MASTER'S THESIS

Immune-modulation effect of *Sargassum horneri* polysaccharides on concanavalin A-stimulated splenocytes and allergic asthma mouse model

Jiwon Yang

Department of Animal Biotechnology
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

August 2022

Immune-modulation effect of Sargassum horneri polysaccharides on concanavalin A-stimulated splenocytes and allergic asthma mouse model

Jiwon Yang

(Supervised by professor Young-Ok Son and Youngheun Jee)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science.

August 2022

This thesis has been examined and approved by

Thesis director, Kyoungtag Do Prof. of Animal Biotechnology, Jeju National University

---

Young-Ok-Son, Prof. of Animal Biotechnology, Jeju National University

---

Youngheun Jee, Prof. of Veterinary Medicine, Jeju National University

August 2022

Department of Animal Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

List of Figures

List of Tables

List of Abbreviations

ABC	Avidin-biotin complex
Con A	Concanavalin A
COX	Cyclooxygenase
DAB	Diaminobenzidine
ELISA	Enzyme linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA binding protein 3
H	Hour
HC	Healthy control
H&E	Hematoxylin and Eosin
HRP	Horseradish peroxidase
HPLC-DAD	High performance liquid chromatography with diode array detector
INOS	Inducible nitric oxide synthase
IL	Interleukin
IgE	Immunoglobulin E
IHC	Immunohistochemistry
LPS	Lipopolysaccharide
LDH	Lactose dehydrogenase
MAPK	Mitogen-activated protein kinase
MUC	Mucin
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B
OVA	Ovalbumin
PM	Particulate matter
PAS	Periodic acid of Schiff

PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
RORγt	Retineic-acid-receptor-related orphan nuclear receptor gamma
S. horneri	*Sargassum horneri*
SHPS	*Sargassum horneri* polysaccharides
SEM	Standard error of mean
SSBs	Sugar-sweetened beverages
T-bet	T-box expressed in T cells
TGF-β	Transforming growth factor beta
Th2	T helper type 2
Th17	T helper type 17
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-α

ABSTRACT

Sargassum horneri (*S. horneri*) is an edible species of large brown algae inhabiting the coasts of northeastern Asia extensively. *S. horneri* has amino acids, polyphenols, and polysaccharides, that have various biological activities such as anti-oxidative, anti-inflammatory, immune-regulatory, anti-viral, and anticoagulant. However, their molecular immunological mechanisms are still unknown. This study investigated the curative effects of *S. horneri* polysaccharides (SHPS) on allergic asthma progression. First, we performed cell viability assays, LDH and ^{3}H-thymidine incorporation of *S. horneri* on splenocytes to examine its cytotoxicity. There was no cytotoxic effects on concentrations 3.9 ~ 250 μg/mL of SHPS. SHPS increased the expression of toll-like receptors (TLRs) in splenocytes of ConA stimulation. SHPS reduced the production of transcription factors and cytokines involved in the differentiation of diverse Th cell types such as Th1 (IL-β, IFN-r, TNF-α), Th2 (IL-4, IL-10), and Th17 (IL-17A, IL-22) in ConA-stimulated splenocytes. Moreover, SHPS significantly decreased the abundance of B cells ($CD19^{+}CD45R/B220^{+)}$ and macrophages ($CD45^{+}CD11b^{+}$) in ConA-stimulated splenocytes. In addition, SHPS suppressed TLRs expression and Th-type cytokines (Th1, Th2, and Th17) in lungs of PM-induced allergic asthma mice. The histopathological results confirmed that SHPS mitigated lung tissue inflammation and suppressed the hypersecretion of mucin in the PM-induced allergic asthma mouse model. The mucin secretion is regulated by genes such as MUC5AC, MUC5B, and SHPS reduced MUC2 in PM-induced allergic asthma mice. In this study, we provide a compelling rationale that SHPS may afford a promising approach for immunotherapy as an alternative for the treatment of allergic asthma induced by ambient PM.

Keywords: *S. horneri* polysaccharides (SHPS), concanavalin A (ConA), inflammation, asthma

I. INTRODUCTION

Allergic asthma is a chronic inflammatory disease caused by inflammation of airways, characterized by mucus overproduction, airway wall remodeling, and bronchial hyperresponsiveness [1]. Common causes of asthma are viral infection, tobacco smoke, particulate matter (PM), and nonsteroidal inflammatory drugs, etc., and symptoms include breathlessness, chest tightness, and coughing due to airway inflammation [2]. Allergic asthma is an abnormal immune response. It is driven mainly by Th2-mediated reactions and cytokine, and is associated with the promotion of immunoglobulin E (IgE) production by allergen-specific B cells [3]. In addition, allergic asthma, especially those of severe form is also known to involve the Th1and Th17 immunity [4]. Th1 and Th17 cells producing IL17A, IL-22, IFN-γ, and TNF-α cause neutrophil recruitment and mucin hypersecretion [5]. Mucin is the first line of defense of our respiratory tract and is essential for homeostasis of airway epithelium [6]. Mucin is a thread-like molecule containing liner peptides, apomucin [7]. However, mucins encoded by genes like MUC2, MUC5B, and MUC5AC form through atypically abundant post-translational glycosylation and constitute mucosal barriers in airways [8]. In support of mucin hypersecretion in allergic asthma, MUC5AC, has been reported to be increased in asthma patients [8]. The ratio of MUC5B and MUC5AC has been observed similar, but MUC2 has been found to present in a small amount in asthma patients [8]. Bronchodilators and anti-inflammatory inhaled corticosteroids have been the mainstay of treating allergic asthma [9]. However, when used for a long time, they have side-effects like weight-gain due to changes in body metabolism, and a reduction in bone mineral density [10]. Therefore, new therapeutic approaches to reduce side effects is necessary urgently needed, and natural products have been regarded as primal candidates for such improved treatments [11].

Marine-based seaweed polysaccharides have been drawing increasing attention owing to their great potential in biomedical and pharmaceutical applications, and brown algae are a great source of such polysaccharides [12]. Brown algae polysaccharides have been found to contain various active components like alginate oligosaccharides, laminarins, fucoidans, and alginic acids [13]. It has been reported that they have beneficial functional properties of antioxidant activity, immune regulation, anticancer, anti-inflammatory, antiviral, antidiabetic functions [13]. For instance, a study of *Sargassum spp* reported that polysaccharides and polyphenolic compounds are about 27% and 20% of all dry weight, respectively [14]. *Sargassum fusiforme* has been reported to inhibit the growth of A549 lung adenocarcinoma cells by promoting IL-1β and TNF-α production in peritoneal macrophages and suggested as an antitumor agent with immunomodulatory activity [15].

Sargassum horneri (*S. horneri*), another member of the *Sargassum f*amily, is an edible brown algae plentiful along the coasts of South Korea, China and Japan [16]. They can grow to more than 7m in length with over 3 kg in fresh weight [16]. Their gas-filled elongated cylindrical vesicles can float long distances away when detached from the substratum [17]. Because of this, they play an important role in providing an environment serving as spawning and nursery grounds for various marine species in the coastal ecosystem, and maintaining local marine biodiversity [18]. Recently, however, too much *S. horneri* afloat is flowing into the Jeju sea, rendering it as a serious threat to biodiversity in local coastal ecosystem, by disturbing aquaculture and fisheries and causing severe damage to local tourism economy [18]. All this makes it urgent to look for the possibility of using *S. horneri* in ways beneficial to inhabitants of Jeju, especially for those who make their living by fishery or tourism. Many studies, including the present one, have examined medicinal ways to use *S. horneri.* In particular, polysaccharides isolated from *S. horneri* have been the main focus of these studies. For instance, it has been reported that lipopolysaccharide (LPS) isolated from *S. horneri* could inhibit pro-inflammatory cytokines like TNF-α and IL-1β and inflammatory factors such as

nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in RAW264.7 cells [19]. Among polysaccharides, laminarins have been shown to reduce inflammation through suppressing TLR-mediated NF-kB and MAPK pathways in RAW264.7 [20]. Even though such inflammation occurs as a physiological defense mechanism against exposures to foreign substances such as microbes or environmental allergens, it can cause various diseases such as asthma, rheumatoid arthritis, and hepatitis if occurs excessively.

In this study, we investigated the immunological response to SHPS and its underlying mechanisms against PM-induced allergic asthma in mice using a high-yield, reusable enzyme digestion method using *S. horneri* ethanol extracts (SHE) under the following research hypotheses.

Research hypothesis

(1) To investigate the immune responses on SHPS in murine splenocytes with or without concanavalin A stimulation

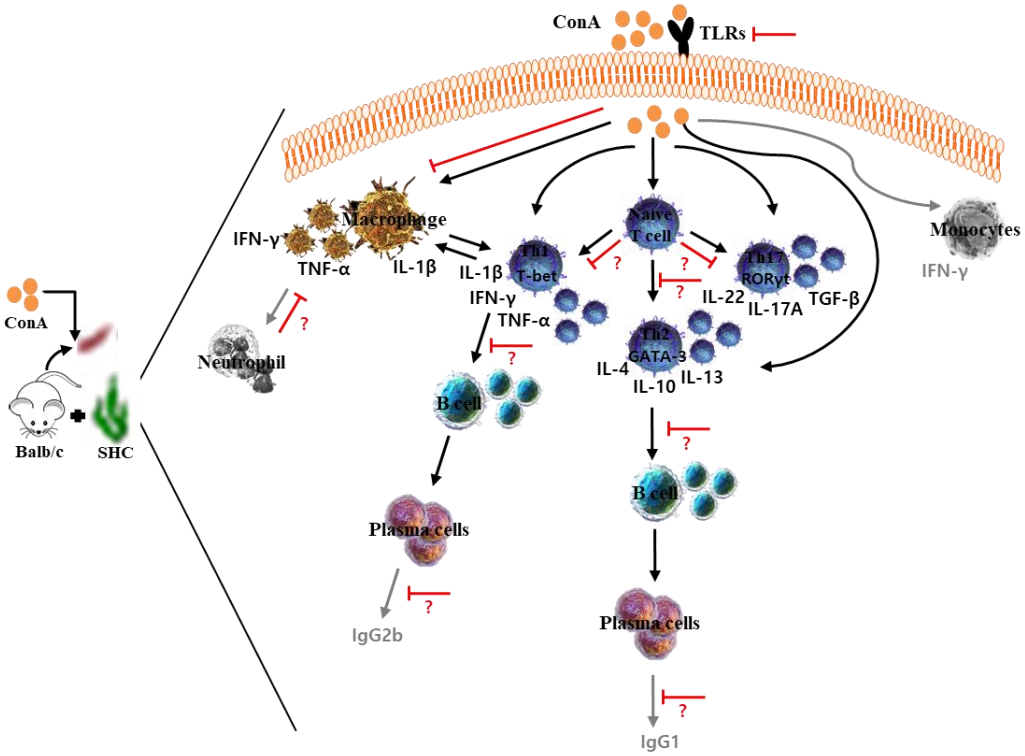


Figure 1. Proposal hypothesis of immune-modulation of SHPS against ConA stimulation. (*In vitro model*)

(2) To investigate the pathological mechanism of SHPS in allergic asthma

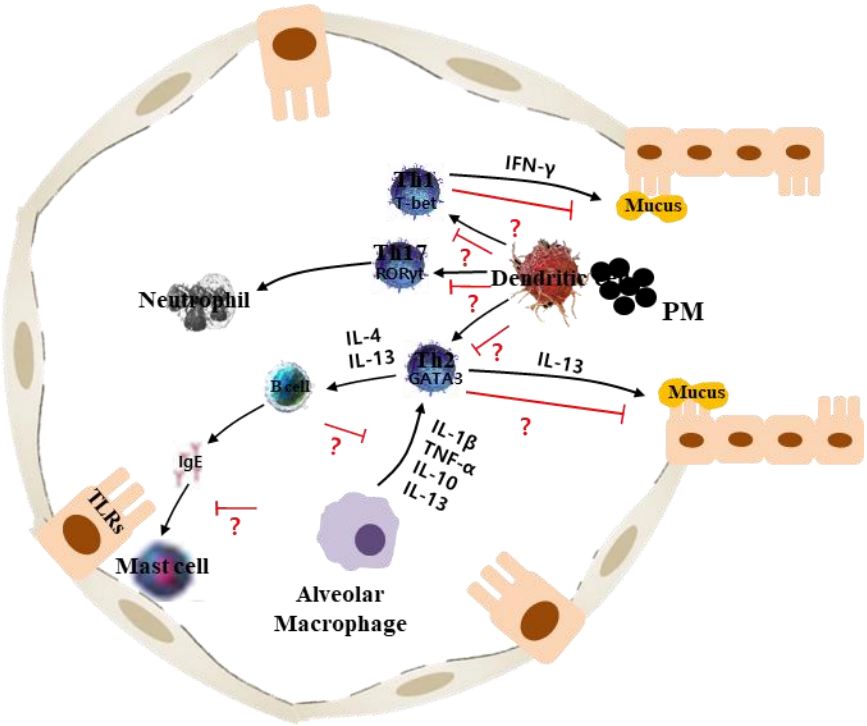


Figure 2. Proposal hypothesis of immune modulation mechanism of SHPS in PM triggered allergic asthma. *(In vivo model)*

II. MATERIALS AND METHODS

Particulate matter

The environmental certified reference material (CRM)-No.28 urban aerosol collected from a central ventilating system of a building in Beijing purchased from National Institute for Environmental Studies (NIES), Japan was used as the PM in this study. When observed under an electronic microscope, the majority of PM was found to be composed of fine dusts with median diameter <2.5 μm containing hazardous heavy metals (Ba, Cr, Pb, Ni and Cu) and polycyclic aromatic hydrocarbons. For details, please refer to a previous publication [21].

Preparation of SHPS

S. horneri was collected from the coasts of Jeju island, South Korea, and SHPS was prepared as shown schematically in Figure 3. In brief, washed *S. horneri* was dried at 50℃, and ground and filtered using a 40-50 mesh. The powdered *S. horneri* (100g) was dissolved in 70% EtOH solution (2L) for 24 hours at room temperature. Arsenic was removed from the solution using citric acid, and SHPS was extracted by adding distilled water with cellulast enzyme 100:1 ratio at room temperature for 24 hours. The supernatants were filtered with 0.45uM filter papers, and the filtered supernatants (SHPS) were collected from the filter paper after dry at 37℃. Afterward, obtained SHPS were freeze-dried and stored at -20℃ until use.

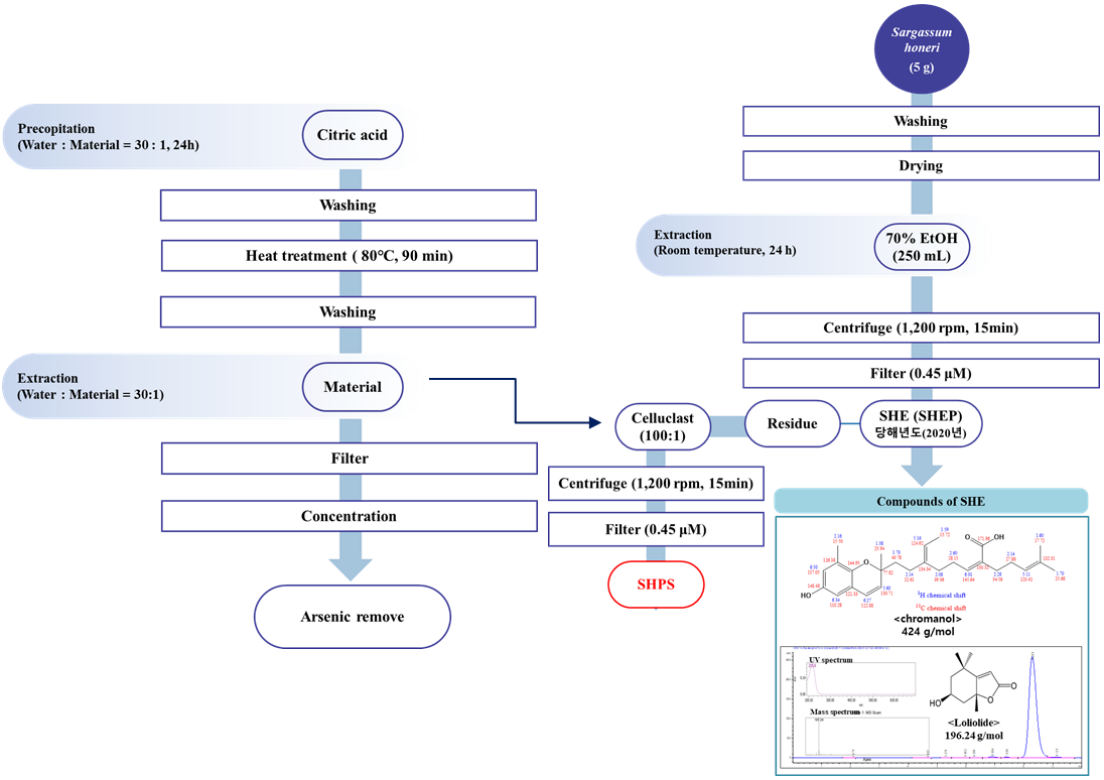


Figure 3. Schematic diagram of extraction.

Analysis of phenolic acid using the HPLC-DAD system

Phenolic acid contents of SHPS were evaluated by using reversed-phase high-performance liquid chromatography using a diode array detector (Agilent 1260 series; Agilent Technologies, USA) and reversed-phase Pursuit XRs C18 column (250 mm × 4.6 mm; 5 μm; Agilent Technologies, USA). The sample analysis was carried out at 27 ℃ with the flow rate 1 mL/min. Waster and 0.1% acetonitrile addition of formic acid was mobile phase. The solvent gradient was as follows: 0-5 min, 20% B isocratic; 5-30 min, linear gradient from 20% to 60% B; 30-35 min, 60% B isocratic; 35-40 min, linear gradient from 60% to 20% B and, finally, washing and reconditioning of the column. Identification of phenolic acids was performed by comparing their retention times and spectra in UV (270 nm: gallic acid, protocatechuic acid, catechin, and vanillic acid; 324 nm: chlorogenic acid, gentisic acid, caffeic acid, coumaric acid,

and ferulic acid: 373 nm, myricetin and quercetin) with those for standards. The SHPS sample, standards, and mobile phases were filtered through 0.45 μm filters (hydrophobic PTFE membrane filter; SciLab Korea Co., Ltd., Seoul, Korea) prior to HPLC injection.

Animals

4-5 weeks old Balb/c mice purchased from Orient Bio (Gwangju, Korea) were used in this study. Before experimentation, they were housed in ventilated cages under pathogen-free conditions at constant temperature 23 ± 1.5°C and 55 ± 15% humidity with a 12 h light-dark cycle. They were fed with approved diet and water ad libitum. All experiments were performed as approved by the Institutional Ethical Committee of Jeju National University (No. 2018-0021).

Preparation of murine splenicytes

Spleens were removed from C57BL/6 mice and treated to red blood cell lysis buffer at room temperature for 10 min and was a centrifuge. After being washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco BRL, Life Technologies, New York, USA), purified cells were used directly for experiments.

Animal model

The mouse model of asthma whose experimental schedule is schematically shown in Figure 4 was based on the OVA-sensitized mouse model following the previously described PM challenge study with some modifications [22]. Female and male Balb/c mice (4-5 weeks, old) were randomly divided in to the following groups (n=4~6 each): healthy control, where no experimental intervention was applied during the full three-week schedule, OVA only where mice were sensitized by an intraperitoneal (i.p) injection of 10 μg OVA with 2 mg AI(OH)3 in 200 μL saline at day one but no other later experimental intervention was applied,

PM only where mice were exposed to sonicated PM only (5 mg/m3, 30 min/day) from day 15 to day 21 without OVA sensitization at day 1 or other intervention of SHPS or Prednison, OVA+PM where mice were sensitized with OVA at day 1 and exposed to sonicated PM from day 15 to 21 but no other intervention of SHPS or Prednison, OVA+PM+SHPS 200 where mice were sensitized with OVA at day 1 and then exposed to sonicated PM and treated orally with SHPS (200mg/kg) simultaneously from day 15 to 21, OVA+PM+SHPS 400 where mice were sensitized with OVA at day 1 and then exposed to sonicated PM and treated orally with SHPS (400mg/kg) simultaneously from day 15 to 21, and OVA+PM+Prednison where mice were sensitized with OVA at day 1 and then exposed to sonicated PM and treated orally with Prednison (5mg/kg) simultaneously from day 15 to 21.

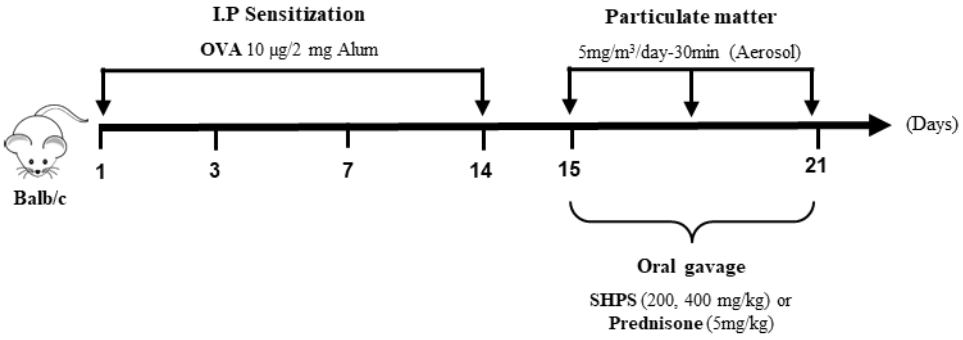


Figure 4. Schematic diagram of experimental setup of PM-induced animal model allergic asthma in mice.

LDH (Lactose dehydrogenase) assay

The cytotoxic effect by SHPS was measured using LDH assay. Splenocytes (1×10^4) were seeded on 96-well plates, treated with varying concentrations (0–250 μg/mL) of SHPS with or without Con A (5 μg/mL), and incubation for 24h or 48h at 37 °C. After incubations, culture supernatants were reacted with similar volume of LDH reaction solution for 30 min according to manufacturer's instructions. The absorbance was measured at a wavelength 490nm, and the

amount of LDH release in treated cells was expressed as a percentage of LDH release in untreated cells.

^{3}H-thymidine incorporation assay

The cell proliferation effect by SHPS was measured using 3H thymidine incorporation assay in the presence or absence of Con A stimulation. Splenocytes (4x105 cells/well) were seeded in 96-well plates and treated with different concentrations of SHPS (0-250ug/mL) with or without ConA (5ug/mL). After 48 h incubation, 3H-thymidine (1μCi/well, 42 Ci/m mol, Amersham Life Science, Arling-ton Heights, IL, USA) was added and incubated for 18 h. Finally, were harvested onto glass fiber filters, and retained radioactivity was measured by liquid scintillation spectrometry (Wallac MicroBeta ® TriLux, Perkin Elmer, Waltham, MA, USA).

Total RNA extraction, cDNA synthesis and q-PCR analysis

Splenocytes (2x107) were seeded in 10cm dishes. And treated with SHPS (0, 62.5, and 125 μg/mL) with or without Con A and incubation for 48h. Then, total RNAs from splenocytes and mice lung tissues were extracted using Trizol reagent (Life Technologies), after adding chloroform (Sigma-Aldrich) and centrifuged at 15,000 g for 15min. After obtaining supernatants, we added isopropanol for RNA precipitation. Resulting RNA pellets were washed with 70% EtOH and dried at room temperature. cDNA was synthesized using Promega A3500kits (St Louis, MO, USA) according to manufacturer's protocols. qPCR was performed with StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the Power SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The mRNA expression level was analyzed using 2-△△CT method and normalized to that of GAPDH. qPCR primers were listed in Table 1.

Table 1. The primer sequences for real-time quantitative PCR

Gene	Sequence	
	Forward (5'-3')	Reverse (5'-3')
TLR1	GTT GTC ACT GAT GTC TTC AGC	CTG TAC CTT AGA GAA TTC TG
TLR2	CAG CTTA AAG GGC GGG TCA GAG	TGG AGA CGC CAG CTC TGG CTCA
TLR3	GAA GCA GGC GTC CTT GGA CTT	TGT GCT GAA TTC CGA GAT CCA
TLR4	AGT GGG TCA AGG AAC AGA AGC A	CTT TAC CAG CTC ATT TCT CAC C
TLR5	GAA TTC CTT AAG CGA CGT AA	GAG AAG ATA AAG CCG TGC GA
TLR6	AGT GCT GCC AAG TTC CGA CA	AGC AAA CAC CGA GTA TAG CG
TLR7	CCT GTT CTA CTG GGG TCC AA	GCC TCA AGG CTC AGA AGA TG
TLR8	GGC ACA ACT CCC TTG TGA TT	CAT TTG GGT GCT GTT GTT TG
TLR9	CCA GAC GCT CTT CGA GAA CC	GTT ATA GAA GTG GCG GTT GT
T-bet	AT GTT TGT GGA TGT GGT CTT GGT	CG GTT CCC TGG CAT GCT
GATA-3	CA AGC TTC ATA ATA CCC CTG ACT ATG	GC GCG TCA TGC ACC TTT T
RORγt	CA CGG CCC TGG TTC TCA T	GC AGA TGT TCC ACT CTC CTC TTC T
IL-1β	GCT ACC TGT GTC TTT CCC GTC G	TTG TCG TTG CTT GGT TCT CCT TG
IFN-γ	AGG TCA ACA ACC CAC AGG TCC A	CCA GAT ACA ACC CCG CAA TCA C
TNF-α	GGC AGC TTC TGT CCC TTT CAC TC	CAC TTG GTG GTT TGC TAC GAC G
TGF-β	GCC ATC TAT GAG AAA ACC AAA G	TTA GTT CAC ACC TCG TTG TAC
IL-4	ACG GAG ATG GAT GTG CCA AAC GTC	CGA GTC ATC CAT TTG CAT GAT GC
IL-10	GCT ATG CTG CCT GGT CTT ACT G	TCC AGC TGG TCC TTT GTT TG
IL-13	CAA TTG CAA TGC CAT CTA CAG GAC	CGA AAC AGT TGC TTT GTG TAG CTG A
IL-17a	TCA ACC GTT CCA CGT CAC CCT GGA C	TCA GCA TTC AAC TTG AGC TCT CAT GC
IL-22	ACC TTT CCT GAC CAA ACT CA	AGC TTC TTC TCG CTC AGA CG
MUC5AC	AAA GAC ACC AGT AGT CAC TCA GCA A	CTG GGA AGT CAG TGT CAA ACC
MUC5B	AAA GAC ACC AGT AGT CAC TCA GCA A	CTG GGA AGT CAG TGT CAA ACC
MUC2	GCT GAC GAG TGG TTG GTG AAT G	GAT GAG GTG GCA GAC AGG AGA C

MUC: Mucin; IL: Interleukin, T-bet: T-box transcription factor TBX21, TNF-α: Tumor necrosis factor-α, TGF-β: Transforming growth factor beta, TLR: Toll like receptor; GATA3: GATA binding protein 3; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; RORγt: Retineic-acid-receptor-related orphan nuclear receptor gamma.

Enzyme-linked immunosorbent assay (ELISA) analysis

Splenocytes were incubated for 48h with varying concentrations of SHPS (0, 62.5, and 125 μg/mL) with or without Con A. Levels of IL-1β, IFN-γ, TNF-α, IL-17A, and IL-22 in culture supernatants and serum were measured using ELISA MAX deluxe set mouse kits (BioLegend, SanDiego, CA, USA) following manufacturer's instructions.

Flow cytometric analysis

Splenocytes ($2x10^6$ cells/well) were seeded in 24-well plates, treated with different concentration (0,125 μg/mL) of SHPS with or without Con A. It incubation for 48h and harvested. After that cells were stained with fluorophore antibody CD45R/B220 (RA3-6B), CD19 (ID3) purchased from Biolegend (Sand Diego, CA, USA) and CD45 (30-F11) purchased from e bioscience (Sand Diego, CA, USA) and CD11b (MI/70) purchased from BD Biosciences (Franklin Lakes, NJ, USA) for 15 min. 20,000 cells per treatment were assayed using CytoFLEX flow cytometer (Beckman Coulter, lnc. CA, USA) and was evaluated using CytExpert software (version 1.2, Beckman Coulter, lnc.).

Histopathological analysis of lung tissues

Mice were sacrificed after exposure to saline, PM, SHPS, and Prednisone for 7 consecutive days from day 15. Lung samples were fixed in 10% formalin and embedded in paraffin. Then the paraffin blots were sectioned with 3 μm thickness, and histological changes were evaluated after hematoxylin and eosin (H&E) staining. The periodic acid of Schiff (PAS) staining was performed to visualize the mucus-secreting cells. Each stained section was

dehydrated in fractionated ethanol and cleared with xylene. Stained sections were observed using a DP-72 microscope camera system (Olympus, Tokyo, Japan).

Immunohistochemistry (IHC)

Paraffin-embedded lung sections were de-waxed and rehydrated in ethanol. Tissues were immersed in 0.3% hydrogen peroxide for 40 min, followed by an incubation in horse serum to block nonspecific binding. Tissues were then incubated with primary anti-mucin 5AC (MUC5AC) antibody (1:500, Abcam, Cambridge, MA, USA), Muc5B antibody (1:1000, Abcam, Cambridge, MA, USA), and MUC2 (1:1000, Abcam, Cambridge, MA, USA) overnight at 4 °C. After washing, tissues were incubated with biotinylated anti-mouse serum for 45 min. Tissues were washed in PBS, and an avidin-biotin-peroxidase complex binding reaction was performed using horseradish peroxidase (HRP)-labeled Vectastain Elite ABC kit (Vector). HRP binding sites were detected with DAB. Counterstaining was performed with hematoxylin, and positive cells were quantified using ImageJ (v1.46) software under the DP-72 microscope (Olympus, Tokyo, Japan).

Statistical analysis

Numerical data are presented as the means ± standard error of S.E.M. Statistical analysis was performed by using Student's t-test for two-group comparisons and using ANOVA for multiple group comparisons. $P < 0.05$ is considered significant in this study.

III. RESULTS

Proximate composition of the SHPS

The chemical composition profile of SHPS is summarized in Table 2. The energy content of SHPS is 164.2 kcal/100g. SHPS is composed of carbohydrate (36.9 %), moisture (50.0 %), ash (9.1 %), protein (3.9 %), and fat (0.1 %) by weight. These results indicate that SHPS is mostly composed of carbohydrates when dried.

Table 2. Proximate composition of general components in SHPS

Components	Composition
Energy (kcal/100g)	164.2
Carbohydrate (%)	36.9
Moistures (%)	50.0
Ash (%)	9.1
Protein (%)	3.9
Fat (%)	0.1

Phenolic acid composition of SHPS

The phenolic acid composition of SHPS is shown in Table 2. The HPLC chromatogram of phenolic acids in Figure 5 were provided. Among 11 types of phenolic acids, gallic acid and protocatechuic acid were detected as 93.53 and 29.24 μg/g, respectively. Additionally, the smaller peaks were detected in HPLC chromatograms, however, they were not identified in current study. Therefore, these results indicated that phenolic acid of SHPS was composed of gallic acid and protocatechuic acid.

Table 3. Total polyphenol content(mg GAE/g) and phenolic acid contents(mg/g) of *Sargassum horneri* Polysaccharide(SHPS)

Components		content
Phenolic acids (μg/g)[b]	Gallic acid	93.53±14.74[c]
	Protocatechuic acid	29.24±8.41
	Catechin	N.D.*
	Vanillic acid	N.D.
	Chlorogenic acid	N.D.
	Caffeic acid	N.D.
	Gentisic acid	N.D.
	p-Coumaric acid	N.D.
	trans-Ferulic acid	N.D.
	Myricetin	N.D.
	Quercetin	N.D.

[a] Total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g SHPS
[b] Gallic acid of SHPS was quantified by establishing calibration curves in the range of 3.13, 6.25, 12.5, 25, 50 μg/mL by HPLC analysis
[c] Result is given as the means ± SD based on triplicate determinations.
*N.D. standards for not detected

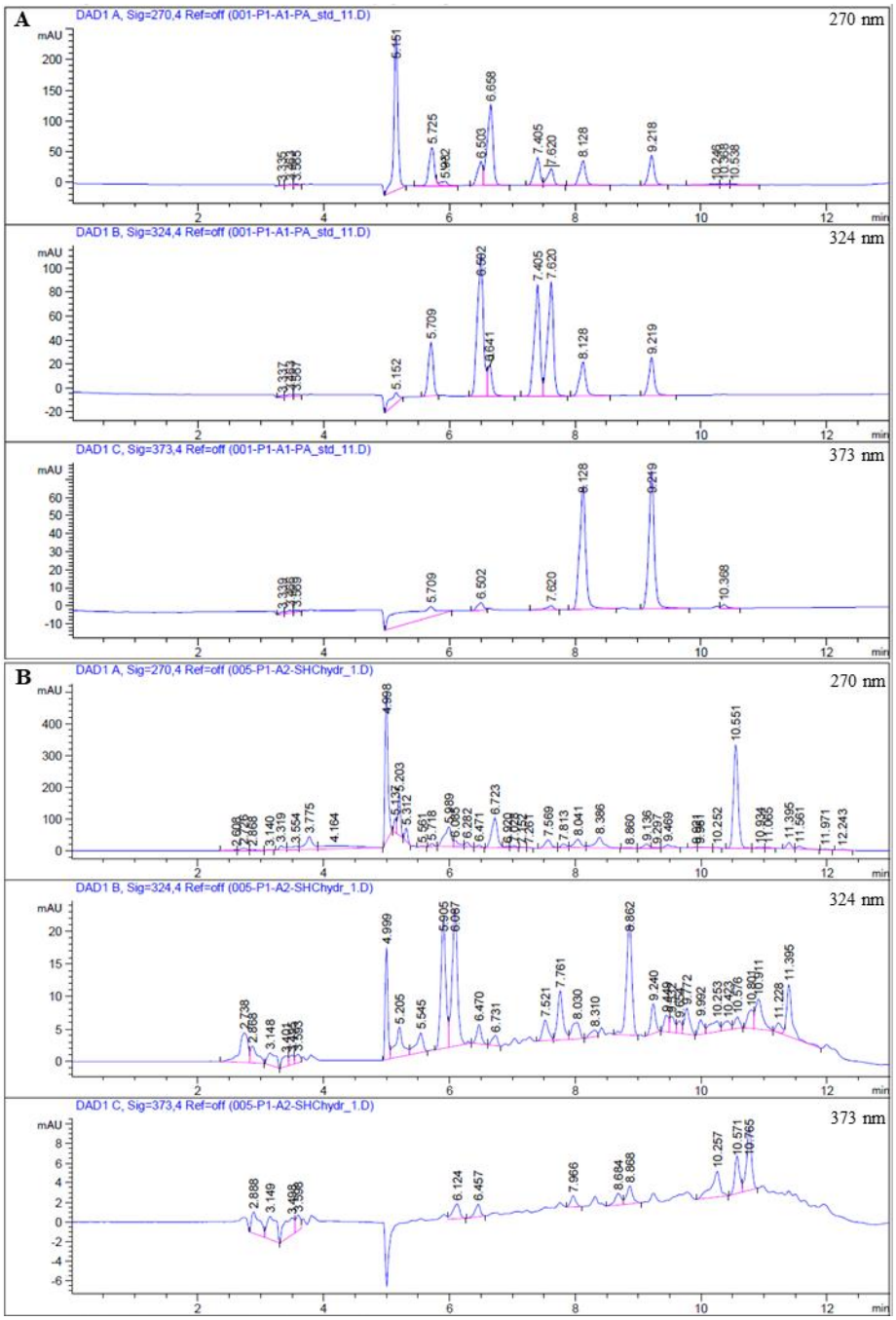


Figure 5. HPLC chromatograms of (A) phenolic acid standards and (B) phenolic acids from SHPS.

The cytotoxicity and proliferation of SHPS in murine splenocytes with or without Con A stimulation

We performed LDH assay to evaluate the cytotoxicity of SHPS on murine splenocytes, and the results are shown in Figure 6. SHPS had no cytotoxic effect at concentrations 0-250 μg/mL after 24 h and 48 h incubation (Fig.6A and 6B). Cytotoxic effect (Fig.6D and 6E) was not observed even in the presence of Con A (5 μg/mL) stimulation when cells were treated with SHPS (0-250 μg/mL). In parallel, the proliferation activating capacity of SHPS was evaluated using ^{3}H-thymidine incorporation assay. When cells were treated with various concentrations of SHPS, the proliferation of splenocytes was significantly increased compared to the control group at concentrations 7.8-125 μg /mL of SHPS (Fig.6C). However, the proliferation was significantly decreased in Con A-stimulated splenocytes when treated with SHPS at concentrations 15.6-250 μg/mL (Fig.6F).

Based on these results, subsequent experiments of SHPS were carried out at concentrations 62.5 and 125 μg/mL.

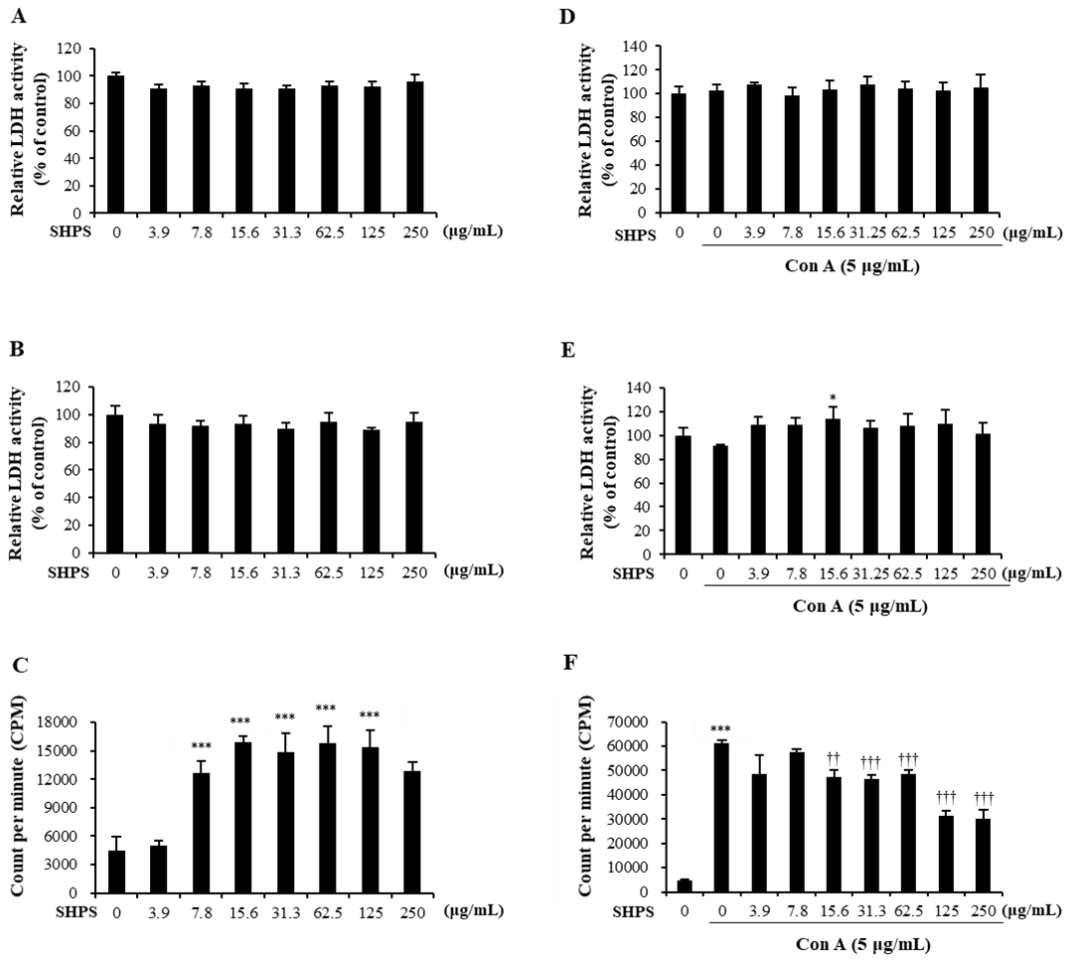


Figure 6. Effect of SHPS on cytotoxicity and proliferation in splenocytes. Relative LDH activity measurements in cells treated with varying concentrations (0–250 μg/ml) of SHPS alone (A) for 24 h and (B) for 48 h or in the presence of Con A (5 μg/ml) (D) for 24 h and (E) for 48 h against that in untreated controls are shown. Also shown is the amount of radioactivity in cells treated with SHPS in the (C) absence or (F) presence of Con A (5 μg/ml) measured after ^{3}H-thymidine incorporation assay. Data are represented as the mean ± SEM of three independent experiments. * ($p < 0.05$) and *** ($p < 0.0005$) indicate significant increase compared to that in untreated control and †† ($p < 0.005$) and ††† ($p < 0.0005$) represent significant decrease compared to that in Con A stimulation only.

Effect of SHPS on Toll-like receptors (TLRs) in murine splenocytes with or without Con A stimulation

TLRs play an important role in innate immunity. They recognize microbes and induce pro-inflammatory mediators and various cytokines. To examine the effect of Con A and/or SHPS on TLRs, we measured mRNA expression level of TLR-1/2/3/4/5/6/7/8/9 using qRT-PCR. After exposing splenocytes to SHPS (62.5 and 125 μg/mL) alone or to Con A (5μg/mL) alone or to SHPS and Con A stimultaneously (Fig.7). The expression of TLRs mRNA in Con A alone group was significantly increased compared to the untreated control. Especially, TLR6 and TLR8 mRNA expression increased up to 3.6 and 4.4 folds in the Con A-stimulated splenocytes compared to untreated control (Fig.7A). Notably, the expression of TLR6 and TLR8 were significantly reduced by treatment of SHPS (62.5 and 125 μg/mL) when compared with Con A treated group (Fig.7A).

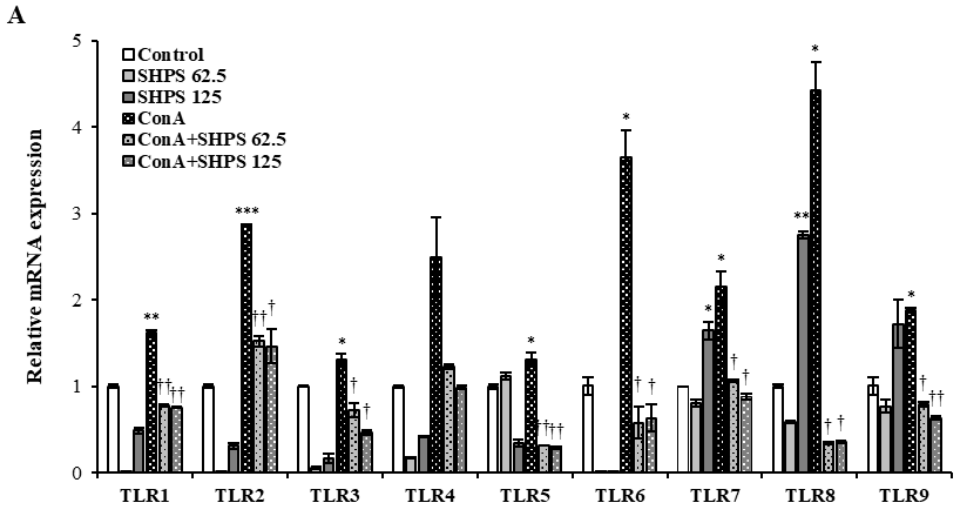


Figure 7. Effect of SHPS on the expression of TLRs in the absence or presence of Con A in splenocytes. The level of mRNA expression of TLR-1/2/3/4/5/6/7/8/9 was measured using real-time PCR with or without Con A stimulation after being exposed to SHPS (62.5 or 125 μg/mL) in splenocytes. * ($p < 0.05$), ** ($p < 0.005$), and *** ($p < 0.0005$) represent significant increase compared to that in untreated control, and † ($p < 0.05$), †† ($p < 0.005$) represent significant decrease compared to that in Con A stimulation only.

The effect of SHPS on the secretion of T helper cell cytokines and the expression of associated transcription factors in murine splenocytes

We evaluated the effect of SHPS on the mRNA expression levels of T-bet (Th1), GATA-3 (Th2), and RORγt (Th17) transcription factors in the presence or absence of Con A stimulation. (Fig.8). When we treated splenocytes with SHPS alone, the mRNA expression levels of T-bet, GATA-3, and RORγt cytokines were increased, though not significant. Among pro-inflammatory cytokines, SHPS (62.5μg/mL and 125μg/mL) increased the mRNA expression of TNF-α (by 1.3 and 1.7 folds, p<0.05 and 0.005, respectively). Also, SHPS (62.5 and 125μg/mL) treatment significantly increased the mRNA expression of Th2 cytokines, IL-4 (1.5 and 2.1 folds, $p < 0.005$ and 0.05, respectivelt; Fig. 7G) and IL-10 (1.5 and 1.7 folds, $p < 0.05$ and 0.005, respectively; Fig.8H), and Th17 cytokines, IL-17A (1.3 folds, $p < 0.05$ at 125 μg/mL; Fig.8J) and IL-22 (3.3 and 3.0 folds, $p < 0.05$ and 0.005, respectively; Fig.8K). In terestingly, when we stimulated T cells with a mitogen, Con A, the level of all cytokines, including those that were not increased significantly when treated with SHPS alone, increased dramatically compared to that in the untreated control. T-bet was increased by 344.6 folds ($p < 0.0005$, Fig.8A), GATA3 by 526.3 folds ($p < 0.0005$, Fig.8B), RORγt by 185.1 folds ($p < 0.0005$, Fig.8C), IL-1β by 22.0 folds ($p < 0.0005$, Fig.8D), IFN-γ by 1061.2 folds ($p < 0.0005$, Fig.8E), TNF-α by 8.8 folds ($p < 0.0005$, Fig.8F), IL-4 by 2.1 folds ($p < 0.005$, Fig.8G), IL-10 by 7.4 folds ($p < 0.005$, Fig.8H), IL-13 by 325.5 folds ($p < 0.0005$, Fig.8I), IL-17A by 258.4 folds ($p < 0.0005$, Fig.8J), IL-22 by 49.1 folds ($p < 0.005$, Fig.8K), and TGF-β by 162.5 folds ($p < 0.05$, Fig.8L), respectively. However, SHPS treatment effectively reduced Con A-induced cytokines significantly. For example, the spiked expression level of Th1 cytokine IL-1β by Con A stimulation was reduced by 11.1 folds ($p < 0.005$) at 62.5 μg/ml and by 13.5 folds ($p < 0.005$) at 125 μg/mL of SHPS (Fig.8D). Con A-stimulated IFN-γ too was attenuated by SHPS by 1.1 folds ($p < 0.005$) at 62.5 μg/mL and by 1.8 folds ($p < 0.005$) at 125 μg/mL. Also, Con

A stimulated Th2 or Th17 cytokines such as IL-4 (by 1.7 and 1.4 folds, $p < 0.05$ and 0.005, respectively; Fig.8G), IL-10 (by 37 and 18.5 folds, $p < 0.0005$ both; Fig.8H), IL-22 (by 1.2 and 1.3 folds, $p < 0.05$ both; Fig.8K), and TGF-β (by 162.0 folds, $p < 0.05$ at 125 μg/mL; Fig.8L) were significantly decreased by SHPS treatment. Furthermore, the expression levels of IL-13 (by 2.0 and 6.3 folds, $p < 0.0005$ both; Fig.8I) and IL-17A (by 1.8 folds, $p < 0.05$ at 62.5 μg/mL; Fig.8J) were dramatically reduced by SHPS in the splenocytes. Finally, Con A stimulated increasing transcription factors such as T-bet (by 1.2 and 1723.0 folds, $p < 0.005$ and 0.0005, respectively; Fig.8A; Th1), GATA3 (by 1.1 and 2552.2 folds, $p < 0.05$ and 0.0005, respectively; Fig.8B; Th2), and RORγt (by 1.8 and 925.5 folds, $p < 0.05$ and 0.005, respectively; Fig.8C; Th17) were also significantly decreased by SHPS.

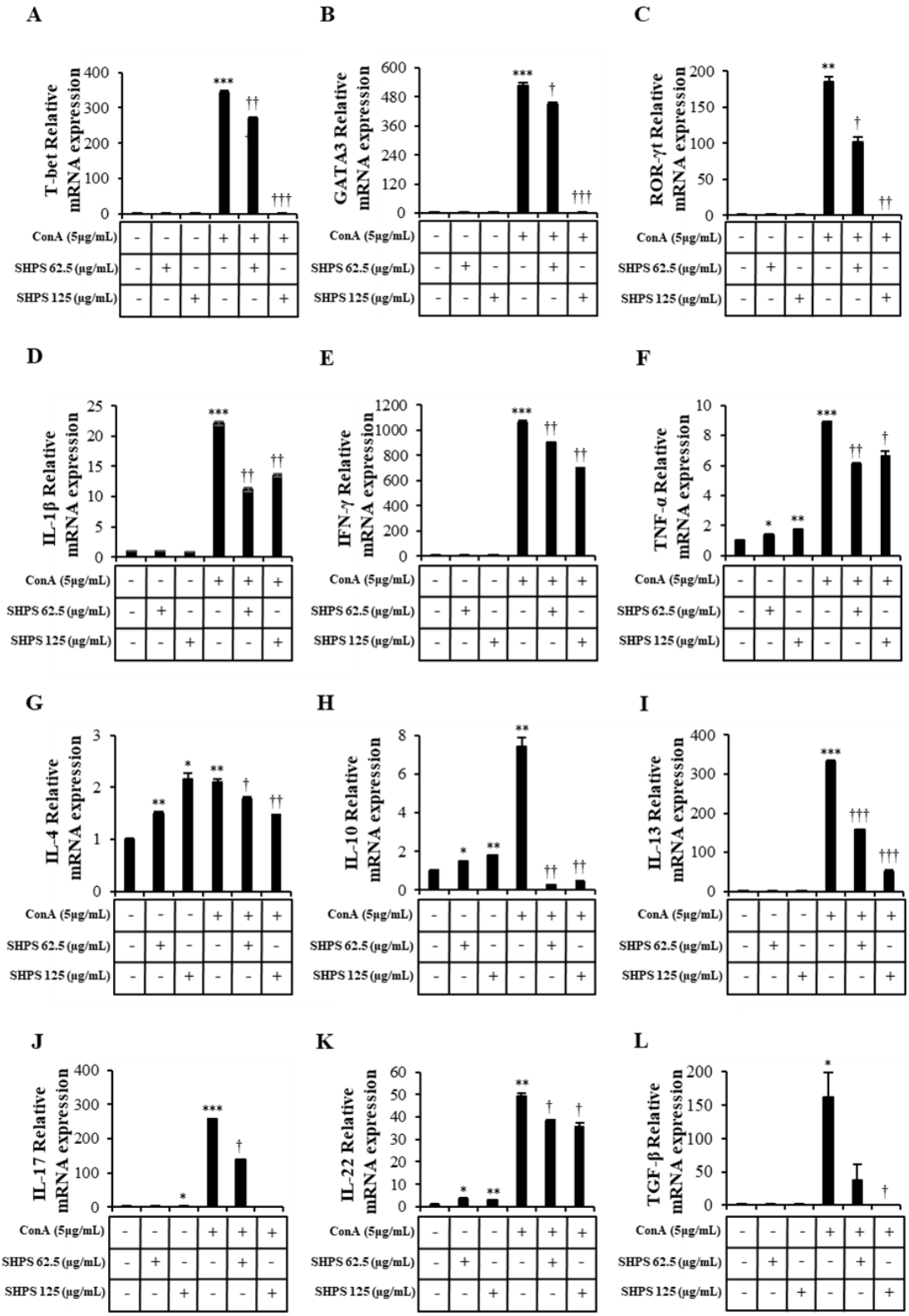


Figure 8. Effect of SHPS on the expression of Th1, Th2, and Th17 cell-derived cytokines and transcription factors in the absence or presence of Con A in splenocytes. The mRNA expression level of (A) T-bet, (B) GATA3, (C) ROR-γt, (D) IL-1β, (E) IFN-γ, (F) TNF-α, (G) IL-4, (H) IL-10, (I) IL-13, (J) IL-17A, (K) IL-22, and (L) TGF-β was evaluated by using real-time PCR with or without Con A. * ($p < 0.05$), ** ($p < 0.005$), and *** ($p < 0.0005$) represent significant increase compared to that in untreated control. † ($p < 0.05$), †† ($p < 0.005$), and ††† ($p < 0.0005$) represent significant decrease compared to that in Con A stimulation only.

Effect of SHPS on the secretion of Th1 (IFN-γ), Th2 (IL-13), and Th17 (IL-17A) cytokines in Con A- stimulated murine splenocytes

The effect of SHPS treatment on cytokine secretion measured using mRNA expression level in Con A-stimulated splenocytes was validated at the protein level as well for three representative cytokines IFN-γ (Th1), IL-13 (Th2), and IL-17A (Th17). The group treated using SHPS alone that recorded very marginal change compared to untreated control in mRNA level did not produce datable cytokines for all three. However, Con A-stimulation spiked the expression of IFN-γ (by 640.0 folds, $p < 0.05$; Fig.9A), IL-13 (by 8.8 folds, $p < 0.05$; Fig.9B), and IL-17A (by 601.6 folds, $p < 0.0005$; Fig.9C), whereas SHPS treatment significantly reduced their expression (IFN-γ by 1.8 folds, $p < 0.05$ at 125 μg/mL, Fig.9A; IL-13 by 8.0 folds, $p < 0.05$ at 125 μg/mL, Fig.9B; IL-17A by 1.1 and 1.7 folds, $p < 0.05$ and 0.005 at 62.5 and 125 μg/mL, respectively, Fig.9C) as in the case of mRNA.

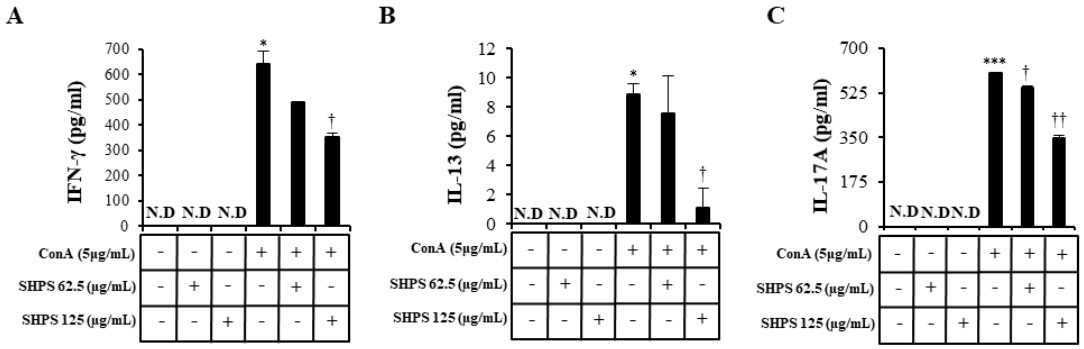


Figure 9. Effect of SHPS on the production of IFN-γ, IL-13, and IL-17A in the absence or presence of Con A in splenocytes. The release of (A) IFN-γ, (B) IL-13, and (C) IL-17A from splenocytes was evaluated by using ELISA. Data are expressed as the mean ± SEM from two individual experiments. * ($p < 0.05$) and *** ($p < 0.0005$) indicate significant increase compared to that in untreated control, and † ($p < 0.05$), †† ($p < 0.005$) indicate significant decrease compared to that in Con A stimulation only.

Effect of SHPS on B cell and macrophage populations in Con A-stimulated murine splenocytes

We also analyzed the effect of SHPS on $CD19^+CD45R/B220^+$ B cell and $CD45^+CD11b^+$ macrophage populations of murine splenocytes. SHPS (125 μg/mL) alone did not affect either B cell or macrophage population (Fig.10). But Con A stimulation significantly increased both $CD19^+CD45R/B220^+$ B cell and $CD45^+CD11b^+$ macrophage populations compared to untreated control by 77.9% and 11.8%, respectively. However, SHPS treatment significantly reduced B cell and macrophage populations by 25.7% ($p < 0.05$) and 4.5% ($p < 0.005$) compared to Con A stimulation alone.

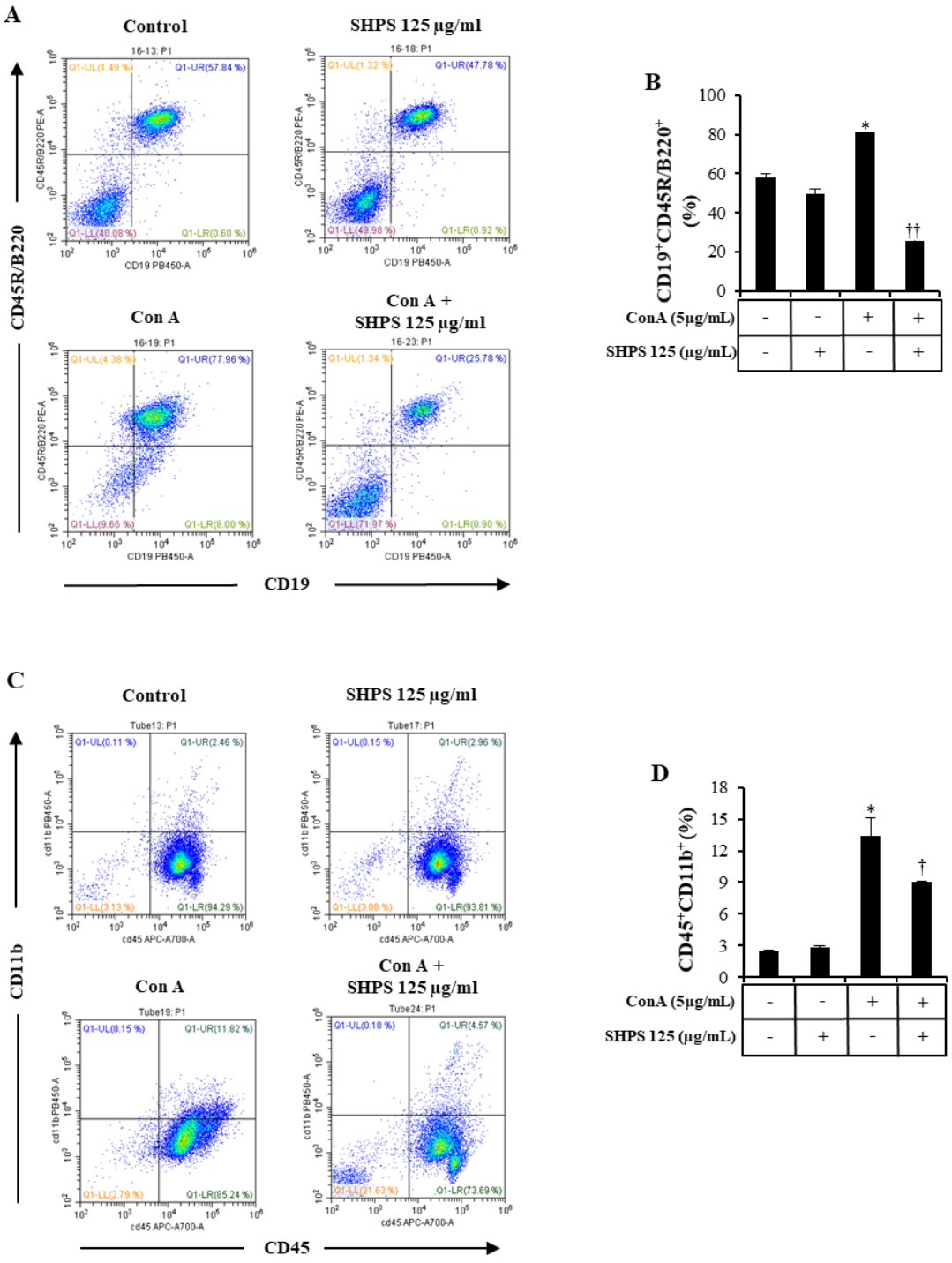


Figure 10. Effect of SHPS on B cell and macrophage population change in the absence or presence of Con A in splenocytes. FACS analysis was performed to measure the effect of SHPS on the populations of (A) $CD19^{+}CD45R/B220^{+}$ B cells and (C) $CD45^{+}CD11b^{+}$ macrophages in the presence of Con A. Proportions of splenocyte population of (B) $CD19^{+}CD45R/B220^{+}$ B cells and (D) $CD45^{+}CD11b^{+}$ macrophages are also shown. Data are expressed as the mean ± S.E.M of two individual experiments. * ($p < 0.05$) indicates significant increase compared to that in untreated control, and † ($p < 0.05$) indicates significant decrease compared to that in Con A stimulation only.

SHPS suppresses expression of Toll-like receptors (TLRs) in lung tissues of PM-induced allergic asthma mice

TLRs play a crucial role in innate immune system to detect structurally conserved molecules from foreign microbes. Considering harmful microbes in PM, we observed the induction of TLRs upon PM challenges and the effect of SHPS after exposure to PM (Fig.11). Expression of TLR2, TLR4, and TLR7 was significantly increased in OVA+PM group compared to that in the the healthy control: by 7.7 folds ($p < 0.05$) for TLR2, 7.6 folds ($p < 0.05$) for TLR4, and 5.0 folds ($p < 0.05$) folds. However, SHPS treatment reduced the mRNA expression of TLRs in a concentration-dependent manner in the PM-induced asthma model. For example, TLR2 mRNA was reduced by 7.0 and 12.8 folds ($p < 0.05$ for both) at 200 and 400mg/kg, respectively; TLR4 mRNA by 6.3 and 10.7 folds ($p < 0.05$ for both); TLR7 mRNA by 5.0 and 12.5 folds ($p < 0.05$ and $p < 0.005$, respectively).

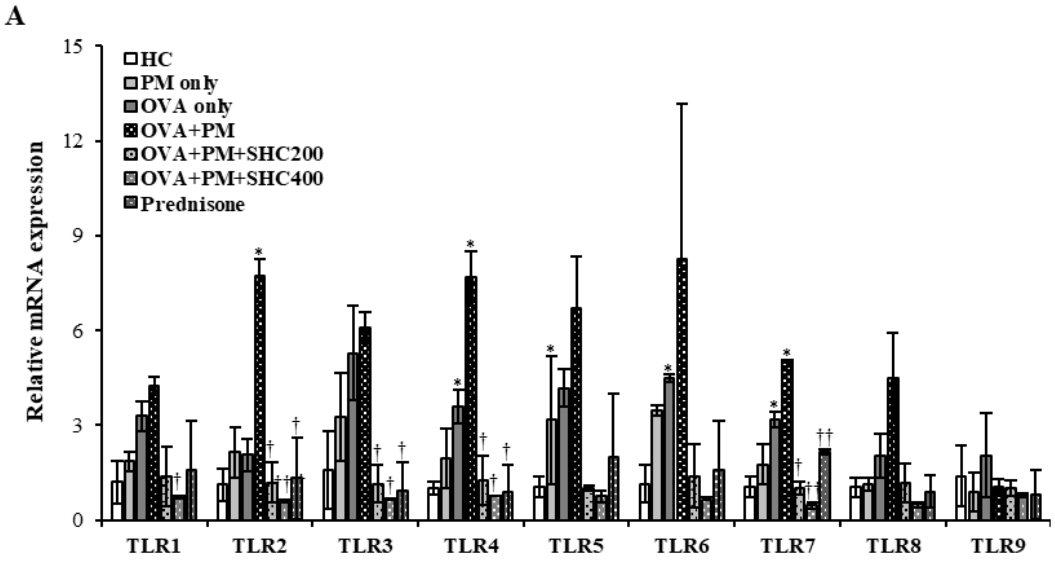
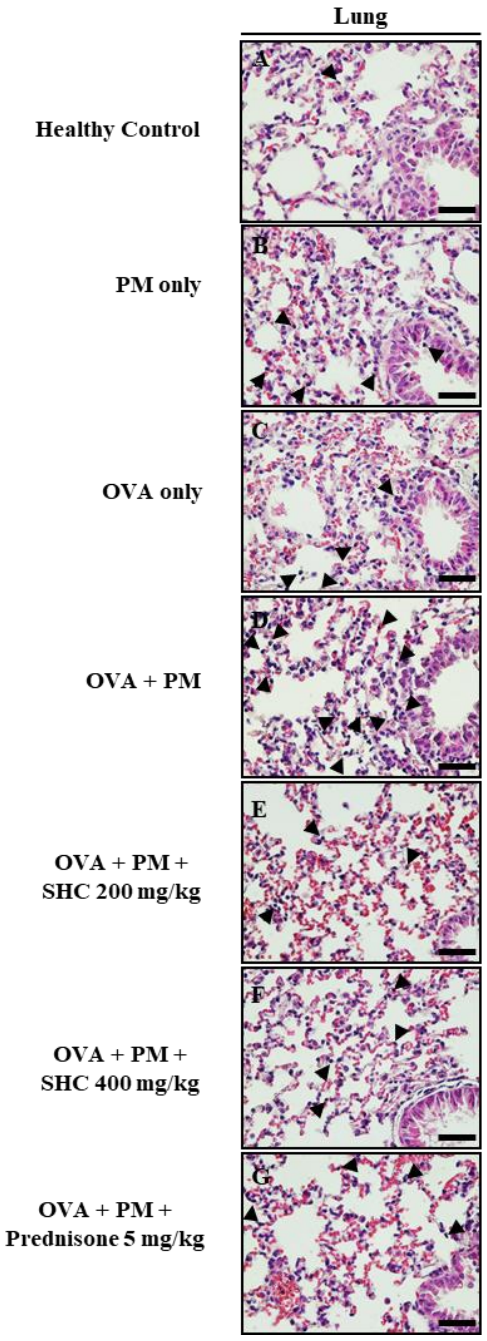


Figure 11. Effect of SHPS on TLR expression in lung tissues of PM-induced allergic asthma mice. The mRNA expression level of TLR-1/2/3/4/5/6/7/8/9 was evaluated by using SYBR green qPCR. Data are expressed as the means ±S.E.M. (n = 3). * ($p < 0.05$) represents significant increase compared to that in healthy control or OVA only group. † ($p < 0.05$), †† ($p < 0.005$), and ††† ($p < 0.0005$) represent significant reduction compared to that in the OVA+PM group.

SHPS attenuates infiltration of inflammatory cells in lung tissues of PM-induced allergic asthma mice

The level of inflammatory cell infiltration into lung tissues is an excellent marker for severity of allergic asthma, and we analyzed the effect of SHPS on the inflammatory cell infiltration in lung tissues of PM-induced allergic asthma mice. Compared to healthy control, OVA sensitization alone or PM challenge alone increased inflammatory cell infiltration, and cells with inflammation score 3 occupied about 40% of all cells in lung sections as a result (Fig.12H). The OVA+PM group showed increase in inflammatory cell infiltration compared to that in the healthy control or even to that in OVA sensitization alone or PM challenge alone group (Figs. 12B~12D), which can be verified by the quantification of the inflammatory score where cells with inflammation score 4 that were absent in OVA alone or PM alone group occupied about 20% of all cells and cells with inflammation score 1 that were about 20% in OVA alone or PM alone group went absent in the OVA+PM group (Fig.12H) However, SHPS treatments with 200 mg/kg and 400 mg/kg concentrations attenuated the inflammatory cell infiltration in the lungs substantially (Figs. 12E and 12F). Remarkably, SHPS treatment with 200mg/kg produced similar effects as prednisone (Fig.12G).



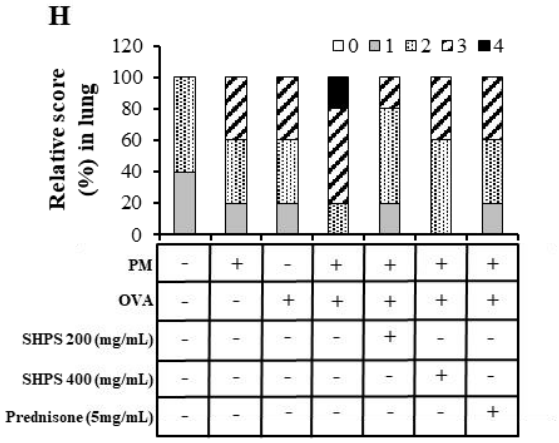


Figure 12. Effect of SHPS on the infiltration of inflammatory cells to lung tissues of PM-induced allergic asthma mice. Representative images of H&E staining of lung sections of (A) healthy control, (B) PM only (C) OVA only, (D) OVA+PM, (E) OVA+PM+SHPS200, OVA+PM+SHPS400, and OVA+PM+Prednisone groups are shown. Examples of inflammatory cell infiltration are indicated arrow heads. The inflammation score for lung section images are shown in (H) (n=5). The degree of peribronchial and perivascular inflammation in each group of lung sections (n = 3~4) was assessed separately on a subjective scale as follows: normal (0), few inflammatory cells infiltrated (1), cell layer deep infiltration of inflammatory cells (2), inflammatory cell infiltration of 2~4 cell layers (3), a ring of inflammatory cells for more than four cell layers (4). Scale bars in (A)~(G) are 25 μm.

SHPS suppressed Th1, Th2, and Th17 cytokines and their transcriptional factors mRNA expression in the lung of PM-induced allergic asthma mice

As intermediary messengers, cytokines play a fundamental role in the propagation of inflammatory signals, and hence their production machinery should be activated or deactivated promptly when necessary. To assess the repertoire of immune responses against PM challenge, we evaluated the expression of T helper cell cytokines and transcription factors in PM-induced allergic asthma mice.

Most of the cytokines and transcription factors did not show significant change in mRNA expression when OVA sensitization or PM challenge was applied alone (Fig.13). However, the mRNA expression of GATA3 (4.2 folds, $p < 0.05$), and RORγt (7.1 folds, $p < 0.05$) that signify the differentiation of Th2, and Th17 was markedly increased in the OVA+PM group compared to untreated control (Figs. 13B~ and 13C). The mRNA expression of T-bet that signifies the differentiation of Th1 was also increased though not statistically significant in OVA+PM group compared with untreated control (Fig.13A). However, SHPS treatment significantly reduced the mRNA expression of GATA3 (1.0 and 0.6 folds, $p < 0.05$ and 0.0005, respectively, at 200 and 400mg/kg; Fig.13B) and RORγt (1.0 And 0.8 folds, $p < 0.05$ both at 200 and 400 mg/kg; Fig.13C) to a level similar or better than the case with prednisone the positive control. T-bet expression was also decreased with SHPS treatment compared to that in OVA+PM group though not statistically significant (Fig.13A).

We also observed cytokines and transcription factors for downstream of Th differentiation. For instance, the mRNA expression of Th1 cytokines IL-1β, IFN-γ, and TNF-α was increased up to 11.6 ($p < 0.05$, Fig.13D), 24.6 ($p < 0.05$, Fig.13E), and 9.2 ($p < 0.05$, Fig.13F) folds in the OVA+PM group compared to the healthy control. However, SHPS treatment significantly inhibited the expression of Th1 cytokines concentration-dependently. The mRNA expression of IL-1β was reduced by 9.6 and 38.6 folds ($p < 0.05$, 0.005), IFN-γ by 22.3 and 27.3 folds

(both $p < 0.05$), and TNF-α by 9.2 and 10.2 folds (both $p < 0.05$) at 200 and 400 mg/Kg of SHPS compared to that in OVA + PM group. Th2 cytokines IL-4, IL-10, and IL-13 were also significantly increased in the OVA+PM group compared to the untreated control (by 7.3 folds, $p < 0.05$ for IL-4; by 31.3 folds, $p < 0.05$ for IL-13, respectively) whereas SHPS decreased those cytokines (by 6.6 and 9.1 folds, $p < 0.05$ both for IL-4 (Fig. 13G); by 16.6 and 55.3 folds, $p < 0.05$ both for IL-10 (Fig. 13H); by 17.3 and 24 folds, $p < 0.005$ for IL-13 (Fig.13I) at 200 and 400 mg/kg of SHPS, respectively compared to the OVA+PM group. Th17 cytokines IL-17A and IL-22 showed a similar pattern of increase in OVA+PM compared to healthy control and suppression in SHPS treatment as well. They were significantly increased in the OVA+PM group compared to the healthy control, (by 880.2 folds, $p < 0.05$ for IL-17A, Fig.13J; by 45.8 folds, $p < 0.05$ for IL-22, Fig.13K, respectively) but suppressed when SHPS was treated compared to the OVA+PM group (by 880.2 and 1257.4 folds, $p < 0.05$ both at 200 and 400 mg/kg for IL-17A; by 16.9 and 229.0 folds, $p < 0.05$ both at 200 and 400 mg/kg for IL-22, respectively). The suppression for SHPS was similar to that observed for positive control prednisone (by 0.9 folds, $p < 0.05$ for IL-17A; by 0.09 folds, $p < 0.05$ for IL-22, respectively).

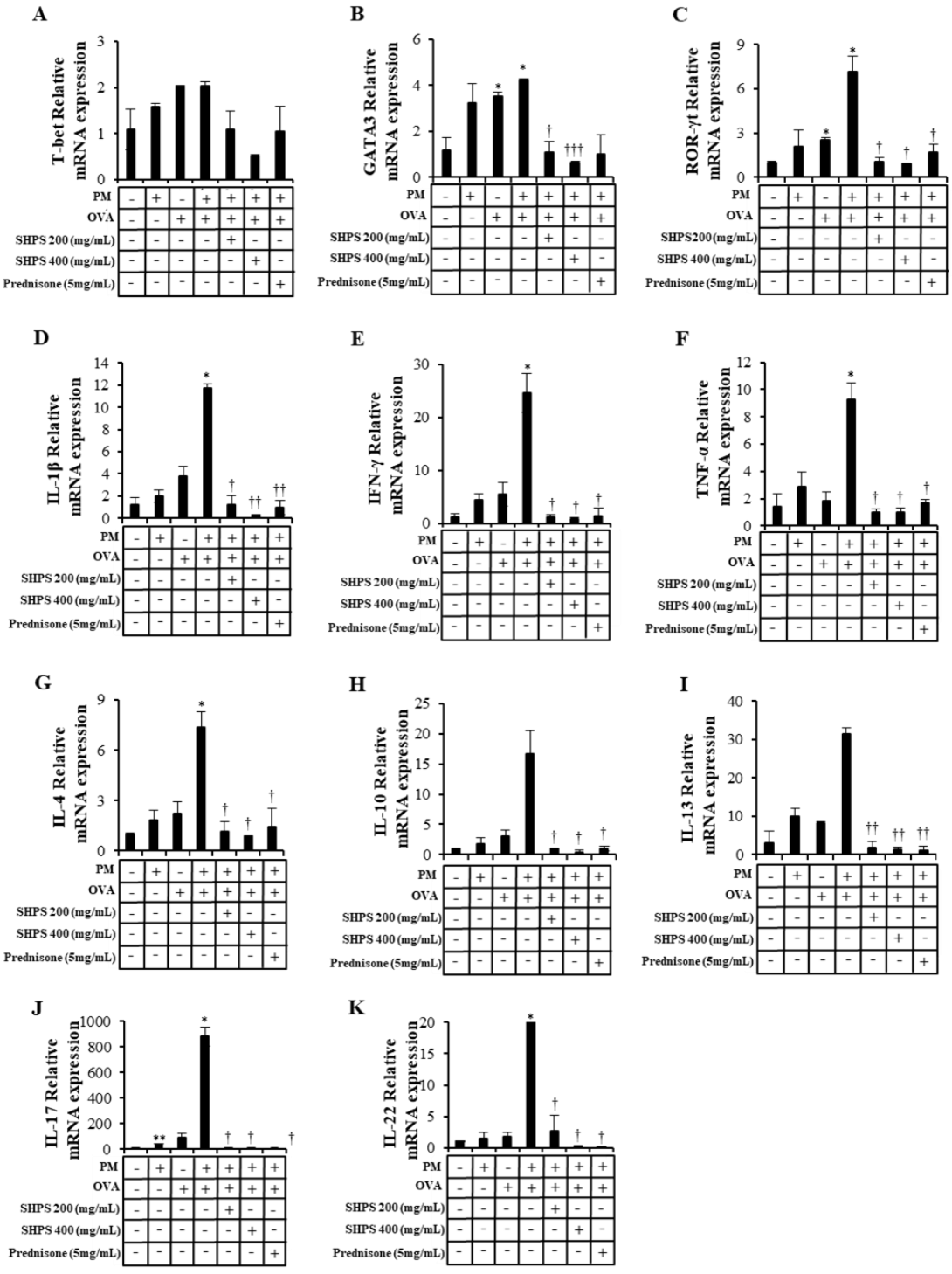


Figure 13. Effect of SHPS on the expression of transcription factors and cytokines in lung tissues of PM-induced allergic asthma mice. The expression of (A) T-bet, (B) GATA-3, (C) ROR-γt, (D) IL-1β, (E) IFN-γ, (F) TNF-α, (G) IL-4, (H) IL-10, (I) IL-13, (J) IL-17A, and (K) IL-22 was evaluated by using qPCR. Data represent the mean ±S.D. of at least three independent experiments. * ($p < 0.05$) and ** ($p < 0.005$) represent significant increase compared to that in healthy control or OVA only group, and † ($p < 0.05$), †† ($p < 0.005$), and ††† ($p < 0.0005$) represent significant decrease compared to that in OVA+PM group.

SHPS suppresses Th1, Th2, and Th17 cytokine secretion in serum of PM-induced allergic asthma mice

We further measured the secretions of IFN-γ, IL-13, and IL-17A in PM-induced allergic asthma murine serum samples by ELISA (Fig.14). The protein level of IFN-γ, IL-13, and IL-17A increased when OVA sensitization (by 77.4 fold, $p < 0.005$ for IFN-γ; by 6.4 fold, $p < 0.05$ for IL-13) or PM challenge (by 82.8 fold, $p < 0.05$ for IFN-γ) was applied alone. They were increased further when OVA and PM were applied together compared to the healthy control (by 102.4 fold, $p < 0.05$ for IFN-γ) However, as expect, SHPS treatment suppressed the PM-induced secreted of IFN-γ (by 1.6, $p < 0.05$ at 200 mg/kg; Fig.14A), and IL-17A (by 27.8 and 6.6 folds, $p < 0.05$ for both, at 200 and 400mg/kg, respectively; Fig.14C).

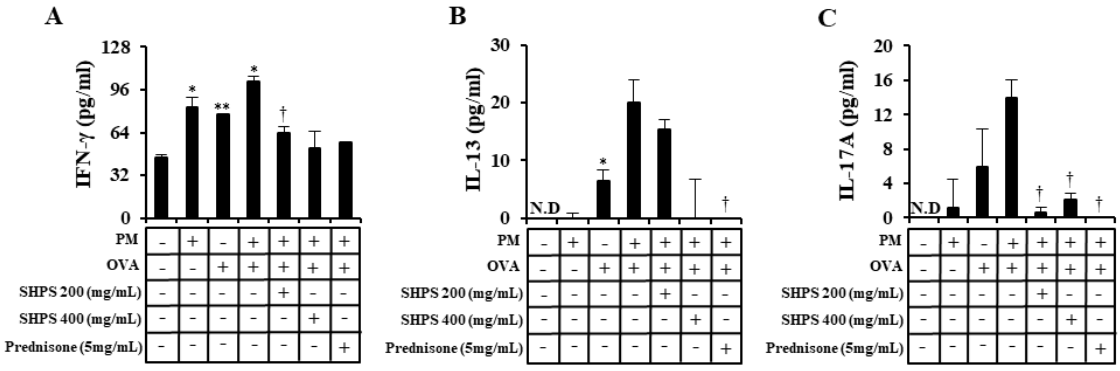


Figure 14. Effect of SHPS on the production of IFN-γ, IL-13, and IL-17A in the serum of PM-induced allergic asthma mice. The protein level of (A) IFN-γ, (B) IL-13, and (C) IL-17A in the serum was evaluated by using ELISA. Data are expressed as mean ± SEM from two individual experiments. * ($p < 0.05$) and ** ($p < 0.005$) represent significant increase compared to that in healthy control or OVA only group, and † ($p < 0.05$) represents significant decrease compared to that in the OVA+PM group.

SHPS mitigates goblet cell metaplasia in lung tissues of PM-induced allergic asthma mice

During asthma, the immune response activates and induces hypersecretion of mucus. We analyzed mucus in goblet cells in PM-induced allergic asthma mice using PAS staining in the lung (Fig.15). The secretion of mucus and goblet cell hyperplasia were significantly increased in the PM group (by 5.4 folds, $p < 0.0005$; Figs. 15B and 15I) or OVA-sensitized group (by 5.6 folds, $p < 0.0005$; Figs. 15C and 15J) compared to those in healthy control (Figs.15A and 15H). The secretion of mucus and goblet cell hyperplasia were further enhanced in the OVA+PM group (Fig. 15 D and 15K) compared to those in the PM-only or OVA-only groups. To the contrary, however, SHPS treatment attenuated the hypersecretion of mucus and goblet cell hyperplasia (by 1.6 and 1.4 folds, $p < 0.005$ and 0.05 at 200 and 400mg/kg, respectively; Figs. 15E and 15L, Figs. 15F and 15M). Interestingly, the efficacy of the 200mg/kg of SHPS was a similar to that of positive control prednisone (by 1.9 folds, $p < 0.05$; Figs.15G and 15N).

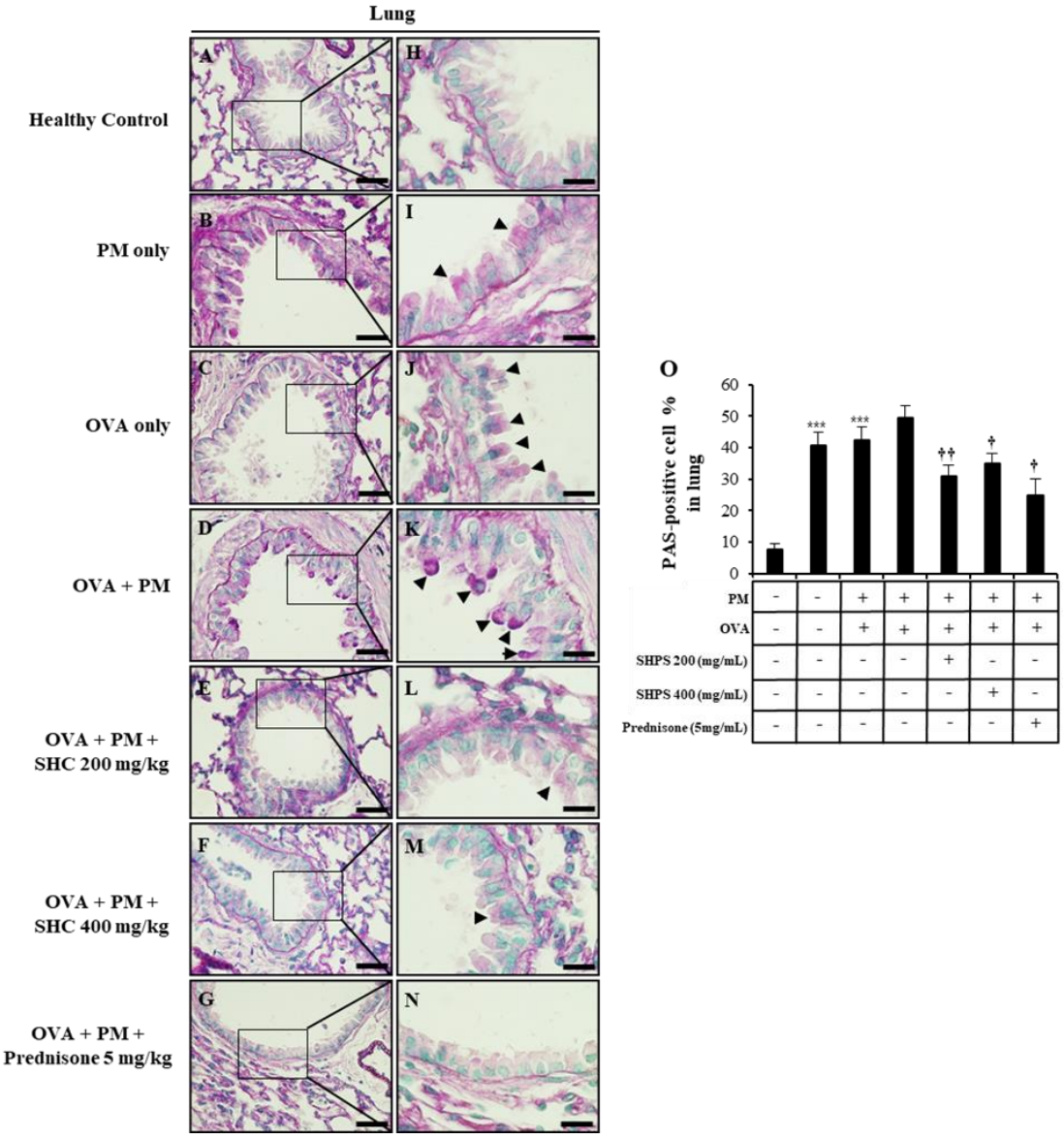


Figure 15. Effect of SHPS on mucus secretion in lungs of PM-induced allergic asthma mice Representative H&E section images of (A, H) healthy control, (B, I) PM only, (C, J) OVA only, (D, K) OVA+PM, (E, L) OVA+PM+SHPS 200, (F, M) OVA+PM+SHPS 400, (G, N) OVA+PM+Prednisone, and (O) PAS-positive cell percentage are shown. Arrow heads indicate goblet cells that secrete mucus. ** ($p < 0.005$) represent significant increase compared to healthy control and † ($p < 0.05$) and †† ($p < 0.005$) represent significant decrease compared to that in OVA+PM group. Scale bars of (A)~(G) are 25 μm, and those in (H)~(N) are 10 μm.

SHPS suppresses mucus hypersecretion in lung tissues of PM-induced allergic asthma mice

The MUC5AC, MUC5B, and MUC2 are the major mucin genes involved in the regulation of mucus secretion expressed most prominently in airway goblet cells in allergic asthma. We evaluated mRNA expression of MUC5AC, MUC5B, and MUC2 in lung tissues of allergic asthma mice (Fig.16). The expression level of MUC5AC was significantly increased by 2.5 and 4.8 folds ($p < 0.05$ for both) compared to that in healthy control when OVA sensitization or PM challenge was applied alone. The expression level of MUC5B, and MUC2 was increased as well when OVA sensitization or PM challenge was applied alone but not statistically significant. In the OVA+PM group, however, the expression level of MUC5AC, MUC5B, and MUC2 was increased by 16.7 ($p < 0.05$), and 7.7 ($p < 0.05$) folds, respectively, compared to the healthy control.

As expected, however, SHPS treatment reduced the mRNA expression of mucin genes substantially. For instance, MUC5AC and MUC2 mRNA expression level was decreased by 1.2 and 1.4 folds ($p < 0.005$ for both) at 200 or 400 mg/kg SHPS treatment, and MUC5B by 7.3 and 10.4 folds ($p < 0.05$ for both) and MUC2 by 7.0 and 19.25 folds ($p < 0.05$ and 0.005, respectively) compared to that in the OVA + PM group. The inhibitory effect of SHPS was similar to that of prednisone for MUC5AC and MUC5B or more efficient for MUC2 than that of prednisone (by 0.8 folds, $p < 0.005$ for MUC5AC; by 0.2 folds, $p < 0.05$ for MUC5B; by 0.8 folds, $p < 0.05$ for MUC2).

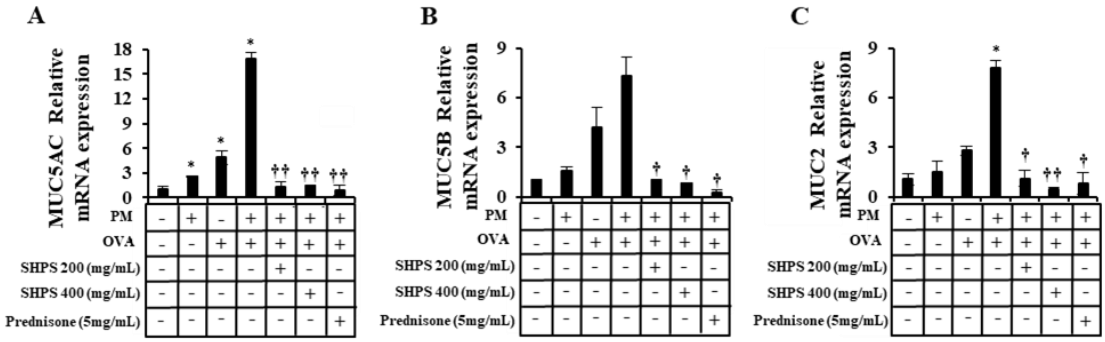


Figure 16. Effect of SHPS on the expression of mucus secretion regulating genes in lung tissues of PM-induced allergic asthma mice.

The mRNA expression of (A) MUC5AC, (B) MUC5B, and (C) MUC2 was measured by using qPCR. Data are represented as the mean ± S.D. of at least three independent experiments. * ($p < 0.05$) indicates significant increase compared to that in healthy control or OVA only group. † ($p < 0.05$) and †† ($p < 0.005$) indicate significant decrease compared to that in OVA+PM group.

SHPS reduces MUC5AC polymeric mucin in lung tissues of PM-induced allergic asthma mice

Of the mucin genes, MUC5AC is of particular interest in association with mucus hypersecretion in the respiratory tract, we analyzed the expression of MUC5AC in lung tissues of PM-induced allergic asthma mice (Fig.17). When OVA sensitization or PM challenge was applied alone, the proportion of MUC5A-positive cells was increased by 2.5 and 2.7 folds ($p < 0.005$ for both) compared to healthy control (Figs.17A~17C and 17H~17J). When OVA sensitization and PM challenge were applied together, the proportion of MUC5AC-positive cells was increased by 3.3 folds ($p < 0.05$) compared to that in the healthy control (Figs.17A, 17H, 17D, and 17K). Furthermore, the proportion of MUC5AC-positive cells in the PM + OVA group was increased by 1.2 and 1.1 folds ($p < 0.005$, both, respectively) compared to the PM or OVA group (Figs.17B, 17I, 17C, and 17J).

Interestingly, however, the proportion of MUC5AC-positive cells was decreased when SHPS treatment was applied as well in a concentration-dependent way: by 1.7 folds ($p < 0.05$) at SHPS 200 mg/kg (Figs.17E and 17L), and 1.5 folds ($p < 0.005$) at SHPS 400mg/kg (Figs. 17F and 17M) compared to that in the OVA+PM group. Intriguingly, the efficacy of the 200 mg/kg SHPS was similar to that of the positive control prednisone that lowered the proportion of MUC5A-positive cells compared to that in OVA+PM group in suppressing the number of PM-induced MUC5AC-positive cells.

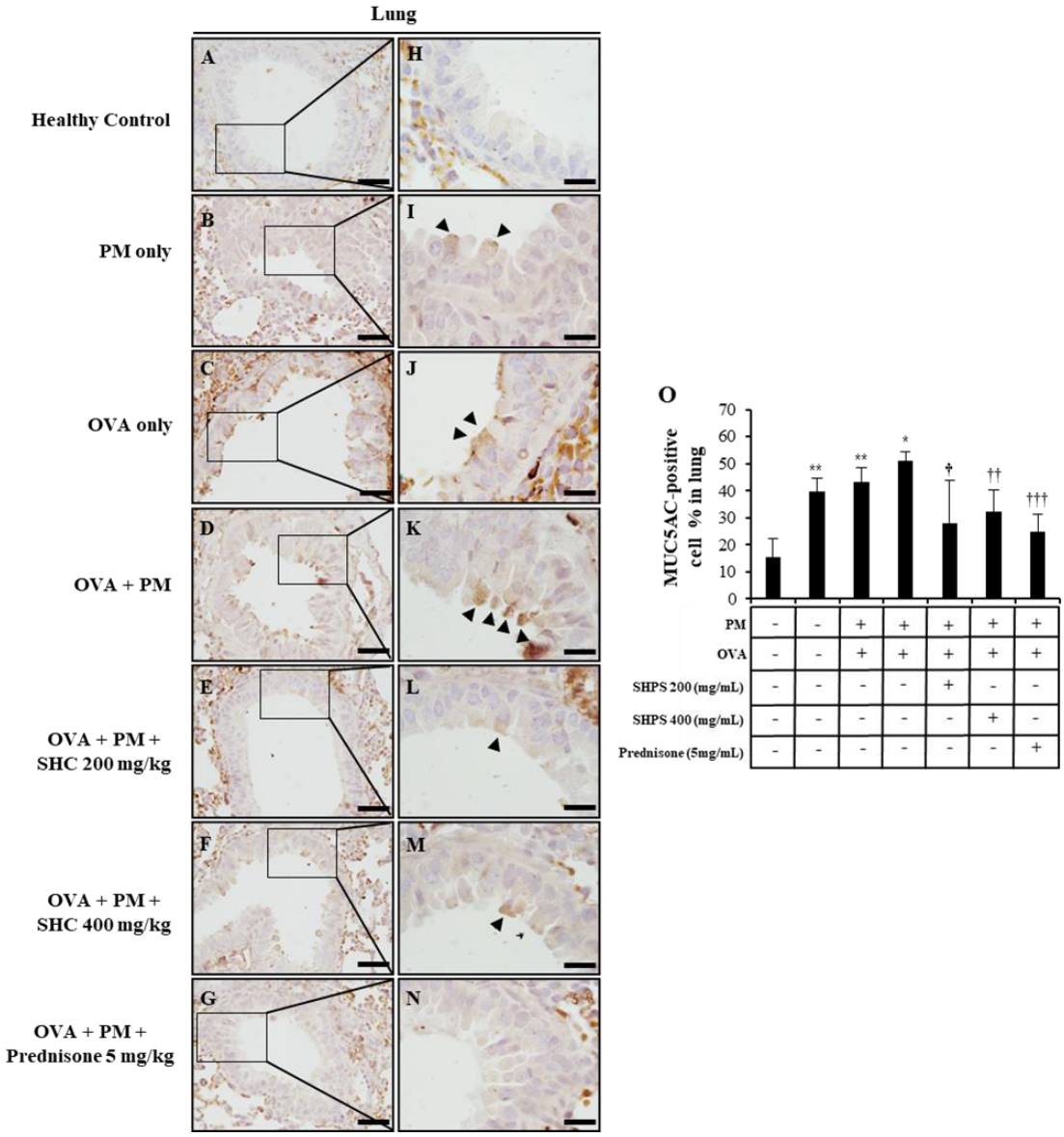


Figure 17. Effect of SHPS on MUC5AC-positive cells in lung tissues of PM-induced allergic asthma mice. Representative IHC-stained section images of (A, H) healthy control, (B, I) PM only, (C, J) OVA only, (D, K) OVA+PM, (E, L) OVA+PM+SHPS 200, (F, M) OVA+PM+SHPS 400, (G, N) OVA+PM+Prednisone, and (O) the percentage of MUC5AC-positive cells are shown. Arrow heads indicate mucus-secreting goblet cells. * ($p < 0.05$) and ** ($p < 0.005$) express significant increase compared to that in healthy control or OVA only group. † ($p < 0.05$), †† ($p < 0.005$), and ††† ($p < 0.0005$) express significant decrease compared to that in OVA+PM group. Scale bars in (A~G) were 25 μm, and those in (H)~(N) are 10μm.

SHPS reduces MUC5B polymeric mucins in lung tissues of PM-induced allergic asthma mice

We also analyzed the expression of MUC5B in lung tissues of PM-induced allergic asthma mice (Fig.18). The proportion of MUC5B positive cells was significantly increased by 2.2 folds ($p < 0.005$) when PM challenge (Fig.18B and 18I) or by 2.1 folds ($p < 0.05$) when OVA sensitization (Fig.18C and 18J) was applied alone compared to that in healthy control (Fig.18A and 18H). Also, the proportion of MUC5B positive cells was increased by 1.2 folds ($p < 0.05$) in the OVA+PM group (Fig.18D and 18K) than that in the OVA group in the bronchiole, the percentage of MUC5B positive cells was significantly decreased in lung tissues by 1.3 and 1.5 folds ($p < 0.05$ for both) when SHPS was treated with 200mg/kg (Fig.18E and 18L) and 400mg/kg (Fig.18F and 18M). Prednisone treatment also decreased the percentage of MUC5B positive cells by 2.3 folds ($p < 0.0005$) compared to that in the OVA+PM group (Fig.18G and 18N).

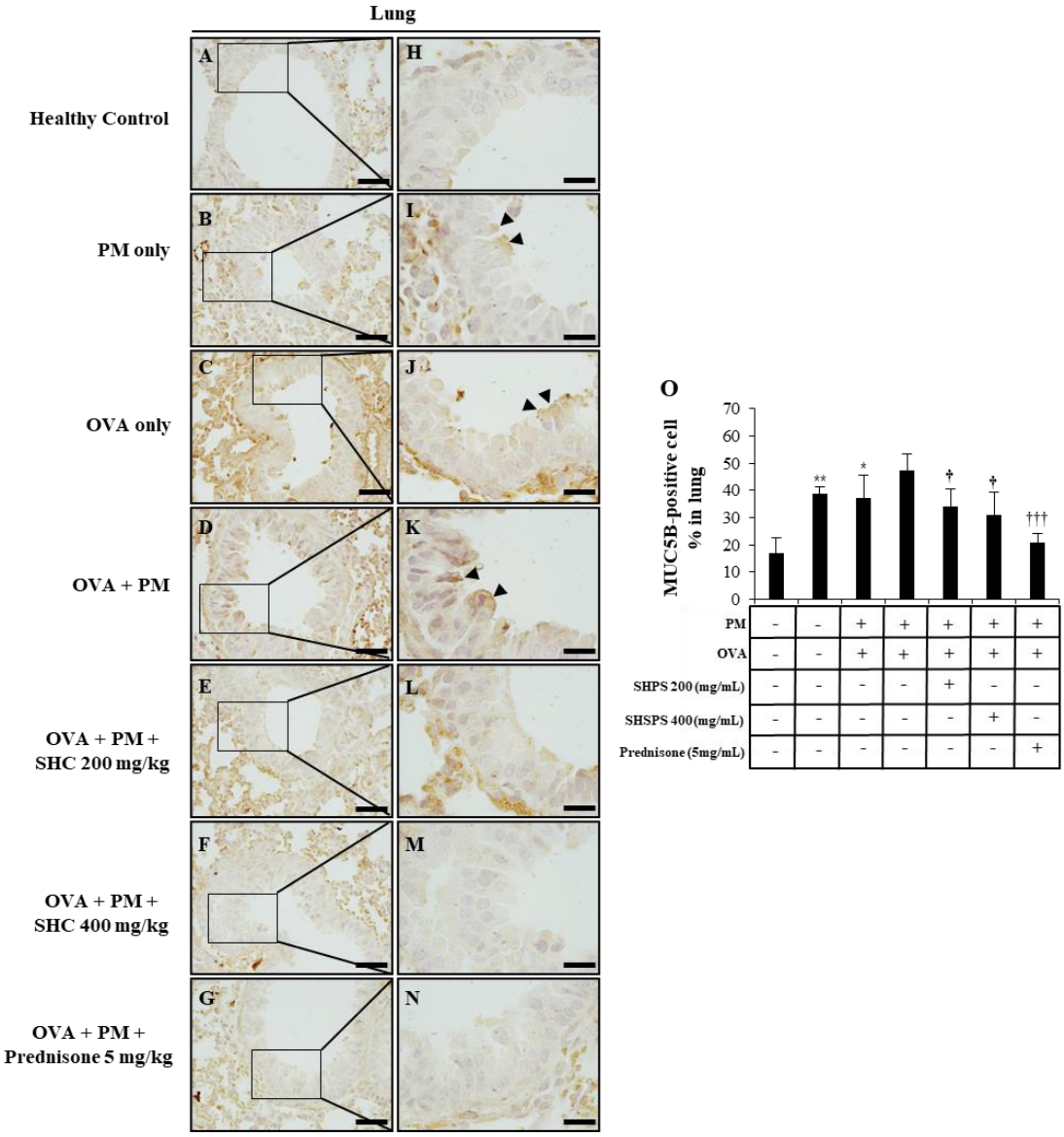


Figure 18. Effect of SHPS on MUC5B-positive cells in lung tissues of PM-induced allergic asthma mice. Representative IHC-stained section images of (A, H) healthy control, (B, I) PM only, (C, J) OVA only, (D, K) OVA+PM, (E, L) OVA+PM+SHPS 200, (F, M) OVA+PM+SHPS 400, (G, N) OVA+PM+Prednisone, and (O) the percentage of MUC5B-positive cells are shown. Arrow heads indicate mucus-secreting goblet cells. * ($p < 0.05$) and ** ($p < 0.005$) express significant increase compared to that in healthy control, and † ($p < 0.05$) and ††† ($p < 0.0005$) express significant decrease compared to that in OVA+PM group. Scale bars in (A)~(G) are 25 μm, and those in (H)~(N) are 10 μm.

SHPS reduces MUC2 polymeric mucins in the lung of PM-induced allergic asthma mice

Finally, we analyzed the expression of MUC2 in the PM-induced allergic asthma lung tissues (Fig.19). The proportion of MUC2 positive cells was significantly increased when PM (Figs.19B and 19I) and OVA (Figs.19C and 19J) was applied alone by 2.9 and 3.1 folds ($p < 0.05$ for both) compared to that in healthy control (Figs.19A and 19H). Also, the proportion of MUC2 positive cells was increased by 3.2 folds ($p < 0.005$) in the OVA+PM group (Figs.19D and 19K) than that in the healthy control group.

However, the proportion of MUC2 positive cells was significantly reduced in the bronchiole by 1.8 and 1.9 folds ($p < 0.005$ for both) when treated with SHPS 200 (Figs.19E and 19L) and 400mg/kg (Figs.19F and 19M), respectively. The mice were treated with positive control prednisone, the proportion of MUC2 was significantly decreased by 2.4 folds ($p < 0.0005$) compared to that in the OVA+PM group (Figs.19G and 19N).

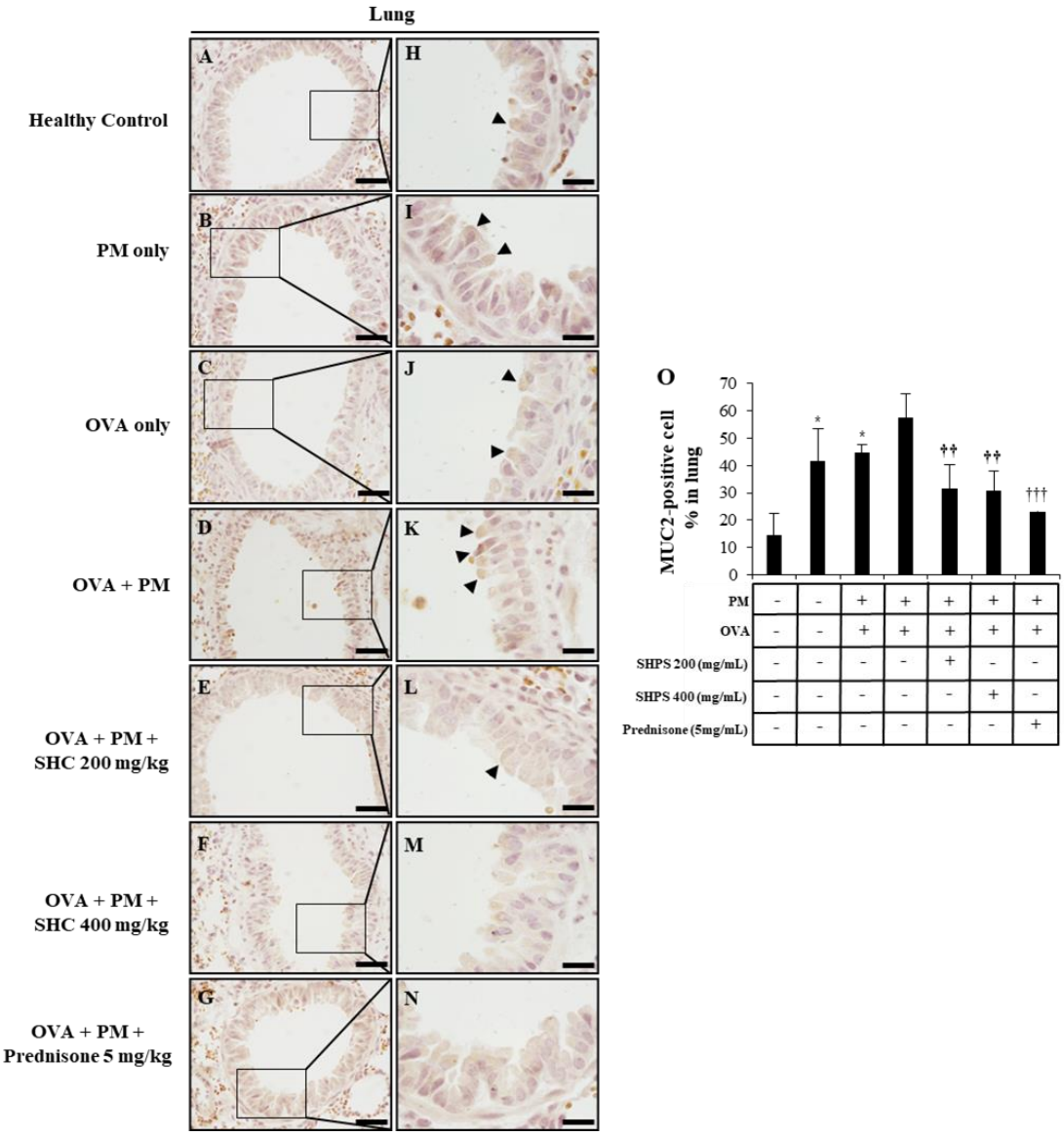


Figure 19. Effect of SHPS on MUC2-positive cells in lung tissue of PM-induced allergic asthma mice. Representative IHC-stained section images of (A, H) healthy control, (B, I) PM only, (C, J) OVA only, (D, K) OVA+PM, (E, L) OVA+PM+SHPS 200, (F, M) OVA+PM+SHPS 400, (G, N) OVA+PM+Prednisone, and (O) the percentage of MUC2-positive cells are shown. Arrow heads indicate goblet cells. * ($p < 0.05$) expresses significant increase compared to that in healthy control. †† ($p < 0.005$), and ††† ($p < 0.0005$) express significant decrease compared to rhat in OVA+PM group. Scale bars of (A)~(G) were 25 μm, and those of (H)~(N) are 10 μm.

IV. DISCUSSION

Allergic asthma, rheumatoid arthritis, and hepatitis are immune disorder disease where immune stimulant or immunosuppressants are needed as drugs or environmental factors [23]. The brown seaweeds have rich polysaccharides and hence pose as a good candidate for such immunomodulators. *Sargassum horneri* (*S. horneri*) contains sulfated polysaccharides [24]. Polysaccharides extracted from *S. horneri* have antioxidant and anti-inflammatory effects [25, 26]. However, the immunological effects of the *S. horneri* polysaccharides remain unknown. This study investigated the immunological effects of *S. horneri* polysaccharides (SHPS) extracted with celluclast in unstimulated or Concanavalin A (Con A) stimulated splenocytes. We further investigate the pathological mechanism of SHPS using an OVA-induced allergic asthma mice model, a model disease of chronic inflammation [27]. Con A is a well-known mitogen and has been used for T cell proliferation and activation in various immunological disease studies [28]. Also, it is known to cause cytotoxicity and inflammation [29]. The present study showed that SHPS treatment up to concentrations 7.8 ~ 125 μg/mL increased the proliferation of splenocytes when used alone up to. We also showed that SHPS treatment at concentrations 15.6~250 μg/mL were able to decrease hyperimmune reaction induced with Con A stimulation. This provides a rationale to use it as an immunostimulator and paves a way for further assessment of the role of SHPS in other immune cells.

Toll-like receptors (TLRs) play an important role in the innate immune response and can recognize both invading pathogens and endogenous risk molecules released by dying cells and damaged tissues. TLRs also play an important role in linking innate and adaptive immunity [30]. Activation of TLRs also leads to acute inflammation, and specific TLRs signaling is implicated in the pathogenesis of asthma and COPD [31]. TLR 2~9 expression has been reported to be increased throuogh Con A stimulation in mouse peritoneal macrophages [32].

Loliolide, one of the *S. horneri* polysaccharides, has been reported to inhibit the expression of activated TLR 2 and TLR 4 in LPS-stimulated macrophages [20]. In this study, SHPS decreased TLRs (1~9) in Con A-stimulated splenocytes (Fig.7). SHPS reduced the expression level of TLR 2, 4, and 7 in PM-induced asthmatic mice (Fig.11). Therefore, SHPS can potentially mitigate PM-induced allergic asthma by inhibition of TLRs signaling.

The immune system is largely divided into innate and adaptive immunity, protecting our bodies from the constantly evolving pathogenic microbes [33]. Previously, it was reported that The the *S. horneri* extracted from ethanol (SHE) increased T-helper cell cytokines or populations, cytotoxic T cells, macrophages, granulocytes, and eosinophils in the spleen [34]. It has also been reported that SHE reversed the activation of T-helper cells, cytotoxic T cells, eosinophils, and mononuclear cells and reduced the secretion of T-helper cell cytokines in Con A-stimulated splenocytes [34]. Furthermore, SHE has been reported to produce Th2 immune suppression under OVA stimulation through GATA3- and NLRP3-dependent IL-4, IL-5, and IL-13 inhibition [35]. In the case of SHPS alone treatment in the present study, there was no T-helper cell cytokine change (Th1: TNF-α, Th2: IL-4 and IL-10, and Th17: IL-17A and IL-22) as opposed to SHE. However, SHPS significantly decreased cytokines and transcription factors (Fig.8), and the populations of B cells and macrophages increased by hyperimmune reactions (Fig.10). Also similarly, SHPS significantly decreased cytokines and transcription factors in PM-induced asthmatic mice (Fig.13). SHE can regulate the immune response, especially the cytokines of Th2 cells. However, T cells stimulated by hyperimmune reactions secrete various cytokines to activate B cells and macrophages, producing more IFN-γ, IL-13, and IL-17A, and we think SHPS regulates the cytokines of mainly Th1 and Th17 cells contrary to SHE.

Airway mucus hypersecretion is a characteristic of many patients with asthma. It has been reported that mucin genes MUC5AC, MUC5B, and MUC2 are highly expressed in patients with asthma. Especially, MUC5AC and MUC5B are crucial in mucus hypersecretions in asthma [8]. In this study, we showed that SHPS mitigated goblet cell hyperplasia and mucus secretion in the OVA+PM group (Fig.15). Also, MUC5AC which was spiked when OVA sensitization and PM challenge were provoked simultaneously was reduced more than MUC5B and MUC2 when SHPS treatment was applied (Figs.16~19). Our findings clearly indicate that SHPS may exert a protective effect against goblet cell metaplasia and mucus overproduction in PM-exacerbated asthma, and it can control the mucin regulator genes.

In conclusion, SHPS reduced the secretion of Th1, Th2, and Th17 cytokines and the numbers of B cells and macrophages in murine splenocytes under hyperimmune status, SHPS significantly mitigated histopathological changes and mucus hypersecretion in PM-induced allergic asthma mice through concomitantly inhibiting Th1/Th2/Th17 immune responses as well. Taken together, our study provides a strong rationale that SHPS may afford a promising approach for immunotherapy as an alternative for conventional treatments of allergic asthma induced by ambient PM.

REFERENC

1. Hammad, H. and B.N. Lambrecht, *The basic immunology of asthma.* Cell, 2021. **184**(6): p. 1469-1485.
2. Mims, J.W., *Asthma: definitions and pathophysiology.* Int Forum Allergy Rhinol, 2015. **5 Suppl 1**: p. S2-6.
3. Athari, S.S., *Targeting cell signaling in allergic asthma.* Signal Transduct Target Ther, 2019. **4**: p. 45.
4. Kalchiem-Dekel, O., X. Yao, and S.J. Levine, *Meeting the Challenge of Identifying New Treatments for Type 2-Low Neutrophilic Asthma.* Chest, 2020. **157**(1): p. 26-33.
5. Pelaia, G., et al., *Cellular mechanisms underlying eosinophilic and neutrophilic airway inflammation in asthma.* Mediators Inflamm, 2015. **2015**: p. 879783.
6. Santus, P., D. Radovanovic, and D.A. Chiumello, *Mucins and Asthma: Are We Headed to the Revolutionary Road?* J Clin Med, 2019. **8**(11).
7. Rogers, D.F. and P.J. Barnes, *Treatment of airway mucus hypersecretion.* Ann Med, 2006. **38**(2): p. 116-25.
8. Rogers, D.F., *Airway mucus hypersecretion in asthma: an undervalued pathology?* Curr Opin Pharmacol, 2004. **4**(3): p. 241-50.
9. Al-Ahmad, M., et al., *Severe asthma treatment patterns: A multicenter observational study in the Gulf region.* World Allergy Organ J, 2022. **15**(5): p. 100647.
10. Wadsworth, S., et al., *IL-13, Asthma and Glycosylation in Airway Epithelial Repair.* 2012: p. 187-228.
11. Amaral-Machado, L., et al., *Use of Natural Products in Asthma Treatment.* Evid Based Complement Alternat Med, 2020. **2020**: p. 1021258.
12. de Jesus Raposo, M.F., A.M. de Morais, and R.M. de Morais, *Marine polysaccharides from algae with potential biomedical applications.* Mar Drugs, 2015. **13**(5): p. 2967-3028.
13. Li, Y., et al., *Brown Algae Carbohydrates: Structures, Pharmaceutical Properties, and Research Challenges.* Mar Drugs, 2021. **19**(11).
14. Rushdi, M.I., et al., *Pharmacological and natural products diversity of the brown algae genus Sargassum.* RSC Adv, 2020. **10**(42): p. 24951-24972.
15. Chen, X., et al., *Antitumor and immunomodulatory activity of polysaccharides from Sargassum fusiforme.* Food Chem Toxicol, 2012. **50**(3-4): p. 695-700.
16. Pang, S.J., et al., *Cultivation of the brown alga Sargassum horneri: sexual reproduction and seedling production in tank culture under reduced solar irradiance in ambient temperature.* Journal of Applied Phycology, 2008. **21**(4): p. 413-422.

17. Zhuang, M., et al., *Sargassum blooms in the East China Sea and Yellow Sea: Formation and management.* Mar Pollut Bull, 2021. **162**: p. 111845.
18. Byeon, S.Y., et al., *The origin and population genetic structure of the 'golden tide' seaweeds, Sargassum horneri, in Korean waters.* Sci Rep, 2019. **9**(1): p. 7757.
19. Sanjeewa, K.K., et al., *Anti-inflammatory activity of a sulfated polysaccharide isolated from an enzymatic digest of brown seaweed Sargassum horneri in RAW 264.7 cells.* Nutr Res Pract, 2017. **11**(1): p. 3-10.
20. Jayawardena, T.U., et al., *Loliolide, isolated from Sargassum horneri; abate LPS-induced inflammation via TLR mediated NF-κB, MAPK pathways in macrophages.* Algal Research, 2021. **56**.
21. Sanjeewa, K.A., et al., *The protective effect of Sargassum horneri against particulate matter-induced inflammation in lung tissues of an in vivo mouse asthma model.* Food & Function, 2019. **10**(12): p. 7995-8004.
22. Herath, K., et al., *Sargassum horneri extract containing mojabanchromanol attenuates the particulate matter exacerbated allergic asthma through reduction of Th2 and Th17 response in mice.* Environ Pollut, 2020. **265**(Pt B): p. 114094.
23. Ahmad, W., et al., *Immunostimulatory effects of the standardized extract of Tinospora crispa on innate immune responses in Wistar Kyoto rats.* Drug Des Devel Ther, 2015. **9**: p. 2961-73.
24. Sanjeewa, K.K.A., et al., *Bioactive potentials of sulfated polysaccharides isolated from brown seaweed Sargassum spp in related to human health applications: A review.* Food Hydrocolloids, 2018. **81**: p. 200-208.
25. Sanjeewa, K.K.A., et al., *Fucoidan isolated from invasive Sargassum horneri inhibit LPS-induced inflammation via blocking NF-κB and MAPK pathways.* Algal Research, 2019. **41**.
26. Shao, P., X. Chen, and P. Sun, *Chemical characterization, antioxidant and antitumor activity of sulfated polysaccharide from Sargassum horneri.* Carbohydr Polym, 2014. **105**: p. 260-9.
27. Casaro, M., et al., *OVA-Induced Allergic Airway Inflammation Mouse Model.* Methods Mol Biol, 2019. **1916**: p. 297-301.
28. Ando, Y., et al., *Concanavalin A-mediated T cell proliferation is regulated by herpes virus entry mediator costimulatory molecule.* In Vitro Cell Dev Biol Anim, 2014. **50**(4): p. 313-20.
29. *<Inflammation Induced by Concanavalin A and Other Lectins.pdf>.*
30. El-Zayat, S.R., H. Sibaii, and F.A. Mannaa, *Toll-like receptors activation, signaling, and targeting: an overview.* Bulletin of the National Research Centre, 2019. **43**(1).
31. Zuo, L., et al., *Molecular Regulation of Toll-like Receptors in Asthma and COPD.* Front Physiol, 2015. **6**: p. 312.

32. Sodhi, A., S. Tarang, and V. Kesherwani, *Concanavalin A induced expression of Toll-like receptors in murine peritoneal macrophages in vitro.* Int Immunopharmacol, 2007. **7**(4): p. 454-63.
33. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
34. Herath, K., et al., *Differential modulation of immune response and cytokine profiles of Sargassum horneri ethanol extract in murine spleen with or without Concanavalin A stimulation.* Biomed Pharmacother, 2019. **110**: p. 930-942.
35. Herath, K., et al., *Polyphenol containing Sargassum horneri attenuated Th2 differentiation in splenocytes of ovalbumin-sensitised mice: involvement of the transcription factors GATA3/STAT5/NLRP3 in Th2 polarization.* Pharm Biol, 2021. **59**(1): p. 1464-1472.