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Immunomodulatory activities of mushroom sclerotial polysaccharides

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ABSTRACT

The fight against cancer cells in the human body involves a defense system that is comprised of the innate and adaptive immunities which is controlled by a series of immune responses mediated by different immune cells and their secretory substances including cytokines and chemokines. Polysaccharides, especially those of fungal origin are well-known immunopotentiators that can boost up the immune system. Polysaccharides isolated from the fruit bodies and mycelia of both medicinal and edible mushrooms have been found to exert immunomodulatory function and antitumor activity in numerous *in vivo* and *in vitro* studies. In contrast, the potential of polysaccharides isolated from mushroom sclerotia as immunomodulators is only emerging. This paper gives a mini review on how the immunomodulatory actions of mushroom polysaccharides can eradicate tumor cells and also includes a recent study on the activation of innate immune cells and T-helper cells in normal and athymic BALB/c mice by three mushroom sclerotial polysaccharides with different structural characteristics extracted from *Pleurotus tuber-regium* and *Polyporus rhinocerus*.

1. Introduction

1.1. Immunity for host defense

In the human body, the immune system is divided into the innate and adaptive immunities. The innate immunity refers to a non-specific immune response in encountering a particular antigen, while the adaptive immunity shows a high specificity to antigens invaded and is responsible for a memory response after the first exposure of the same antigen (Martin & Dodds, 2006). The two systems do not work separately, in fact, they act closely together to help fight against the external threats by triggering a series of immune responses (Goldsby, Kindt, & Osborne, 2000).

The central function of the innate immune system is the ability to distinguish between self and non-self (Janeway, 1992) and cells of the innate immune system recognize the antigens of different pathogens through a wide range of pattern recognition receptors located on their surface (Fearon & Locksley, 1996; Medzhitov & Janeway, 1997a).

In addition to discriminating between self and non-self, it is vital for the adaptive immune system to further distinguish between innocuous non-self and pathogenic non-self (Medzhitov & Janeway, 1997a). The development of the adaptive immune system is due to the inflexibility of the innate immune system that cannot cope with the high mutation rate of pathogenic microbes (Hoffmann, 1995). In addition to the capacity of providing a large repertoire of antigen-specific responses, the adaptive system is powerful due to its ability to confer the immunologic memories on the second encounter of the same pathogen (LaRosa & Orange, 2008). Generally, the adaptive immune system is made up of cell-mediated immunity directed by T lymphocytes (Mills, 1989) and humoral immunity mainly contributed by B lymphocytes (McNeela & Mills, 2001).

1.2. Cooperation between innate and adaptive immune systems to help fight against cancer

The uptake of antigens should be the first step in our defense system to help in tumor surveillance since the adaptive immunity depends greatly on antigen presenting cells (APCs) from the innate immunity (Hoebe, Janssen, & Beulter, 2004). The innate immunity is superior to and have an instructive role on the adaptive immunity (Janeway, 1989) due to the reason that cells and molecules of the innate immune system have the ability to distinguish between self and non-self molecules to elicit tailor-made response by activating different effectors mechanisms according to different pathogens encountered; however, the lymphocytes of the adaptive immunity are quite the opposite that they are not having any specialization before activation (Medzhitov & Janeway, 1997b).

Phagocytic cells are powerful in eliminating the advanced stage of tumors because of their strong infiltrating ability to carry out functions such as phagocytosis, antibody-dependent cell-mediated

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cytotoxicity (ADCC) and secretion of cytokines that contribute to direct and/or indirect antitumor activities (DiCarlo et al., 2001). Apart from the presentation of antigens from APCs to cells of the adaptive immune system, the secretion of mediators by innate cells plays a pivotal role in activation of the adaptive immunity and among all, cytokines and chemokines are playing centralized roles in coordinating various immune responses (Jakóbisiak, Lasek, & Gołąb, 2003; Luster, 2002). Cytokines are low-molecular-weight proteins to regulate the intensity and duration of the immune responses by applying different effects on various kinds of immune cells, while chemokines are small polypeptides responsible for chemotaxis and the subsequent regulation of the migration, adhesion and activation of the leukocyte populations (Goldsby et al., 2000).

Interleukin-12 (IL-12) is one of the crucial cytokines for transition of the immune response from innate to adaptive one since the monocyte-derived dendritic cells (DCs) produce a substantial amount of IL-12 that primes the T-helper lymphocytes (T_H1) response (Cellar et al., 1996). This in turn induces the production of IFN-γ and IL-2 by the T_H1 cells to further stimulate the downstream cell-mediated immunity (Qin & Blankenstein, 2000). On the other hand, macrophages and DCs are able to secrete IL-8, interferon inducible protein-10 (IP-10) and macrophage inflammatory protein 1α, 1β (MIP-1α, MIP-1β) upon the binding of antigens to the receptors, and IL-8 helps recruit neutrophils while IP-10 involves in guiding activated T cells in to infected tissues (Khan et al., 2000) and MIPs help induce the influx of natural killer (NK) cells. All together, the production of various kinds of cytokines and chemokines can specify the immune response according to different antigens encountered (Luster, 2002).

1.3. Previous studies on mushroom polysaccharides and their immunopotentiating effects

The medicinal efficacies of *Ganoderma lucidum* (Fr.) Karst (commonly known as Lingzhi) have been reported in one of the most well-known classical Chinese pharmacopoeia "Ben Cao Gang Mu" in 1552. In 1968, it was first reported that the hot water extract from the fruit body of some edible fungi from Polyporaceae family, had a remarkable host-mediated antitumor activity against Sarcoma, S-180 (Ikekawa, Nakanishi, Uehara, Chihara, & Fukuoka, 1968). Since then, scientists are interested in investigating the antitumor substances in mushrooms and many patients from China and Japan have consumed mushroom polysaccharides as part of their treatment.

The major component in the biologically active polysaccharides was identified as β-D-glucan, in which it could only produce D-glucose with acid hydrolysis (Mizuno, Saito, Nishitoba, & Kawagishi, 1995). The well-known chemical structure of this β-D-glucan has been shown to be a (1 → 6)-β-D-glucosyl branched (1 → 3)-β-D-glucopyran, possessing specific rotation of $[\alpha]_D$ +80° to −20° and a mean molecular weight of 500,000−2,000,000. However, different forms of β-D-glucan can provide quite different antitumor effects as their biological activities are influenced by their structures − molecular weight, water solubility, degree of branching and conformation (Mizuno et al., 1995).

It is noted from above that a typical structure−function relationship can be found between the antitumor activities and structural characteristics of β-D-glucans. A wide range of polysaccharides with different molecular masses ranging from 1×10^4 to 1×10^6 kDa can be isolated during extractions. This difference in molecular weight would cause diverse immunomodulatory effects. It is generally found that those polysaccharides with higher molecular weight (1×10^5 kDa or above) would exert stronger antitumor effects and higher immunomodulatory activities. This is particularly noticeable in the case of *Grifola frondosa* in which there was a change of biological activities with respect to fractions of different molecular weights (Adachi, Ohno, Ohsawa, Oikawa, & Yadomae, 1990; Kim, Sakagami, Tanuma, & Konno, 1990). Due to the high molecular weight of mushroom polysaccharides, it is unusual for them to exert a direct cytotoxic effect against tumor cells, but instead, they would enhance a host-mediated immunomodulatory response by activating the immune system to fight against cancer (Wasser & Weis, 1999).

Heterogenous polysaccharides having various kinds of glycosidic linkages can be obtained as a mixture when being extracted. In mushrooms, main chains of glucans with β-1,3-linkages having β-1,6-linkages as branches are most responsible for antitumor activities. In general, β-glucans with more 1,6-linkages would have less antitumor activities (Franz, 1989). Apart from the linkages of the main chain and side branches, degree of branching greatly contributed on the immunotherapeutic effects of polysaccharides. Degree of branching refers to the ratio of occurrence of branches to the main-chain unit (Yadomae & Ohno, 1996). It has been suggested that if the branching ratio of β-glucans were less than 1/2 (branch-to-main-chain unit), its *in vivo* antitumor activity against Sarcoma S-180 tumor cells would become more significant than that with more branching (Ohno et al., 1995). On the contrary, the highly branched glucan called OL-2 with a branching ratio of 2/3 extracted from *Omphalia lapidescens* showed pronounced antitumor activity against Sarcoma S-180 (Saito, Nishijima, Ohno, Yadomae, & Miyazaki, 1992). OL-2 was also found to be effective to stimulate the expression of various kinds of cytokine genes like IL-1α and IL-1β (Nemoto, Ohno, Saito, Adachi, & Yadomae, 1993, 1994).

Lentinan is one of the most well-known immunomodulators extracted from *Lentinus edodes* that could trigger the production of tumor necrosis factor (TNF)-α from macrophages (Abel, Szollosi, Chihara, & Fachet, 1989). Activity of NK cells was stimulated when lentinan was administered *in vivo* to the host that helped suppressing tumor growth (Miyakoshi & Aoki, 1984). Cytotoxicity of peritoneal macrophages was enhanced by lentinan against metastatic tumor cells in mice (Ladanyi, Timar, & Lapis, 1993) and lentinan could increase the production of cytotoxic T lymphocytes (CTL), macrophages and induction of other non-specific immune responses (Wasser & Weis, 1999).

G. lucidum, being regarded as "mushroom of immortality", has been widely used by people from China and Japan in treating various kinds of diseases (Shiao, Lee, Lin, & Wang, 1994). Water extract from *G. lucidum*, with β-D-glucan as the major component, has been shown to be effective in stimulating T cells to release cytokines like IL-1β, IFN-γ, TNF-α, IL-2, IL-6 which are proven to be effective in combating tumor cells (Mao, van De Water, & Keen, 1999; Wang, Hsu, & Hsu, 1997).

Apart from β-D-glucan, the polysaccharide−protein complexes (PSPC) from *Trametes versicolor* serve as immunostimulators specific to T cells and APCs such as macrophages (Tzianabos, 2000). The PSPC is effective in inducing T-helper cells and thus increasing the ratio of T-helper cell/T-killer cells ($CD4^+/CD8^+$) (Liang & Wang, 1996).

It was most recently shown that SCG, a purified soluble β-(1 → 3)-glucan isolated from an edible mushroom *Sparaciss crispa*, could stimulate the cytokine production by splenocytes from various strains of mice. This activity was triggered by both granulocyte macrophage-colony-stimulating factor (GM-CSF) and the β-glucan receptor dectin-1 (Harada & Ohno, 2008). Such cytokine-producing ability was eliminated in the dendritic cells obtained from dectin-1 knockout mice (Harada & Ohno, 2008).

1.4. Previous studies on sclerotial polysaccharides

Poria cocos (PC) is one of the most well-known traditional Chinese medicinal mushrooms. It is also known as hoelen or Fu ling

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and the sclerotium can be collected throughout the year. Its sclerotium can be in the shape of spherical, oval or irregular size with a diameter of 10–30 cm. PC is a subterranean fungus, which grows together with the roots of various conifers, especially Chinese red pine, Taiwan pine and oaks (Liu & Bau, 1980).

As a traditional Chinese medicine, PC is considered to be with a mild sweetness and bland. It is claimed to be effective in treating edema and clear febrile illnesses. Different portions of PC have different medicinal uses in which its cortex is used as a diuretic and a decoction for cough, while the internal white portion is responsible for relieving uneasiness arising from pregnancy and the heat discomfort (Hobbs, 1995). In particular, the polysaccharides of PC such as the highly branched pachyman and debranched pachymaran have long been proven to exhibit strong antitumor and immunomodulatory activities. However, pachyman had relatively lower antitumor activity than pachymaran which was debranched by Smith degradation (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970). On the other hand, the low-molecular-weight tetracyclic triterpenes are also claimed to have immunostimulating and antiviral activities (Hobbs, 1995).

Pleurotus tuber-regium (PT), commonly found in Africa, is a kind of mushroom that is both edible and medicinal (Wong & Cheung, 2008). It is not only used as a food substitute, but also used as a kind of medicine in treating headache, stomachache, fever and colds (Oso, 1977). The fruit body of PT is rich in protein (Kadiri & Fasidi, 1990) while its sclerotium is rich in fiber, especially, non-starch polysaccharides (Cheung & Lee, 1998). In particular, the bioactive β-glucan could be found in large amount (more than 60% dry weight) (Cheung & Lee, 2000). The hot water extract of PT was shown to possess both host-mediated antitumor activity on murine model using BALB/c mice implanted with allogenic solid tumor cell (Sarcoma 180) (significant inhibition of 55.3%) and direct cytotoxic effect on various mammalian cancer cell lines but were non-cytotoxic to normal kidney cells from Monkey (VERO) when administered intraperitoneally (Zhang, Zhang, Cheung, & Ooi, 2004). On the other hand, carboxymethylated hot alkali fractions from PT were found to have significant *in vivo* inhibition of Sarcoma, S-180 on BALB/c mice and enhanced *in vitro* inhibition of HL-60 when compared to native ones (Zhang, Cheung, Zhang, Chiu, & Ooi, 2004). When lipopolysaccharides (LPS) were not injected into mice implanted with S-180, there was no significant increase in the plasma TNF-α of mice treated with carboxymethylated hot alkali fractions (Zhang, Cheung, et al., 2004). However, when LPS was injected, TNF-α production was significantly induced by more than 2-fold. This suggested that the antitumor activity of β-glucan isolated from PT was mediated by TNF-α production under the influence of LPS. Sulfated β-glucan obtained from PT exhibited higher *in vivo* inhibition of Sarcoma tumor in BALB/c mice and *in vitro* antiproliferation of liver cancer HepG2 cells which could possibly be due to the increase of solubility in the chemically modified polysaccharide (Tao, Zhang, & Cheung, 2006).

Polyporus rhinocerus (PR) is a novel mushroom sclerotium belonging to the Polyporacea family. The mycelium of PR was inoculated and cultivated on potato dextrose agar (PDA) plate with a yellowish-brown appearance. After three to five months of growing in an incubator, primodia and even sclerotia appeared. PR is a traditional Chinese medicine in which there has been some preliminary data showing their effectiveness in treating liver cancer, chronic hepatitis and gastric ulcer (Huang, 1999). However, there is very little in-depth scientific research data in supporting their biological functions.

A recent study of this mushroom sclerotium had indicated that various fractions of sclerotial polysaccharides including water-soluble polysaccharide–protein complex and alkali-soluble β-glucan isolated from PR not only possessed remarkable host-mediated antitumor activity on the Sarcoma 180 implanted BALB/c mice (tumor regression was also observed), but also had notable direct cytotoxic effect on various human leukemic cell lines including HL-60, K562 and THP-1 (Lai, Wong, & Cheung, 2008). Flow cytometric analysis on cell cycle revealed the onset of apoptosis of the HL-60 cells by hot water extract of PR should be related to the cell cycle arrest at G_1 phase (Lai et al., 2008).

1.5. Objectives of study

In vivo immunomodulatory effect of three mushroom sclerotial polysaccharides was studied in healthy BALB/c mice and athymic nude mice in terms of their serum cytokine profile and immune cell population of the splenocytes or spleen mononuclear cells (MNCs) and peritoneal exudate cells (PECs).

2. Materials and methods

2.1. Materials

2.1.1. Mushroom sclerotia

Sclerotia of *P. tuber-regium* (Fries) Singer (PT) and *P. rhinocerus* Cooke (PR) were supplied by the Sanming Mycological Institute in Fujian, China. The sclerotia were peeled and milled into powder by a mechanical grinder (MF10, IKA) through a 0.5 mm sieve.

2.1.2. Sclerotial polysaccharides

Three sclerotial polysaccharides including the hot water-soluble one from *P. tuber-regium* (PTW), the hot water and sonication-assisted cold alkali-soluble sclerotial polysaccharides from *P. rhinocerus* (PRW and PRS, respectively) were chosen based on their previous promising antitumor effects (Lai, 2005; Zhang, Cheung, et al., 2004; Zhang, Zhang, et al., 2004). Extraction protocols of these sclerotial polysaccharides were mentioned elsewhere (Lai et al., 2008). PRW and PRS were chemically characterized previously as polysaccharide–protein complex and β-glucan, having a molecular weight of 4×10^5 kDa and 1.5×10^4 kDa, respectively (Lai et al., 2008) while PTW was found to be a polysaccharide–protein complex with the largest molecular weight of 1×10^6 kDa (Zhang, Zhang, et al., 2004).

2.1.3. Mice

Male BALB/c mice and athymic BALB/c nude mice aged 7–8 weeks old were obtained from the Laboratory Animal Service Centre of The Chinese University of Hong Kong and housed under controlled room conditions at $21 \pm 2\,^{\circ}C$ with an alternating 12 h light and dark cycle. The athymic nude mice were housed in sterile rodent microisolator cages. The mice were provided with sterile water and rodent chow *ad libitum*. All the mice were allowed to acclimate in the laboratory for one week before the start of each experiment.

2.1.4. Antibodies

All the antibodies used for immunophenotyping of PECs and spleen MNCs were purchased from BD PharMingen including the blocking antibody, CD16/32; the staining of macrophages by PE-anti-mouse Mac-3(M3/84) and PE-rat IgG1, κ as its isotype control; staining of natural killer cells by PE-anti-mouse NK1.1 and PE-mouse IgG2a, κ as its isotype or by PE-anti-mouse CD56 and PE-mouse IgG1, κ as its isotype; staining of dendritic cells by PE-anti-mouse CD11c and PE-hamster IgG1, λ1 as its isotype; staining of B lymphocytes by FITC-anti-mouse CD45R/B220 and FITC-rat IgG2a, κ as its isotype. For the staining of T lymphocytes, FITC-anti-mouse CD3 molecular complex was applied followed by the addition of either PE-anti-mouse CD4 or PE-anti-mouse CD8a for $CD4^+$

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T-helper cells or $CD8^+$ T-killer cells, respectively while PE-rat IgG2a, κ was added as isotype control.

Primary antibodies applied in immunohistochemistry to stain macrophages were anti-mouse F4/80 antigen-pan macrophage marker (clone BM8) and NK cells were anti-mouse CD49b (integrin α-2) (clone HMa2) (all from eBioscience).

2.2. Methods

2.2.1. In vivo immunomodulation in normal and athymic BALB/c mice

Twenty milligrams per kilogram of each mushroom sclerotial polysaccharides was given to both normal and athymic BALB/c mice daily by intraperitoneal injection for 10 consecutive days. PBS was given to the control group. One hour after the last injection on day 10, blood of the mice was collected by heart puncture. The sera were separated by centrifugation at 12,000 rpm for 5 min and stored for cytokine profile determination. Three days before the end of experiment, 1 ml of 3% thioglycollate broth were injected i.p. into the mice to induce sterile peritonitis. After termination, the PECs were collected by washing the peritoneum of the mouse with 6 ml cold PBS. The collected cells were washed twice with cold PBS with centrifugation at 1000 rpm for 5 min and resuspended in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin with constant shaking.

After termination, the spleen of each mouse was isolated aseptically in a laminar flow cabinet and weighed. Spleens were then placed in a 60 mm Petri-dishes, cut into small pieces, minced and pressed through a sterile stainless steel mesh pre-wetted with 3 ml cold RPMI-1640 to obtain a single cell suspension of MNCs using sterile syringe plug. The spleen MNCs were then obtained using the Ficoll-Paque centrifugation gradient. In brief, cell debris of isolated MNCs was settled down by centrifugation at 2000 rpm for 30 s. The single cell suspension was mixed with Ficoll solution. The resultant narrow yellow band between the upper medium layer and lower MNC suspension layer was carefully transferred to a new polythene tube. These cells were washed twice with cold RPMI-1640 by centrifugation at 1800 rpm for 10 min and for another 5 min. The collected MNCs were resuspended in cold RPMI-1640.

The whole immunophenotyping process was carried out on ice and the staining of cell surface antigens was carried out in darkness. Both PECs and spleen MNCs isolated were firstly counted for cell viability by trypan blue exclusion assay. One million cells were used for each antigen staining. The cells were then washed with 3 ml wash buffer, PBS containing 0.1% sodium azide and 1% bovine serum albumin, followed by centrifugation at 2000 rpm for 5 min. Afterwards, cells were resuspended in 25 μl wash buffer and the non-specific binding were blocked by incubating with CD16/32 (1 μg/10^6 cells) for 10 min. Different antibodies as mentioned were then added with the concentration of 1 μg/10^6 cells in 25 μl wash buffer for binding cell surface antigens of particular immune cell type presence in PECs and MNCs. All cells were incubated with the antibodies for 30 min with constant shaking. Finally, 0.5 ml wash buffer was added to each sample before analyzing by flow cytometry (Beckman Coulter, XL-MCL, USA).

2.2.2. Determination of cytokine profile using cytokine antibody array

The serum isolated from the athymic BALB/c mice was analyzed for the cytokine profile using RayBio® Mouse Cytokine Antibody Array II (RayBiotech). Different cytokine antibodies were precoated on array membranes. The membrane was first incubated with 1× blocking buffer for 30 min and then with 1 m1 diluted serum (1:10 in 1× blocking buffer) for 2 h at room temperature. Mouse serum was then removed and the membrane was washed 3 times with 1× wash buffer I and then twice with 1× wash buffer II. One milliliter of diluted biotin-conjugated anti-cytokines was added to each membrane and was allowed to incubate for 2 h. Then, the membrane was washed again with wash buffer I and II followed by incubating with 1000-fold diluted HRP-conjugated streptavidin for 2 h. After incubation, the membrane was washed with wash buffer I and II and prepared for subsequent detection processes. Five hundred microliters of a mixture of two different detection buffers was added to each membrane and the membrane was incubated for 2 min at room temperature. The membrane was then exposed to X-ray film and signals were detected using film developer.

2.3. Statistical analysis

All analyses were performed in triplicate unless otherwise stated. The data were recorded as mean ± standard deviations and analyzed by ANOVA. Differences between means were significant ($p < 0.05$) by Tukey's multiple comparison.

3. Results and discussion

3.1. In vivo immunomodulation on normal BALB/c mice

All the spleens from treatment groups showed a significant increase in size when compared with control group injected with PBS only (Table 1). The increase in the size of spleen was an indicator for enhancement of mitogenic activities which would lead to the maturation of lymphocytes.

The MNCs and PECs isolated from the BALB/c mice were analyzed for their cell surface antigens to identify the different immune cell populations by flow cytometric analysis. Different types of antibodies were used as cell surface markers for staining various immune cell populations in MNCs (Table 2) namely NK cells (NK1.1), macrophages (Mac-3), B lymphocytes ($CD45R/B220^+$), T-helper cells ($CD3^+/CD4^+$) and T-killer cells ($CD3^+/CD8^+$). Within spleen MNCs, it was found that there were nearly no expression of $NK1.1^+$ natural killer cells in both the control and PTW treated groups, but the mice injected with PRW and PRS had a significant increase ($p < 0.05$) of 10.0% and 18.6%, respectively. Macrophage population in PTW was significantly lower ($p < 0.05$) than that of the others (Table 2). There was a significant increase ($p < 0.05$) of T-helper cells population in the MNCs of all the treated mice, and in particular, the T-helper cells population increased from 6.74% in the control group to more than 30% in the PRW and PRS groups (Table 2). In general, the increase in % T-helper cells of the three treatment groups contributed to a large increase of CD4/8 ratio when compared with that of the control group where the normal ratio should only be around 2:1. The exceptionally high CD4/8 ratio for the group injected with PTW was caused by the smaller T-killer cells population present.

On the other hand, only mice injected with PTW showed a slight increase (though not statistical significant) in the NK cells

Table 1
Weight and % increase of spleens isolated from healthy BALB/c mice injected with different mushroom polysaccharides compared with control group.

	Spleen weight (g)	% of Increase
PTW	0.250 ± 0.018^c	145%
PRW	0.152 ± 0.015^b	49.0%
PRS	0.112 ± 0.004^a	9.80%
Control	0.102 ± 0.008^a	–

Data were means ± S.D. ($n = 5$). Different letters represent significant differences between means ($p < 0.05$) analyzed by ANOVA and Tukey's multiple comparison.

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Table 2
Identification of various immune cell populations (%) within spleen mononuclear cells (MNCs) of healthy BALB/c mice treated with sclerotial polysaccharides using different cell surface antigens.

	PTW	PRW	PRS	Control
$NK1.1^+$	n.d.	10.0 ± 1.20^b	18.6 ± 1.83^c	0.42 ± 0.10^a
$Mac\text{-}3^+$	0.70 ± 0.14^a	3.08 ± 0.43^b	2.58 ± 0.33^b	2.94 ± 0.41^b
$CD45R/B220^+$	47.2 ± 6.44^a	57.8 ± 7.80^a	47.7 ± 5.24^a	51.2 ± 6.71^a
$CD3^+/CD4^+$	21.2 ± 3.56^a	37.5 ± 5.51^b	37.6 ± 4.87^b	6.74 ± 1.12^c
$CD3^+/CD8^+$	1.38 ± 0.63^a	4.59 ± 1.80^a	5.30 ± 2.65^a	3.17 ± 1.23^a
CD4/8 ratio	15.4:1	8.17:1	7.10:1	2.13:1

n.d.: Level of the targeted antibody was not determined as it was the same as the isotypic control.
Data were means ± S.D. ($n = 5$). Different letters represent significant differences between means ($p < 0.05$) analyzed by ANOVA and Tukey's multiple comparison.

population in PECs. All three mushroom sclerotial polysaccharides showed an increase in the $Mac\text{-}3^+$ macrophage population in the PECs with PTW and PRS having a significant higher population ($p < 0.05$) (Table 3).

Proliferation of $CD4^+$ and $CD8^+$ T-cell population may be one of the causes to the enlargement of spleen (Goldsby et al., 2000). $CD3^+$ positive cells represent the T cells and two subsets of $CD4^+$ and $CD8^+$ are two common T lymphocytes important for adaptive immunity. Many studies reported the cytotoxic effects of $CD8^+$ and non-cytotoxic $CD4^+$ cells because they are lymphocytes responsible for releasing pro-inflammatory cytokines that recruit different effector cells, including macrophages, neutrophils, eosinophils, etc. (Forni et al., 1988). Therefore, different kinds of cytokines would be released when these effector cells are stimulated. It is very common to study the central role of $CD4^+$ cells because they are particularly required for modulating immunity by striking a balance between T_H1/T_H2 subset. The T_H1 subset is mainly responsible for classic cell-mediated function, for example, activation of cytotoxic T lymphocytes, while the T_H2 subset is more responsible for activation of B cells (Goldsby et al., 2000). Therefore, the percentage of $CD4^+$ or $CD8^+$ cells treated by the sclerotial polysaccharides increased substantially when compared with the control (Table 2), and the significant increase in the weight of spleen may be due to the increase of T-cell population. Over 95% of peripheral T cells (Goldsby et al., 2000) were $CD3^+$ and only 25% of them were found in murine splenocytes (Li, Minami, Hanaoka, & Yamamura, 1999).

A high percentage of B cells are normally present in splenocytes and cell surface markers are used to test if B lymphocytes (CD45R/B220) are also induced. However, the peripheral blood cytokine profile showed that only T_H1 subset was activated and the expression of $CD45R/B220^+$ B cells was not augmented so that B cell activation in murine spleen should not have taken place (Hardy, Carmack, Shinton, Kemp, & Hayakawa, 1991).

The three mushroom sclerotial polysaccharides were responsible for increasing the peritoneal exudate cells (data not shown) and this was due to the stimulatory effect of polysaccharides present on the host immune response by targeting the macrophages derived from peritoneal cavity (Figueras, Santarem, & Novoa, 1998; Tomoda et al., 1993). Macrophages are known to be the first defense line in preventing foreign invasion and neoplastic diseases (Kiho, Ito, Nagai, Hara, & Ukai, 1987). The release of peritoneal macrophages by polysaccharides was due to the liberation of monocytes from bone marrow into peripheral blood, followed by the activation of adherence to endothelial cells (Roesler et al., 1991). β-glucans from lentinan and grifolan had no proliferative effects of peritoneal macrophages *in vitro* by measuring their H_2O_2 production (Adachi, Ohno, & Yadomae, 1993), but polysaccharides from *Phellinus linteus* enhanced the peritoneal macrophages production through determining the augmentation of phagocytosis (Kim, Choi, Lee, & Park, 2004).

Only PECs but not MNCs were stimulated by the three mushroom sclerotial polysaccharides to have an increase of $Mac\text{-}3^+$ population with PTW and PRS having a significantly ($p < 0.05$) larger increase (Table 3). There was even a significant ($p < 0.05$) down-regulation of Mac-3 in MNCs treated with PTW (Table 2). $Mac\text{-}3^+$ is used as a marker for determining the presence of murine macrophages as it is used to recognize the mouse macrophage differentiation antigen. This marker is therefore adopted as a general marker for macrophages that can be distinguished from lymphocytes (Ho & Springer, 1983). In addition, $NK1.1^+$ NK cells were significantly ($p < 0.05$) up-regulated in MNCs treated by PRW and PRS (Table 2) but no in PECs (Table 3).

Generally speaking, T-helper cells were being activated in the MNCs while both NK cells and macrophages were activated in MNCs as well as PECs from the present results. By using this *in vivo* model, it was shown that all the mushroom sclerotial polysaccharides might be involved in activating both the adaptive and innate immunities to different extent.

Table 4
Weight and % increase of spleen isolated from healthy athymic nude mice injected with different mushroom sclerotial polysaccharides compared with control group.

	Spleen weight (g)	% of Increase
PTW	0.142 ± 0.021^b	30.3%
PRW	0.135 ± 0.016^b	23.9%
PRS	0.122 ± 0.022^a	11.9%
Control	0.109 ± 0.009^a	–

Data were means ± S.D. ($n = 7$). Different letters represent significant differences between means ($p < 0.05$) analyzed by ANOVA and Tukey's multiple comparison.

3.2. Immunomodulation on athymic nude mice

To obtain a clearer picture on how the mushroom sclerotial polysaccharides activated the innate immune system and whether the immunomodulatory effects of these sclerotial polysaccharides depended on the adaptive immunity, healthy athymic nude mice were employed as another *in vivo* model. Nude mice are the BALB/c mice having the *nu* gene knocked out, resulting in hairless athymic

Table 3
Identification of various immune cell populations (%) within peritoneal exudate cells (PECs) of healthy BALB/c mice treated with sclerotial polysaccharides using different cell surface antigens.

	PTW	PRW	PRS	Control
$NK1.1^+$	11.0 ± 4.2^a	n.d.	5.72 ± 1.65^a	7.96 ± 2.65^a
$Mac\text{-}3^+$	9.80 ± 3.54^b	6.32 ± 1.87^{ab}	8.28 ± 2.96^b	3.50 ± 1.24^a

n.d.: Level of the targeted antibody was not determined as it was the same as the isotypic control.
Data were means ± S.D. ($n = 5$). Different letters represent significant differences between means ($p < 0.05$) analyzed by ANOVA and Tukey's multiple comparison.

Table 5
Identification of various immune cell populations (%) within spleen mononuclear cells (MNCs) of healthy athymic nude mice treated with sclerotial polysaccharides using different cell surface antigens.

	PTW	PRW	PRS	Control
$NK1.1^+$	n.d.	n.d.	n.d.	n.d.
$Mac\text{-}3^+$	6.45 ± 1.23^a	5.05 ± 0.61^a	6.71 ± 1.13^a	6.31 ± 1.69^a
$CD11c^+$	2.48 ± 0.41^a	2.96 ± 0.55^a	3.09 ± 0.49^a	3.66 ± 0.51^a

n.d.: Level of the targeted antibody was not determined as it was the same as the isotypic control.
Data were means ± S.D. ($n = 7$). Different letters represent significant differences between means ($p < 0.05$) analyzed by ANOVA and Tukey's multiple comparison.

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Table 6
Identification of various immune cell populations (%) within peritoneal exudate cells (PECs) of healthy athymic nude mice treated with sclerotial polysaccharides using different cell surface antigens.

	PTW	PRW	PRS	Control
NK1.1$^+$	n.d.	n.d.	n.d.	n.d.
Mac-3$^+$	n.d.	n.d.	n.d.	n.d.
CD11c$^+$	2.67 ± 0.33^a	3.67 ± 0.49^a	2.48 ± 0.39^a	6.36 ± 1.16^a

n.d.: Level of the targeted antibody was not determined as it was the same as the isotypic control.
Data were means ± S.D. ($n = 7$). Different letters represent significant differences between means ($p < 0.05$) analyzed by ANOVA and Tukey's multiple comparison.

animals which are unable to generate T lymphocytes and part of the maturation of B lymphocytes are affected as well.

Similar to the conditions used in healthy BALB/c mice, the athymic nude mice were treated with i.p. injection of the three mushroom sclerotial polysaccharides. It was observed that the degree of tissue proliferation of spleen in athymic nude mice was less than that of BALB/c mice though the spleen weight (in control group) for these two kinds of mice were similar (Table 4). All three sclerotial polysaccharides contributed to the weight increase of spleens, but only the two hot water-soluble sclerotial polysaccharides, PTW and PRW, showed a significant increase in spleen weight ($p < 0.05$).

As shown in Tables 5 and 6, while there was no subpopulation of NK1.1$^+$ cells detected in both MNCs and PECs isolated from the nude mice, there was no Mac-3$^+$ macrophage population found in PECs (Table 6). For the CD11c$^+$ dendritic cells population in the MNCs, while there were no significant difference ($p > 0.05$) in the treatment groups and the control (Table 5), PECs treated with all the sclerotial polysaccharides had a significantly lower ($p < 0.05$) CD11c$^+$ population than that of the control (Table 6). It was observed that although the same treatment was applied to healthy BALB/c mice and healthy athymic nude mice, these two kinds of mice responded differently in a way that the increment of spleen weight was less in nude mice and the NK1.1$^+$ NK cells as well as the Mac-3$^+$ macrophages were not detected in nude mice. This could be due to the reason that the enlargement of spleen in BALB/c mice might be related to the extensive increase of T-cell population and NK cells. Therefore, the absence of thymus in nude mice that caused the deficiency of T lymphocytes might be responsible for the relatively small increase of spleen weight in healthy athymic nude mice. However, the size of spleen of treatment groups still had significant increase ($p < 0.05$) when compared with that of the control group and this must be related to the increase of innate immune cell populations. Therefore, the sera from the healthy athymic nude mice were assessed for their cytokine profile to determine which kinds of innate immune cells were involved in relation to the immunomodulatory effects exerted by the mushroom sclerotial polysaccharides.

The sera drawn from healthy athymic nude mice injected intraperitoneally with PTW, PRW, PRS and PBS at a dose of 20 mg/kg were used for the determination of cytokine profile using the mouse cytokine array (see Appendix 1 for full list of cytokines) and the results are shown in Fig. 1. The cytokine profile from sera of mice injected with PRW had a slight differential pattern with those injected with the other two mushroom sclerotial polysaccharides. It

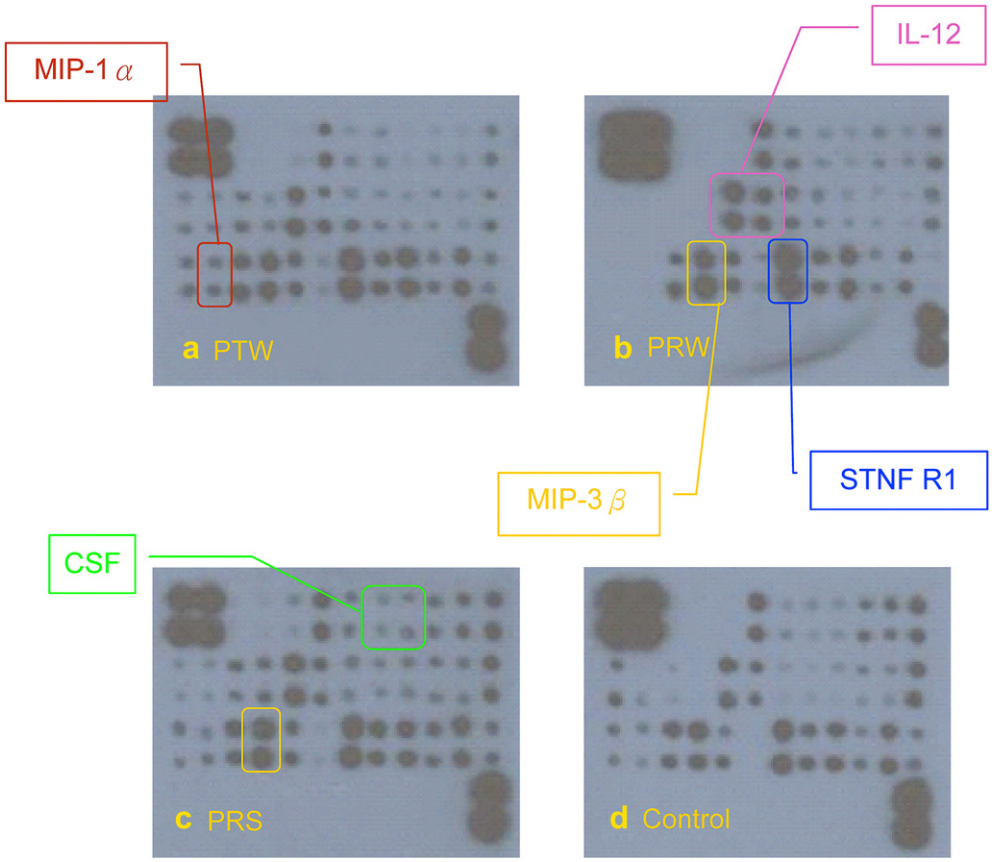

Fig. 1. The RayBio® mouse antibody array showing cytokine profile of sera from healthy athymic nude mice injected intraperitoneally with (a) PTW, (b) PRW, (c) PRS and (d) PBS as control at a dose of 20 mg/kg body weight for 10 consecutive days.

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was found that IL-5, IL-6, IL-9, IL-10, monocyte chemoattractant protein (MCP-5), macrophage inflammatory protein-1 (αMIP-1α) and vascular endothelial growth factor (VEGF) were absent in the sera of mice injected with PRW (Fig. 1). However, it was shown that the expression of the two forms of IL-12 (IL-12p40p70 and IL-12p70), MIP-3β and sTNFR1 in mice injected with PRW was the highest among the three sclerotial polysaccharides. On the other hand, mice injected with PRS had the sera cytokine profile showing a higher expression of eotaxin, G-CSF, GM-CSF, IL-13, IL-17, IFN-γ, KC and leptin, with similar expression levels of IL-2, IL-3, IL-4 and MCP-1 as that of control. Sera of mice injected with PTW showed a stronger expression on MCP-5 and MIP-1α (Fig. 1).

The absence of IL-5, IL-6, IL-9 and IL-10 in mice injected with PRW indicated the lack production of the T_H2 subset of cytokines and thus B lymphocytes production was also hindered. In addition, IL-5, the eosinophil-selective cytokine, is produced to activate eosinophils and in return secrete an array of cytokines such as IL-2, IL-6 and IL-10 to promote T-cell proliferation and activation as well as T_H1/T_H2 polarization (Rothenberg & Hogan, 2006) affecting the production of these cytokines. The deficiency of T lymphocytes therefore affected the production of eosinophils in athymic nude mice injected with PRW and thus a range of cytokines and chemokines like IFN-γ and MIP-1α as detected in the serum cytokine profile.

IL-12 was present in all groups but its expression was found to be the highest in nude mice injected with PRW (Fig. 1). In general, IL-12 is produced by APCs such as monocytes and dendritic cells, and it can induce the T cells to produce IFN-γ (Trinchier, 1998). A high level of IL-12 (IL-12p40p70 and IL-12p70) present in the sera indicated that the APCs were activated in the nude mice injected with PRW, but no IFN-γ was found to be induced accordingly owing to the deficiency of matured T lymphocytes. One of the reasons was that IL-10, produced by monocytes (Hsieh, Heimberger, Gold, O'Garra, & Murphy, 1992), was known to have a suppressive effect on IL-12 production and in this case, the absence of IL-10 helped facilitating the production of IL-12 in mice injected with PRW (Hsieh et al., 1993). More importantly, IL-12 secreted by dendritic cells had a function in modulating the T_H1/T_H2 ratio and polysaccharides from *G. lucidum* were shown to increase the polarization to the T_H1 cytokine production (Lin, Lee, Hou, & Chiang, 2006). The three sclerotial polysaccharides thus might have a structure resembling this extract of *G. lucidum* in having the branched (1 → 6)-β-D-glucan moiety (Wang, Hsu, Lee, Shiao, & Ho, 1997) and thus possessing similar biological functions.

Furthermore, the absence of VEGF in the serum of mice injected with PRW indicated that PRW might effectively suppress the angiogenesis which usually took place in tumor development, since VEGF was a pro-angiogenic factor produced by tumor-infiltrating macrophages that are responsible for enhancing the invasion of murine and human tumor xenografts (Barbera-Guillem, Nyhus, Wolford, Friece, & Sampsel, 2002).

For the mice injected with PRS, it could be seen that there was a different serum cytokine profile detected, though PRW and PRS were both from *P. rhinocerus* and the different immune responses were thus owing to the structural differences of the two sclerotial polysaccharides. The relatively higher expression of the two colony-stimulating factors (G-CSF and GM-CSF) and IL-17 was responsible for the recruitment of neutrophils in which the CSFs were used for production and differentiation of bone marrow progenitor cells to form neutrophils (Cannistra & Griffin, 1988) while IL-17 was a potent cytokine for recruitment and proliferation of neutrophils (Bettelli, Oukka, & Kuchroo, 2007). The murine KC expressed here was analogous to growth-regulated oncogene (GRO) (Oquendo et al., 1989), so they shared the same function of being chemoattractant to neutrophils (Nasu et al., 2001).

In the mice injected with PTW, the higher expression of MIP-1α might increase the influx of NK cells, macrophages and dendritic cells (Salazar-Mather, Hamilton, & Biron, 2000).

Therefore, it was generally concluded that the three mushroom sclerotial polysaccharides were able to activate the innate immune system even with the absence of an intact adaptive immune system. In particular, PRS was mainly responsible for activating neutrophils while the two hot water-soluble sclerotial polysaccharides PRW and PTW stimulated the functions of a wide range of innate immune cells.

4. Conclusions

Sclerotial polysaccharides isolated by hot water extraction from *P. tuber-regium* and *P. rhinocerus* (PTW and PRW, respectively) and by sonication-assisted alkali extraction (PRS) having different chemical structures (polysaccharide–protein complex and pure β-glucan, respectively) demonstrated distinctive immunomodulatory effects. The two hot water extracts exerted significant ($p < 0.05$) increase in spleen weight of both healthy BALB/c mice and healthy athymic nude mice while this effect by sonication fraction was less significant. *In vivo* immunomodulatory study had shown that innate immune cells and T-helper cells are activated by the mushroom sclerotial polysaccharides. A more detailed mechanistic study is required to explain how the innate immune cells such as macrophages are activated by the sclerotial polysaccharides to destroy the tumor cells. Investigation of the receptor-mediated pathways for the immunomodulatory activity of the immune cells by the sclerotial polysaccharides is underway.

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Appendix 1

RayBio® Mouse Cytokine Antibody Array II.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	6Ckine	CTACK	Eotaxin	GCSF	GM-CSF	IL-2	IL-3	IL-4
2	Pos	Pos	Neg	Neg	6Ckine	CTACK	Eotaxin	GCSF	GM-CSF	IL-2	IL-3	IL-4
3	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13	IL-17	IFN-γ	KC	Leptin	MCP-1
4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13	IL-17	IFN-γ	KC	Leptin	MCP-1
5	MCP-5	MIP-1a	MIP-2	MIP-3β	RANTES	SCF	STNF R1	TARC	TIMP-1	TNF-α	Tpo	VEGF
6	MCP-5	MIP-1a	MIP-2	MIP-3β	RANTES	SCF	STNF R1	TARC	TIMP-1	TNF-α	Tpo	VEGF
7	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos
8	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos

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