

Studies on the immuno-modulating and anti-tumor activities of *Ganoderma lucidum* (Reishi) polysaccharides

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Abstract—We describe here the isolation of Reishi polysaccharides for the study of their effect on cytokine expression in mouse splenocytes. A fraction (F3) has been shown to activate the expression of IL-1, IL-6, IL-12, IFN- γ , TNF- α , GM-CSF, G-CSF, and M-CSF, and from this three subfractions have been prepared where F3G1 activates IL-1, IL-12, TNF- α , and G-CSF, F3G2 activates all the cytokines as F3 does, and F3G3 activates only IL-1 and TNF- α . Together with previous studies, the mode of action on macrophages has been proposed where F3 binds to TLR4 receptor and activates extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 to induce IL-1 expression.

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1. Introduction

Ganoderma lucidum (Reishi or Ling-Zhi) has been used as health-promotion supplement owing to its anti-tumor and immuno-modulating effects.¹ The activities of liver protection, hypoglycemia, and platelet-aggregation inhibition have also been demonstrated from the fruiting bodies and cultured mycelia of Reishi,^{1–4} in addition to its use for hypertension and neoplasia.⁵ Previous studies have shown that the water soluble, polysaccharide components of Reishi exhibit anti-tumor activity^{6,7} and reduce tumor metastasis.⁸ When Reishi polysaccharides (RPS) were given alone or in combination with cytotoxic and anti-tumor drugs, the lifespan of tumor-implanted mice was prolonged,^{5b} presumably due to the activation of host immune response.⁹ Furthermore, it is interesting that RPS are able to increase the CD4

and T-cell counts during or after chemotherapy.^{6,7} The saccharides were known to contain either a β -1,3-glucan¹⁰ or α -1,4-mannan backbone,¹¹ both of which have side chains of unknown structure. Although the receptor complement receptor type three (CR3) has been shown to bind the β -glucan polysaccharides,^{7b} the real receptor, and carbohydrate epitope responsible for the anti-tumor activity remain to be established.

Recently we reported the identification of a glycoprotein fraction which, isolated from the water-soluble extract of *G. lucidum*,¹² can stimulate spleen cell proliferation and cytokine expression. Herein we present further purification of the active fraction to enrich the immuno-modulating activity, as well as the detailed analyses of cytokine expression, especially that of GM-CSF and IFN- γ . Together with our previous studies on the signaling pathway of RPS, we propose its mode of action on macrophages that stimulates the immune response.

2. Results and discussion

The crude extract of Reishi is currently commercially available from Pharmanex Co., as a dietary supplement for the immune system. To identify the active

Abbreviations: RPS, Reishi polysaccharides; CR3, complement receptor type three; LPS, lipopolysaccharide; TLR, Toll-like receptor; PTK, protein-tyrosine kinase; PKC, protein kinase C; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PAK, p21-activated kinase; p38, p38-mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; WSR, water soluble extract of Reishi.

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components in this crude extract, gel filtration chromatography of this crude extract was carried out by using a Sephacryl S-500 column eluted with 0.1N Tris buffer (pH 7.0), and the sugar content of each fraction was determined by anthrone analysis or phenol–sulfuric acid method. Among five fractions collected, the main fraction was designated as F3 (30% yield based on the water-soluble extract of Reishi, WSR).¹² On the basis of the colorimetric assay using MTT for cell proliferation and RT–PCR experiments to detect the cytokines expressed in mouse spleen cells, our previous results indicated that the active component of Reishi is highly located in F3.¹² Ten cytokines were tested including IL-1, IL-2, IL-4, IL-6, IL-12, IFN- γ , TNF- α , GM-CSF, G-CSF, and M-CSF. Most of them were up-regulated significantly (Fig. 1), when compared with the negative (no treatment) and positive (treated with Con A) controls. The results implied that F3 was able to stimulate the inflammatory response due to the expression of IL-1, IL-6, and TNF- α . The expression of IFN- γ and TNF- α was relevant to the anti-tumor activity, and that of GM-CSF, G-CSF, and M-CSF was possibly associated with hematopoiesis. The former finding is consistent with the prior documented evidence that the elevated release of TNF- α and IFN- γ from the lymphocytes contributes to the anti-tumor effect of RPS.^{7a} The latter discovery is significant according to the recent reports that the anti-apoptotic effect of RPS on neutrophils primarily relies on the expression of GM-CSF.¹³

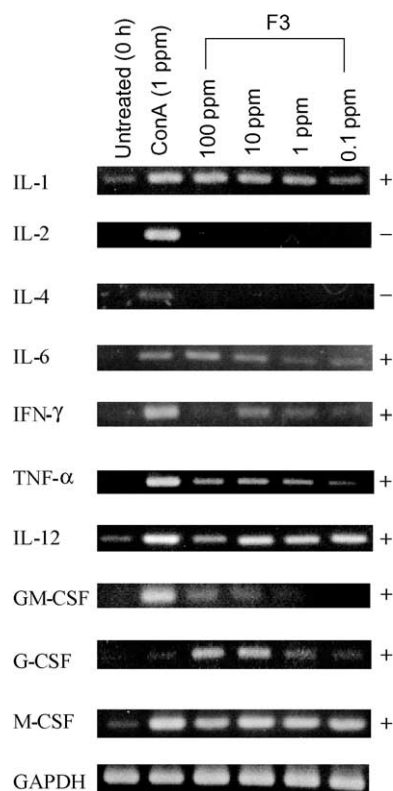


Figure 1. Gel electrophoresis of the RT–PCR studies to show the expression of 10 different cytokines by the incubation of mouse spleen cells with or without F3 at the concentration of 0.1, 1, 10, 100 ppm for 6h.

Since IL-12, instead of IL-4, was expressed in the splenocyte incubation with F3, the development of the T_H1 subset was likely involved in the cell-mediated response. Based on the consideration that IFN- γ up-regulates IL-12 production and activates the IL-12 receptor on activated T cells, T_H1 development was also dependent on IFN- γ . Moreover, although the crude Reishi extract and F3 both induced a similar pattern of cytokine expression at the same dosage (0.1–100 μ g/mL, as shown in Table 1), the latter showed the expression at a much higher level, especially in the expression of IL-1 β , IL-6, IFN- γ , TNF- α , GM-CSF, and G-CSF, which supported the previous conclusion that the major activity of crude Reishi extract is concentrated in F3.

When F3 was subjected to anion exchange chromatography with a Diaion–WA30 column, two fractions were produced by eluting the column with 0.2 and 0.8M NaCl and designated as F3G1 (11% yield based on F3) and F3G2 (10% yield based on F3), respectively (Fig. 2). Further elution with 2M NaOH gave another fraction as F3G3 (11% yield based on F3). According to the RT–PCR studies for the cytokine expression (Table 1), the treatment with F3G2 led to significant expression of the eight cytokines aforementioned, indicating that this fraction contains the enriched active components of F3. Because the expression of TNF- α and IL-1 were detectable in the studies of F3G1 and F3G3, it is of interest that both fractions can trigger only the inflammatory pathway, unlike F3 or F3G2.

The additional gel-filtration chromatography of F3G2 on a TSK HW-75 column resulted in two fractions—G2H1 (19% yield based on F3G2) and G2H2 (69% yield based on F3G2), as shown in Figure 3. The preliminary result from the RT–PCR studies revealed that the former fraction contains much higher activity than the same dosage of F3G2 and G2H2 in the expression of IL-1 β , IL-6, IFN- γ , TNF- α , and GM-CSF. The structural and compositional investigations of G2H1 are currently in progress.

On the basis of colorimetric sandwich ELISA, the quantitative measurement for the expressed cytokines revealed an intriguing feature. As shown in Figure 4, the individual treatment of mouse splenocytes with crude Reishi extract, F3 and F3G2 led to the GM-CSF production at the concentration of 0.91, 10.4, and 24.0 pg/mL, respectively, after incubation for 72h. Similar enhancement was observed when the ELISA assay was carried out for the IFN- γ expression (Fig. 5). The treatment with F3G2 and F3 was able to stimulate the spleen cells to generate 143 and 8.6 pg/mL IFN- γ , respectively, after the 48-h incubation.

It is important to distinguish if our purified fractions from RPS were contaminated with lipopolysaccharides (LPS), the well-known immunogenic glycoconjugates. Figure 6 demonstrated the dose dependent profile of F3G2 and LPS. It appeared that LPS reached a plateau at the concentration of 10 μ g/mL. On the other hand, a sigmoidal curve was obtained for F3G2. In particular, F3G2 was more active than LPS at the dosage higher than 100 μ g/mL.

Table 1. Cytokine expression of mouse splenocytes treated with different Reishi samples^a

Entry	Samples	Cytokine expression ^b									
		IL-1 β	IL-2	IL-4	IL-6	INF- γ	IL-12	TNF- α	GM-CSF	G-CSF	M-CSF
1	WSR ^c	+	–	–	±	±	+	+	–	+	+
2	F3	+	–	–	+	+	+	+	+	+	+
3	F3G1	+	–	–	–	–	+	+	–	+	±
4	F3G2	+	–	–	+	+	+	+	+	+	+
5	F3G3	+	–	–	–	–	±	+	–	±	±

^a Each sample was evaluated at 10^2 , 10^1 , 10^0 , and 10^{-1} $\mu\text{g/mL}$ with the splenocytes (3×10^6 cells/mL) incubated at 37°C with 5% CO_2 .

^b +, indicating a significant increase of cytokine expression; –, indicating no increase of cytokine expression; ±, showing an increase but not significant of cytokine mRNAs.

^c WSR: water soluble extract of Reishi.

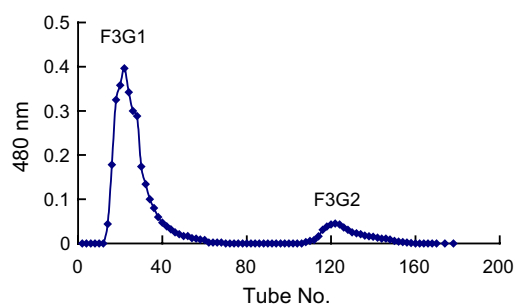


Figure 2. Anion exchange chromatography was carried out by using a column of Diaion-WA30 (Cl^- form, $40 \times 3.5\text{cm}$) eluted with 0.2 and 0.8 M NaCl at a flow rate of 0.5 mL/min to obtain fractions F3G1 and F3G2, respectively. Further elution with 2 M NaOH afforded the collective fractions defined as F3G3.

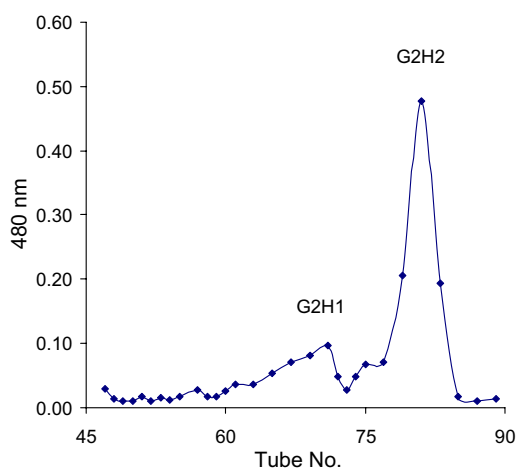


Figure 3. Chromatographic diagram to obtain fractions G2H1 and G2H2 from F3G2. The gel-filtration column TSK HW-75 ($130 \times 2.6\text{cm}$) was employed and eluted with double distilled water at a flow rate of 0.5 mL/min.

The carbohydrate composition analyses of crude Reishi extract, F3, F3G1, F3G2, and F3G3 all pinpointed that glucose and mannose exist as the major components together with smaller components of other sugars including fucose, galactose, *N*-acetylglucosamine, and xylose (Table 2). Interestingly the percentage of galactose is apparently less in F3G2 and F3G3 than others.

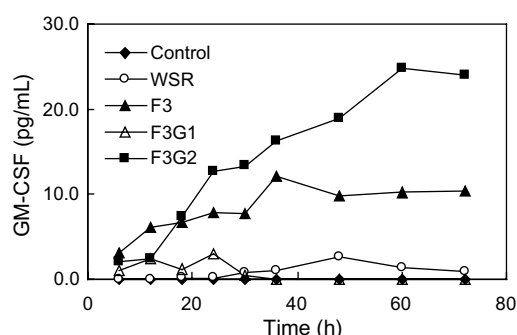


Figure 4. In vitro GM-CSF production of mouse splenocytes (3.0×10^6 cells/mL) after treatment with Reishi crude extract (WSR), F3, F3G1, and F3G2 ($100\mu\text{g/mL}$). Sample supernatants were collected at indicated time intervals and the concentration of GM-CSF released was measured by ELISA.

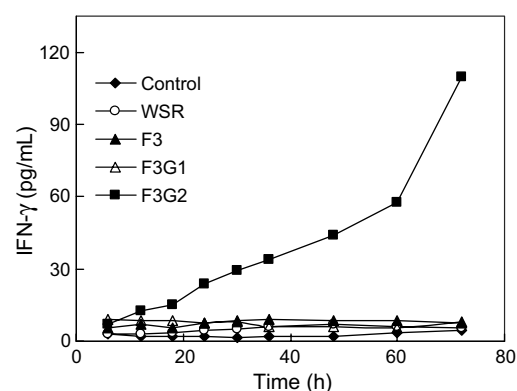


Figure 5. In vitro IFN- γ production of mouse splenocytes (3.0×10^6 cells/mL) after treatment with Reishi crude extract (WSR), F3, F3G1, and F3G2 ($100\mu\text{g/mL}$). Sample supernatants were collected at indicated time intervals and the concentration of IFN- γ released was measured by ELISA.

Whether it is associated with activity still remains to be further investigated.

Our current data indicate that RPS can enhance both adaptive and innate immunity. The former process is initiated by the production of $\text{T}_\text{H}1$ response, which is in agreement with the previous observation on the increase of CD4 and T-cell counts during or after chemotherapy.^{6,7} During the event of innate immunity, Toll-like

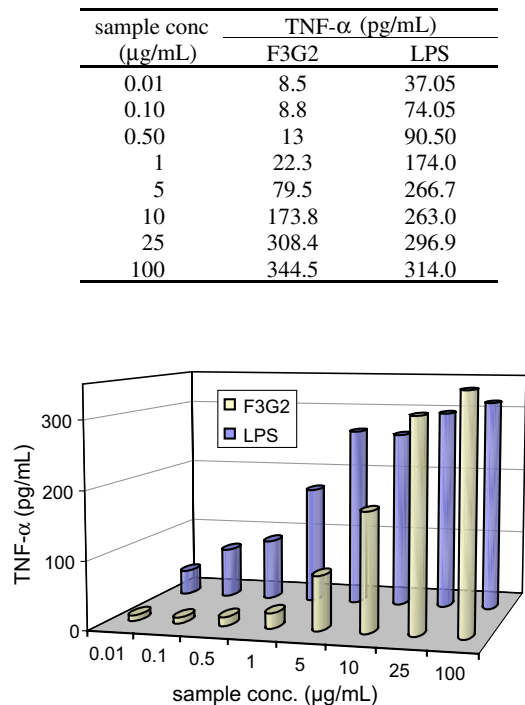


Figure 6. The expression of TNF-α under the influence of either RPS or LPS at various concentrations. Sample supernatants were collected by centrifuging at 300g for 10min and the activity was determined using the Quantokine® Murine ELISA kit (RD System Inc, Minneapolis, USA).

receptors (TLRs) have been well documented to play an indispensable role in the recognition of molecular structures that are shared by many microbial pathogens.¹⁴ The immune response triggered by TLRs primarily occurs via the activation of the transcription factor NF-κB.^{14,15} Upon stimulation, TLRs bind to the adaptor protein MyD88 based on the interaction of their Toll/IL-1 receptor homology domain (TIR). MyD88 then associates with IRAK, the serine threonine kinase for the subsequent activation.^{14–16} Meanwhile, our concurrent investigations¹⁷ have established these signaling pathways initiated by F3, which binds to the TLR4 on macrophages: PTK(Src)/PLCγ1/PKC/MEK1/ERK, PTK(Src)/Rac1/PAK/p38 and PTK/Rac1/PAK/JNK (PTK: protein-tyrosine kinase, PKC: protein kinase C, MEK1: mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, ERK: extracellular signal-regulated kinase, PAK: p21-activated kinase, p38: p38-mitogen activated protein kinase, JNK: c-Jun N-terminal kinase), as illustrated in Figure 7. These pathways were shown to induce the inflammatory cytokines

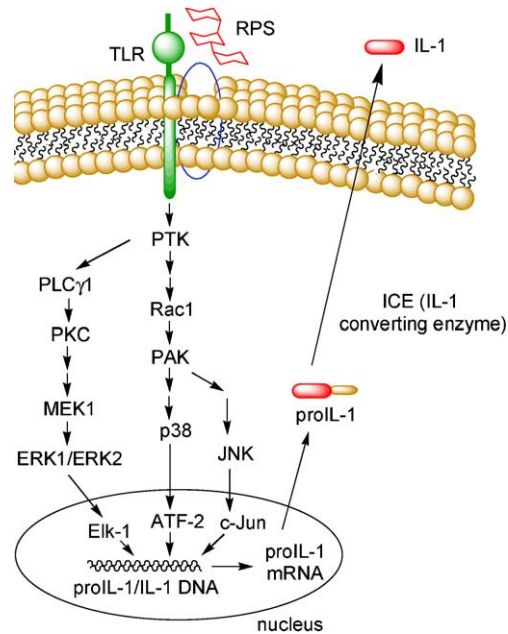


Figure 7. The signaling pathways triggered by the proposed binding event of RPS with TLR.

IL-1. In addition, our study exhibited obvious differences between F3- and LPS-mediated signaling pathways albeit both lead to the same outcome—the expression of IL-1. The ERK, JNK, and p38-mediated pathways have an equal contribution to the F3-induced IL-1 expression, whereas uneven participations were found in LPS-stimulated macrophages. PI3-kinase is upstream of JNK and p38 phosphorylation in LPS-stimulated macrophages, but not in the case of F3.

Kataoka et al., recently reported that the purified polysaccharide fractions from safflower petals activate the NF-κB signaling pathway via TLR4.¹⁸ As for the lipopolysaccharide (LPS)-induced macrophage activation, the *Salmonella* lipid A and the polysaccharide portion were found to be inactive individually. In contrast, the sugar portion covalently bound to lipid A exhibited strong activity for TLR4-mediated activation of NF-κB.¹⁹ Our study also showed that the activation of IL-1 by the F3 fraction of Reishi is not inhibited by polymyxin B, indicating that the cytokine activation is not due to the contamination of LPS. In summary, we have further purified the active fraction of Reishi, investigated their effect on cytokine activation, and elucidated the mode of action on macrophages, including the signaling pathways in association with immune response.

Table 2. Carbohydrate compositions of F3, F3G1, F3G2, and F3G3

	Percentage (%)						
	L-Fuc	D-Xyl	D-Man	D-Gal	D-GlcNAc	D-Glc	Unknown
F3	7.1	3.1	15.1	13.5	1.20	58.1	1.90
F3G1	8.0	5.7	10.2	12.6	0.25	63.2	0.05
F3G2	6.2	4.5	18.3	5.3	0.78	64.9	0.02
F3G3	8.4	7.2	14.5	2.9	1.18	65.7	0.12

3. Experimental section

3.1. Materials

Crude Reishi extract (prepared via alkaline extraction (0.1 N NaOH), neutralization and ethanol precipitation) was obtained from Pharmanex Co., (CA, USA). All the chemicals and reagents were from Sigma Co., (St. Louis, MO, USA) unless indicated.

3.2. Purification of Reishi extract

Crude Reishi extract (100 g) was dissolved in 3 L of double distilled water, stirred at 4°C for 24 h, and centrifuged for 1 h to remove the insoluble. The resulting solution was concentrated at 35°C to give a small volume and lyophilized to generate 70 g powder of dark-brown color, 2.5 g of which were dissolved in a small volume of Tris buffer (pH 7.0, 0.1 N), and purified by gel filtration chromatography using a Sephacryl S-500 column (95 × 2.6 cm) with 0.1 N Tris buffer (pH 7.0) as the eluent. The flow rate was set at 0.6 mL/min, and 7.5 mL per tube was collected. After the chromatography, each fraction was subjected to anthrone analysis or the phenol–sulfuric acid method to detect sugar components. Five fractions were collected (fractions 1–5), each dialyzed to remove excessive salt and lyophilized to give 450 mg of F3.

F3 was further subjected to a column of Diaion-WA30 anion exchanger (Cl[−] form, 40 × 3.5 cm) eluted with 0.2 and 0.8 M NaCl at a flow rate of 0.5 mL/min, and two fractions were designated as F3G1 (11% yield based on F3) and F3G2 (10% yield based on F3), respectively (Fig. 2). Another fraction (F3G3, 11% yield based on F3) was generated when the column was further eluted with 2 M NaOH.

The gel-filtration chromatography of F3G2 was carried out on a TSK HW-75 column (130 × 2.6 cm) eluted with double distilled water at a flow rate of 0.5 mL/min. There were two fractions collected; that is, G2H1 (19% yield based on F3G2) and G2H2 (69% yield based on F3G2), as shown in Figure 3.

3.3. Anthrone colorimetric method²⁰

Each 1.5 mL of anthrone (9,10-dihydro-9-oxoanthracene) solution (0.2 g anthrone dissolved in 100 mL of concd sulfuric acid) in a series of test tubes immersed in an ice water bath was carefully overlaid with 1.5 mL of sample (20–40 µg/mL of D-glucose or equivalent). After all additions had been made, the tubes were shaken rapidly, and then cooled down in an ice water bath. The tubes were heated for 5 min in a boiling water bath and then cooled; the optical densities were read within an hour at 625 nm against distilled water. Standards, reagent blanks, and unknowns were run in triplicate because of likely contamination by other carbohydrate sources. Calculations were made on the basis that the optical densities are directly proportional to the carbohydrate concentration.

3.4. Reverse transcription (RT) and polymerase chain reaction (PCR)²¹

Mouse spleen cells were aseptically removed from healthy mice (BALB/c male mice, 6 weeks old), adjusted to an ideal cell concentration (3×10^6 cells/mL), and incubated in RPMI-1640 medium containing 10% of FCS (fetal calf serum) at 37°C with 5% CO₂. After 6 h, the cells were subjected to RNA extraction using Qiagen RNAeasy mini kit to obtain ~1 µg of the desired RNA. Reverse transcription (RT) was performed using the Thermoscript R/T PCR System, and the Thermoscript system protocol I, from Gibco BRL. The reaction was carried out as follows: 1 µg of RNA, 1 µL of primer (Oligo(dT)₂₀), and 2 µL of 10 mM dNTP Mix were added to each 0.2 mL tube and the total volume was adjusted to 12 µL with DEPC H₂O (0.1% diethylpyrocarbonate-treated H₂O). The mixture was incubated at 65°C for 5 min and immediately chilled on ice. The following was added to each tube as an 8 µL mixture: 4 µL of 5 × cDNA buffer, 1 µL of 0.1 M dithiothreitol (DTT), 1 µL of RNaseOut (a ribonuclease inhibitor) and 1 µL of Thermoscript R/T, and 1 µL of DEPC water. The mixture was incubated at room temperature for 10 min and then 50°C for 1 h to allow the first strand of cDNA synthesis. Enzyme activity was terminated by incubating the reactions at 85°C for 5 min and the tubes were then placed on ice for 10 min. The samples were stored at −20°C until used for PCR.

Each sample (2 µL) was added to each reaction tube and the following reagents were added as a 25 µL mix: 2.5 µL of 10 × PCR buffer, 2 µL of 10 mM dNTP Mix, 2.5 µL of 10 mM each primer (sense and anti-sense), 13 µL of DEPC H₂O, and 1 µL of ProZyme[®] (DNA polymerase, from PROtech Technology). The reaction tubes were placed in a Strategene PCR Robocycler (Gradient 96) and run under the following condition: one cycle at 94°C for 2 min (initial denaturation), then 25 consecutive cycles of 94°C for 1 min (denaturation), primer annealing (various temperatures depending on primers, see Table 3 for the details) for 1 min and 72°C for 1 min (primer extension). The reactions were analyzed by gel electrophoresis.

3.5. Detection of cytokine activity by colorimetric sandwich ELISA

Fresh spleen cells were harvested from BALB/c mice (6–10 weeks old), treated with RBC lysis buffer to destroy red blood cells, and adjusted to 3×10^6 cells/mL. Splenocytes were then subject to the treatment of either F3 or F3G2 at 100 µg/mL and incubated in RPMI-1640 medium (GIBCO) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS at 37°C, 5% CO₂. Supernatants were collected at indicated time intervals by centrifugation at 300g for 10 min. In vitro IFN-γ and GM-CSF activity was determined using the Quantokine[®] Murine ELISA kit (RD System Inc., Minneapolis, USA) based on the manual provided. The procedure was briefly described as follows. Assay diluents (50 µL) and 50 µL of sample supernatants,

Table 3. Primer sequences used in RT-PCR experiments

Cytokine	Sequences (S: sense, A: anti-sense)	Annealing temp (°C)	Size of PCR products (bp)
IL-1 β	S 5'-CAACCAACAAGTGATATTCTCCATG-3' A 5'-GATCCACACTCTCCAGCTGCA-3'	55	152
IL-2	S 5'-TGATGGACCTACAGGAGCTCCTGAG-3' A 5'-GAGTCAAATCCAGAACATGCCGAG-3'	59	167
IL-4	S 5'-ACGAGGTCACAGGAGAAGGGACGCCATGCA-3' A 5'-TCATTCATGGAGCAGCTTATCGATGAATCC-3'	71	221
IL-6	S 5'-GTGACAACCACGGCCTTCCCTACT-3' A 5'-GGTAGCTATGGTACTCCA-3'	53	313
IL-12	S 5'-TGTTGTAGAGGTGGACTGG-3' A 5'-TGGCAGGACACTGAATACTT-3'	67–65	483
IFN- γ	S 5'-TGGAGGAAGTGGCAAAAGGATGGT-3' A 5'-TTGGGACAATCTCTTCCCCAC-3'	63	336
TNF- α	S 5'-GCGACGTGGAAGTGGCAGAAG-3' A 5'-GGTACAACCCATCGGCTGGCA-3'	65	383
GM-CSF	S 5'-TGTGGTCTACAGCCTCTCAGCAC-3' A 5'-CAAAGGGGATATCAGTCAGAAAGGT-3'	65	368
G-CSF	S 5'-ATCCAGGCCAGCGGCTCGG-3' A 5'-GGGCTTTCTGCTCAGGTCTAG-3'	63–65	467
M-CSF	S 5'-GTAGCCACATGATTGGGAATGG-3' A 5'-TCATGGAAAGTTCGGACACAGG-3'	67–65	304
GAPDH	S 5'-ACGACCCCTTCATTGACC-3' A 5'-AGACACCAGTAGACTCCACG-3'	57	203

standard and control, were gently mixed and loaded into individual wells. After 2h of incubation at room temperature, each well was aspirated, washed five times with 400 μ L of Wash Buffer, loaded with 100 μ L of secondary antibody solution (conjugated with horseradish peroxidase) at room temperature for 2h. The same aspiration and wash procedures were performed; 100 μ L of substrate solution was then added to each well and incubated in darkness at room temperature for 30 min. The enzymatic reaction was finally terminated by the addition of 100 μ L of Stop Solution. The optical density was determined using a microplate reader set at 450 nm with the correction wavelength at 540 or 570 nm. The concentration of cytokine released was determined by plotting the sample reading against the standard curve.

3.6. Sugar composition analysis—TMS method

For monosaccharide analysis, the polysaccharide extracts/fractions were methanolized with 0.5 M methanolic-HCl (Supelco) at 80 °C for 16 h, re-N-acetylated with 500 μ L of methanol, 10 μ L of pyridine and 50 μ L of acetic anhydride, and then treated with the Sylon HTP[®] trimethylsilylating reagent (Supelco) for 20 min at room temperature, dried, and redissolved in hexane. GC-MS analysis of the trimethylsilylated derivatives was carried out using a Hewlett-Packard (HP) Gas Chromatograph 6890 connected to an HP 5973 Mass Selective Detector. Samples were dissolved in hexane prior to splitless injection into an HP-5MS fused silica capillary column (30 m $\times$ 0.25 mm i.d., HP). The column head pressure was maintained at around 8.2 psi to give a constant flow rate of 1 mL/min using helium as carrier gas. The initial oven temperature was held at 60 °C for 1 min, increased to 140 °C at 25 °C/min, to 250 °C at 5 °C/min, and then increased to 300 °C at 10 °C/min.

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