# Immunostimulatory activity of soybean hull polysaccharide on macrophages

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**Abstract.** Water-soluble polysaccharide isolated from soybean hull and fractionated using ion-exchange chromatography were investigated to determine their molecular characteristics and immunostimulating activity. In the present study, soybean hull polysaccharide (SHP) was separated and purified to obtain three main fractions (F1, F2 and F3), and their chemical and monosaccharide compositions were analyzed. SHP was mainly composed of carbohydrates (64.3%), proteins (16.2%) and sulfates (12.5%), with minor levels of uronic acid (3.2%), and predominantly contained glucose and mannose as monosaccharides. Moreover, when compared with cells treated with RPMI medium, SHP was revealed to promote the proliferation and pinocytosis of RAW264.7 cells, and to enhance the production of nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. Furthermore, flow cytometry demonstrated that CD11b and CD40 were involved in the immune regulation of RAW264.7 cells by SHP. Moreover, western blotting and other experiments revealed that SHP, a type of pathogen-associated molecular pattern, was specifically recognized by the Toll-like receptor 2, which, in turn, upregulated the expression levels of proteins downstream of the mitogen-activated protein kinase and nuclear factor κB pathways. Notably, the immune activity of the F2 fraction was markedly higher than that of the crude polysaccharides. In summary, the purified F2 fraction of SHP may be an effective nutritional supplement for human disorders associated with low immunity.

## Introduction

The immune system is made up of two parts: Innate and acquired immunity. Innate immunity represents the first line of host defense, and provides resistance against foreign, and potentially harmful, pathogens or organisms (1). Macrophages are considered important target cells for immunomodulatory agents among the components of innate immunity, which include macrophages, monocytes and granulocytes (2). Immune cell activation is, in general, a direct and effective way to improve immunity (3).

Natural polysaccharides have recently attracted a lot of attention due to their low toxicity and potential immunomodulatory properties. They activate immune cells either indirectly or directly to produce immune effects (4). Soybean is the most important oil crop worldwide and a source of high-protein food, which is widely planted alongside rice, wheat and corn, as the four major economic crops. Current research on soybeans has not only focused on soybean-derived products, but also the development and utilization of various food industries and functional food materials, such as soy protein meat, isoflavones, lecithin and peptides, which have the functions of regulating immunity, lowering cholesterol and anti-oxidation (5). As a result, there has been an increasing number of soybean by-products. Soybean hulls, which are the seed coat of soybeans (~8%), are one of the major by-products released during the initial cracking process in the production of soybean oil. The vast majority are discarded or used in animal feed (6). The soybean hull contains variable amounts of cellulose (29-51%), hemicellulose (10-25%), lignin (1-4%), pectins (4-8%) and proteins (11-15%) (7). Soybean hulls are considered a source of novel polysaccharides, which have been shown to exert hypoglycemic and hypolipidemic effects (8). However, few reports have explored the functional properties of these polysaccharides, especially in immune regulation.

RAW264.7 murine macrophages are useful to study the molecular mechanisms of macrophages in immune regulation (8) and the pattern recognition receptors (PRRs) on the surface of immune cells, such as Toll-like receptors (TLRs) that recognize pathogen-related molecular patterns (PAMPs). As a type of PAMP, plant polysaccharides can activate macrophages by specifically binding to their cell membrane receptors and can subsequently activate the mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways to generate an immune response. In the present study, SHP was extracted, and three fractions were separated. The three fractions were obtained and designated F1, F2 and F3. Their effects on the proliferation and pinocytosis, as well as

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nitric oxide (NO) and cytokine secretion, of RAW264.7 cells were evaluated to study SHP immunomodulatory activity and to clarify its underlying molecular mechanisms.

## Materials and methods

*Experimental material*. Soybean hulls were purchased from Jinzhou Beiwang Bean Products Co., Ltd. Lipopolysaccharide (LPS), phosphate-buffered saline (PBS), trypsin solution and polymyxin B (PMB) were purchased from Damao Chemical Reagent Factory. Roswell Park Memorial Institute (RPMI)-1640 medium, Griess reagent, fetal bovine serum (FBS) and penicillin were purchased from Qingdao Haiyang Chemical Co. Ltd. Anti-TLR2 (cat. no. ab209216), anti-TLR4 (cat. no. ab22048), anti-Dectin-1 (DC; cat. no. ab169783) and anti-mannose receptor (MR; cat. no. ab64693) antibodies used to treat cells and inhibit PRRs were obtained from Abcam. Rabbit monoclonal antibodies against phosphorylated (p)-Erk at Thr202/Tyr204 (cat. no. 9101S), p-p38 at Thr180/Tyr182 (cat. no. 9211S), p-SAPK/JNK at Thr183/Tyr185 (cat. no. 4668T), p-p65 (cat. no. 3033S), p38 MAPK (cat. no. 8690S), NF-κB p65 (cat. no. 8242S), Erk1/2 (cat. no. 4695S), SAPK/JNK (cat. no. 9252S), α-tubulin (cat. no. 2144S) and horseradish peroxidase-labeled secondary antibody (cat. no. 7074P2) were purchased from Cell Signaling Technology, Inc. All of the antibodies for western blotting were diluted in 5% skim milk solution at a 1:2,000 dilution from a 1 mg/ml stock solution.

*Preparation of polysaccharides*. SHP was extracted from the soybean hull (9). Briefly, dried soybean hull (20 g) was treated with 85% ethanol (EtOH, 200 ml) overnight at 20˚C with constant mechanical stirring. The residual part was separated by centrifugation (10˚C, 1,500 x g, 10 min), rinsed in acetone and dried at room temperature. The dried biomass (10 g) was extracted with distilled water (200 ml) and stirred for 2 h at 65˚C. The extracts were centrifuged at 3,000 x g for 10 min at room temperature, and the supernatants were collected and concentrated to ~200 ml using reduced pressure evaporation at 60˚C for 1 h. EtOH (99%) was added to the supernatants to obtain a final concentration of 70%, and the solution was incubated at 4˚C overnight. The polysaccharide was obtained by filtration of the solution through a membrane (0.45-$\mu$m pore size; Cytiva), was washed with EtOH (99%), followed by acetone and then dried at room temperature for 24 h. The precipitated polysaccharide was called the crude polysaccharide, and the yield was calculated (weight of crude extract/weight of seaweed powder) according to the dried biomass obtained after treating the milled sample with 85% EtOH and drying at room temperature for 24 h.

To obtain different fractions, SHP (200 mg) was dissolved in 10 ml distilled water, filtered through an ion-exchange chromatography system, equipped with a DEAE Sepharose Fast Flow column (cat. no. 17-0709-01; Cytiva) and eluted with distilled water to obtain a non-absorbed fraction F1. Subsequently, a stepwise NaCl gradient (0.5-1 M) was used to wash the highly charged anionic macromolecules, for which elution with a 0.5 M NaCl gradient was performed to obtain fraction F2 and elution with a 1 M NaCl gradient was performed to obtain fraction F3, and the unbound samples was washed with 2 M NaCl gradient. The fractions were obtained at a flow rate of 1.5 ml/min for 7 h. The carbohydrate-positive fractions were pooled, concentrated, dialyzed and lyophilized (9).

*Chemical composition analysis*. Using the phenol-sulfuric acid method and dextrose as the standard, the total carbohydrate content of SHP was quantified (10). With FBS as the standard, the protein content was determined using a Bradford method (11). The sulfate content was determined using the $BaCl_2$-gelatin method with $K_2SO_4$ as the standard following SHP hydrolysis using 0.5 M HCl (12). The uronic acid content was determined using the sulfamate/m-hydroxy diphenyl assay with glucuronic acid as the standard (13).

*Monosaccharide composition analysis*. The composition of SHP monosaccharides was determined as previously described (14). SHP was hydrolyzed using 4 M trifluoroacetic acid at 100˚C for 6 h, reduced in water using $NaBD_4$, acetylated with acetic anhydride. TFA was removed by evaporation with a dried stream of nitrogen (nitrogen gas temperature, 350˚C; nebuliser pressure, 40 psi; flow rate, 8 l/min). The hydrolysates were injected into the high-performance liquid chromatography system that consisted of a pump (Waters 510; Waters Corporation), an injection valve (Model 7010; Rheodyne) with a 20-l sample loop, a column (carbohydrate analysis column, 4.6x250 mm; Waters Corporation) and a refractive index detector (Waters 2414; Waters Corporation). A mixture of acetonitrile and water (80:20, v/v) was used as a mobile phase at a flow rate of 2 ml/min. The following neutral monosaccharides were used as references: Rhamnose, xylose, mannose, galactose, and glucose.

*Cell line and cell culture*. The murine macrophage cell line RAW264.7 was obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. RAW264.7 macrophages were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. The cells were incubated at 37˚C and 5% $CO_2$ (15).

*NO production and cell proliferation analysis*. RAW264.7 cells were seeded in 96-well microplates ($1x10^6$ cells/ml) and cultured in a $CO_2$ incubator. After 24 h, the medium was removed and replaced by 100 $\mu$l culture medium containing different concentrations (25, 50 and 100 $\mu$g/ml) of SHP (Crude, F1, F2 and F3) or LPS (1 $\mu$g/ml); cells treated with RPMI were used as the negative control group and LPS as the positive control group. After an additional 24 h of culture, the cell NO production and cell proliferation were determined using the Griess reaction and water-soluble tetrazolium-1 (WST-1) assays (cat. no. ab65475; Abcam), respectively, according to the manufacturer's instructions. The optical density was measured using a microplate reader (EL-800; BioTek Instruments, Inc.) (16).

*Endotoxin contamination and reactive oxygen species (ROS) analyses*. RAW264.7 cells were seeded in 96-well microplates

(1x10$^5$ cells/ml) and cultured in a $CO_2$ incubator. After 24 h, the medium was removed and replaced with 100 $\mu$l medium containing SHP (100 $\mu$g/ml) or LPS (1 $\mu$g/ml); cells treated with RPMI were used as the negative control group and LPS as the positive control group, in the presence or absence of PMB (50 $\mu$g/ml) to assess endotoxin contamination. After an additional 24 h of culture, the NO content of the supernatant was measured as aforementioned (17).

RAW264.7 cells were seeded in a 96-well microplate (1x10$^5$ cells/ml) and cultured in a $CO_2$ incubator. After 24 h, the medium was removed and replaced with 100 $\mu$l medium containing different concentrations (25, 50 and 100 $\mu$g/ml) of SHP or LPS (1 $\mu$g/ml); cells treated with RPMI were used as the control group. After an additional 24 h of culture, all media were removed and 100 $\mu$l 2',7'-dichlorofluorescin diacetate (10 $\mu$M) was added, and the cells were incubated in the dark for 20 min at 37˚C. The supernatant was removed and the cells were washed three times with PBS. Fluorescence intensity was immediately detected and recorded at 488 nm excitation and 525 nm emission using an Infinite M200 Pro microplate reader (Tecan Group, Ltd.) (18).

*Pinocytic activity analysis*. RAW264.7 cells were seeded in a 96-well microplate (2x10$^4$ cells/ml). After 6 h of culture, the medium was removed and replaced with 100 $\mu$l medium containing different concentrations (25, 50 and 100 $\mu$g/ml) of SHP or LPS (1 $\mu$g/ml); cells treated with RPMI were used as the negative control group and LPS as the positive control group. After an additional 24 h of culture, the medium was removed, 100 $\mu$l PBS containing 0.08% neutral red (cat. no. ab146365; Abcam) was added, and the cell plate was incubated for 1 h at 37˚C. Subsequently, the medium was removed, the cells were washed three times with PBS and 100 $\mu$l cell lysis buffer (acetic acid: ethanol=1:1) was added to each well. The absorbance was recorded at 540 nm using a VersaMax microplate reader (Molecular Devices) (19).

*Quantitative analysis of mRNA expression*. RAW264.7 cells were seeded in a 24-well microplate (1x10$^6$ cells/ml) and were cultured for 24 h in the presence of SHP (100 $\mu$g/ml) or LPS (1 $\mu$g/ml); cells treated with RPMI were used as the negative control group and LPS as the positive control group. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA generation was carried out in a total volume of 10 $\mu$l, containing 5 $\mu$l RNA (100 ng/ml), 0.5 $\mu$l 10 pmol oligo-(dT)20 primer, 0.5 $\mu$l dNTP (10 mM) and 4 $\mu$l superscript III RT (Takara Biotechnology, Co., Ltd.). The primers used are shown in Table I, and β-actin was used as the internal standard. Quantitative PCR (qPCR) was performed using the CFX connect Real-time system (Bio-Rad Laboratories, Inc.) with a Fast Start DNA Master TB Green II kit (Takara Biotechnology Co., Ltd.), with the following program: 1 cycle of initial PCR denaturation at 95˚C for 15 min, 2 cycles of primer annealing at 60˚C for 0.5 min, 32 cycles of denaturation at 95˚C for 0.5 min, 1 cycle of final extension at 60˚C for 1 min and 1 cycle of melting curve analysis at 95˚C for 0.5 min. The results were calculated using the $2^{-\Delta\Delta Cq}$ method (20) and are expressed relative to β-actin.

Table I. Primer sequences used for reverse transcription-quantitative PCR analysis.

| Gene | Primer sequence (5'-3') |
|---|---|
| TNF-α | F: ATGAGCACAGAAAGCATGATC |
| | R: TACAGGCTTGTCACTCGAATT |
| iNOS | F: CCCTTCCGAAGTTTCTGGCAGCAGC |
| | R: GGCTGTCAGAGCCTCGTGGCTTTGG |
| IL-1β | F: ATGGCAACTATTCCAGAACTCAACT |
| | R: CAGGACAGGTATAGATTCTTTCCTTT |
| IL-6 | F: TTCCTCTCTGCAAGAGACT |
| | R: TGTATCTCTCTGAAGGACT |
| IL-10 | F: TACCTGGTAGAAGTGATGCC |
| | R: CATCATGTATGCTTCTATGC |
| β-actin | F: TGGAATCCTGTGGCATCCATGAAAC |
| | R: TAAAACGCAGCTCAGTAACAGTCCG |

iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin.

*Inhibition of NO production using antibodies against PRR*. Cells were pretreated with medium containing different antibodies against PRR (10 $\mu$g/ml) for 2 h at 37˚C, prior to treatment with SHP-F2 (100 $\mu$g/ml) or LPS (1 $\mu$g/ml) for 24 h at 37˚C to stimulate the macrophages; cells treated with RPMI were used as the negative control group and LPS as the positive control group. The levels of NO in the supernatant were measured using the aforementioned procedure (20).

*Flow cytometry*. RAW264.7 cells (2x10$^6$ cells/ml) were cultured for 24 h in the presence of SHP (100 $\mu$g/ml) or LPS (1 $\mu$g/ml); cells treated with RPMI were used as the negative control group and LPS as the positive control group. Subsequently, cells were harvested and washed with FACS buffer containing 1% bovine serum albumin and 0.1% sodium azide (Damao Chemical Reagent Factory). Cells were then stained with 10 $\mu$g/ml anti-mouse CD40 conjugated with APC (cat. no. ab272271) and CD11b conjugated with FITC (cat. no. ab24874) (both from Abcam) for 30 min at 4˚C Parallel sets of cells were incubated without the antibodies and their autofluorescence intensity served as a non-specific negative control. A total of 100,000 viable cells per treatment (as determined using light scatter profiles) were analyzed using a FACS Symphony A5 flow cytometer (BD Biosciences) and CytExpert cytometry software (version 1.0) (Beckman Coulter, Inc.).

*Western blot analysis*. RAW264.7 cells (2x0$^6$ cells/ml) were cultured for 24 h in the presence of crude SHP or SHP fractions (100 $\mu$g/ml), or LPS (1 $\mu$g/ml); cells treated with RPMI were used as the negative control group and LPS as the positive control group. The cells were then washed three times with PBS, and the protein was extracted using lysis reagents [1 ml RIPA lysis buffer (cat. no. ab156034) + 1 $\mu$l inhibitor cocktail + 1 $\mu$l EDTA; Abcam). The protein content was measured using a BCA protein kit. The extracted protein (30 $\mu$g) was loaded, separated by SDS-PAGE on a 10% gel, and then transferred

Table II. The chemical compositions of polysaccharides of soybean hull.

| | SHP | | | |
|---|---|---|---|---|
| Components | Crude | F1 | F2 | F3 |
| Yield, % | $9.2±0.4^a$ | $33.4±1.2^b$ | $47.1±0.5^c$ | $20.2±1.3^d$ |
| Total carbohydrate, % | $64.3±1.4^d$ | $81.2±0.4^c$ | $69.5±0.8^b$ | $51.2±2.1^a$ |
| Protein, % | $16.2±0.3^c$ | $12.5±0.5^b$ | $8.3±0.2^a$ | $11.6±0.6^d$ |
| Uronic acid, % | $3.2±0.5^c$ | $2.3±0.1^b$ | $1.6±0.3^d$ | $3.4±0.2^c$ |
| Sulfate, % | $12.5±0.8^c$ | $11.6±1.2^c$ | $8.6±0.4^b$ | $7.2±0.6^b$ |
| Monosaccharide content, % | | | | |
| Arabinose | $9.4±0.6^b$ | $7.2±1.2^d$ | $12.3±0.9^c$ | $6.7±0.6^d$ |
| Glucose | $36.5±1.3^b$ | $42.3±0.8^c$ | $28.4±1.1^d$ | $28.1±0.5^d$ |
| Galactose | $18.7±0.3^c$ | $14.3±0.4^d$ | $17.2±0.2^b$ | $14.0±0.5^d$ |
| Mannose | $41.7±1.1^c$ | $10.2±0.2^a$ | $34.2±0.6^b$ | $22.5±0.4^d$ |

[a-d]Different letters denote significant differences among groups (P<0.05). Yield, (weight of crude/weight of seaweed powder) or (weight of fractions/weight of crude injected into ion-exchange chromatography) x100. SHP, soybean hull polysaccharide; F1, fraction 1; F2, fraction 2; F3, fraction 3.

onto a PVDF membrane (0.22 $\mu$m; MilliporeSigma). The membranes were incubated with primary antibodies at 4˚C overnight after blocking with Blocking One (Nacalai Tesque, Inc.) for 1 h at 4˚C. Finally, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled secondary antibodies. Antibodies for western blotting were diluted in 5% skim milk solution at a 1:2,000 dilution from a 1 mg/ml stock solution. The protein was detected using an ECL kit (Takara Bio, Inc.).

*Statistical analysis*. Excel 2016 (Microsoft Corporation) and SPSS software (v19.0; IBM Corp.) were used for the statistical analysis of the data, and Origin 8.0 (OriginLab) was used for generating the related graphics. Data are presented as the mean ± standard deviation from at least three independent experiments. Significant differences were statistically analyzed by two-way analysis of variance (ANOVA) or one-way ANOVA followed by Tukey's post hoc test. P<0.05 was used to indicate a statistically significant difference.

## Results and Discussion

*Isolation and chemical composition analysis of SHP*. Soybean hull was extracted using hot water and crude polysaccharides were obtained using ethanol precipitation (yield, 9.2%). Ion-exchange chromatography on a DEAE Sepharose Fast Flow column was used for separation and purification to obtain three fractions: F1 (distilled water), F2 (0.5 M NaCl) and F3 (1 M NaCl). Chemical analysis of the soybean hull crude polysaccharides revealed that they were mainly composed of carbohydrates (64.3%), protein (16.2%) and sulfate (12.5%), as well as a small amount of uronic acid (3.2%) (Table II).

The monosaccharide composition of SHP, F1, F2 and F3 was determined using gas chromatography-mass spectrometry. The crude polysaccharide and the fractions were primarily composed of mannose (22.5-41.7%) and glucose (28.1-42.3%), whereas F1 was primarily composed of glucose (42.3%), galactose (14.3%) and mannose (10.2%) (Table II; Fig. S1).

*Effects of SHP on NO production and cell proliferation*. In the present study, crude SHP and fractions were used to stimulate NO production in RAW264.7 cells and assess the ability of SHP to induce immune activity. Overall, the purified fraction F2 was more effective than crude SHP at promoting NO secretion in RAW264.7 cells (Fig. 1A).

Previous reports have indicated that NO may induce the apoptosis of bacteria, microorganisms and tumor cells in the body (21,22). The purified component F2 produced 41.21-43.57 $\mu$M NO, which was significantly higher than that produced by crude polysaccharides and the other purified components (Fig. 1A), indicating that F2 had the highest ability to stimulate RAW264.7 cell activation. According to a previous study, the cell membrane is torn during apoptosis, and the intracellular nutrients are dissolved out, resulting in a false-positive increase in the measurement of the NO value (21). To verify whether SHP increases NO values due to sample apoptosis, a cytotoxicity test was performed.

Macrophages are known to serve a vital role in innate and acquired immune responses (23); therefore, active ingredients that increase macrophage proliferation have a certain significance for the immune system response. Herein, the proliferation of RAW264.7 cells was stimulated by crude SHP and the isolated fractions (Fig. 1B). After being treated with increasing SHP concentrations (25, 50 and 100 $\mu$g/ml), cell proliferation was 100.44-126.02% compared with that in the RPMI negative control group (100%), which indicated that SHP (25, 50 and 100 $\mu$g/ml) exhibited a nontoxic effect on RAW 264.7 cells, further verifying that soybean hull polysaccharides-induced stimulation of NO production is not due to sample toxicity leading to cell apoptosis. Notably, F2 had the highest immunostimulatory potential among the three fractions, making it a good source of natural immune modulators.

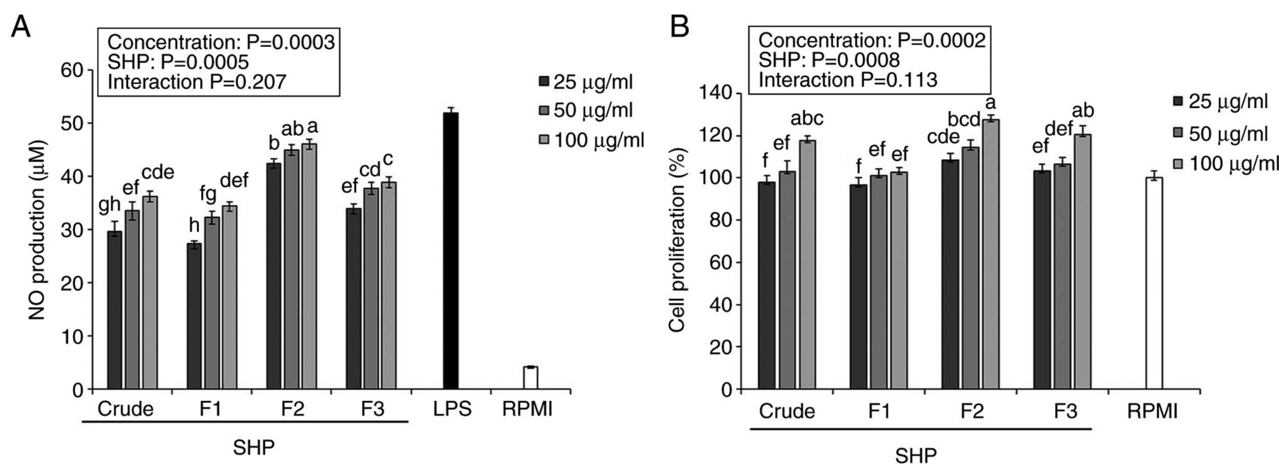


Figure 1. Effects of SHP on (A) NO production and (B) cell proliferation. Data are expressed as the mean ± SD. Main effects and interactions (SHP and concentration) were analyzed by two-way ANOVA, followed by Tukey's post hoc test. [a-h]Different letters denote significant differences among groups (P<0.05). SHP, soybean hull polysaccharide; NO, nitric oxide; LPS, lipopolysaccharide; RPMI, Roswell Park Memorial.

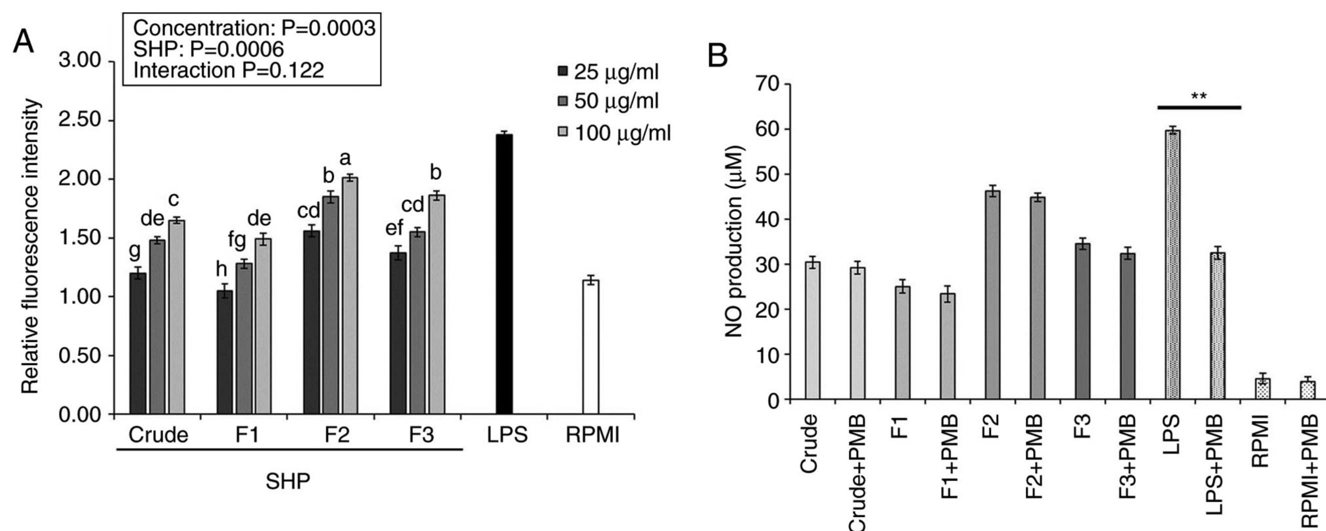


Figure 2. Effects of SHP on (A) ROS production and (B) endotoxin contamination. RAW264.7 cells were treated with crude SHP and fractions of SHP (100 $\mu$g/ml), RPMI or LPS (1 $\mu$g/ml) with or without the presence of PMB (50 $\mu$g/ml). Data are expressed as the mean ± SD. (A) Main effects and interactions (SHP and concentration) were analyzed by two-way ANOVA, followed by Tukey's post hoc test. [a-h]Different letters denote significant differences among the groups (P<0.05). (B) Statistical analysis was carried out using one-way ANOVA followed by Tukey's post hoc test. **P<0.01, LPS vs. LPS + PMB. SHP, soybean hull polysaccharide; NO, nitric oxide; LPS, lipopolysaccharide; RPMI, Roswell Park Memorial Institute; PMB, polymyxin B.

*Effects of SHP on ROS production and endotoxin contamination.* ROS are important intracellular signaling molecules that participate in the secretion and synthesis of inflammatory factors in macrophages, making them important in the pathophysiology of inflammation, immune diseases and pathogen defense. Therefore, analysis of ROS is one of the main interests for several researchers (24,25). ROS content produced by the RAW264.7 macrophages, as measured by fluorescence intensity, increased as the concentrations of crude SHP and SHP fractions increased (25-100 $\mu$g/ml), with significant differences observed between the SHP-treated and negative control (RPMI) groups (Fig. 2A). Following treatment with crude SHP and SHP fractions, ROS content in cells treated with F2 (100 $\mu$g/ml) was ~2-fold higher than that in the control group. The results indicated that SHP could mediate the upregulation of intracellular ROS production, with the F2 fraction having the greatest impact.

A recent study indicated that polysaccharides isolated from different natural sources have the high possibility of being contaminated by LPS and show false-positive results in immune-stimulation assays (17). To overcome this limitation, some studies have demonstrated that PMB blocks the biological effects of gram-negative LPS through binding to lipid A, the toxic component of LPS, which is negatively charged (26). Therefore, in the present study, to rule out the possibility of LPS (endotoxin) contamination in SHP, RAW 264.7 cells were treated for 24 h with SHP or LPS (positive control group) in the absence or presence of PMB. As shown in Fig. 2B, the presence of PMB did not affect NO production in SHP-treated macrophages. PMB, on the other hand, significantly inhibited LPS-induced NO production in macrophages,

indicating that SHP-induced activation of macrophages was not caused by endotoxin contamination.

*Effect of SHP on pinocytic activity.* Macrophages play an immunomodulatory role in the body through biological activities, such as phagocytosis or pinocytosis (27). Therefore, enhancement of the pinocytic activity of macrophages is an important sign of macrophage activation, which can be indirectly evaluated by the absorption of neutral red solution (28). The pinocytic indexes of the SHP-treated groups were significantly higher than those of the negative control (RPMI) samples in a dose-dependent manner (Fig. 3). Following treatment with 25 $\mu$g/ml F2, the pinocytotic indices of RAW264.7 cells exceeded 1.0, and F2 upregulated the pinocytotic activity of RAW264.7 cells in a dose-dependent manner. In addition, LPS (positive control group) significantly promoted pinocytosis at a lower concentration (1 $\mu$g/ml) when compared with RPMI group, and the F2-enhanced cell pinocytosis index was significantly higher than that in the crude, F1 and F3 groups.

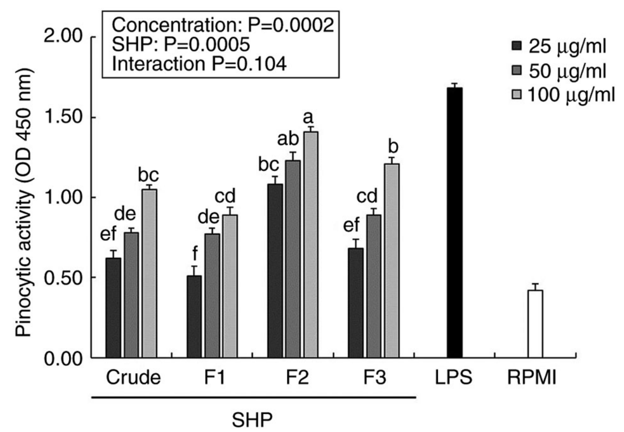


Figure 3. Effect of SHP on pinocytic activity. Data are expressed as the mean ± SD. Main effects and interactions (SHP and concentration) were analyzed by two-way ANOVA, followed by Tukey's post hoc test. [a-f]Different letters denote significant differences among the groups ($P<0.05$). SHP, soybean hull polysaccharide; LPS, lipopolysaccharide; RPMI, Roswell Park Memorial Institute; OD, optical density.

*Effect of SHP on the mRNA expression levels of inflammatory factors.* To further explore the effect of SHP on RAW264.7 cells at the molecular level, qPCR was used to analyze the gene expression of inflammatory factors. Inducible nitric oxide synthase (iNOS) is one of the three key enzymes that catalyze the production of NO from L-arginine (29). Proinflammatory cytokines have been shown to play an important role in immune regulation. When foreign antigens are detected by the host immune cells, proinflammatory factors aid T cells in developing an immune response and quickly initiate the innate immune defense (30). Therefore, the expression levels of TNF-α, IL-1β, IL-6, and IL-10 were determined in the present study. The results showed that the SHP fractions were able to upregulate the mRNA expression levels of inducible nitric oxide synthase (iNOS), as well as those of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 (Fig. 4). Compared with in the RPMI (negative control group), the mRNA expression levels of iNOS and cytokines were significantly increased following SHP (F2 and F3) or LPS (positive control group) stimulation. Notably, although the inflammatory cytokines (TNF-α, IL-1β, IL-6) are beneficial at appropriate amounts, their excessive production in a deregulated fashion is toxic and may cause severe inflammatory responses (30). As an anti-inflammatory agent, IL-10 can protect cells from enduring severe inflammatory reactions and cell death by inhibiting the overexpression of inflammatory factors such as TNF-α, IL-1β and IL-6. Therefore, the potential production of IL-10 was examined in the present study and the gene expression levels were significantly increased (Fig. 4), indicating the capability of SHP to tightly mediate the inflammatory process. Specifically, the mRNA expression levels of iNOS, IL-6, IL-1β, TNF-α, and IL-10 following treatment with 100 $\mu$g/ml F2 were 9.72-, 12.2-, 5.10-, 2.42- and 4.12-fold higher than those in the negative control group (RPMI), but were lower than those in the positive control group (LPS). These results indicated that F2 could upregulate the secretion and related gene expression of NO and cytokines in murine macrophages.

*Effect of specific antibodies against PRR on NO production of SHP-stimulated macrophages.* PRRs are known to be used by macrophages to identify pathogens and exert immune effects. In particular, glycans and glycol-conjugates (as PAMPs) are recognized by PRRs on macrophages (29), which include TLRs, DC and MR. The activation of PRRs causes cells to transmit related signals, thus stimulating the secretion of inflammatory factors and macrophage activation (30). SHP was used as a PAMP to verify whether it could be recognized by specific PRRs to exert immune effects. Following the addition of inhibitors of TLR2, TLR4, DC and MR during RAW264.7 cell culture, cells were stimulated with the F2 fraction and NO production was detected, with cells treated with RPMI being used as a negative control group. The addition of a TLR2 inhibitor to the culture medium of F2-stimulated RAW264.7 cells significantly reduced NO production compared with that in cells not exposed to the inhibitor (Fig. 5). Hence, these results suggested that the F2 fraction (100 $\mu$g/ml) stimulated the production of NO through signals from TLR2. This finding differed from the results of a previous study (31), which may be related to the structural characteristics of the polysaccharides, as TLR2 is more easily recognized by polysaccharides with a high content of glucose and mannose (18). These findings indicated that the F2 fraction, as a type of PAMP, was specifically recognized by TLR2.

*Effect of SHP on the expression of surface molecules in RAW264.7 cells.* CD11b and CD40 are important inflammatory regulators that not only protect the activity of immune cells but also induce cytokine expression (32). CD40 is a member of the TNF receptor family that is expressed in antigen-presenting cells (33). Therefore, the high-intensity signals from CD11b and CD40 indicate that macrophages have been activated to induced an inflammatory responses.

Expression of CD11b and CD40 in macrophages was increased in response to stimulation with crude SHP and SHP fractions when compared with the RPMI group (negative

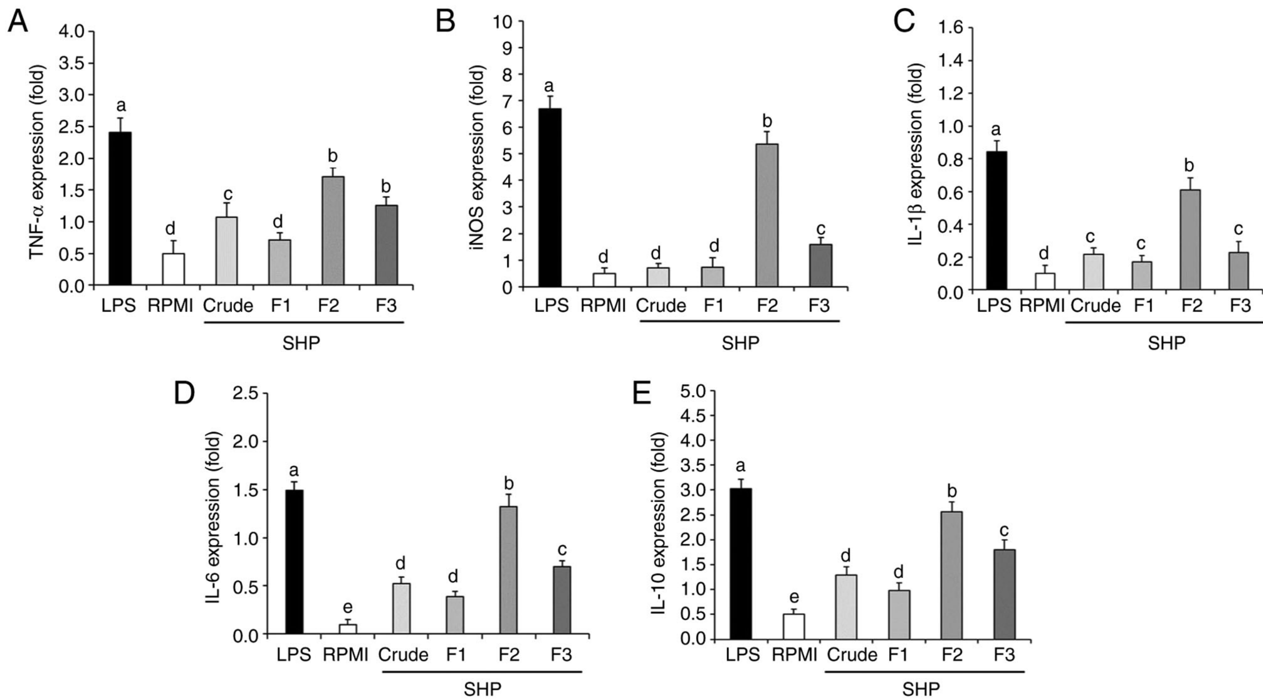


Figure 4. Effect of SHP on the mRNA expression levels of inflammatory factors. RAW264.7 cells were treated with crude SHP and fractions of SHP (100 µg/ml), RPMI or LPS (1 µg/ml) for 24 h. mRNA expression levels of (A) TNF-α, (B) iNOS, (C) IL-1β, (D) IL-6 and (E) IL-10. Data are expressed as the mean ± SD. [a-e]Different letters denote significant differences among groups (P<0.05). SHP, soybean hull polysaccharide; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL-interleukin; RPMI, Roswell Park Memorial Institute.

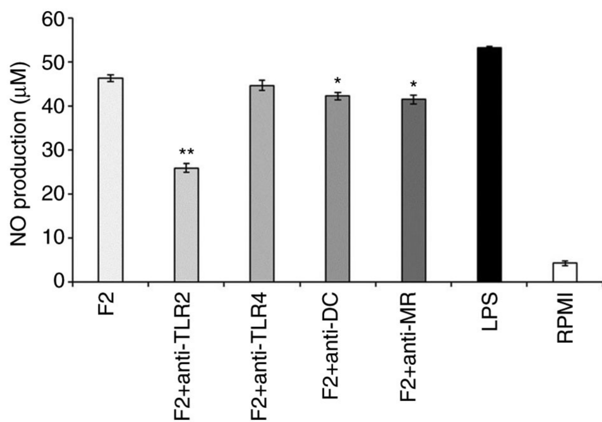


Figure 5. Effect of specific PRR inhibitors on the production of NO by SHP-stimulated macrophages. RAW264.7 cells were pretreated with PRR-specific inhibitors (10 µg/ml) for 2 h, before adding the SPH sample (100 µg/ml), RPMI or LPS (1 µg/ml) to stimulate the macrophages *P<0.05; **P<0.01 vs. F2 group. Values are expressed as the mean ± standard deviation (n=3). PRR, pattern recognition receptors; F2, fraction 2; NO, nitric oxide; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; DC, dectin-1; MR, mannose receptor; LPS, lipopolysaccharide;. RPMI, Roswell Park Memorial Institute.

control), but was significantly lower than that detected in the LPS group (positive control) (Fig. 6A-D). Notably, no difference in the expression of the inflammatory regulator CD40 was observed between F2- and F3-treated cells; however, CD11b levels were significantly different when the F2 group was compared with the F3-treated group. These findings indicated that CD11b and CD40 may be involved in the SHP-promoted immune regulation of RAW264.7 cells.

*Effects of SHP on the activation of MAPK and NF-κB signaling pathways in macrophages.* A previous study demonstrated that PRRs can specifically recognize PAMPs and activate the NF-κB pathway, thereby inducing the transcription of downstream genes, as well as promoting the production of inflammatory factors and NO (27). IκB-α, as an inhibitor of NF-κB, encapsulates NF-κB to form a complex; thus, macrophages will be inactive. When IκB-α is phosphorylated, it is degraded, which, in turn, releases NF-κB and enhances the translocation of p65 from the cytoplasm to the nucleus (34). MAPKs are important regulators of cell proliferation, differentiation and stress response, as well as the secretion of cytokines, chemical factors and other regulators. Thus, phosphorylation of MAPKs directly affects the activated stress response of macrophages (35).

Under SHP fraction stimulation, the levels of p-p65 were increased when compared with those in the RPMI group (negative control), indicating that SHP activated RAW264.7 cells via the NF-κB pathway to exert its immune activity (Fig. 7). Moreover, the F2 group exhibited markedly increased protein expression levels of p-JNK, p-ERK and p-p38 compared with those in the negative control group (RPMI), but not as much as positive control group (LPS) (Fig. 7). Furthermore, the expression levels of these proteins were higher in F2-treated cells than in cells treated with the other fractions, indicating that F2 had higher immunostimulatory ability. Collectively, these results demonstrated that SHP activated macrophages through the NF-κB and MAPK pathways to support its immune effects. In the present study, a detailed analysis of these pathways indicated that SHP induced macrophages to secrete inflammatory factors via TLR2 and consequent activation of the NF-κB and MAPK pathways.

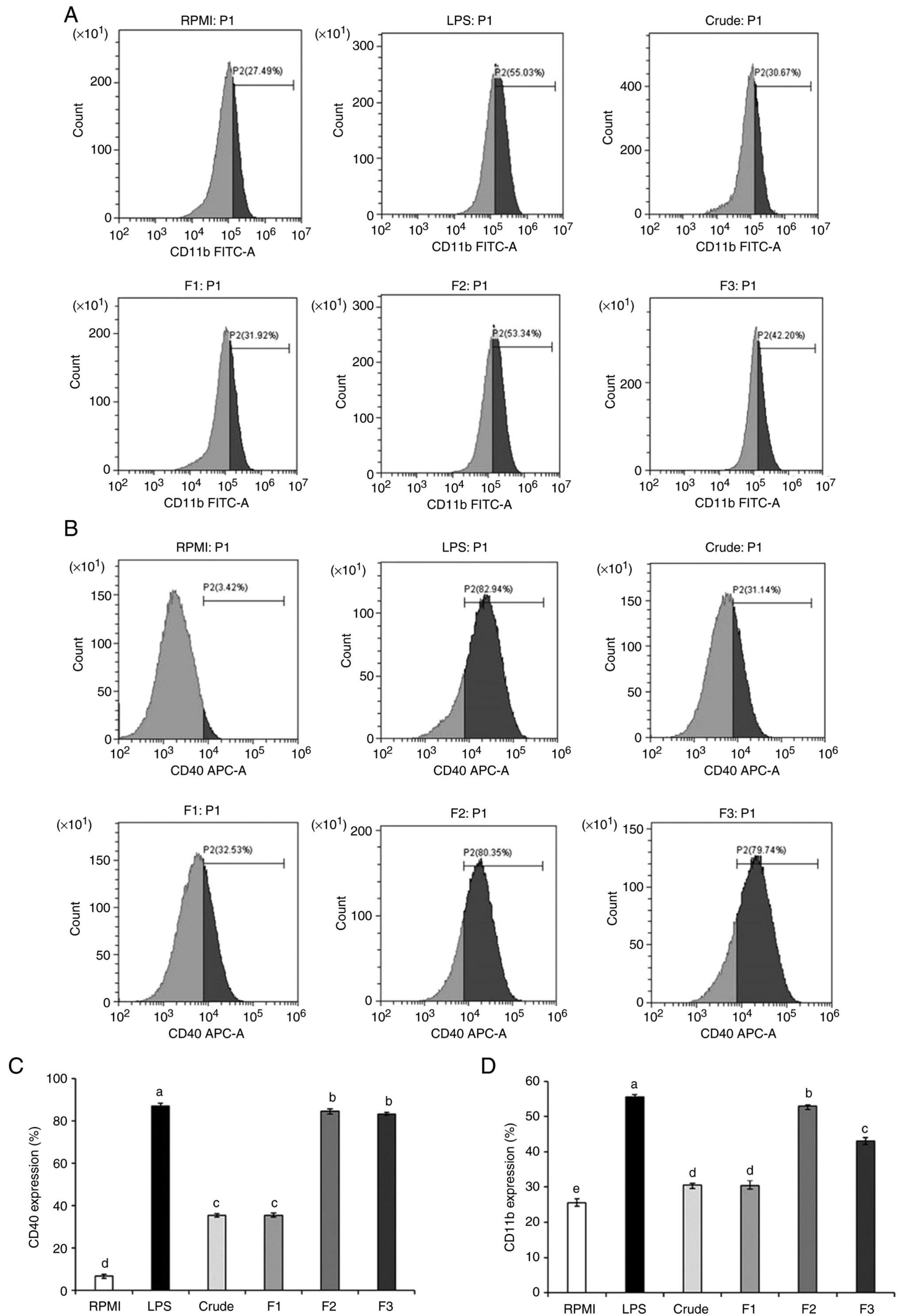


Figure 6. Effect of SHP on the expression levels of surface molecules in RAW264.7 cells. Black colors and percentage in histogram is the expression levels of surface molecules in RAW264.7 cells. (A and C) CD11b, (B and D) CD40 expression. Data are expressed as the mean ± SD. [a-e]Different letters denote a significant differences among the groups ($P<0.05$). SHP, soybean hull polysaccharide; LPS, lipopolysaccharide; RPMI, Roswell Park Memorial Institute; FITC, fluorescein isothiocyanate; APC, alkaline phosphatase.

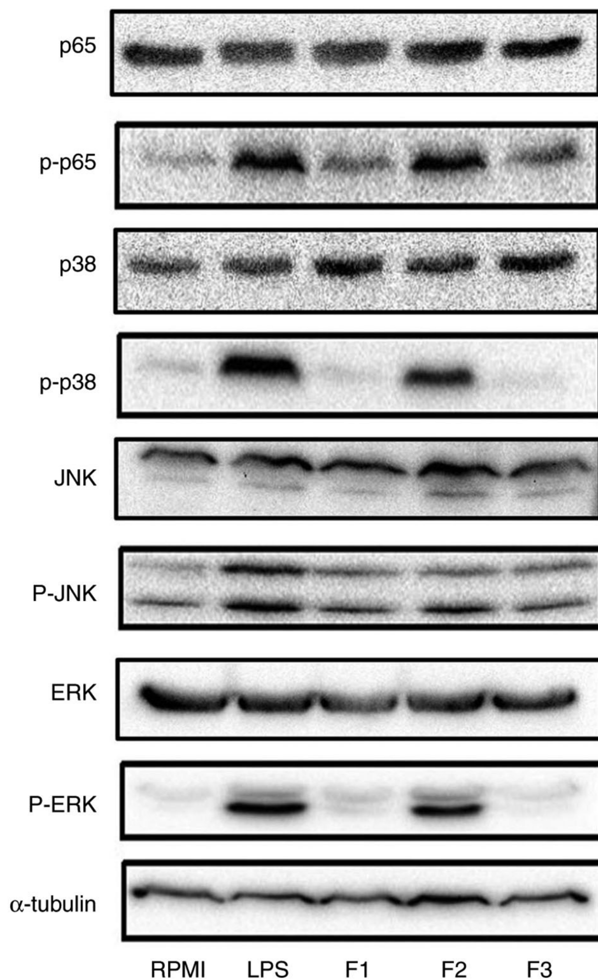


Figure 7. Western blot analysis showing the effects of SHP on the activation of MAPK and NF-κB signaling pathways in macrophages. LPS, lipopolysaccharide; RPMI, Roswell Park Memorial Institute; p-, phosphorylated.

In summary, following extraction of natural plant polysaccharides from soybean hull and purification of fractions (F1, F2 and F3), monosaccharide composition analysis demonstrated that glucose and mannose were the repeating unit of the polysaccharide backbone of SHP after hot water extraction (Table II), which is consistent with the findings of Li *et al* (36). SHP-purified component F2 was able to regulate the immune activity of murine macrophages, by promoting cell proliferation, enhancing pinocytosis, and inducing the secretion of NO and proinflammatory cytokines, such as TNF-α. In addition, in comparison with a previous study, F2 was shown to exert stronger immunostimulatory effects on macrophages compared with rice bran; for example, high levels of NO and cytokines were secreted in response to low F2 concentrations (37). Moreover, CD11b and CD40 were found to be involved in the SHP-mediated immune regulation of RAW264.7 cells. TLR4 is considered an essential receptor in the binding of β-glucan to the macrophage surface, and in activating the MAPK and NF-κB signaling pathways; however, in the present study, following incubation of cells with SHP and antibodies against PRRs, TLR2 was shown to activate the immune response in macrophages through the NF-κB and MAPK pathways, which in turn may induce the production of NO and the proinflammatory cytokines, including iNOS, IL-1β and IL-6. The results of this experiment differ from those in the study by Hsu *et al* (38), which may be due to the different experimental materials used: Hsu *et al* (38) extracted polysaccharides from *Ganoderma lucidum* (*reishi* or *lingzhi*), whereas the present study used soybean hulls as a source of polysaccharides. In addition, DC was investigated in the present study and the results regarding this PRR were similar to those found in the study by Zheng *et al* (39), which indicated that RAW264.7 cells only express low levels of DC. However, another study demonstrated the synergistic stimulation of primary human monocytes and macrophages by combined stimulation with TLR-4 and DC ligands (40). Even though the possibility is low, the immune responses activated by SHP could be amplified through the synergy between DC and TLR4, which requires further investigation.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

MW was involved in the conceptualization, investigation and writing the original draft of this study; CF was involved in the investigation, and the data validation and analysis; MZ conceived and designed the experiments, and wrote, reviewed and edited the manuscript; YZ and LC were involved in the conceptualization, methodology, supervision, writing, reviewing and editing the manuscript, and funding acquisition. MW and LC confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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