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Effects on cytokine-stimulating activities of EPS from *Tremella mesenterica* with various carbon sources

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Abstract

The effects on cytokine-stimulating activities of extracellular polysaccharides from submerged cultures with various carbon sources of *Tremella mesenterica* were investigated in vitro. The predominant sugars in EPS identified in this study were glucose, mannose, xylose, and galactose. The results indicated that the component sugar and uronic acids within the EPS vary with the different carbon sources, a variation which also affects cytokine (interlukin-6 and tumor necrosis factor-α) and nitric oxide production in RAW 264.7 macrophage cells. Xylose and glucose were better carbon sources from the viewpoint of immunomodulatory activity due to the relatively high mannose content in EPS.

Keywords: *Tremella mesenterica*; Cytokine-stimulating; Extracellular polysaccharides; Xylose

1. Introduction

The *Tremella* species is a common food and folk medicine, widely used in several Asian countries as a tonic for weakness. The pharmaceutical products, especially acidic polysaccharides, from the genus *Tremella* have received extensive attention. Zhou et al. (1989) reported that *Tremella fuciformis* polysaccharides exert an anti-aging effect by increasing the superoxide dismutase (SOD), a key antioxidant enzyme in brain and liver cells. Other interesting pharmacological activities of the *Tremella* species have been reviewed by Reshetnikov, Wasser, Nevo, Duckman, and Tsukor (2000), including cytokine-stimulating, anti-diabetic, anti-inflammatory, vascular-stimulating, hypocholesterolemic, anti-allergic and hepatoprotective effects.

Many mushroom polysaccharides, such as (1 → 3)-β-D-glucans, are known to possess immunomodulatory characteristics and may contribute to various therapeutic effects, such as anti-tumor or anti-inflammatory activities (Adachi, Okazaki, Ohno, & Yadomae, 1994; Borchers, Stern, Hackman, Keen, & Gershwin, 1999). As compared to other mushroom polysaccharides, the (1 → 3)-β-D-glucans and all of the heteroglycans produced by the *Tremella* species consist of a (1 → 3)-

Abbreviations: DMEM, Dulbecco's modifided Eagle's medium; EPS, Extracellular polysaccharides; EPS-G, EPS isolated from glucose; EPS-M, EPS isolated from mannose; EPS-X, EPS isolated from xylose; EPS-C, EPS isolated from a semi-chemically defined medium; IL-1, interlukin-1; IL-2, interlukin-2; IL-6, interlukin-6; LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor-α; YMA, yeast malt agar.

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α-mannan backbone with small xylose- and glucuronic acid-containing side chains (Slodki, Wickerham, & Bandoni, 1966). Gao, Jiang, Chen, Jesen, and Seljelid (1996) found that four kinds of acidic heteroglycans isolated from the fruiting body of *T. fuciformis* Berk. possess cytokine-stimulating activities (IL-1, IL-6 and TNF) in human monocytes, and they demonstrated that xylosyl and glucuronic acid residues, as well as acetyl groups, are not important for these activities. Cui and Lin (1996) observed that *Tremella* polysaccharides can increase the activity of IL-2 in the supernatant of murine splenocytes and the activities of IL-6 and TNF-α in the supernatant of murine macrophage, by promoting the mRNA expression of these cytokines. It has been reported that *Tremella* polysaccharides significantly inhibit cancer-cell DNA synthesis and growth in mice. However, the phenomenon was not observed in vitro, thus indicating that the anti-tumor effect might occur by activating immune responses in the host (Zhou, Wu, & Hou, 1987).

Khondkar, Aidoo, and Tester (2002) isolated the heteropolysaccharides from liquid cultures of *Tremella* species and found that all of the polysaccharides contain essentially the same sugars but in different ratios. Moreover, some of the polysaccharides contained additional carbohydrates, such as fucose, ribose, arabinose, galactose and glucose. The authors also demonstrated that different carbon sources (xylose, arabinose, glucose, fructose and galactose) affect the sugar (including uronic acid content) ratio within the polysaccharides.

Most established data for the pharmaceutical properties of the *Tremella* polysaccharides focus on the *T. fuciformis* and *Tremella aurantia* species. However, there are few data published on the effects of immune responses by the extracellular polysaccharides from *Tremella mesenterica*, an important species of the so-called "Jelly Mushroom" genus *Tremella* Pers. (Tremellales). Wasser, Tan, and Elisashvili (2002) reported that a tremellastin, containing 40–45% of an acidic polysaccharide, glucuronoxylomannan, isolated from the submerged culture of *T. mesenterica*, appeared to be an active interferonogen and the activator of oxygen-dependent macrophage biocide activity.

It is clear that the yields and compositions of the extracellular polysaccharides (EPS) are affected by culturing conditions, especially when using different carbon sources (De Baets, Laing, Francois, & Vandamme, 2002; Khondkar et al., 2002; Slodki et al., 1966) which might also affect the immunomodulatory property. Therefore, the objective of this study was to evaluate the effects on cytokine-stimulating (IL-6 and TNF-α) and nitric oxide (NO) in RAW 264.7 macrophage of the EPS of *T. mesenterica* from submerged cultures with various carbon sources.

2. Materials and methods

2.1. Microorganisms

T. mesenterica CCRC 36028 was purchased from the Bioresources Collection and Research Center, Hsinchu, Taiwan. The cultures were maintained on yeast malt agar (YMA) plates. The plates were inoculated and incubated at 25 °C for seven days, and stored at 4 °C.

2.2. Reagents

D-Glucurono-6,3-lactone was obtained from the Aldrich Chemical Co., Stinheim, Germany. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone, Logan, Utah, USA. Sodium nitrite, Griess reagent and lipopolysaccharide (*Escherichia coli*, Serotype 055:B5) were purchased from the Sigma Chemical Co., St. Louis, MO, USA. The concentrations of IL-6 and TNF-α were determined by using mouse IL-6 and a TNF-α ELISA kit from e-Bioscience, CA, USA. All other reagents were purchased from Sigma unless indicated otherwise.

2.3. Fermenter cultures

Seed cultures on the flask were obtained 500 ml Erlenmeyer flasks filled with 200 ml of YM media. The sterilized media were inoculated with liquid seven-day-old precultures (2.5% vol/vol), and incubated at 25 °C for seven days on a rotary shaker at 150 rpm. Fermenter cultures were obtained in 5 l fermenters (from Bio-top BTF-600T, Taiwan) with 3 l of medium with various carbon sources. The fermenters were sterilized by autoclaving for 30 min at 121 °C. The sterilized media were inoculated with 150 ml (cell number adjusted to ca 1.5×10^8 yeast-like cells/ml) seven-day-old seed cultures. The fermentation was run at 25 °C for seven days, with the aeration set at 1.5 l/min and the agitation at 150 rpm without pH control. The media consisted of the following components (in g/l): glucose, 20; yeast extract, 3; malt extract, 3; peptone, 5. To study the effect of different carbon sources on extracellular polysaccharide production, the glucose was replaced by xylose and mannose. In addition, the following chemical media (in g/l) were used: glucose, 50; yeast extract, 2.5; KH_2PO_4, 0.5; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5.

2.4. Determination of biomass and extracellular polysaccharides

The biomass was obtained by centrifuging samples at 9000 rpm for 15 min, washing the sediment three times with water, and drying to a constant weight. All supernatants were collected; then, the crude EPS was precipitated with the addition of 4 vols. of 95% ethanol.

The precipitated EPS was collected by centrifugation at 9000 rpm for 15 min and then lyophilized. The total EPS in the culture medium was determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), using glucose as the standard.

2.5. Sugar analysis

The EPS (0.1 g) was completely hydrolyzed in a sealed tube with 2 ml of 2 M trifluoroacetic acid (TFA) at 95 °C for 16 h; the amount of neutral sugars in the polysaccharides was analyzed by HPLC (Ajlouni, Beelman, Thompson, & Mau, 1995). An HPLC system (Jasco PU-2080 Plus) equipped with a refractive detector (RI-2031 PLUS) and a column (8 mm × 300 mm) of SUGAR SP0810 (Shodex) was used. A solution of de-ionized water was used as the eluent at a flow rate of 0.8 ml/min. The temperature of the column was maintained at 80 °C. The standards were ribose, glucose, mannose, xylose and galactose. The amount of uronic acid was estimated calorimetrically by using the method described in the literature (Bitter & Muir, 1962). A calibration curve was constructed with D-Glucurono-6,3-lactone as the standard.

2.6. Cell cultures and activation of macrophages

The murine macrophage cell line, RAW 264.7, was purchased from the Bioresources Collection and Research Center (Hsinchu, Taiwan). The RAW 264.7 cells were regularly cultured at 37 °C in a 5% CO_2 incubator in a DMEM medium without phenol red, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The lipopolysaccharide (LPS) preparation was dissolved in the DMEM at 20 μg/ml by vortexing and then filter sterilizing (millipore 0.2 μm). The EPS was dissolved by the complete medium and applied to the cell cultures at final concentrations of 100, 250 and 500 μg/ml, alone or with 5 μg/ml of LPS. The RAW 264.7 cells were seeded in 24-well plates (2.5×10^5 cells/500 μl), and cultured for 24 h in a humidified atmosphere containing 5% CO_2 and 95% air; each (of LPS or EPS) solution was added to appropriate RAW 264.7-containing (500 μl) wells in a volume of 250 μl. The final volume in wells was adjusted to 1 ml by adding DMEM. The control wells, RAW 264.7 cells only, were added to a 500 μl DMEM complete medium. The cultures were incubated for a further 48 h. At the end of the incubation, the supernatants were harvested into Eppendorf tubes. A 100 μl portion was used for an NO assay; the remainder was aliquoted and then frozen at −70 °C until the assays for cytokine contents were performed.

2.7. Measurement of nitrite production as an assay of NO release

The NO production by RAW 264.7 cells was determined by assaying the cultured supernatants for nitrite by the Griess reaction (Green et al., 1982). After an incubation of 48 h 100 μl of the supernatant were mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% NED/2.5% H_3PO_4) in a 96-well flat-bottomed microtitre plate and incubated at room temperature for 15 min. The absorbance was read at 540 nm by using a microplate reader (μQuant, from BIO-TEK Instruments, Inc., USA). The nitrite concentration in the cultured supernatants was determined by comparison with a sodium nitrite standard curve.

2.8. Cytokine assay

The concentrations of cytokines present in the cultured supernatants were determined by commercially available cytokine ELISA kits. The cytokine levels in the cultured supernatants were assayed by using these ELISA kits according to the manufacturer's instructions.

2.9. Statistical analysis

The data were subjected to a one-way ANOVA by using the Statistical Analysis System version 8.0. The treatment means were compared by using Duncan's multiple-range test, wherein the differences were considered to be significant if the associated P value was <0.05.

3. Results

3.1. Effects of carbon sources in medium and SCDM on biomass and EPS production

The results of the biomass and EPS of *T. mesenterica* from the submerged culture with various carbon sources are shown in Fig. 1. After a fermentation period of seven days, no significant differences in biomass yield were observed. The highest EPS yield was obtained by using a semi-chemically defined medium (EPS-C; 7.6 ± 0.4 g/l), compared with EPS-G (3.1 ± 0.3 g/l), EPS-M (2.3 ± 0.1 g/l), and EPS-X (1.8 ± 0.2 g/l). Khondkar et al. (2002) found that YM broth-based media (the same medium as in this study) seem to be the best broths for EPS production from several *Tremella* species, although the carbon source was changed. They also found that xylose, as the carbon source, produced the highest EPS yield; however, a similar result was not observed in our study.

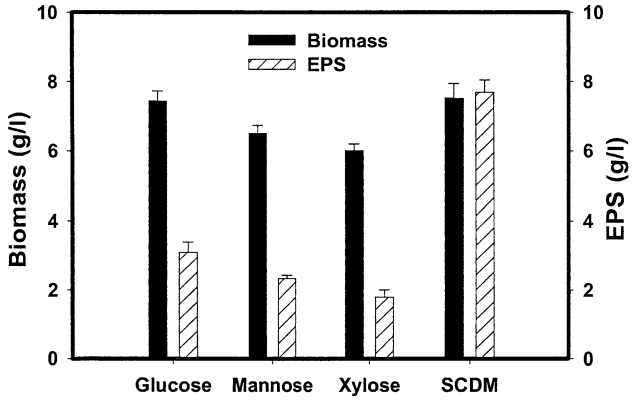

Fig. 1. Effects of different carbon sources (glucose, mannose, and xylose) and a semi-chemically defined medium (SCDM) on production of biomass and extracelluar polysaccharide (EPS) by *Tremella mesenterica*. The composition of the semi-chemically defined medium is described in Section 2. The data are expressed as means ± SD ($n = 3$).

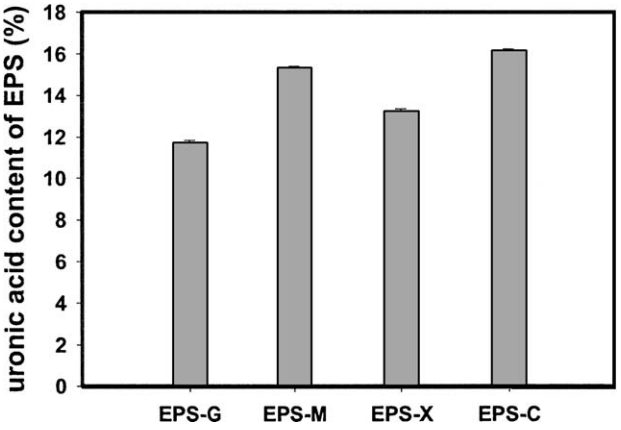

Fig. 2. Total uronic acids content of extracellular polysaccharides (EPS) from *Tremella mesenterica* with different media. The data are expressed as means ± SD ($n = 3$).

3.2. Analysis of sugar composition

As expected, the component sugar (molar ratio) within the EPS varies with the different carbon sources, as shown in Table 1. Nevertheless, the predominant sugars in EPS, identified in this study, were glucose, mannose, xylose, and galactose. A similar sugar composition was reported by Khondkar et al. (2002). The total uronic acid content of EPS from *T. mesenterica* varied according to the carbon sources (Fig. 2). The uronic acid contents of EPS-C (16.2 ± 1.0%) and EPS-M (15.4 ± 0.6%) were relatively high compared with EPS-G (11.2 ± 0.1%) and EPS-X (13.3 ± 0.2%).

3.3. Effects on cytokine-stimulating activities of EPS from different carbon sources

To investigate the effects on cytokine-stimulating activities of EPS from different carbon sources, resting cells or cells activated with LPS (5 μg/ml) were treated with each EPS from different carbon sources at concentrations of 100, 250 and 500 μg/ml, after which the cytokine contents (TNF-α and IL-6) in the cultured supernatants were determined. As shown in Table 2, the EPS from *T. mesenterica* submerged cultures with various carbon sources demonstrated cytokine-stimulating activities against murine macrophage RAW 264.7 cells in vitro. The contents of TNF-α and IL-6 in the cultured supernatants of untreated cells (complete medium only) were 1.2 ± 0.1 and 0.36 ± 0.01 ng/ml, respectively. The RAW 264.7 cells stimulated by LPS were able increase the level of the production for TNF-α and IL-6 to 4.8 ± 0.1 and 10.6 ± 0.7 ng/ml, respectively. All TNF-α contents except EPS-M (100 μg/ml) and all IL-6 contents from EPS-only treatments indicated significant differences, compared with the untreated cells ($P < 0.05$). Furthermore, all the various EPS from different carbon sources in this study, when co-incubated with LPS, were able to increase the cytokine response (TNF-α and IL-6) from RAW 264.7 cells. It is worth noting that these co-incubated EPS could enhance the IL-6 response from RAW 264.7 cells by 2–3 fold, in comparison with LPS-only treatments.

Table 1
Component sugars (molar ratios) within extracellular polysaccharides (EPS) from submerged culture with various carbon sources of *Tremella mesenterica*

EPS	Molar ratio			
	Mannose	Xylose	Galactose	Glucose
EPS-G[a]	2.0	2.8	0.5	3.3
EPS-M[b]	1.6	1.1	0.2	1.6
EPS-X[c]	2.5	2.0	0.4	3.0
EPS-C[d]	1.1	0.7	0.2	0.5

[a] EPS-G: isolated from glucose.
[b] EPS-M: isolated from mannose.
[c] EPS-X: isolated from xylose.
[d] EPS-C: isolated from a semi-chemically defined medium, composition described in Section 2.

3.4. Effects on nitric oxide production of EPS from different carbon sources

Macrophages can be activated by LPS to produce nitric oxide, which can react with oxygen to form the stable products nitrate and nitrite, processes which can be determined by the Griess reagent. As shown in Table 3, the nitrite content in the supernatants of untreated RAW 264.7 cells (complete medium only) was 2.7 ± 0.2 μM. After stimulation by LPS-only (5 μg/ml) and incubation for 48 h, the nitrite content in the supernatants of RAW 264.7 cells increased to 36.0 ± 0.4 μM. All EPS-only treatments (at concentrations of 100, 250 and 500 μg/ml), except for EPS-X (250 and 500 μg/ml), showed no significant difference when compared

Table 2
Contents of TNF-α and IL-6 secretion in RAW 264.7 macrophages incubated with different concentrations of extracellular polysaccharides (EPS) from submerged culture with various carbon sources of *Tremella mesenterica*

Sample	Concentration (μg/ml)	Content of cytokine (ng/ml ± SD)			
		TNF-α[a]		IL-6	
		EPS only	EPS + LPS	EPS only[b]	EPS + LPS
EPS-G	500	5.3 ± 0.1	8.3 ± 0.2*	0.78 ± 0.03	36.7 ± 0.3*
	250	4.0 ± 0.1	9.7 ± 0.3*	0.15 ± 0.01	33.3 ± 0.4*
	100	3.1 ± 0.1	9.1 ± 0.1*	0.52 ± 0.01	25.3 ± 0.1*
EPS-M	500	4.1 ± 0.1	5.8 ± 0.2*	0.60 ± 0.01	22.6 ± 0.9*
	250	2.4 ± 0.1	6.0 ± 0.2*	0.51 ± 0.01	22.5 ± 1.1*
	100	1.3 ± 0.1	7.6 ± 0.1*	0.49 ± 0.01	23.4 ± 1.2*
EPS-X	500	5.2 ± 0.1	9.4 ± 0.1*	0.79 ± 0.02	24.9 ± 1.1*
	250	3.9 ± 0.1	9.8 ± 0.1*	0.53 ± 0.01	32.1 ± 0.4*
	100	3.4 ± 0.1	9.4 ± 0.2*	0.52 ± 0.06	33.8 ± 0.5*
EPS-C	500	10.0 ± 0.2	5.6 ± 0.3*	1.58 ± 0.01	22.2 ± 1.2*
	250	6.7 ± 0.2	7.2 ± 0.3*	1.04 ± 0.02	23.1 ± 0.1*
	100	3.8 ± 0.1	7.4 ± 0.1*	0.62 ± 0.01	24.9 ± 0.1*

The cytokine content (TNF-α and IL-6) stimulated by LPS-only (5 μg/ml) treatment and untreated cells (completely medium only) are 4.8 ± 0.1 and 1.2 ± 0.1 ng/ml;10.6 ± 0.7 and 0.36 ± 0.01 ng/ml, respectively. The data are expressed as means ± SD ($n = 3$).
[a] Significant difference at $P < 0.05$, except for EPS-M(100 μg/ml).
[b] Significant difference at $P < 0.05$.
* Significant difference from the LPS-only treated cells ($P < 0.05$).

Table 3
Contents of nitric oxide secretion in RAW 264.7 macrophages incubated with different concentrations of extracellular polysaccharides (EPS) from submerged culture with various carbon sources of *Tremella mesenterica*

Sample	Concentration (μg/ml)	Content of nitrite (μM ± SD)	
		EPS only[a]	EPS + LPS
EPS-G	500	2.5 ± 0.2	47.4 ± 0.1*
	250	2.3 ± 0.4	46.7 ± 0.3*
	100	1.9 ± 0:1	47.6 ± 0.3*
EPS-M	500	2.8 ± 0.5	38.7 ± 0.5
	250	2.2 ± 0.2	42.2 ± 0.1*
	100	1.8 ± 0.2	47.7 ± 0.5*
EPS-X	500	3.8 ± 0.5	56.4 ± 0.6*
	250	3.5 ± 0.7	51.7 ± 0.6*
	100	1.6 ± 0.4	41.9 ± 0.9*
EPS-C	500	1.7 ± 0.2	34.7 ± 0.5
	250	1.2 ± 0.4	36.0 ± 0.7
	100	0.9 ± 0.2	39.0 ± 0.8

Nitrite content stimulated by LPS-only (5 μg/ml) treatment and untreated cells (completely medium only) are 36.0 ± 0.4 and 2.7 ± 0.2 μM, respectively. The data are expressed as means ± SD ($n = 3$).
[a] No significant differences, compared with untreated cells, except for EPS-X (250 and 500 μg/ml).
* Significant difference from the LPS-only treated cells ($P < 0.05$).

with the untreated cells. However, when LPS was co-incubated with various EPS from different carbon sources, the EPS-G, EPS-M and EPS-X, but not EPS-C, indicated significantly increasing effects on nitric-oxide production of LPS ($P < 0.05$).

4. Discussion

In the present study, we found that the component sugar (molar ratio) within the EPS varied with the different carbon sources, a variation which also affected nitric oxide (NO) and cytokine (IL-6 and TNF-α) production in RAW 264.7 macrophage cells. The evidence generally indicates that the principal pharmacologically-active substance from *Tremella* is the polysaccharide glucuronoxylomannan, which is mainly composed of a linear backbone of 1,3-linked α-D-mannose with xylose and glucuronic acid in the side chains (Reshetnikov et al., 2000). As mentioned earlier, the cytokine-stimulating activities of *Tremella* polysaccharides are mainly related to the mannan backbone, and the side-chain residues are not important for promoting the cytokine response (Gao et al., 1996). Furthermore, Karaca, Sharma, and Nordgren (1995) reported a complex carbohydrate extracted from *Aloe vera*, acemannan, which can stimulate chicken macrophages to produce nitric oxide. They suggested that those effects on macrophages might result from the combination of mannose in the structure of acemannan with mannose receptors on the membrane of the macrophages. Indeed, the molar ratios of mannose in EPS-X and EPS-G were relatively high, compared with EPS-M and EPS-C (Table 1). The EPS-X and EPS-G also performed better in the production of nitric oxide (NO) and cytokine (IL-6 and TNF-α) in RAW 264.7 macrophage cells, compared with EPS-M and EPS-C while co-incubated with LPS (Tables 2 and 3). Moreover, the EPS from a semi-chemically defined medium (EPS-C) of *T. mesenterica* with the lowest

mannose content (Table 1) exhibited the lowest activity in NO production and cytokine stimulations (Tables 2 and 3). By way of contrast, the total uronic acid content of *T. mesenterica* varied according to the carbon sources (Fig. 2); however, it seems that the content of uronic acids in EPS was not related to the promotion of the cytokine response.

Human health is related to immune responses, but an inappropriate prolongation of inflammation will cause diseases (Kuo et al., 1998). Therefore, the use of immunomodulators must be cautious and discreet. In this study, we found that most EPS-only treatments showed no significant difference when compared with the untreated RAW 264.7 macrophage cells (Table 3). In addition, the TNF-α and IL-6 contents from EPS-only treatments were relatively low compared with an LPS-only treatment, thus indicating that EPS themselves are milder immunostimulants, although EPS-only treatments showed significant differences when compared with the untreated cells (Table 3). However, in this study all the various EPS from different carbon sources, when co-incubated with LPS, were able to increase the cytokine response (TNF-α and IL-6) and NO production from RAW 264.7 cells by 2–3 fold in comparison with the LPS-only treatment. This finding suggests that EPS from *T. mesenterica* might be immunity enhancers and play important roles against bacterial infection and in anti-tumor activities (Ukai, Hirose, Kiho, Hara, & Irikura, 1972). It is believed that the anti-tumor mechanisms of polysaccharides isolated from many mushrooms are mediated largely by T cells and macrophages, although it remains unclear what mechanisms are involved (Borchers et al., 1999).

In conclusion, the study presented here has demonstrated that the component sugar within the EPS varies with the different carbon sources, a variation which also affects NO and cytokine (IL-6 and TNF-α) production in RAW 264.7 macrophage cells. Xylose and glucose are better carbon sources from the viewpoint of immunomodulatory activity due to the relatively high mannose content in EPS.

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