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Immunomodulatory effect of intracellular polysaccharide from mycelia of *Agaricus bitorquis (QuéL.) Sacc.* Chaidam by TLR4-mediated MyD88 dependent signaling pathway

Ximeng Lin [a], Wenxia Li [b], Hywel Yuen [b], Michael Yuen [b], Qiang Peng [a,*]

[a] College of Food Science and Engineering, Northwest A&F University, Yangling 712100, PR China
[b] Puredia Limited, No.12, Jing'er road (North), Biological Technology Park, Chengbei District, Xining, Qinghai, PR China

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ABSTRACT

Agaricus bitorquis (QuéL.) Sacc. Chaidam is a valuable edible fungus in Qinghai-Tibet plateau and ABSP is a novel intracellular polysaccharide from its mycelia. GC and NMR analysis determined ABSP is galactoglucomannan-like polysaccharide that may have immunomodulatory effect. This study used RAW264.7 as model cell to determine immunomodulatory effect of ABSP. After ABSP treatment, viability and phagocytic ability promoted, and NO, ROS, TNF-α levels also raised which proved ABSP had immune regulation to RAW264.7. WB and qRT-PCR determined the key proteins and genes expression of TLR4, MyD88, TRAF-6 and NF-κB significantly increased while protein and gene expression of TRAM had no significant increase. Also, TNF-α level extremely decreased by adding inhibitors of TLR4 and MyD88 which confirmed ABSP could immunologically regulate RAW264.7 byTLR4-MyD88 dependent pathway. This study would provide theoretical basis for further study on ABSP and be helpful for development of beneficial functionally foods and exploitation of this resource.

1. Introduction

Edible fungi, is a kind of mushroom that has huge and edible fruit body. They have had a long research history in China. In the world's first medicine monograph 'Shengnong Book of Herbs', some edible fungi such as ganoderma lucidum have used as medicines [1]. There have abundant resources of edible fungus in China such as Lentinus edodes, Pleurotus ostreatus, Volvariella vovlaca, Flammulina velutipes, Hericium erinaceus [2]. Qinghai-Tibet plateau is an area with the highest distribution of macro-fungi in the world which grows more than 300 kinds macro-fungi. *Agaricus bitorquis (Quél.) Sacc.* Chaidam, as a representative variety in fungal resources of this area, has publicly recognized as one of the most valuable wild edible mushrooms [3]. It has huge fruit body, strong resistance ability and rich nutrition in carbohydrate (34.91%), proteins (24%), fiber (17.32%) [4]. However, there only have few researches on this valuable mushroom before.

Since 1970s, scientific researches have determined carbohydrates are not only the important energetic resource and indispensable structural material to maintain life for all living organism, but also the significant participants for various activities of cells in life sciences, such as regulation of immune function, cell-to-cell recognition, transport of material between cells and so on [5]. Polysaccharides from Edible fungi are biologically active substances that have immunomodulatory, antitumor, and antioxidant activities [6], which are internationally called 'Biological Response Modifier'. Many researches have proved polysaccharides extracted from edible fungi like pleurotus eryngii [7,8], grifola frondosa [9], collybia radicata [10], and paxillus involutus [11] have showed abilities on tumor inhibitory, antioxidant and immunomodulatory. However, there existed only few studies on polysaccharides from *Agaricus bitorquis (Quél.) Sacc.* Chaidam about their antioxidant and anti-hypoxia activities [3,4,12]. No study on immunomodulatory function of polysaccharide from *Agaricus bitorquis (Quél.) Sacc.* Chaidam has been studied. Also, immunomodulatory effect of mycellial polysaccharide of this fungus is no exception.

This study aimed at immunomodulatory effect of a novel intracellular polysaccharide from mycelium of *Agaricus bitorquis (QuéL.) Sacc.* Chaidam on RAW264.7 cells and determined its structural features by chemical methods and its immunomodulatory pathway on RAW264.7 macrophages through Western blots and qPCR.

2. Materials and methods

2.1. Materials and chemicals

The mushroom, *Agaricus bitorquis (QuéL.) Sacc.* Chaidam, was provided by Food Laboratory of Qinghai University (China). The mycelium

* Corresponding author.
E-mail addresses: ximenglin423@nwsuaf.edu.cn (X. Lin), lwx010508@163.com (W. Li), hywel@puredia.com (H. Yuen), michael@puredia.com (M. Yuen), pengqiang@nwsuaf.edu.cn (Q. Peng).

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was obtained from *Agaricus bitorquis (QuéL.)*, then dried and grinded into powder. The standard monosaccharides including rhamnose, arabinose, xylose, mannose, glucose and galactose were purchased from Aladdin Biochemical Technology Co., China. Deuterium oxide (D_2O) was provided by Sigma-Aldrich Co., German. TAK-242 (TLR4 inhibitor) and ST-2825 (specific MyD88 dimerization inhibitor) were from MedChemExpress Co., USA. All chemicals are analytical grade.

2.2. Extraction and purification

The crude mycelia polysaccharide was extracted by deionized water ($\sigma = 2.92$ μS). At first, hot distilled water (80 °C, 1.5 h) was applied to mycelium powder for extraction, then repeating this extraction after removing the supernatants. Mixed all supernatants and then concentrated to half volume. Subsequently, four volumes of absolute ethanol was added to the concentrate and standing for 8–12 h. After centrifugation and lyophillzation, crude mycelium polysaccharide was obtained.

For purification, 800 mg crude polysaccharide was dissolved in 35 mL Tris-HCl buffer (0.1 mol/L, pH = 8.0), then treated with Pronase E (100:1, mg/mg) at 37 °C for 24 h [13]. After deactivation of enzyme (100 °C, 5 min), dialysis and lyophillzation, 100 mg crude polysaccharide was dissolved in 10 mL deionized water, then injected to a gel chromatography column of Sephadex G-100 (1.6 cm × 60 cm), eluted with deionized water at a flow rate of 0.3 mL/min [14]. The most symmetrical eluting peak was collected, concentrated, and lyophilled as the purified polysaccharide (ABSP).

2.3. Monosaccharide analysis

The monosaccharide composition was determined by GC [15,16]. In Brief, 10 mg ABSP was dissolved in 10 mL of 2 mol/L trifluoroacetic acid at 121 °C for 2 h, then derived by pyridine and acetic anhydride. The derived sample was detected by a GC-2014 (Shimadzu Co., Japan) with a capillary column of DB-17 (30 m × 0.25 mm × 0.25 μm). The temperature program was as follows: raised from 130 °C to 160 °C at a rate of 30 °C/min, then raised to 180 °C at a rate of 10 °C/min, maintained for 2 min, then raised to 210 °C at a rate of 3 °C/min, maintained for 2 min, and finally raised to 220 °C at a rate of 1 °C/min, maintained for 4 min. The carrier gases were nitrogen (100 kPa, 1.5 mL/min), hydrogen (75 kPa, 60 mL/min) and air (50 kPa, 450 mL/min). Mixed standard monosaccharides (rhamnose, arabinose, xylose, mannose, glucose, galactose) were derived in the same way as ABSP.

2.4. NMR analysis

Dried ABSP (50 mg) was dissolved in D_2O and lyopohilized, then repeated this step for three times. The prepared ABSP was redissolved in 0.5 mL D_2O. NMR spectra of ^1H, ^{13}C, COSY, HSQC were recorded by a Bruker BioSpin GmbH 600 MHz NMR spectrometer at room temperature with standard pulse sequences.

2.5. Cell culture

RAW264.7 macrophages of mice were purchased from ATCC. The macrophages were cultured in RPMI1640 medium (BasalMedia Co., China) with 10% FBS (ThermoFisher co., Shanghai, China), 100 U/mL penicillin (Beyotime co., Shanghai, China), and 100 μg/mL streptomycin (Beyotime co., China) and in a 5% CO_2 atmosphere at 37 °C.

2.6. Cell viability assay

The cell viability was evaluated by CCK-8 (EnoGene Co., China). Briefly, RAW264.7 cells (5×10^4 cells/mL) were cultured in a 96-well plate and incubated for 24 h (37 °C, 5% CO_2). Then the cells were treated by different concentrations of ABSP (25, 50, 100, 200, 400, 800 and 1000 μg/mL) for 24 h. After treatment, 10 μL CCK-8 solution was added and then these cells were incubated at same environment for 1 h. The cell viability of RAW264.7 was determined by a multifunctional microplate reader (victorX3, PerkinElmer Co., USA) at wavelength of 450 nm. LPS (0.1 μg/mL) was used for positive control and the cell viability was expressed as percentage of control group.

2.7. Assay for phagocytic ability

The effect of ABSP on macrophages phagocytic ability was investigated by neutral red experiment. 5×10^4 cells/mL of RAW264.7 were seeded in a 96-well plate then cultured for 24 h. After incubation with different concentrations of ABSP (25, 50, 100, 200 μg/mL) for 24 h, neutral red staining solution was added to this plate, then cultured for 4 h. Subsequently, 150 μL cell lysis solution was added to the cell plate and absorbance was measured by a multifunctional microplate reader (victorX3, PerkinElmer Co., USA) at wavelength of 540 nm [17]. The results were expressed as a relative percentage to control group.

2.8. NO and TNF-α concentrations analysis

RAW264.7 cells at density of 5×10^4 cells/mL were incubated in a 96-well plate at 37 °C with 5% CO_2 for 24 h, then treated with different concentrations of ABSP (25, 50, 100, 200 μg/mL) for 24 h. The intracellular NO level was determined by a Nitric Oxide Assay Kit (Beyotime, Biotechnology, China), using 0.1 μg/mL LPS as positive control. The TNF-α levels in supernatants were determined by a Mouse TNF-α ELISA Kit (MultiSciences Co., China) following its instruction. TNF-α concentrations of sample were calculated from the standard curve.

2.9. ROS production determination

The incubated RAW264.7 cells were treated with 50 and 200 μg/mL ABSP for 24 h. Then DCFH-DA at the concentration of 10 μmol/L was added and cultured together for 30 min. After removing the extra DCFH-DA by PBS washing, ROS level of each group was determined by a flow cytometer (CytoFLEX, Beckman co., America) at 488 nm of DCF excitation wavelength and 525 nm of emission wavelength. LPS (0.1 μg/mL) was used as positive control.

2.10. Western blot analysis

Briefly, RAW264.7 cells were washed with PBS (ice-cold) and collected after ABSP treatment. Then cell pellets were suspended in the RIPA lysis (Bioworld technology co., USA) for 30 min. After centrifugation (12,000 rpm) for 5 min, the supernatant was collected. A BCA protein quantitative kit (Biotopped co., China) was used to evaluate total protein content in sample. According to quantitative result, different groups were mixed with 5 times protein loading buffer by equal quality. After centrifugation, the samples were electrophoresed on SDS polyacrylamide gels and transferred to PVDF membranes (Millipore, USA). The blots were blocked in PBS (containing 0.02% sodium azide, 5% nonfat milk and 0.2% Tween 20). After being incubated with the primary antibodies for 1 h (at room temperature), the membranes were rinsed with PBS (containing 0.2% Tween 20), and incubated with the corresponding secondary antibodies (conjugated with horseradish peroxidase) for another 1 h. With the chemiluminescent method, the proteins were finally detected after washing.

2.11. qRT-PCR analysis

After cultured for 24 h, the cells were treated with 0.1 μg/mL LPS and different concentrations of ABSP (50, 100, 200 μg/mL) for

24 h. The total RNAs were extracted by TRIZOL reagent (TaKaRa co., China). The quantitative RT-PCR commercial kit (Beyotime co., China) and cDNA reverse transcription commercial kit (Bioer co., China) was used for qRT-PCR. The condition for qRT-PCR is: predenaturation at 95 °C for 2 min, denaturation at 95 °C for 15 s, annealing/extension at 60 °C for 15–30 s (40 cycles), with an ABI 7900HT fluorescence quantitative PCR (ABI co., USA) was used for evaluation.

2.12. Assay for TNF-α production after inhibition of TLR4 or MyD88

RAW264.7 cells (5×10^4 cells/mL) at logarithmic growth were cultured in a 96-well plate. After incubated for 24 h, the cells were treated with 100 μg/mL ABSP, 0.1 μg/mL LPS, 100 μg/mL of ABSP with 20 μg/mL TAK-242, 0.1 μg/mL LPS with 20 μg/mL TAK-242, 100 μg/mL of ABSP with 20 μg/mL ST-2825 and 0.1 μg/mL LPS with 20 μg/mL ST-2825 for 12 h. The TNF-α levels in supernatants were determined by a Mouse

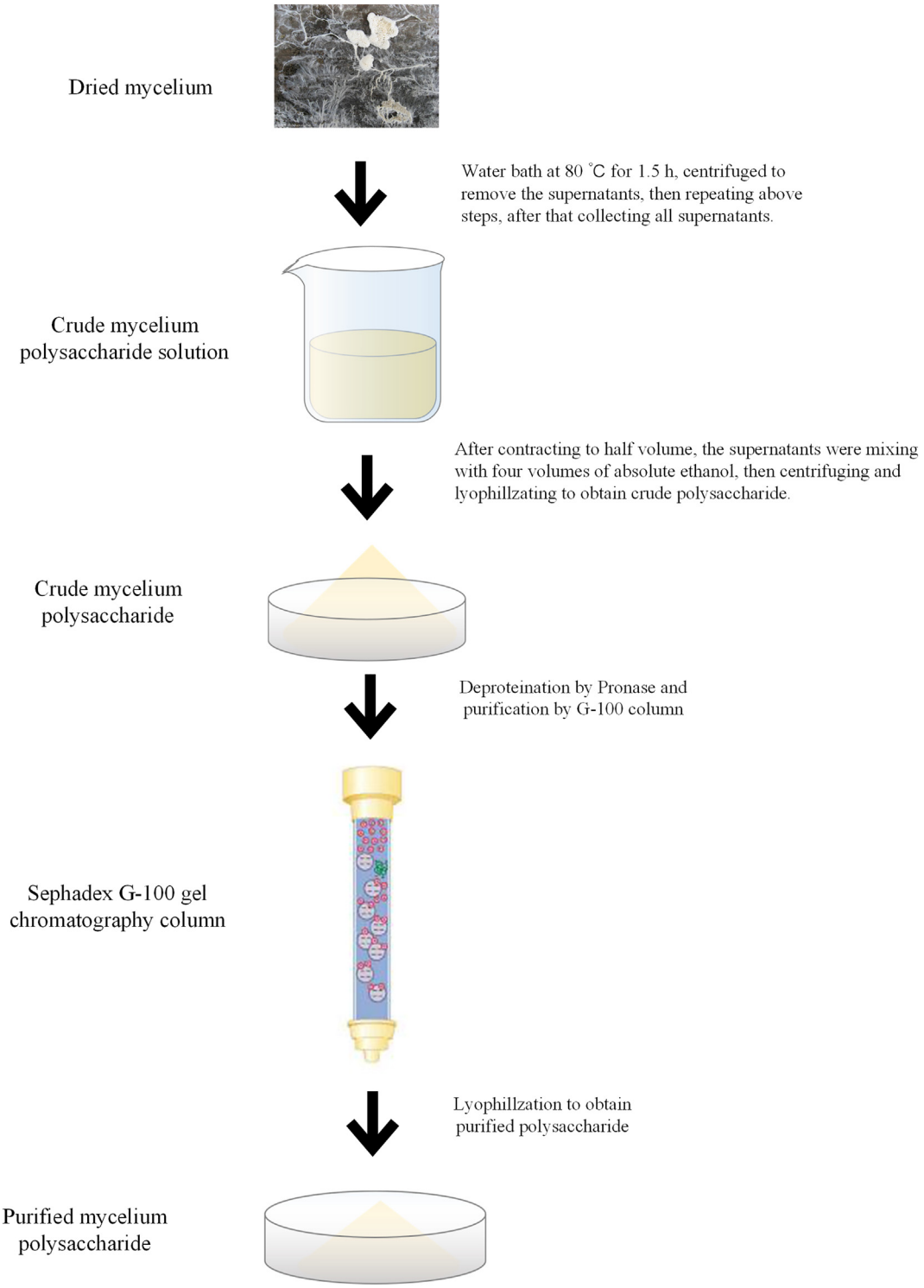

Fig. 1. The flow diagram of extraction and purification.

TNF-α ELISA Kit (MultiSciences Co., China) following its instruction. TNF-α concentrations of sample were calculated by standard curve.

2.13. Data analysis

All tests results of this study were expressed as means ± SD from no fewer than triplicates determinations and analyzed with variance (ANOVA). SPSS version 26.0 was used to all statistical analysis. The $p < 0.05$ was considered to be significant and $p < 0.01$ was considered to be extremely significant.

3. Results

3.1. Extraction and purification of ABSP

The extraction and purification flow diagram displayed in Fig. 1. After extraction, the yield of crude intracellular polysaccharide was 21.5% compared to mycelia of *Agaricus bitorquis (QuéL.) Sacc.* Chaidam. Then purified by G-100 column, a novel intracellular polysaccharide (ABSP) was obtained and its yield was 6.3% relative to crude polysaccharide. Sugar content of ABSP was 96.5% as determined by phenol sulfuric acid method. In addition, there were no absorption peaks at 260 nm or 280 nm in UV–Vis spectra of ABSP which determined no nucleic acid and protein (<3%) were in this polysaccharide. These results were similar with other mycelia polysaccharides [18,19].

3.2. Monosaccharide composition of ABSP

The gas chromatogram was showed in Fig. 2. It showed that ABSP was a neutral polysaccharide, consisted of Glucose (59.95%), Galactose (22.94%), Mannose (14.51%), Xylose (1.21%), Arabinose (0.91%) and Rhamnose (0.48%). This polysaccharide might take glucose and galactose as main chain and mannose, xylose, arabinose and rhamnose as side chain. Usually, structure of polysaccharides has some connection with their functions. As previous research reported [20,21], polysaccharides had composition like ABSP would probably have immunomodulatory effect. Therefore, the following experiments used RAW264.7 cells as model to determine if ABSP had immunomodulatory effect.

3.3. NMR analysis

The NMR spectra were displayed in Fig. 3. In ^{1}H NMR spectrum (Fig. 3A), the four anomeric proton signals (δ 5.15, 5.03, 4.44 and 4.35) were respectively found and tabbed A, B, C and D. The corresponding anomeric carbon signals (δ 98.70, 100.75, 103.40, 103.73) were also found in ^{13}C spectra. These results showed residues of A and B were α- pyran type and residues of C and D were β-pyran type. According to the anomeric proton signals, the other correlative proton signals were found in COSY spectrum. Then the corresponding carbon signals were found in HSQC spectrum on the basis of proton signals. The results were showed in Table 1. Also, all signals were marked in NMR spectra. Based on NMR spectra of standard monosaccharides, residue A, B, C and D were determined as 1,2,4-α-Rha, 1,6-α-Gal, 1,4-β-Glu and 1,4-β-Man. Hence, the back bone of ABSP were made by 1,4-β-Glu and 1,4-β-Man and the side chains included 1,2,4-α-Rha and 1,6-α-Gal, combined with GC results, which determined ABSP is galactoglucomannan-like polysaccharide. Galactoglucomannan considered having immunomodulatory effect in recent researches [22,23].

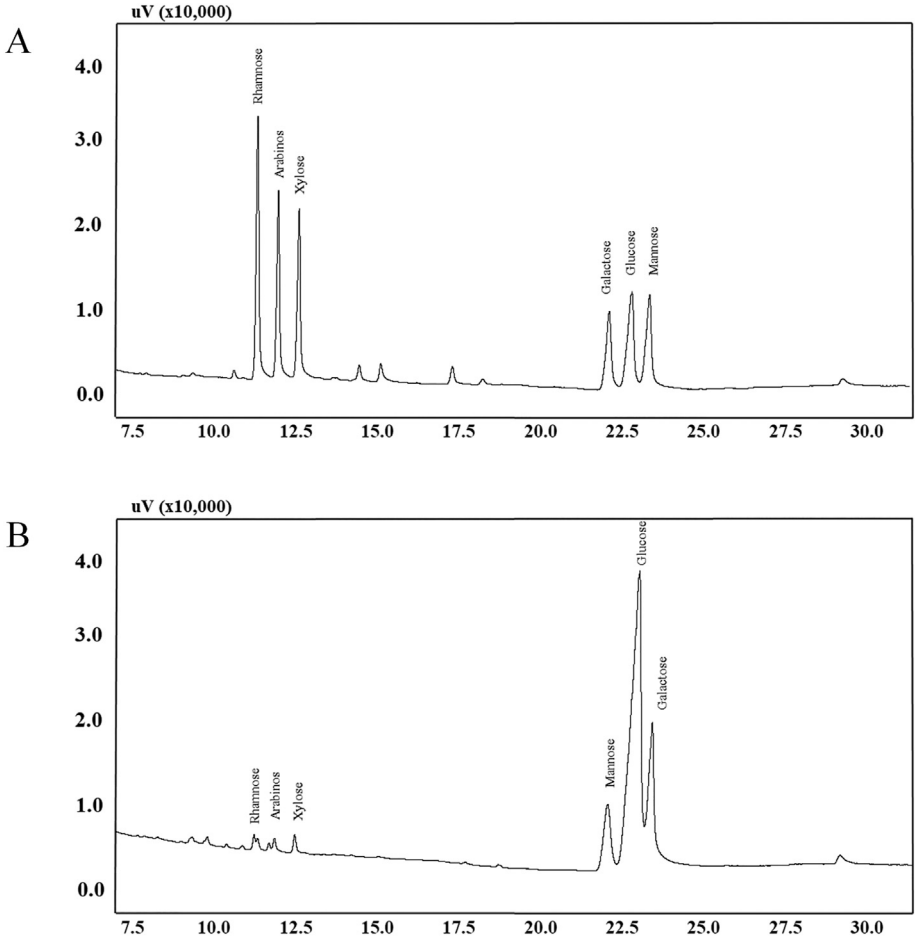

Fig. 2. Gas chromatograms. The monosaccharide standards (A), ABSP (B).

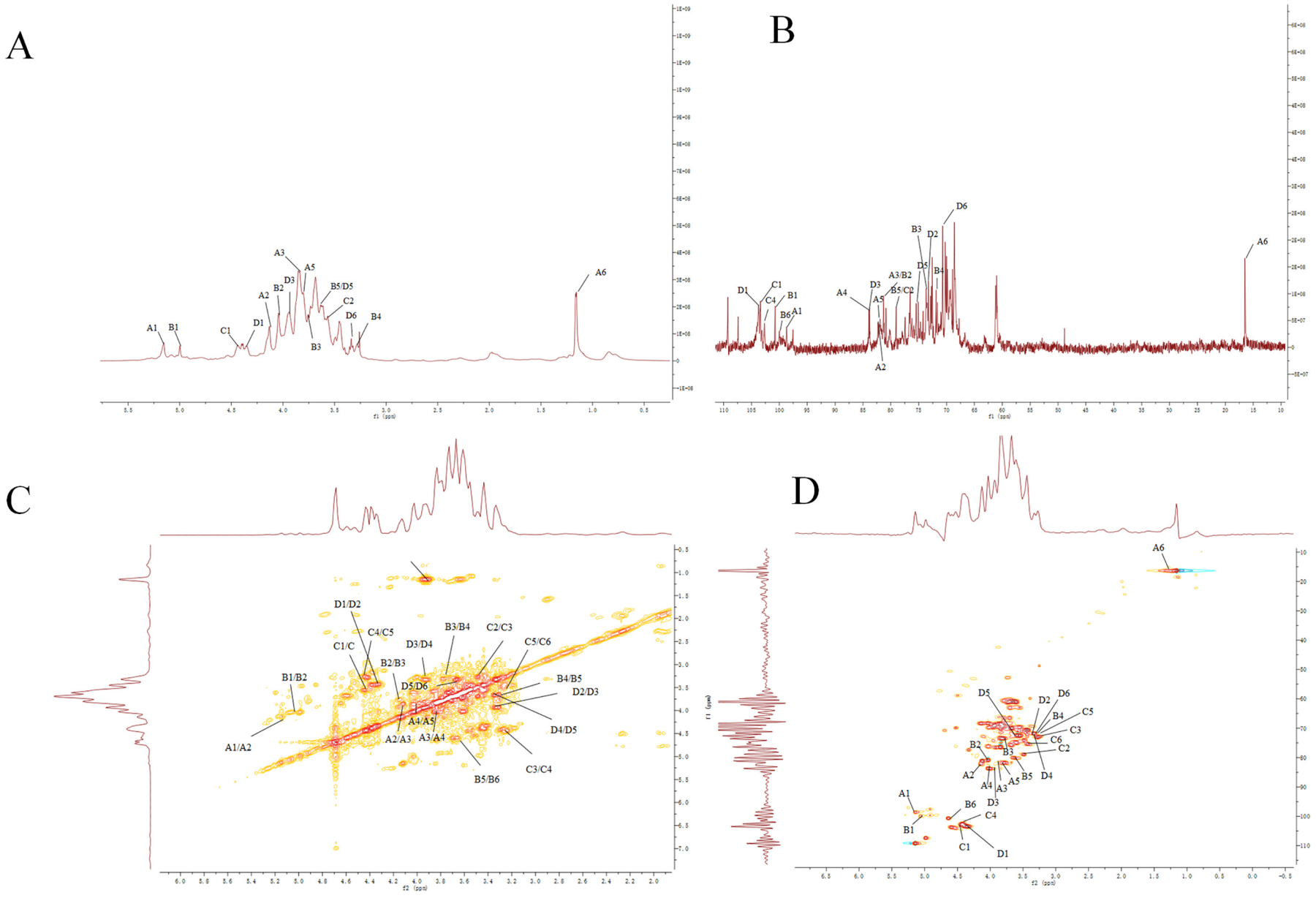

Fig. 3. The NMR spectra of ABSP in D_2O. ^1H spectrum (A); ^{13}C spectrum (B); COSY spectrum (C); HSQC spectrum (D).

Table 1
Assignments of ^{1}H and ^{13}C NMR spectra of ABSP.

	Residues		1	2	3	4	5	6
A	1,2,4-α-Rha	C	98.70	82.31	80.90	83.72	81.78	16.50
		H	5.15	4.14	3.86	4.01	3.82	1.22
B	1,6-α-Gal	C	100.75	80.89	73.51	71.92	79.89	99.83
		H	5.03	4.03	3.77	3.29	3.63	4.61
C	1,4-β-Glu	C	103.40	78.96	73.51	102.35	72.82	75.44
		H	4.44	3.52	3.24	4.47	3.21	3.43
D	1,4-β-Man	C	103.73	72.52	83.75	72.18	75.77	70.26
		H	4.35	3.38	3.91	3.35	3.63	3.33

3.4. Cell viability of RAW264.7

Cell viability effects of RAW 264.7 under different concentrations of ABSP was evaluated by CCK-8 commercial kit. In Fig. 4B, ABSP below 200 μg/mL ABSP had no significant influence on viability of RAW264.7 cells compared to control group. Hence, four concentrations (25, 50, 100 and 200 μg/mL) of ABSP were chosen for following experiments. As shown in Fig. 4A, it was obvious that RAW264.7 cells had increases and a degree of differentiation after treatment of ABSP. This demonstrated ABSP could promote proliferation of RAW264.7 cells.

3.5. Phagocytic ability analysis

Neutral red uptake assay was used to determine phagocytic ability of RAW264.7 cells. As shown in Fig. 4C, ABSP can significantly intensify phagocytic ability of macrophages. Also, this result displayed a dose-dependent response. As treatment concentration increased, phagocytic ability of cells was stronger. Especially, the strongest ability exhibited at concentration of 200 μg/mL compared to blank control though it was much lower than positive control. Phagocytosis is an important way for immune response because it can have responsibility for clearing

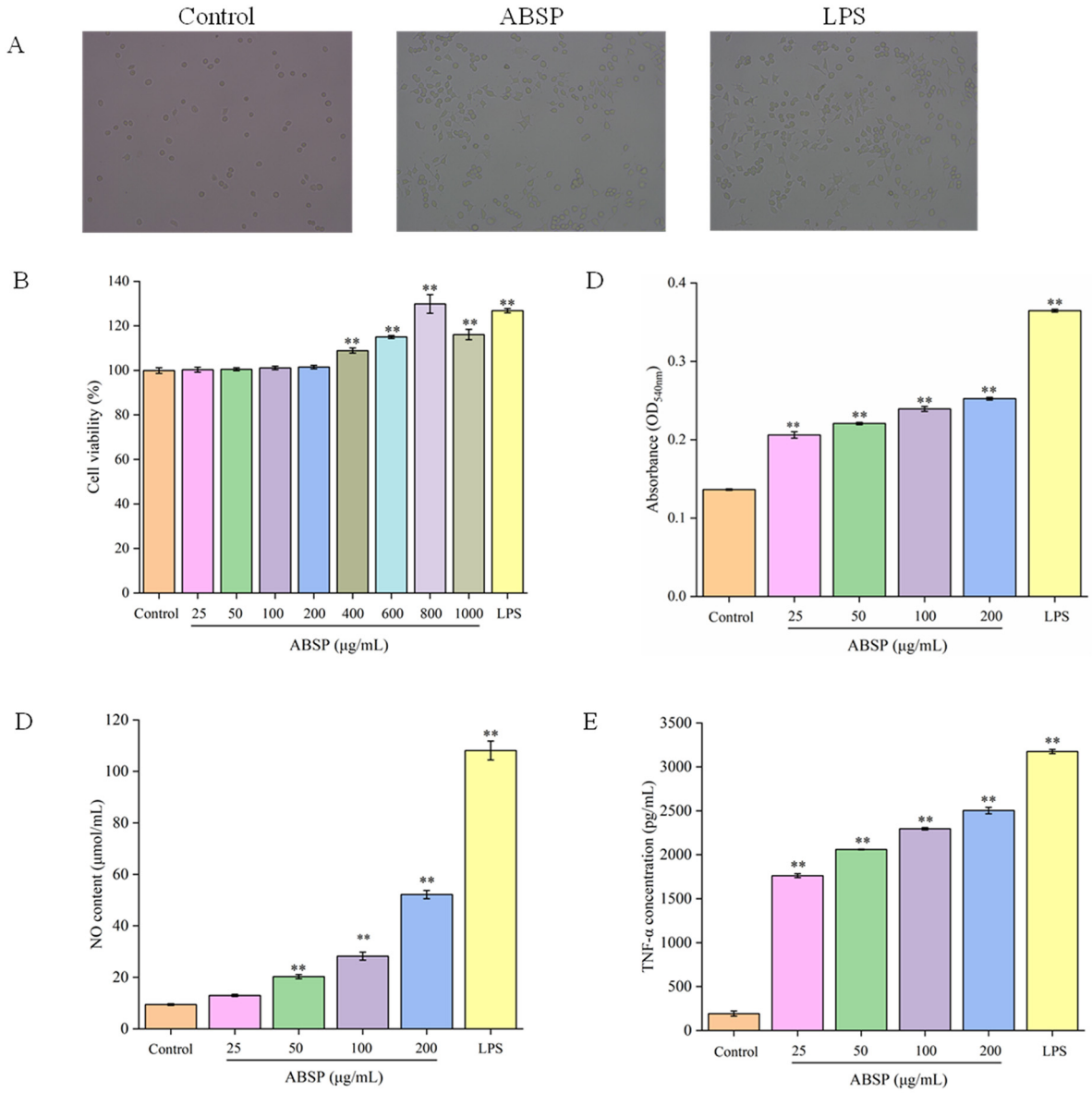

Fig. 4. Microscope images (×200) of RAW264.7 cells in different treatments (A); The cell viability of RAW264.7 after treatment by different concentrations of ABSP (B); The phagocytic ability analysis (C); the NO level analysis (D); the TNF-α production analysis (E). The results were expressed as the mean ± SD ($n = 4$). (*) $p < 0.05$ and (**) $p < 0.01$ compared to blank control group.

apoptotic cells and adventitious pathogens [24]. The macrophages engulf and digest these cells and pathogens [25]. This result proved ABSP could promote cells proliferation and differentiation to enhance its phagocytic ability.

3.6. NO and TNF-α levels in RAW264.7

NO and TNF-α levels were determined by corresponding commercial kits and the results were displayed in Fig. 4D and E. In Fig. 4F, three concentrations (50, 100, 200 μg/mL) of ABSP showed extremely significant difference to control group. The NO content increased together with the concentration of ABSP increased and NO level reached to 52 μmol/mL at 200 μg/mL ABSP. Also, TNF-α level showed similar dose-dependent response as NO level. At 200 μg/mL ABSP, the TNF-α concentration attained to 2503 pg/mL. The production of NO and TNF-α demonstrated ABSP could activate RAW264.7 to produce biological chemicals which help exert immunomodulatory function. This is an indispensable way for RAW264.7 to exterminate invading pathogens in body [26].

3.7. ROS level in RAW264.7

ROS levels of each group were evaluated by flow cytometer. The results of ROS levels were showed in Fig. 4. In Fig. 5A, ABSP could significantly stimulate RAW264.7 cells to produce ROS as a dose dependent manner, but the ROS levels of ABSP groups were lower than LPS group. NO, TNF-α and ROS levels were all showed that ABSP exerted its immune ability by irregulating RAW264.7 cells to produce these biological chemicals [26]. However, how ABSP to exert immunomodulatory effect on RAW264.7 was still a mystery. Hence, the corresponding proteins and mRNAs expressions were determined by Western blots and qRT-PCR.

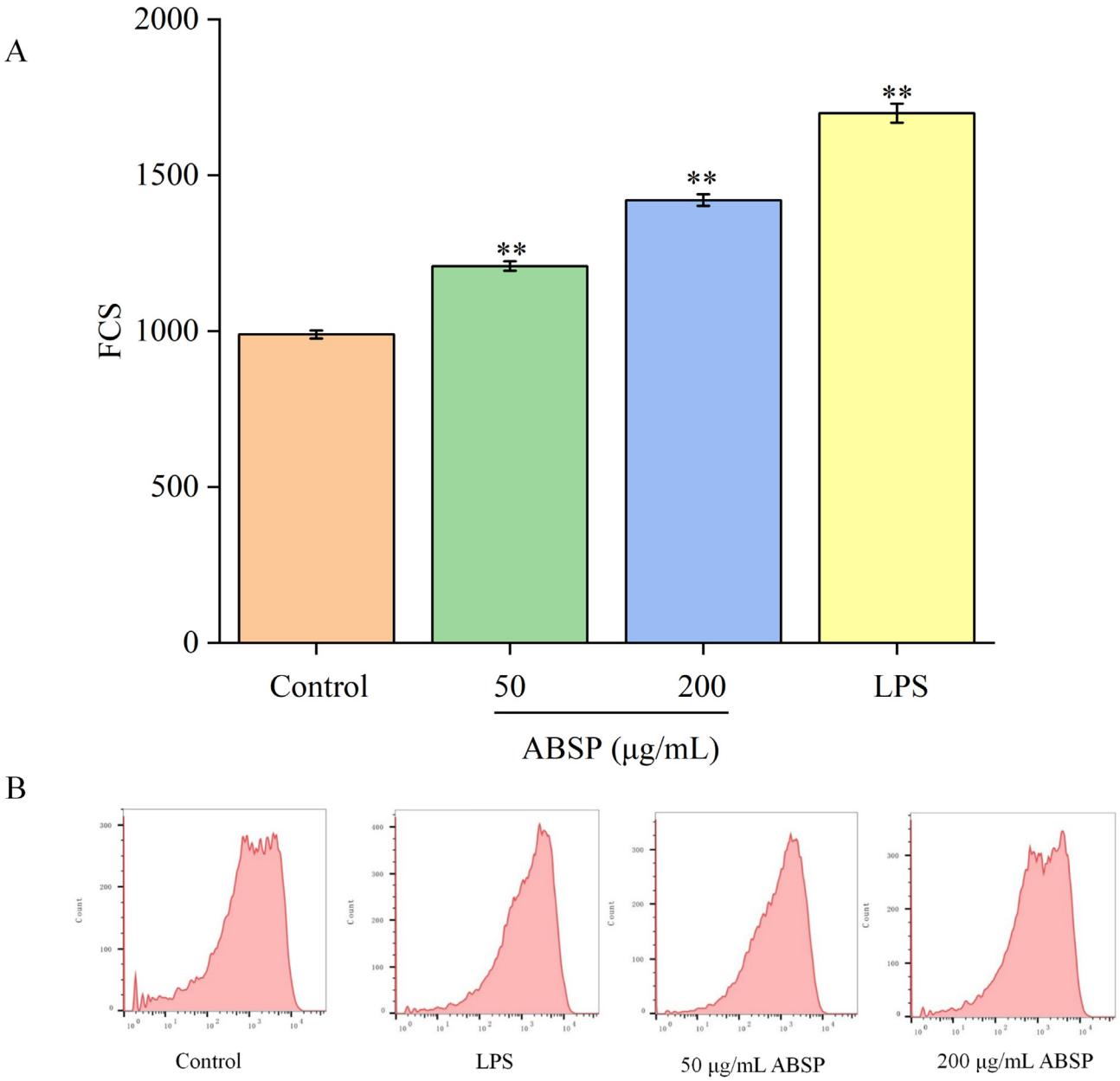

Fig. 5. The ROS level of each group (A). The picture of each group from flow cytometer (B). The results were expressed as the mean ± SD ($n = 3$ p). (*) $p < 0.05$ and (**) $p < 0.01$ compared to blank control group.

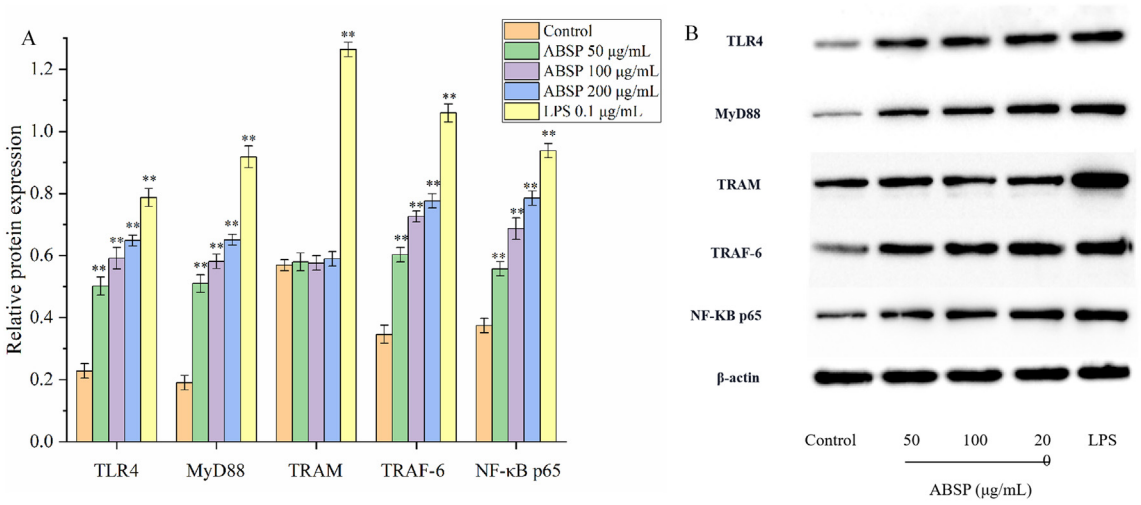

Fig. 6. Relative proteins expression analysis (A). The results were expressed as the mean ± SD (n = 3 p). (*) $p < 0.05$ and (**) $p < 0.01$ compared to blank control group. The chemiluminescent pictures of each group (B).

3.8. Relative proteins production analysis

In order to determine the signaling pathway of ABSP in RAW264.7 cells, Western blots were used to investigate the corresponding proteins production (Fig. 6). TLR4 content increased with the ABSP concentration raised and its relative content reached the maximum (0.65) at ABSP of 200 μg/mL. Also, MyD88 content showed the similar does-dependent response. However, all concentrations of ABSP showed no significant effect on TRAM content. Besides, downstream proteins like TRAF-6 and NF-κB p65 increased with the raising concentration of ABSP. This result possibly showed that ABSP could activate RAW264.7 cells through TLR4-MyD88 dependent signaling pathway [27]. However, all protein expression levels induced by each dose of ABSP and these induced by LPS had statistically significant differences and the protein expression levels induced by LPS were much higher (almost twice times) than these induced by each does of ABSP. This demonstrated ABSP could immunological regulate RAW264.7 but probably would not cause inflammatory reaction like LPS.

3.9. Relative mRNAs expression analysis

Relative mRNAs expression was determined by qRT-PCR. As shown in Fig. 7, upstream mRNAs expression including TLR4 and MyD88 were increased with the ABSP concentration raised, so as the downstream mRNAs like TRAF-α and NF-κB p65 were increased by raising concentration of ABSP. However, the mRNA of TRAM was not significant raised compared to blank control group. All results were consistent with results of Western blots. Therefore, PCR results showed that ABSP activated RAW264.7 by TLR4-mediated MyD88 dependent signaling pathway rather than TLR4 pathway [28]. Similarly, all genes expression levels induced by LPS were much higher (almost twice times) than these induced by each does of ABSP and they had significant differences in statistics which indicated ABSP had immunomodulatory effect on RAW264.7 cells instead inflammatory response caused by LPS in RAW264.7.

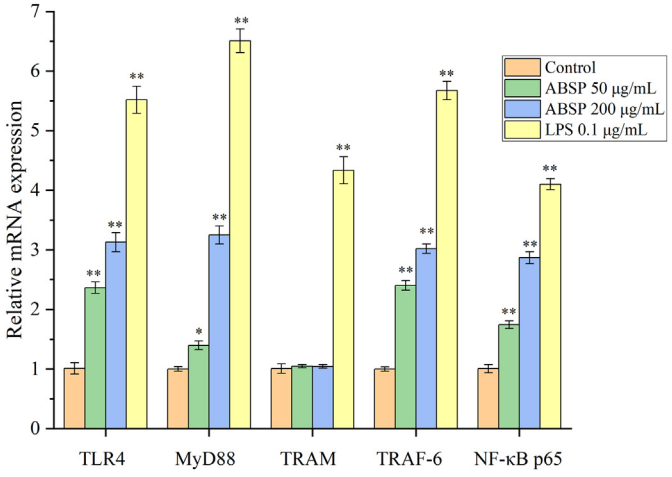

Fig. 7. Relative mRNAs expression analysis. The results were expressed as the mean ± SD (n = 3). (*) $p < 0.05$ and (**) $p < 0.01$ compared to blank control group.

3.10. TNF-α levels after inhibiting TLR4 or MyD88

For determining ABSP activated RAW264.7 cells throughTLR4-MyD88 signaling pathway, TNF-α content was evaluated after inhibiting TLR4 or MyD88 by their corresponding inhibitors. In Fig. 8,

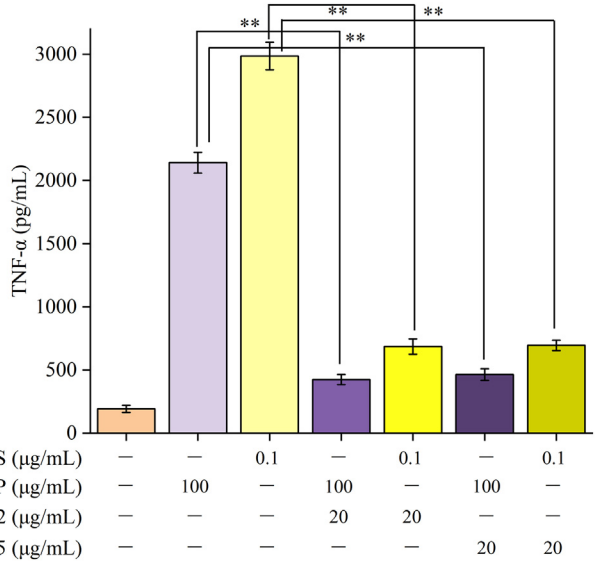

Fig. 8. TNF-α level after inhibiting TLR4 or MyD88 analysis. The results were expressed as the mean ± SD (n = 4). (*) $p < 0.05$ and (**) $p < 0.01$ compared to related group without inhibitor.

it was obvious that TNF-α content of ABSP group was extremely decreased after adding TAK242 or ST-2825 compared to the group without inhibitors. This demonstrated that ABSP could not activate RAW264.7 cells without TLR4 or MyD88 which determined TLR4-MyD88 pathway is the way for ABSP to exert immunomodulatory effect [27,28].

4. Discussion

Polysaccharides are one of mainly biological substances extracted from *Agaricus bitorquis (QuéL.) Sacc.* Chaidam. Recent researches have shown they have antioxidant and anti-hypoxia activities [3,4,12]. However, few works on its immune ability have been published. Macrophages are important in immune system. They have many functions which can exert their ability in specific immunity and non-specific immunity. The functions of macrophages include antigen presentation, phagocytic ability, cytotoxic chemicals and cytokines secretion. Macrophages can swallow invading pathogens and pathological cells which is a part of non-specific immunity. Also, the phagocytosis level can demonstrate immune level of body. Each dose of ABSP significantly enhanced phagocytic ability and higher concentration of ABSP enhanced higher level of phagocytosis which indicated ABSP can strengthen immune ability of body through enhancing phagocytosis level of macrophages.

In addition, previous researches determined polysaccharides can activate macrophages to produce immune chemicals like NO, TNF-α and ROS that can exert important roles in nonspecific and specific immunity [29,30]. NO, an important non-specific chemical, is produced by activated macrophages through iNOS catalysis [40]. It can kill or stop various pathogens growing. ROS is also an important non-specific chemical produced by mitochondria in cells. It can oxidize cell lipids to kill cells and cross-link amino acids to inactivate proteins. TNF-α is a cytokine mainly produced by macrophages which can directly kill cancer cells [41]. In this study, we found mycelia polysaccharide from *Agaricus bitorquis (QuéL.) Sacc.* Chaidam (ABSP) could induce secretions of NO, ROS and TNF-α by macrophages. And ABSP can enhance phagocytosis and proliferation of macrophages and these promotions have positive does-dependent response. These results have displayed ABSP can promote phagocytosis and proliferation at beginning of pathogens invasion to boost natural immune response of body. In addition, other polysaccharides from *Salicornia herbacea*, *Laminaria digitata* can strengthen phagocytic ability, too [31,32].

ABSP can activate macrophages to enhance phagocytosis, produce more cytokines for immune regulation. However, polysaccharides are macromolecules. They cannot across the cell membrane without receptors. TLR4, located on the surface of macrophages, can regulate activation of macrophages by transmitting extracellular signals and induce cytokines production (NO and TNF-α) [33,34]. In this study, protein and mRNA expressions of TLR4 have significant increase. At the same time, TNF-α production has extremely decreased after treatment with TLR4 inhibitor which determined TLR4 can mediate some polysaccharides to induce nonspecific immunity [35,36]. Recent researches proved TLR4 can participate macrophages activation of polysaccharides [27,28]. The downstream signing pathways of TLR4 mainly include MyD88 dependent and MyD88 independent pathways [37]. MAPKs can regulate intracellular signal transduction. NF-κB is one of MAPKs. It is composed of p50 and p65 subunits. Once TLR4 combined with polysaccharides, TIR, intracellular structure domain of TLR4, would interact with adaptive protein (MyD88) to activate IRAK and phosphorylate. Then IRAK would separate with MyD88 and interact with TRAF6 to activate NIK in downstream. Activated NIK would activate IKK and IKK phosphorylation would make inhibitory subunit of NF-κB (IκB) separate with NF-κB. Then NF-κB would be activated and transfer into nucleus to induce NO and TNF-α production. In this study, protein and mRNA expression of MyD88 have significantly up-regulated. Meanwhile, the production of TNF-α have extremely decrease by adding inhibitor of MyD88 which confirmed ABSP active macrophages by TLR4-mediated MyD88-dependent pathway. This pathway can also activate MAPK/NF-κB pathway to induce cytokines secretions [38]. This work also showed protein and mRNA expression of NF-κB p65 significantly up-regulated which proved ABSP can active macrophages by TLR4-mediated MyD88-dependent pathway, then activating MAPK/NF-κB pathway to promote production of NO and TNF-α. Researches also reported some other polysaccharides exerted immunomodulatory effect by this signaling pathway [26–28,39] (Fig. 9).

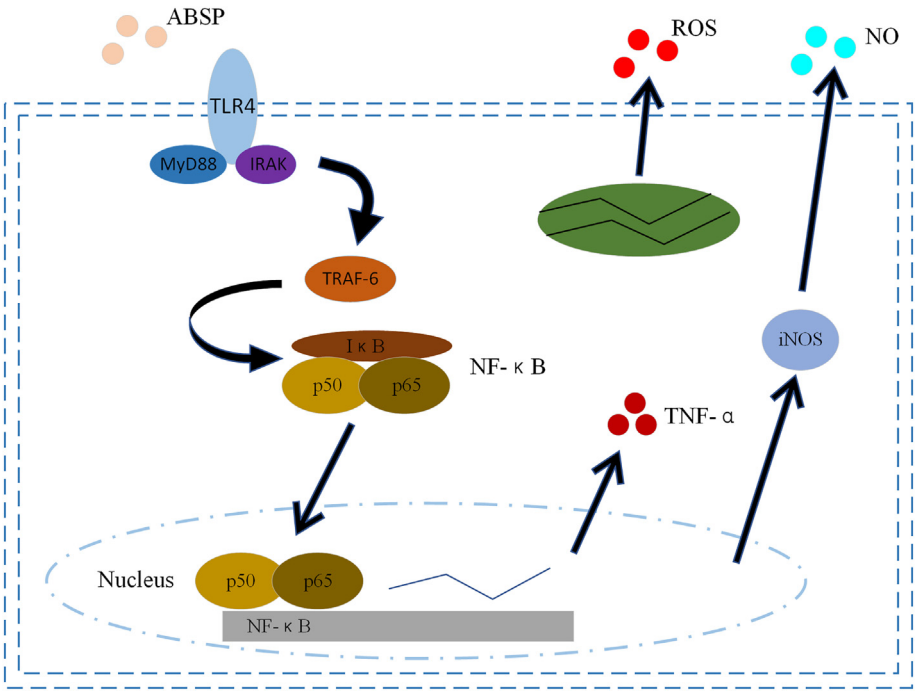

Fig. 9. Immunomodulatory pathway of ABSP in RAW264.7 cells.

5. Conclusion

This study determined ABSP can significantly activate RAW264.7 cells by TLR4-mediated MyD88 dependent signaling pathway to induce biological cytokines. However, it just simply proved the key proteins and mRNAs expression in this signaling pathway which can provide theoretical basis for further study on immune mechanism of ABSP. Furthermore, this study will offer a new way to exploit *Agaricus bitorquis (QuéL.) Sacc.* Chaidam and develop beneficial functionally foods.

Abbreviations

ABSP	purified mycelium polysaccharide from *Agaricus bitorquis (QuéL.) Sacc.*
ATCC	American Type Tissue Culture Collection
GC	gas chromatography
LPS	lipopolysaccharide
NO	nitric oxide
TNF-α	tumor necrosis factor-α
ROS	reactive oxygen species
qRT-PCR	real-time quantitative PCR
TLR4	toll-like receptors 4
MyD88	myeloid different factory 88
TRAF-6	TNF receptor-associated factor-6
TRAM	TRIF-related adapter molecule
NF-κB	nuclear factor kappa-B
TAK-242	molecule-specific inhibitor of TLR4
ST-2825	MyD88 pharmacologic inhibito

CRediT authorship contribution statement

Ximeng Lin: Investigation; Softwar; e Writing—original draft.
Wenxia Li: Resources; Data curation.
Hywel Yuen: Validation; Conceptualization;
Michael Yuen: Project administration; Methodology; Formal analysis
Qiang Peng: Funding acquisition; writing—review & editing; Supervision

Declaration of competing interest

All authors declare there have no personal or financial conflicts of interest.

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References

[1] R. Hua, W. Zhang, Main species and medicinal value of wild edible (medicinal) fungi in Yunnan Province, Med. Plants 9 (2018) 1–4.
[2] X. Wang, H. Liu, J. Zhang, T. Li, Y.-Z. Wang, Evaluation of heavy metal concentrations of edible wild-grown mushrooms from China, J. Environ. Sci. Health Part B 52 (2017) 178–183.
[3] H. Kuang, Y. Jiao, W. Wang, F. Wang, Q. Chen, Characterization and antioxidant activities of intracellular polysaccharides from Agaricus bitorquis (QueL.) Sacc. Chaidam ZJU-CDMA-12, Int. J. Biol. Macromol. 156 (2020) 1112–1125.
[4] Y. Jiao, H. Kuang, J. Hu, Q. Chen, Structural characterization and anti-hypoxia activities of polysaccharides from the sporocarp, fermentation broth and cultured mycelium of Agaricus bitorquis (Quél.) Sacc. Chaidam in mice, J. Funct. Foods 51 (2018) 75–85.
[5] K. Ingólfsdóttir, K. Jurcic, H. Wagner, Immunomodulating polysaccharides from aqueous extracts of *Cetraria islandica* (Iceland moss), Phytomedicine 5 (1998) 333–339.
[6] P. Gong, S. Wang, M. Liu, F. Chen, W. Yang, X. Chang, N. Liu, Y. Zhao, J. Wang, X. Chen, Extraction methods, chemical characterizations and biological activities of mushroom polysaccharides: a mini-review, Carbohydr. Res. 494 (2020), 108037, .
[7] S. Li, N.-P. Shah, Characterization, antioxidative and bifidogenic effects of polysaccharides from Pleurotus eryngii after heat treatments, Food Chem. 197 (2016) 240–249.
[8] D. Ren, N. Wang, J. Guo, L. Yuan, X. Yang, Chemical characterization of Pleurotus eryngii polysaccharide and its tumor-inhibitory effects against human hepatoblastoma HepG-2 cells, Carbohydr. Polym. 138 (2016) 123–133.
[9] Y. Chen, Y. Liu, M. Sarker, X. Yan, C. Yang, L. Zhao, X. Lv, B. Liu, C. Zhao, Structural characterization and antidiabetic potential of a novel heteropolysaccharide from Grifola frondosa via IRS1/PI3K-JNK signaling pathways, Carbohydr. Polym. 198 (2018) 452–461.
[10] Y. Wang, Y. Tian, J. Shao, X. Shu, J. Jia, X. Ren, Y. Guan, Macrophage immunomodulatory activity of the polysaccharide isolated from Collybia radicata mushroom, Int. J. Biol. Macromol. 108 (2018) 300–306.
[11] Y. Liu, Y. Zhou, M. Liu, Q. Wang, Y. Li, Extraction optimization, characterization, antioxidant and immunomodulatory activities of a novel polysaccharide from the wild mushroom Paxillus involutus, Int. J. Biol. Macromol. 112 (2018) 326–332.
[12] Y. Jiao, H. Kuang, J. Wu, Q. Chen, Polysaccharides from the edible mushroom Agaricus bitorquis (Quel.) Sacc. Chaidam show anti-hypoxia activities in pulmonary artery smooth muscle cells, Int. J. Mol. Sci. 20 (2019) 637–652.
[13] S. Xu, P. Zhang, L.-J. Huang, Z.-F. Wang, Pronase E digestion of N-glycans in glycoprotein and its fluorescent derivatives analysis by HPLC-ESI/MS, Chem. J. Chin. Univ.-Chin. 31 (2010) 1992–1998.
[14] Y. Cai, Z. Wei, X. Liao, Y. Ding, J. Sun, Characterization of three extracellular polysaccharides from Shiraia sp. Super-H168 under submerged fermentation, Carbohydr. Polym. 82 (2010) 34–38.
[15] J. Lehrfeld, Simultaneous gas-liquid chromatographic determination of aldoses and alduronic acids, J. Chromatogr. A 408 (1987) 245–253.
[16] Q. Peng, J. Song, X. Lv, Z. Wang, L. Huang, Y. Du, Structural characterization of an arabinogalactan-protein from the fruits of Lycium ruthenicum, J. Agric. Food Chem. 60 (2012) 9424–9429.
[17] Y. Wang, N. Liu, X. Xue, Q. Li, D. Sun, Z. Zhao, Purification, structural characterization and in vivo immunoregulatory activity of a novel polysaccharide from Polygonatum sibiricum, Int. J. Biol. Macromol. 160 (2020) 688–694.
[18] F. Wu, C. Zhou, D. Zhou, S. Ou, X. Zhang, H. Huang, Structure characterization of a novel polysaccharide from Hericium erinaceus fruiting bodies and its immunomodulatory activities, Food Funct. 9 (2018) 294–306.
[19] J. Cheng, J. Song, H. Wei, Y. Wang, X. Huang, Y. Liu, N. Lu, L. He, G. Lv, H. Ding, S. Yang, Z. Zhang, Structural characterization and hypoglycemic activity of an intracellular polysaccharide from Sanghuangporus sanghuang mycelia, Int. J. Biol. Macromol. 164 (2020) 3305–3314.
[20] Y. Konkol, H. Vuorikoski, J. Tuomela, B. Holmborm, J. Bernoulli, Galactoglucomannan-rich hemicellulose extract from Norway spruce (Picea abies) exerts beneficial effects on chronic prostatic inflammation and lower urinary tract symptoms in vivo, Int. J. Biol. Macromol. 101 (2017) 222–229.
[21] A. Ebringerová, Z. Hromádková, V. Hříbalová, C. Xu, B. Holmbom, A. Sundberg, S. Willför, Norway spruce galactoglucomannans exhibiting immunomodulating and radical-scavenging activities, Int. J. Biol. Macromol. 42 (2008) 1–5.
[22] W. Wei, L. Feng, W.R. Bao, D.L. Ma, C.H. Leung, S.P. Nie, Q.B. Han, Structure characterization and immunomodulating effects of polysaccharides isolated from Dendrobium officinale, J. Agric. Food Chem. 64 (2016) 881–889.
[23] J.-B. Lee, T. Tanikawa, K. Hayashi, M. Asagi, Y. Kasahara, T. Hayashia, Characterization and biological effects of two polysaccharides isolated from Acanthopanax sciadophylloides, Carbohydr. Polym. 116 (2015) 159–166.
[24] C. Rosales, E. Uribequerol, Phagocytosis: a fundamental process in immunity, Biomed. Res. Int. 10 (2017) 1–18.
[25] J. Miao, P. Ye, J. Lan, S. Ye, J. Zhong, A. Gresham, S. Li, A. You, X. Chen, X. Liu, H. Li, Paeonol promotes the phagocytic ability of macrophages through confining HMGB1 to the nucleus, Int. Immunopharmacol. 89 (107068) (2020).
[26] N.G. Geum, H.J. Eo, H.J. Kim, G.H. Park, H.J. Son, J.B. Jeong, Immune-enhancing activity of Hydrangea macrophylla subsp. serrata leaves through TLR4/ROS-dependent activation of JNK and NF-κB in RAW264.7 cells and immunosuppressed mice, J. Funct. Foods 73 (2020), 104139, .
[27] H. Tian, Z. Liu, Y. Pu, Y. Bao, Immunomodulatory effects exerted by Poria Cocos polysaccharides via TLR4/TRAF6/NF-kappaB signaling in vitro and in vivo, Biomed. Pharmacother. 112 (108709) (2019).
[28] Z. Xu, R. Lin, X. Hou, J. Wu, W. Zhao, H. Ma, Z. Fan, S. Li, Y. Zhu, D. Zhang, Immunomodulatory mechanism of a purified polysaccharide isolated from Isaria cicadae Miquel on RAW264.7 cells via activating TLR4-MAPK-NF-kappaB signaling pathway, Int. J. Biol. Macromol. 164 (2020) 4329–4338.
[29] C. Milk, Molecular basis of suppressor macrophages arginine metabolism via the nitric oxide synthase pathway, J. Immunol. 146 (1991) 2719–2723.
[30] J. MacMicking, Q. Xie, C. Nathan, Nitric oxide and macrophage function, Annu. Rev. Immunol. 15 (1997) 323–350.
[31] S. Ima, K. Kim, C.-K. Lee, Immunomodulatory activity of polysaccharides isolated from Salicornia herbacea, Int. Immunopharmacol. 6 (2006) 1451–1458.
[32] V. Vetvicka, J.-C. Yvin, Effects of marineβ-1,3 glucan on immune reactions, Int. Immunopharmacol. 4 (2004) 721–730.
[33] D. Werling, T.-W. Jungi, Toll-like receptors linking innate and adaptive immune response, Vet. Immunol. Immunopathol. 91 (2003) 1–12.

[34] M. Franchini, M. Schweizer, P. Matzener, Evidence for dissociation of TLR mRNA expression and TLR agonist-mediated functions in bovine macrophages, Vet. Immunol. Immunopathol. 110 (2006) 37–49.
[35] S.-B. Han, S.-K. Park, H.-J. Ahn, Characterization of B cell membrane receptors of polysaccharide isolated from the root of Acanthopanax koreanum, Int. Immunopharmacol. 3 (2003) 683–691.
[36] B. Beutler, K. Hoebe, X. Du, How we detect microbes and respond to them the Toll-like receptors and their transducers, J. Leukoc. Biol. 74 (2003) 479–485.
[37] Y. Pu, Z. Liu, C. Zhong, X. Zhang, Y. Bao, Immunomodulatory effects of a polysaccharide from Solanum nigrum Linne through TLR4-MyD88 signaling pathway, Int. Immunopharmacol. 88 (106973) (2020).
[38] Y. Qiao, E.-G. Giannopoulou, C.-H. Chan, S.-H. Park, S.-C. Gong, J. Chen, X.-Y. Hu, O. Elemento, L.-B. Ivashkiv, Synergistic activation of inflammatory cytokine genes by interferon-gamma-induced chromatin remodeling and toll-like receptor signaling, Immunity 39 (2013) 454–469.
[39] D.-D. Yoon, J.-S. Kang, S.-B. Han, Activation of mitogen-activated protein kinases and AP-1 by polysaccharide isolated from the radix of Platycodon grandiflomm in RAW264.7 cells, Int. Immunopharmacol. 4 (2004) 1477–1487.
[40] C.-D. Mills, Molecular basis of suppressor macrophages arginine metabolism via the nitric oxide synthase pathway, J. Immunol. 146 (1991) 2719–2723.
[41] A. Rojas, J. Padron, L. Caveda, Role of nitric oxide pathway in the protection against lethal endotoxemia afforded by low doses of lipopolysaccharide, Biochem. Biophys. Res. Commun. 191 (1993) 441–446.