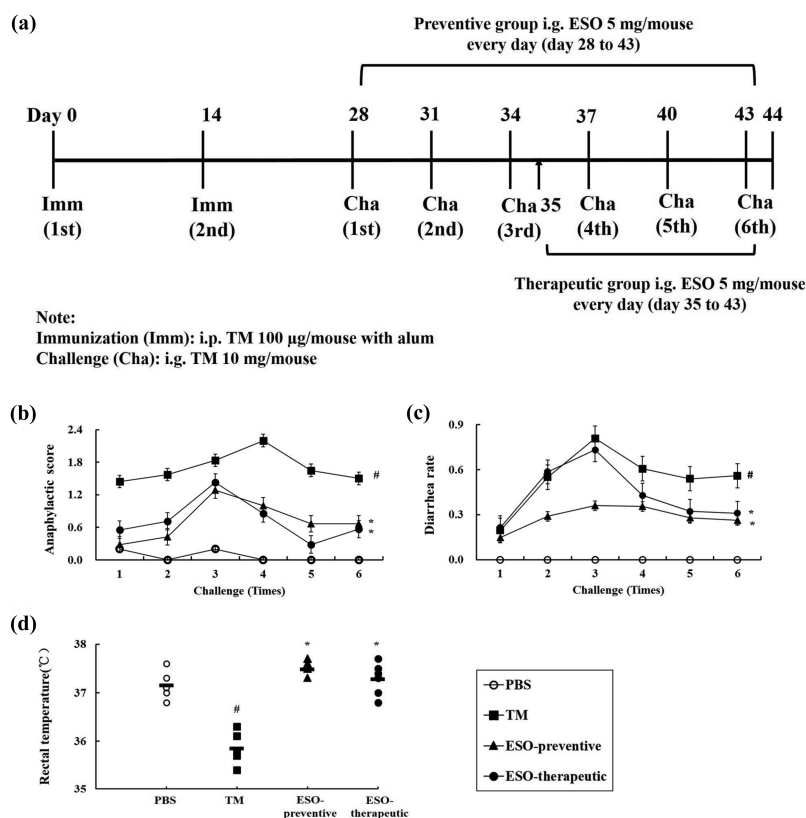


Figure 2. ESO attenuated food allergy symptoms in a TM-induced mouse food allergy model. (a) Groups of female BALB/c mice (4–6 weeks age, six mice in each group) were immunized with 100 µg of TM adsorbed in 0.5 mg/mL alum by intraperitoneal injection on days 0 and 14. Starting at day 28, the immunized mice were orally challenged with 10 mg of TM in PBS every 3 days, for six doses. Mice were treated by intragastric administration with 5 mg of ESO every day from days 28 to 43 (regarded as ESO-preventive group) or from days 35 to 43 (regarded as ESO-therapeutic group). (b) Symptom scores of anaphylactic response for 1 h after challenge with TM. (c) Diarrhea of TM-induced mice (%) 1 h after each TM challenge. (d) Rectal temperature of mice 1 h after the sixth challenge with TM. i.p., intraperitoneal injection; i.g., intragastrically; TM, tropomyosin. Each value is presented as the mean ± SD. Statistical differences are indicated by *P* values (one-way ANOVA). (*) *P* < 0.05 and (**) *P* < 0.01, compared with the TM-treated group. (#) *P* < 0.01, compared with the PBS group.

following the regulations for the treatment of live animals at Jimei University (Xiamen, China), SCXK 2012-0005.

TM was purified from *Scylla paramamosain* as previously described.⁴ Mice were sensitized and monitored according to the method of Liu with some modification.⁸ In brief, four groups (phosphate buffer saline (PBS) group, TM group, ESO-preventive group (pretreated with ESO), and ESO-therapeutic group (sensitized with TM and then treated with ESO)) of mouse experiments were established to investigate the anti-food allergic activity of ESO. Twenty-four mice were divided into the four groups by randomization (six mice in each group). For the sensitization, mice were immunized with 100 µg of TM in 150 µL of PBS emulsified with inject alum by intraperitoneal injection on days 0 and 14. Then mice were challenged six times from days 28 to 43 by intragastric administration with 10 mg of TM in 200 µL of PBS every 3 days to establish the food allergy model. ESO (5 mg/mouse in 200 µL of PBS) was intragastrically administered daily from days 28 to 43 (the ESO-preventive group), and ESO (5 mg/mouse in 200 µL of PBS) intake daily in mice already challenged with TM three times (from days 35 to 43) to evaluate the therapeutic effect of ESO on food allergy (the ESO-therapeutic group). Meanwhile, the negative control mice (PBS group) were immunized with 150 µL of PBS emulsified with inject alum at days 0 and 14 and intragastrically given 200 µL of PBS at days 28, 31, 34, 37, 40, and 43.

Anaphylactic Symptoms and Measurement of Immunoglobulins (IgE/IgG1/IgG2a), Histamine, and mMCP-1 Levels in Mouse Serum. The anaphylactic symptoms were scored as follows: 0, no symptom; 1, reduced activity, trembling of limbs; 2, loss of consciousness, no activity upon prodding; 3, convulsions; 4, death.²⁶

Diarrhea rates (the proportion of diarrheal mice in each group) were measured/calculated 1 h after each challenge. Rectal temperatures were measured <1 h after the sixth challenge using a rectal electronic thermometer (Prosper, Shanghai, China). The concentrations of histamine and mMCP-1 were determined by ELISA kits according to the manufacturer's instructions.

The concentrations of IgE, IgG1, and IgG2a were determined by indirect ELISA according to the method of Shi.⁷ Briefly, mouse sera of four groups (diluted 1:5) were taken as the first antibody. Goat anti-mouse IgE–HRP (diluted 1:1000), goat anti-mouse IgG1 heavy chain (HRP) (diluted 1:5000), and goat polyclonal secondary antibody to mouse IgG2a-heavy chain (HRP) (diluted 1:5000) were secondary antibodies. Furthermore, Western blot analysis with TM-specific serum from mice was performed as described by Yamaki.²⁷ Briefly, proteins (TM) were resolved by SDS-PAGE and transferred to a nitrocellulose filter membrane. Mouse serum (diluted 1:5) was taken as the first antibody, and anti-IgG1, anti-IgE, and anti-IgG2a (diluted 1:1000) were the secondary antibody.

Splenic Lymphocytes and Mesenteric Lymph Node Cell (MLN) Restimulation for Cytokine Measurement and Real-Time Quantitative PCR (qPCR) of Transcription Factors. Splenic lymphocytes and MLNs were prepared by aseptic removal. Splenic lymphocytes were obtained using a nylon mesh filter and mouse lymphocyte separation medium (Da Ke Wei, Shenzhen, China) by density gradient centrifugation, providing lymphocytes including bits of dendritic cells. Numbers of splenic lymphocytes and MLNs were adjusted to a cell density of 2×10^5 cells/mL in RPMI 1640 medium with 10% FBS and 1% penicillin–streptomycin solution at day 44. Splenic lymphocytes and MLNs were then cultured and restimulated

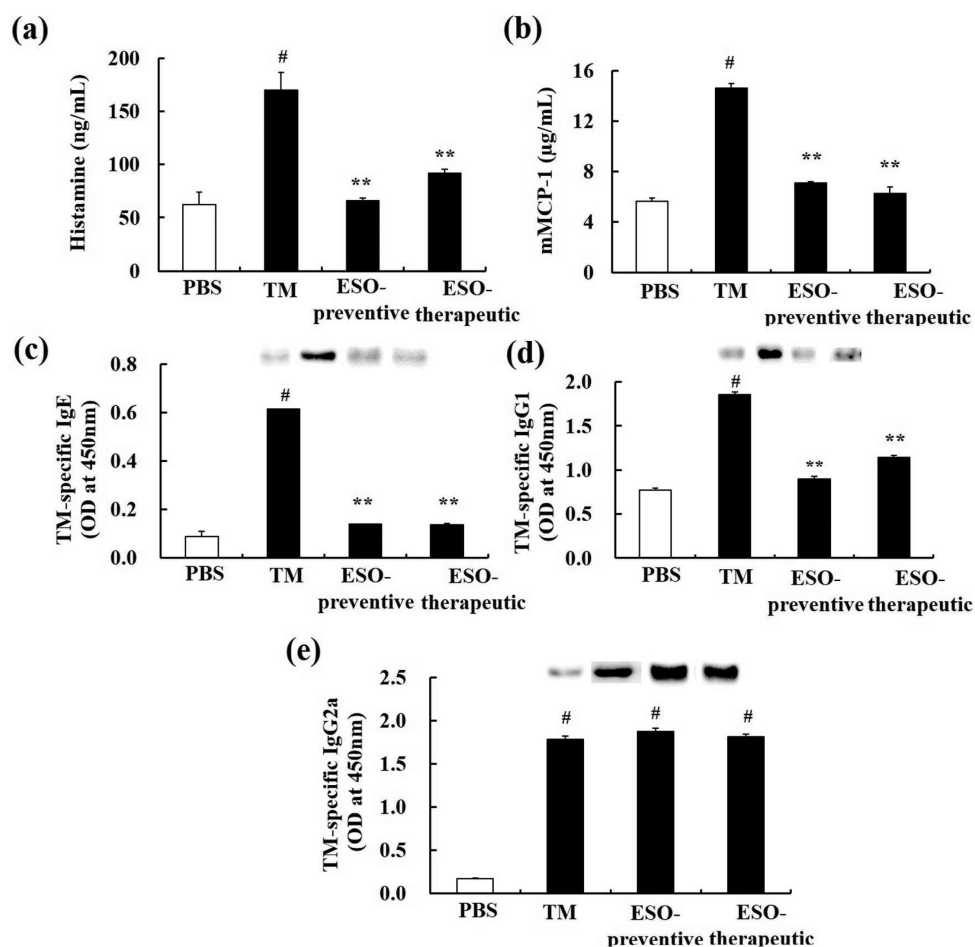


Figure 3. Effects of ESO on histamine, mMCP-1, and TM-specific antibodies in serum of TM allergic mice. To measure levels of histamine, mMCP-1, and immunoglobulin, serum samples from each group were obtained 1 h after the last challenge. (a–e) The levels of histamine, mMCP-1, TM-specific antibodies IgE, IgG1, and IgG2a were measured by ELISA, and the OD at 450 nm (y-axis) for each group (x-axis) is shown. (c–e) The levels of TM-specific antibodies IgE, IgG1, and IgG2a were measured by Western blot. All experiments were repeated three times. Statistical differences are indicated by *P* values (one-way ANOVA). (*) *P* < 0.05 and (**) *P* < 0.01, compared with the TM-treated group. (#) *P* < 0.01, compared with the PBS group.

with the antigen TM (10 μg/mL) and negative control with PBS for 3 days in a humidified incubator with 5% CO₂.

The cell supernatants were collected to measure the concentrations of IL-4, IL-13, IFN-γ, and IL-10 using ELISA kits following the manufacturer's instructions. The cells were collected for mRNA preparation and qPCR of transcription factors according to Liu.⁸ Briefly, a real-time PCR was performed on the cDNA by using the SYBR Green PCR Master Mix (Tiangen, Beijing, China) according to the manufacturer's instructions. The housekeeping gene β-actin was used to normalize the values obtained for all transcripts under examination. The program of the thermal cycler was 15 min at 95 °C and 40 times a cycle of 10 s at 95 °C and 30 s at 60 °C (Applied Biosystem 7300).

RBL-2H3 Assay. The rat basophil cell line (RBL-2H3) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RBL-2H3 cells were grown in minimum essential medium with Eagle's salts (EMEM) and supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator with a 5% CO₂/95% air atmosphere. β-Hexosaminidase and histamine released by RBL-2H3 cells were measured using the model of the IgE-mediated mast cell allergic reaction, which was sensitized by anti-DNP-IgE and stimulated by DNP-BSA.^{8,28} Briefly, different concentrations of ESO (25–100 μg/mL) and PBS as control were added to the culture medium of anti-DNP-IgE (1 μg/mL) sensitized RBL-2H3 cells for 1 h, then stimulated with DNP-BSA (1 μg/mL) for 15 min (histamine),

4 h (IL-4), or 6 h (β-hexosaminidase, TNF-α), which were evaluated as described by Liu.⁸

Flow Cytometry Assay. Splenic lymphocytes and MLNs (2 × 10⁵ cells/well) were isolated from mice in the food allergy model at day 44 and analyzed by FACS.

BMMCs were generated as described by Kim.¹² Bone marrow cells from the thighbone of BALB/c mice were cultured in RPMI 1640 medium in the presence of mIL-3 (10 ng/mL; R&D Systems, Minneapolis, MN, USA) and mSCF (stem cell factor; 50 ng/mL, R&D Systems) for 2–4 weeks, and the differentiated BMMCs (c-kit⁺FcεRI⁺) were sorted using FACS.

The unactivated BMMCs were incubated with ESO (100 μg/mL) for 7 h and then analyzed by FACS. To detect the influences of ESO on the TM-activated BMMCs. Cells were sensitized with TM-specific IgE for 16 h. ESO was added to the medium at a final concentration of 100 μg/mL for 1 h, followed by stimulation with 10 μg/mL TM for 6 h, and then BMMCs were analyzed by FACS.

To analyze cell surface markers by flow cytometry, cells were washed and stained for 30 min at 4 °C with the following monoclonal antibodies according to the manufacturer's protocols (Biollegend Pharmingen, San Diego, CA, USA): PerCP-Cy5.5-conjugated anti-mouse CD4, PE-conjugated anti-mouse c-kit, APC-conjugated anti-mouse FcεRIα. Staining for intracellular Foxp3 was performed according to the manufacturer's instructions using the Foxp3 staining buffer set (eBioscience, San Diego, CA, USA) and Alexa Fluor647-conjugated anti-mouse Foxp3 (Biollegend). Cells were then washed with PBS-BSA and permeabilized

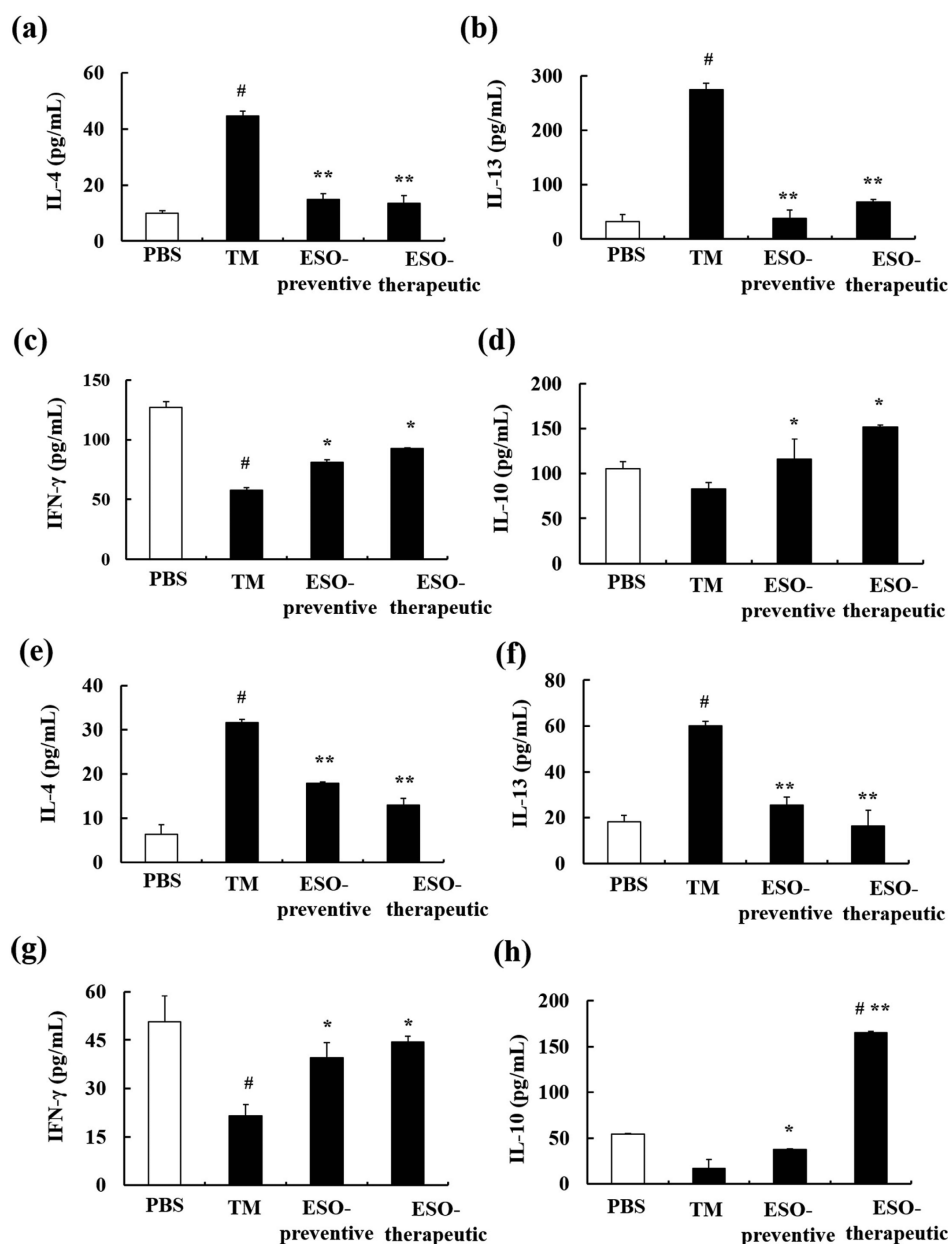


Figure 4. Immunomodulatory effects of ESO on cytokine patterns in splenic lymphocytes and MLN lymphocytes in mice. Mice were sacrificed by cervical dislocation on day 44, and lymphocytes isolated from the spleen and mesenteric lymph node (MLN), respectively, were cultured in the presence of TM (10 $\mu\text{g/mL}$) for 72 h. The secretion of cytokines (IL-4, IL-13, IFN- γ , and IL-10), from cell culture supernatants was measured by ELISA, respectively. (a–d) Levels of IL-4, IL-13, IFN- γ , and IL-10 secreted from splenic lymphocytes; (e–h) levels of IL-4, IL-13, IFN- γ , and IL-10 secreted from MLN lymphocytes. Statistical differences are indicated by *P* values (one-way ANOVA). (*) *P* < 0.05 and (**) *P* < 0.01, compared with the TM-treated group. (#) *P* < 0.01, compared with the PBS group.

for 1 h in fixation/permeabilization working solution (eBioscience). Then, cells were washed and stained for 45 min at 4 °C with the anti-mouse Foxp3 in permeabilization working solution. All FACS experiments were performed with a Guava easyCyte 6-2L system and analyzed with GuavaSoft 3.1.1 software (Millipore, Billerica, MA, USA).

Statistical Analysis. Data are presented as means $\pm$ SD. Differences between means were analyzed by ANOVA of the Duncan test. A *P* < 0.05 was considered statistically significant. In the animal model study, samples from individual mice were processed and analyzed separately. Each experiment was repeated at least three times.

RESULTS

Analysis of the Composition of ESO. The carbohydrate, 3,6-anhydrogalactose, and sulfate contents of pure ESO were

98.88, 5.37, and 18.17%, respectively. The results of the limulus amoebocyte lysate assay showed that the lipopolysaccharide content of ESO was <0.03 EU/mg. The results of the IC diagram for ESO are shown in Figure 1a and suggest that the main monosaccharide of ESO was galactose and its content was about 86.58%. The FTIR spectrum of the sample is shown in Figure 1b. ESO exhibited a characteristic broad stretching vibration peak at around 3380 cm^{-1} for –OH, a weak stretching region around 2935 cm^{-1} for C–H,²⁹ and intense absorption peaks around 1072 cm^{-1} for C–O. The solid arrow represents the absorption bands at 1253 and 849 cm^{-1} for the sulfate group, which were also present in a previously reported IR spectrum.⁹ The dashed arrows showed the absorption band at 929 cm^{-1} of

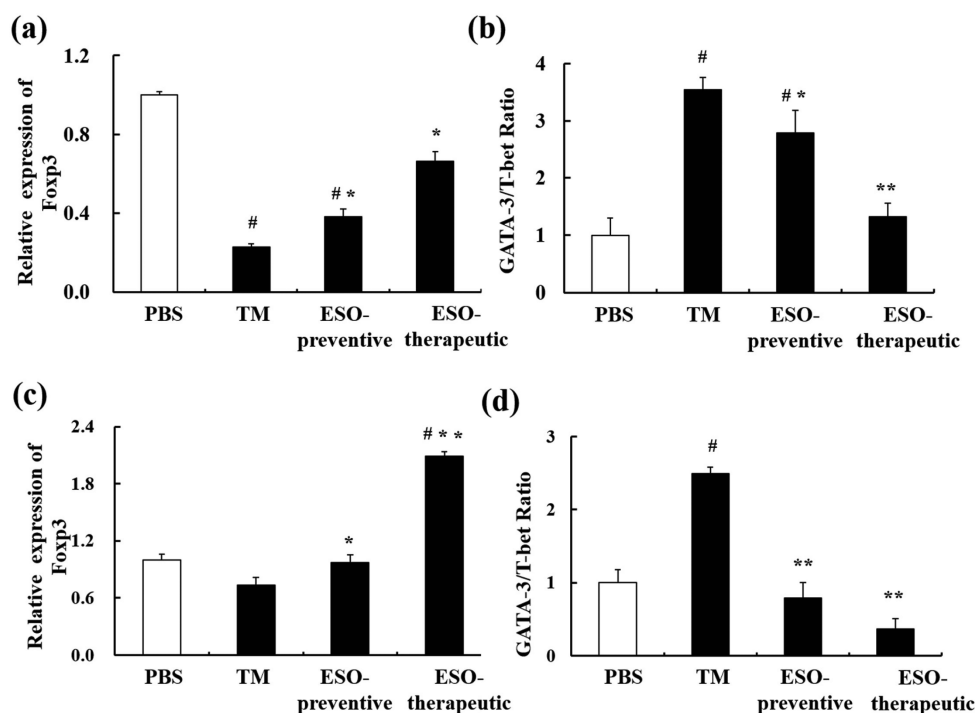


Figure 5. mRNA of transcription factors in splenic lymphocytes and MLN lymphocytes in mice. Splenic lymphocytes and MLN lymphocytes obtained from mice at day 44 were cultured with TM (10 $\mu\text{g/mL}$) for 72 h (2×10^5 cells/well). The transcription factors (Foxp3, GATA-3, and T-bet) were detected by qPCR. QPCR was performed with the following primers: β -actin (5'-AGC TGC GTT TTA CAC CCT TT-3', 5'-AAG CCA TGC CAA TGT TGT CT-3'); T-bet (5'-CCC CTG TCC AGT CAG TAA CTT-3', 5'-CTT CTC TGT TTG GCT GGC T-3'); GATA-3 (5'-AGG AGT CTC CAA GTG TGC GAA-3', 5'-TTG GAA TGC AGA CAC CAC CT-3'); Foxp3 (5'-CTC ATG ATA GTG CCT GTG TCC TCA A-3', 5'-AGG GCC AGC ATA GGT GCA AG-3'). (a) Relative expression of Foxp3 from splenic lymphocytes; (b) GATA-3/T-bet ratio of splenic lymphocytes; (c) relative expression of Foxp3 from MLN lymphocytes; (d) GATA-3/T-bet ratio of MLN lymphocytes. Statistical differences are indicated by *P* values (one-way ANOVA). (*) *P* < 0.05 and (**) *P* < 0.01, compared with the TM-treated group. (#) *P* < 0.01, compared with the PBS group.

3,6-anhydrogalactose.³⁰ (Figure 1b). The results of TLC and IC show that ESO is composed of tetroses and hexoses, 45.67 and 40.16%, respectively. (Figure 1c).

Preventive and Therapeutic Effects of ESO on TM-Induced Mice. To monitor the possible preventive and therapeutic effects of ESO on food allergy in vivo. An experimental schedule of food allergy induced by TM in a mouse model was followed and is shown in Figure 2a. The two ESO-treated groups decreased in the anaphylactic response scores (Figure 2b) and diarrhea rates (Figure 2c) compared to the TM group. At the same time, Figure 2d shows that the average rectal temperature of ESO-preventive (37.5 °C) and ESO-therapeutic (37.3 °C) groups was higher by 1.5–1.7 °C than the TM group (35.8 °C). In addition, the concentrations of histamine, mMCP-1, and TM-specific antibodies (IgE, IgG1) dramatically decreased (Figure 3a–d), whereas IgG2a showed no change (Figure 3e). Especially the levels of specific IgE was inhibited by 77.7%. Serum samples were also used for Western blot analysis (Figure 3c–e) to measure TM-specific IgE, IgG1, and IgG2a. The results confirmed the ELISA results.

Immunomodulatory Effects of ESO on Cytokine Patterns from Splenic Lymphocytes and MLNs in Mice. Cytokine patterns were investigated in splenic lymphocytes and MLNs isolated from mice of TM-induced food allergy. In splenic lymphocytes, the levels of IL-4 and IL-13 were higher in TM group but significantly decreased in the two ESO-treated groups (Figure 4a,b). The levels of IFN- γ were decreased in the TM group and increased in the two ESO groups (Figure 4c) by 38.5–58.2%. Moreover, IL-10 was dramatically increased in the

ESO groups (Figure 4d) by 38.0–60.3%. These results suggest that ESO may suppress Th2 cell polarization and up-regulate Treg cells. In MLNs, the results (Figure 4e–h) of Th2-related cytokines (IL-4, IL-13), Th1-related cytokines (IFN- γ), and regulatory T-related cytokines (IL-10) were similar to the results observed in splenic lymphocytes.

These results show that ESO suppressed the production of Th2-related cytokines and promoted Treg-related cytokines in both preventive and therapeutic protocols. Furthermore, the expressions of the transcription factors, T-bet, GATA-3, and Foxp3, as a reflection of Th1, Th2, and Treg cells, were investigated by qPCR, respectively (Figure 5). The qPCR results for splenic lymphocytes and MLNs show that the relative expression levels of Foxp3 increased in the two ESO-treated groups (Figure 5a,c), confirming the cytokine results. Meanwhile, the ratios of the relative expressions of GATA-3/T-bet significantly decreased from the TM group (3.5; 2.5) to the ESO-preventive (2.8; 0.8) and ESO-therapeutic groups (1.3; 0.4) in (splenic lymphocytes; MLNs), respectively (Figure 5b,d).

The effects of ESO on Treg cells in mice were further investigated by FACS. The data are shown in Figure 6a. In splenic lymphocytes, the percentage of CD4+Foxp3+ Treg cells dramatically increased from 3.85% (TM group) to 7.59% (ESO-therapeutic group). Meanwhile, the percentage of CD4+Foxp3+ Treg cells of the ESO-preventive group (5.12%) also increased compared to the TM group (3.85%). As shown in Figure 6b, all of the results for MLNs were similar to the results observed in splenic lymphocytes. Thus, the ESO may

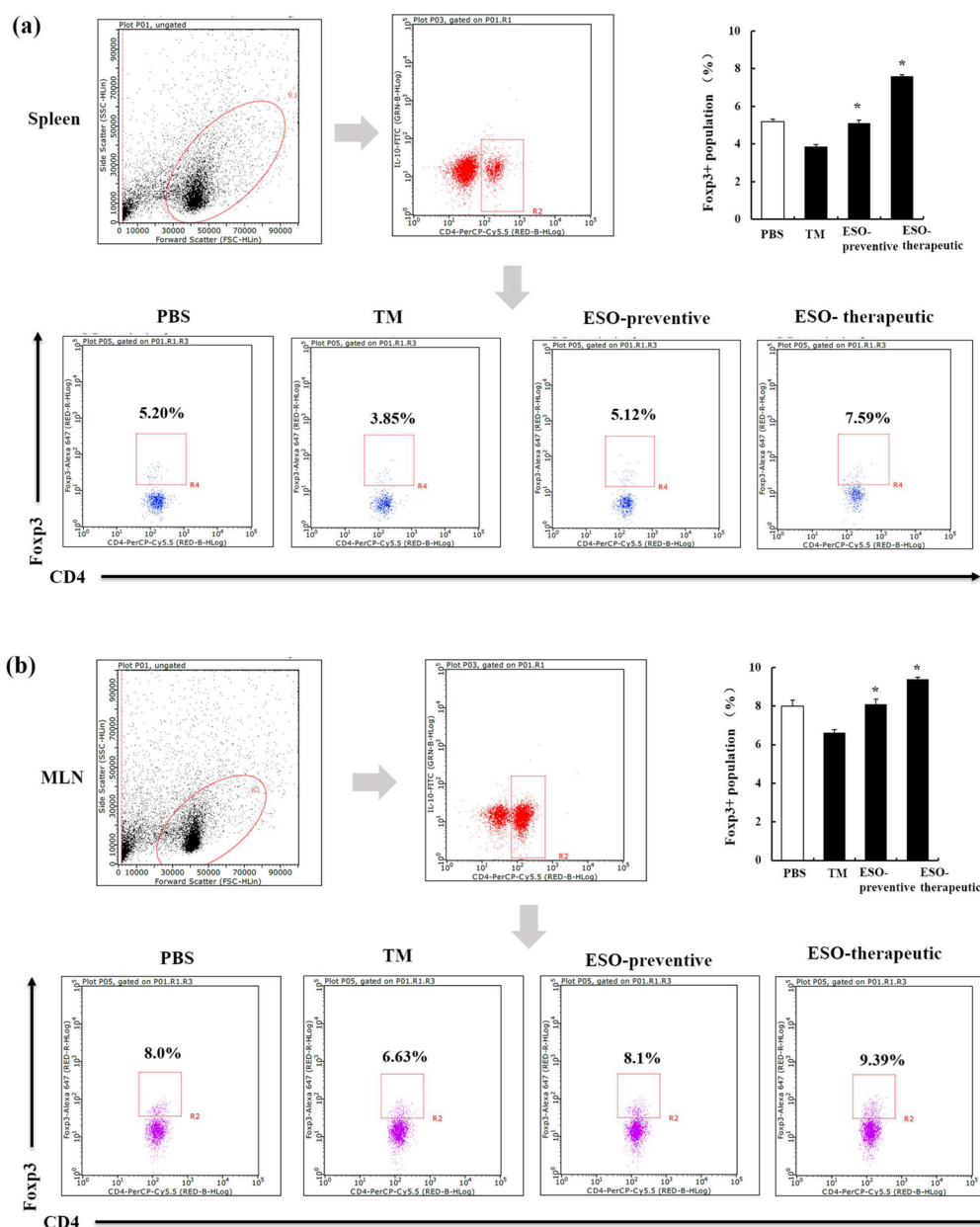


Figure 6. Flow cytometric analysis of splenic and MLN lymphocytes for CD4+ and Foxp3+ cells. Levels of CD4+Foxp3+ T cells were analyzed by flow cytometry after the sixth intragastric challenge of PBS, TM, or the two ESO-treated groups (indicated in the figure) on cells that were isolated from spleen (a) and mesentery (b). The graphic demonstration of the percentages of CD4+Foxp3+ T cells is shown at the top right corner of panels a and b. The flow cytometric analysis chart was the result of just a single experiment, and the histogram at the top right corner is the average of three independent experiments. All experiments were repeated three times. Statistical differences are indicated by *P* values (one-way ANOVA). (*) *P* < 0.05 and (**) *P* < 0.01, compared with the TM-treated group. (#) *P* < 0.01, compared with the PBS group.

promote the polarization of CD4+Foxp3+ Treg cells from both splenic lymphocytes and MLNs by 33.0–97.1%.

Inhibition Effect of ESO on IgE-Mediated RBL-2H3 Cells. The RBL-2H3 cell line had typically been used as a mast cell model for IgE-mediated cellular studies.³¹ IL-4 and TNF- α are the important cytokines in allergic inflammation.³² Anti-DNP-IgE sensitized RBL-2H3 cells were stimulated with the presence and absence of DNP-BSA, and allergic mediators' secretion were assessed. The results showed that ESO (20–100 μ g/mL) had no measurable cytotoxic effect on unactivated RBL-2H3 cells (vitality = 99.87%). ESO (20–100 μ g/mL) significantly diminished DNP-induced release of β -hexosaminidase (Figure 7a) by 64.40%, histamine (Figure 7b) by 61.11%,

IL-4 by 44.08%, and TNF- α by 40.91% (Figure 7c,d) in a concentration-dependent manner in IgE-sensitized RBL-2H3 cells treated with DNP-BSA.

ESO Specifically Induced Apoptosis of TM-Activated BMMCs. Mast cells are important effector cells in food allergy; once activated, they can release prostaglandin, proteases, and leukotrienes and effect degranulation.³³ BMMCs were activated with or without TM, the apoptosis and the surface marker Fc ϵ RI of BMMCs measured by FACS. ESO had shown no significant effects on the high-affinity receptor Fc ϵ RI of either unactivated BMMCs (Figure 8a) or TM-activated BMMCs (Figure 8b). However, ESO could induce apoptosis of TM-activated BMMCs, increasing it from about 5.51 to

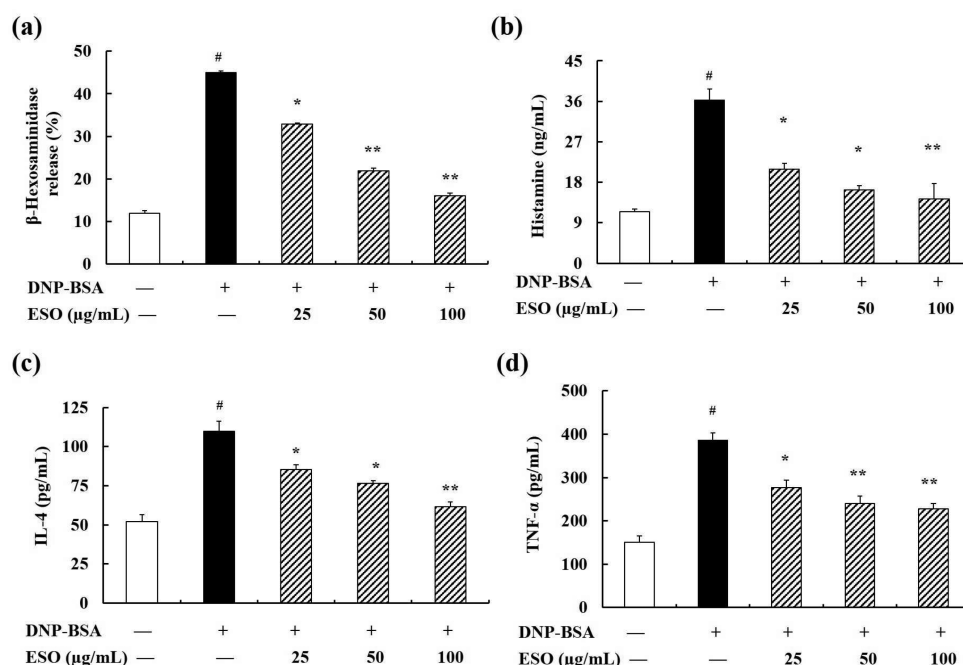


Figure 7. Effects of ESO on IgE-mediated allergic responses in RBL-2H3. Different concentrations of ESO and PBS as control were added to the culture medium of anti-DNP-IgE (1 μ g/mL) sensitized RBL-2H3 cells, and degranulation was induced by the addition of DNP-BSA (1 μ g/mL). (a) Released β -hexosaminidase was measured at 6 h and (b) histamine at 15 min. (c, d) Antigen-stimulated RBL-2H3 cells were incubated in medium containing anti-DNP-specific IgE (1 μ g/mL) in the presence or absence of ESO (25–100 μ g/mL) for 4 h for IL-4 (c) or for 6 h for TNF- α measurements (d). The amounts of IL-4 and TNF- α released into the culture supernatant were measured by ELISA. DNP, DNP-BSA. Each value is presented as the mean $\pm$ SD from three independent experiments. Statistical differences are indicated by *P* values (one-way ANOVA). (*) *P* < 0.05 and (**) *P* < 0.01, significantly different from DNP-BSA. (#) *P* < 0.01, compared with the PBS group.

9.56% (Figure 8d), while having no effects on unactivated BMMCs (Figure 8c).

DISCUSSION

In recent years, algal products have attracted much attention as a source of bioactive compounds for the development of healthy foodstuffs.³⁴ It has been reported that carrageenan oligosaccharides separated from *E. cottonii* possess antiangiogenic, antitumor, and immunomodulatory activities.^{35,36}

The purified oligosaccharides, referred to as ESO, were obtained from *E. cottonii* by enzymatic treatment with κ -carrageenase, which was produced according to our patented methodology (patent 2015100841980). In the purification process of ESO, the solution of samples had been applied to a 200 Da membrane, so some small molecules may not exist in ESO, including monosaccharides and minerals. ESO was composed of tetroses and hexoses, which were more soluble than polysaccharides. It showed a similar monosaccharide composition (galactose) with sulfated polysaccharides purified from *P. haitanensis* and *G. lemaneiformis*, but a higher sulfate content (18.17%) than the sulfate content.^{7,8} The sulfate plays a crucial role in suppressing the activation and function of RBL-2H3 cells.^{8,17}

Splenic lymphocytes and MLNs were commonly investigated in a mouse model of food allergy. Pabst reported that antigen taken up into afferent intestinal lymph will pass through the MLNs in the presence of DCs and eventually enter the bloodstream to the spleen circulation.³⁷ In other words, splenic lymphocytes were closely related with the whole immune system, and MLNs were tightly related with gastrointestinal reactions. The effects of ESO on splenic lymphocytes and MLNs were both investigated in this study. Food allergy is an

immunologically adverse reaction caused by food protein, which induces a range of IgE-mediated disorders including anaphylaxis, a decrease in body temperature, and gastrointestinal adverse reactions.⁸ The characteristic Th2 phenotype immune response plays a critical role in the development of allergic disorders through the release of cytokines such as IL-4 and IL-13; these cytokines induce mastocytosis and IgE class-switching in the intestine.^{7,15} Brandt reported that the decrease of Th2 cytokines (IL-4 and IL-13) could attenuate food allergy.³⁸ In this study, both preventive and therapeutic group administrations of ESO were shown to attenuate food allergy symptoms and decrease the levels of histamine, mMCP-1, and TM-specific antibodies (IgE, IgG1) in TM-sensitized mice serum. ESO had an obvious suppression of IL-4 and IL-13 and an increase in the IFN- γ of splenic lymphocytes and MLNs. In addition, the qPCR results showed that the ratio of GATA-3/T-bet was obviously reduced in the two ESO-treated groups compared to the TM group. These results demonstrated that ESO had the same effects as other polysaccharides or oligosaccharides from algae, which could diminish the imbalance of the Th1/Th2 immune response to relieve food allergy symptoms in the whole immune system and gastrointestinal reactions.^{7,8,18}

It was reported that Treg cells play an important role in the suppression of immune responses and are crucial in preventing the development of food allergy diseases.^{38–40} Treg cells are known to release IL-10 and TGF- β that could block IgE produced by B cells, down-regulating Th2 cells and suppressing mast cells, basophils, and eosinophils.^{41,42} In the present study, levels of IL-10 significantly increased in the two ESO-treated groups, especially in the ESO-therapeutic group compared to the TM group. It was reported that Foxp3 protein regulates

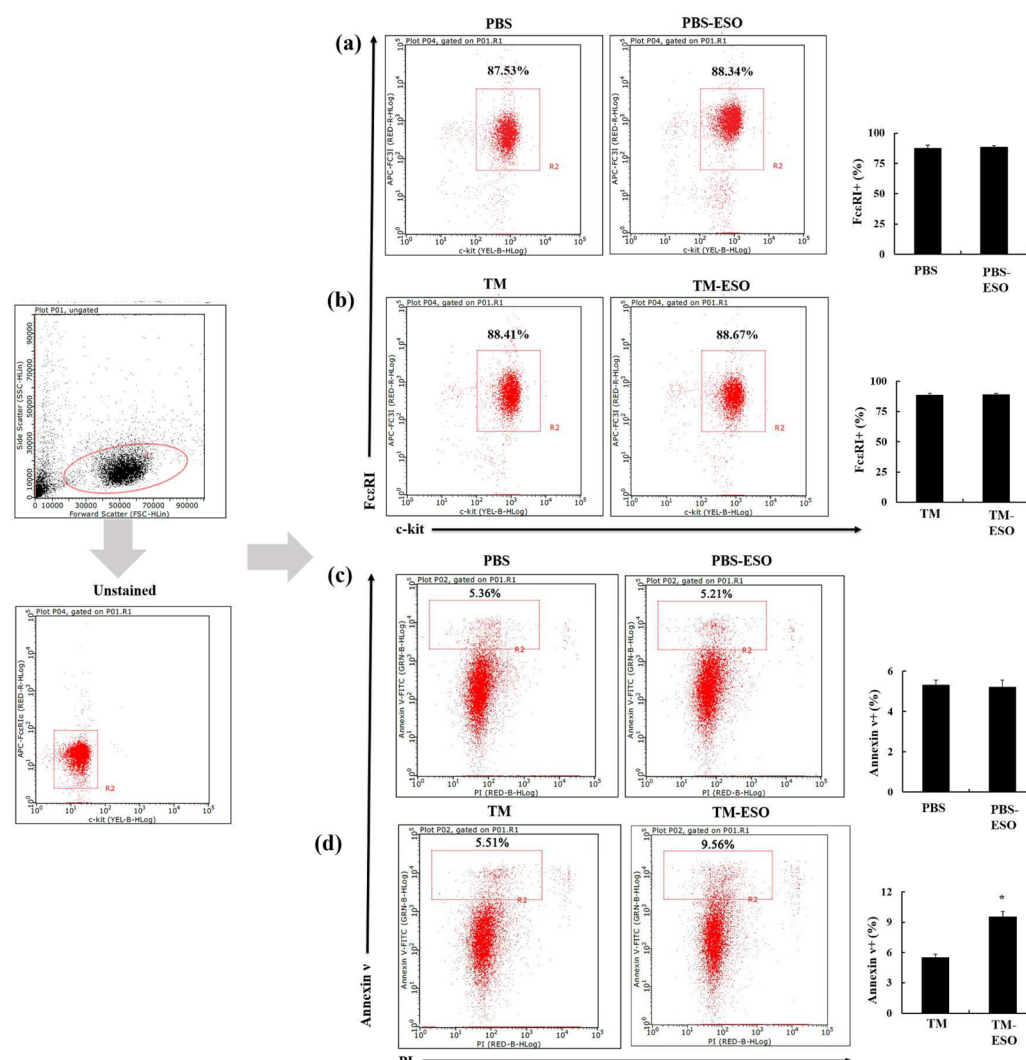


Figure 8. Effect of ESO on TM-activated BMMCs. BMMCs from thighbone of BALB/c mice were incubated with and without ESO (100 $\mu\text{g/mL}$). (a, b) Effects of ESO on c-kit⁺FcεRI⁺ of the unactivated BMMCs and TM-activated BMMCs, respectively. (c, d) Effects of ESO on apoptosis of the unactivated BMMCs and TM-activated BMMCs, respectively. The percentages of FcεRI⁺ and annexin-V⁺ expression are shown in the graphs on the right. The flow cytometric analysis chart on the left was the result of just a single experiment, and the histogram on the right was the average of three independent experiments. All experiments were repeated three times. Statistical differences are indicated by *P* values (one-way ANOVA). (*) *P* < 0.05 and (**) *P* < 0.01, compared with the TM-treated group. (#) *P* < 0.01, compared with the PBS group.

some immune cells such as regulatory T cells and, during point mutation in the *Foxp3* gene, will decrease the function of Treg cells in the intestine by sensitization.⁴³ The expression of *Foxp3* in mouse splenic lymphocytes and MLNs were increased in the two ESO-treated groups by qPCR and FACS. The ESO was thus speculated to promote *Foxp3* to induce Treg, which could suppress Th2 cells. Shin reported that the effect of curcumin positively affects food allergy symptoms through an immunosuppressive effect via induction of Treg cells, and the results of curcumin on induction of CD4⁺*Foxp3*⁺T cells from splenocytes by FACS showed that curcumin group (3.74%) was higher than the allergic group (2.45%).²⁶ Both ESO and curcumin are natural extract products and up-regulate Treg induction, but ESO has a stronger effect on induction of Treg cells than curcumin. In this study, the ESO-therapeutic group (7.59%) was much higher than allergic group (3.85%). It was reported that the aryl hydrocarbon receptor (AhR) could be activated by some extraction of natural product (sinomenine, baicalein, curcumin) to induce Treg cells.^{44–46} ESO is an extraction of a natural product, and it was speculated that ESO

might have the ability to activate AhR and induce Treg cells. In addition, Liu⁴⁷ reported that sulfated polysaccharide purified from *P. haitanensis* up-regulates the subpopulation of Tregs, as well as the levels of serum cytokines IL-10, and Courtade⁴⁸ reported that treatment with human milk oligosaccharides did lead to a significant increase in CD4⁺CD25⁺ Treg cells in OVA-induced mice. These results of saccharides in inducing Treg cells were consistent with ESO.

The polarization of CD4⁺*Foxp3*⁺ Treg cells in both preventive and therapeutic protocol mice was promoted by ESO, further suggesting that the oligosaccharides could induce the Treg cell population that protects against allergy development and enhanced the regulatory response to the allergic reaction. Therefore, ESO might decrease food allergic immune responses by regulating the Th1/Th2 balance, due to suppression by up-regulating Treg cells.

Mast cells are an important component of the innate immune system.⁴⁹ In allergic diseases, mast cells express FcεRI, and after cross-linking of surface-bound IgE by the allergen, β-hexosaminidase, histamine, and other mediators were released.^{50,51} The lower

levels of serum mMCP-1 of the ESO-treated groups suggest that ESO reduced mast cell activation. Thus, the influence of ESO on mast cells was investigated. In vitro studies, the results of RBL-2H3 cells showed that ESO significantly inhibited (64.40%) DNP-induced β -hexosaminidase and the release of histamine (61.11%), IL-4 (44.08%), and TNF- α (40.91%) by DNP-BSA activated RBL-2H3 cells. Meanwhile, BMMCs are useful for the analysis of intestinal and other mast cell functions in response to various agents.⁵² Effects of ESO (100 μ g/mL) on BMMCs were analyzed by FACS in this study. The results showed that ESO (100 μ g/mL) could specifically induce apoptosis of activated BMMCs by TM and caused no damage to unactivated BMMCs. Kim et al. reported that extracellular vesicle-derived protein from *Bifidobacterium longum* alleviates food allergy through apoptosis of the mast cell.¹² In this study, ESO was also shown to induce apoptosis of the TM-activated BMMCs to some degree. Thus, it is likely that apoptosis of activated mast cells caused by ESO might contribute to the suppression of a food allergic response.

Forward et al. reported that activated mast cells down-regulate CD4+CD25+ T regulatory cell suppressor function via histamine H1 receptor interaction.⁴⁹ It was speculated that ESO could also reduce the secretion of serum histamine in allergic mice and RBL-2H3 cell, which inhibited the interaction of H1 receptor, to rescue the Treg function. Gri et al. reported that CD4+CD25+ regulatory T cells suppressed mast cell degranulation and allergic responses through OX40–OX40L interaction.⁴² It seems that mast cells and Treg cells could suppress each other's function. The present results suggested that the polarization of Treg cells was promoted and that apoptosis of the TM-activated BMMCs was induced to some degree by ESO. It was speculated that ESO promoted polarization of Treg cells through inducing apoptosis of mast cells.

In conclusion, the antiallergic activity of purified ESO was evaluated systematically through the RBL-2H3 cell model in vitro and the TM-induced BALB/c mouse food allergy model in vivo. The results suggest that dietary intervention with ESO could alleviate the allergic symptoms of TM-allergic mice by up-regulating polarization of Treg cells by 22.2–97.1%. Our research suggests that sulfated oligosaccharide derived from *E. cottonii* might provide a novel preventive or therapeutic option for allergic diseases.

AUTHOR INFORMATION

Corresponding Author

*(G.-M.L.) Phone: +86-592-6180378. Fax: +86-592-6180470. E-mail: gmlu@jmu.edu.cn.

ORCID

Guang-Ming Liu: 0000-0002-8689-0504

Author Contributions

[†]S.-S.X. and Q.-M.L. contributed equally to this study as lead authors.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BMMCs, bone marrow-derived mast cells; DNP, dinitrophenyl; ESO, *Eucheuma cottonii* sulfated oligosaccharide; FACS, flow cytometry; FBS, fetal bovine serum; Foxp3, Forkhead box protein 3; MLNs, mesenteric lymph node cells; mMCP, mouse mast cell protease; PBS, phosphate buffer saline; TGF- β , transforming growth factor- β ; TM, tropomyosin; Treg, regulatory T

REFERENCES

- (1) Turnbull, J. L.; Adams, H. N.; Gorard, D. A. Review article: the diagnosis and management of food allergy and food intolerances. *Aliment. Pharmacol. Ther.* **2015**, *41*, 3–25.
- (2) Wang, J.; Sampson, H. A. Food allergy: recent advances in pathophysiology and treatment. *Allergy, Asthma Immunol. Res.* **2009**, *60*, 19–29.
- (3) Kim, J. S.; Ouyang, F.; Pongracic, J. A.; Fang, Y.; Wang, B.; Liu, X.; Xing, H.; Caruso, D.; Liu, X.; Zhang, S.; Xu, X.; Wang, X. Dissociation between the prevalence of atopy and allergic disease in rural China among children and adults. *J. Allergy Clin. Immunol.* **2008**, *122*, 929–935.
- (4) Liang, Y. L.; Cao, M. J.; Su, W. J.; Zhang, L. J.; Huang, Y. Y.; Liu, G. M. Identification and characterisation of the major allergen of Chinese mitten crab (*Eriocheir sinensis*). *Food Chem.* **2008**, *111*, 998–1003.
- (5) Ying, L.; Ohshima, T.; Ushio, H.; Hamada, Y.; Shiomi, K. Immunological characteristics of monoclonal antibodies against shellfish major allergen tropomyosin. *Food Chem.* **2007**, *100*, 1093–1099.
- (6) Liu, Q. M.; Wang, Y. Z.; Cao, M. J.; Pan, T. M.; Yang, Y.; Mao, H. Y.; Sun, L. C.; Liu, G. M. Anti-allergic activity of R-phycoerythrin from *Porphyra haitanensis* in antigen-sensitized mice and mast cells. *Int. Immunopharmacol.* **2015**, *25*, 465–473.
- (7) Shi, C. L.; Pan, T. M.; Cao, M. J.; Liu, Q. M.; Zhang, L. J.; Liu, G. M. Suppression of Th2 immune responses by the sulfated polysaccharide from *Porphyra haitanensis* in tropomyosin-sensitized mice. *Int. Immunopharmacol.* **2015**, *24*, 211–218.
- (8) Liu, Q. M.; Yang, Y.; Maleki, S. J.; Alcocer, M. J.; Xu, S. S.; Shi, C. L.; Cao, M. J.; Liu, G. M. The anti food allergic activity of sulfated polysaccharide from *Gracilaria lemaneiformis* is dependent on immunosuppression and inhibition of p38 MAPK. *J. Agric. Food Chem.* **2016**, *64*, 4536–4544.
- (9) Helm, R. M.; Burks, A. W. Mechanisms of food allergy. *Curr. Opin. Immunol.* **2000**, *12*, 647–53.
- (10) Ozdemir, C.; Akdis, M.; Akdis, C. A. T-Cell response to allergens. *Prog. Allergy* **2010**, *95*, 22–44.
- (11) Cianferoni, A.; Spergel, J. M. Food allergy: review, classification and diagnosis. *Allergol. Int.* **2009**, *58*, 457–466.
- (12) Kim, J. H.; Jeun, E. J.; Hong, C. P.; Kim, S. H.; Jang, M. S.; Lee, E. J.; Moon, S. J.; Yun, C. H.; Lm, S. H.; Jeong, S. G.; Park, B. Y.; Kim, K. T.; Seoh, J.; Kim, Y. K.; Oh, S. J.; Ham, J. S.; Yang, B.; Jang, H. M. Extracellular vesicle-derived protein from *Bifidobacterium longum* alleviates food allergy through mast cell suppression. *J. Allergy Clin. Immunol.* **2015**, *137*, 507–516.
- (13) Brandenburg, S.; Takahashi, T.; de la Rosa, M.; Janke, M.; Karsten, G.; Muzzulini, T.; Orinska, Z.; Bulfone-Paus, S.; Scheffold, A. IL-2 induces in vivo suppression by CD4 + CD25 + Foxp3 + regulatory T cells. *Eur. J. Immunol.* **2008**, *38*, 1643–1653.
- (14) Sicherer, S. H. Food allergy. *J. Allergy Clin. Immunol.* **2010**, *125*, S116–S125.
- (15) Rueter, K.; Haynes, A.; Prescott, S. L. Developing primary intervention strategies to prevent allergic disease. *Curr. Allergy Asthma Rep.* **2015**, *15*, 1–12.
- (16) Nika, K.; Mulloy, B.; Carpenter, B.; Gibbs, R. Specific recognition of immune cytokines by sulphated polysaccharides from marine algae. *Eur. J. Phycol.* **2003**, *38*, 257–264.
- (17) Ngo, D. H.; Kim, S. K. Sulfated polysaccharides as bioactive agents from marine algae. *Int. J. Biol. Macromol.* **2013**, *62*, 70–75.

- (18) Yoshida, T.; Hirano, A.; Wada, H.; Takahashi, K.; Hattori, M. Alginic acid oligosaccharide suppresses Th2 development and IgE production by inducing IL-12 production. *Int. Arch. Allergy Immunol.* **2004**, *133*, 239–247.
- (19) Yoshida, T.; Sasahara, Y.; Miyakawa, S.; Hattori, M. Reduced T cell response to β -lactoglobulin by conjugation with acidic oligosaccharides. *J. Agric. Food Chem.* **2005**, *53*, 6851–6857.
- (20) Uno, T.; Hattori, M.; Yoshida, T. Oral administration of alginic acid oligosaccharide suppresses IgE production and inhibits the induction of oral tolerance. *Biosci., Biotechnol., Biochem.* **2007**, *70*, 3054–3057.
- (21) Zhang, L.; Ran, Y.; Chen, W. Purification and characterization of algae *Eucheuma* polysaccharide degrading enzymes from *Microbacterium*. *Int. J. Mol. Sci.* **2015**, *2*, 103–106.
- (22) Omokawa, Y.; Miyazaki, T.; Walde, P.; Akiyama, K.; Sugahara, T.; Masuda, S.; Inada, A.; Ohnishi, Y.; Saeki, T.; Kato, K. In vitro and in vivo anti-tumor effects of novel Span 80 vesicles containing immobilized *Eucheuma serra* agglutinin. *Int. J. Pharm.* **2010**, *389*, 157–167.
- (23) Mou, H.; Jiang, X.; Guan, H. A κ -carrageenan derived oligosaccharide prepared by enzymatic degradation containing anti-tumor activity. *J. Appl. Phycol.* **2003**, *15*, 297–303.
- (24) Xiao, A. F.; Xu, C. Y.; Lin, Y.; Ni, H.; Zhu, Y. B.; Cai, H. N. Preparation and characterization of κ -carrageenase immobilized onto magnetic iron oxide nanoparticles. *Electron. J. Biotechnol.* **2016**, *19*, 1–7.
- (25) Zhang, Z.; Xie, J.; Zhang, F.; Linhardt, R. J. Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides. *Anal. Biochem.* **2007**, *371*, 118–120.
- (26) Shin, H. S.; See, H. J.; Sun, Y. J.; Choi, D. W.; Kwon, D. A.; Bae, M. J.; Sung, K. S.; Shon, D. H. Turmeric (*Curcuma longa*) attenuates food allergy symptoms by regulating type 1/type 2 helper T cells (Th1/Th2) balance in a mouse model of food allergy. *J. Ethnopharmacol.* **2015**, *175*, 21–29.
- (27) Yamaki, K.; Yoshino, S. Tyrosine kinase inhibitor sunitinib relieves systemic and oral antigen-induced anaphylaxes in mice. *Allergy* **2012**, *67*, 114–122.
- (28) Kim, M.; Lim, S. J.; Lee, H. J.; Nho, C. W. Cassia tora seed extract and its active compound aurantio-obtusin inhibit allergic responses in IgE-mediated mast cells and anaphylactic models. *J. Agric. Food Chem.* **2015**, *63*, 9037–9046.
- (29) Yao, Y.; Zhu, Y.; Ren, G. Immunoregulatory activities of polysaccharides from mung bean. *Carbohydr. Polym.* **2016**, *139*, 61–66.
- (30) Zhang, Q.; Li, N.; Liu, X.; Zhao, Z.; Li, Z.; Xu, Z. The structure of a sulfated galactan from *Porphyra haitanensis* and its in vivo antioxidant activity. *Carbohydr. Res.* **2004**, *339*, 105–111.
- (31) Passante, E.; Ehrhardt, C.; Sheridan, H.; Frankish, N. RBL-2H3 cells are an imprecise model for mast cell mediator release. *Inflammation Res.* **2009**, *58*, 611–618.
- (32) Bradding, P.; Roberts, J. A.; Britten, K. M.; Montefort, S.; Djukanovic, R.; Mueller, R.; Heusser, C. H.; Howarth, P. H.; Holgate, S. T. Interleukin-4, -5, and -6 and tumor necrosis factor- α in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am. J. Respir. Cell Mol. Biol.* **1994**, *10*, 471–480.
- (33) Shea-Donohue, T.; Stiltz, J.; Zhao, A.; Notari, L. Mast cells. *Curr. Gastroenterol. Rep.* **2010**, *12*, 349–357.
- (34) Qi, H.; Zhao, T.; Zhang, Q.; Li, Z.; Zhao, Z. Q.; Xing, R. Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (*Chlorophyta*). *J. Appl. Phycol.* **2005**, *17*, 527–534.
- (35) Yuan, H.; Song, J.; Li, X.; Li, N.; Liu, S. Enhanced immunostimulatory and antitumor activity of different derivatives of κ -carrageenan oligosaccharides from *Kappaphycus striatum*. *J. Appl. Phycol.* **2011**, *23*, 59–65.
- (36) Yao, Z.; Wu, H.; Zhang, S.; Du, Y. Enzymatic preparation of κ -carrageenan oligosaccharides and their anti-angiogenic activity. *Carbohydr. Polym.* **2014**, *101*, 359–367.
- (37) Pabst, O.; Mowat, A. M. Oral tolerance to food protein. *Mucosal Immunol.* **2012**, *5*, 232–239.
- (38) Brandt, E. B.; Munitz, A.; Orekov, T.; Mingler, M. K.; McBride, M.; Finkelman, F. D.; Rothenberg, M. E. Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea. *J. Allergy Clin. Immunol.* **2009**, *123*, 53–58.
- (39) Hartl, D.; Koller, B.; Mehlhorn, A. T.; Reinhardt, D.; Nicolai, T.; Schendel, D. J.; Griesse, M.; Krauss-Etschmann, S. Quantitative and functional impairment of pulmonary CD4+CD25+ regulatory T cells in pediatric asthma. *J. Allergy Clin. Immunol.* **2007**, *119*, 1258–1266.
- (40) Wing, K.; Sakaguchi, S. Regulatory T cells as potential immunotherapy in allergy. *Curr. Opin. in Immunol.* **2006**, *6*, 482–488.
- (41) Akdis, M. Healthy immune response to allergens: T regulatory cells and more. *Curr. Opin. Immunol.* **2006**, *18*, 738–744.
- (42) Gri, G.; Piconese, S.; Frossi, B.; Manfroi, V.; Merluzzi, S.; Tripodo, C.; Viola, A.; Odom, S.; Rivera, J.; Colombo, M. P.; Pucillo, C. E. CD4 + CD25 + regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* **2008**, *29*, 771–781.
- (43) Devaraja, G.; Bhavyashree, G.; Rashmi, B. S. Food allergy: immunological mechanisms and prevention strategies. *EC Nutr.* **2016**, *5*, 1058–1066.
- (44) Tong, B.; Yuan, X. S.; Dou, Y. N.; Wu, X.; Wang, Y. H.; Xia, Y. F.; Dai, Y. Sinomenine induces the generation of intestinal Treg cells and attenuates arthritis via activation of aryl hydrocarbon receptor. *Lab. Invest.* **2016**, *96*, 1076.
- (45) Bae, M. J.; Shin, H. S.; See, H. J.; Jung, S. Y.; Kwon, D. A.; Shon, D. H. Baicalein induces CD4+Foxp3+ T cells and enhances intestinal barrier function in a mouse model of food allergy. *Sci. Rep.* **2016**, *6*, 1–10.
- (46) Ma, C.; Ma, Z.; Fu, Q.; Ma, S. Curcumin attenuates allergic airway inflammation by regulation of CD4+CD25+ regulatory T cells (Tregs)/Th17 balance in ovalbumin-sensitized mice. *Fitoterapia* **2013**, *87*, 57.
- (47) Liu, Q. M.; Xu, S. S.; Li, L.; Pan, T. M.; Shi, C. L.; Liu, H.; Cao, M. J.; Su, W. J.; Liu, G. M. In vitro and in vivo immunomodulatory activity of sulfated polysaccharide from *Porphyra haitanensis*. *Carbohydr. Polym.* **2017**, *165*, 189–196.
- (48) Castillo-Courtade, L.; Han, S.; Lee, S.; Mian, F. M.; Buck, R.; Forsythe, P. Attenuation of food allergy symptoms following treatment with human milk oligosaccharides in a mouse model. *Allergy* **2015**, *70*, 1091–1102.
- (49) Forward, N. A.; Furlong, S. Y. Mast cells down-regulate CD4+CD25+ T regulatory cell suppressor function via histamine H1 receptor interaction. *J. Immunol.* **2009**, *183*, 3014–3022.
- (50) Marshall, J. S. Mast-cell responses to pathogens. *Nat. Rev. Immunol.* **2004**, *4*, 787–799.
- (51) Bischoff, S. C. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat. Rev. Immunol.* **2007**, *7*, 93–104.
- (52) Yamaki, K.; Yoshino, S. Preventive and therapeutic effects of rapamycin, a mammalian target of rapamycin inhibitor, on food allergy in mice. *Allergy* **2012**, *67*, 1259–1270.