

# Structural analysis and macrophage activation of a novel $\beta$ -glucan isolated from *Cantharellus cibarius*

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**Abstract.** The present study was designed to investigate the structure and immunomodulatory activity of a polysaccharide. A novel acidic  $\beta$ -glucan (WCCP-A-b; molecular weight, 7.3 kDa) was purified from the fruiting bodies of the edible mushroom *Cantharellus cibarius*, which possesses high nutritional values. WCCP-A-b was composed primarily of glucose (89.7%) and glucuronic acid (8.8%). Methylation and nuclear magnetic resonance analysis suggested that WCCP-A-b contained  $\beta$ -D-1,6-glucan as its main chain, which was substituted at O-3 by  $\beta$ -1,3-D-Glcp oligosaccharides or a single-unit of  $\beta$ -Glcp residues. Minor  $\beta$ -1,4-D-GlcpA residues may also be present in the side chains. The degree of branching was ~20.9%. Moreover, WCCP-A-b possessed a macrophage activating effect by promoting the secretion of nitric oxide, TNF- $\alpha$  and IL-6 in a dose-dependent manner. At a cellular mechanistic level, WCCP-A-b activated macrophages via the MAPK signaling pathway. The present results provided useful information for supporting further investigations on the structure-activity association of polysaccharides from *C. cibarius*, and indicated that the novel  $\beta$ -glucan may be a potent natural immunomodulator, thus promoting the application of *C. cibarius* as a valuable source for functional food.

## Introduction

Mushrooms have been widely used and consumed as part of the human diet in numerous countries for centuries due to their high nutritional values, since they contain high protein and fiber contents with small amounts of fat (1). Additionally,

mushrooms are sources of minerals, such as calcium, zinc and magnesium, and vitamins (2). Recently, the medicinal properties of mushrooms have attracted increasing attention. Polysaccharides are the main active ingredients of edible mushrooms (3). Moreover, mushroom polysaccharides, which have been isolated from the fruiting bodies, mycelia and culture media, serve an important role in the growth and development of fungal organisms and have abundant activities, such as antioxidant, anti-inflammatory, anticancer, antidiabetic and immunomodulatory effects (4).

Glucans are considered as one of the most essential polysaccharides in mushrooms, based on their diversified chemical structures and marked medicinal effects (5). Glucans have been discovered in numerous *Basidiomycetes* in the form of  $\alpha$ -1,3-D-glucans (6),  $\alpha$ -1,4-D-glucans (7),  $\beta$ -1,3-D-glucans (8) and  $\beta$ -1,6-D-glucans (9). The differences in structure among these glucans may affect their biological properties. A recent study has revealed that  $\alpha$ -1,6-D-glucans and  $\beta$ -1,3-D-glucans isolated from the basidiome and the mycelium of *Pleurotus albidus* differentially inhibit lipid-induced inflammation and pro-inflammatory lipid-laden macrophage (foam cell) formation in macrophage-like cells (10). Additionally, it has been demonstrated that linear  $\beta$ -(1 $\rightarrow$ 3)-D-glucan from *Cordyceps militaris* exerts an anti-inflammatory effect *in vitro* and *in vivo* (11). The linear (1 $\rightarrow$ 6)- $\beta$ -D-glucan of *Agaricus bisporus* has an effect on the expression levels of pro-inflammatory genes and significantly inhibits the production of inflammatory cytokines caused by lipopolysaccharide (LPS) (9). Furthermore, a branched 1,3- $\beta$ -D-glucan, which is obtained from *Sparassis crispa*, stimulates leukocytes of DBA/2 mice and secretes cytokines *in vitro*, indicating its immunological activity (12). Previous studies have reported that  $\beta$ -1,3-D-glucan with a branching degree (DB) between 0.20 and 0.33 present the most potent antitumor effects (13-15), while the  $\beta$ -1,3-D-glucan with a DB that is too high or too low is not effective (16). The linear  $\alpha$ -1,3-glucan and branched  $\beta$ -1,3/1,6-glucan from the stems of *Pleurotus eryngii* and *Pleurotus ostreatus* exhibit potential prebiotic activity by regulating the growth of *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (17).

Macrophages are innate immune cells present in every tissue and are necessary for normal tissue development, homeostasis and repair of damaged tissues (18). Activated macrophages can phagocytose and neutralize cancer cells

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by secreting nitric oxide (NO), TNF- $\alpha$  and IL-6 cytokines. Polysaccharides have been reported to exert immune and anti-tumor activities by regulating macrophage activities (19,20). For example, a polysaccharide from *Ganoderma sinense* (GSP-2) specifically upregulates the protein expression levels of Toll-like receptor 4 and activates the MAPK signaling pathway in RAW246.7 macrophages. Moreover, GSP-2 exerts its immunomodulatory activity by inducing the secretion of the cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (21). Additionally, it has been reported that a water-soluble  $\beta$ -D-glucan obtained from *Hericium erinaceus* is able to induce the proliferation of lymphocytes and improve the expression levels of inflammatory cytokines produced by THP-1 macrophages (22).

*Cantharellus cibarius* is a common wild edible mushroom belonging to the phylum *Basidiomycota*. Polysaccharides are one of the main active ingredients of *C. cibarius* (23). A linear  $\alpha$ -1,6-D-mannan and a branched  $\alpha$ -1,6-D-mannan substituted with mannan side chains can be isolated from the fruiting bodies of *C. cibarius* (24-26). Our previous study indicated that linear 3-O-methylated galactan isolated from *C. cibarius* activated macrophages and modulated the antitumor immune response by converting tumor-associated macrophages towards an M1-like phenotype (27,28). In addition, *C. cibarius* contains various types of glucans.  $\alpha$ -1,6-D-glucan with  $\beta$ -1,4-D-Glcp side chains is extracted using boiling water, and  $\beta$ -1,3-D-glucan branched at O-6 and a  $\beta$ -1,6-D-glucan with single and short side chains can be separated from the hot aqueous NaOH fraction (26,29). The present study reported the extraction and purification of a novel acidic  $\beta$ -glucan from the fruiting bodies of *C. cibarius* and characterized its structure. The immunomodulatory activity of the acidic  $\beta$ -glucan was further investigated.

## Materials and methods

**Materials.** Fruiting bodies of *C. cibarius* were purchased from the Guilin Road Market (Changchun, China) and were identified using rDNA-Internal Transcribed Spacer sequencing analysis (27). Anion-exchange chromatography (DEAE-cellulose) was purchased from Amersham (Cytiva). Sepharose CL-6B was purchased from Cytiva, while LPS and Polymyxin B sulfate (PMB) were obtained from Sigma-Aldrich (Merck KGaA). TNF- $\alpha$  (cat. no. EK0527) and IL-6 ELISA kits (cat. no. EK0411) were obtained from Boster Biological Technology. Nembutal was acquired from Sinopharm Chemical Reagent Co., Ltd. SB203580 (cat. no. S1863), U0126 (cat. no. S1901) and SP600125 (cat. no. S1876) were acquired from Beyotime Institute of Biotechnology. All of the other reagents were of analytical grade or higher.

**Extraction and purification of the polysaccharides.** Fruiting bodies of *C. cibarius* were first extracted with distilled water at 100°C for 4 h (1:25 w/v), followed by another extraction at 100°C for 2 h (1:20 w/v). The hot water extracts were concentrated under a vacuum at 60°C and precipitated using 4X volumes of 95% ethanol at room temperature for 12 h. The precipitate was collected via centrifugation (3,200 x g for 15 min) at 25°C and re-dissolved in water, dialyzed and lyophilized to obtain the polysaccharide named water-soluble *C. cibarius* polysaccharide (WCCP).

WCCP was dissolved in distilled water, added to a DEAE-cellulose column (8.0x20 cm; Cl<sup>-</sup>) pre-equilibrated with distilled water for anion-exchange chromatography (27) and eluted with distilled water to yield a neutral polysaccharide fraction (WCCP-N) or 0.3 M NaCl to obtain an acidic polysaccharide fraction (WCCP-A). WCCP-A was further purified via gel-permeation chromatography (Sephacrose CL-6B), the eluate was collected and the absorbance was measured at 490 nm for total sugar to give a homogeneous fraction (WCCP-A-b), as previously described by Yang *et al.* (27).

**General methods.** The total carbohydrate content was determined using the phenol-sulfuric acid protocol with glucose as the standard (30). Uronic acid content was determined using the colorimetric method with glucuronic acid as the standard (31). Protein content was determined using the Bradford assay with BSA (VWR International, LLC) as the standard (32).

**Homogeneity and molecular weight determination.** Molecular weight distributions were determined using high-performance gel permeation chromatography (HPGPC) with a TSK-gel G-3000PWXL (7.8x300 mm; Tosoh Corporation) coupled to a Shimadzu high-performance liquid chromatography (HPLC) system (LC-10ATvp pump and refractive index RID-10A detector) as described by Zhang *et al.* (33). The column was pre-calibrated using standard dextrans (50, 25, 12, 5 and 1 kDa) and linear regression.

**Monosaccharide composition analysis.** Monosaccharide composition was determined using HPLC as described by Zhang *et al.* (33). Briefly, polysaccharide samples (2 mg) were first hydrolyzed with 1 ml anhydrous methanol containing 2M HCl at 80°C for 16 h and then with 1 ml 2M trifluoroacetic acid at 120°C for 1 h. Following derivatization with 1-phenyl-3-methyl-5-pyrazo-lone, the derivatives were analyzed using a Shimadzu HPLC system.

**Fourier transform infrared (FT-IR) spectroscopy.** Polysaccharides were ground with KBr powder and turned into a pellet that was ~1 mm for subsequent FT-IR analysis, as previously described (34). FT-IR spectra were obtained with a Spectrum Two FT-IR spectrometer in the range of 4,000-400 cm<sup>-1</sup> (PerkinElmer, Inc.).

**Methylation analysis.** Methylation analysis was conducted according to the method of Needs and Selvendran (35). Uronic acid was firstly reduced into neutral sugars using NaBD<sub>4</sub> prior to methylation analysis. Subsequently, the reduced polysaccharide (5 mg) was dissolved in DMSO (0.5 ml) and methylated with a suspension of NaOH/DMSO (0.5 ml) and iodomethane (1.0 ml). The reaction mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub>, and then the solvent was removed via vacuum evaporation. Complete methylation was confirmed by the disappearance of the -OH band (3,200-3,400 cm<sup>-1</sup>) in the FT-IR spectrum. The per-O-methylated polysaccharide was subsequently hydrolyzed using HCOOH (85%; 1 ml) for 4 h at 100°C and then CF<sub>3</sub>COOH (2 M; 1 ml) for 6 h at 100°C. The partially methylated sugars in the hydrolysate were reduced using NaBH<sub>4</sub> and were then acetylated. The resulting alditol acetates were analyzed using gas chromatography-mass

spectrometry (GC-MS; 7890B-5977B; Agilent Technologies, Inc.) with a DB-35 ms capillary column (30 m x 0.32 mm x 0.25 mm), as previously described (27). The oven temperature was programed from 120°C (hold for 1 min) to 210°C (hold for 2 min) at 3°C/min, then up to 260°C (hold for 4 min) at 10°C/min. The temperature of both the inlet and detector was 300°C. Helium was used as the carrier gas. The mass scan range was 50.0-500.0 m/z.

**Nuclear magnetic resonance (NMR) analysis.**  $^1\text{H}$ ,  $^{13}\text{C}$ , heteronuclear singular quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) NMR spectra were recorded at 20°C on a Bruker Avance 600 MHz spectrometer (Bruker Corporation) with a Bruker 5-mm broadband probe, operating at 600 MHz for  $^1\text{H}$  NMR and 150 MHz for  $^{13}\text{C}$  NMR. Polysaccharides (20.0 mg) were dissolved in  $\text{D}_2\text{O}$  (0.5 ml) and centrifuged at  $10,462 \times g$  for 3 min at 25°C to remove any undissolved polysaccharide. Data were analyzed using standard Bruker software (MestReNova v10.0; Bruker Corporation).

**Cell culture.** RAW264.7 cells were purchased from the American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) high glucose medium supplemented with 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin and 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.). RAW264.7 cells were maintained at 37°C in a 5%  $\text{CO}_2$  incubator.

**Animals.** Specific-pathogen-free female Balb/c mice (10 mice; age, 6-8 weeks; weight, 16-18 g) were obtained from Beijing HFK Bioscience Co., Ltd. Animal experiments were conducted in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Animal Care and Use Committee of Northeast Normal University (Changchun, China). All animals were kept at a constant temperature of 21°C, relative humidity of 55% and under a 12-h light-dark cycle. The animals were maintained under pathogen-free conditions and allowed access to food and water *ad libitum*. Nembutal (50 mg/kg) was injected intraperitoneally into Balb/c mice. After being anesthetized, mice were sacrificed by cervical dislocation. A single cell suspension of peritoneal macrophages and bone marrow macrophages were prepared from Balb/c mice under aseptic conditions by frosted slides in PBS. The suspension was centrifuged at  $400 \times g$  for 3 min at 4°C to obtain the cell pellet. After two washes in PBS, the cells were resuspended in complete DMEM for subsequent examinations.

**Cell viability assay.** RAW264.7 cells were seeded at a density of  $5 \times 10^4$  cells/well in a 96-well plate overnight at 37°C and were then treated with various concentrations (0, 25, 50, 100 and 200  $\mu\text{g}/\text{ml}$ ) of WCCP-A-b or 1  $\mu\text{g}/\text{ml}$  LPS at 37°C for 24 h. The medium was removed, and 100  $\mu\text{l}$ /well of MTT solution (0.5 mg/ml) was added. After a 4-h incubation at 37°C, supernatants were discarded and the resulting formazan was dissolved in 100  $\mu\text{l}$  DMSO. The absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc.; Agilent Technologies, Inc.). Cell proliferation was expressed as the percentage of the control, which was set to 100%. To

examine the effect of endotoxin contamination, the polysaccharides were pre-treated with PMB (30  $\mu\text{g}/\text{ml}$ ) for 24 h at 37°C and then used for cell viability assays.

**Measurement of NO.** RAW264.7 cells, mouse peritoneal macrophages and bone marrow macrophages were seeded at a density of  $1 \times 10^5$  cells/well in a 48-well plate overnight at 37°C and were then treated with various concentrations (0, 25, 50, 100 and 200  $\mu\text{g}/\text{ml}$ ) of WCCP-A-b or 1  $\mu\text{g}/\text{ml}$  LPS at 37°C for 24 h. After incubation, supernatants were collected and reacted with Griess reagent as previously described (36).

**Measurement of TNF- $\alpha$  and IL-6.** RAW264.7, mouse peritoneal macrophages and bone marrow macrophages were seeded at a density of  $1 \times 10^5$  cells/well in a 48-well plate overnight at 37°C and were then treated with various concentrations of WCCP-A-b (0, 25, 50, 100 and 200  $\mu\text{g}/\text{ml}$ ) or 1  $\mu\text{g}/\text{ml}$  LPS at 37°C for 24 h. The concentrations of TNF- $\alpha$  and IL-6 in the supernatants were assessed using the aforementioned TNF- $\alpha$  and IL-6 ELISA kits according to the manufacturer's instructions.

**Western blotting.** RAW264.7 cells were seeded at a density of  $8 \times 10^5$  cells/well in a 6-well plate, and were then pretreated with or without specific inhibitors (25  $\mu\text{M}$  SB203580, 25  $\mu\text{M}$  U0126 or 20  $\mu\text{M}$  SP600125) for 2 h at 37°C, followed by incubation with WCCP-A-b (200  $\mu\text{g}/\text{ml}$ ) at 37°C for 24 h. Western blot analysis was performed as described by Meng *et al* (36). RAW264.7 cells were rinsed twice with cold PBS and lysed in lysis buffer (50 mM Tris/acetate, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, 150 mM sodium chloride, 0.1 mM PMSF and Roche incomplete protease inhibitor cocktail). Protein concentration was measured using the Bradford method. Equal amounts of protein (30  $\mu\text{g}/\text{lane}$ ) were separated via 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in 3% BSA at room temperature for 1 h and then blotted with specific antibodies, and proteins were detected using an electrochemiluminescence reagent (Tanon Science and Technology Co., Ltd.). Both the primary and secondary antibody incubations were 1 h at room temperature. Primary antibodies (1:1,000) against phosphorylated (p)-JNK (cat. no. 4668s), JNK (cat. no. 9252s), p-ERK (cat. no. 9101s), ERK (cat. no. 9102s), p-p38 (cat. no. 9215s) and p38 (cat. no. 9212s) were obtained from Cell Signaling Technology, Inc. The antibody against  $\beta$ -actin (cat. no. 612657) was purchased from BD Biosciences. HRP-conjugated goat anti-rabbit IgG (cat. no. AS014) and goat anti-mouse IgG (cat. no. AS003) secondary antibodies (1:5,000) were obtained from ABclonal Biotech Co., Ltd.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD from  $\geq 3$  independent experiments. Data were analyzed using one-way ANOVA followed by Dunnett's test for comparisons among multiple groups. Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Prism, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results and Discussion

**Preparation of polysaccharide from *C. cibarius*.** WCCP was extracted from fruiting bodies of *C. cibarius* using boiling hot

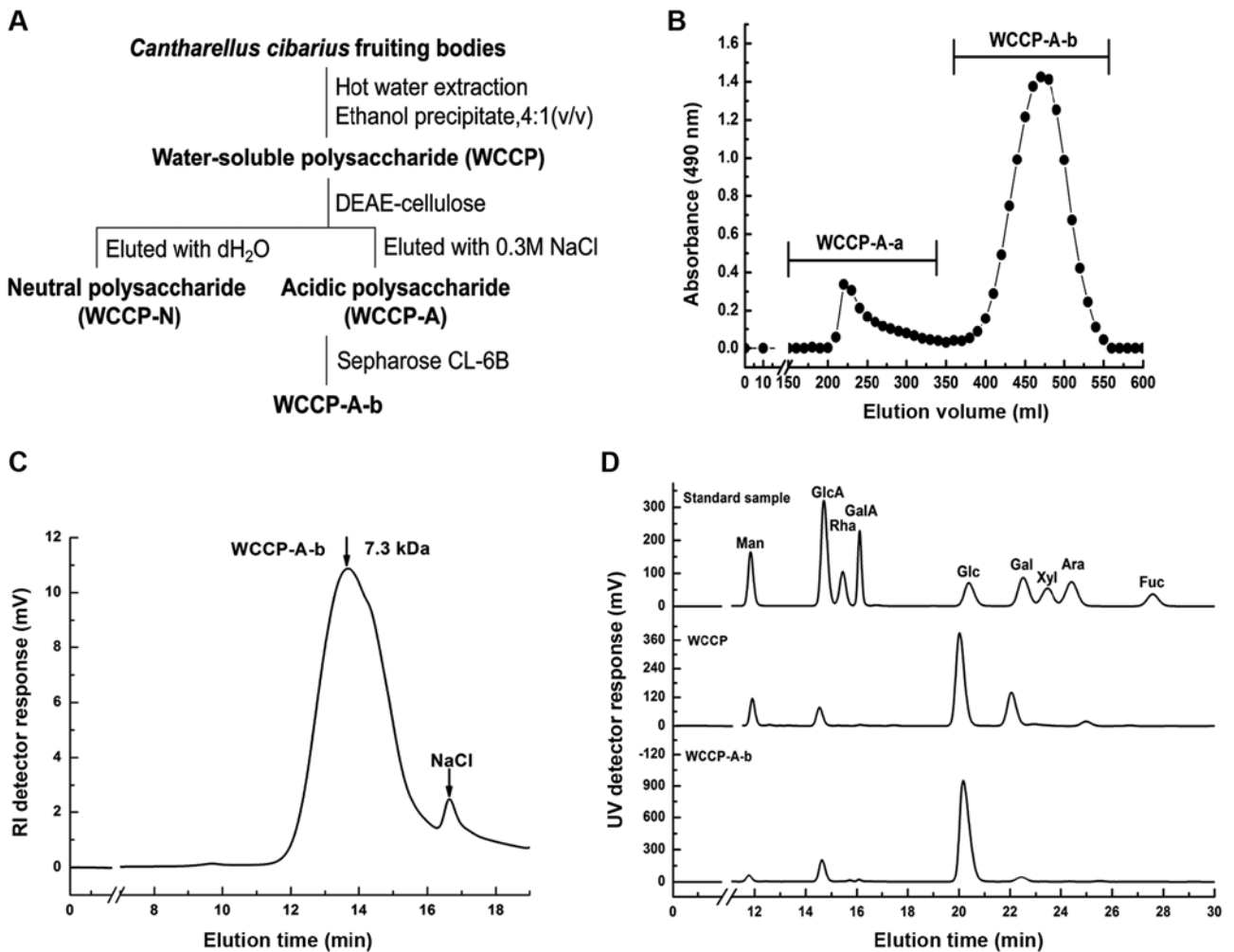


Figure 1. Extraction and purification of polysaccharide. (A) Preparation scheme of WCCP-A-b from *Cantharellus cibarius*. (B) Elution profile of WCCP-A on Sepharose CL-6B column. (C) Elution profile of WCCP-A-b via high-performance gel permeation chromatography. (D) Monosaccharide composition of WCCP and WCCP-A-b. WCCP, water-soluble *C. cibarius* polysaccharide; A, acidic polysaccharide fraction; A-b, homogenous fraction; RI, refractive index.

water, and the yield was 5.5% relative to the dry weight of the material. WCCP contained 79.2% of total sugar, 4.2% of uronic acids and 2.8% of protein (data not shown). Moreover, it was composed of 56.0% glucose, 21.8% galactose, 10.2% mannose, 8.6% glucuronic acid and minor 3-methyl-galactose (3.4%) (Fig. 1D). WCCP was separated into WCCP-N and WCCP-A using anion-exchange chromatography (Fig. 1A). WCCP-A was further purified using gel-permeation chromatography (Fig. 1B), and a homogeneous fraction WCCP-A-b was obtained with the yield of 64.2% relative to WCCP-A. The molecular weight of WCCP-A-b was ~7.3 kDa, as determined via HPGPC (Fig. 1C). This contained glucose as the major sugar (89.7%), followed by minor of glucuronic acid (8.8%) (Fig. 1D).

**FT-IR spectrum analysis.** The FT-IR spectrum of WCCP-A-b is presented in Fig. 2. The strong absorption band at 3,381  $\text{cm}^{-1}$  was attributed to the stretching vibration of O-H. The weak band near 2,894  $\text{cm}^{-1}$  indicated C-H stretching vibration. The stretching bands at ~1,607  $\text{cm}^{-1}$  were observed as the bending vibration of O-H. The band near 1,048  $\text{cm}^{-1}$  suggested the presence of pyranose ring and the weak bands at ~900  $\text{cm}^{-1}$  was associated with the presence of  $\beta$ -linked glycosyl residues (34).

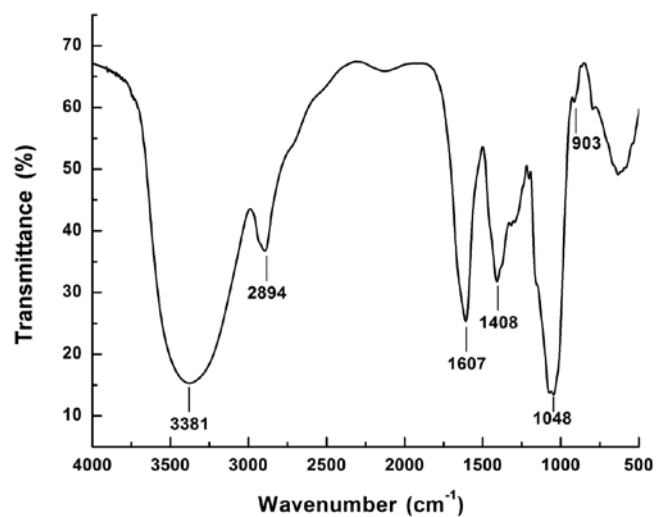


Figure 2. Fourier transform infrared spectrum of the water-soluble *C. cibarius* polysaccharide homogenous fraction (WCCP-A-b).

**Methylation analysis.** To determine the glycosidic linkages in WCCP-A-b, methylation analysis was performed. As

Table I. Glycosidic linkages of water-soluble *C. cibarius* polysaccharide homogenous fraction (WCCP-A-b) analyzed by GC-MS.

Methylated sugars	Linkages	Molar %	Mass fragments. m/z
2,3,4-Me3-Glcp	1,6-	57.4	101,117,129,161,173,189,233
2,4-Me2-Glcp	1,3,6-	15.2	117,129,159,189,233,261,305
2,4,6-Me3-Glcp	1,3-	5.0	101,117,129,161,189,233,277
2,3,4,6-Me4-Glcp	1-	17.3	101,117,129,145,161,205
2,3,6-Me3-GlcAp <sup>a</sup>	1,4-	5.1	101,117,129,161,235

<sup>a</sup>2,3,6-Me3-GlcAp was deduced from 2,3,6-Me3-Glcp (GC-MS ions at m/z 101, 117, 129, 161, 235), which was produced due to the reduction of GlcpA by NaBD<sub>4</sub> to Glc. GC-MS, gas chromatography-mass spectrometry.

Table II. Chemical shift assignments of H and C signals for water-soluble *C. cibarius* polysaccharide homogenous fraction (WCCP-A-b).

Linkage type	1	2	3	4	5	6
$\beta$ -1,6-D-Glcp						
H	4.44	3.25	3.41	3.41	3.55	4.14;3.78
C	102.92	73.01	75.56	69.44	74.85	68.74
$\beta$ -1,3,6-D-Glcp						
H	4.44	3.44	3.67	3.39	3.55	4.14;3.78
C	102.89	72.84	84.57	69.42	74.85	68.74
$\beta$ -1,3-D-Glcp						
H	4.67	3.25	3.73	3.32	3.55	3.85;3.66
C	102.79	73.01	83.50	69.54	74.85	60.67
t- $\beta$ -D-Glcp						
H	4.67	3.35	3.44	3.44	3.34	3.85;3.66
C	102.79	73.04	75.51	68.15	75.63	60.67
$\beta$ -1,4-D-GlcAp						
H	4.97	3.67	3.79	3.66	3.87	-
C	102.18	73.21	75.26	80.55	75.03	172.32

H, proton; C, carbon.

WCCP-A-b contained minor glucuronic acid, it was firstly reduced by NaBD<sub>4</sub>, then methylated, hydrolyzed and acetylated. The partially methylated alditol acetates were analyzed using GC-MS. As presented in Table I, the glycosidic linkage of glucose in WCCP-A-b was mainly in the form of 1,6-linked (57.4%) and 1,3,6-linked (15.2%), suggesting that its backbone was 1,6-glucan, which was branched at O-3. The DB was ~20.9%. Terminal glucose (17.3%) and 1,3-linked glucose (5.0%) were detected as side chains (Table I). It was indicated that terminal glucose may be linked to the backbone through O-3 of 1,6-linked glucose or through short 1,3-linked glucose. Moreover, 1,4-linked glucuronic acid (5.1%) residues were detected in WCCP-A-b, which may be present in side chains (Table I).

**Structure analysis by NMR spectra.** The 1D/2D NMR spectra of WCCP-A-b are presented in Fig. 3 and the chemical shifts are listed in Table II. In the <sup>1</sup>H-NMR spectrum (Fig. 3A), there were three anomeric proton signals at

4.97, 4.67 (the signal peaks overlapped with HOD peaks) and 4.44 ppm, which were assigned to anomeric protons of  $\beta$ -1,4-D-GlcAp (37), t- $\beta$ -D-Glcp/ $\beta$ -1,3-D-Glcp and  $\beta$ -1,6-D-Glcp/ $\beta$ -1,3,6-D-Glcp (38), respectively. The proton chemical shifts occurring in the 3.25-4.14 ppm region were H2-H6 of each sugar residue. In the <sup>13</sup>C-NMR spectrum (Fig. 3B), six obvious signals at  $\delta$ 102.92, 73.01, 75.56, 69.44, 74.85 and 68.74 ppm arose from C-1, C-2, C-3, C-4, C-5 and C-6 of  $\beta$ -1,6-D-Glcp residues (39). The signals at 102.79 and 60.67 ppm were assigned to C-1 and C-6 of t- $\beta$ -D-Glcp ( $\beta$ -1,3-D-Glcp), respectively. The weak signal at 172.32 ppm was assigned to carboxyl group of GlcpA.

Other proton and carbon signals of WCCP-A-b were assigned according to the HSQC spectrum (Fig. 3C). The strong cross H1/C1 signal at 4.44/102.92 ppm, H3/C3 signal at 3.41/75.56 ppm and H6/C6 signal at 4.14;3.78/68.76 ppm arose from 1,6-linked  $\beta$ -D-Glcp. The strong cross H1/C1 signal at 4.44/102.92 ppm, H3/C3 signal at 3.67/84.57 ppm and H6/C6 signal at 4.14;3.78/68.76 ppm were attributed to 1,3,6-linked

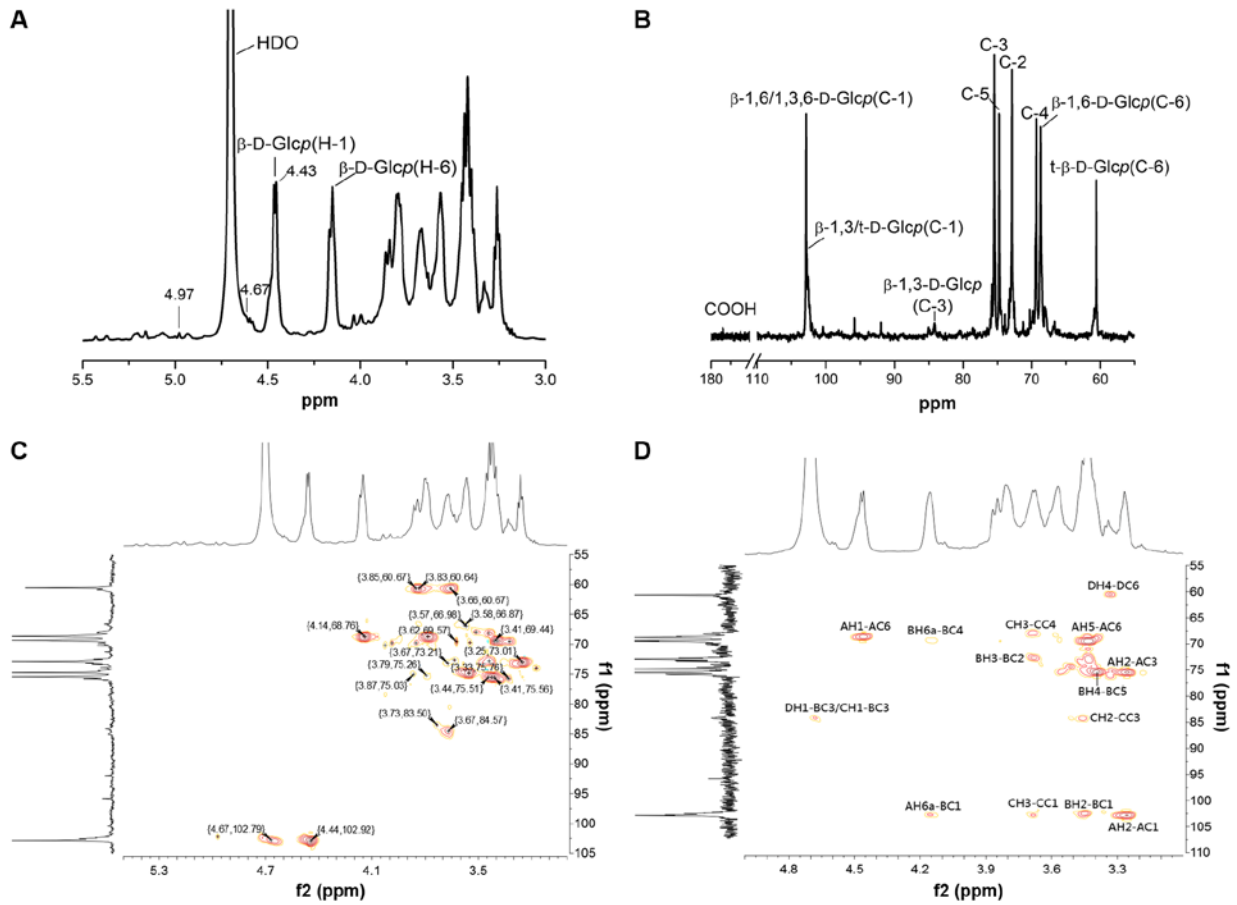


Figure 3. NMR spectra of the water-soluble *C. cibarius* polysaccharide homogenous fraction (WCCP-A-b). (A)  $^1\text{H}$  NMR spectrum. (B)  $^{13}\text{C}$  NMR spectrum. (C) Heteronuclear singular quantum correlation spectrum. (D) heteronuclear multiple bond correlation spectrum. NMR, nuclear magnetic resonance.

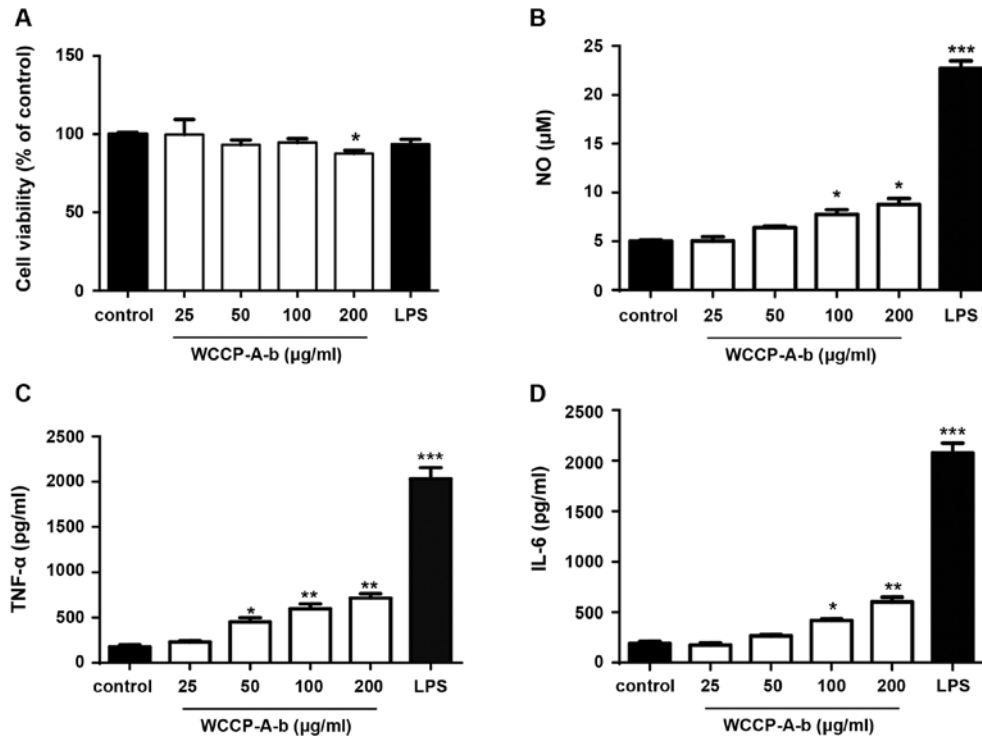


Figure 4. WCCP-A-b promotes macrophage activation. RAW264.7 cells were treated with the indicated doses of WCCP-A-b for 24 h. (A) Cell viability was determined using the MTT assay. (B) NO concentration was analyzed using Griess reagents and the secretion of the cytokines (C) TNF- $\alpha$  and (D) IL-6 was examined via ELISA. The data are presented as the mean  $\pm$  SD ( $n=3$  independent experiments). \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$  vs. control. WCCP-A-b, water-soluble *C. cibarius* polysaccharide homogenous fraction; LPS, lipopolysaccharide; NO, nitric oxide.

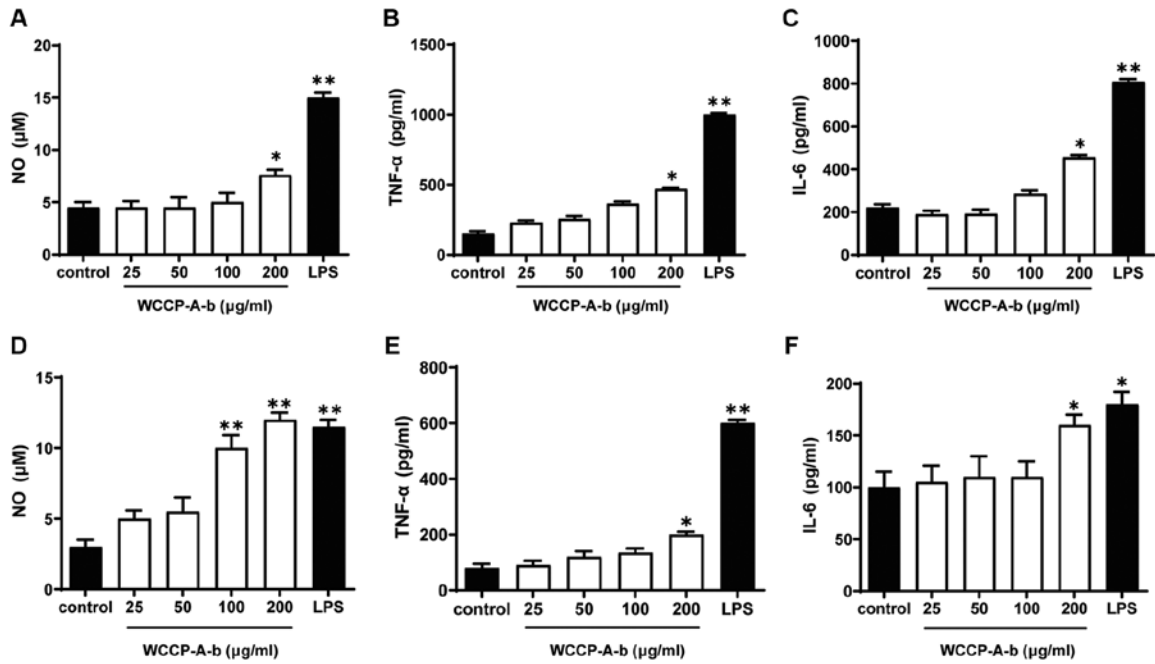


Figure 5. Immunomodulatory activities of WCCP-A-b *in vitro*. Effect of WCCP-A-b on (A) NO, (B) TNF- $\alpha$  and (C) IL-6 release from mouse peritoneal macrophages and on (D) NO, (E) TNF- $\alpha$  and (F) IL-6 release from bone marrow macrophages. The data are presented as the mean  $\pm$  SD (n=3 independent experiments). \*P<0.05 and \*\*P<0.01 vs. control. WCCP-A-b, water-soluble *C. cibarius* polysaccharide homogenous fraction; LPS, lipopolysaccharide; NO, nitric oxide.

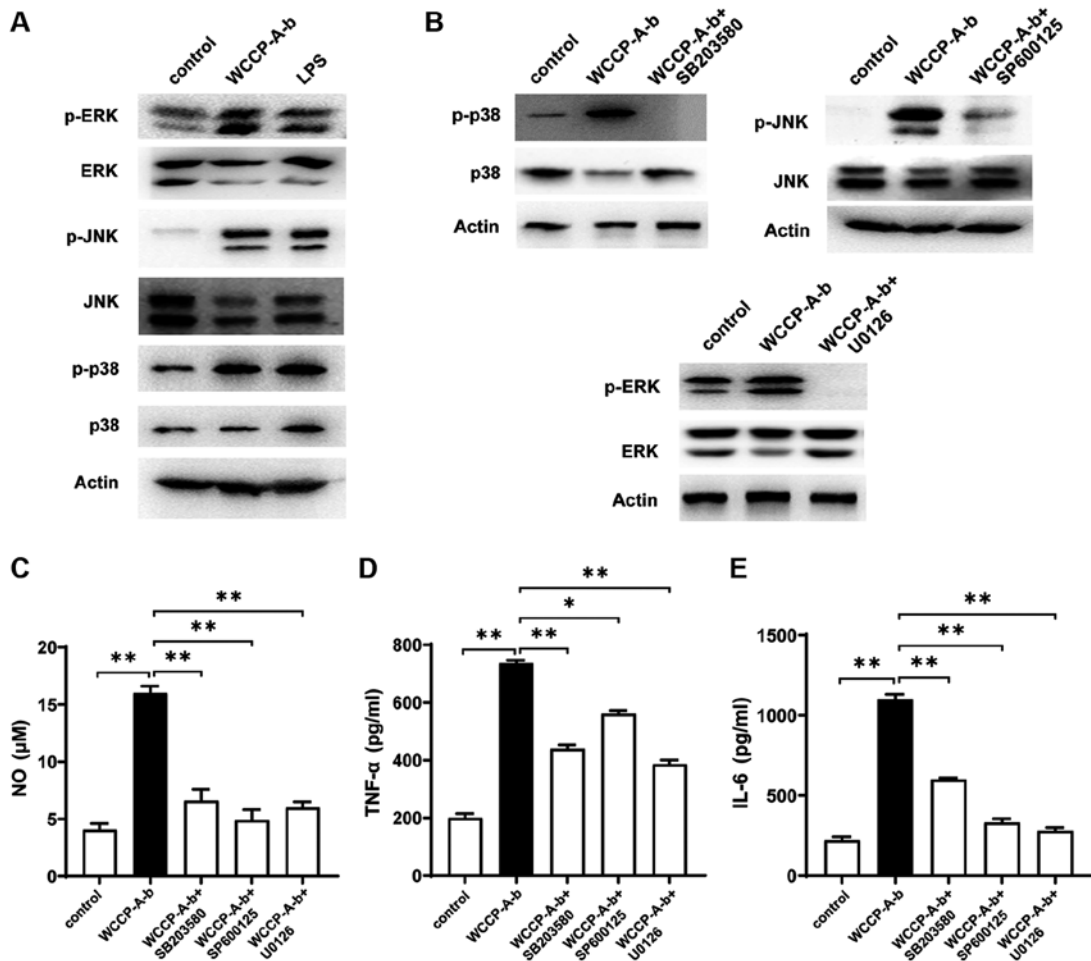


Figure 6. Effect of WCCP-A-b on MAPK signaling pathways. (A and B) Protein expression levels of p38, JNK and ERK, and their phosphorylation. (C) NO production, (D) TNF- $\alpha$  and (E) IL-6 secretion was detected using Griess reagents and ELISAs, respectively. The data are presented as the mean  $\pm$  SD (n=3 independent experiments). \*P<0.05 and \*\*P<0.01. WCCP-A-b, water-soluble *C. cibarius* polysaccharide homogenous fraction; LPS, lipopolysaccharide; NO, nitric oxide, p-, phosphorylated.

$\beta$ -D-Glcp. The cross-peak at 4.67/102.79 and 3.85;3.66/60.67 ppm were from H1/C1 and H6a;b/C6 of the terminal- $\beta$ -D-Glcp, while the down-field shift at 3.73/83.50 ppm was from H3/C3 of 1,3- or 1,3,6-linked  $\beta$ -D-Glcp (40,41). Furthermore, the weak signal peak of H1/C1 (4.97/102.18 ppm) and down-field shift of H4/C4 (3.66/80.55 ppm) confirmed the existence of 1,4-linked  $\beta$ -D-GlcpA (42,37).

In the HMBC spectrum (Fig. 3D), the cross peaks of both anomeric protons and carbons of glycosyl residues AH1/AC6, AH2/AC1, AH6a/BC1, BC3/CH1, BC3/DH1, CC1/CH3 and DC1/DH2 were observed. Due to the low amount of GlcA in WCCP-A-b, no obvious cross peaks were observed in HMBC for GlcA. Combined with the methylation analysis results, the possible structure of WCCP-A-b was proposed as a  $\beta$ -1,6-D-glucan, which was branched at O-3 of  $\beta$ -1,6-D-Glcp by short  $\beta$ -1,3-D-Glcp oligosaccharides or single-unit terminal  $\beta$ -Glcp residues. Small amounts of  $\beta$ -1,4-D-GlcpA may also exist in side chains. According to the aforementioned analysis, WCCP-A-b may be a novel  $\beta$ -1,6-D-glucan structure containing  $\beta$ -1,4-D-GlcpA side chains, which to the best of our knowledge has not been previously reported.

Acidic  $\beta$ -glucans have been previously reported (37,43); however, their structures are different from the WCCP-A-b that was identified in the present study. A polysaccharide fraction (PSG-1-F0.2), isolated from *Ganoderma atrum*, also mainly consisted of glucose and glucuronic acid (37). The backbone of PSG-1-F0.2 was identified to be composed of  $\beta$ -(1 $\rightarrow$ 3)-glucose, which was different from WCCP-A-b. One glucan fraction (WPOPA) obtained from *Pleurotus ostreatus* had a similar main chain structure to WCCP-A-b, but most of the GlcpA residues were in the form of t- $\beta$ -D-GlcpA, which was notably distinguished from WCCP-A-b (43).

**WCCP-A-b promotes macrophage activation.** Macrophages can defend against pathogen invasion, can kill tumor cells and can improve the immune capabilities by releasing inflammatory mediators (27). NO, TNF- $\alpha$  and IL-6 serve an important role in the immune process (34). In order to investigate the immunomodulatory activity of WCCP-A-b, RAW264.7 cells were treated with WCCP-A-b. The MTT assay indicated that WCCP-A-b exhibited no cytotoxicity towards RAW264.7 cells at concentrations of 0-100  $\mu$ g/ml, but that there was a significant decrease in cell viability at 200  $\mu$ g/ml (Fig. 4A). ELISA assays and Griess reagent were used to quantify NO, TNF- $\alpha$  and IL-6 concentrations in the conditioned medium of RAW264.7 cells. As the positive control, LPS significantly stimulated NO, TNF- $\alpha$  and IL-6 production. Moreover, WCCP-A-b induced NO, TNF- $\alpha$  and IL-6 production in RAW264.7 cells in a dose-dependent manner (Fig. 4B-D), with a significant difference for all at 100 and 200  $\mu$ g/ml. To further confirm these results, the aforementioned experiments were repeated using mouse peritoneal macrophages and bone marrow macrophages (Fig. 5). The results demonstrated that WCCP-A-b also induced NO, TNF- $\alpha$  and IL-6 production in mouse peritoneal macrophages (Fig. 5A-C) and bone marrow macrophages (Fig. 5D-F), with significant increases at 200  $\mu$ g/ml, indicating that WCCP-A-b may promote macrophage activation.

$\beta$ -D-glucans obtained from mushrooms are effective immunomodulators and are considered as modifiers of biological responses (4). A neutral branched  $\beta$ -glucan

extracted from the fruiting bodies of *Amillariella mellea*, containing  $\beta$ -D-(1 $\rightarrow$ 6)-linked Glcp as its main chain, can promote macrophage phagocytosis and increase production of NO, reactive oxygen species, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (34). Another polysaccharide, containing a higher content of (1 $\rightarrow$ 6)-linked  $\beta$ -glucan and lower content of  $\alpha$ -glucan, purified from *Agaricus brasiliensis* increases the secretion of the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  in phorbol myristate acetate-differentiated THP-1 cells, while it decreases pro-inflammatory effects caused by LPS, indicating promising immune activity (44). However, a branched  $\beta$ -1,3-glucan, derived from the fruiting bodies of *Lentinus squarrosulus*, is able to activate macrophages, splenocytes and thymocytes at certain concentrations (45). Therefore, the novel  $\beta$ -1,6-D-glucan isolated from *C. cibarius* in the present study may be used as a potential natural immunostimulatory agent.

**MAPK signaling pathway is involved in macrophage activation.** MAPKs, including ERKs, JNKs and p38-MAPKs, have been found to be associated with macrophage activation (36). The present study investigated whether the MAPK signaling pathway was associated with WCCP-A-b-induced macrophage activation. The results indicated that WCCP-A-b increased the phosphorylation of ERK, JNK and p38 (Fig. 6A). Pre-treatment of cells with inhibitors of JNK (SP600125), ERK (U0126) and p38 (SB203580) markedly decreased the phosphorylation of ERK, JNK and p38, respectively (Fig. 6B). In addition, the secretion of NO, TNF- $\alpha$  and IL-6 was significantly suppressed following the addition of the aforementioned inhibitors (Fig. 6C-E). Therefore, it was suggested that the MAPK signaling pathway may be involved in the macrophage activation by WCCP-A-b.

**Conclusion.** In the present study, an acidic  $\beta$ -glucan (WCCP-A-b) was purified from hot water extracted polysaccharides from the fruiting bodies of *C. cibarius* using anion exchange and gel-permeation chromatography. The backbone of WCCP-A-b was a  $\beta$ -D-1,6-glucan, which was branched at O-3 of Glcp by  $\beta$ -1,3-D-Glcp short chains or single-unit of  $\beta$ -Glcp residues. Furthermore, small amounts of  $\beta$ -1,4-D-GlcpA may be present in the side chains. WCCP-A-b possessed a macrophage activatory effect by promoting the secretion of NO, TNF- $\alpha$  and IL-6. On a cellular mechanistic level, WCCP-A-b activated macrophages via the MAPKs signaling pathway. However, the structure-activity association of the glucan was not deeply discussed in the present study. Therefore, more studies, such as *in vivo* animal experiments, on the investigation of the immunomodulatory activity should be further performed. The present data indicated that the identified novel  $\beta$ -glucan may be used as a potent immunomodulator.

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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

LS and YZ conceived the study and revised the manuscript. YQ purified and characterized the polysaccharides and drafted the manuscript. XZ and HG performed the polysaccharide extraction. YM and YW performed the macrophage activation experiments and confirmed the authenticity of the data. All authors have read and approved the final manuscript.

#### Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Northeast Normal University (approval no. AP20151009) and was conducted in accordance with the National Standards of the People's Republic of China Laboratory Animal-Guideline for Ethical Review of Animal Welfare.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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