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Inducement of Cytokine Release by GFPBW2, a Novel Polysaccharide from Fruit Bodies of *Grifola frondosa*, through Dectin-1 in Macrophages

Ying Wang,[†,§] Jianping Fang,[†,§] Xinyan Ni,[†] Jie Li,[†] Qin Liu,[†] Qun Dong,[†] Jinyou Duan,*[,‡] and Kan Ding*[,†]

[†]Glycochemistry & Glycobiology Lab, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, People's Republic of China

[‡]College of Science, Northwest A&F University, Yangling 712100, Shanxi, People's Republic of China

Supporting Information

ABSTRACT: Polysaccharides, especially β-glucans isolated from various species of mushrooms, are considered as biological response modifiers (BRMs) to be widely used in the treatment of cancer, especially due to their immunostimulatory activity. We herein characterized the structure of a novel water-soluble homogeneous polysaccharide (GFPBW2) from the fruit bodies of mushroom *Grifola frondosa* and investigated its immunomodulatory activity in vitro. GFPBW2 was purified from the alkali-extracted fractions by stepwise elution with a molecular weight of 26.2 kDa. On the basis of infrared and NMR spectroscopy, methylation and monosaccharide composition analysis, partial acid hydrolysis, and Smith degradation, its structure was elucidated to possess a backbone consisting of β-D-1,3- and β-D-1,4-linked glucopyranosyl residues, with branches attached to *O*-6 of β-D-1,3-linked glucopyranosyl residues. Functionally, it is an effective inducer of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) secretion in murine resident peritoneal macrophages. Using quartz crystal microbalance (QCM) analysis, we found that GFPBW2 could bind dendritic cell-associated C-type lectin-1 (Dectin-1) with an affinity constant (K_d) value of 1.08×10^{-7} M, while it could activate Syk and enhance TNF-α production in RAW264.7 cells overexpressing wild type but not mutant Dectin-1. Furthermore, Syk, NF-κB signaling, and cytokine release in resident peritoneal macrophages induced by GFPBW2 could be significantly inhibited by a specific Dectin-1 blocking reagent, Laminarin. These data suggested that GFPBW2 might be a potential ligand of Dectin-1, and the potential of GFPBW2 to activate macrophage through triggering cytokine secretion might be attributed, at least in part, to the involvement of Dectin-1.

KEYWORDS: *antitumor activity, β-glucan, biological response modifier, maitake*

INTRODUCTION

Cancer, also known as a malignant tumor, is one of the leading causes of death worldwide despite newly developed drugs and tools for diagnosis and treatment. One of the major problems is damage to immune systems during tumor treatment. Therefore, it is necessary to investigate novel substances that enhance immunity potential in combination with other drugs or alone for treatment of tumors. Many polysaccharides, especially β-glucans, isolated from various species of mushrooms have been intensively investigated in the medical field for many decades due to their immunomodulatory activity. Now, β-glucans, considered as biological response modifiers (BRMs), are widely used in treatment of tumors for their ability to improve/modulate immunity by activation of immune cells, particularly macrophages.[1−3] In China and Japan, mushroom-derived extracts rich in β-glucans have been used for over 30 years as adjuncts to standard chemotherapy against cancer.[4] Recently, a great deal of research has focused on the molecular mechanisms underlying the clinical beneficial immunomodulatory effects of β-glucans, and many receptors on the immune cells for recognition of β-glucans, such as dendritic cell associated C-type lectin (Dectin-1) and toll like receptor 2 (TLR2), have been identified.[4,5] In vitro, β-glucan activates macrophages, leading to protein phosphorylation and production of reactive oxygen species and proinflammatory cytokines.[6] Dectin-1, the major leukocyte receptor for β-(1,3)-glucans, preferentially expressed on macrophages and dendritic cells, plays a crucial role in β-glucan-mediated activation of immunity. Accumulated evidence has demonstrated that β-glucan binding to Dectin-1 results in the phosphorylation of the receptors by Src family kinases and then the activation of spleen tyrosine kinase (Syk), followed by the formation of the caspase recruitment domain 9 (CARD9) adapter complex and subsequently the activation of the nuclear factor κB (NF-κB) pathway to produce cytokines.[6−8]

Grifona frondasa (Fr.) S. F. Gray (*G. frondosa*), a species of basidiomycetes, namely maitake, is a popular edible mushroom in Asia countries. In recent years, many polysaccharide fractions with immunomodulatory and antitumor activities have been obtained from the fruit bodies of *G. frondasa*.[9,10] Grifolan (GRN), a β-D-1,3-linked glucan with a single β-D-1,6-linked glucopyranosyl residue branched at C-6 on every third residue, shows strong antitumor and immunostimulatory activities, such

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as activation of macrophages to produce cytokines and NO products.[11,12] Maitake D (MD) fraction containing β-1,6-glucan with β-1,3-branched chains exhibits potent antitumor activity by increasing the activity of immunocompetent cells.[13,14]

β-Glucan polysaccharides derived from different sources vary in backbone linkage, degrees of branching, and branch linkage, and the fine structural characteristics of β-glucans subsequently affect their effectiveness as BRMs.[3] Thus, it is of great interest to identify and characterize novel β-glucans with better immunostimulatory activity and better therapeutic potential in the clinic. Moreover, few of the polysaccharides from the fruit bodies of *G. frondosa* are homogeneous, although many have been purified. The structures of these polysaccharides have not been unambiguously characterized. In a previous study, we characterized the structure of a homogeneous polysaccharide, named GFPBW1, isolated from the fruit bodies of *G. frondosa*, and investigated its immunostimulatory effects and antitumor activity.[15] Here, we report another novel water-soluble polysaccharide, named GFPBW2, extracted from the same source. Its structure was elucidated, and the binding ability of GFPBW2 with Dectin-1 was analyzed by quartz-crystal microbalance (QCM). In addition, the immunostimulatory effect of GFPBW2 was evaluated and the underlying mechanisms were also investigated.

MATERIALS AND METHODS

Reagents and Animals. Dried fruit bodies of *G. frondosa* were bought from Dashanhe Group Company (Shanghai, People's Republic of China). DEAE-cellulose 32 was purchased from GE Healthcare Biosciences (Pittsburgh, PA, USA). Standard monosaccharides, T-series Dextran, sodium borohydride, iodomethane, trifluoroacetic acid (TFA), lipopolysaccharide (LPS, isolated from *E. coli* strain 055: B5), polymyxin B (PMB), and Laminarin (Lam) were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Merck KGaA (Darmstadt, Hesse, Germany). All reagents used were of analytical grade unless otherwise claimed.

Female ICR mice, 6−8 weeks old, purchased from Shanghai SLAC Laboratory Animal Center were maintained in a specific pathogen-free (SPF) environment. All animal experiments were performed according to the guidelines for the Institutional Animal Care and Use Committees of Shanghai Institute of Materia Medica, Chinese Academy of Sciences (IACUC-SIMM) (Approval ID: 2010-11-DK-03). Antibodies against IKKβ, p-IKKβ, p-IκBα, IκBα, p-Syk, and Syk were obtained from Cell Signaling Technology (Danvers, MA, USA). The β-actin antibody used as internal reference was purchased from Sigma, and the Myc-Tag antibody was obtained from Abmart (Shanghai, People's Republic of China). DMEM, RMPI 1640 medium, and fetal bovine serum (FBS) were obtained from Gibco (Gaithersburg, MD, USA). IL-6 ELISA kit was purchased from Jingmei Biotech (Shanghai, People's Republic of China), while recombinant murine Dectin-1 containing the extracellular domain (69−244 aa) from mouse myeloma cell line NS0 and TNF-α ELISA kit were obtained from R&D Systems (Minneapolis, MN, USA).

General Methods. All evaporations were carried out at 45 °C under reduced pressure. Optical rotations were determined with a Perkin-Elmer 241 M digital polarimeter (Shanghai Physical Optics Co., People's Republic of China). Gas chromatography (GC) was conducted on a Shimadzu GC-14 B instrument, equipped with a 3% OV-225-packed glass column (3.2 mm × 2 m) and an FID detector (Shimadzu, Japan). The column temperature was kept at 210 °C for monosaccharide composition analysis and at 190 °C for methylation analysis, while the carrier gas was N_2 at a flow rate of 25 mL/min. The injection and detection temperatures were 250 and 240 °C, respectively. Electrospray ionization mass spectrometry (ESI-MS) was carried out on a Finnigan LCQ Deca instrument (Waltham, MA, USA), which was operated with a spray voltage of 4.5 kV and a capillary temperature of 275 °C. The samples were dissolved in MeOH/H_2O (1/1, v/v) solution (200 μL) containing 1% (v/v) formic acid at a concentration of 0.5 mg/mL.

Isolation and Fractionation of GFPBW2. The fruit bodies of *G. frondosa* (3 kg) were defatted twice (for 1 week each time) with 95% EtOH, air-dried, and then extracted with boiling water four times under intermittent stirring (6 h each time). The resultant residues were treated with 5% NaOH at 4 °C for 4 h. After neutralization by 2 M HCl, the alkali extraction was dialyzed against running water for 2 days. After centrifugation, the retentate was concentrated and precipitated with three volumes of 95% ethanol at 4 °C for 12 h to give the crude polysaccharide GFPB (50 g, yield 1.7%). A portion of GFPB (10 g) was dissolved in water and loaded on a DEAE−cellulose chromatography column (6.0 × 60 cm), followed by stepwise elution with deionized water, 0.1 M NaCl, and 0.2 M NaCl. Measured by the colorimetric total carbohydrate test using the phenol−sulfuric acid method, the fraction that eluted with 0.2 M NaCl was pooled, dialyzed, and lyophilized to give GFPBW2 (120 mg, 1.2% of GFPB). The LPS content of GFPBW2 was less than 1 ng/mg, as determined by LPS-specific chromogenic Limulus Amebocyte Lysate test using Pyrochrome (Associates of Cape Cod, MA, USA).

Homogeneity and Molecular Weight. The homogeneities and molecular weights of polysaccharides were determined using a high-performance gel-permeation chromatography (HPGPC) method which was performed with an Agilent 1200 instrument (Agilent, USA), including a G1311A Qudra pump, a G1315D RI dectector, and a G1362A dual λ absorbance detector. Tandem linked Ultrahydrogel 2000 and Ultrahydrogel 500 columns (Waters, Japan) were used in this process. The solvent for samples was 0.2 M $NaNO_3$, and the flow rate was 0.5 mL/min. The column temperature was kept at 25 ± 0.1 °C. The column was calibrated by reference to the MW (molecular weight)-known T-series Dextran (T-700, T-580, T-110, T-80, T-40, T-11) from Sigma (USA).

Monosaccharide Composition Analysis. A 2 mg portion of GFPBW2 was hydrolyzed in 2 M TFA at 110 °C for 2 h in a sealed test tube. After evaporation to completely remove the TFA, the resultant monosaccharides were converted into alditol acetates as described previously[15] and then analyzed by GC.

NMR Spectroscopy. For NMR analysis, the sample (35 mg) was dissolved in 0.5 mL of D_2O (99.8% D), and ^{13}C NMR, ^{1}H NMR, heteronuclear single quantum coherence (HSQC), and heteronuclear single quantum coherence (HMBC) spectra were obtained at room temperature on a Bruker AM-400 NMR spectrometer. All chemical shifts were referenced to Me_4Si (TMS).

Methylation Analysis. GFPBW2 (10 mg) was dissolved in 2.0 mL of DMSO before 20 mg of powdered NaOH was added. The mixture was then placed in an ultrasonic bath and treated with an ultrasonic wave for 5 min, followed by cooling in ice−water. Iodomethane (0.3 mL) was added over 15 min. The mixture was kept for 30 min before 2 mL of distilled water was added to stop the reaction. Then the mixture was dialyzed against deionized water for 24 h, concentrated, and extracted three times with 1 volume of chloroform, followed by treatment with anhydrous Na_2SO_4 and drying with an N_2 stream. After the complete methylation was confirmed by the disappearance of OH bands (3200−3700 cm^{-1}) in the IR spectrum, the methylated polysaccharide was depolymerized and converted into partially methylated alditol acetates and analyzed by gas chromatography mass spectra (GC-MS).[15]

Partial Acid Hydrolysis. GFPBW2 (100 mg) was dissolved in 0.1 M TFA (20 mL) and kept at 100 °C for 1 h. After evaporation to remove TFA, the residue was dissolved in water and dialyzed. The retentate was lyophilized and then analyzed by HPGPC, while the dialysate was concentrated and analyzed by ESI-MS.

Smith Degradation Analysis. GFPBW2 (50 mg) was dissolved in 0.02 M $NaIO_4$ (30 mL) and kept in the dark at 4 °C. The absorption at 224 nm was determined each day. After the oxidation was complete, ethylene glycol (1 mL) was added, and the mixture was stirred for 0.5 h. The solution was dialyzed against distilled water, and the nondialysate was reduced with $NaBH_4$ (100 mg) at 4 °C for 12 h. After the pH was adjusted to 5.0 with 50% HOAc, the solution was

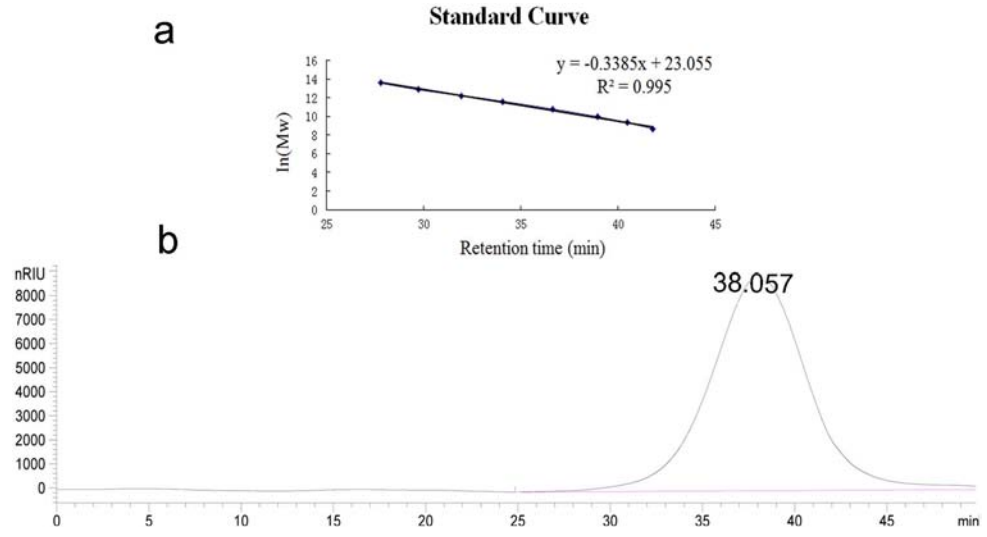

Figure 1. HPGPC chromatogram of GFPBW2 (a) and standard curve between retention volume and MW (b).

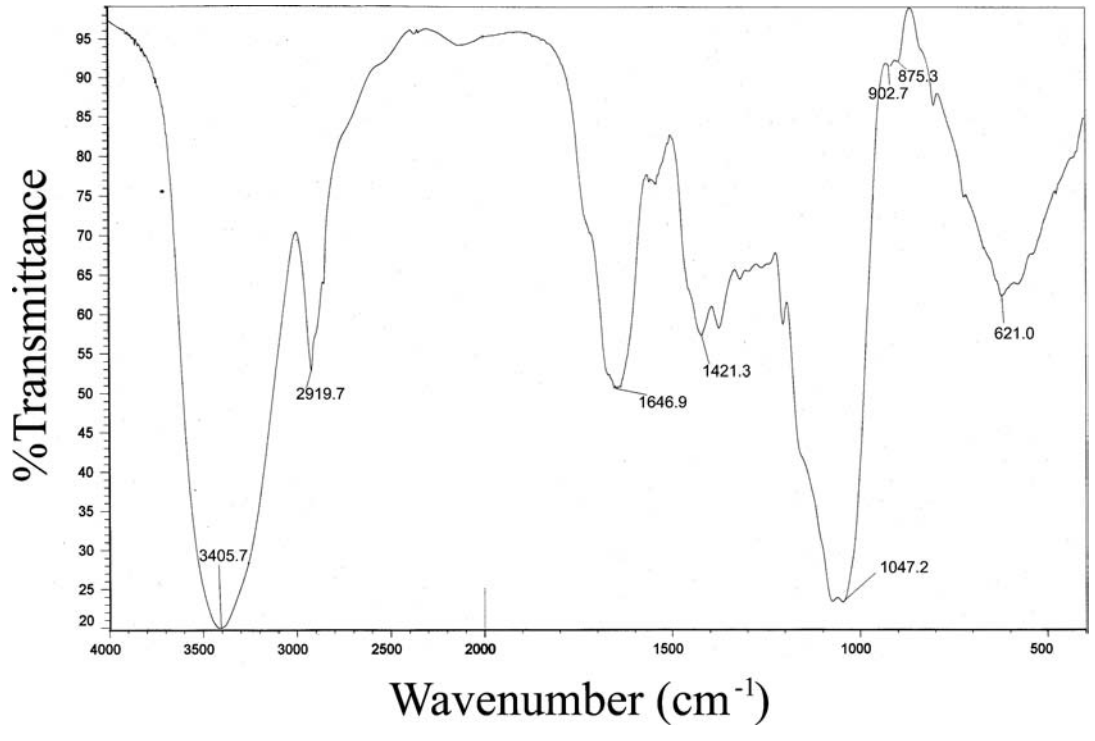

Figure 2. IR spectrum of GFPBW2.

dialyzed against distilled water to give the retentate, which was lyophilized and then hydrolyzed with 0.2 M TFA at 40 °C for 24 h. The hydrolysate was dialyzed, and the nondialysate portion was lyophilized and subjected to analysis by HPGPC and methylation analysis.

Cell Culture and Growth Conditions. All the cells were cultured in media supplied with 10% FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. RAW264.7 macrophages obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China) were cultured in DMEM medium. Resident peritoneal macrophages from ICR mice were isolated by peritoneal lavage as described previously[15] and maintained in RPMI 1640 medium. After being precultured overnight, the prepared resident peritoneal macrophages were used for the experiments. RAW264.7 cells stably expressing wild type (WT), active site mutant (Y15S), and truncated (Δ38) Dectin-1 tagged with a Myc-His epitope at the COOH terminus were obtained according to the method described previously,[15] while RAW264.7 cells infected with lentiviral particles bearing control vector pLVX-IRES-ZsGreen1 were used as mock control (ZSG).

QCM Analysis. The binding ability of GFPBW2 to Dectin-1 was examined by QCM analysis. Briefly, recombinant mouse Dectin-1/CLEC7A (R&D, USA) was biotinylated with NHS-PEG_{12}-Biotin (Thermo Scientific, Rockford, IL, USA) for 30 min at room temperature according to the manufacturer's instructions, followed by purification with a desalting column. Then biosensor experiments were carried out on an Attana A200 QCM instrument (Attana AB, Stockholm, Sweden) to study the binding capability of GFPBW2 to the recombinant Dectin-1. First, the Attana biotin sensor surfaces were mounted in the QCM system and equilibrated with phosphate buffer solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.005% Tween 20, pH 7.4). Subsequently, the streptavidin solution (100 μg/mL) was injected, and then the biotinylated Dectin-1 was immobilized on the streptavidin surface to produce a Dectin-1

biosensor surface. Laminarin (L9634-1G, Sigma, USA) and Dextran (31389-25 G, Sigma, USA) were also tested as control. Laminarin, a β-1,3-glucan, was proved to interact with Dectin-1. We used Laminarin as a positive control. Dextran is a α-1,6-glucan, and there was no report about the binding between Dectin-1 and Dextran. We used Dextran as a negative control. A continuous flow of running buffer at a flow rate of 25 μL/min was used throughout, and the samples were prepared in the same buffer. The frequency responses produced from the interactions were monitored by frequency logging with Attester 1.1 (Attana), where the mass changes from the bound or released ligands were recorded as the resulting frequency shifts (Δf). The K_D values of affinity for GFPBW2-Dectin-1 were calculated by Attana evaluation software.

Determination of Cytokines. The cytokine contents in the medium were determined using ELISA kits for TNF-α and IL-6, following the manufacturer's instructions. Briefly, cells were treated with various concentrations of GFPBW2 or 50 ng/mL of LPS in the absence or presence of PMB (10 μg/mL) for 4 h, followed by collection and centrifugation (500g) for 5 min. The supernatant was subjected to ELISA assay.

Western Blotting. After being treated with GFPBW2 or other reagents, cells were harvested and lysed by the addition of SDS-PAGE loading buffer. Then the lysate supernatant was resolved by SDS-PAGE and incubated with specific antibodies. After three washes, secondary antibodies were applied and blots were developed.

Statistical Analysis. All data are expressed as means ±SEM. Statistical analysis was performed with GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) using one-way ANOVA. A p value of less than 0.05 was taken as significant.

RESULTS

Isolation and Structural Elucidation of GFPBW2. The alkali-extracted crude polysaccharide GFPB (10 g) dissolved in distilled water (100 mL) was applied to a DEAE−cellulose column and eluted stepwise with water and 0.2 M NaCl to give GFPBW1 (300 mg, 3%) and GFPBW2 (120 mg, 1.2%) (Supplemental Figure 1, Supporting Information). The homogeneity of GFPBW2 was estimated by HPGPC, which showed a symmetric peak (Figure 1b). The mean molecular mass of GFPBW2 was estimated to be 26.2 kDa according to the standard curve (Figure 1a). Monosaccharide composition analysis indicated that GFPBW2 was mainly composed of D-glucose. The specific rotation was −11° (c 2.5, H_2O). The IR spectrum of the GFPBW2 exhibited an absorption peak at 902.7 cm^{-1}, which is characteristic of the β anomeric configuration,[15] while the absorption at 1047.2 cm^{-1} was typical for the pyranose form (Figure 2).

Next, GFPBW2 was subjected to methylation analysis to determine the glycosyl linkage types. The methylated polysaccharide was depolymerized and converted into partially methylated alditol acetates followed by GC-MS analysis. The results showed that the linkage type of GFPBW2 included mainly terminal Glc (T-Glc) and 1,3-, 1,4-, 1,6-, and 1,3,6-linked Glc, in a molar ratio of 3:3:1:4:3 (Table 1) .

Table 1. Identities and Molar Ratios of Methylated D-Glucose in GFPBW2 and GFPBW2-Smith

		molar ratio (%)	
methyl position	linkage indicated	GFPBW2	GFPBW2-Smith
2,3,4,6-tetra	Glcp(1→	18.89	5.73
2,4,6-tri	→3)-Glcp(1→	21.98	27.16
2,3,6-tri	→4)-Glcp(1→	7.12	14.41
2,3,4-tri	→6)-Glcp(1→	30.45	10
2,4-di	→3,6)-Glcp(1→	19.69	32.91

To ascertain more detailed structural features, GFPBW2 (100 mg) was partially hydrolyzed with 0.1 M TFA. After partial degradation, GFPBW2 was separated into a nondialysate fraction named GFPBW2-de and dialysate fraction named GFPBW2-O. GFPBW2-de was shown to be a homogeneous polysaccharide (molecular weight 17.7 kD) evaluated by HPGPC (data not shown). In comparison with the native glucan, the MW of the glucan decreased. An ESI-MS analysis of GFPBW2-O showed that the molecular weight of GFPBW2-O was below 1000 Da. It contained abundant trisaccharide according to the m/z at 520.8 $[M + H_2O - H]^-$ (Supplemental Figure 2, Supporting Information). Therefore, it indicated the presence of short chains of glucosyl units in the native polysaccharide. HPGPC analysis of this oligosaccharide mixture also showed that it contained trisaccharide, disaccharide, and monosaccharide (data not shown).

In order to determine whether GFPBW2 was a 1,3-linked or 1,6-linked glucan, periodate oxidation and Smith degradation were carried out. After Smith degradation, the derived polysaccharide (GFPBW2-Smith) was obtained as the retentate (yield 20%) with an average MW value of 8000 Da. Methylation analysis of GFPBW2-Smith showed that the percentage of 1,6-linked Glc decreased to 10% and 1,4-linked Glc increased to 14.41% (Table 1). The oxidized residues would be hydrolyzed and completely removed after Smith degradation under mild conditions. However, the 1,3-linked backbone would remain intact.[16] Therefore, the degraded glucan (GFPBW2-Smith) with high molecular weight clearly showed that GFPBW2 might be a 1,3-linked glucan. Since the ratio of 1,4-linked glucan increased after the Smith degradation, the backbone of GFPBW2 might consist of 1,3- and 1,4-linked glucans. The methylation analysis results showed that the ratio of 1,3-linked glucosyl residues of the Smith-degraded glucan increased, which indicated that the monosaccharide side chains and (or) short chains of 1,6-linked glucosyl units may be present in GFPBW2.[17] In comparison with the ^{13}C NMR spectra of GFPBW2 (Figure 3a), in the ^{13}C NMR spectrum

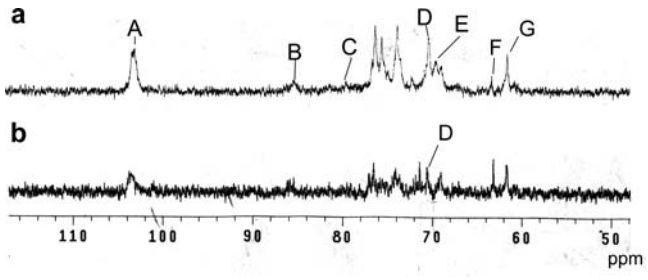

Figure 3. ^{13}C NMR spectra of GFPBW2 (a) and its Smith-degraded product GFPBW2-Smith (b): (A) anomeric carbon; (B) C-3 signal of the 1,3- and 1,3,6-linked glucosyl residues; (C) C-4 signal of the 1,4-linked glucosyl residues; (D) D-4 signal of the branched chains; (E) C-6 signal of the 1,3,6-linked glucosyl residues; (F) C-6 signal of 1,4-linked glucosyl residues; (G) C-6 signal of 1,3-linked glucosyl residues.

(Figure 3b) of GFPBW2-Smith, the intensity of the C-4 signal of side chains (peak D) and the C-6 signal of (1→3,6)-linked glucosyl residues (peak E) decreased remarkably. Hence, there was more linear structure in the degraded glucan than in the native glucan.[17] However, the increased intensity of the C-6 signal of the 1,4-linked glucan at δ 62.68 (peak F) demonstrated that the 1,4-glucosyl linkage was in the backbone.

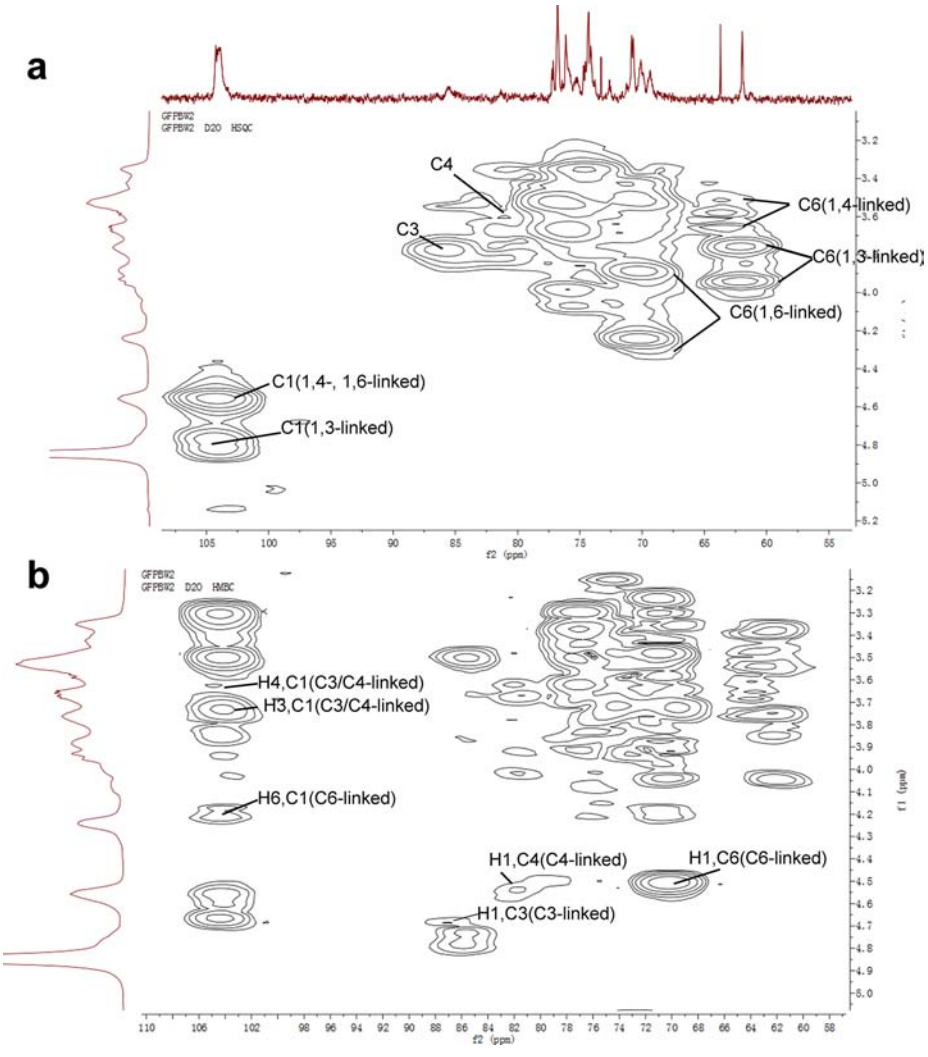

Figure 4. HSQC and HMQC spectrum of GFPBW2.

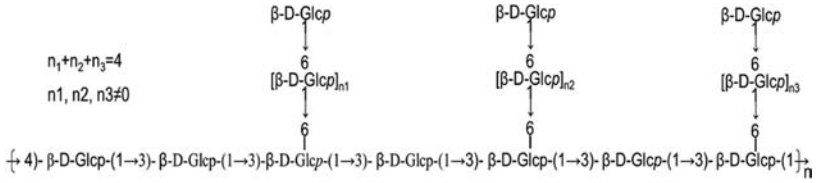

Figure 5. Putative structure of GFPBW2.

The signal at δ 85.6−86 (peak B) showed that GFPBW2-Smith was mainly a β-1,3-glucan.

It was difficult to assign all signals without any ambiguities due to the severe overlaps of signals in the ^{13}C NMR spectrum of GFPBW2 (Figure 3a). The anomeric carbon signal of Glc is at around δ 104 (peak A), which suggested that the configuration of polysaccharide was β-glucan. There was no signal at 5.2 ppm (Figure 3a) which was consistent with the result above. The signal of the O-substituted carbon atoms at δ 69.83 showed that there are branching points at C-6 (peak E). The signals of unsubstituted C-6 of 1,3-linked and 1,4-linked species were at δ 61.73 (peak G) and δ 62.68 (peak F), respectively.[18,19] The multiplicity of the signals and the broad C-3 signals in the region at δ 85.6−86 (peak B) could be ascribed to the presence of 1,3- and 1,3,6-linked glucose. The signal at 81.23 (peak C) was assigned to substituted C-4. An HMBC (Figure 4b) experiment showed that H1 (δ 4.57) of 1,4-linked Glc was transglycosidically correlated to C4 (δ 81.73) and H1 (δ 4.55) of 1,6-linked Glc was correlated to C6 (δ 70.03), while H1 (δ 4.71) of 1,3- and 1,3,6-linked Glc was correlated to C3 (δ 85.57). The cross peaks at δ 104.03/3.55, 103.91/3.78, and 104.0/4.26 were assigned to transglycosidic correlations of C1/H3, C1/H4, and C1/H6.[20] A previous article established that the cross peak at δ 104/4.2 in HSQC (Figure 4a) belonged to the signal of C1/H6, which was ascribed to the presence of the side chain of nonreducing end units of β-D-glucan substituted at O-6 at the 1,3-linked main chain.[21,22] Since there was no cross peak at δ 104/4.2 in the HSQC of GFPBW2, we deduced that the nonreducing end units of β-D-glucan were not substituted at O-6 at the 1,3-linked main chain directly. From the information above, we propose a putative structure for GFPBW2 in Figure 5.

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Inducement of Cytokine Release from Resident Peritoneal Macrophages by GFPBW2. β-Glucans from the cell walls of fungi, one of the main classes of bioactive molecules, have been shown to exhibit broad immunomodulatory effects.[4] Experiments were then carried out to evaluate the biological activities of GFPBW2. Cytokine secretion is an important characterization of activated macrophages and an effect exerted by macrophages to modulate the immune system.[23] As shown in Figure 6, GFPBW2 significantly

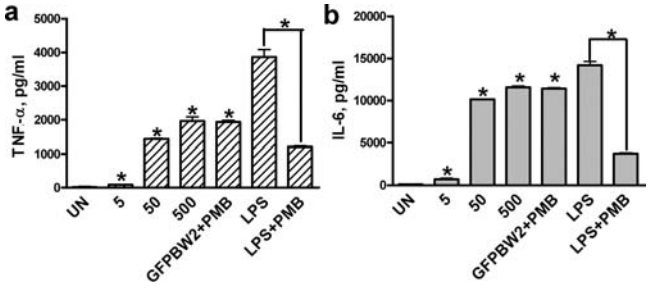

Figure 6. Cytokine secretion induced by GFPBW2 in resident peritoneal macrophages from ICR mice. Cells (2×10^5) were left untreated (UN), treated with indicated concentration of GFPBW2 (5, 50, 500 μg/mL), 500 μg/mL of GFPBW2 in the presence of PMB (10 μg/mL), or 50 ng/mL of LPS in the absence or presence of PMB (10 μg/mL) for 4 h. The supernatant was collected and subjected to determination of TNF-α (a) and IL-6 (b) by ELISA assay. Similar results were obtained in three separate experiments. Asterisks indicate $p < 0.05$ vs untreated group.

stimulated TNF-α (Figure 6a) and IL-6 (Figure 6b) secretion in a dose-dependent manner in resident peritoneal macrophages from ICR mice after the cells were treated with GFPBW2 for 4 h. To exclude the contamination of LPS in GFPBW2, PMB, an antibiotic that binds the lipid A in LPS and sequesters LPS from its receptor, toll like receptor 4 (TLR4), to block its activity, was used. Indeed, PMB could significantly inhibit the production of TNF-α and IL-6 in the resident peritoneal macrophages induced by LPS, although it could not completely block the cytokine secretion induced by LPS to the level of that in the untreated cells, which might be caused by the high dose of LPS and relatively low dose of PMB used. However, treatment of PMB had no effect on GFPBW2-timulated cytokine secretion (Figure 6), which ruled out the possibility of LPS contamination in GPBW2. These results indicated that GFPBW2 could trigger cytokine secretion in the absence of LPS. Additionally, no absorption was observed at 280 nm and the Lowry method revealed a negative response, which suggested that GFPBW2 was free of proteins (data not shown).

Possible Binding of GFPBW2 to Dectin-1 and Activation of Macrophages. Dectin-1 was an NK-cell-receptor-like C-type lectin which was preferentially expressed on the macrophages and dendritic cells. It was reported to be specifically recognized by water-soluble β-1,3-linked glucans.[24,25] Furthermore, the ability of β-1,3-linked glucan binding to Dectin-1 would be strongly influenced by its chain length and side-chain modification.[26] GFPBW2 is a water-soluble complicated β-glucan with a backbone consisting of β-1,3-linked and β-1,4-linked glucopyranosyl residues, which might influence its recognition by Dectin-1. Experiments should be done to investigate whether Dectin-1 could bind GFPBW2 and mediate the immunomodulatory effect of GFPBW2. QCM analysis was employed to study on the binding affinity of GFPBW2 to Dectin-1. As shown in Figure 7a, GFPBW2 and Laminarin (a linear β-(1,3),(1,6)-glucan) but not Dextran (an α-1,6-glucan) showed a persistent frequency response when injected into the Dectin-1-immobilized biosensor surface, suggesting that Dectin-1 could specifically recognize glucans with β but not α configuration, which was consistent with the report that Dectin-1 is a β-glucan receptor.[25] When various concentrations of GFPBW2 (100, 80, and 50 μg/mL) were mounted onto the Dectin-1-immobilized biosensor surface, further global fit analysis of the frequency response data with attana evaluation software gave the affinity constant (K_d) of GFPBW2 with Dectin-1 as 1.08×10^{-7} M (Figure 7b), and GFPBW2 binding to Dectin-1 was concentration-dependent. All of the above results indicated that GFPBW2 could directly bind to Dectin-1 and might be a potential ligand of Dectin-1.

We next examined whether Dectin-1 could mediate the biological activity of GFPBW2, when GFPBW2 was bound to Dectin-1. The cytoplasmic tail of Dectin-1 contains an immunoreceptor tyrosine-based activation motif (ITAM), which becomes tyrosine-phosphorylated after receptor engagement. Then the phosphorylated ITAM motif activates Syk kinase to induce an intracellular signaling cascade including NF-κB activation, leading to expression of cytokines.[7,27] Therefore, we used RAW264.7 cells stably expressing myc-tagged wild-type and mutant Dectin-1 to examine whether Dectin-1 could activate Syk upon stimulation with GFPBW2. As shown in Figure 7c, RAW264.7 macrophage cells stably expressing different types of myc-tagged Dectin-1 were stimulated with GFPBW2 for 30 min. Western blotting with antiphosphorylated Syk antibody revealed that Syk phosphorylation was induced by GFPBW2 only in RAW 264.7 cells overexpressing wild-type Dectin-1 (WT) but not in the cells overexpressing truncated (Δ38), active site mutant (Y15S) Dectin-1 or in the control RAW264.7 cells which were infected with lentiviral particles bearing control vector (ZSG) when stimulated by GFPBW2 (50 μg/mL).

Then we examined whether GFPBW2 could trigger macrophages activation through Dectin-1. As shown in Figure 7d, TNF-α secretion was relatively low in GFPBW2-stimulated control RAW264.7 cells (ZSG) which express trace amounts of endogenous Dectin-1,[6] and TNF-α secretion was slightly but significantly enhanced in RAW264.7 cells stably expressing wild type Dectin-1. In contrast, signaling deficient Dectin-1 (Δ38) and active site mutant Dectin-1 (Y15S) failed to enhance TNF-α secretion, although TNF-α was greatly produced in GFPBW2-treated manipulated RAW264.7 cells. These data suggested that GFPBW2 could partially employ Dectin-1 to activate macrophages, and other receptors might also be involved in the induction of cytokine secretion in macrophages by GFPBW2.

The above results suggested that GFPBW2 could be a potential ligand of Dectin-1. It could bind to Dectin-1, activate Dectin-1/Syk signaling, and then induce cytokine release in macrophages.

Possible Inhibition of the Syk/NF-κB Signaling Activation and Cytokine Secretion Stimulated by GFPBW2 by Laminarin in Resident Peritoneal Macrophages from ICR Mice. Once bound to β-glucans, Dectin-1 can stimulate cytokine production via the Syk/NF-κB signaling pathway.[7,27] Laminarin, a soluble β-glucan, has been reported to be a specific inhibitor of Dectin-1 activity induced by particulate

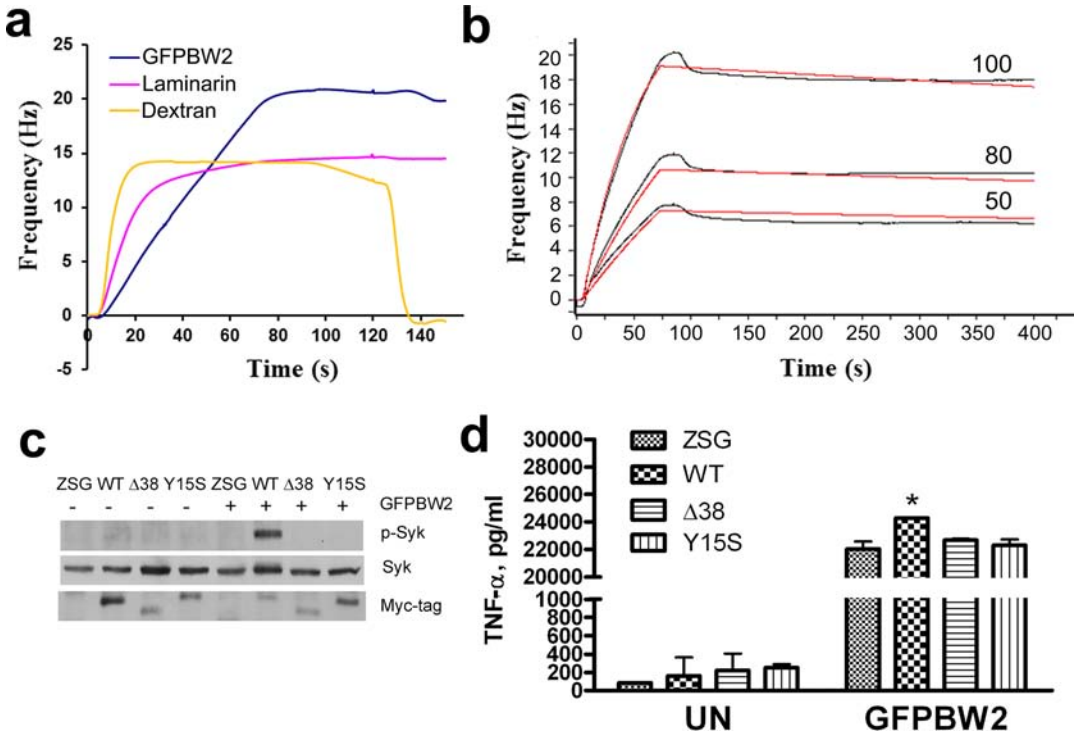

Figure 7. Involvement of Dectin-1 in GFPBW2-induced activation of macrophages. (a, b) QCM analysis of the binding ability of GFPBW2 to Dectin-1. The GFPBW2−Dectin-1 interaction was tested by injecting 100 μg/mL of GFPBW2, Laminarin, or Dextran in running buffer onto the Dectin-1 biosensor surface, and the frequency response is recorded. As controls, the frequency responses by the Laminarin (100 μg/mL) or Dextran (100 μg/mL) interaction with Dectin-1 surface were measured (a). The interactions between GFPBW2 and Dectin-1 were measured by injecting various concentrations of GFPBW2 (100, 80, 50 μg/mL) in running buffer on to the Dectin-1 biosensor surface, and the frequency responses were recorded (black curves). Then the frequency response curves were fitted to binding curves (red curves) and the affinity constant (K_d) of GFPBW2 with Dectin-1 was calculated to be 1.08×10^{-7} M using Attana evaluation software (b). (c, d). RAW264.7 cells overexpressing wild type (WT), mutant (Δ38, Y15S) Dectin-1 tagged with a Myc-His epitope at the COOH terminus or mock control (ZSG) cells were left untreated (UN) or treated with 50 μg/mL GFPBW2 (c) for 30 min followed by cell lysing. Then immunoblotting was conducted to detect phosphorylated form of Syk in the total cell lysate, and total Syk served as internal control. Meanwhile, Myc-tag antibody was used to detect the expression of the epitope fused Dectin-1 or (d) for 4 h. Then the supernatant was collected, and TNF-α content was assayed by ELISA. The asterisk indicates $p < 0.05$.

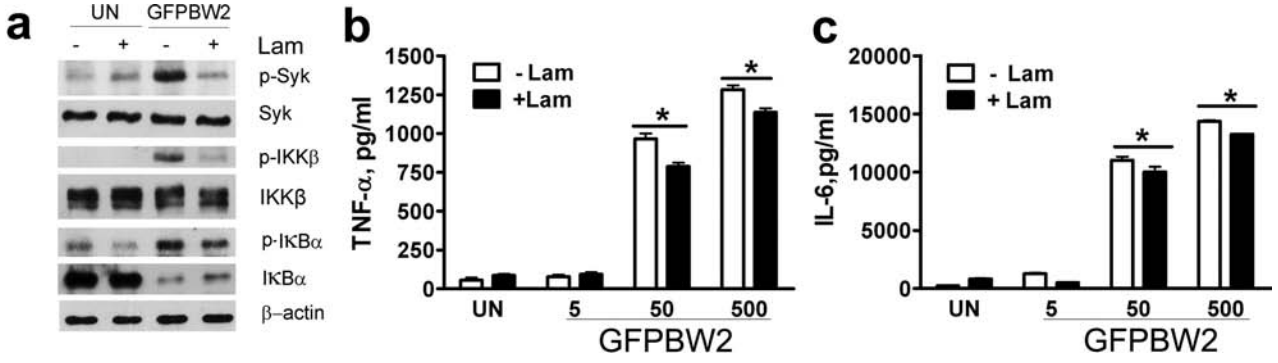

Figure 8. Laminarin could reduce activation of Syk and NF-κB signaling activation and cytokines secretion trigged by GFPBW2. (a) Lysates of resident peritoneal macrophages from ICR mice (2×10^5) treated with GFPBW2 (50 μg/mL) for 30 min after pretreatment with or without Lam (500 μg/mL) for 1 h were subjected to Western blotting analysis with antibodies against IκBα, phosphorylated forms of Syk, IKKβ, and IκBα. Total Syk, IKKβ, or β-actin served as reference, respectively. (b, c) Resident peritoneal macrophages from ICR mice (2×10^5) were left untreated (−Lam), or treated with 500 μg/mL of Lam (+Lam) 1 h prior to treatment with the indicated concentration of GFPBW2 (5, 50, 500 μg/mL) for 4 h. The amounts of TNF-α (b) and IL-6 (c) secreted into the medium were determined by ELISA assay. Similar results were obtained from three independent experiments. Asterisks indicated $p < 0.05$.

or water-soluble β-glucans such as zymosan, Curdlan, and GRN.[6,28,29] Then Laminarin was employed to further study the potential role of Dectin-1 in GFPBW2-induced activation of macrophages. Because GFPBW2 could bind to Dectin-1, we examined whether downstream Syk/NF-κB signaling was activated by GFPBW2 in primary resident peritoneal macrophage. As shown in Figure 8a, an increased level of phosphorylated Syk was observed after cells were treated with GFPBW2 (50 μg/mL) for 30 min. Meanwhile, the phosphorylation levels of IKKβ and IκBα in cells were increased when cells were treated with GFPBW2, while the total IκBα level was impaired accordingly, indicating that GFPBW2 could activate

the NF-κB signaling pathway.[30] In addition, Syk and NF-κB signaling were not significantly activated when cells were treated with Laminarin (500 μg/mL) alone. However, in comparison with that in macrophages treated with GFPBW2 alone, the phosphorylation levels of Syk, IKKβ, and IκBα were decreased, while the total IκBα level was enhanced, when cells were pretreated with Laminarin (500 μg/mL) for 1 h prior to the addition of GFPBW2 (50 μg/mL) for another 30 min (Figure 8a). Moreover, the levels of TNF-α and IL-6 secretion induced by various concentrations of GFPBW2 were also significantly reduced by Laminarin pretreatment. However, there was no apparent TNF-α and IL-6 detected when cells were treated with Laminarin alone (500 μg/mL) (Figure 8b,c), which was consistent with the previous report.[28]

Taken together, these data demonstrated that Laminarin could inhibit the activation of Syk/NF-κB signaling pathway and cytokine secretion induced by GFPBW2 in resident peritoneal macrophages from ICR mice.

DISCUSSION

In the present study, we have isolated a novel water-soluble homogeneous polysaccharide, GFPBW2, from the fruit bodies of *G. frondosa*, which was shown to bind Dectin-1 and induce cytokine production in macrophages partially through Dectin-1.

Polysaccharide extracted from *G. frondosa* mushroom has been intensively investigated because of its immunomodulatory effects.[10] However, few polysaccharides, except GRN and MD fractions, are reported to be homogeneous and their structures have been characterized. In a previous study, we obtained a homogeneous β-glucan named GFPBW1 purified from the fractions of 5% NaOH extraction with distilled water elution on a DEAE column. However, GFPBW2 was elucidated to be a β-D-1,3-, β-D-1,4-linked glucan with branches attached to *O*-6 of β-D-1,3-linked glucopyranosyl residues, while GFPBW1 is a 1,3-β-D-glucan with a single 1,6-β-D-glucosyl side branching unit on every third residue. In addition, the structure of GFPBW2 is also different from that of two β-glucans, GRN and MD fractions, mentioned above. Therefore, GFPBW2 is a novel water-soluble homogeneous β-glucan purified from the fruit bodies of *G. frondosa*.

β-Glucans have been shown to possess immunostimulatory effects and employed as BRMs to treat various diseases.[3] GFPBW2 could induce TNF-α and IL-6 secretion in resident peritoneal macrophages from ICR mice, and the immunostimulatory effect of GFPBW2 was not due to the contamination of endotoxin, suggesting that GFPBW2 could activate macrophages through inducing cytokine release. As Dectin-1 is expressed in murine resident peritoneal macrophages and is a major β-glucan receptor on macrophages,[24] we thus investigated the role of Dectin-1 in the GFPBW2-mediated activation of macrophages. Unlike indirect competitive binding experiments used by most others,[6,25,31,32] we employed QCM analysis to investigate the binding capability of GFPBW2 to Dectin-1. QCM measures a change in resonance frequency (Δ*f*) as a mass accumulates/eliminates on a quartz crystal,[33] for investigating biological interactions in real time.[34] Theoretically, the macromolecules added or removed on the sensor surface can cause alteration in the resonance frequency corresponding to the change in mass on the surface. To test this, the target molecule was first immobilized to the sensor chip. Then the interacting molecules by injection were flowing over the surface, while the interaction could be recorded in real time. The kinetic, affinity and specific data for the interaction were readout by the software. The model used for data analysis assumed a 1:1 binding between the mobilized molecule and the analyte (http://www.attana.com/wp-content/uploads/2013/01/AE07-02- KineticaAffinity_PAbs.pdf). Figure 7 clearly showed that GFPBW2 could directly bind to Dectin-1. Dectin-1 is a highly specific acceptor for glucans with a pure β-D-1,3-linked backbone structure. However, the chain length and side chain modification of β-1,3-linked glucan strongly influence its binding ability to Dectin-1. Additionally, Dectin-1 does not interact with mixed-linkage polymers.[26] Although GFPBW2 has a backbone with mixed β-1,3- and β-1,4-linkages, the sufficiently long regions of the β-1,3-linked backbone may facilitate Dectin-1 recognition. As a result, GFPBW2 could be recognized by Dectin-1, which is similar to scleroglucan.[26]

In addition, although many β-glucans can bind to Dectin-1, they could not exert their immunostimulatory activity through Dectin-1. For instance, Laminarin, the antagonist of Dectin-1, possibly due to its low molecular weight (7.7 kDa), could not significantly activate the Dectin-1-dependent signaling and induce cytokine secretion at a relatively low concentration despite its high affinity for Dectin-1.[26,28] GFPBW2 could directly bind to Dectin-1. The wild type rather than truncated (Δ38) or site mutant (Y15S) Dectin-1 could activate Syk and enhance TNF-α secretion stimulated by GFPBW2 in RAW 264.7 cells which only express trace amount of endogenous Dectin-1. These results suggested that GFPBW2 could employ Dectin-1 to activate macrophages. Additionally, Laminarin could inhibit activation of Syk and NF-κB signaling and release of TNF-α and IL-6 stimulated by GFPBW2 in murine resident peritoneal macrophages expressing a high level of Dectin-1,[24] further demonstrating the requirement of Dectin-1 in GFPBW2-mediated activation of macrophages. Hence, the above evidence showed that GFPBW2 was a potential ligand of Dectin-1, and it could activate the Dectin-1/Syk/NF-κB signaling pathway and subsequently initiate the transcription and secretion of cytokines when GFPBW2 was recognized by Dectin-1 which is expressed in murine resident macrophages.

Notably, GFPBW2 could induce the phosphorylation of Syk only in RAW264.7 cells expressing wild type Dectin-1 but not in other manipulated RAW264.7 cells (ZSG, Δ38, Y15S) (Figure 7c). Moreover, Laminarin could almost entirely block the phosphorylation of Syk and cytokine release in primary macrophages triggered by GFPBW2 (Figure 8a). These data suggested that activation of Syk by GFPBW2 is dependent on Dectin-1 and cytokine induction is Syk-dependent on Dectin-1, which is consistent with a previous report.[7] Additionally, although GFPBW2 could activate Syk only in RAW264.7 cells expressing wild-type Dectin-1, all of the manipulated RAW264.7 cells (ZSG, WT, Δ38, Y15S) could greatly produce TNF-α stimulated by GFPBW2 (Figure 7d). Furthermore, Laminarin could only partially but not completely abrogate the GFPBW2-induced NF-κB signaling (Figure 8a) and cytokine release in resident peritoneal macrophages from ICR mice (Figure 8b,c). All of the above data suggested that some other Syk-independent receptor in addition to Dectin-1 might also play a more important role in the GFPBW2-mediated activation of macrophages and it might have a crosstalk with Dectin-1/Syk/NF-κB signaling at the point of NF-κB activation. Recently, many receptors inducing immune responses through β-glucan have been identified.[4] Many receptor signalings such as TLR2 converging at NF-κB signaling have been found.[8] Future studies in our laboratory will investigate whether Dectin-1 and other receptors such as

TLR2 can collaboratively recognize GFPBW2 in mediation of NF-κB activation and the production of cytokines, just as the particulate β-glucan zymosan did.[6,35]

In summary, we showed that the novel water-soluble polysaccharide GPBW2, isolated from the fruit bodies of *Grifola frondosa*, was a potential ligand of Dectin-1 and an effective inducer of macrophage activation through triggering cytokine secretion, at least in part, via Dectin-1. Our data provided evidence that GFPBW2 might be a potential biological response modifier for antitumor therapies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures giving additional characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*J.D.: tel, +86-29-87080351; fax, +86-29-87080351; e-mail, jduan@nwsuaf.edu.cn.

*K.D.: tel, +86-21-50806928; fax, +86-21-50806928; e-mail, dingkan@simm.ac.cn.

Author Contributions

§These author contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BRMs, biological response modifiers; Dectin-1, dendritic cell-associated C-type lectin-1; GRN, Grifolan; HPGPC, high-performance gel-permeation chromatography; MW, molecular weight; NK, natural killer cells; QCM, quartz crystal microbalance; DCs, dendritic cells; NF-κB, nuclear factor κB; MD fraction, maitake D fraction; GC-MS, gas chromatagraphy mass spectrum; TLR4, toll like receptor 4; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple-bond correlation; LPS, lipopolysaccharide; PMB, Polymyxin B; Syk, spleen tyrosine kinase; TNF-α, tumor necrosis factor-α; IL-6, Interleukin-6

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