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Protein chip analysis of cytokines reveals a key mechanism of the antitumor and immunostimulatory activities of polysaccharide from *Lactarius deliciosus* Gray.

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Abstract

Fungal polysaccharides had been approved to show manifold biological activities in the medical and health fields, including antitumor, antioxidant and immune stimulatory. The aim of this paper is to study the mechanism of the antitumor and immune-stimulatory activities of the polysaccharides from *Lactarius deliciosus* Gray (LDG-A) acting on macrophages. A fungal polysaccharide was fractionated and purified by using the DEAE-Cellulose 52 to fast flow column. After that the Sevag method was used for de-proteinization. The LDG-A was used to stimulate macrophages to secrete cytokines. The cytokines that were secreted by macrophages were detected by protein chips. The results indicated that 96 cytokines were up-regulated and 56 cytokines were down-regulated. All cytokines were imported into KEGG PATHWAY Database and NCBI to carry out analysis of the signalling pathway and biological function, respectively. The results showed that LDG-A could induce the immune response of macrophages through a series of signalling pathways. The major signalling pathways were JAK-STAT, PI3K-AKT and NF-kappa B. In addition, ELISA experiment was used to further verify that macrophages stimulated by LDG-A could secrete cytokines through the three major signalling pathways to regulate the antitumor and immunostimulatory activities.

Keywords: Fungal polysaccharide, *L. deliciosus* Gray, Protein chip, Macrophage, Mechanism.

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Introduction

Fungal polysaccharide, also called as polysaccharide, was a kind of bio macromolecule that could be lightly separated from the mycelium, fruiting body, and fermentation broth originating of the large edible and medicinal fungi [1-3]. Since Lentina polysaccharide with antitumor activity was first reported by Japanese scholars, the fungal polysaccharide had aroused the widespread concern of the society, and its chemical structure and biological activity were carried on thorough and meticulous research. The massive researches showed that fungal polysaccharides were polymers with more than 10 monosaccharides are joined by glycosidic bonds. The structures of fungal polysaccharides usually were linear, but some of them presented a high degree of branching [4]. Generally speaking, the molecular weight of fungal polysaccharide was bigger and branching degree of the polysaccharide chain was higher. The ability of immune regulation and anti-tumor activity of fungal polysaccharides was stronger [5]. Fungal polysaccharide was known as the "biological response modifier" in the world. Fungal polysaccharides were not only involved in the formation of cytoskeleton, but also were the important components of many endogenous bioactive molecules. A great deal of researches had shown that fungal polysaccharides had many physiological activities, for instance, anti-tumor, antioxidant and immunostimulant activity [2,6-10]. Fungal polysaccharides would not be digested, decomposed, and absorbed when they entered into the body. They could combine with the receptors on the cell membrane to play pharmacological activity. As a kind of exogenous biological macromolecule, fungal polysaccharide could stimulate macrophages, which released a variety of cytokines to effectively kill tumor cells [11]. As an immunomodulatory agent, fungal polysaccharides had been used in the clinical treatment of various diseases, including autoimmune diseases, tumors, viral infections, etc.

L. deliciosus Gray, named the saffron milk cap and red pine mushroom, belonged to *Basidiomycotina*, *Agaricales*, *Hymenomycetes*, Russulaceae and *Lactarius*. It was a kind of

precious, rare and wild edible fungus. *L. deliciosus* Gray was distributed in the southern Pyrenees as well as all over the Mediterranean basin, in Spain, Bulgaria, Greece, Cyprus, France, Italy etc., [12]. In China, it was mainly distributed in the middle and lower regions of the Yangtze River and coastal areas. In addition, *L. deliciosus* Gray was also distributed in Yunnan, Sichuan, Hainan, Liaoning and Jilin. The fruiting bodies of *L. deliciosus* Gray could produce a large amount of physiological active substances, such as polyisoprene, organic acids, phenols, polysaccharides etc. In view of this, *L. deliciosus* Gray exhibited tremendous potential in biological medicine and biological control of forest diseases and insect pests. In our previous research, a water-soluble polysaccharide (LDG-A) was isolated and purified from *L. deliciosus* (L. ex Fr.) Gray. Its structure was analyzed by spectroscopy technology. The result indicated that the LDG-A was composed of α-L-mannopyranose and α-D-xylopyranose with the ratio of 3:1. Its average molecular weight was about 11 kD. It had a back bone of 1, 6-disubstituted-α-L-mannopyranose which branched at O-2 and the branches were mainly composed of a-3)-α-D-xylopyranose residue (Figure 1) [13]. Further research showed that LDG-A had obvious inhibitory effect on BALB/c mouse solid tumor (S180) *in vivo*. At the same time, it could significantly increase the weight of immune organs (liver, spleen, thymus, etc.) of the test mice, and had no obvious damage to the organ of mice. *In vitro*, the LDG-A could enhance phagocytosis and phagocytic capacity of macrophages (RAW264.7). The antitumor activity of fungal polysaccharide was generally achieved by immune regulation, but the molecular biological mechanism that the LDG-A regulated the immune activity of the macrophage was still unclear. In this paper, the LDG-A was used to stimulate macrophages, and the secretion of the cells was detected by protein chips. The biological functions were analyzed by related bioinformatics tools. The ELISA was used to further verify the protein chip experiment. The aim of this study was to provide scientific basis for the development of polysaccharide targeting immune modulator.

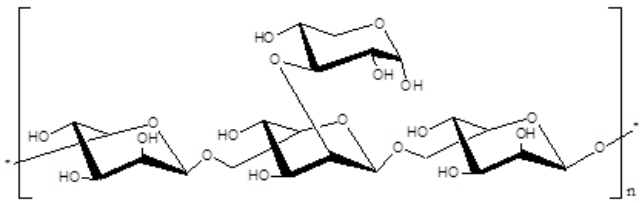

Figure 1. *The structure of polysaccharide from L. deliciosus Gray.*

Materials and Methods

Materials and chemicals

The fruiting bodies of *L. deliciosus* Gray were obtained from Xiaojing country, Sichuan, China. They were authenticated by Prof. Xiang Ding coming from College of Life Sciences, China West Normal University, Nanchong, China. At the same time, a voucher specimen had been preserved in Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), College of Life Sciences, China West Normal University. DEAE-cellulose 52 and Sephadex G-200 were purchased from Sigma-Aldrich (Mainland, China). Monosaccharide standards were purchased from Beijing Biodee Biotechnology Co., Ltd. (Beijing, China). Mouse IL-10 ELISA Kit, Mouse IL-12 (P70) ELISA Kit and Mouse MCP-1 ELISA Kit were purchased from Boster Biological Technology Co., Ltd. (Wuhan city, Hubei Province, China). The other reagents from our own laboratory were of analytical grade.

Fungal polysaccharides isolation from L. deliciosus Gray

Dried comminuted fruiting bodies (200 g) of *L. deliciosus* Gray were soaked with 95% ethyl alcohol for 6 h, and then filtrated to remove lipids. The precipitates were dried to remove ethyl alcohol, and then were extracted with boiling distilled water for three times, 6 h each time. The aqueous extract was retained by concentrating and centrifuging at 12000 g/min for 10 min. The supernatant was added 4-fold volume of 95% ethyl alcohol to precipitate the crude polysaccharides overnight. The precipitate was dried *in vacuo* at 45°C, re-dissolved in the double distilled water, centrifuged at 12000 g/min for 10 min. Sevage method was used to remove the proteins of the crude polysaccharides [14,15]. The crude polysaccharide fluid was fractionated and purified by using DEAE-Cellulose quickly flow column. The purified *L. deliciosus* Gray polysaccharide was named LDG-A.

Cell culture and stimulation

Macrophage cells (RAW264.7, bought from Chengdu Golden Kay Biological Technology Co., Ltd.), were incubated to logarithmic growth phase in culture medium. The media components were as follows: the final concentration of culture solution (200 ml) was 90% RPMI-1640 culture medium, 10% foetal bovine serum and 1% double antibiotic (200 mM L-Glutamine, 10,000 U/ml Penicillin and 10 mg/ml Streptomycin). The cells were diluted to 2×10^5/ml. The cell suspension was plated in 6-well plates, and cultured for 3 d at 37°C, in 5% carbon dioxide incubator. The drug groups were stimulated with serum free medium with LDG-A, and the blank group was added serum free medium. The cells were cultured for the indicated time points. The supernatant was analyzed by protein microarray.

Protein chip experiment

The cell supernatants were sent to The Protein Array Pioneer Company (Ray Biotech, Inc., Guangzhou) for testing.

Expression and quantification analysis of the cytokines

After the completion of the protein chip experiment, all the experimental data were extracted by Array Vision. Quantibody Q-Analyzer software was used to carry out the quantitative data analysis.

KEGG enrichment analyses

All the cytokines were imported into KEGG to carry out the cell signalling pathway analysis. The map of the identified signal pathway was plotted by Pathway Builder Tool 2.0. The biological functions of the related cell factors were analyzed by using NCBI (National Center for Biotechnology Information).

Data processing

In view of the large difference of the experimental data, we took the histogram of the original data on the graph to make it more clearly.

Detection of the IL-10, IL-12p70 and MCP-1 by ELISA

The macrophages (RAW264.7) were cultured at 37°C, in 5% carbon dioxide incubator for 24 h. Serum free medium (2 ml), serum free medium with LPS (2 ml) and serum free medium with LDG-A (2 ml) were used as negative control group, positive control group and drug group, respectively. The supernatants were collected after 24 h and the IL-10, IL-12 (P70) and MCP-1 were detected according to the kit operating instructions.

Statistical analysis

All data were presented as mean ± standard deviation (SD) of three replications. Statistical analyses were performed using student's t-test and one-way analysis of variance. Values of $P<0.05$ were considered to be a statistically significant finding.

Results

Protein microarray experiment

Protein chip was also named protein array. Over the years, in a number of ways, microarray methodology and protein chip had shown its infinite potential. The results were shown in Figure 2. The intensity of fluorescence signal represented the content of cytokines. Figure 2(a) presented the results of protein chip scans of the 200 cytokines. The fluorescence signal intensity comparing the LDG-A group with the blank group was enhanced, which indicated the amount of cytokines was up-regulated. Figure 2(b) exhibited the sketch map of 200 cell factor standard, which included TNF alpha, IL-6, CD40 etc. The results indicated the LDG-A could increase or decrease the levels of cytokines secreted in varying degrees in macrophages, and participated in immune regulation and anti-tumor.

Comparison of two types of cytokines expression in macrophages stimulated by LDG-A

Macrophages belonged to Antigen-Presenting Cell (APC), which played an indispensable role in specific immunity. In order to study the mechanism in macrophages stimulated by LDG-A, the 200 cytokines were tested by protein chips. Through the comparison of expression of cytokines between the LDG-A group and the blank group, all cytokines were divided into two types, which were the down-regulation of cytokines and the up-regulation of cytokines. The results were showed that the down-regulated cytokines were 56 and the up-regulated cytokines were 96 in macrophages induced by LDG-A (Figure 3), which stated clearly that the LDG-A could raise or inhibit the secretion of cytokines by stimulating macrophages to achieve immune regulation.

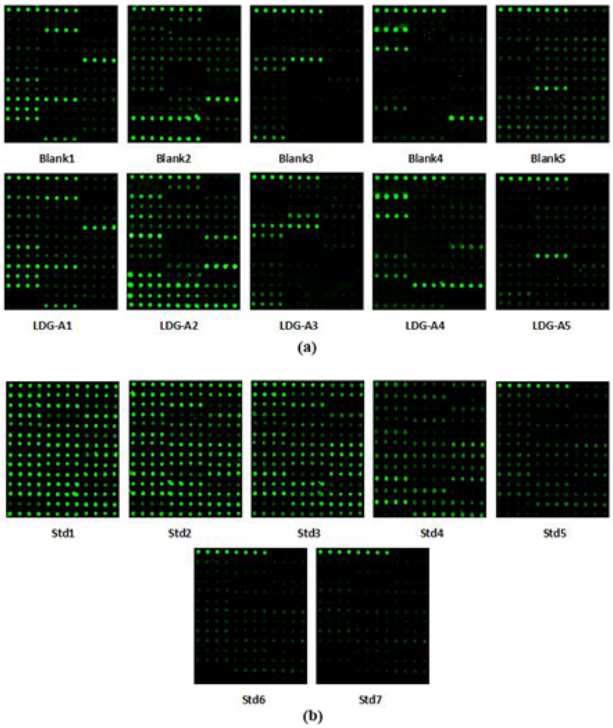

Figure 2. *(a) Original diagram of different protein microarray experiment in macrophages stimulated in LDG-A groups comparing with blank groups; (b) MStandard sketch map of cell factors.*

The up-regulation of cytokines in macrophages stimulated by LDG-A

Cytokines as immune-modulating agents played an important role in health and disease, specifically in immune responses, inflammation. The expression levels of different cytokines in macrophages were significantly different. A comparison was made between the blank group and the LDG-A group in terms of the content of cytokines. The secretions of cytokines increased by 5 times or more were shown in Table 1. Among them, RANTES (2047.46 times), G-CSF (1503.17 times), LOX-1 (1360.61 times) and TNFα (1208.31 times) were increased by more than 1000 times. Lipocalin-2 (943.24 times) and PlGF-2 (767.91 times) were up-regulated between 500 times and 1000 times. A total of 5 cytokines were up-regulated between 100 times and 500 times, which were IL-6 (353.22 times), IL-23 (343.97 times), TARC (201.39 times), MCP-5 (133.44 times) and NOV (111.09 times). The cytokines that were increased between 10 times and 100 times included TREM-1 (65.61 times), GM-CSF (62.94 times), IL-1α (61.17 times), IL-28 (58.30 times), CD40 (43.33 times) etc. Others were up-regulated between 5 times and 10 times. The sketch

map was as Figure 4(a). The results of less than five-fold up-regulation of cytokines in macrophages induced by LDG-A group comparing with the blank group were shown in Table 2, such as TSLP (4.67 times), 4-1BB (4.48 times), MCP-1 (4.39 times), IGFBP-2 (4.32 times), HGF (4.24 times) and so on. Figure 4(b) presented the sketch map of them. These cytokines consisted of interleukins, interferons, tumor necrosis factor, colony stimulating factor, chemotactic cytokine. They played different roles in the immune response, inflammatory response and anti-tumor activity. The results showed the LDG-A could regulate human immune system through inducing macrophages secrete different cytokines.

The down-regulation of cytokines in macrophages stimulated by LDG-A

The results of the protein microarray experiments stated that the cytokines of the up-regulation were 56, such as IL-17B, CD27, etc. the results were showed at Table 3 and Table 4, respectively.

The sketch map was as follow (Figure 5). It could be seen from the Table 3 that the effect of LDG-A on the expression level of cytokines was obviously different. The IL-17B (1662.77 times), Chemerin (404.69 times), TremL1 (224.33 times), Endocan (199.74 times) and IL-17Rα (137.92 times) were down-regulated more than 100 times.

Among these cytokines, Endocan was a kind of endothelial cell-specific molecule 1, which involved in angiogenesis and migration of tumor cells. IL-17B and IL-17Rα belonged to interleukin 17 family. They could act on specific targets in the genome to produce pro-inflammatory chemokine's and promote the development of autoimmune diseases. Under the stimulating of LDG-A, the IL-17B, IL-17Rα and Endocan were down-regulated by 1662.77 times, 137.92 times and 199.74 times, respectively. There were also 5 cytokines that were down regulated with the multiples between 50 times and 100 times.

They were CD27 (96.87 times), Epiregulin (64.01 times), Granzyme B (57.38 times), Fas (52.03 times) and ANGPTL3 (51.47 times). The cytokines that were decreased between 10 times and 50 times was 15, including IL-17B R (46.51 times), IL-7 (42.40 times), EGF (40.00 times) etc.

While the Table 4 presented the cytokines were decreased by less than 10 times in macrophages induced by LDG-A, such as VEGF-B (8.97 times), TCK-1 (8.35 times), IL-15 (8.10 times), TRANCE (8.08 times), 6Ckine (7.62 times), RAGE (6.87 times) etc.

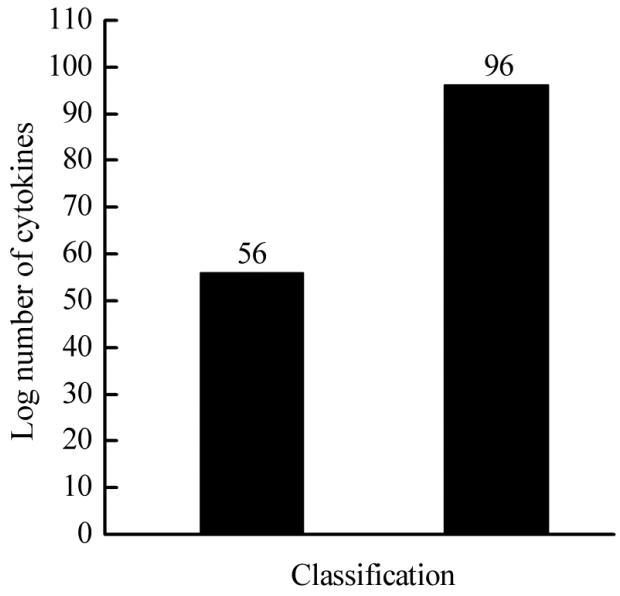

Figure 3. *Down-regulation and up-regulation of cytokines in macrophages stimulated by polysaccharide from L. deliciosus Gray.*

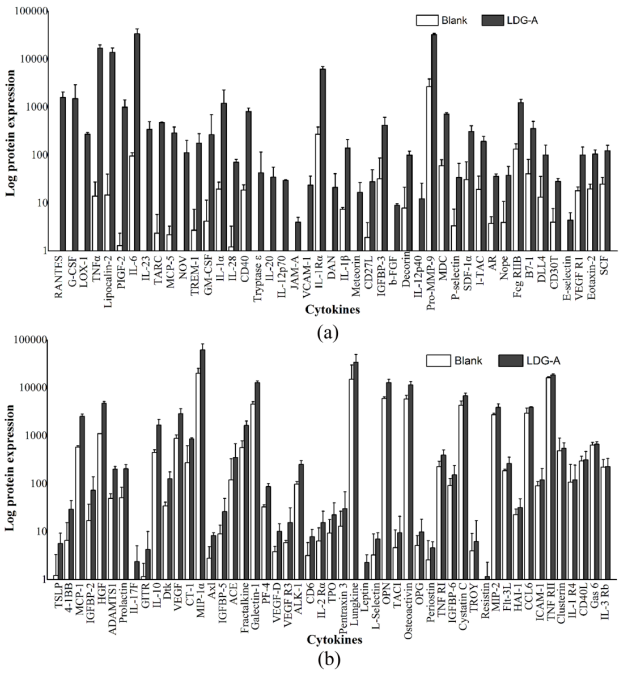

Figure 4. *Up-regulation of the cytokines in macrophages stimulated in the LDG-A group compared with the blank group. (a) The secretion of cytokines was increased by five times or more in the LDG-A group compared with the blank group; (b) The secretion of cytokines was increased less than five times in the LDG-A group compared with the blank group.*

Table 1. *Table showing the more than five-fold up-regulation of cytokines in macrophages induced by LDG-A group comparing with the blank group.*

Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple

RANTES	0.77	1585.55	2047.46	CD40	18.68	809.27	43.33	Pro-MMP-9	2685.23	32272.79	12.02
G-CSF	0	1503.17	1503.17	Tryptase ε	0	42.45	42.45	MDC	59.64	711.82	11.94
LOX-1	0.2	270.54	1360.61	IL-20	0	34.57	34.57	P-selectin	3.35	34.17	10.2
TNFα	13.99	16898.32	1208.31	IL-12p70	0	29.68	29.68	SDF-1α	30.7	308.61	10.05
Lipocalin-2	14.68	13843.93	943.24	JAM-A	0.14	3.97	27.58	I-TAC	19.3	191.76	9.94
PIGF-2	1.3	995.91	767.91	VCAM-1	0	23.58	23.58	AR	3.72	35.78	9.61
IL-6	95.49	33728.7	353.22	IL-1Rα	270.53	6203.88	22.93	Nope	3.95	37.41	9.46
IL-23	0	343.97	343.97	DAN	0	21.13	21.13	Fcg RIIB	132.53	1230.91	9.29
TARC	2.36	475.7	201.39	IL-1β	7.36	139.47	18.94	B7-1	40.49	357.64	8.83
MCP-5	2.15	287.49	133.44	Meteorin	0	16.67	16.67	DLL4	13.18	100.72	7.64
NOV	0	111.09	111.09	CD27L	1.91	27.92	14.62	CD30T	3.96	28.18	7.11
TREM-1	2.7	177.35	65.61	IGFBP-3	31.81	419.94	13.2	E-selectin	0.72	4.37	6.08
GM-CSF	4.22	265.32	62.94	b-FGF	0.68	8.93	13.08	VEGF R1	18.1	100.5	5.55
IL-1α	19.62	1200.32	61.17	Decorin	7.83	99.63	12.72	Eotaxin-2	19.68	104.6	5.32
IL-28	1.21	70.74	58.3	IL-12p40	0	12.34	12.34	SCF	24.79	124.06	5

Note: Each factor set up three repetitions, and included three repeat of blank.

Table 2. *Table showing the less than five-fold up-regulation of cytokines in macrophages induced by LDG-A group comparing with the blank group.*

Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple
TSLP	1.21	5.66	4.67	Fractalkine	562.01	1641.29	2.92	Periostin	2.57	4.58	1.78
4-1BB	6.51	29.16	4.48	Galectin-1	4576.92	12754.23	2.79	TNF RI	228.3	392.4	1.72
MCP-1	579.63	2547.11	4.39	PF-4	32.58	86.88	2.67	IGFBP-6	91.23	151.96	1.67
IGFBP-2	16.94	73.14	4.32	VEGF-D	3.82	10.18	2.66	Cystatin C	4301.28	6816.39	1.58
HGF	1108.69	4706.05	4.24	VEGF R3	5.86	15.39	2.63	TROY	3.95	6.22	1.57
ADAMTS1	49.11	199.96	4.07	ALK-1	97.59	252.24	2.58	Resistin	0.74	1.15	1.55
Prolactin	50.89	205.27	4.03	CD6	3.17	7.87	2.48	MIP-2	2742.07	3888.7	1.42
IL-17F	0.61	2.36	3.89	IL-2 Rα	6.36	15.51	2.44	Flt-3L	186.28	263.98	1.42
GITR	1.14	4.23	3.7	TPO	9.29	22.36	2.41	HAI-1	22.67	31.74	1.4
IL-10	450.25	1657.67	3.68	Pentraxin 3	12.94	29.9	2.31	CCL6	2934.83	3935.3	1.34
Dtk	34.45	125.71	3.65	Lungkine	14921.21	34275.46	2.3	ICAM-1	90.52	119.71	1.32
VEGF	878.73	2857.51	3.25	Leptin	0	2.26	2.26	TNF RII	16214.61	18397.3	1.13
CT-1	273.05	855.86	3.13	L-Selectin	3.26	7.04	2.16	Clusterin	488.92	544.61	1.11
MIP-1α	20041.37	61610.49	3.07	OPN	6019.7	12699.73	2.11	IL-1 R4	107.6	119.67	1.11
Axl	2.77	8.29	2.99	TACI	4.64	9.43	2.03	CD40L	297.73	314.05	1.05
IGFBP-5	8.91	26.09	2.93	Osteoactivin	5831.09	11490	1.97	Gas 6	638.56	667.16	1.04
ACE	120.03	350.69	2.92	OPG	5.12	9.85	1.92	IL-3 Rβ	220.75	228.24	1.03

Note: Each factor set up three repetitions, and included three repeat of blank.

Table 3. *Table showing the more than ten-fold down-regulation of cytokines in macrophages induced by LDG-A group comparing with the blank group.*

Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple
IL-17B	1662.77	0	1662.77	ANGPTL3	51.48	0	51	Fetuin A	400.6	27.11	14.78
Chemerin	404.7	0	404.69	IL-17B R	1010.74	21.73	46.51	TCA-3	3.16	0.22	14.53
TremL1	224.33	0	224.33	IL-7	2.45	0.06	42.4	TACI	278.95	20.09	13.89
Endocan	199.74	0	199.74	IL-22	40.38	0	40	CCL28	254.74	19.58	13.01
IL-7 Rα	137.92	0	137.92	Chordin	216.46	6.64	32.59	SLAM	2400.27	201.55	11.91
CD27	96.88	0	96.87	VEGF-R2	78.29	3.36	23.3	EDAR	11.21	0	11.2
Epiregulin	2121.16	33.14	64.01	EGF	18.52	0	18.51	Shh-N	122.86	11.96	10.27
Granzyme B	132.2	2.3	57.38	TWEAK	491.08	28.85	17.02				
Fas	20.42	0.39	52.03	Activin A	50.62	3.34	15.14				

Note: Each factor set up three repetitions, and included three repeat of blank.

Table 4. *Table showing the less than ten-fold down-regulation of cytokines in macrophages induced by LDG-A group comparing with the blank group.*

Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple	Cytokines	Blank	LDG-A	Multiple
VEGF-B	416.03	46.39	8.97	Galectin-7	931.27	166.85	5.58	Marapsin	138.47	34.45	4.02
TCK-1	88.44	10.6	8.35	P-Cadherin	5.41	0	5.4	CD36	2656.42	710.83	3.74
IL-15	8.09	0	8.1	Adiponectin	5.32	0	5.3	BLC	6.2	1.94	3.2
TRANCE	468.35	57.93	8.08	MIP-1b	14.52	2.74	5.3	MMP-2	208.15	66.17	3.15
6Ckine	175.79	23.08	7.62	IL-3	7.29	1.38	5.27	CRP	5.17	1.66	3.11
RAGE	6.87	0	6.87	Artemin	5.16	0	5.1	Leptin R	16.76	5.73	2.93
IFN-γ R1	9.42	1.38	6.83	IL-17E	5.11	0	5	IL-17	7.24	3.18	2.28
MMP-3	170.95	27.39	6.24	Persephin	66.69	13.62	4.89	IL-4	9.17	4.2	2.19
ANG-3	170.29	27.69	6.15	gp130	4.48	0	4.5	TECK	523.22	249.51	2.1
M-CSF	21.77	3.58	6.09	Epigen	85.22	20	4.26				
sFRP-3	128.63	21.58	5.96	E-Cadherin	86.11	20.53	4.19				

Note: Each factor set up three repetitions, and included three repeat of blank.

Signalling pathway analysis in macrophages stimulated by LDG-A

The KEGG pathway analysis indicated LDG-A induced the macrophage immune response by a large number of signalling pathways, for instance, Fc-epsilon RI signalling pathway, JAK-STAT signalling pathway, NF-kappa B signalling pathway, Inflammatory Bowel Disease (IBD), PI3K-Akt signalling pathway and Toll-like receptor signalling pathway.

Among them, three main pathways were selected from all signalling pathway. They were PI3K-Akt signalling pathway (Figure 6), JAK-STAT signalling pathway (Figure 7) and NF-kappa B signalling pathway (Figure 8).

PI3K-Akt signalling pathway (Figure 6) was the most important signal transduction about cell survival at present. It was activated by two approaches. One of them was interacted with connection protein or growth factor receptor of phosphorylated tyrosine residues to lead the conformational change of the polymers, and then the PI3K was activated. The other one was the PI3K activation by direct binding of p110 and p85. Phosphatidylinositol 3-Kinase (PI3K) could phosphorylate the D3 in the lipid kinase. Through various growth factors, including EGF (decreased by 18.5 times

comparing the LDG-A group with the blank group), HGF (4.25 times), VEGF (3.25 times) etc., acted on the membrane receptors, the PI3K signalling pathway was activated.

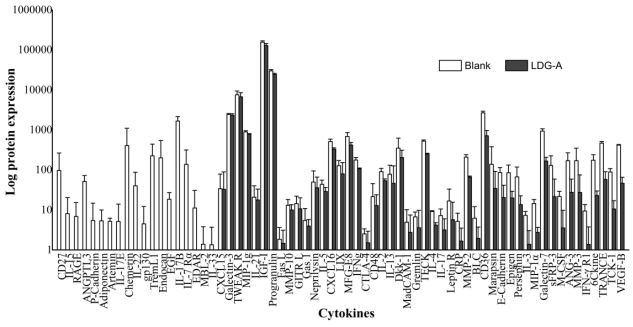

Figure 5. *Down-regulation of the cytokines in macrophages stimulated in the LDG-A group compared with the blank group.*

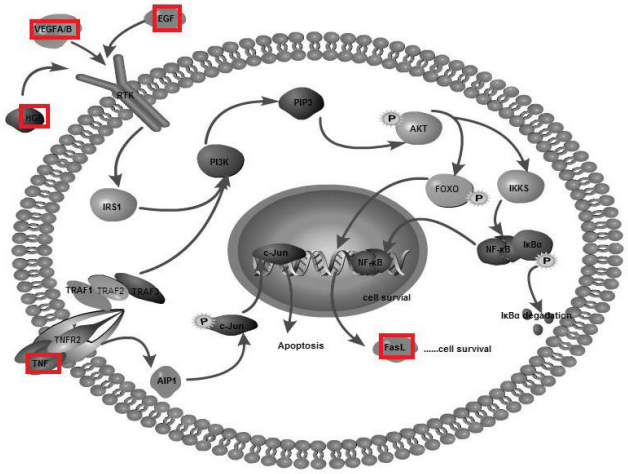

Figure 6. *PI3K-Akt signalling pathway in macrophages stimulated by polysaccharides from L. deliciosus Gray. Note: Red box presented cytokines from experimental data.*

Figure 7 indicated the ligands (IL6, TSLP, IL23 and G-CSF) bonded with special receptors and leaded to receptor dimerization. Then the receptor combined with JAK kinase to cause phosphorylation of tyrosine residues of the receptor cytoplasmic tail. The tyrosine residues of phosphorylation would serve as a binding site for stat. STAT was phosphorylated by Src homology domain 2 (SH2) in the domain of a tyrosine phosphate interaction to dimerization and transferred into the nucleus. As a transcription activator, it promoted gene expression in the nucleus.

Figure 8 represents NF-kappa B signal pathway, which included two activation pathway. One of them belonged to MyD88 dependent signal transduction pathway. IL-1β boned with membrane receptor. The signal would be transmitted to the cell interior across the cytomembrane. The MyD88 combined with serine/threonine of Irak kinase, and further activated TRAF-6. The TRAF-6 that was activated could phosphorylate NF kappa B inhibitor kinase. The I kappa B was degraded eventually by the proteasome. The NF kappa B was released from the inactivated state I kappa B/NF-kappa B trimer, and then was transferred into the nucleus from the cytosol. It combined with unique kappa B sequence in the nucleus and promoted the expression of MIP-2, COX2 and TNFα. The other one belonged to the MyD88 un-dependent signal transduction pathway. The TNF-α combined with TNF-R1 and further promoted the boned between RIP1 and TRADD, which could activate TRAF2/5. The TRAF2/5 that was activated could phosphorylate NF kappa B inhibitor kinase. The next process was similar with the signal transduction pathway that MyD88 was dependent. Eventually, the BLC and SDF-1α were expressed by NF-kappa B signal pathway. The pathway of CD40 activated TRAF2/3, which stimulated the NF-kappa B-Inducible Kinase (NIK) to induce BLC, SDF-1α.

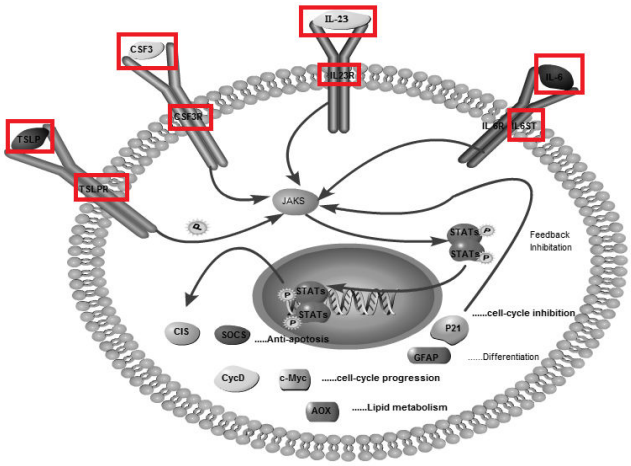

Figure 7. *JAK-STAT signalling pathway in macrophages stimulated by polysaccharides from L. deliciosus Gray. Note: Red box presented cytokines from experimental data.*

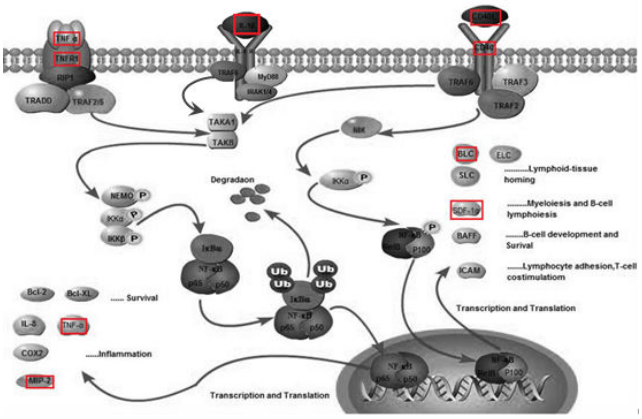

Figure 8. *NF-kappa B signalling pathway in macrophages stimulated by polysaccharides from L. deliciosus Gray. Note: Red box presented cytokines from experimental data.*

Detection of the IL-10, IL-12 (P70) and MCP-1 by ELISA

The ELISA was used to detect the expression of IL-10, IL-12 (P70) and MCP-1. The result was shown in Figure 9. The expression levels of IL-10, IL-12 (P70) and MCP-1 were very significantly increased under the stimulating of LDG-A ($P<0.01$). Among them, the secretion of IL-10 in the blank

group, LPS group and LDG-A group were 1327.35 pg/ml, 3135.03 pg/ml and 13791.47 pg/ml, respectively.

Although the secretion of IL-10 in the drug group was significantly less than that in the positive control group, it was significantly increased comparing with the blank group. This showed that LDG-A could significantly promote the secretion of IL-10 in macrophages. It was worth noting that IL-10 belonged to important anti-inflammatory cytokines, which could prevent and inhibit tissue damage caused by strong specific and non-specific immune responses.

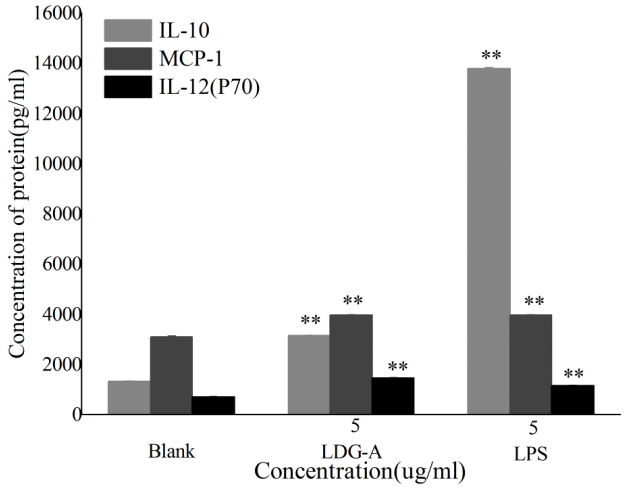

Figure 9. *ELISA analysis of the expression of IL-10, IL-12p70 and MCP-1 in macrophages stimulated by LDG-A.*

Studies had shown that IL-10 was produced in macrophages *via* the PI3K-Akt pathway, which indicated that LDG-A could stimulate macrophages to secrete cytokines through PI3K-Akt pathway to regulate immune-stimulatory activities [1].

The secretion of IL-12 (P70) in LDG-A group (1453.46 pg/ml) was significantly higher than that in blank group (706.66 pg/ml) and slightly higher than that in LPS group (1133.78 pg/ml), which clearly indicated that LDG-A could significantly promote the secretion of IL-12 (P70) in macrophages, and the effect was better than LPS. IL-12 could stimulate macrophages to secrete large amounts of TNF- gamma, TNF-alpha and NO, which directly killed target cells, induced tumor cell apoptosis or non-specifically enhanced killing function of macrophages.

Studies had shown that the biological function of IL-12 was achieved mainly *via* the JAK-STAT4 signalling pathway [16]. It suggested that LDG-A could regulate antitumor activity *via* JAK-STAT pathway. In addition, the concentration of MCP-1 was 3089.48 pg/ml (blank group), 3961.39 pg/ml (LDG-A-treated group) and 3960.18 pg/ml (LPS-treated group), respectively. Compared with the blank group, the increase of the secretion of MCP-1 was also very significant ($P<0.01$). A large number of studies had confirmed that the NF kappa B signalling pathway was critical for regulation of the expression of MCP-1 [17].

For example, in the mouse model of liver and colon cancer, the combination of suicide gene and MCP-1 could effectively enhance the antitumor activity of the organism [18]. In recent years, there were experiments pointed out that mouse breast cancer cell metastasis ability was significantly increased after *MCP-1* gene was knocked [19]. It indicated that LDG-A could stimulate macrophages to secrete MCP-1 by NF-κ B signalling pathway to regulate the antitumor and immune-stimulatory activities. Under the stimulating of LDG-A, the expression levels of IL-10, IL-12 (P70) and MCP-1 were very significantly increased ($P<0.01$), which indicated that macrophages stimulated by LDG-A could secrete cytokines to regulate antitumor and immune-stimulatory activities through JAK-STAT signal pathway, PI3K-Akt signal pathway and NF-kappa B signal pathway.

Discussion

Fungal polysaccharides had been attracted widespread attentions, because of the broad spectrum of the therapeutic properties. The functions of LDG-A had been reported at the field of human diseases, but the molecular biological mechanism that the LDG-A regulated the immune system was still seldom. In this study, we used the LDG-A to stimulate macrophages and then the supernatant was selected to carry out biological analysis by protein microarrays. The results showed the cytokines of up-regulation were up to 96 and cytokines of down-regulation were up to 56. In order to further study the mechanism of the LDG- A for the human immune system, all cytokines were imported into KEGG software to do the signal pathway analysis. The results indicated the LDG-A could induce the macrophage immune response and anti-tumor by JAK-STAT signalling pathway, PI3K-Akt signalling pathway and NF-kappa B signalling pathway. ELISA experiment was carried out to further verify the three signal pathways.

Janus Kinase (JAK) belonged to a intracellular, non-receptor Protein Tyrosine Kinase (PTK) that could mediate the activation of cytokines and its receptor *via* JAK/STAT pathway in the signal protein cascade [20]. JAK/STAT signalling pathway was a significant signal transduction pathway, which involved in cell proliferation and migration, apoptosis and immune regulation. It was essential for growth, development, and homeostasis, such as haematopoiesis, immune cell development, the development of stem cells the growth of an organism, mammary gland development etc. [21]. JAK-ATAT signalling pathway was mainly composed of three parts, including cell surface receptors, Janus Kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) protein. The JAK-STAT signal pathway initiates transcription of related genes by transferring extracellular chemical signals into the cell. It is involved in the regulation of multifarious biological activity, such as immune regulation, proliferation, differentiation, apoptosis and carcinogenesis [22]. In this experiment, the IL6, IL23, TSLP and G-CSF were secreted by macrophages stimulated by LDG-A were increased by 353.22 times, 343.97 times, 4.67 times and 1503.17 times, respectively. The pathway analysis indicated that LDG-A could regulate the immune and antitumor activity, including cell proliferation, differentiation, apoptosis, immune dysfunction

and tumor formation by activating JAK-STAT signalling pathway in macrophages.

Phosphoinositide 3 Kinase, PI3K/AKT, was a kind of signal transduction pathway that could promote cell survival or block apoptosis by transcriptional regulation or direct phosphorylation. It was the most important signal transduction about cell survival at present. After the stimulation of LDG-A, EGF, HGF and VEGF were decreased by 18.5 times, 4.25 times, 3.25 times, respectively, comparing the LDG-A group with the blank group. The results indicated the PI3K-AKT signalling pathway was activated. It showed that LDG-A could induce the immune response of macrophages through PI3K-Akt signalling pathway.

NF kappa B was a pleiotropic nuclear transcription factor that regulated immune responses of the body, including angiogenesis, atherosclerosis, cell proliferation and apoptosis, inflammation and acute response, etc. [23]. The main functions some of them were to regulate cell proliferation, apoptosis, immune inflammatory reaction, which played a key role in macrophages activation [24-31]. As shown in picture 8, LDG-A could effectively promote activation macrophages to secrete immunity factors by NF-κB signal pathway, such as CD40, TNF-α, CD40L, BLC, SDF-1α etc.

CD40 (Cluster of Differentiation 40), this gene belonged to the TNF-receptor superfamily, which encoding protein was a kind of receptor on the surface of antigen-presenting cell of the body immune system. It was essential that the protein mediated a wide variety of immune and inflammatory reaction. Such as memory B cells development, T cell-dependent immunoglobulin class switching, and germinal center formation.

TNF-α (Tumor Necrosis Factor α) was a family of the Tumor Necrosis Factor (TNF) superfamily, which encoded a multifunctional cytokine to pro-inflammatory. It played an important role in the innate immune response as well as regulated homeostasis and also implicated in diseases of chronic inflammation. In our research, after the LDG-A stimulated macrophages, the secretion of the IL-1β, CD40 and TNFα was increased by 8.94 times, 43.33 times, 1208.31 times, respectively. The results showed that LDG-A could secrete tumor necrosis factor and interleukin in macrophages to regulate the body's anti-tumor immunity and inhibit tumor cells or lead to tumor cells necrosis by NF-kappa B signal pathway.

Meanwhile, we selected some cytokines for discussion. They were MCP-5, RANTES, GM-CSF, LOX-1, IL-17B, IL-17E and RAGE. It is worth noting that these factors did not appear in the above pathway, but played an important role.

Both MCP-5 and RANTES belonged to chemokine's. Comparing with the blank group, the RANTES was increased 2047.46 times and MCP-5 was increased by 133.44 times. RANTES (chemokine ligand 5) was also named CCL5 (Chemokine (C-C motif) ligand 5), which was a protein that regulated and activated normal T cell expressed and secreted. It could enhance the antigen specific immune response by inducing the production of specific antibodies. In addition, under the induction of cytokine receptors and co-stimulatory molecules as well as assist of Th1 and Th2 cytokines, RANTES could enhance mucosal immunity and systemic humoral immunity. It was produced by Toll-like receptor signalling pathway in macrophages. In this study, compared with the blank group, the RANTES increased 2047.46 times, which indicated indirectly that the LDG-A could promote the expression of RANTES by activating Toll-like receptor signalling pathway in macrophages to regulate the immune response.

MCP-5 (133.44 times) might be the downstream target gene of *TNF-gamma* gene. TNF-γ activated JAK-STAT1 signalling pathway to produce MCP-5. It could induce myeloid immature dendritic cells infiltration and aggregation in tumor epithelial layer. The synergistic effect of MCP-5 and ICAM2 inhibited the occurrence and development of tumor. The results showed that LDG-A could activate JAK-STAT1 signalling pathway to secret MCP-5 in macrophages, and regulate the immune stimulation and antitumor activity.

LOX-1 (1360.61times) belonged to the C type lectin superfamily, whose genes were regulated by cyclic AMP signalling pathway. LOX-1 bound, internalized and degraded the oxidized low density lipoprotein. The protein might be involved in Fas induced apoptosis. Studies had shown that TNF could enhance the expression of LOX-1, and activate the Mitogen Activated Protein Kinase (MAPK) and Protein Kinase (PK), which leaded to the activation of the transcription factor nuclear factor (NF-kappa B). NF kappa B that was activated released ROS. ROS played an important role in the body's defense system, for instance, killing bacteria or parasites. Macrophages could kill tumor cells by reactive oxygen species. LOX-1 was increased by 1360.61 times. The results showed the LDG-A could increase the levels of the cytokines secreted by macrophages to enhance the immune activity and inhibit the growth of tumor cells or kill the tumor cells directly.

LDG-A also has a certain influence on the interleukin family. Lipocalin-2 belonged to a protein encoded by *LCN2* gene, which involved in innate immunity through sequestrating iron that in turn limited bacterial growth. It was also used as biomarker of kidney injury to help patient avoid kidney dialysis.

It is worth noting that GM-CSF was a cell colony factor. It could promote the proliferation, differentiation and functional maturation of granulocytes and macrophages, and improved host defences. Under the action of LDG-A, the expression of GM-CSF was significantly up-regulated, indicating that LDG-A could enhance the immune function of the body by inducing the high expression of GM-CSF in macrophages.

IL-17B and IL17-E belonged to the interleukin-17 family, which were pro-inflammatory cytokine. IL-17B and IL17-E exerted biological effects by binding to specific receptors to activate IL-17 signalling pathway. These functions included inflammation, immune response, immune rejection, and so on. Under the action of LDG-A, IL-17B was reduced by 1662.77

times compared with the blank group, and IL-17E was reduced by 5.11 times, which showed that LDG-A could enhance the immune function and anti-inflammatory by inhibiting interleukin 17 signalling pathway.

Macrophages could participate in specific immune response, and played an anti-tumor effect by cooperating with sensitized T cells, specific antibodies and complement. Fas belonged to a kind of tumor necrosis factor receptor. Fas could bind to the ligand (FasL) on the surface of macrophages, and activate the Fas/FasL signalling pathway, which induced apoptosis of macrophages, and thereby decreased the specific immune response and antitumor effect. In our research, the secretion of the Fas was decreased by 52.03 times, which indicated the LDG-A could also enhance the immune response and antitumor effect by Fas/FasL signalling pathway in macrophages.

RAGE was reduced by 6.87 times. It belonged to the receptor of advanced glycation end products, and could activate multiple signal transduction pathways after binding with its ligand AGEs, for instance, P21ras Mitogen Activated Protein Kinase (MAPK) and nuclear factor Kappa B. Especially the NF- kappa B was activated. Studies had reported that activation of NF-Kappa B activated by RAGE was closely associated with diabetes mellitus. The activated pathway leaded to the release of a variety of cytokines and growth factors, resulting in vascular endothelial injury, hemodynamic, abnormal blood rheology, abnormal cell proliferation and other pathological changes. Therefore, it could be concluded that LDG-A could regulate the immune function through the RAGE inhibited the NF-Kappa B signalling pathway.

In conclusion, immunocytes could be activated by LDG-A, and then secreted tumor necrosis factor and interleukin to regulate the body's anti-tumor immunity and other related aspects. This research indicated that macrophages induced by LDG-A, secreted cytokines through three major signalling pathways, which were JAK-STAT signalling pathway, PI3K-Akt signalling pathway and NF-kappa B signalling pathway. The ELISA results further proved that the major signalling pathways were JAK-STAT, PI3K-Akt and NF-kappa B.

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References

1. Du Q, Huang H, Wang TT, Zhang XJ, Chen Y, Cui BB, Li DL, Zhao XM, Zhang WL , Chang LL, Tong DW. Porcine circovirus type 2 activates PI3K/Akt and p38 MAPK pathways to promote interleukin-10 production in macrophages via Cap interaction of gC1qR. Oncotarget 2016; 7: 17492-17507.
2. Hou Y, Ding X, Hou W, Song B, Wang T, Wang F, Zhong J. Immunostimulant activity of a novel polysaccharide isolated from Lactarius deliciosus (L. ex Fr.) Gray. Indian J Pharm Sci 2013; 75: 393-399.
3. Zong A, Cao H, Wang F. Anticancer polysaccharides from natural resources: a review of recent research. Carbohydr Polym 2012; 90: 1395-1410.
4. Bertozzi CR, Kiessling LL. Chemical glycobiology. Sci 2001; 291: 2357-2364.
5. Zhu ZY, Liu N, Si CL, Liu Y, Ding LN. Structure and anti-tumor activity of a high-molecular-weight polysaccharide from cultured mycelium of Cordyceps gunnii. Carbohydr Polym 2012; 88: 1072-1076.
6. Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. Mushrooms, tumors and immunity. Proc Soc Exp Biol Med 1999; 221: 281-293.
7. Chang ST, Wasser SP. The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. Int J Med Mushrooms 2012; 14: 95-134.
8. Chen Y, Xie MY, Nie SP, Li C, Wang YX. Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of Ganoderma strum. Food Chem 2008; 107: 231-241.
9. Wasser SP. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Apl Microbiol Biotechnol 2002; 60: 258-274.
10. Wasser SP. Medicinal mushroom science: Current perspectives, advances, evidences, and challenges. Biomed J 2014; 37: 345-356.
11. Liu Q, Zhu MG, Geng XR, Wang HX, Tzi BN. Characterization of polysaccharides with antioxidant and hepatoprotective activities from the edible mushroom Oudemansiella radicata. Molecules 2017; 22: 234.
12. Gevelt TV. Community-based management of Tricholoma matsutake (S. Ito and S. Imai) Singer: a case study of South Korean mountain villages. Int J Commons 2014; 8: 134-154.
13. Ding X, Hou YL, Hou WR. Structure feature and antitumor activity of a novel polysaccharide isolated from Lactarius deliciosus Gray. Carbohydr Polym 2012; 89: 397-402.
14. Bode L. Human milk oligosaccharides: prebiotics and beyond. Nutr Rev 2009; 67: 183-191.
15. Ding X, Hou YL, Hou WR, Zhu YX, Fu L, Zhu HQ. Structure elucidation and anti-tumor activities of water-soluble oligosaccharides from Lactarius deliciosus (L. ex Fr.) Gray. Pharmacogn Mag 2015; 11: 716-723.
16. Park WR, Park CS, Tomura M. CD28 costimulation is required not only to induce IL-12 receptor but also torender janus/STAT4 responsive to IL-12 stimulation in TCR-triggered T cell. Eur J Immunol 2001; 31: 1456-1464.
17. Chang KK, Liu LB, Li H, Mei J, Shao J, Xie F, Li MQ, Li DJ. TSLP induced by estrogen stimulates secretion of MCP-1 and IL-8 and growth of human endometrial stromal

cells through JNK and NF-κB signal pathways. Int J Clin Exp Pathol 2014; 7: 1889.

18. Tsuchiyama T, Nakamoto Y, Sakai Y, Mukaida N, Kaneko S. Optimal amount of monocyte chemoattractant protein-1 enhances antitumor effects of suicide gene therapy against hepatocellular carcinoma by M1 macrophage activation. Cancer Sci 2008; 99: 2075.
19. Takahashi M, Miyazaki H, Furihata M, Sakai H, Konakahara T, Watanabe M, Okada T. Chemokine CCL2/MCP-1 negatively regulates metastasis in a highly bone marrow-metastatic mouse breast cancer model. Clin Exp Metas 2009; 26: 817-828.
20. Mukthavaram R, Ouyang X, Saklecha R, Jiang P, Nomura N, Pingle SC, Guo F, Makale M, Kesari S. Effect of the JAK2/STAT3 inhibitor SAR317461 on human glioblastoma tumorspheres. J Transl Med 2015; 13: 269.
21. Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. Immunol Rev 2009; 228: 273-287.
22. Duzagac F, Inan S, ElaSimsek F, Acikgoz E, Guven U, Khan SA, Rouhrazi H, Oltulu F, Aktug H, Erol A. JAK/STAT pathway interacts with intercellular cell adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) while prostate cancer stem cells form tumor spheroids. J Buon 2015; 20: 1250-1257.
23. Pamukcu B, Lip GY, Shantsila E. The nuclear factor-kappa B pathway in atherosclerosis: a potential therapeutic target for atherothrombotic vascular disease. Thromb Res 2011; 128: 117-123.
24. Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am J Physiol Lung Cell Mol Physiol 2006; 290: 622-645.
25. Chen JC, Tian S, Shu XY, Du HT, Li N, Wang JR. Extraction, characterization and immunological activity of polysaccharides from Rhizoma gastrodiae. Int J Mol Sci 2016; 1: 1011.
26. Førland DT, Johnson E, Tryggestad AM, Lyberg T, Hetland G. An extract based on the medicinal mushroom Agaricusblazei Murill stimulates monocyte-derived dendritic cells to cytokine and chemokine production in vitro. Cytokine 2010; 49: 245-250.
27. Hetland G, Johnson E, Lyberg T, Bernardshaw S, Tryggestad AM, Grinde B. Effects of the medicinal mushroom Agaricusblazei Murill on immunity, infection and cancer. Scand J Immunol 2008; 68: 363-370.
28. Hou Y, Ding X, Hou W, Song B, Wang T, Wang F, Li J, Zeng Y, Zhong J, Xu T, Zhu H. Pharmacological evaluation for anticancer and immune activities of a novel polysaccharide isolated from Boletus speciosus Frost. Mol Med Rep 2014; 9: 1337-1344.
29. Klupp NL, Chang D, Hawke F, Kiat H, Cao H, Grant SJ, Bensoussan A. Ganoderma lucidum mushroom for treating cardiovascular risk factors. Cochrane Database Syst Rev 2015; 17: CD007259.
30. Xiao JH, Zhong JJ. Secondary metabolites from Cordyceps species and their antitumor activity studies. Recent Pat Biotechnol 2007; 1: 123-137.
31. Yue K, Ye M, Zhou Z, Sun W, Lin X. The genus Cordyceps: a chemical and pharmacological review. J Pharm Pharmacol 2013; 65: 474-493.

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