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Suppression of Th2 immune responses by the sulfated polysaccharide from *Porphyra haitanensis* in tropomyosin-sensitized mice

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ABSTRACT

The sulfated polysaccharide from *Porphyra* was hypothesized to exhibit immunoregulatory, anti-tumor and anti-inflammatory activity, but its anti-allergic activity is not fully understood. Therefore, the aim of this study was to isolate sulfated polysaccharide from *Porphyra haitanensis* (PHPS) and investigate its anti-allergic potential using a tropomyosin (TM)-induced mouse allergy model. Intraperitoneal injection of PHPS suppressed the allergic reaction by modulating serum IgE, IgG1 and IgG2a levels in mice. In particular, when PHPS was injected prior to the first immunization with TM, the IgE level decreased by 34.2% compared with the control (PBS) group. Oral therapeutic administration of PHPS to TM-sensitized mice decreased histamine release and repaired the pathology in the jejunum of the small intestine. *In vitro*, the mRNA expressions of the TM-induced Th2 cytokines (interleukin-4 (IL-4), IL-5 and IL-13) in splenic lymphocytes were reduced by PHPS; however, the expression of Th1 and regulatory cytokines (interferon gamma (IFN- γ) and IL-10) were up-regulated in PHPS-treated splenic lymphocytes. In the splenic lymphocyte supernatant, the IL-4, IL-13 and IFN- γ levels were also regulated by PHPS. Moreover, PHPS induced IFN- γ secretion via the Jun N-terminal kinase (JNK) and Janus kinase 2 (JAK2) signaling pathways. Therefore, these results suggest that PHPS suppresses the TM-induced allergic reaction, possibly by modulating the imbalance of the Th1/Th2 immune response.

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1. Introduction

Food allergy is an abnormal immunologic disease affecting nearly 8% of children and 5% of adults, with growing evidence of an increase in prevalence [1]. Milk, eggs, peanuts and crustaceans are common allergenic foods, and crustaceans are one of the eight major allergenic foods [2]. In the USA, shellfish is the number one cause of IgE-mediated food allergy in persons older than 6 years [3]. Shellfish allergy in the Asia-Pacific region ranks among the highest in the world and is the most common cause of food-induced anaphylaxis [4]. In China, 16.7% of the rural population is sensitized to shellfish [5]. The major allergen in the shellfish has been identified as the muscle protein tropomyosin (TM) [6]. With the rapid growth of crab consumption, researchers all over the world are paying much more attention to the crab allergens. Our laboratory has purified and identified the TM moiety in *Eriocheir sinensis* [7], and we have proved that TM is an allergen in crab through digestion experiments and a mouse model [8,9].

Food allergy is a representative of IgE-mediated type I hypersensitivity and is characterized by the development and activation of T helper 2 (Th2) cells [1,10]. The differentiation of Th2 cells leads to the production of IL-4, IL-5 and IL-13, which results in the binding of allergens to mast cells and basophiles [11,12]. When Th1 cells produce IFN- γ , the allergy symptoms may be relieved since IFN- γ can suppress Th2 differentiation and proliferation [12,13]. Therefore, an allergen-induced Th1/Th2 imbalance leads to allergic inflammation.

Porphyra has been used as a drug in traditional Chinese medicine to relieve sore throat, fever and oedema [14]. *Porphyra* is a red alga and is widely consumed in Asia. In Japan, *Porphyra* is usually used to prepare nori, which is a component of the traditional Japanese food, sushi. However, most investigations to date have focused on *Porphyra yezoensis*, a species widely distributed in East Asia. In China, *Porphyra haitanensis* is a top-grade *Porphyra*, and it has been used as an imperial tribute to royalty since the Song dynasty. We investigated *P. haitanensis*, which is an important economic alga in south China nowadays [15].

Polysaccharide accounts for approximately 25–40% of the total mass of dried *Porphyra* [16]. Sulfated polysaccharide from *P. haitanensis* (PHPS) comprises the hot-water soluble portion of the cell wall in *Porphyra*. PHPS is composed of sulfated polygalactans consisting of galactose and 3,6-anhydrogalactose units, which are sometimes substituted with galactose-6-sulfate and 6-O-methyl-galactose [17]. During the

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past few years, many studies have investigated the immune-modulating activities of the polysaccharide in *Porphyra*. Yoshizawa et al.[18,19] have shown that the polysaccharides in *Porphyra yezoensis* possess macrophage-stimulating activity *in vivo* and *in vitro*. Ishihara et al.[20] have confirmed that the sulfated polysaccharides in nori had the ability to suppress the contact hypersensitivity reaction by suppressing IgE and promoting IFN- γ in serum. Bhatia et al.[21] have found that the sulfated polysaccharides in *Porphyra vietnamensis* possess potential immunomodulatory activity and have therapeutic potential for the prevention of autoimmune diseases.

The aim of this study was to isolate sulfated polysaccharide from *P. haitanensis* and explore its anti-allergic activity with respect to the specific immune responses of mice to the allergen TM. To explore our hypothesis, we investigated the *in vivo* therapeutic potential of intraperitoneal injection or oral administration of PHPS on the TM-induced Th2 response in the mouse model. Moreover, we analyzed cytokine production by spleen lymphocytes isolated from TM-sensitized mice that were re-stimulated *in vitro* by the allergen in the presence or absence of PHPS.

2. Materials and methods

2.1. Animals

Specific pathogen-free (SPF) 6-week-old female BALB/c mice 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 °C with a relative humidity of $55 \pm 10\%$. All protocols were approved by the Fisheries College, Jimei University.

Scylla paramamosain was purchased from a local market in Xiamen (China) and used for the purification of TM.

2.2. Purification of PHPS

P. haitanensis that grew in Dadeng Island (Xiamen, China) were purchased from a local market in Xiamen. PHPS was extracted and purified according to the procedures of Zhou et al. [22] and Zhang et al. [23]. Dried material (25 g) was crushed and boiled in distilled water (1 L) for 4 h. The mixture was centrifuged. The volume of the supernatant was reduced to 1/3 of the initial volume at 65 °C by rotary evaporation. The crude polysaccharides were precipitated by adding 4 volumes of 95% ethanol. The crude polysaccharides were dissolved in distilled water and applied to a column (3.5 $\times$ 50 cm) of DEAE-cellulose 52 (Whatman, New York, USA) that had been equilibrated with distilled water. Distilled water was added to remove the components that did not bind to the column, such as the neutral polysaccharides, proteins, inorganic substances and pigments. The absorbed materials were eluted with a 2.0 M NaCl solution. The carbohydrate content of the eluate was detected by the anthrone-sulfate acid method [24]. The eluate containing carbohydrate was collected and dialyzed to obtain the PHPS. The lipopolysaccharide (LPS) was removed from PHPS with Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, USA). The LPS content of PHPS was measured and found to be < 0.03 EU/mg as measured by the Limulus amoebocyte lysate assay (Associates of Cape Cod, New York, USA).

2.3. Chemical analysis of PHPS

The carbohydrate content, 3,6-anhydrogalactose (3,6-AG), sulfate, uronic acid and protein contents were measured by the anthrone-sulfate method [24], ester sulfate-methoxyl method [25], barium chloride-gelatin method [26], sulfate-carbazole method [27] and the Lowry method [28], respectively, using galactose, fructose, potassium sulfate, glucuronic acid and bovine serum albumin (BSA) as standards, respectively. To determine the monosaccharide composition of PHPS, the PHPS acid hydrolysate was applied to an ion chromatography

(IC) (ICS-3000, Dionex, Sunnyvale, CA, USA) column (CarboPac PA10 [4 $\times$ 250 mm]) and separated using 0.02 M NaOH at a flow rate of 0.6 mL/min.

2.4. Immunization of mice

TM was extracted and purified from *S. paramamosain* as previously described [7]. In the preliminary experiments, the effect of PHPS on the mice was investigated. The mice of PHPS group were intraperitoneally injected with PHPS (100 μ g/mouse) three times every week from days 0 to 14 and bled on day 15. After the intraperitoneally injection with PHPS, the mice were fed with PHPS (5 mg/mouse) on days 28, 30, 32 and 34. The fecal samples were collected to evaluate the histamine levels 1 h after treatment. The same volume of PBS was regarded as the unimmunized group (PBS group).

Mice were immunized intraperitoneally with 0.15 mL phosphate buffer solution (PBS) containing the purified TM (100 μ g/mouse) and alum adjuvant (Pierce, Rockford, USA) on days 0 and 14 (Fig. 1A). The negative control mice were given intraperitoneal injection of 0.15 mL PBS and regarded as the unimmunized group (PBS group). During the course of TM sensitization, mice were intraperitoneally injected with 100 μ g/mouse PHPS from 7 days before (regarded as the PHPS preventive group) or 1 day after the first immunization (regarded as the PHPS treatment group) three times every week. The TM-sensitized mice injected with PBS instead of PHPS were referred to as the positive control (TM group) in which an allergic reaction was maintained. These mice were bled on day 15. The blood was centrifuged at 4000g for 10 min at 4 °C. The serum samples were collected and stored at -80 °C for future assay.

From day 28, each mouse from the TM group and PHPS preventive group was challenged 10 times at 2- to 3-day intervals by gavages of 10 mg TM in a final volume of 200 μ L PBS (Fig. 1A) according to the procedure of Costa et al. [29]. Before each gavage, mice were deprived of food for 3 h, and 1 h before, each TM-challenged mouse from the PHPS preventive group was treated with 5 mg PHPS/mouse. On day 49, mice were re-challenged as described above, and fecal samples were collected to evaluate the histamine levels 1 h after treatment. On day 50, mice were killed to collect the jejunum.

2.5. Measurement of the TM-specific IgE, IgG1 and IgG2a in sera

The TM-specific IgE level was measured by indirect ELISA [30]. Briefly, 96-well plates were coated with 100 μ L purified TM solution and maintained overnight at 4 °C. The wells were washed with 300 μ L wash buffer (20 mM Tris, 0.145 M NaCl, 0.05% Tween 20, pH 7.5) and then blocked with 300 μ L blocking solution (5% skim milk in PBS) at 37 °C for 2 h. After washing, 100 μ L diluted sera (1:6 dilution) were added to each well, and the wells were incubated overnight at 4 °C. After the wells were washed, 100 μ L of 1:1000 dilution for goat anti-mouse IgE-HRP secondary antibody (Southern Biotech, Birmingham, USA), 1:5000 dilution for goat anti-mouse IgG1 heavy chain (HRP) secondary antibody or 1:5000 dilution for goat polyclonal secondary antibody to mouse IgG2a-heavy chain (HRP) (Abcam, Cambridge, UK) was added to the wells, and the wells were incubated at 37 °C for 2 h. The wells were then washed before the addition of 100 μ L TMB substrate solution (Tiangen, Beijing, China). The wells were incubated at 37 °C for 20 min. The color reactions were stopped with 100 μ L stop solution (2 M H₂SO₄). The absorbance at 450 nm was read with an ELISA plate reader (Bench mark 96, Bio-Rad, Hercules, CA, USA). The TM-specific serum IgE level was also detected by Western blotting analysis according to the method of Huang et al. [8].

2.6. Measurement of the histamine in fecal extracts

Histamine in fecal samples was measured using high-performance liquid chromatography (HPLC) according to the procedure of Hu et al.

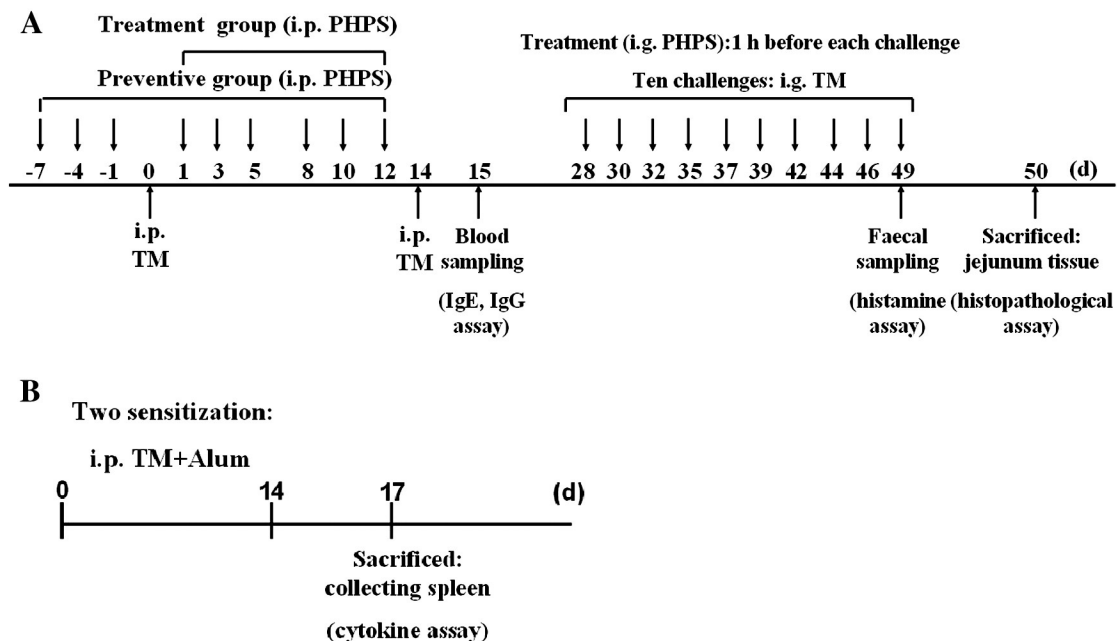


Fig. 1. Experimental design. (A) *In vivo* sensitization and PHPS treatment. Groups of mice ($n = 6$ mice per group) were sensitized two times by intraperitoneal injection of TM (100 μ g/mouse) followed by ten challenges of TM (10 mg/mouse) by gavage. During the intraperitoneal injection of TM, mice were intraperitoneally injected with 100 μ g/mouse PHPS from 7 days before (regarded as PHPS preventive group) or 1 day after the first immunization (regarded as PHPS treatment group) three times every week. One hour before each TM-gavage, mouse from the PHPS preventive group was treated with 5 mg PHPS/mouse. (B) Experimental set up for TM-stimulated allergic cell model *in vitro*.

[31] with some modification. Briefly, 0.8-g fecal samples were added to 2 mL 5% trichloroacetic acid and crushed. After centrifugation at 8000g for 20 min, the supernatant was adjusted to pH 9.5 using NaOH. The sample (300 μ L) was mixed with 600 μ L dansyl chloride and heated at 40 $^{\circ}$ C for 20 min. Then 100 μ L ammonia water was added to remove the excess dansyl chloride. The final volume of each sample was brought up to 4 mL by adding acetonitrile. Each sample (20 μ L) was injected onto the HPLC (1260, Agilent, Santa Clara, CA, USA) with an Agilent ZORBAX Extend-C18 column (50 $\times$ 4.6 mm) and separated using 70% acetonitrile at a flow rate of 1 mL/min.

2.7. Histopathological examination

The jejunum of the small intestine was cut and washed with PBS. Jejunum tissues were fixed with 4% formaldehyde in 0.1 M PBS (pH 7.4) and embedded in paraffin. The jejunum sections (5 μ m thick) were hydrated through an ethanol gradient to water and stained with hematoxylin and eosin. The sections were analyzed for histological changes by light microscopy (Leica RM2235, Solms, Germany).

2.8. Analysis of the cytokines in the splenic lymphocytes

According to the Fig. 1B, mice were immunized by intraperitoneal injection of 100 μ g/mouse purified TM together with alum adjuvant in a total of 150 μ L of PBS on day 0, 14. On day 17, mice were sacrificed to collect spleen tissues. Spleen cells were obtained by using a nylon mesh filter and mouse lymphocyte separation medium (Dakewe, Shenzhen, China). Spleens were isolated from mice and minced in RPMI 1640 medium (Invitrogen, New York, USA) with 10% fetal calf serum (Hyclone, Logan, USA) and 1% penicillin-streptomycin solution (Gibco, New York, USA). Cells were cultured in 24-well plates and treated with the antigen TM (10 μ g/mL) alone or in combination with PHPS (80 μ g/mL) and maintained at 37 $^{\circ}$ C in an atmosphere containing 5% CO₂ (Forma 3111, Thermo, Waltham, MA, USA). Co-culture with PHPS (80 μ g/mL) alone was also set as a control. Co-culture with PBS regarded as the negative control. Three days later, the culture supernatants were harvested by centrifugation at 4000g for 10 min. The levels of the

cytokines IFN- γ , IL-4 and IL-13 in the supernatants were determined by sandwich ELISA kits (Perprotech, New Jersey, USA), according to the manufacturer's instructions. The cell precipitate was then subjected to RNA extraction. Total RNA of the cultured cells was extracted using TRizol reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Extracted RNA was subjected to first-strand cDNA synthesis by a TIANScript RT Kit (Tiangen, Beijing, China) for RT-PCR (PTC-100, Bio-Rad, Hercules, CA, USA). The expressions of IL-4, IL-5, IL-13, IL-10 and IFN- γ in splenic lymphocytes were evaluated by a SuperReal PreMix kit (Tiangen, Beijing, China) with an ABI PRISM 7300 sequence detector (Applied Biosystems, CA, USA). The measurement of hypoxanthine guanine phosphoribosyl transferase (HPRT) and β -actin mRNA levels served as internal standards for calibration. Real-time quantitative PCR (QPCR) was performed with the following primers: HPRT (5'-CTG GTG AAA AGG ACC TCT CG-3'; 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'), β -actin (5'-AGC TGC GTT TTA CAC CCT TT-3'; 5'-AAG CCA TGC CAA TGT TGT CT-3'), IL-4 (5'-ACA GGA GAA GGG ACG CCA T-3'; 5'-GAA GCC CTA CAG ACG AGC TCA-3'), IL-5 (5'-GAG GTT ACA GAC ATG CAC CAT T-3'; 5'-TCA GTT GGT AAC ATG CAC AAA G-3'), IL-13 (5'-ACA GGA GAA GGG ACG CCA T-3'; 5'-GAA GCC CTA CAG ACG AGC TCA-3'), IL-10 (5'-TGG ACA ACA TAC TGC TAA CC-3'; 5'-GGA TCA TTT CCG ATA AGG CT-3') and IFN- γ (5'-TGG CAT AGA TGT GGA AGA AAA GAG-3'; 5'-TGC AGG ATT TTC ATG TCA CCA-3').

2.9. Signaling pathways involved in PHPS-induced IFN- γ

Spleen cells were obtained as in previous experiments and were pretreated with SP600125 (an inhibitor of JNK), SB 203580 (an inhibitor of p38 MAPK) or SD1008 (an inhibitor of JAK2) for 1 h and subsequently

Table 1

Chemical analysis of PHPS.

Composition	3,6-AG	Sulfate	Uronic acid	Monosaccharide composition (galactose)
Content (%)	6.25	14.26	14.02	60.09

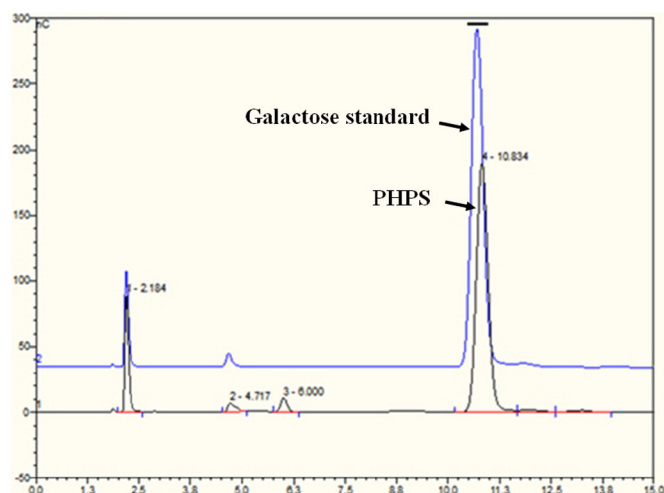


Fig. 2. IC analysis of the monosaccharide from PHPS. Peak number 1, solvent; Peak number 2, unknown; Peak number 3, unknown; Peak number 4, galactose.

stimulated with PHPS for 48 h. The production of IFN- γ was measured by means of ELISA.

2.10. Statistical analysis

Results of three separate experiments performed in triplicates for the *in vitro* studies, or six mice ($n = 6$) for each group of the *in vivo* studies were expressed as mean $\pm$ SD. Data were analyzed by general linear model and one-way ANOVA using SPSS statistics 14.0 software. Differences measured by Duncan's multiple range test between groups were considered significant at $p < 0.05$.

3. Results

3.1. Composition of PHPS

The carbohydrate, 3,6-AG, sulfate, uronic acid and protein contents are shown in Table 1. Fig. 2 provides the IC diagram for PHPS. The monosaccharide in PHPS is galactose (peak number 4) determined through IC analysis, and the galactose content was 60.09%. The elution

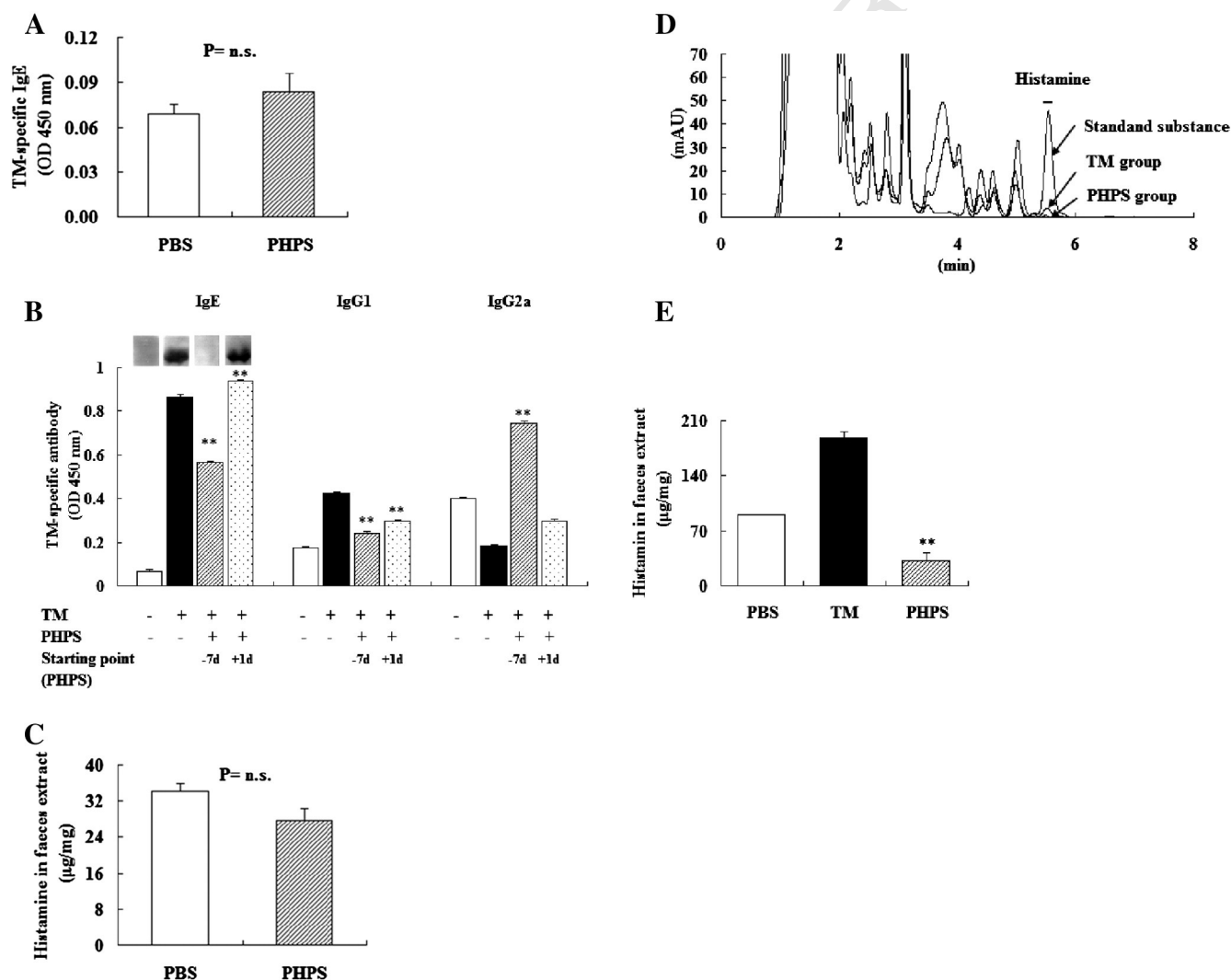


Fig. 3. Effects of PHPS on the TM-specific antibody and histamine levels. (A) Effects of PHPS on the TM-specific IgE. The mice were intraperitoneally injected with PHPS (100 μ g/mouse) three times every week from days 0 to 14 and bled on day 15. The TM-specific IgE level was measured by indirect ELISA. (B) Sera at 15 days after the second TM sensitization were collected to measure the IgE, IgG1 and IgG2a levels through ELISA and Western blotting analysis (for IgE). (C) Effect of PHPS on the histamine levels was measured by HPLC. After the intraperitoneally injection with PHPS, the mice were fed with PHPS (5 mg/mouse) on days 28, 30, 32, 34. The fecal samples were collected to evaluate the histamine levels 1 h after treatment. The same volume of PBS was regarded as the unimmunized group (PBS group). (D) HPLC analysis of histamine. (E) Histamine in fecal samples was collected within 60 min of the last gavage on day 49. The histamine in fecal samples was measured using HPLC. Three replicates for each measurement were performed. The results represent the mean $\pm$ SD of $n = 6$ mice per group. $^{**}p < 0.01$; n.s. = no significance. $^{*}p < 0.05$ vs. the positive control (the TM group).

times of peak numbers 2 and 3 are inconsistent with the elution time of a standard monosaccharide peak, and they are too small to be significant.

3.2. PHPS regulates TM-specific IgE, IgG1 and IgG2a in sera

As shown in Fig. 3A, the absorbance of the IgE of the PHPS group was 0.069 and similar to that (0.083) in the PBS group. The practical application of this work is to use PHPS as a preventative or complementary treatment for shellfish allergy. We commenced the administration of PHPS 7 days before (PHPS preventive group) or 1 day after (PHPS treatment group) the first immunization with TM. As shown in Fig. 3B, TM-specific IgE in sera was significantly suppressed ($p < 0.01$) by injections of PHPS that were started before the immunization with TM (PHPS preventive group). However, there was no effect of injections that were started after immunization with TM (PHPS treatment group). The TM-specific IgG1 levels in sera were all significantly suppressed ($p < 0.05$) by PHPS, whereas TM-specific IgG2a levels were only significantly promoted ($p < 0.05$) in mice injected with PHPS before TM sensitization (PHPS preventive group). Moreover, TM-specific IgE levels were also detected by Western blotting analysis. Fig. 3B shows some examples of the immunoblot experiments. Specific serologic reactions were observed in both the TM group and the PHPS treatment group. The sera from both PBS group and PHPS preventive group did not react with the protein band of TM on immunoblot.

3.3. PHPS reduces TM-induced histamine levels

We also measured the histamine levels in the fecal samples by HPLC. As shown in Fig. 3C, the histamine level (34.22 $\mu\text{g}/\text{mg}$) of PHPS group was similar to that (27.68 $\mu\text{g}/\text{mg}$) of PBS group. As shown in Fig. 3D and E, the histamine level in the TM group significantly increased to 187.48 $\mu\text{g}/\text{mg}$, which was 2-fold higher than the PBS group (90.68 $\mu\text{g}/\text{mg}$). In the PHPS preventive group, the mice were gavaged with PHPS before every oral administration of TM, and their histamine levels significantly decreased ($p < 0.01$) to basal level (32.48 $\mu\text{g}/\text{mg}$).

3.4. Histopathological changes of the TM-specific allergy

We conducted histopathological examinations to assess the jejunum changes associated with PHPS challenged and unchallenged TM-sensitized mice. As shown in Fig. 4, oedema and lymphocytic

infiltration of the jejunum were observed in the TM-sensitized mice, whereas remarkable histopathology was absent in the PBS group and the PHPS preventive group.

3.5. PHPS modulates the Th1/Th2 cytokines in splenic lymphocytes

In this study, we evaluated the potential of PHPS to modulate TM-induced cytokine production by splenocytes *in vitro*. Splenocytes from mice immunized with TM were re-stimulated *in vitro* by the same allergen alone or in combination with PHPS. In order to evaluate the effects of PHPS therapeutic treatment on the expression of cytokines, we measured mRNA expressions of the cytokines by QPCR, the splenocytes co-cultured with PHPS were also set as a control. As shown in Fig. 5A, the production of the regulatory cytokine IL-10 ($p < 0.01$) and Th1 cytokine IFN- γ ($p < 0.05$) was significantly accelerated in splenocytes stimulated with TM in the presence of PHPS compared with the TM group. The PHPS was also able to significantly increase IL-10 ($p < 0.05$) and IFN- γ ($p < 0.05$) production in the absence of the allergen compared with the PBS group. However, the levels of Th2 cytokines IL-4 ($p < 0.05$), IL-5 ($p < 0.01$) and IL-13 ($p < 0.01$) were significantly reduced in the splenocytes co-incubated with PHPS during TM stimulation compared with the TM group and were not affected by the presence of PHPS alone *in vitro* (Fig. 5B). We also measured the levels of cytokines IFN- γ , IL-4 and IL-13 in the splenocyte supernatants using sandwich ELISA kits. As shown in Fig. 5C, the TM-induced IL-4 ($p < 0.01$) and IL-13 ($p < 0.05$) levels in the supernatant from the splenocytes that were co-cultured with PHPS preparation were significantly reduced compared with the TM group, while the level of IFN- γ was significantly higher ($p < 0.01$) in the supernatant of the splenocytes stimulated with TM in the presence of PHPS compared with the splenocytes stimulated with TM alone. Altogether, these results indicate that PHPS is able to modulate the Th1/Th2 balance.

3.6. PHPS induces IFN- γ secretion via the JNK and JAK2 signaling pathways

We investigated whether PHPS-induced secretion of IFN- γ was affected by inhibitors of JNK, p38 MAPK or JAK2. As Fig. 6 shows, PHPS-induced IFN- γ production was significantly reduced ($p < 0.01$) by the inhibitors of JNK and JAK2 compared with the level of IFN- γ obtained from the splenocytes treated with PHPS containing DMSO (0.1%). In contrast, PHPS-induced IFN- γ production was not affected by SB203580 (an inhibitor of p38 MAPK) in splenocytes.

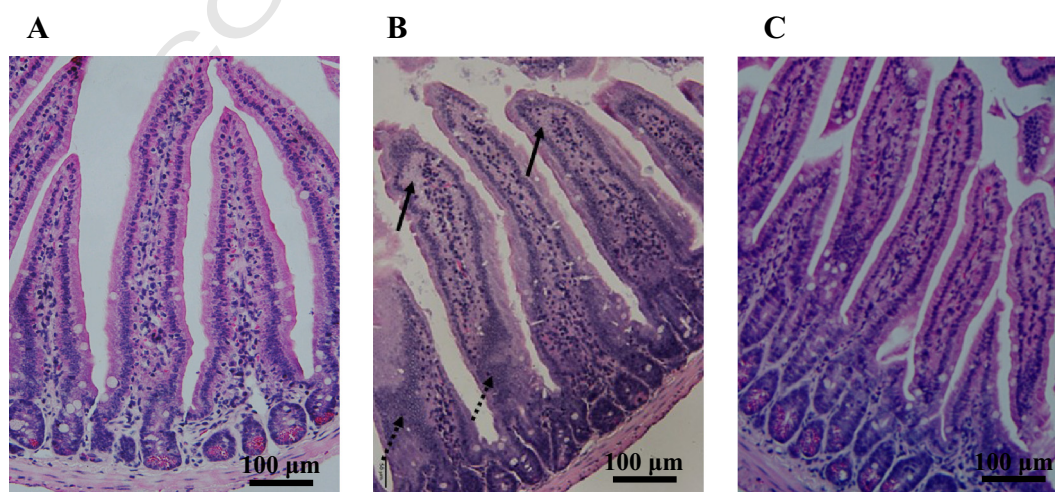


Fig. 4. Effects of PHPS on the impaired allergic jejunum tissue. Twenty-four hours after the last challenge, jejunum tissues were fixed in 4% formalin, embedded in paraffin and sectioned. Tissue section slides were stained with hematoxylin-eosin. The solid arrows indicate oedema; broken arrows indicate lymphocytic infiltration. (A) The negative control (PBS group); (B) the positive control (the TM group); (C) the PHPS preventive group.

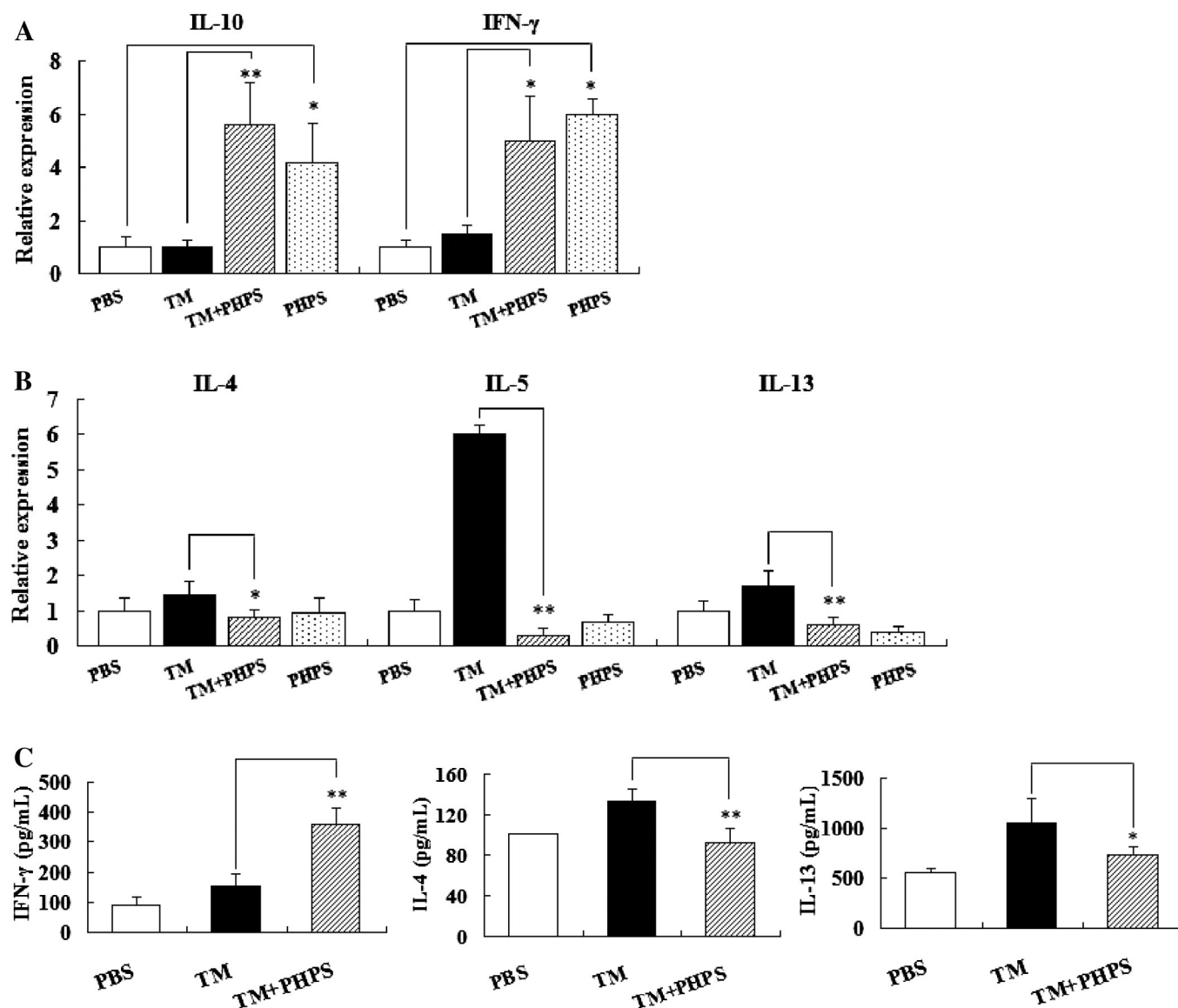


Fig. 5. Therapeutic treatment with PHPS down-regulates the Th2 response in the splenic lymphocytes, whereas it induces the Th1 cell response. (A) The expression of IL-10 and IFN- γ in the splenic lymphocytes from individual mice was assessed by QPCR. (B) The expressions of IL-4, IL-5 and IL-13 in the splenic lymphocytes from individual mice were assessed by QPCR. All results normalized against HPRT and β -actin expression are reported as mean $\pm$ SD. (C) IFN- γ , IL-4 and IL-13 in the supernatants of cultured splenic lymphocytes from mice were measured. Three replicates for each measurement were performed. The results represent the mean $\pm$ SD. * p < 0.05; ** p < 0.01.

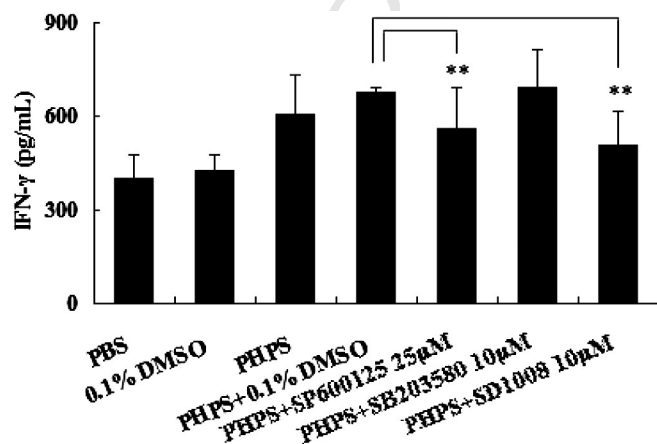


Fig. 6. JNA pathway and JAK2 pathway involved PHPS-induced IFN- γ production in mouse splenic lymphocytes. Three replicates for each measurement were performed. The results represent the mean $\pm$ SD. ** p < 0.01.

4. Discussion

346

This study led to the purification of PHPS, which is a sulfated polysaccharide from *P. haitanensis*. PHPS contained 98.85% of carbohydrate, 14.26% of sulfate and 6.25% of 3,6-AG. Zhang et al. reported that the sulfated polysaccharides isolated from *P. haitanensis* showed antioxidant activity, and that their carbohydrate content was 41.2–60.4%, which is less than that of PHPS (98.85%); sulfate content was 17.4–33.5%, which is more than that of PHPS; and 3,6-AG content was 1.5–12.4%, which is the same as that of PHPS. Galactose was the main sugar unit of the sulfated polysaccharides. Considering the sulfated polysaccharides obtained from Zhang et al. contained less carbohydrate than that of PHPS, it is possible that, in the process of removal of the protein or fat, the use of organic solvent caused loss of the carbohydrate. The sulfate content is mainly related to the growing environment and the harvesting season of *P. haitanensis*.

In order to assess the effects of the injection time (7 days before or 1 day after the first immunization) of PHPS *in vivo* on its anti-allergy activity, we used the mouse model of TM sensitization previously

established in our laboratory [9]. IgE is an important indicator of type I hypersensitivity. IgE is bound to the surface of mast cells and basophils that express high-affinity IgE receptors. Cross-linking of surface-bound IgE on these cells leads to the release of inflammation mediators and a large variety of cytokines, including IL-4, IL-5 and IL-13. Hence, the specific serum IgE is the hallmark of a Th2 immune response [33]. We have demonstrated that the TM-induced IgE level was significantly reduced by repetitive injection of PHPS 7 days before immunization. However, when PHPS was injected after immunization with TM, it had no effect on the IgE level. These results are similar to those of a previous study by Yanase et al. [34] on fucoidan, which is also a type of algal polysaccharide. It was reported that the total IgE level was significantly suppressed by constant injections of fucoidan before immunization with ovalbumin (OVA), and only a small effect on IgE was seen when fucoidan was injected after immunization. Furthermore, we studied the IgG1 and IgG2a levels of the TM-sensitized mice injected with PHPS at different times. The IgG1 levels in the sera were significantly suppressed by injection of PHPS before or after the first immunization. However, IgG2a only increased in the PHPS preventive group. In mice, the Th2 response results in IgE and IgG1 production, while the Th1 response leads to IgG2a production [35]. As shown in Fig. 3B, the TM-specific antibody detected by ELISA showed an increase in PHPS preventive groups, however, as to the western blot, the PHPS preventive group showed negative. This is because the TM used in Western blotting analysis is SDS-denatured, its structure is different from that of the natural TM used in ELISA. And there is the sensitivity difference between western and ELISA, for the enhanced chemiluminescent (ECL) is not as sensitive as ELISA, the concentration of specific IgE antibody was lower than the detection limit of ECL, thus the result was negative. These results suggest that the immunoglobulins and imbalance of the Th1/Th2 immune response were significantly modulated only by injection of PHPS before immunization with TM. To act as an allergic preventative, PHPS needs to be administered prior to allergen sensitization, such as one week before.

The aim of the gavage experiment was to investigate the effect of PHPS on histamine level in fecal and the impaired allergic jejunum tissue. The experimental design of *in vivo* sensitization and PHPS treatment referred to the scheme described by Costa et al. [29]. The antibodies were regulated of the PHPS preventive group in the intraperitoneally experiment, so the mice in the PHPS preventive group was investigated in the following gavage experiment. While the PHPS treatment group was not effective. It illustrated that immune response were not modulated when injection of PHPS after immunization with TM, so further investigation of this group was put aside.

Histamine release is a clinical indicator in type I hypersensitivity. In this study, the fecal histamine level was significantly inhibited by PHPS compared with the TM group ($p < 0.05$). A similar result was reported by Schiavi et al. [36], who found that oral therapeutic treatment with a probiotic mixture in a murine model decreased the fecal histamine level compared with the level found before therapeutic treatment, and the inhibition rate of histamine was the same as ours.

In the histopathological examination, some oedema and lymphocytic infiltration of the jejunum were observed in the TM-sensitized mice. These results are similar to those of previous studies by Birmingham et al. [37] and Chen et al. [38]. Birmingham et al. [37] found that mice sensitized with nuts displayed severe oedema in the jejunum, and Chen et al. [38] reported that lymphocytic infiltration was observed in the jejunum of mice sensitized with rice seed protein.

We demonstrated the *in vitro* anti-allergy activity of PHPS on splenocytes. We re-stimulated spleen cells from TM-sensitized mice with TM in the presence or absence of PHPS. IL-4 is not only critical to the development of Th2 cells but also mediating antibody isotype switching to IgE synthesis [39]. Moreover, IL-4 and IL-13 are important in directing the B-cell growth and differentiation and secretion of IgE and IgG1 [40]. Therefore, we determined the effects of PHPS on the IL-4, IL-5 and IL-13 levels in splenocytes. The IL-4, IL-5 and IL-13 levels

tended to decrease when co-cultured with PHPS. Th1 cells, characterized by the production of large amounts of IFN- γ , control inflammatory immune responses and mediate the defense against intracellular pathogens [41]. IFN- γ can suppress the production of IL-4 and IL-5 by resting Th2 cells, inhibit the secretion of IgE and histamine and increase the production of IgG2a to further suppress allergic reactions [42]. IL-10 was reported to have anti-inflammatory and suppressive effects on most hematopoietic cells, and it suppresses cytokine production and proliferation of antigen-specific CD4⁺ T effector cells indirectly [43]. IL-10 is the T regulatory type 1 (Tr1) cytokine. In our research, IL-10 increased by PHPS could down-regulate the TM-induced Th2 cell response. Moreover, IL-10 has reported as an important role in the regulation of Th2 responses and allergic responses, and an important therapeutic candidate for the treatment of allergy and asthma [44]. IL-10 might have side effects only when it reaches a certain concentration. In our research, only the mRNA relative expression of IL-10 was detected, further research on the protein level of IL-10 need to be detected. Our results of IL-10 were in accordance with those of a previous study by Schiavi et al. [36], that VSL#3 bacteria were also able to significantly increase IL-10 production both in the presence or in the absence of the antigen; thus, higher IL-10 levels in our study might reflect that the PHPS has the ability to regulate the TM-induced allergic reaction. Transforming growth factor- β (TGF- β) and IL-10 both were produced by IL-10-secreting T regulatory type 1 (Tr1) cells. TGF- β is a pleiotropic cytokine with a potent regulatory capacity that has a very important role in the suppression of immune responses, especially during induction and maintenance of peripheral tolerance to allergens [45]. Hence, further research will be needed to clarify the effect of PHPS on the TGF- β *in vitro* and *in vivo*. In our study, the expressions of Treg/Th1 cytokines such as IL-10 and IFN- γ were increased by PHPS in the presence or in the absence of the allergen. These results support the idea that PHPS could suppress the allergic reaction through modulation of the Th1/Th2 imbalance by promoting the Th1 cell response and down-regulating the TM-induced Th2 cell response.

During these experiments, we found that IFN- γ expression was significantly promoted by PHPS. Therefore, inhibitors of JNK, p38 MAPK and JAK2 were used to determine which signaling pathways might be involved in the PHPS-induced secretion of IFN- γ . We observed that pre-treatment of cells with JNK and JAK2 inhibitors blocked the increase in IFN- γ . In contrast, SB203580 had no effect. This is a novel finding that may partially explain how PHPS induces IFN- γ protein expression. Other signaling pathways of IFN- γ and the signaling pathways of relevant allergic cytokines such as IL-4 and IL-13 need further investigation.

In conclusion, we have purified the sulfated polysaccharide (PHPS) from *P. haitanensis*, and its major ingredients are galactan and sulfate. We have shown that intraperitoneal injection of PHPS can redirect allergen-specific Th2-polarized immune responses toward Th1 regulatory responses in established murine Th2 immune responses; oral therapeutic administration of PHPS to TM-sensitized mice significantly reduced histamine release and alleviated the pathology in the jejunum in a murine model of food allergy. These results suggest that PHPS is able to regulate the allergic response to the allergen (TM) in mice. Therefore, to develop PHPS into a functional food, detailed studies of the effects of PHPS from different species of porphyra, the molecular weight of PHPS and the effects of active sulfate groups must be conducted.

5. Uncited references

[32].

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