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Stimulation of Human Innate Immune Cells by Medicinal Mushroom Sclerotial Polysaccharides

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ABSTRACT: Polysaccharides extracted from the sclerotia of medicinal mushrooms *Pleurotus tuber-regium* (PT) and *Polyporus rhinocerus* (PR), including two water-soluble polysaccharide-protein complexes (PTW and PRW) and a β-glucan (PRG), were incubated with human innate immune cells, and the extracellular cytokines released by the immune cells to the culture medium were analyzed by using the RayBio® human cytokine antibody array. PRG significantly stimulated the proliferation of NK-92MI cells with a corresponding increase in the expression of cytokines IL-2 and I-309, which belong to the chemokine subfamily and are known to be chemotactic for monocytes. All three sclerotial polysaccharides stimulated the proliferation of $CD56^+$ natural killer (NK) and human normal spleen monocytes/macrophages (MD) cells. Cell surface β-glucan receptors on the immune cells treated with the sclerotial polysaccharides were stained with antibodies of dectin-1 and its isotypes before flow cytometric analysis. Dectin-1 expression on NK-92MI and MD cells, but not on $CD56^+$ NK cells, was upregulated by the three sclerotial polysaccharides. The above results suggest the stimulatory effect of mushroom sclerotial polysaccharides on human innate immune cells. These results provide some insight into the mechanism of immunomodulation of mushroom polysaccharides and their potential development into antitumor agents.

KEY WORDS: medicinal mushrooms, innate immune cells, mushroom sclerotium, polysaccharides, stimulation

I. INTRODUCTION

Fungal polysaccharides isolated from the fruit body and/or mycelium of medicinal mushrooms such as *Ganoderma lucidum* and culinary-medicinal mushrooms such as *Lentinus edodes* and *Grifola frondosa* are known for their immunomodulatory and antitumor activities.[1–3] The mechanism of immunomodulation by these mushroom polysaccharides involves stimulation of immune cells such as macrophages, natural killer (NK) cells, and T lymphocytes to release cytokines that eventually result in the death of tumor cells.[4,5] It has been demonstrated that β-glucans isolated from the yeast cell wall can interact with some surface receptors, such as dectin-1 and CR3, in human monocytes and

ABBREVIATIONS

α-MEM: alpha minimum essential medium; **CR3:** complement receptor 3; **Dectin-1:** dendritic-cell-associated C-type lectin-1; **DMSO:** dimethyl sulfoxide; **FBS:** fetal bovine serum; **GC:** gas chromatography; **IMDM:** Iscove's modified Dulbecco's medium; **IL-2:** interleukin 2; **MD:** human normal spleen monocytes/macrophages; **NK:** natural killer; **PR:** *Polyporus rhinocerus*; **PRG:** β-glucan from *Polyporus rhinocerus*; **PRW:** water-soluble polysaccharide–protein from PR; **PT:** *Pleurotus tuberregium*; **PTW:** water-soluble polysaccharide–protein from PT.

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macrophages.[6–8] This, in turn, triggers a series of immune responses, including cytokine production and proliferation of innate immune cells.[5,8] Polysaccharides isolated from mushroom sclerotia, which are a compact mass of fungal hyphae, represent a novel source of unconventional immunomodulatory agents.[9] Recent studies have demonstrated *in vitro* antitumor activities of some sclerotial polysaccharides isolated from *Pleurotus tuberregium* (PT) and *Polyporus rhinocerus* (PR).[10–14] Although some evidence suggests that this antitumor effect seemed to be mediated by immunomodulation and direct cytotoxicity, the exact mechanisms still remain largely unknown. For decades, mechanistic studies on the immunomodulatory effect of fungal β-glucans via β-glucan receptors were limited to the yeast-derived β-glucan rich particulate (zymosan), and no cell surface β-glucan receptor specific for mushroom sclerotial β-glucans has been identified on human immune cells.

The objective of the present study is to investigate the immunomodulatory effect of mushroom sclerotial polysaccharides on some human innate immune cells, including NK cells (NK-92MI and primary $CD56^+$ NK) and MD cells in terms of their *in vitro* proliferation and extracellular cytokine production. Moreover, the expression levels of cell surface β-glucan receptors (Dectin-1) on these innate human immune cells were investigated by flow cytometric analysis. This study provides new insights into how mushroom sclerotial polysaccharides carry out their remarkable immunomodulating and antitumor effects.

II. MATERIALS AND METHODS

A. Materials

Three structurally characterized sclerotial polysaccharides, including two hot-water-soluble polysaccharide–protein complexes from PT and PR (PTW and PRW, respectively) and one sonication-assisted cold alkali-soluble β-glucan from PR (PRG) were chosen for this study based on their immunomodulatory and antitumor activities, reported previously.[11–14] The medicinal mushrooms strains studied were obtained from the Sanming Mycological Institute, Fujian Province (China).

MD (CRL-9850, ATCC, Manassas, VA, USA) and human NK cells—NK-92MI (CRL-2408, ATCC)—were grown in tissue-culture flasks (NUNC, Roskilde, Denmark) in different media, accordingly. MD cells were maintained in IMDM with 4 mM L-glutamine and the addition of 0.05 mM 2-mercaptoethanol, 0.1 mM hypoxanthine, 0.016 mM thymidine (HT supplement, Cat. # 11067-030, GIBCO, Grand Island, NY, USA), and 10% FBS. NK-92MI cells were maintained in alpha minimum essential medium (α-MEM) without ribonucleosides and deoxyribonucleosides but with 2 mM L-glutamine (Cat. # 12000-022, GIBCO, USA), 0.2 mM inositol (Cat. # I7508, Sigma, St. Louis, MO, USA), 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid (Cat. # F8758, Sigma) and 25% FBS. All media were then adjusted to pH 7.4 with the addition of 1.5 g/L sodium bicarbonate and supplemented with 1% penicillin-streptomycin (Cat. # SV30010, HyClone, Logan, UT, USA). All of the above cell cultures were seeded at a concentration ranging from 1×10^5 to 1×10^6 cells/mL and incubated at 37°C in a humidified atmosphere of 5% of CO_2. The cultures were maintained with replacement of fresh medium at 48-hour intervals during subculture.

Human peripheral blood $CD56^+$ NK cells (Cat. # PB012-P-F, StemCell Technologies, Vancouver, BC, Canada) were cryopreserved in 1.8 mL solution of 50% IMDM, 40% FBS, and 10% dimethyl sulfoxide (DMSO). IMDM supplemented with 10% FBS was brought to room temperature to act as the thawing medium after the vial containing frozen cells was thawed in a 37°C water-bath. Cells were ready for immediate use once they were thawed due to the finite life span of primary cells.

B. Monosaccharide Determination by Gas Chromatography (GC)

Monosaccharide profiles of the sclerotial polysaccharides were obtained by GC analysis of their alditol acetate derivatives. The preparation of alditol acetates from sclerotial polysaccharides involved acid hydrolysis by sulfuric acid, reduction by sodium borohydride, and acetylation by anhydrous acetic anhydride, as described previously.[14] A gas

chromatograph (Hewlett-Packard 6890, USA) equipped with an Alltech DB-225 capillary column (15 m × 0.25 mm i.d., 0.25 μm film) was used to analyze the alditol acetate derivatives. A temperature program for the GC oven involved an initial temperature of 170°C, followed by a temperature rise of 2°C/min to 220°C, with a final hold of 10 minutes. The injector and detector temperatures were at 270°C. The carrier gas was helium, and detection was by flame ionization. Individual sugars were quantified by reference to standard sugar mixture and corrected for losses during hydrolysis and derivatization, and for the response to the flame ionization detector using allose as the internal standard. The values for monosaccharides were expressed as polysaccharide residues (anhydrosugars) by multiplying the number of pentoses by a factor of 0.88, hexoses by a factor of 0.90, and deoxypentoses by a factor of 0.89.

C. *In Vitro* Proliferation of Human Innate Immune Cells by Sclerotial Polysaccharides

1. NK-92MI and MD Human Immune Cells

One hundred microliters of 2.5×10^4 cells/mL of MD cells (in IMDM) and NK-92MI cells (in α-MEM) were seeded on a flat-bottomed 96-well plate (Cat. # 83.1835, Sarstedt, Germany) for 24 hours. The immune cells were incubated with 6 concentrations of the 3 sclerotial polysaccharides (from 12.5 μg/mL to 400 μg/mL) with medium alone as control. After 72 hours of incubation, trypan blue exclusion assay and MTT assay were used for testing the cell viability (as % inhibition ratio) and cell proliferation, respectively. The *in vitro* assays were performed in 5 replicates (detailed procedures have been described previously).[14]

2. CD56⁺ Human Primary NK Cells

$CD56^+$ human primary NK cells were used immediately after thawing in a 37°C water bath and resuspended in IMDM. The cells were seeded in triplicates into the 6-well plate and incubated with various concentrations of the 3 sclerotial polysaccharides and IMDM as control, similar to the innate immune cells above, for 72 hours. The trypan blue exclusion assay was used to compare the cell density between the treatment and control groups.

D. Determination of Cytokine Profile Using Cytokine Antibody Array

The cytokines released to the media from the *in vitro* immunomodulatory experiments from Section II.C above were analyzed for their expression profile using the RayBio® Human Cytokine Antibody Array 3 (Cat. # AAH-CYT-3-8, RayBiotech, Norcross, GA, USA) according to the protocols in the array kit, which are based on a simultaneous multiple detection of cytokines.[15]

E. Determination of the Expression of Cell Surface β-Glucan Receptors

The immune cells were first counted for cell viability by the trypan blue exclusion method, and their cell concentration was adjusted to 1×10^6 cells/mL in each well. They were preincubated with 25 μL of CD16/32 (1 μg/10^6 cells; Cat. # 553142, PharMingen, San Jose, CA, USA) for 10 minutes in order to block for any nonspecific binding. The immune cells were then stained with antibodies (from Serotec) for cell surface β-glucan receptors, dectin-1, and its isotypes as control by incubation with 10 μL of prediluted antimouse dectin-1 (clone 2A11, Cat. # MCA 2289EL; 0.5 mg/mL, 1:20) and rat-IgG2b (Cat. # MCA1125; 1.0 mg/mL, 1:40), respectively. After incubating with primary antibodies for 60 minutes on ice, the cells were washed with wash buffer twice by centrifugation at 1300 rpm for 5 minutes each. Then, 10 μL prediluted FITC (Cat. # STAR69, UK; 1:50) was added to the cells and incubated in darkness at 4°C for another 60 minutes before washing the cells in wash buffer twice as previously mentioned. Finally, 0.5 mL of wash buffer was added to each tube before analyzing by flow cytometry (Beckman Coulter, XL-MCL, CA, USA). Data from 10,000 cells were acquired, and their distribution was analyzed using Multicycle

Analysis AV software (Phoenix Flow Systems, San Diego, CA, USA).

F. Statistical Analysis

The data were recorded as mean ± standard deviation and analyzed by SPSS (Version 13.0 for Windows XP, SPSS Inc.). The mean values between treatment and control were analyzed by Student's *t* test. Differences between means at 5% ($p < 0.05$) level were considered to be significant.

III. RESULTS

A. Monosaccharide Composition of Sclerotial Polysaccharides

As previously shown, PRG was the polysaccharide, and PTW and PRW were the polysaccharide–protein complexes, with about 40%–60% protein.[12,14] From the GC results, PRG was confirmed to be a homoglucan, whereas PTW and PRW were heteroglucan–protein complexes (Table 1). The 60%–70% polysaccharide moiety of PTW and PRW consisted mainly of glucose together with other monosaccharides, including arabinose, xylose, galactose, glucosamine, galactosamine and over 10% of mannose (Table 1).

TABLE 1
Monosaccharide Profile (normalized % by weight ratio) of Mushroom Sclerotial Polysaccharides

Monosaccharides	PTW	PRW	PRG
Arabinose	2.82	5.74	0.00
Xylose	2.25	4.38	0.00
Mannose	14.1	17.1	0.00
Galactose	2.67	3.30	0.00
Glucose	70.7	59.7	99.0
Glucosamine	4.00	4.87	1.02
Galactosamine	3.53	4.89	0.00

Note: PTW = water-soluble polysaccharide–protein from *Pleurotus tuberregium*; PRW = water-soluble polysaccharide–protein from *Polyporus rhinocerus*; PRG = β-glucan from *Polyporus rhinocerus*.

B. Immunomodulation on Human Innate Immune Cells

NK-92MI cells responded differently to the 3 sclerotial polysaccharides in which PTW and PRW exerted no or insignificant proliferative effect ($p > 0.05$), whereas PRG conferred a significant proliferative effect ($p < 0.05$) (Table 2). The cytokine expression profiles of the medium obtained from the cell culture of NK-92MI cells treated with the 3 sclerotial polysaccharides did not show a noticeable difference, in general. However, there was an obvious upregulated expression of IL-2 present in the medium incubated with PRW and PRG (Fig. 1), and it was suspected that the IL-2 secreted was responsible for the proliferation of NK cells. It was shown that there was virtually no expression of IL-2 in the medium of NK-92MI incubated with PTW, which might partially explain the unusual cell inhibition by this sclerotial polysaccharide.

In addition, there was a higher expression of I-309 in the extracellular medium of NK-92MI incubated with PRG compared to PRW and PTW (Fig. 1). I-309 was from the CC chemokine subfamily and was found to be chemotactic for monocytes but not neutrophils.[16]

Unlike in NK-92MI cells, all 3 sclerotial polysaccharides exerted a proliferative effect on MD cells (Table 3), with PRW and PRG showing a significantly stronger effect ($p < 0.05$). However, the cytokine expression profiles of the medium of MD cells incubated with the 3 sclerotial polysaccharides did not differ greatly from that of the control (data not shown).

C. Immunomodulation on CD56⁺ Human Primary NK Cells

The human primary CD56⁺ NK cells were thawed and immediately incubated for 72 hours with or without (as control) the 3 sclerotial polysaccharides. It is normal for apoptosis to take place once the primary cells are thawed, so that relative proliferation can be compared with the control. The cell population of primary CD56⁺ cells incubated with

TABLE 2
***In Vitro* Proliferation Ratio (%)# of NK-92MI Cells by Different Concentrations of Sclerotial Polysaccharides**

Concentrations of sclerotial polysaccharides	PTW	PRW	PRG
400 μg/mL	n.d.	n.d.	29.0 ± 19.8*
200 μg/mL	n.d.	15.7 ± 24.9	28.2 ± 14.8*
100 μg/mL	n.d.	n.d.	21.8 ± 14.6*
50 μg/mL	n.d.	n.d.	13.4 ± 24.0
25 μg/mL	n.d.	n.d.	n.d.
12.5 μg/mL	n.d.	3.01 ± 4.48	18.6 ± 14.7*

Note: Data were means ± S.D. (n = 5). # = (number of cells in treatment group/number of cells in control) × 100%. * represents significant difference between treatment and control groups (Student's *t* test with $p < 0.05$). n.d. = no difference from control group. PTW = water-soluble polysaccharide–protein from *Pleurotus tuberregium*; PRW = water-soluble polysaccharide–protein from *Polyporus rhinocerus*; PRG = β-glucan from *Polyporus rhinocerus*.

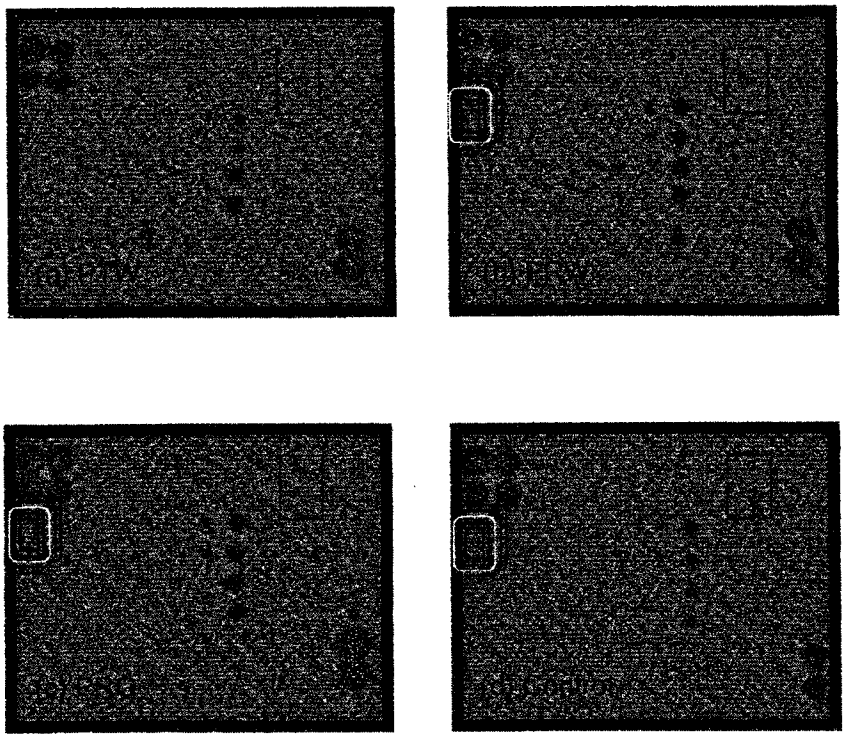

FIGURE 1. The RayBio® human antibody array showing the extracellular cytokine profile of media isolated from NK-92MI incubated with (a) water-soluble polysaccharide–protein from *Pleurotus tuberregium* (PTW); (b) water-soluble polysaccharide–protein from *Polyporus rhinocerus* (PRW); (c) β-glucan from *Polyporus rhinocerus* (PRG); and (d) α-MEM medium as control at a dose of 400 μg/mL for 72 hours. The dots within white and black squares represent the expression of IL-2 and I-309, respectively.

TABLE 3
***In Vitro* Proliferation Ratio (%)# of MD Cells by Different Concentrations of Sclerotial Polysaccharides**

Concentrations of sclerotial polysaccharides	PTW	PRW	PRG
400 µg/mL	18.2 ± 17.7	22.9 ± 10.8*	42.7 ± 9.82*
200 µg/mL	20.8 ± 8.75*	33.0 ± 16.7*	21.2 ± 5.63*
100 µg/mL	8.72 ± 15.5	37.2 ± 19.5*	30.5 ± 6.81*
50 µg/mL	11.0 ± 9.16	25.8 ± 24.0*	34.6 ± 10.1*
25 µg/mL	15.8 ± 18.1	13.5 ± 8.63	21.3 ± 11.8*
12.5 µg/mL	21.8 ± 18.5	45.4 ± 16.5*	47.8 ± 26.6*

Note: Data were means ± S.D. (n = 5). # = (number of cells in treatment group/number of cells in control) × 100%. * represents significant difference between treatment and control groups (Student's *t* test with $p < 0.05$). PTW = water-soluble polysaccharide–protein from *Pleurotus tuberregium*; PRW = water-soluble polysaccharide–protein from *Polyporus rhinocerus*; PRG = β-glucan from *Polyporus rhinocerus*.

the 3 sclerotial polysaccharides at 400 µg/mL for 72 hours all increased, with PRW and PRG showing a significant increase ($p < 0.05$) by 4-fold and 2-fold, respectively (Fig. 2). It was thus believed that PRW and PRG were able to delay the apoptosis of the human primary NK cells and might be further involved in stimulating their growth.

Though there was no conclusive pattern for the cytokine profile in the NK cells (data not shown), it was believed that the 3 sclerotial polysaccharides exerted their actions on primary $CD56^+$ cells via some other cytokines yet to be determined.

D. Expression of Dectin-1 on Human Innate Immune Cells

All sclerotial polysaccharides showed a trend of increase in dectin-1 expression on NK-92MI cells when compared with the control, and PTW showed significant upregulation ($p < 0.05$) of dectin-1 expression up to 32.9% on NK-92MI cells (Table 4). For MD cells, there was a similar trend indicating that all 3 sclerotial polysaccharides upregulated the dectin-1 expression, and the 2 polysaccharide-protein complexes PTW and PRW showed significant upregulation ($p < 0.05$) but to a lesser extent than that on NK-92MI cells (Table 4).

It was observed that there is a trend toward increase in the proliferation of MD cells and an upregulation of dectin-1 on these cells. This might suggest that dectin-1 might be the sole β-glucan receptor present for the binding of sclerotial polysaccharides and that it significantly enhances cell proliferation.

When $CD56^+$ NK cells were incubated with the 3 sclerotial polysaccharides, no significant upregulation of dectin-1 expression could be found when compared with the control (Table 4).

IV. DISCUSSION

NK-92MI cells were NK-92 cells transfected with human IL-2 so that its high cytotoxicity could be maintained independently of exogenous IL-2 administration.[17] MD cells were established from human spleen cells that had monocyte/macrophage characteristics.[18] Unlike tumor cells, these human immune cell lines were being assessed for any proliferation after incubation with the 3 sclerotial polysaccharides because this would be an indicator for the augmented cellular functions.

As a large biopolymer, it was very unlikely that the sclerotial polysaccharides would enter the immune cells to activate the biological responses;

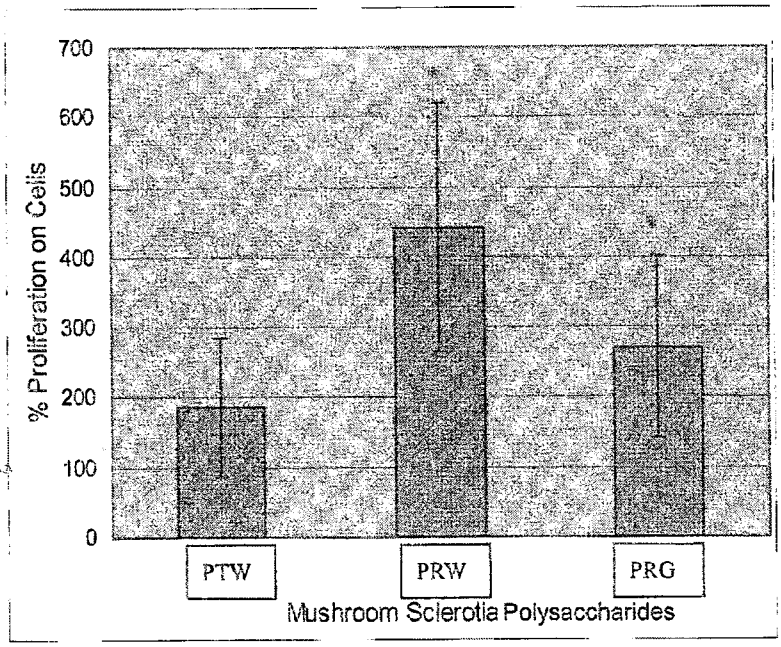

FIGURE 2. The *in vitro* proliferation (%) of 3 mushroom sclerotial polysaccharides (400 µg/mL) on human primary CD56⁺ natural killer (NK) cells comparing with the control (number of cells in the treatment group compared with the number of cells in the control group). Data were means ± S.D. (n = 5). * represents significant difference between treatment and control groups (Student's *t* test with $p < 0.05$). PTW = water-soluble polysaccharide–protein from *Pleurotus tuberregium*; PRW = water-soluble polysaccharide–protein from *Polyporus rhinocerus*; PRG = β-glucan from *Polyporus rhinocerus*.

TABLE 4
Percentage Expression of Dectin-1 on Human Innate and Primary Immune Cells Incubated with Mushroom Sclerotial Polysaccharides (400 µg/mL for 72 hours) and Analyzed by Flow Cytometry

Immune cells	PTW	PRW	PRG	Control #
NK-92MI	32.9 ± 3.43*	21.5 ± 4.65	18.5 ± 2.81	13.5 ± 2.66
MD	12.0 ± 0.25*	14.3 ± 1.18*	15.4 ± 4.90	9.38 ± 0.84
CD56⁺ NK	24.4 ± 4.95	27.3 ± 0.325	23.4 ± 1.91	21.9 ± 2.24

Note: Data were means ± S.D. (n = 3). * represents significant difference between treatment and control groups (Student's *t* test with $p < 0.05$). # = medium used as control for immune cells: α-MEM for NK-92MI; IMDM for both human normal spleen monocytes/macrophages (MD) and CD56⁺ natural killer (NK) cells. PTW = water-soluble polysaccharide–protein from *Pleurotus tuberregium*; PRW = water-soluble polysaccharide–protein from *Polyporus rhinocerus*; PRG = β-glucan from *Polyporus rhinocerus*.

rather, they would likely bind to the receptors on the cell surfaces and then activate the downstream cell-signaling cascade and subsequent events. This suggests that receptors play a vital role in enabling mushroom polysaccharides to be effectively linked to the immune cells in order to exert various biological functions. The present findings demonstrate the increase in expression of Dectin-1 receptors on the immune cells which might be closely related to the cellular functions resulting from the activation of the immune cells. Although it was observed that PRG can activate the upregulation of Dectin-1, there is no direct evidence to support the receptor-mediated activation of the innate immune cells in the present study.

Both PTW and PRW had a high protein content; therefore, they were most likely to be polysaccharide–protein complexes similar to protein-bound β-glucan (PSK) or glycan–protein complexes (PSPC). PSK referred specifically to the polysaccharide–protein complex extracted from *Trametes versicolor* (=*Coriolus*), whereas PSPC referred to a wide range of compounds formed with polysaccharides and proteins.[19,20] Many kinds of PSPC isolated from mushrooms have potent immunomodulatory and antitumor activities, such as those from *Tricholoma lobayense*[21] and from *Ganoderma tsugae*.[22] Mushroom polysaccharide–protein complexes with high glucose and mannose content have been found to possess strong immunopotentiating and antitumor activities, including those from *Lentinus edodes*[23] and from *Agaricus brasiliensis* (=*A. blazei* sensu Heinem.).[24] This might suggest that the potent immunomodulatory and antitumor effects exerted by PTW and PRW were mediated by stimulation of immune cells via other non-Dectin-1 receptors, such as CR3.[25–27]

The interesting observations on the different extents of proliferation of the innate immune cells by mushroom sclerotial polysaccharides might be mediated by the enhanced synthesis of growth factors yet to be determined. More investigations are required to find out the related underlying mechanisms, including the identification of the cell surface β-glucan receptor on human immune cells and the effect of the structure of sclerotial polysaccharides on the recognition and binding to these receptors.

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