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Structure of a β-glucan from *Grifola frondosa* and its antitumor effect by activating Dectin-1/Syk/NF-κB signaling

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Abstract A soluble homogeneous β-glucan, GFPBW1, with a molecular mass of 300 kDa was purified from the fraction of the fruit bodies of *Grifola frondosa* extracted with 5 % NaOH. Using various methods, such as infrared spectroscopy, NMR, methylation and monosaccharide composition analysis, its structure was determined to be a β-D-(1-3)-linked glucan backbone with a single β-D-(1-6)-linked glucopyranosyl residue branched at C-6 on every third residue. It induced TNF-α and IL-6 production and the activation of Syk and NF-κB signaling in resident peritoneal macrophages from ICR mice, which could be significantly inhibited by the blocking reagent laminarin. A competitive phagocytosis assay with FITC-zymosan indicated that GFPBW1 could bind to DC-associated C-type lectin 1 (Dectin-1). The TNF-α secretion and activation of Syk/NF-κB signaling triggered by GFPBW1 were enhanced in RAW264.7 cells overexpressing wild but not mutant (Δ38 and Y15S) Dectin-1. Furthermore, GFPBW1 potentiated the Concanavalin A-induced proliferative response of splenocytes and inhibited Sarcoma-180 growth allografted in ICR mice but not in immunodeficient BALB/c nu/nu mice. These results suggested that the antitumor activity of GFPBW1 was partially associated with the activation of macrophages via the Dectin-1/Syk/NF-κB signaling pathway. This molecule could be a promising biological response modifier with clear application for antitumor therapies.

Keywords β-glucan · Grifola frondosa · Biological response modifier · Dectin-1

Abbreviations

BRMs	Biological response modifiers
CARD9	Caspase recruitment domain 9
ConA	Concanavalin A
CR3	Complement receptor 3
CTX	Cyclophosphamide
DCs	Dendritic cells
Dectin-1	DC-associated C-type lectin 1
DMSO	Dimethyl sulfoxide
FACS	Fluorescence-activated cell sorting
GC	Gas chromatography
G. frondosa	*Grifola frondosa* (Fr.) S. F. Gray
GRN	Grifolan
HPGPC	High performance gel permeation chromatography
HRP	Horseradish peroxidase
HSQC	Heteronuclear single quantum coherence
IR	Infrared
ITAM	Immunoreceptor tyrosine-based activation motif
LPS	Lipopolysaccharide
MS	Mass spectrometry
NF-κB	Nuclear factor κB
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
PMB	Polymyxin B
POD	Peroxidase
PRRs	Pattern recognition receptors
Syk	Spleen tyrosine kinase
TFA	Trifluoroacetic acid
TLR2	Toll like receptor 2
SDS	Sodium dodecyl sulfate

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Introduction

Polysaccharides, especially β-glucans, isolated from mushrooms are typical biological response modifiers (BRMs) and important tools for cancer immunotherapy [1]. Mushrooms have been recognized for their medicinal properties for five millennia in China. Mushrooms such as *Poria cocos* (FR.) Wolf and *Polyporus umbellatus* (Pers.) Fries were originally compiled in a compendium of material medica "Shen Nong Ben Cao Jing" around 200 BC to 200 AD, and they are still used extensively in Chinese herbal medicine today. Currently, there are three major β-glucans clinically used as BRMs for anticancer therapy in Japan: krestin, generated from the cultured mycelia biomass of *Trametes versicolor* (Turkey Tail); lentinan, generated from the fruiting bodies of *Lentinus edodes* (Shiitake); and schizophyllan, generated from the liquid cultured broth product of *Schizophyllum commune* (Split Gill). These polysaccharides may induce an antitumor effect through boosting the immune system without side effects. Moreover, they can reduce the leukopenia induced by chemotherapy [2].

Grifola frondosa (Fr.) S. F. Gray (*G. frondosa*), called maitake in Japanese, is an edible and medicinal mushroom that has been consumed in some Asian countries for thousands of years. Since its cultivation in 1981, the study of its medicinal applications has been ongoing, and the activity of polysaccharides purified from this mushroom has been highlighted [3]. Over the past three decades, many polysaccharides have been isolated from the fruit bodies of *G. frondosa* and showed antitumor activity. Most of these polysaccharides were β-glucans with different chain linkage types such as β-(1-3)-, β-(1-6)- or α-(1-4)- heteroglucans [4]. Grifolan (GRN) and MD-fraction are two polysaccharides that have been intensively investigated for antitumor activities. GRN is a (1-3)-β-D-glucan backbone with a single (1-6)-β-D-glucosyl side branching unit on every third residue isolated from the cultured fruit bodies [5], matted mycelia [6] and liquid culture supernatant [7] of *G. frondosa* [8]. It has potent antitumor and immunomodulatory activities, such as activation of the complement system and induction of cytokine production and NO synthesis from macrophages *in vitro* and *in vivo* [9]. The MD-fraction is an orally effective fraction obtained from *G. frondosa* by Namba *et al*. through a proprietary extraction procedure [10, 11]. It is a protein-bound β-glucan, consisting of β-(1-6)-linked glucose residues with β-(1-3)-linked glucose branches. MD-fraction was demonstrated to have antitumor activity via the activation of the host immune system such as activating macrophages, dendritic cells, and natural killer cells and the induction of Th-1 dominant responses [12]. The immunological effects of MD-fraction have also been demonstrated in a phase I/II clinical trial in breast cancer patients [13].

Although β-glucans from mushrooms modulate the immune system *in vivo* and some are used clinically for tumor immunotherapy in several countries [14], their cellular mechanism of action remains unclear, despite significant therapeutic implications [10]. Recently, the molecular mechanism of fungal pathogen recognition by innate immune cells, such as macrophages and DCs, has become clearer with the identification of the pattern recognition receptors (PRRs) for β-glucan, which reside in the fungal cell wall. The recognition of pathogenic fungi by PRRs, such as DC-associated C-type lectin (Dectin-1), toll like receptor 2 (TLR2), complement receptor 3 (CR3), and the mannose receptor on macrophages and DCs, triggers the innate immune response [15, 16]. Dectin-1, the major leukocyte receptor for β-(1,3)-glucans, plays a crucial role in recognizing pathogenic fungi [17] and particulate β-glucans, such as zymosan [18]. In studies using zymosan, Dectin-1 has been shown to collaborate with TLR2 to amplify TLR2-mediated NF-κB activation and cytokine production by macrophages [19]. Moreover, Dectin-1 also can directly trigger cytokine production through an immunoreceptor tyrosine-based activation motif (ITAM)-like motif in its cytoplasmic tail in bone marrow-derived dendritic cells, resident peritoneal macrophages and other specific cell types [15]. Stimulation of the receptor by the binding of β-glucans leads to the activation of spleen tyrosine kinase (Syk) and then the activation of the canonical NF-κB pathway, driving cytokine production via the caspase recruitment domain 9 (CARD9) adapter [15, 18, 20, 21].

Most of the information regarding the molecular mechanism has been generated using live fungi and particulate β-glucan, whereas few studies have examined the mechanism of purified, soluble β-glucan used as a biological response modifier to augment the host immune response [10]. Furthermore, although numerous polysaccharides have been obtained from *G. frondosa*, mainly in 1980s, few of them, except GRN, were homogeneous. The primary structures of the polysaccharides have not been unambiguously elucidated either. Novel polysaccharides are still frequently isolated, purified and evaluated [22]. In this study, we report the structure of a polysaccharide named GFPBW1, isolated from the fruit bodies of *G. frondosa*, its immunostimulatory effect and underlying molecular mechanism *in vitro*, and its immunomodulatory and antitumor activities *in vivo*.

Material and methods

Reagents and animals

Dried fruit bodies of *G. frondosa* were purchased from Dashanhe Group Company (Shanghai, China). DEAE-cellulose 32 was from Whatman (Piscataway, NJ, USA). Standard monosaccharides, sodium borohydride, dimethyl sulfoxide (DMSO), iodomethane, cyclophosphamide (CTX),

lipopolysaccharide (LPS), polymyxin B (PMB), laminarin (Lam) and Concanavalin A (ConA) were all Sigma products (St. Louis, MO, USA). Other reagents were of analytical grade unless otherwise claimed.

Female ICR and BALB/c nu/nu mice (body weight 18±1 g) purchased from Shanghai SLAC Laboratory Animal Center were maintained in a specific pathogen-free 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). Antibodies against total or phosphorylated forms of IKKβ, IκBα, and Syk were from Cell Signaling Technology (Danvers, MA, USA). The β-actin antibody used as an internal reference was from Sigma, and the anti-Myc antibody was from Abmart (Shanghai, China).

General methods

All evaporations were carried out at <45 °C under reduced pressure. Infrared spectra were determined with a Perkin-Elmer 591 B spectrophotometer using KBr pellets (native polysaccharides) or Nujol films (permethylated polysaccharides). The optical rotations were determined with a Perkin-Elmer 241 M digital polarimeter. Gas chromatography (GC) was performed on a Shimadzu GC-14 B instrument equipped with a 3 % OV-225-packed glass column (3.2 mm×2 m) and a FID detector. GC-MS was performed with a Shimadzu QP-5050A apparatus equipped with a DB-1 capillary column (0.25 mm×30 m).

Isolation and fraction of GFPBW1

The fruit bodies of *G. frondosa* (3 kg) were defatted twice (for 1 week each time) with 95 % EtOH, air dried, and extracted with boiling water four times with intermittent stirring (6 h each time). The resulting 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. Three volumes of 95 % EtOH were added to the concentrated retentate to precipitate the crude polysaccharide GFPB (50 g, yield 1.7 %). Ten grams of GFPB was dissolved in water and subjected to fractionation on a DEAE-cellulose column, which was equilibrated and eluted with distilled water to give one major fraction when monitored with phenol-sulfuric acid analysis. The fraction was pooled and lyophilized to give GFPBW1 (300 mg, 3 % GFPB). The LPS content of GFPBW1 was less than 1 ng/mg as determined by LPS-specific chromogenic Limulus Amebocyte Lysate test using Pyrochrome (Associates of Cape Cod, East Falmouth, MA, USA). GFPBW1 was dissolved in PBS at 5 mg/ml as a stock solution and diluted with medium to suitable concentrations in the bioactivity experiment.

Homogeneity and molecular weight

The homogeneity and molecular weight of the polysaccharide were estimated by high performance gel permeation chromatography (HPGPC) with a Waters HPLC module consisting of a 515 pump, a 2410 RI detector, a 2487 dual λ absorbance detector and series-connected Ultrahydrogel^{TM} 2000 and Ultrahydrogel^{TM} 500 columns. The sample solvent and eluent was 10 mM NaOH, and the flow rate was kept at 0.5 ml/min. The column temperature was kept at 30±0.1 °C. The column was calibrated using the T-series dextran standards of known molecular weights (T-700, T-580, T-110, T-80, T-40, T-11) from Sigma (USA). Data were processed by GPC software (Millennium^{32}).

Monosaccharide composition analysis

The monosaccharide composition of the polysaccharide was analyzed as described previously [23]. Briefly, the polysaccharide (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 2 ml) at 110 °C for 2 h in a sealed test tube. After evaporation to completely remove the TFA, the hydrolysate was converted into alditol acetates, followed by GC analysis.

NMR spectra

The sample (35 mg) was dissolved in 0.5 ml of DMSO-d_6. The ^{1}H-NMR, ^{13}C-NMR and heteronuclear single quantum coherence (HSQC) spectra were measured at RT on a Bruker AM-400 NMR spectrometer. All chemical shifts were referenced to Me_4Si (TMS).

Methylation analysis

Methylation of vacuum-dried polysaccharide (5 mg) with iodomethane and powdered sodium hydroxide in DMSO was carried out as described by Needs and Selvendran [24]. The completeness of methylation was confirmed by the disappearance of the hydroxyl absorption in IR (Nujol). The permethylated polysaccharide was depolymerized, reduced and derivatized into the partially methylated alditol acetates and analyzed by GC-MS [23].

Cell culture and growth condition

RAW264.7 macrophages, Sarcoma 180 (S-180), HEK293 and its variant HEK293T cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM medium (Gibco, Gaithersburg, MD, USA). Murine resident peritoneal macrophages were isolated by peritoneal lavage using prechilled PBS. The lavage fluids were pooled and centrifuged at 100× g for 5 min. After washing with HBSS buffer (Gibco), the adherent cells were enriched by plastic adherence for 2 h and cultured

in RPMI 1640 medium (Gibco) at 37 °C in 5 % CO_2 to achieve nearly pure macrophages. After being pre-cultured overnight, the prepared resident peritoneal macrophages were used for experiments. All the cells were cultured in media supplied with 10 % FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2.

Plasmids and cell transfection

The murine dectin-1 expression vector pCDNA3.1-Dectin-Myc in was constructed as described previously with some modifications [19]. In brief, the full coding region of murine Dectin-1 (GenBank ID: AF262985) was amplified from RAW 264.7 cDNA [sense primer 5′ GGGGAATTCG CCACCATGAAATATCACTCTCATATAG 3′ (EcoR I) and anti-sense primer 5′ AATTCTAGACTCAGTTCCTTC TCACAGATACGTAT 3′ (Xba I)] and subcloned into pCDNA3.1/Myc-His B (Invitrogen, Carlsbad, CA, USA), thus adding a Myc-His epitope tag to the COOH terminus of the expressed protein. Decitn-1 was expressed in HEK293 cells by transfection of pCDNA3.1-Dectin-Myc with lipofectAMINE 2000 (Invitrogen). Cells transfected with pCDNA3.1/Myc-His B were used as control (mock).

The lentivirus expression vector carrying wild-type Dectin-1 (pLVX-DectinWT) was generated by subcloning Myc-His tag fused Dectin-1 fragment from pCDNA3.1-Dectin-Myc into pLVX-IRES-ZsGreen1 (Takara). The NH-terminal 38 amino acids were replaced with a new ATG start codon (pLVX-DectinΔ38) by PCR, and tyrosine 15 was mutated to serine (pLVX-DectinY15S) using a QuickChange site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA). All three types of Dectin-1 were tagged with a Myc-His epitope at the COOH terminus. The identities of all expression vectors were confirmed by sequencing (Sangon, Shanghai, China). Lentivirus particles were produced with the expression vectors constructed above, a psPAX2 packaging plasmid and the VSV-G-encoding plasmid pMD2.G as described previously [25], followed by concentration and titration. After being infected with lentivirus particles and consecutively passaged for 2 weeks, RAW264.7 cells were enriched by fluorescence-activated cell sorting (FACS) to obtain cells expressing three types of murine Dectin-1 (WT, Δ38, and Y15S) and control ZSG. All types of Dectin-1 were verified by immunoblotting with anti-Myc antibody.

Immunoblotting

Cells were harvested and lysed with SDS-PAGE sample loading buffer (50 mM Tris, pH 6.8, 2 % SDS, 1 % β-mercaptoethanol, 10 % glycerol, 0.1 % bromophenol blue) followed by denaturation at 95 °C for 10 min. The lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (PALL, Ann Arbor, MI, USA). After blocking with 5 % nonfat milk in TBST buffer [20 mM Tris, pH 8.0, 150 mM NaCl and 0.1 % Tween-20], the membranes were incubated with a specific antibody at 4 °C overnight. The membranes were washed with TBST and incubated with the appropriate HRP-conjugated secondary antibody (Jackson, West Grove, PA, USA) for 1 h at RT. After being washed again, the bands were detected by SuperSignal West Dura Substrate (ThermoScientific, Rockford, IL, USA) according to manufacturer's instructions.

Immunodetection of cytokines

Cytokine contents were determined using sandwich ELISA kits for TNF-α and IL-6, following the manufacturers' instructions (R&D systems, Minneapolis, MN, USA). Briefly, cells were treated by compounds or LPS for 4 h, followed by collection and centrifugation (500 x g) for 5 min. The supernatant was subjected to ELISA assays.

Phagocytosis assay

The flow cytometry assay of FITC-zymosan phagocytosis was performed as described previously with minor modification [26]. Briefly, after washing three times with pre-chilled PBS, cells were pretreated with GFPBW1 or Lam at 37 °C for 30 min. Then, FITC-zymosan particles (Invitrogen) were fed to cells at a ratio of cell: particle of 1: 25 for 1 h at 37 °C. Subsequently, cells were washed with pre-chilled PBS four times, followed by digestion with trypsin EDTA and centrifugation at 500× g for 10 min. After being fixed with 1 % formaldehyde at RT for 10 min, cells were resuspended in PBS and subjected to analysis with a FACS Calibur (BD Bioscience, Bedford, MA, USA).

Splenocytes proliferation assay

Splenocyte proliferation was quantified using an ELISA BrdU colorimetric kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Briefly, after *i.p.* administration of normal saline or GFPBW1 for 10 days, the spleen was dissected from ICR mice and strained with a 100-mesh sieve. After centrifugation and elimination of erythrocytes using low osmotic Gey's solution (0.15 M NH_4Cl, 10 mM $KHCO_3$), 5×10^{5} splenocytes were transferred into 96-well plates (4 wells per sample) and stimulated with 5 μg/ml ConA for 48 h at 37 °C followed by the addition of BrdU for another 12 h. Cells were then collected, dried, fixed, denatured and incubated with anti-BrdU-POD antibody for 90 min. The BrdU incorporation was measured at 450 nm with a NOVOstar Microplate Reader (BMG Labtech, Offenburg, Germany).

Tumor inhibition assay

Sarcoma 180 cells that were maintained serially in ascite form by weekly passage in ICR mice were harvested and

suspended in normal saline to obtain a cell density of 2×10^7 cells/ml followed by subcutaneous (*s.c.*) injection into the right groin of ICR or BALB/c nu/nu mice (day 0). Beginning the next day, vehicle (normal saline), GFPBW1 or CTX was injected *i.p.* once daily for successive 10 days. On day 11, all mice were sacrificed. The tumors and spleens were dissected out and weighed. The splenic index was calculated as follows: Splenic Index = spleen weight (mg)/body weight (g).

Statistical analysis

All data were managed with software GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) and expressed as the mean ± SEM. The significance (p value) of difference was evaluated according to an unpaired student's t-test for comparison of two groups or one-way ANOVA analysis method in multiple comparisons. Values of $p<0.05$ were considered statistically significant.

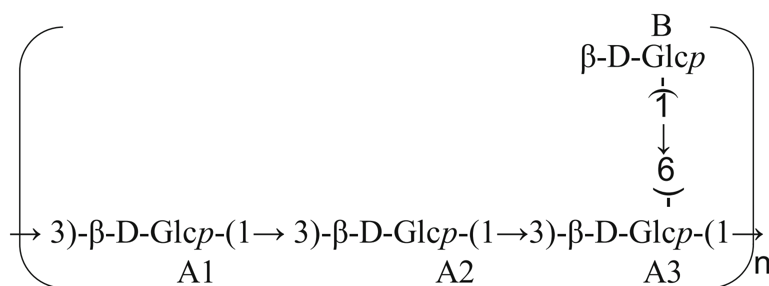
Results

Isolation and structural characterization of GFPBW1

GFPBW1 (3 % of the alkali-extracted crude polysaccharide GFPB) was isolated by anion-exchange chromatography on a DEAE cellulose column and eluted with water from the alkali-extracted crude polysaccharide obtained from the fruit bodies of *G. frondosa* with 5 % NaOH extraction followed by alcohol precipitation. The homogeneity of GFPBW1 was estimated by HPGPC with 10 mM NaOH as the mobile phase, which showed one symmetrical peak (data not shown). The mean molecular mass of GFPBW1 was estimated to be 3.0×10^5 Da, and the specific rotation was +23° (*c* 2.5, H_2O). When GFPBW1 was redissolved in water and precipitated with different concentrations of ethanol (30 %, 40 %, and 60 %, respectively), each of the resulting polysaccharides was shown to have almost the same specific rotation as that of GFPBW1 (data not shown). This result also confirmed the homogeneity of GFPBW1. After complete hydrolysis with 2 M TFA, GFPBW1 was shown to contain no detectable uronic acid by thin layer chromatography (TLC) (data not shown). The monosaccharide composition analysis indicated that GFPBW1 was composed only of glucose. The IR spectrum of GFPBW1 (Fig. 1) showed absorption at 902 cm^{-1}, which is a characteristic of the β anomeric configuration [27]. The absorptions at 1076.1 cm^{-1} and 1039.0 cm^{-1} were typical for the pyranose form of the glucose residue, while the absorptions at 3425.0, 2921.7 and 1639.2 cm^{-1} were due to the hydroxyl stretching vibration, C-H stretching vibration and associated water, respectively [28]. No absorption at approximately 850 cm^{-1} suggested that GFPBW1 most likely does not contain an α anomeric configuration. The above results indicated that GFPBW1 might be a β-D-glucan.

To determine the glycosidic linkage types, GFPBW1 was subjected to methylation analysis. The results showed that GFPBW1 consisted of terminal Glc*p* (T-Glc), 1, 3- linked Glc*p* and 1, 3, 6-linked Glc*p*, in a molecular ratio of 1 : 1.8 : 1 (data not shown). This result was similar to that reported by Ohno [7]. ^{13}C NMR (Fig. 2a) and HSQC (Fig. 2B) spectra analysis were carried out to better characterize the structure of GFPBW1. In the anomeric signal area, δ 103.69 in the ^{13}C-NMR spectrum is characteristic of a β-glycosidic linkage [29] (Fig. 2a). No resonance around δ 175 further indicated that GFPBW1 did not contain uronic acid (Fig. 2a). To assign the ^{13}C NMR signals, we compared the result of ^{13}C-NMR of GFPBW1 with those reported by Tada [8, 30] and Dong [23]. The signals at δ 87.72, δ 86.86 and δ 86.38 arose from the substituted C-3 of glucose. The signal at δ 69.36 overlapped with the neighboring C-4 signal at δ 69.41, and was assigned to substituted C-6 of glucose, indicating that the branches were attached to O-6. Other signals were assigned according to the values reported in the literature [8] as shown in Table 1. The ^{1}H NMR resonances were assigned with the help of the HSQC spectrum (Fig. 2b). The anomeric signals at δ 4.2 and δ 4.6 in the ^{1}H NMR spectrum of GFPBW1 showed that the glucose residues adopt β-configuration [29], which is in good agreement with the results of IR and ^{13}C NMR data.

Taken together, the putative structure of GFPBW1 is suggested to be the following:



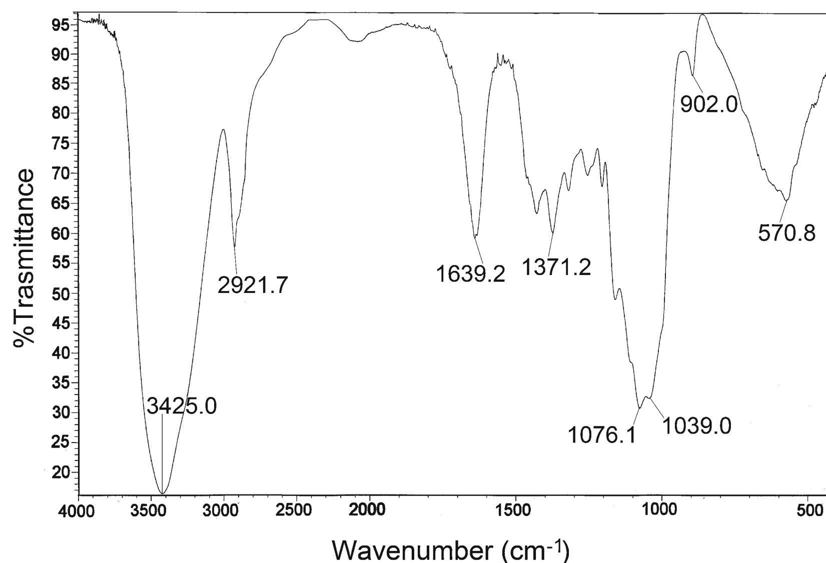


Fig. 1 IR spectrum of GFPBW1

GFPBW1 triggers cytokines release in resident peritoneal macrophages from ICR mice

Cytokine secretion is an important characterization of activated macrophages, which modulate the immune system [31]. To evaluate the biological activity of GFPBW1, peritoneal macrophages were treated with the polysaccharide at different concentrations (5, 50, 500 μg/ml) for 4 h. As shown in Fig. 3, GFPBW1 significantly induced dose-dependent TNF-α (Fig. 3a) and IL-6 (Fig. 3b) secretion compared with untreated cells. To exclude LPS contamination, PMB, which binds with lipid A in LPS to block LPS activity, was used. Indeed, LPS induced TNF-α (Fig. 3a) and IL-6 (Fig. 3b) secretion, though this induction could be blocked by PMB. However, the cytokine secretion induced by GFPBW1 was not inhibited by PMB (Fig. 3a and B). These results suggested that GFPBW1 could induce cytokine secretion in the absence of LPS.

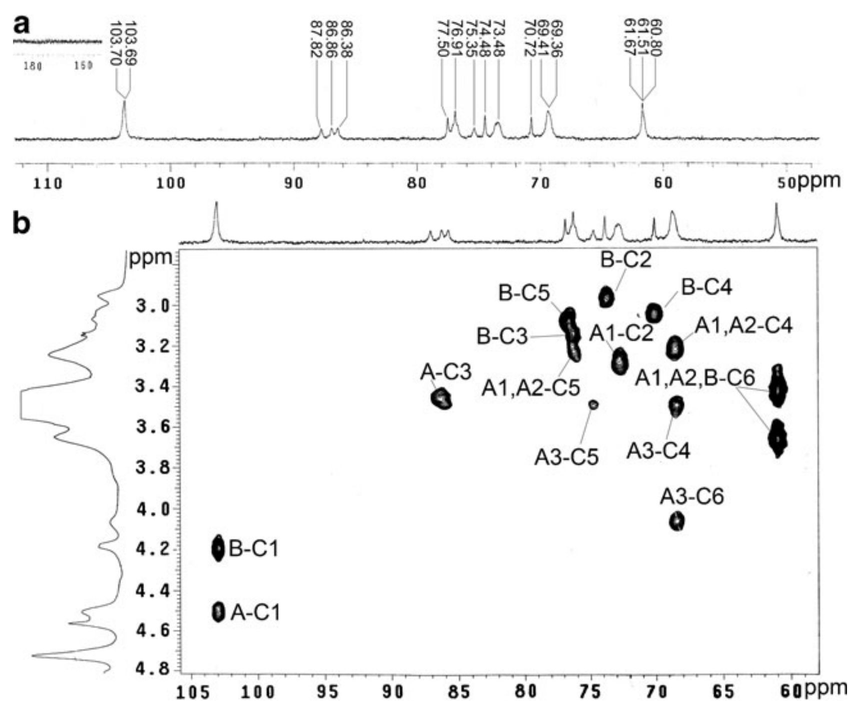


Fig. 2 ^{13}C-NMR (**a**) and HSQC (**b**) spectra of GFPBW1. A1, A2, A3, and B refer to the residues at different positions in the proposed structure of GFPBW1, while A includes A1, A2 and A3

Table 1 ^{13}C NMR chemical shift assignments of the polysaccharide GFPBW1[a]

Sugar residue	C1	C2	C3	C4	C5	C6
→3)-β-D-Glcp-(1→A1	103.70	73.63	87.72	69.36	76.89	61.51
→3)-β-D-Glcp-(1→A2	103.70	73.48	86.86	69.31	76.91	60.80
→3,6)-β-D-Glcp-(1→A3	103.70	73.83	86.38	69.38	75.35	69.41
β-D-Glcp-(1→B	103.69	74.48	76.72	70.72	77.50	61.67

[a] A1, A2, A3, B refer to the position in the structure of GFPBW1.

Blockade of the β-glucan receptor on macrophages reduces GFPBW1 trigged cytokines production and activation of Syk and NF-κB signaling

To further study the potential role of the β-glucan receptor in GFPBW-triggered cytokine production, resident peritoneal macrophages prepared from ICR mice were pretreated for 1 h with the soluble glucan laminarin, a β-glucan receptor-blocking reagent and specific inhibitor of Dectin-1 activity [19, 32], and then exposed to different concentrations of GFPBW1 for another 4 h *in vitro*. Cytokine production was then measured in culture supernatants. Although laminarin could not trigger the release of TNF-α, the TNF-α production induced by different concentrations of GFPBW1 was significantly reduced by laminarin treatment (Fig. 4a). A similar inhibition by laminarin was also observed for IL-6 (Fig. 4b). These results indicated that the TNF-α and IL-6 production in resident peritoneal macrophages that is triggered by GFPBW1 could be blocked by laminarin.

Dectin-1 is a C-type lectin-like receptor that has been reported to be a major receptor for β-glucan expressed on macrophages. It plays an important role in the induction of inflammatory cytokines by β-(1-3)-linked D-glucan [16, 33]. The attenuation of TNF-α and IL-6 production by GFPBW1 by laminarin suggested that a β-glucan receptor, likely Dectin-1, might be involved in the production of cytokines. Therefore, we examined whether Syk/NF-κB signaling was activated by GFPBW1. As shown in Fig. 4c, an increase of phosphorylated Syk was detected after primary resident macrophages were treated with GFPBW1 (50 μg/ml) for 30 min. While the phosphorylation of IKKβ and IκBα were enhanced by the polysaccharide, the total IκBα decreased accordingly, suggesting that GFPBW1 activates the NF-κB signaling pathway [34]. Furthermore, Lam (500 μg/ml) alone could not activate Syk and NF-κB signaling. However, the phosphorylation of Syk, IKKβ and IκBα were impaired, while the total IκBα was increased compared with that in macrophages treated with GFPBW1 alone. Treatment with Lam (500 μg/ml) was applied for 1 h before the addition of GFPBW1 (50 μg/ml) for another 30 min. These data demonstrated that laminarin inhibits the Syk/NF-κB signaling pathway activation stimulated by GFPBW1 in resident peritoneal macrophages from ICR mice.

The above results suggested that the cytokine production induced by GFPBW1 in resident peritoneal macrophages might be mediated through the polysaccharide binding to Dectin-1 and activating Syk/NF-κB signaling.

Dectin-1 could bind and promote GFPBW1 triggered TNF-α production

Because our glucan inhibition experiments suggested Dectin-1 involvement in the resident peritoneal macrophage response to GFPBW1, we next examined whether Dectin-1 could bind GFPBW2. Although Dectin-1 is a specific receptor for β-(1-3)-linked D-glucans, it does not recognize all glucans equally. Accumulated evidence indicated that the chain length and side-chain motif of β-(1-3)-linked D-glucan strongly influence its binding capacity to Dectin-1[32]. GRN has been reported to be capable of binding to

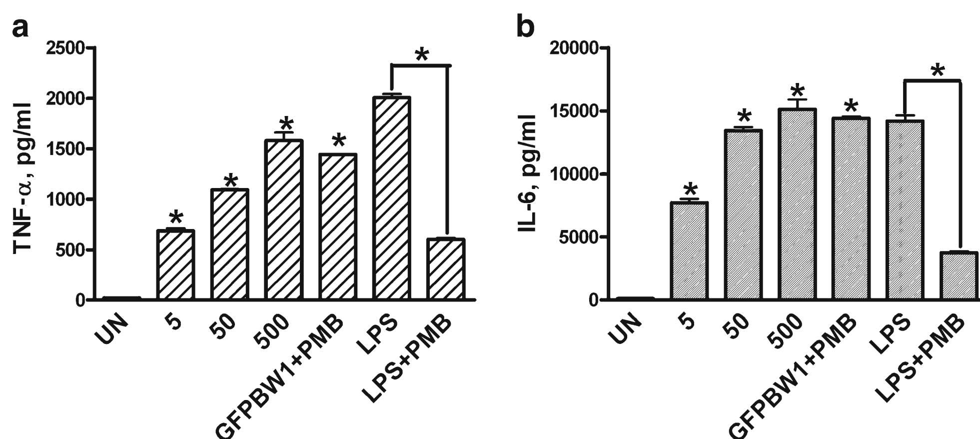


Fig. 3 Cytokine secretion induced by GFPBW1 in resident peritoneal macrophages from ICR mice. Cells (2×10^5) were left untreated (UN), treated with various concentrations of GFPBW1 (5, 50, 500 μg/ml), 500 μg/ml GSG in the presence of 10 μg/ml PMB, or 50 ng/ml LPS in the absence or presence of 10 μg/ml PMB for 4 h. Induction of TNF-α (**a**) and IL-6 (**b**) in the medium was assayed by ELISA. *, $p<0.05$ *vs.* untreated group

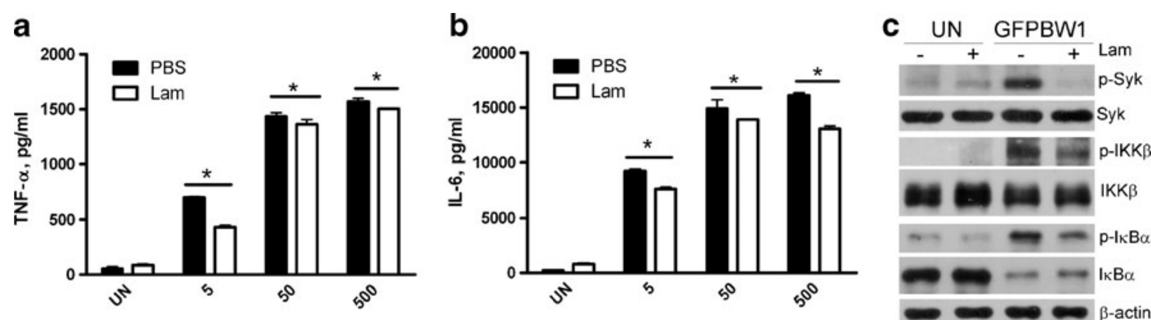


Fig. 4 Laminarin reduced GFPBW1-triggered TNF-α and IL-6 production and activation of Syk and NF-κB signaling. **a**–**b**) Resident peritoneal macrophages from ICR mice were left untreated (PBS) or pretreated with 500 μg/ml laminarin (Lam) 1 h prior to treatment with the indicated concentrations of GFPBW1 (5, 50, 500 μg/ml) for 4 h. The amount of TNF-α (**a**) and IL-6 (**b**) secreted to medium was determined by ELISA. *, $p<0.05$. **c** Lysates of resident peritoneal macrophages from ICR mice stimulated with GFPBW1 (50 μg/ml) for 30 min after pretreatment with or without Lam (500 μg/ml) for 1 h were subjected to immunoblot analysis and probed with antibodies for the phosphorylated (p-) forms of Syk, IKKβ, IκBα and expression of IκBα. Total Syk, IKKβ or β-actin served as references, respectively

Dectin-1 in a competitive binding assay [35]. Although the repeating unit is the same as GRN, as reported by Ohno [8], the molecular weight of GFPBW1 was lower than that of GRN (3×10^{5}Da in 10 mM NaOH *vs.* 2×10^{6}Da in 0.2 M NaOH) [5]. This difference may lead to a significant discrepancy in the ability of these two glucans to bind to Dectin-1. We tested whether Dectin-1 could bind GFPBW1 using a competitive phagocytosis assay. As particulate β-glucan zymosan bound to Dectin-1, it could be internalized through activating an ITAM-like motif in the Dectin-1 [36]. We assessed the ability of GFPBW1 to inhibit Dectin-1 from binding zymosan with the FACS technique. As shown in Fig. 5a, HEK293 cells transiently transfected with Dectin-1 rather than the vector (mock) significantly bound and internalized FITC-zymosan (34.21 % *vs.* 1.47 %), suggesting that internalization of FITC-zymosan was specific to Dectin-1 receptor. However, when preincubated with GFPBW1 (500 μg/ml), the amount of cells with phagocytosed FITC-zymosan was reduced to 17.51 %, while it decreased to 14.96 % with laminarin, which was used as a positive control. These results showed that GFPBW1 could block the internalization of FITC-zymosan binding to Dectin-1 just as Lam did. They also provided indirect evidence suggesting that GFPBW1 might bind to Dectin-1.

Dectin-1 is expressed in murine resident peritoneal macrophages [33], but only a trace amount of Dectin-1 is expressed in RAW264.7 cells [19]. Furthermore, the membrane-proximal tyrosine (Tyr15) in the cytoplasmic domain of Dectin-1 plays a critical role in the activation of Dectin-1/Syk/NF-κB signaling by β-glucans [18]. We constructed signal domain-deficient and active site mutant Dectin-1 expression vectors to examine whether overexpression of Dectin-1 could enhance cytokine secretion and the activation of Syk/NF-κB signaling. As shown in Fig. 5b, TNF-α secretion was relatively low in GFPBW1-stimulated control RAW264.7 cells (ZSG), but this secretion was enhanced in RAW264.7 cells stably expressing wild-type Dectin-1 (WT). In contrast, overexpression of the mutant Dectin-1 (Δ38 and Y15S) failed to enhance TNF-α secretion (Fig. 5b). Furthermore, Syk phosphorylation was induced by GFPBW1 in RAW 264.7 cells overexpressing wild-type Dectin-1 but not in the cells overexpressing mutant Dectin-1 when stimulated by GFPBW1 (50 μg/ml), although phosphorylated IKKβ and IκBα and decreased total IκBα were observed in all manipulated RAW264.7 cells (Fig. 5c). However, the relative levels of phosphorylation of IKKβ and IκBα were enhanced, and total IκBα was relatively weaker in cells expressing wild-type Dectin-1 (WT) compared with other RAW264.7 cells without expressing (ZSG) or expressing truncated (Δ38) and site-mutated (Y15S) Dectin-1 (Fig. 5c). These data suggested that GFPBW1 could employ Dectin-1 to activate macrophages through Syk/NF-κB signaling and further demonstrated the role of Dectin-1 in the activation of macrophages by the secretion of cytokines induced by GFPBW1.

GFPBW1 has immunostimulatory and anti-tumor activities *in vivo*

GFPBW1 is a β-(1-3)- linked glucan with a β-(1-6) linked terminal glucose residue. Evidence has suggested that β -glucans containing a β-(1-3) linked backbone with a β -(1-6)-linked glucan side chain usually have antitumor effect through activating the immune system [16]. Therefore, it is reasonable to ask whether GFPBW1 also has immunostimulatory activity that could impair tumor cell growth. To address this question, the immunological bioactivity of GFPBW1 was first investigated by a splenocyte proliferation assay *in vivo*. As shown in Fig. 6a, the ConA-induced proliferative response of splenocytes from mice intraperitoneally treated with GFPBW1 (0.2, 1 and 5 mg/kg) were significantly increased compared with that from vehicle-treated mice after 10 days of treatment. These results suggested that GFPBW1 might activate the immune system *in vivo*. Second, to evaluate the antitumor activity

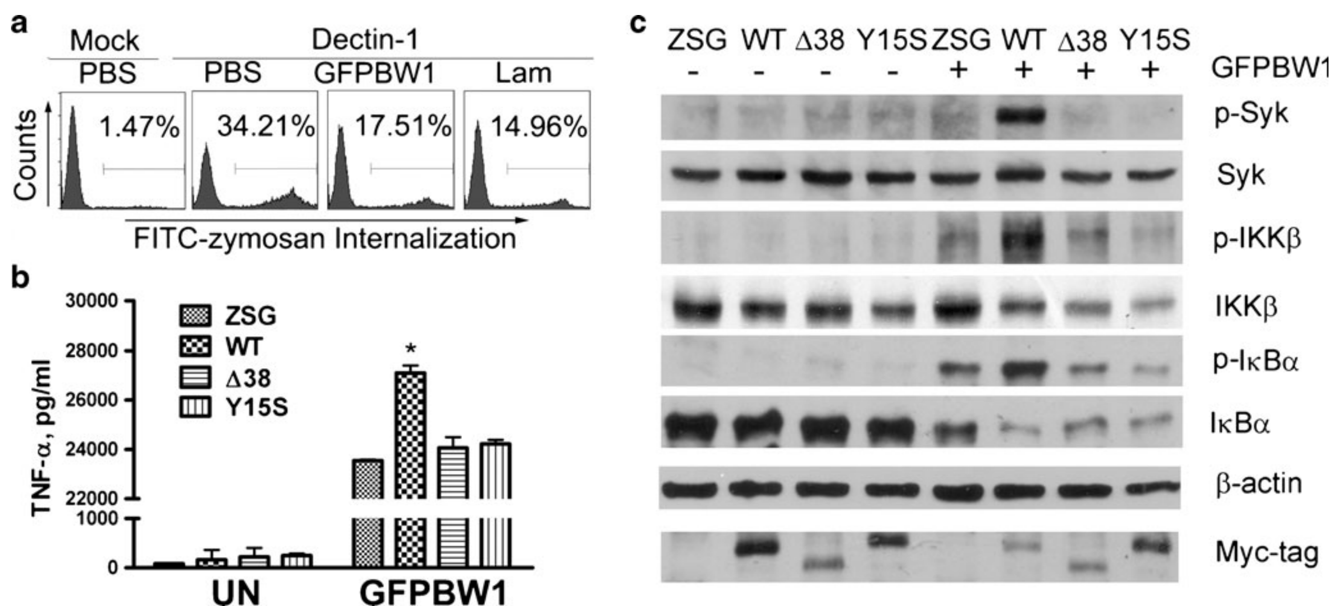


Fig. 5 Dectin-1 mediates the macrophage activation triggered by GFPBW1. **a** HEK293 cells transiently transfected with pCDNA3.1/myc-HisB (Mock) or Dectin-1 expression vector pCDNA3.1-Dectin-Myc were preincubated with PBS, GFPBW1 (500 μg/ml) or Lam (500 μg/ml) for 30 min and then incubated with FITC-zymosan for 1 h. Phagocytosis of FITC-zymosan by cells was assessed by FACS. Data shown were taken from one of three representative experiments. **b** RAW264.7 cells infected with lentivirus packed with mock (ZSG), wild-type (WT), truncated (Δ38) or mutant (Y15S) Dectin-1 expression vectors were left unstimulated (UN) or stimulated with 50 μg/ml GFPBW1 for 4 h. The TNF-α content in the medium was assayed by ELISA. *, $p<0.05$. **c** RAW264.7 cells (ZSG, WT, Δ38, Y15S) were left unstimulated (UN) or stimulated with 50 μg/ml GFPBW1 for 30 min. The cell lysates were subjected to an immunoblotting assay to detect phosphorylated (p-) forms of Syk, IKKβ, IκBα and expression of IκBα. Total Syk, IKKβ or β-actin served as references, respectively

of GFPBW1, sarcoma 180 cells allografted into ICR mice were employed. As shown in Fig. 6b, S-180 tumor growth was significantly inhibited by GFPBW1 (0.2, 1 and 5 mg/kg once daily intraperitoneally) accompanying an increased splenic index in a dose-dependent manner after the tumor was transplanted. The activity of GFPBW1 at 5 mg/kg was comparable to that of the positive control CTX at 30 mg/kg, which decreased the splenic index of the mice (Fig. 6c). However, GFPBW1 did not mediate tumor regression and had no significant change in splenic index in immunodeficient nude mice that were athymic, whereas CTX inhibited tumor growth and induced a decreased splenic index (Fig. 6d, e), which suggested that a complete host immune system was required for GFPBW1 to mediate tumor regression *in vivo*. Furthermore, the body weight of mice treated with different doses of GFPBW1 had no significant changes compared with that of the vehicle control, suggesting this polysaccharide had no apparent toxicity in mice (data not shown). All the results *in vivo* support the hypothesis that the antitumor activities of GFPBW1 are dependent on the stimulation of host defense responses.

Discussion

The healing and immunostimulating properties of mushrooms have been known for thousands of years. The extracts of these mushrooms are widely used for treatment purposes in Eastern countries [15]. Bioactive constituents derived from medicinal mushrooms are a potentially important new source of immunomodulatory agents [37, 38]. The fruit bodies of *G. frondosa* contain a large amount of bioactive substances and many polysaccharides, some of which were shown to have immunomodulatory and antitumor activities and have been isolated [3]. However, few of them were homogeneous, and their structural characterizations were not unambiguously determined. Meanwhile, most studies on polysaccharides prepared from *G. frondosa* have focused on the phenomena of stimulating the immune cells, but few have considered the underlying molecular mechanisms [10]. In the present study, we have isolated a water-soluble homogeneous polysaccharide, GFPBW1, which was shown to induce cytokine production in macrophages through Dectin-1/Syk/NF-κB signaling and to have immunopotentiating and antitumor activities.

Using a simple alkali extraction procedure, a homogeneous polysaccharide, GFPBW1, was isolated from the fruit bodies of *G. frondosa*. Although the repeating unit of GFPBW1 was the same as that of GRN, which was reported by Ohno *et al*. [8], some physicochemical properties of these two polysaccharides were quite different. For example, the molecular size of GRN (2×10^{6}Da in 0.2 M NaOH) was much larger than that of GFPBW1 (3×10^{5}Da in 10 mM NaOH) [5]. In appearance, GFPBW1 was a white powder,

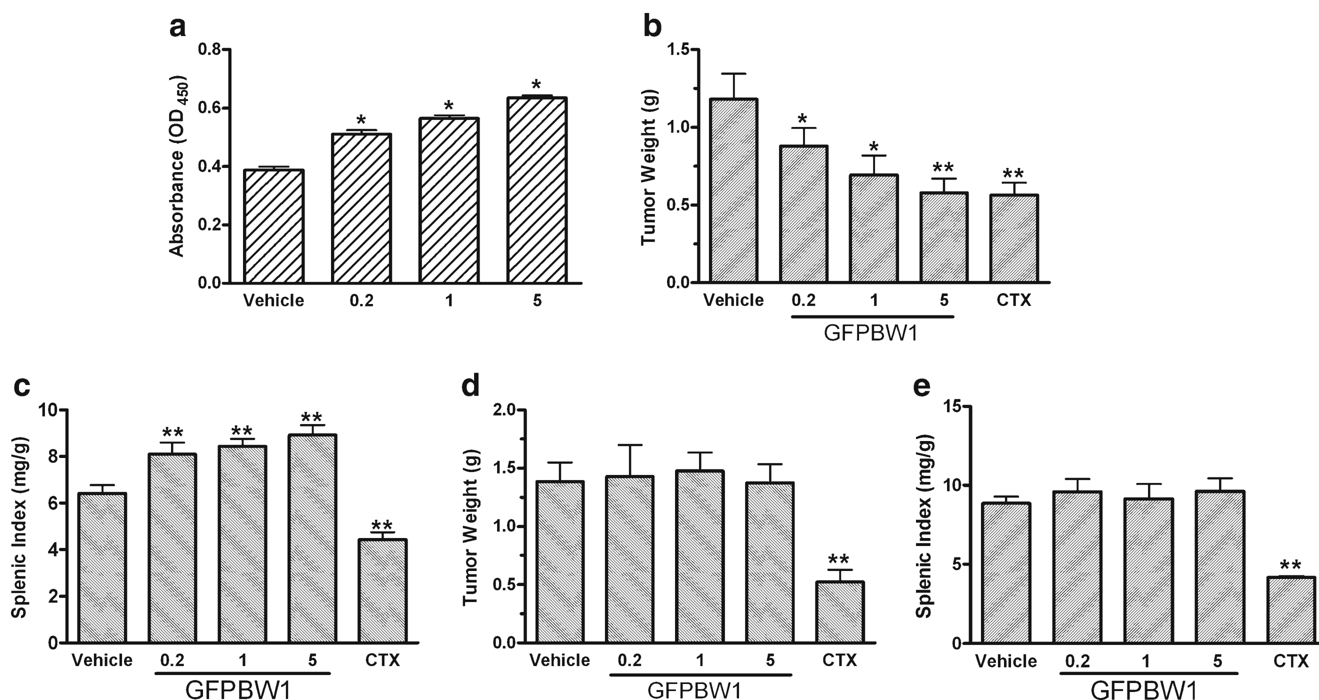


Fig. 6 Immunostimulatory and antitumor activities of GFPBW1 *in vivo*. **a** ICR mice were intraperitoneally injected with normal saline (Vehicle) or the indicated doses of GFPBW1 (0.2, 1, 5 mg/kg) daily for 10 days. 5×10^{5} splenocytes from the mice were transferred to a 96-well plate and stimulated with 5 μg/ml ConA for 48 h followed by the addition of BrdU to label for another 12 h. The relative incorporated BrdU was determined by colorimetric immunoassay. *, $p<0.05$ *vs.* vehicle treatment. The data represent the means±S.E. from two independent experiments. ICR **b**–**c** and BALB/c nu/nu **d**–**e** mice bearing sarcoma 180 allografts were intraperitoneally injected with various doses of GFPBW1 (0.2, 1, 5 mg/kg) for daily 10 days. The weight of the tumors **b** and **d** and spleens were measured. Splenic index was calculated (c and e). Vehicle (normal saline) and CTX (cyclophosphamide, 30 mg/kg) were used as negative and positive controls, respectively. $n=12$. *$p<0.05$, **$p<0.01$, *vs.* vehicle control group

while GRN was weak brown. GFPBW1 was water-soluble, and the intrinsic viscosity of GFPBW1 was relatively low (data not shown). The differences in molecular weight and properties might be due to the different isolation procedures (GFPBW1 with cold 5 % NaOH; GRN with 10 % NaOH containing 5 % urea [5]), strain or growth conditions, environment, harvest time or storage conditions of *G. frondosa* [2]. It is well known that molecular weight, conformation and solubility influence the bioactivity of polysaccharide. Because there are so many obvious differences in the physico-chemical properties of GFPBW1 and GRN, GFPBW1 might have different bioactivity than GRN. In addition, although GRN was reported to be capable of binding to Dectin-1 in a competitive assay [35], the precise mechanisms of signaling transduction was still unclear [8]. Therefore, we investigated bioactivity of GFPBW1 *in vitro* and *in vivo*.

Dectin-1 plays a central role in the β-glucan recognition and immune response to fungi. Dectin-1-knockout mice showed impaired inflammation in fungal infection, and Dectin-1-deficient macrophages demonstrated significantly impaired recognition of and responses to particle β-glucan zymosan and live fungi, such as reduced production of TNF-α [17, 39]. Dectin-1 was also shown to induce NF-κB activation and cytokine production using particle β-glucan or live fungi via interaction with TLR2 or through a direct mechanism. However, few studies have examined the immune response mechanism of water-soluble β-glucan mediated by Dectin-1. It was reported that an 8000 Da water-soluble glucan containing β-(1-3)-linked glucosyl residues, GSG, could employ Dectin-1 to activate macrophages [40]. TNF-α secretion was relatively low in GSG-stimulated parental RAW264.7 cells, which express trace amount of endogenous Dectin-1, but TNF-α secretion was enhanced in RAW264.7 cells stably expressing wild-type Dectin-1. In contrast, signaling-deficient Dectin-1-Δ38 expression failed to enhance TNF-α secretion [40]. Recently, another water-soluble β-glucan MD-fraction from *G. frondosa* was reported to induce Dectin-1/Syk signaling in resident macrophages. Using a Dectin-1 neutralization antibody, they showed that Dectin-1 is required for MD-fraction-induced TNF-α induction [10]. In our study, GFPBW1 could induce the release of TNF-α and IL-6 in mouse resident peritoneal macrophages. The immunostimulating effect of GFPBW1 was not due to the contamination

of endotoxin. Competitive phagocytosis assay indirectly showed that Dectin-1 could bind GFPBW1, which is consistent with the results of studies of GRN [35]. Furthermore, the release of TNF-α and IL-6 and the activation of Syk and NF-κB signaling stimulated by GFPBW1 in mouse resident peritoneal macrophages could be inhibited by the blocking reagent laminarin, similar to the report by Okazaki *et al*. that laminarin could not induce cytokine production from macrophages but inhibited GRN-induced TNF-α release from macrophages [41]. Moreover, RAW264.7 cells expressing wild-type but not truncated Δ38 or mutant Y15S Dectin-1 could enhance the secretion of TNF-α and activation of Syk and NF-κB signaling stimulated by GFPBW1. Therefore, these results showed that GFPBW1 could activate macrophages through the secretion of cytokines partially via the Dectin-1/Syk/NF-κB signaling pathway.

Administration of GFPBW1 potentiated the ConA-induced proliferative response of splenocytes and induced antitumor activity against sarcoma in mice accompanied by an enhanced weight of spleens *in vivo*. The *in vivo* tumor regression properties of GFPBW1 were not due to direct toxicity against tumors. The direct toxicity of the polysaccharide fractions was excluded because the antitumor effect was only observed *in vivo* and not *in vitro* (data not shown). Furthermore, the antitumor effects of GFPBW1 were not observed in immunodefficient nude mice (Fig. 6), which suggested that GFPBW1 mediated tumor regression *in vivo* by the activation of host immune systems. All results *in vivo* supported the conclusion that the major mechanism for the antitumor activities of GFPBW1 was the stimulation of host defense responses. The antitumor efficacy of GFPBW1 was similar to that of GRN, which was also shown to have high potency at 2 mg/kg [7].

Macrophages expressing Dectin-1 may play a critical role in the antitumor activity of β-glucans through activating the immune system. Administration of carrageenan, an inhibitor of macrophage function, reduced the antitumor activity of β-glucan GRN administered *i.p. in vivo* [42]. When using the anti-Dectin-1 neutralizing antibody to block the binding of (1-3)-β-glucan to Dectin-1, the antitumor activity of the β-(1-3)-glucan schizophyllan was significantly attenuated *in vivo* [43]. Furthermore, recent investigations showed that when some β-glucans were orally or *i.p*. administered, they could be taken up by macrophages and then transported to circulation system and bone marrow, where they further boosted the immune system against tumor growth [44, 45]. Activation of macrophages that express Dectin-1 might be a first step to trigger the immunomodulatory and antitumor activities of β-glucan *in vivo* [10, 33]. GFPBW1, as a typical (1-3)-β-glucan, could bind Dectin-1 and activate macrophages partially through the Dectin-1/Syk/NF-κB signaling pathway. Furthermore, GFPBW1 mediated tumor regression activity *in vivo* by the activation of host immune system. Hence, the data suggested that the antitumor activity of GFPBW1 partially resulted from the activation of macrophages via the Dectin-1/Syk/NF-κB signaling pathway.

Notably, laminarin could not completely block the GFPBW1-induced cytokine release in murine resident peritoneal macrophages (Fig. 4a, b). GFPBW1 could also induce TNF-α secretion in RAW264.7 cells that did not express wild-type Dectin-1 (Fig. 5a). These data suggest that other receptors in addition to Dectin-1 might also be involved in the activation of macrophages induced by GFPBW1. Moreover, laminarin could almost completely abrogate the phosphorylation of Syk, but only partially inhibite NF-κB signaling induced by GFPBW1 in primary macrophages (Fig. 4c). GFPBW1 could activate Syk only in RAW264.7 cells that expressed wild-type Dectin-1 (WT). However, NF-κB signaling was activated by GFPBW1 in all manipulated RAW264.7 cells (ZSG, WT, Δ38, Y15S) (Fig. 5b). All above data suggested that activation of Syk induced by GFPBW1 in macrophages might be in linear arrangement with Dectin-1, while some other Syk-independent signaling might crosstalk with the Dectin-1/Syk/NF-κB pathway at the point of NF-κB activation. Many receptors, such as TLR2, might also be involved in the GFPBW1-mediated activation of NF-κB [16]. Whether Dectin-1 collaborates with other receptors or directly induces NF-κB activation and the production of cytokines by GFPBW1 requires further investigation.

In conclusion, the present study showed that a soluble 300 kDa homogeneous polysaccharide named GFPBW1 was obtained from the fruit bodies of *G. frondosa* using an alkali extraction process. GFPBW1 was determined to have a β-D-(1-3)-linked glucan backbone with a single β-D-(1-6)-linked glucopyranosyl residue on every third residue. The bioactivtity study showed that GFPBW1 has immunomodulatory effects and antitumor activity. GFPBW1 could activate macrophages through Dectin-1/Syk/NF-κB signaling and might further mobilize the immune system to achieve its antitumor effect. Although the repeating unit of GFPBW1 is the same as that of GRN, its lower molecular weight, greater solubility, and easier isolation procedure suggests that it has promise to be utilized as biological response modifier that may activate the immune system against tumor growth.

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