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A galactomannoglucan derived from *Agaricus brasiliensis*: Purification, characterization and macrophage activation via MAPK and IκB/NFκB pathways

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A B S T R A C T

In this study, a novel galactomannoglucan named as TJ2 was isolated from *Agaricus brasiliensis* with microwave extraction, macroporous resin, ion exchange resin and high resolution gel chromatography. TJ2 is composed of glucose, mannose and galactose in the ratio 99.2:0.2:0.6. Infrared spectra (IR), methylation analysis and nuclear magnetic resonance spectra indicated that TJ2 mainly contained a β-(1 → 3) – linked glucopyranosyl backbone. Interestingly, TJ2 significantly promoted RAW264.7 cell proliferation, and was able to activate the cells to engulf *E. coli*. In addition, TJ2 induced the expression of Interleukin 1β (IL-1β), Interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and cyclooxygenase-2 (Cox-2) in the cells. TJ2 also promoted the production of nitric oxide (NO) by inducing the expression of inducible nitric oxide synthase (iNOS). Moreover, TJ2 is a potent inducer in activating the mitogen-activated protein kinase (MAPK) and inhibitor of nuclear factor-kappa B (IκB)/nuclear factor-kappa B (NFκB) pathways.

1. Introduction

Agaricus brasiliensis is originally native to southern Brazil and produced on an industrial scale in some countries such as China and Japan nowadays (Kuroiwa et al., 2005; Wasser et al., 2005). It is one of the most important edible and medicinal mushrooms in the world (Akiyama et al., 2011). In recent years, it has been demonstrated that the mushroom exhibits various bioactivities, for example, immunoregulation (Goncalves et al., 2011), anti-cancer (Ishii et al., 2011; Kaneno et al., 2004), liver protection (Al-Dbass, Al-Daihan, & Bhat, 2012), anti-diabetes (Oh et al., 2010) and antioxidant activity (Carneiro et al., 2013; Kozarski, Klaus, Niksic, & Jakovljevic, 2011; Stojković et al., 2014). Thus, it shows the great potential of being developed as a novel functional food additive for health care.

Agaricus brasiliensis contains a variety of chemical compounds including polysaccharides, proteins, lipids, and sterols (Jia et al., 2013; Kawagishi et al., 1988; Takaku, Kimura, & Okuda, 2001). Among them, polysaccharides are commonly believed to be the main component responsible for the various bioactivities (Liu, Miao, Wen, & Sun, 2009). Studies have shown that *Agaricus brasiliensis* polysaccharides exert certain immunostimulation activities (Cui et al., 2013; Wang, Fu, & Han, 2013). For example, the polysaccharide extracts from *Agaricus brasiliensis* play a crucial role in stimulating immunity and induce the expression of IL-1β, IL-6, TNF-α and IFN-γ in murine immune cells (Huang et al., 2012; Yamanaka et al., 2013). However, the immuneregulatory mechanism of the polysaccharides, especially their structural characteristics key to the bioactivity, is still vague.

In the present study, we isolated and purified a polysaccharide designated as TJ2 from *Agaricus brasiliensis*. Its purity, monosaccharide composition, and structure characters were determined with high performance size-exclusion chromatography, GC–MS, IR and NMR. The effect of TJ2 on proliferation and phagocytosis of RAW264.7 macrophages were assayed to evaluate its immuneregulatory activities. Moreover, the relevant mechanisms, such as cytokine production, NO production and signaling pathway mediating the events, were investigated.

2. Materials and methods

2.1. Materials and reagents

Agaricus brasiliensis (strain AbML11) was from Jinsheng agricultural technology development company (Tianjin, China). It is identified by Doctor Junbo Xie, and the voucher specimen was deposited in the College of Biotechnology and Food Science, Tianjin University of Commerce. Dextran standards were obtained from Ampolymer (Mentor, USA). Chromatographic pure water was from J. T. Baker (Center Valley, USA). Glucose was purchased from Troody Technology, Ltd. (Shanghai, China). Rhamnose was purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Mannose, arabinose and galactose were obtained from Lanji Technology Development, Co. (Shanghai, China). HPLC grade acetonitrile and methanol were from Merck (Darmstadt, Germany). DMEM culture medium, fetal bovine serum (FBS), Trypsin-EDTA, Trizol reagent, Penicillin-Streptomycin and FITC-E.coli particles were purchased from Gibco/Invitrogen (Eugene, USA). 3-(4,5-dimethylthiazol-2-yl) – 2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) and the Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylene diamine dihydrochloride in 5% phosphoric acid] were obtained from Sigma (St. Louis, MO, USA). FastStart Universal SYBR Green Master (Rox) was obtained from Roche (Indianapolis, USA) and the High Capacity cDNA Reverse Transcription Kit was from Applied Biosystems Pty, Ltd (Foster City, CA, USA). The protein extraction commercial kit was obtained from Sangon Biotechnology, Ltd (Shanghai, China), and the polyvinylidenedifluoride (PVDF) membrane was from Millipore (Billerica, USA). The ECL™ primer western blotting detection reagent was from Pierce (Rockford, USA). The antibodies to p-ERK, p-IκB, iNOS and β-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Cox-1 and Cox-2 antibodies were purchased from Cayman (Ann Arbor, USA). Raw 264.7 cells were obtained from ATCC (Manassas, USA).

2.2. Isolation and purification of TJ2

The freeze-dried mushrooms were crushed and mixed with distilled water at a ratio of 1/50 (*W/V*, g/mL). The crude polysaccharide was then extracted with the microwave method (640 W, 5 min). Through macroporous resin (AB-8, The Chemical Plant of NanKai University, China) and ion exchange resin (D280, the Chemical Plant of NanKai University, China) columns, the pigment and the proteins were removed from the extracting solution. After dialysis (molecular weight cut-off 10,000 Da), the crude polysaccharide was further separated and purified through a Sephacryl™ S-300 High Resolution gel column (80 cm × 3.0 cm) with ultrapure water as eluent. A main single fraction (TJ2) was obtained and then freeze-dried.

The purity of TJ2 polysaccharide was determined by using the Agilent 1100 Series system (Agilent Technologies, USA) equipped with an Alltech3300 ELSD (Grace Technologies, USA). The sample was assayed using an Agilent PL aquael-OH column (4.6 × 150 mm, 3 μm) with the temperature at 25 °C. Isocratic elution was applied with water (HPLC grade, 100%). The flow rate was 0.8 mL/min, and the injection volume was 20 μL. The temperature of the ELSD drift tube was 75 °C, and the gas flow rate was 1.8 L/min. Each fraction was dissolved in water (1 mg/mL), and then measured under the chromatographic conditions described above.

2.3. Characterization of the polysaccharide

The molecular weight of TJ2 polysaccharide was determined with the same HPLC-ELSD method described above. The linear regression was calibrated by using dextrans (DXT 91K-1900K) as standards. A standard curve was prepared based on the retention time (RT) *versus* the log molecular weight.

Monosaccharide composition was determined by using HPLC with precolumn derivatization (Song & Du, 2012). Briefly, the polysaccharide was hydrolyzed with 4 M trifluoroacetic acid (TFA) at 120 °C for 4 h. After being dried with N_2, the residue was dissolved in 0.3 M NaOH and derivatized with 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP, methanol solution) at 70 °C for 100 min. Then, 0.3 M HCl was sucked to neutralize the solution, and an equal volume of chloroform was added and shaken. After layering, the supernatant was filtered with a 0.45 μm microporous membrane. Then, it was analyzed by HPLC using a SUPEL COSIL LC_{18} column (4.6 × 150 mm, 3 μm) (Agilent Technologies, USA) with a mobile phase consisting of phosphate buffer-acetonitrile (83:17, v/v) at a flow rate of 0.5 mL/min. The UV detection wavelength was 250 nm. The monosaccharide composition of the polysaccharide was identified by comparing retention times with those of PMP-labeled standard monosaccharides (glucose, galactose, rhamnose, arabinose, mannose), and the content of each monosaccharide was calculated using the corresponding peak areas and response factors.

Methylation analysis was performed according to the reported method with some modification (Ciucanu & Kerek, 1984). Briefly, the polysaccharide was O-methylated with NaOH in DMSO –MeI. After being treated with 50% sulfuric acid (1 h, 0 °C), the resulting solution was diluted with H_2O to 5.0% (v/v) and hydrolyzed at 100 °C for 18 h. The solution was neutralized with $BaCO_3$ and filtered. The filtrate was evaporated with N_2 to a small volume. The partially O-methylated aldoses were converted to alditol derivatives with $NaBD_4$. The sample was analyzed by Trace DSQ -II GC–MS (Themo, USA), and the TR-5MS column was programmed as 160–210 °C at 5 °C/min, and then 210–260 °C at 8 °C/min.

Infrared spectra (IR) spectrum was performed by using a NEXUS 670 FT-IR (Thermo Nicolet, USA) spectrophotometer in the range of 4000–400 cm^{-1}. The ^1H NMR and ^{13}C NMR spectra were recorded on a Brucker Avance 600 MHz spectrometer (Brucker, Germany). Dimethyl Sulfoxide-D6 (DMSO-D6) was used as the solution.

2.4. Cell proliferation assay

The viability of RAW264.7 cells was determined using the colorimetric CellTiter 96 aqueous cell proliferation assay (MTS) according to the instruction provided by the manufacturer (Promega). Briefly, cells (1 × 10⁴ cells per well) were seeded in 96 wells plates. One day after seeding, they were treated with or without different concentrations of TJ2 and 1 μg/mL of LPS for 24 h. At the end of incubation, 50 μL CellTiter 96 Aqueous reagent (40% v/v dilution in 1 × PBS) was added to each well. Plates were incubated at 37 °C for 2 h, and absorbance was measured at 490 nm with a 96-well plate reader (model Spectra Max 340; Molecular Devices).

2.5. Reverse transcription PCR (RT-PCR) and Real-time quantitative PCR (qPCR)

After the cells were treated with 1 μg/mL LPS or various concentrations of TJ2 for 24 h, the total RNAs were isolated with Trizol reagent (Invitrogen, USA). The Superscript One-Step RT-PCR kit (Invitrogen, USA) was used for RT-PCR. cDNA synthesis was performed with the following condition: 55 °C for 30 min (1 cycle), and 94 °C for 2 min (1 cycle). The condition of PCR amplification is: denaturing at 94 °C (15 s), annealing at 55 °C (30 s), and extension at 72 °C for 1 min (25 cycles), then at 72 °C for 8 min. The PCR primers are designed as the previous reports (Yi et al., 2013). Cox-2 sense: 5′ GCA AAT CCT TGC TGT TCC AAT C 3′, antisense: 5′ GGA

GAA GGC TTC CCA GCT TTT G 3'; IL-6 sense: 5' CAT GTT CTC TGG GAA ATC GTG G 3', antisense: 5' AAC GCA CTA GGT TTG CCG AGT A 3'; TNF-α sense: 5' GAT CTC AAA GAC AAC CAA CTA GTG 3', antisense: 5' CTC CAG CTG GAA GAC TCC TCC CAG 3'; IL-1β sense: 5' GGG ATG ATGATG ATA ACC TG 3', antisense: 5' TTG TCG TTG CTT GGT TCT CCT 3'; β-actin sense: 5'TCA CCC ACA TG TGC CCA TCT ACG A3', antisense: 5' CAG CGC AAC CGC TCA TTG CCA ATG G3'. With the FastStart Universal SYBR Green Master Kit (Rox), qPCR for these molecules was carried out in a Thermal Cycler (Model: DNA Engine 2/Opticon 2).

2.6. Phagocytosis assay

Phagocytosis of macrophages was assessed by FITC-conjugated E. coli particles (Invitrogen, USA). After the macrophages were activated with LPS (0.5 μg/mL) and different concentrations of TJ2 for 48 h, the cells were rinsed with cold PBS and incubated in RPMI 1640 medium (containing 25 mM Hepes and 10% FBS) with FITC-E. coli (bacterium/macrophage ratio = 50/1) at 37 °C for 1 h. With 4% paraformalhyde, the cells were fixed for 10 min after incubation. Then, the extracellular fluorescence particles were rinsed out with ice cold PBS and quenched with 0.02% trypan blue for 3 min. Finally, the cold anti-fade reagent (Invitrogen, USA) was utilized as mounting solution for the slides. The phagocytosis was investigated under a microscope.

2.7. Determination of NO production

RAW264.7 cells (2 × 10^5 cells/mL) were seeded on 96-well plates with 100 μL/well and cultured for 24 h. The cells were incubated 24 h in the presence of LPS (1 μg/mL) or TJ2 at a concentration of 0, 1, 5 and 10 μg/mL. The equal volume of the Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl) – ethylenediamine dihydrochloride in 5% phosphoric acid] was added to the culture supernatants. The absorbance was read at 540 nm and the NO concentration was calculated by using NaNO$_2$ to generate a standard curve.

2.8. Western blot analysis

The analysis was performed according to the procedure previously described (Yi et al., 2013). Briefly, the cells were washed with PBS (ice-cold) and collected after treatment. The cell pellets were suspended in the NP-40 lysis buffer, and the cytoplasmic extracts were obtained. After centrifugation (10,000×g) at 4 °C for 10 min, the samples (100 μg protein/per sample) were electrophoresed on SDS-10% 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.

2.9. Statistical analysis

All the data were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Student's-test were performed to evaluate the significant differences. Statistical significance was considered when p value is less than 0.05. All statistical analyses were performed by using SPSS 16.0 software.

3. Results

3.1. Isolation, purification and structural characterization of TJ2

To determine the active component in Agaricus brasiliensis, which displays any immunoregulatory activities, a water-soluble polysaccharide designated as TJ2 was isolated and purified. The chromatogram of HPLC-ELSD with an Agilent PL aquael-OH column showed a single symmetrical peak, indicating that the polysaccharide was homogeneous. No any absorption at 280 and 260 nm suggested the absence of protein and nucleic acid (Hall, 2013). The relative molecular weight (Mw) of the polysaccharide was calculated as 1150 KDa according to the equation: lg Mw = 14.50–0.950 RT (R^2 = 0.998), which was from the standard curve of molecular size distribution, where Mw was the molecular weight and RT was the retention time of the polysaccharide.

The monosaccharide composition of TJ2 was determined by HPLC after it was hydrolyzed in the presence of TFA. The chromatogram of monosaccharides was compared with the one generated by using the standard monosaccharides (glucose, galactose, rhamnose, arabinose and mannose). The major monosaccharides in TJ2 were glucose (99.2%), although a trace amount of mannose (0.2%) and galactose (0.6%) was also detected. The results suggest that glucose is the main sugar residues in TJ2 (Fig. 1A).

The infrared spectra of TJ2 (Fig. 1B) showed a strong and wide stretching peak around 3394.40 cm^{-1} for O–H stretching vibration, and a moderate intensity absorption peak at 2923.50 cm^{-1} generated by C–H stretching vibration. The intense peak which appeared at 1632.52 cm^{-1} was the characteristic absorption of sugar hydration, while the band at 1411.49 cm^{-1} was attributed to the bending vibration of C–H or O–H (Cao, Yuan, Sun, & Sun, 2011). The absorbance in the range of 1160–1000 cm^{-1} (1155.21, 1078.33 and 1017.47) are due to their C–O–C and C–O–H linkages of typical pyranose form sugar units. The peak at 930.98 cm^{-1} was the characteristic of a β-configuration (Huang et al., 2016), suggesting the glucose units in TJ2 are mainly joined by β-linkages. In addition, the weak peak at 848.75 indicated that there is also a little α-linkages in the structure.

The results of methylation analysis (Table 1) demonstrated that (1→3) linked glucose was main backbone component of the structure (2,4,6-tri-O-Me-Glc). In addition, a small amount of (1→6) linked glucose (2,3,4-tri-O-Me-Glc), (1→4) linked galactose (2,3,6-tri-O-Me-Gal), (1→6) linked mannose (2,3,4-tri-O-Me-Man) and (1→) linked glucose terminal (2,3,4,6-tetra-O-Me-Glc) were found, which might be the residues in the branch structure (Liu et al., 2016).

The ^{1}H NMR spectrum of the polysaccharide clearly showed that chemical shifts of anomeric H-1 were less than 5.0 ppm, indicating that the polysaccharide was mostly composed of β-configuration. The signal at 4.66 ppm was assigned to the β-glucose unit on the basis of its broad singlet appearance (He, Zhang, Ru, Dong, & Sun, 2014). The extremely weak signal at 5.17 and 5.06 ppm implied the presence of a little α-configuration, and this is in accordance with the infrared spectra results. The ^{13}C NMR spectrum showed no signal at 160–180 ppm, illustrating TJ2 does not contain uronic acid. The carbon signals demonstrated there is mainly β-anomeric configuration of glucopyranose moieties (Zhang et al., 2010). As shown in Fig. 1C, three anomeric C at 92.14 ppm, 95.59 ppm and 99.58 ppm, were in corresponding with the anomeric carbon atoms of mannopyranose, galactopyranose and glucopyranose, respectively (Liu et al., 2016). These were all in accordance with the results of monosaccharide composition analysis and IR spectrum.
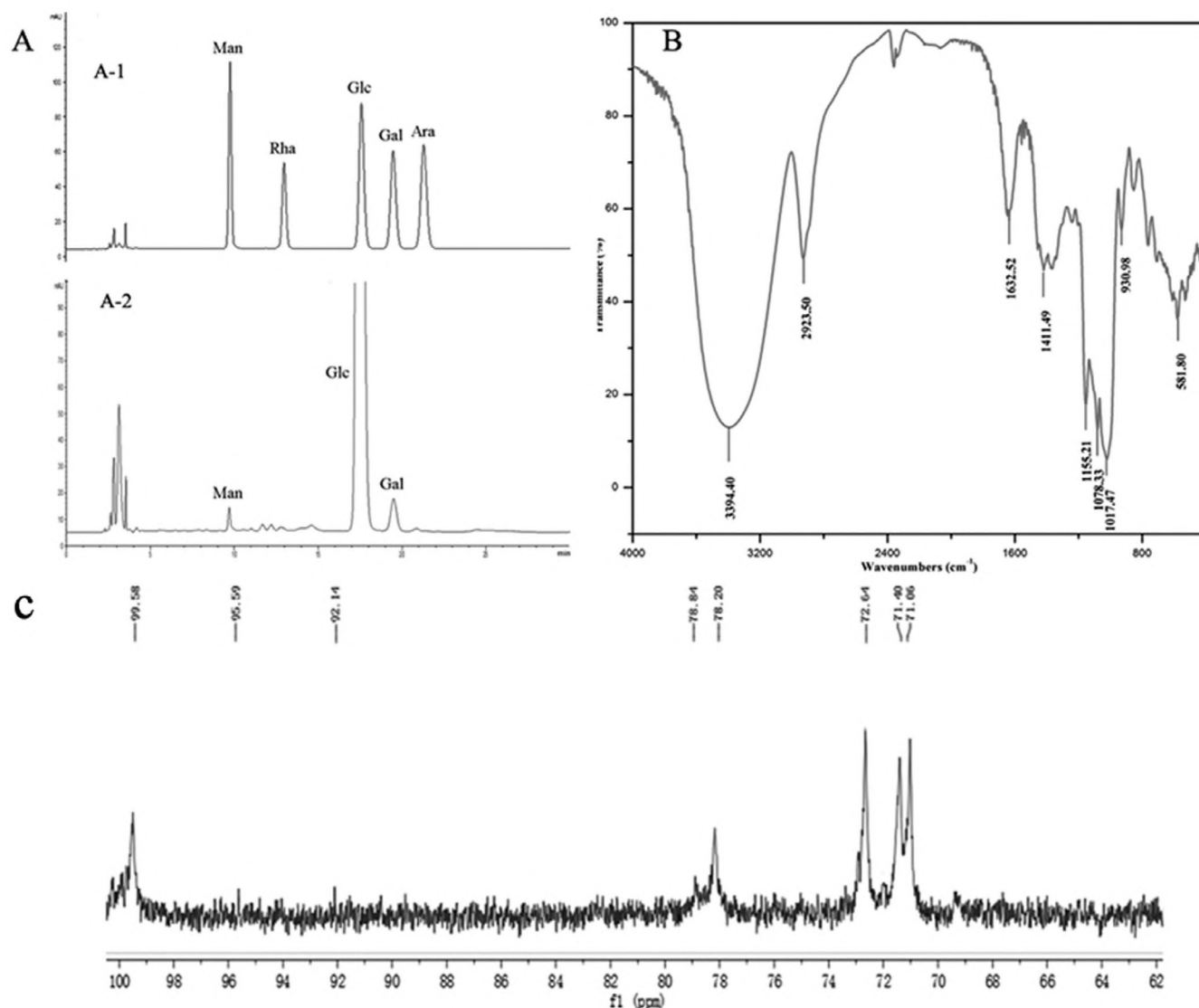
Fig. 1. Characterization of TJ2 from *Agaricus brasiliensis*. (A) The monosaccharide composition analysis of TJ2. The chromatogram of mixed standard monosaccharides (A-1); The chromatogram of TJ2 after hydrolyzed (A-2). (B) The FT-IR spectra of TJ2; (C) ^{13}C NMR spectrum of TJ2 (60–100 ppm).

Table 1
Methylation analysis of TJ2.

Methylation sugar residues	Linkage types	Major mass fragments (*m/z*)	Peak area percentage (%)
2,3,4-tri-O-Me-Glc	→6)-glc-(1→	43.1,59.3,71.0,87.1,118.5,129.2	8.9
2,3,6-tri-O-Me-Gal	→4)-gal-(1→	43.1, 88.0	0.5
2,3,4-tri-O-Me-Man	→6)-man-(1→	43.1,87.2,189.0	0.3
2, 4-di-O-Me-Glc	→3,6)-glc-(1→	43.1,87.0,102.4,189.1,305.2	1.2
2,3,4,6-tetra-O-Me-Glc	T-	43.1, 45.0, 101.6	3.8
2,4,6-tri-O-Me-Glc	→3)-glc-(1→	43.1,71.0, 87.1, 118.2, 161.5	80.1

3.2. TJ2 promoted macrophage proliferation

In this study, we treated RAW264.7 cells, a murine macrophage cell line, with different concentrations of TJ2 and examined its effect on cell proliferation. Interestingly, TJ2 treatment significantly promoted macrophage proliferation. As shown in Fig. 2, the population of the cells after treatment with TJ2 for 48 h was significantly higher than that in the control. Quantitative analyses revealed the cell numbers were nearly double in the presence of 2 μg/mL TJ2 compared to the control at the same condition. The results suggest that TJ2 can stimulate immune cell proliferation.

3.3. TJ2 activated the phagocytosis of macrophages

Macrophages are professional phagocytes able to ingest a pathogen after they are activated. To determine if TJ2 can activate macrophages, the cells were incubated with FITC-labeled *E. coli* after incubated with different concentrations of TJ2. Lipopolysaccharine (LPS) was used as a positive control. Fluorescence of engulfed FITC-labeled *E. coli* was observed and analyzed under a microscope after washing to remove the rest of them in the media and quenching the extracellular signals. As shown in Fig. 3A, TJ2 treatment significantly increased phagocytosis of the cells for
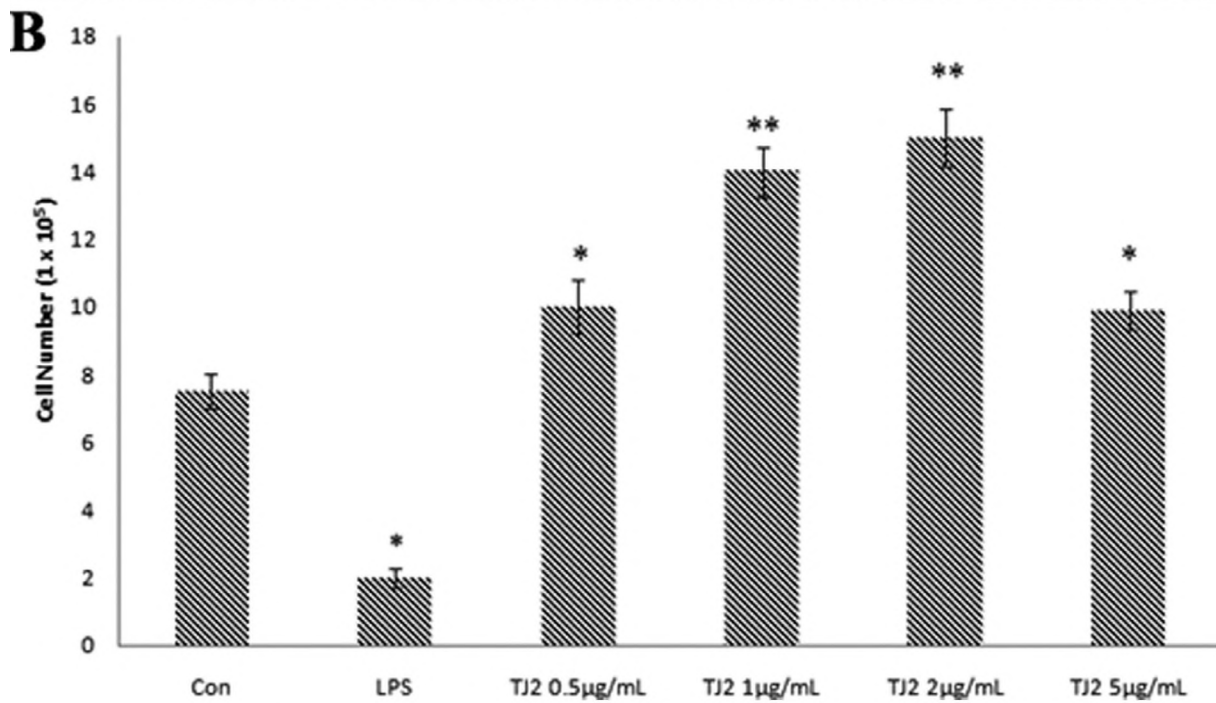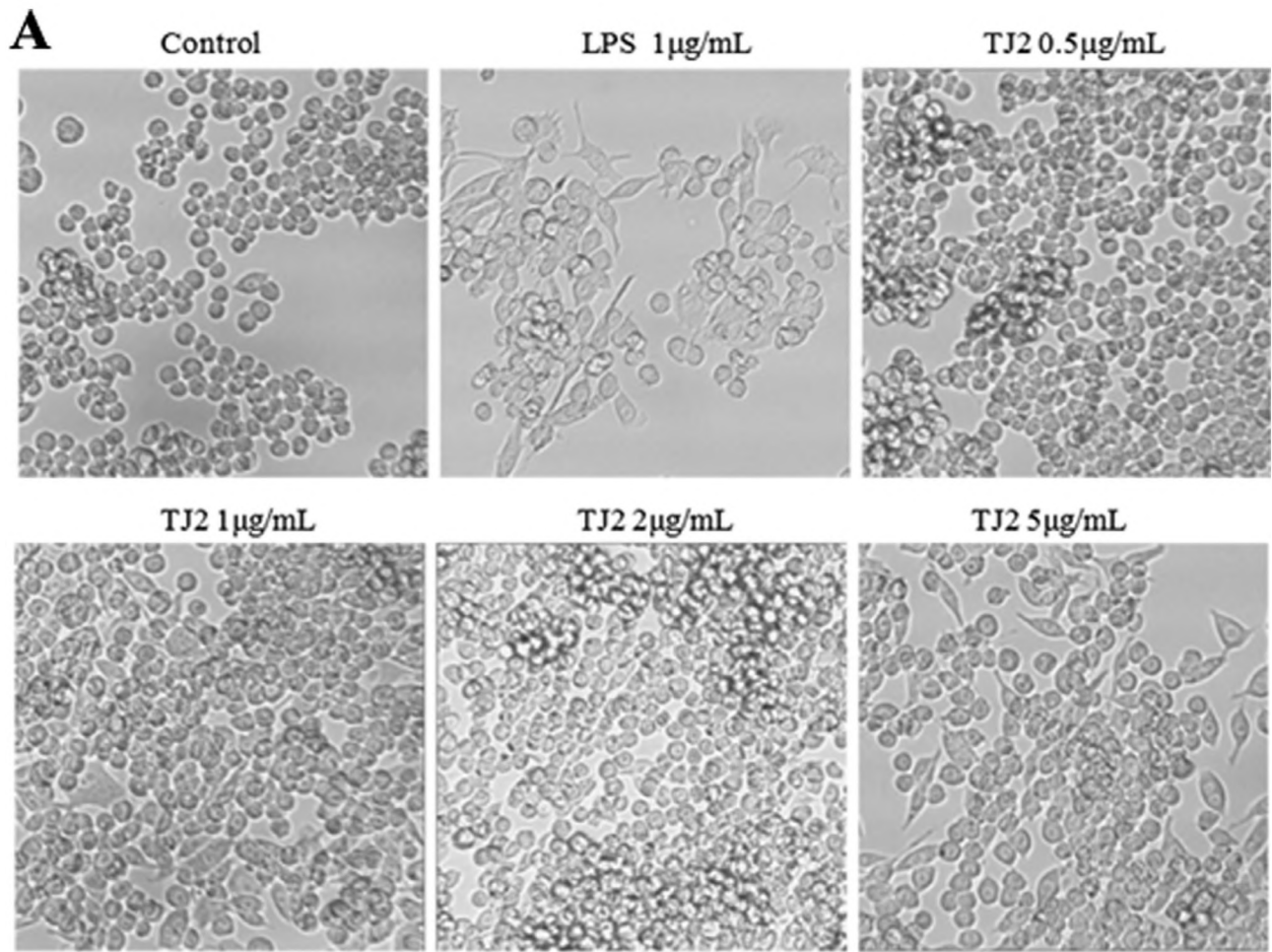
Fig. 2. The effect of TJ2 on cell proliferation. (A) Cellular growth state of RAW264.7 cells (20×) treated with 0.5, 1, 2 and 5 μg/mL TJ2 for 48 h. (B) The viability of RAW264.7 cells measured by MTS. All the experiments were performed in triplicates. Data are presented as mean ± SD. *p < 0.05 and **p < 0.01.
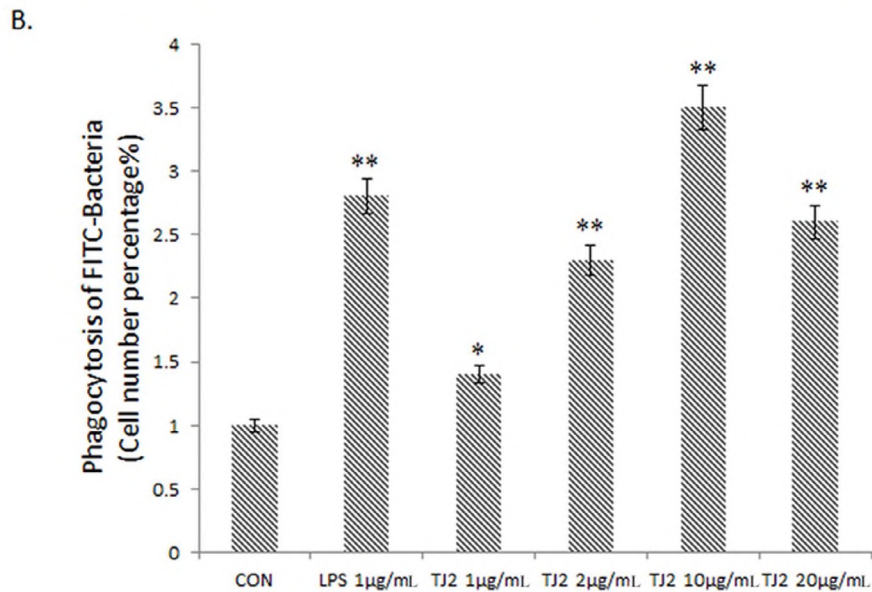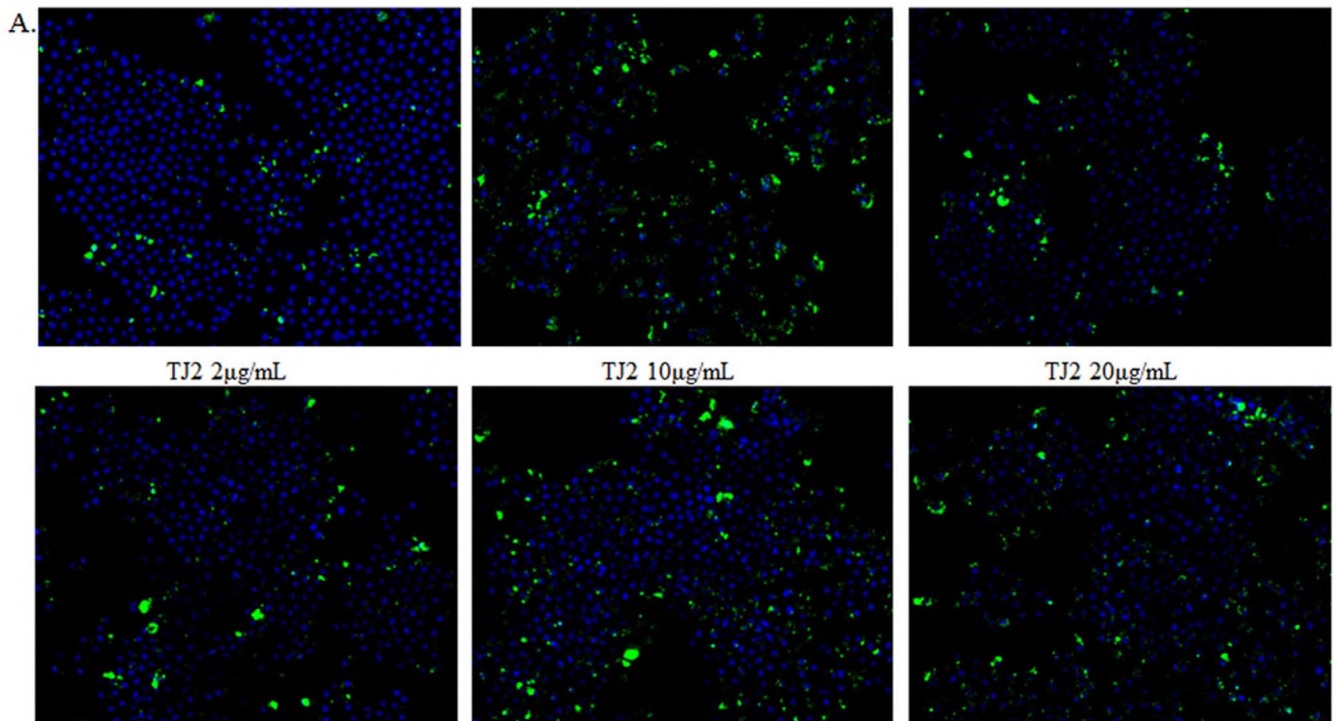
Fig. 3. Effect of TJ2 on macrophage phagocytosis. (A) The cells were activated with TJ2 at a concentration of 1, 2, 10 and 20 μg/mL for 48 h, followed by incubating with FITC-*E. coli* (bacterial to macrophage ratio = 50:1) at 37 °C for 1 h. LPS (1 μg/mL) was used as a positive control. (B) The fluorescent cell numbers were averaged from five fields per well and present as mean ± SD. $^*p < 0.05$ and $^{**}p < 0.01$.

E. coli in a dose-dependent manner. The average number of the cells with fluorescence to the total cells from 5 fields was 3.5-fold higher in the cells (treated with 10 μg/mL of TJ2) compared to the control (Fig. 3B). The result indicates that TJ2 is able to activate macrophages. However, the molecular mechanism remains largely unknown.

3.4. TJ2 induced the production of IL-6, IL-1β, TNF-α and Cox-2 in macrophages

It has been well known that macrophages are capable to secret a large number of cytokines, chemokines and growth factors under stimulation, which in turn regulate their own functions and pro-

mote adaptive immunity (Schultze & Schmidt, 2015). Since our results showed that TJ2 was able to activate macrophages, we were intrigued to determine if TJ2 induces the production of the relevant cytokines contributing to the function of macrophages and mediating adaptive immunity. The cells were treated with TJ2 for 24 h, and the expression of IL-6, IL-1β, TNF-α and Cox-2 genes was determined by both RT-PCR and qPCR (Fig. 4A and B). The results indicated that TJ2 remarkably induced the expression of IL-1β, IL-6, TNF-α and Cox-2 at 1 μg/mL although its role was slightly weaker than that with the same concentration of LPS. It is well known that two forms of cyclooxygenase (COX) exist, i.e. Cox-1 and Cox-2. Cox-1 is present in most tissues and remains a constant level, whereas Cox-2 is primarily present at sites of inflammation
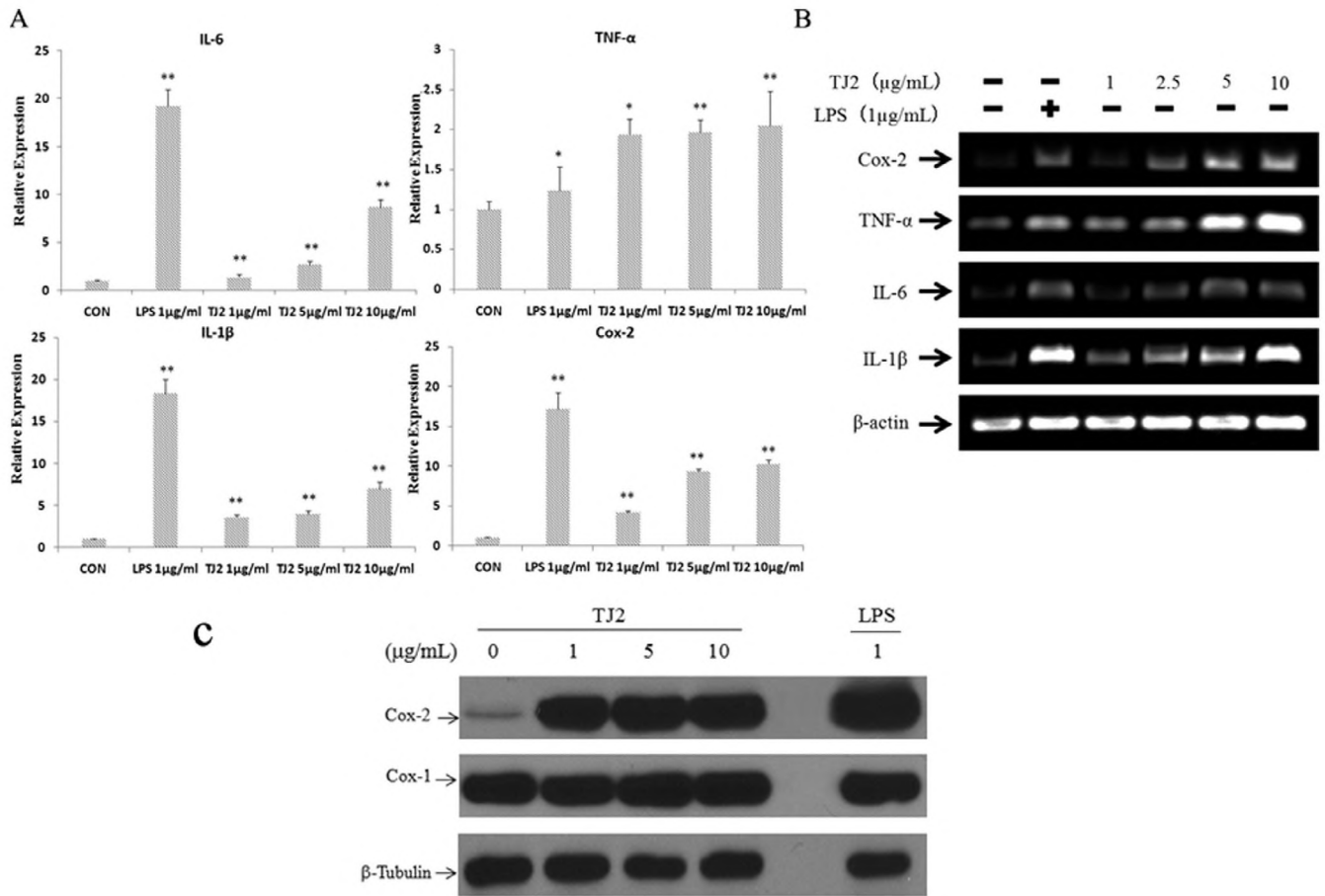
Fig. 4. TJ2 induced the expression of IL-6, IL-1β, TNF-α and Cox-2 in RAW264.7 cells. The cells were treated with 1, 5 and 10 μg/mL of TJ2 for 24 h. (A) The expression of IL-1β, IL-6, TNF-α and Cox-2 was measured by RT-PCR (mean ± SD, n = 3), $^*p < 0.05$ and $^{**}p < 0.01$. (B) The expression of IL-1β, IL-6, TNF-α and Cox-2 was measured by qPCR. (C) Western blot analysis of the Cox-2 expression in RAW264.7 cells.

and can be inducible. They both participate in the physiological events, but have different functional roles. Apparently, TJ2 significantly induced the expression of Cox-2 in macrophages (Fig. 4C). Taken together, the results suggest that TJ2 stimulates the immune responses.

3.5. TJ2 induced the production of NO

Nitric Oxide (NO) is a pleiotropic regulator involved in different molecular and biological pathways. The NO production of activated macrophages plays a vital role in protective host responses to infectious pathogens (Volman et al., 2010). To further elucidate immunological effect of TJ2, the cells were treated with various concentrations of TJ2, and NO production was measured according to the corresponding method. As shown in Fig. 5A, TJ2 dose-dependently induced NO production in macrophages. At the same time, TJ2 promoted the expression of iNOS in a dose-dependent manner. Apparently, increased production of NO was the result of iNOS induction by TJ2.

3.6. TJ2 activated the MAPK and IκB/NFκB pathways

Obviously TJ2 was capable to induce the expression of inflammatory genes and promote macrophage proliferation and phagocytosis. To determine the molecular mechanism responsible for the activation of macrophages by TJ2, we examined its effect on the MAPK and IκB/NFκB pathways in macrophages. The cells were treated with TJ2 at a concentration of 10 μg/mL for various times,

and the activation of the pathways was examined by performing Western blot analyses for p-ERK and p-IκB proteins. As shown in Fig. 5B, TJ2 treatment effectively induced the phosphorylation of ERK and IκB, suggesting that activation of the two pathways may mediate the biological functions of the polysaccharide.

4. Discussion

Agaricus brasiliensis is well known as an edible medicinal mushroom. Its crude extracts and partially purified ingredients, such as beta glucans and proteoglucans, are widely used as popular complementary and alternative medicine to treat cancer and viral infection (Fujimiya, Suzuki, Katakura, & Ebina, 1999). Studies have revealed that beta-glucan and crude extracts obtained from *Agaricus brasiliensis* are able to induce cancer cell apoptosis *in vitro* and suppress tumor growth and metastasis *in vivo* for a variety of cancer types including lung cancer, ovarian cancer, liver cancer, leukemia, and fibrosarcoma (Hetland et al., 2008). In addition, the extracts of *Agaricus brasiliensis* also display antiviral activity against type I poliovirus and western equine encephalitis (WEE) virus (Faccin et al., 2007; Grinde, Hetland, & Johnson, 2006).

In this study, we isolated and purified a polysaccharide named as TJ2 from *Agaricus brasiliensis* and characterized its structure and composition. The polysaccharide consisted of glucose, mannose and galactose, and its relative molecular weight was 1150 KDa. Its structure characters differed obviously from those of the reported polysaccharides in *Agaricus brasiliensis*, indicating that TJ2 is a new polysaccharide derived from this mushroom. Our find-
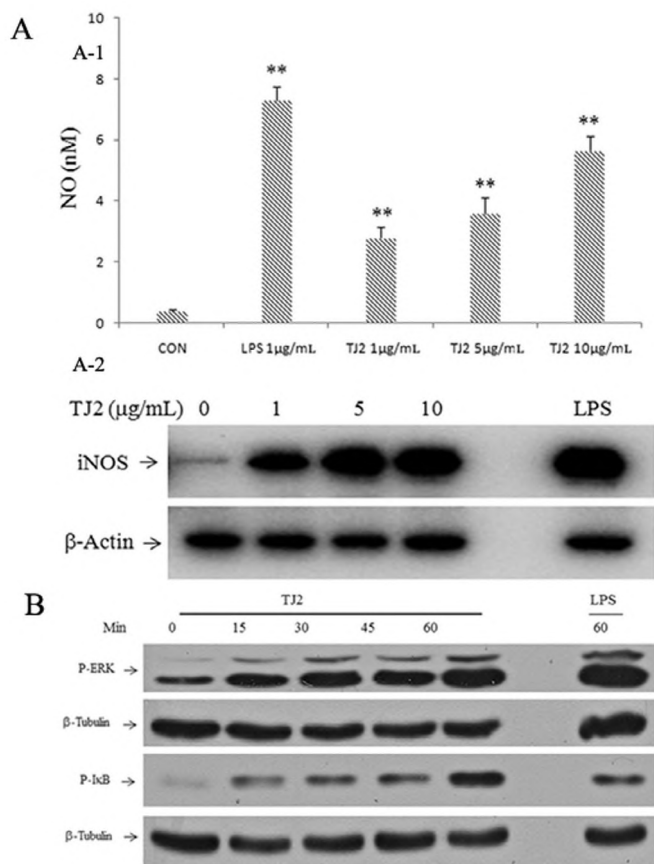
Fig. 5. (A) Effect of TJ2 on NO production in RAW264.7 cells. The cells were treated with different concentrations of TJ2 and 1 µg/mL of LPS for 24 h and the production of NO in the cells were measured by using the Griess reagent. The absorbance was read at 540 nm and the NO concentration was calculated by using $NaNO_2$ to generate a standard curve. The values are presented as mean ± S.D. (n = 6), [**]p < 0.01 (A-1). The cells were treated as described above. The expression of Cox-2 in cell extracts were analyzed by Western blot, and β-actin was used to normalize the protein level (A-2). (B) TJ2 activated the MAPK and IκB/NFκB pathways. The cells were treated with 1 µg/mL of LPS for 60 min or 10 µg/mL of TJ2 for various times. Phosphorylation of ERK and IκB was examined by Western blot analysis with antibodies for p-ERK and p-IκB. β-Tubulin was used as a control.

ings showed that TJ2 is a potent stimulator of macrophage proliferation and activation. In general, medicinal mushrooms upregulate the immune system through impacting phagocytic cells such as macrophages and NK cells. For example, macrophages activated by the active ingredients in mushrooms ingest invading pathogens, and secrete cytokines and chemokines to further promote innate and adaptive immunity. In our study, TJ2 was demonstrated to be able to activate macrophages obviously. It has been reported that the extract of *Agaricus brasiliensis* significantly induces the expression of IL-1β, IL-6 and TNF-α in human monocytes and vein endothelial cells (Hetland et al., 2008). IL-1β, IL-6 and TNF-α are pro-inflammatory cytokines involved in the regulation of the immune response. COX-2 plays a key role in the synthesis of prostaglandins that induce inflammation *in vivo*. Apparently our results demonstrated that TJ2 has the same role in induction of proinflammatory gene expression including Cox-2 in immune cells. NO produced by activated macrophages plays a vital role in protective host responses to infectious pathogens, and inhibits iron-sulfur-dependent enzymes involved in cellular respiration, energy production, and reproduction as well (Green, Mellouk, Hoffman, Meltzer, & Nacy, 1990). TJ2 treatment significantly induced the production of NO and the expression of iNOS in macrophages. As a common effector molecule, NO may be responsible for the

destruction of invaded pathogens in macrophages after TJ2 treatment. In the recent years, it has been reported that the mushroom extract mainly from *Agaricus brasiliensis* could reduce IL-1β and IL-6 levels in human blood (Forland et al., 2011). Furthermore, the polysaccharide extract of *Agaricus brasiliensis* exhibited obvious inhibition effect on the synthesis of these cytokines induced by LPS in human monocytic THP-1 cells (Smiderle et al., 2011). Whether TJ2 also has the same effect on RAW264.7 macrophages needs to be further investigated in the future study.

TJ2 could promote macrophage proliferation, induce the expression of certain cytokines and Cox-2, and activate macrophages to engulf bacteria. The cellular functions of TJ2 remain to be further investigated. LPS induces the expression of proinflammatory genes in cells through activating the TLR4 signaling pathway (Doyle & O'Neill, 2006). Although TJ2 displayed the similar functions as LPS in induction of the expression of proinflammatory genes, the level of TLR4 and MYD88, the downstream component of the pathway, was not affected by TJ2 treatment (data not shown). Furthermore, the cell morphology was clearly distinctive in the presence of LPS or TJ2, suggesting a different role of the two molecules in cell biology. The MAPK pathway can integrate stimuli signals and mediate a variety of physiological phenotypes, such as cellular proliferation, differentiation and inflammatory responses. Activation of the NF-κB pathway is closely related to the process of tumor development, growth and metastasis. Interestingly, we found that TJ2 was able to activate the MAPK and IκB/NFκB pathways, which may partially explain the role of TJ2 in promoting macrophage proliferation and activation. After all, the contribution of the two pathways to cell proliferation and other functions has been well established (Lake, Corrêa, & Müller, 2016; Ukaji & Umezawa, 2014). As a novel polysaccharide derived from *Agaricus brasiliensis*, TJ2 showed obvious effects on macrophage activation, and the process was involved in the MAPK and IκB/NFκB pathways. This result will help to reveal the specific immunoregulatory mechanism of *Agaricus brasiliensis*.

5. Conclusions

In the present study, we isolated and purified a polysaccharide designated as TJ2 from *Agaricus brasiliensis* and determined its purity and structure character. Analyses of its biological functions revealed that TJ2 was able to induce the expression of IL-1β, IL-6, TNF-α, Cox-2 and iNOS, and promote the proliferation and phagocytosis of RAW264.7 macrophages. Furthermore, TJ2 may function through activating the MAPK and IκB/NFκB pathways.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

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