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Effects of Selenizing *Codonopsis pilosula* Polysaccharide on Macrophage Modulatory Activities

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The purpose of the present study was to investigate the immune-enhancing activity of selenizing *Codonopsis pilosula* polysaccharide ($sCPPS_5$) in nonspecific immune response. In in vitro experiment, the results showed that $sCPPS_5$ could promote the phagocytic uptake, NO production, and TNF-α and IL-6 secretion of RAW264.7 cells. $sCPPS_5$ could also strongly increase the IκB-α degradation in the cytosol and the translocation of NF-κB p65 subunit into the nucleus of RAW264.7 cells. In the vivo experiment, $sCPPS_5$ at medium doses could significantly improve the phagocytic index of peritoneal macrophages and induce the secretion of TNF-α and IL-6. Moreover, the effect of $sCPPS_5$ was significantly better than *Codonopsis pilosula* polysaccharide (CPPS). These results indicated that selenylation modification could significantly enhance the immune-enhancing activity of CPPS in the nonspecific immune response.

Keywords: Macrophages, selenizing *Codonopsis pilosula* polysaccharide, nonspecific immune response

Introduction

Macrophages exist in almost all tissues of the body. They play important roles in the immune system and provide a bridge between innate and adaptive immunity [16]. The phagocytosis of activated macrophages would be enhanced during inflammation, and they can kill pathgens directly by phagocytosis and indirectly by the secretion of inflammatory cytokine (TNF-α and IL-6), nitric oxide (NO), and other mediators against microorganisms in order to effectively defend against the invasion that would cause inflammation as well as tissue injury [2, 3, 31]. Current studies have demonstrated that some immunomodulators can act by inducing the immunomodulatory effects of macrophages and enhance the host defense response [8]. Therefore, macrophages were thought be the important target cells of some immunomodulatory drugs.

Codonopsis pilosula (CP) is an herb that has been used in traditional Chinese medicine for thousands of years. It exhibits similar therapeutic effect to *Panax ginseng* and is sometimes used as a substitute of the much more costly *P. ginseng* [22]. The main constituents of CP are very complex, including polysaccharides, saponins, sesquiterpenes, polyphenolic glycosides, alkaloids, polyacetylenes, essential oils, and phytosteroids [6, 15, 26, 28]. *Codonopsis pilosula* polysaccharide (CPPS) is one of the major bioactive components of *Codonopsis pilosula*. According to modern pharmacology, CPPS possesses antioxidant, antitumor, immune-enhancing, and other effects [12, 29, 30].

In recent years, using selenylation modification to enhance the biological activities of polysaccharide has proved to be effective. In our previous research, CPPS was selenizingly modified by the HNO_3-Na_2SeO_3 method according to $L_9(3^4)$ orthogonal design of three factors, the amount of sodium selenite (Na_2SeO_3), reaction temperature, and reaction time each at three levels. Nine selenizing CPPSs (sCPPSs) named $sCPPS_1$-$sCPPS_9$ were obtained, and we found that $sCPPS_5$ presented a variety of immune regulatory functions, including promoting serum antibody titer and lymphocyte proliferation. However, the effect of

$sCPPS_5$ in nonspecific immune response was not investigated.

Thus, the present study sought to investigate the activity of CPPS and $sCPPS_5$ on enhancing nonspecific immune response by macrophages in vitro and vivo. We also attempted to identify whether selenylation modification could further improve the immune-enhancing activity of CPPS, and the results may provide insights into the underlying mechanisms of activity.

Materials and Methods

Materials

RPMI-1640 and fetal bovine serum were purchased from Gibco (USA). Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (USA). Cyclophosphamide (CY) was purchased from Jiangsu Hengrui Medicine Co. (China). RAW264.7 cells were obtained from the Institute of Cell Biology, Chinese Academy of Sciences (China). Giemsa stain, assay kits for interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), immunoglobulin, and BCA were all obtained from Nanjing Jiancheng Bioengineering Institute (China). Antibodies (I-κBα, NF-κB p65, Lamin A/C, and β-actin) were purchased from Santa Cruz Biotechnology Inc. (USA). All other chemicals and solvents used were of analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (China).

Preparation of the Se-Polysaccharide

CPPS and $sCPPS_5$ were prepared in our laboratory. Briefly, CPPS was extracted by water decoction and ethanol precipitation, purified through eliminating protein by the Sevage method and column chromatography of DEAE Cellulose-52 (2 cm × 50 cm), and its carbohydrate content was 98.19% as determined by the phenol-sulfuric acid method [11, 32]. $sCPPS_5$ was prepared by the nitric acid-sodium selenite method (Li GY, Miu JL, Liu JL. 2001. Selenium polysaccharide compounds and their preparation methods: China: 1288899A) under the optimal modification conditions obtained in our previous research: the amount of sodium selenite used was 300 mg, the reaction temperature was 70°C, and the reaction time was 8 h. Their selenium contents were 11.86 mg/g as measured by atomic fluorescence spectrometry [4] and carbohydrate contents were 56.2% as measured by the phenol-sulfuric acid method.

For the in vitro test, CPPS and $sCPPS_5$ were diluted to 400 μg/ml with PBS (pH 7.4). Then, CPPS and $sCPPS_5$ were diluted with RPMI-1640 2-fold serially from 800 to 6.25 μg/ml.

For the in vivo test, CPPS and $sCPPS_5$ were diluted to 1, 2, and 3 mg/ml with PBS (pH 7.4). The diluted preparations were sterilized by pasteurization and the endotoxin was assessed with an E-TOXATE kit (Sigma). When the endotoxin amount was up to the standard of *Chinese Veterinary Pharmacopoeia* (less than 0.5 EU/ml) [24], they were stored at 4°C for the test [9].

Infrared Spectroscopy Analysis

The FT-IR spectra of CPPS and $sCPPS_5$ in a wavenumber range of 4,000−400 cm^{-1} were recorded by the KBr pellet method with a Nicolet 200 Magna-IR spectrometer (Nicolet Instrument Corp, USA).

Molecular Weight Analysis

The average M_W was measured through GPC on an ultrahydrogel linear column at 25°C. The eluent (flow rate: 1.0 ml/min) was a 0.1 M sodium acetate solution. Pullulan standards were used as the standards for M_W measurement.

Animals and Cell Line

ICR mice weighing 20 ± 2 g were obtained from Shanghai Slac Laboratory Animal Center of Chinese Academy of Sciences (China). Prior to the experiment, the mice were acclimatized at controlled environment for a period of 2−3 days. Half were males and the others were females. During the experiment, the mice were fed under controlled environmental conditions and temperature (24 ± 1°C) with a normal day/night cycle and humidity (55−60%). The mice were provided with a basal diet and free access to drinking water. All procedures involving animal care were approved by the Ethics Committee of the Chinese Academy of Agricultural Sciences.

RAW264.7 cells were obtained from the Institute of Cell Biology, Chinese Academy of Sciences (China). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 g/ml streptomycin, and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell Viability Assay

The effect of CPPS and $sCPPS_5$ on the viability of RAW264.7 cells was determined by the MTT method. RAW264.7 cells were seeded at 1 × 10^6 cells/ml in a 96-well plate and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 24 h, the various concentrations of CPPS and $sCPPS_5$ were added into each well and these cells were incubated at 37°C for 24 h. Each concentration was repeated in four wells. At 4 h prior to incubation end, 20 μl of MTT stock solution (2 mg/ml) was added to each well. The supernatant was removed carefully and 100 μl of DMSO was added into each well to dissolve the formazan crystals. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance values of cells in each well was measured by a microliter enzyme-linked immunosorbent assay reader (Model DG-3022; East China Vacuum Tube Manufacturer) at a wave length of 570 nm (A_{570} value) [23, 25]. When the A_{570} values of polysaccharide groups were not significantly lower than that of the cells control group, it indicated that the polysaccharides had no cytotoxicity, and the corresponding concentrations were considered as its maximal safe concentration for RAW264.7 cells.

Nitric Oxide Production Assay

The RAW264.7 cells were plated at 1 × 10^6 cells/ml in the 96-

well plate and treated with the various concentrations of $sCPPS_5$ and CPPS. A solution of 50 ng/ml LPS was used as a positive control. The cells were incubated at 37°C in a 5% CO_2 incubator for 24 h. Next, the culture supernatants (50 µl) were collected and mixed with Griess reagent (50 µl). After incubation for 30 min at room temperature, nitrite production was determined by measuring the absorbance at 540 nm. The NO concentration was calculated according to the regression equation of the standard curve.

Determination of Phagocytic Uptake

RAW264.7 cells were seeded at a density of 1×10^6 cells/ml in the 96-well plate and treated with the various concentrations of CPPS and $sCPPS_5$ for 48 h. The cells were washed and neutral red (50 mg/ml) was added and incubated for 3 h. Then, the cells were washed with PBS to remove excess dye. The cells were resuspended in 50% ethanol containing 1% glacial acetic acid, and the absorbance values at 570 nm were measured in a microplate reader.

Cytokine Assays

The culture supernatants of RAW264.7 cells treated with CPPS and $sCPPS_5$ for 24 h were collected for the detection of TNF-α and IL-6 levels, using commercial ELISA kits according to the instruction of the manufacturer.

Western Blot Analysis

After treated with the 50 µg/ml of CPPS and $sCPPS_5$ for 30 min, RAW264.7 cells were washed twice with cold PBS and lysed with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Pierce, USA) for 30 min on ice. Lysates were then centrifuged at 12,000 ×*g* for 20 min at 4°C. The protein contents were measured with the BCA protein assay kit using bovine serum albumin as a standard.

The protein extract was then boiled with isotonic loading buffer for 10 min, and an appropriate amount of the protein samples was separated on a 12% SDS-PAGE, and was then transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). After blocking with 5% non-fat milk for 1 h at room temperature, the membrane was incubated with rabbit monoclonal antibody I-κBα and NF-κB p65, or with rabbit anti-β-actin and Lamin A/C to incubate at 4°C overnight. Subsequently, the blots were incubated with an HRP-conjugated anti-rabbit antibody. The protein band was visualized using the ECL-detection with chemiluminescence system.

Animal Treatment and Experimental Design in Vivo

The mice were randomly divided into eight groups of 20 mice each. All animals were allowed 1 week to adapt to their environment before treatment. One group of healthy mice was used as the normal group (sterile physiological saline), for 18 days. From days 1 to 3, the other seven groups of mice were given 80 mg/kg/day CY via intraperitoneal injection. From days 4 to 18, the mice were given the following treatments: physiological saline solution (0.2 ml), 1, 2, and 3 mg/ml of $sCPPS_5$ and CPPS (0.2 ml). These regimens were administered via subcutaneous injection. Twenty-four hours after the last dose, the animals were weighed and then killed via decapitation. The liver and spleen were immediately weighed.

Phagocytic Activity of Peritoneal Macrophages Assay in Vivo

The 10 mice were selected from each group. Mononuclear phagocytic system function assay was carried out as previously described [27]. Each mouse was injected with India ink (0.1 ml/10 g body weight) intravenously through a lateral tail vein. Blood samples (20 µl) were collected at 2 min (t_2) and 10 min (t_{10}) intervals after India ink staining injection and mixed with 2 ml of 0.1% Na_2CO_3. The optical densities of the samples were then determined at 600 nm (OD_2 for 2 min and OD_{10} for 10 min). The phagocytic index (α) was calculated according to the following formula:

$$K = (\lg OD_2 - \lg OD_{10})/(t_{10} - t_2)$$

Phagocytic index $\alpha = \sqrt[3]{K} \times A/(B + C)$, where A is the body weight, B is the liver weight, and C is the spleen weight.

Immunoglobulin and Cytokine Quantitation in Serum in Vivo

The 10 mice were selected from each group. Blood samples were collected and centrifuged at 3,000 rpm for 15 min, and TNF-α and IL-6 in the serum were measured using the manufacturer's kit instructions.

Statistical Analysis

Data are expressed as means ± SD. Duncan's multiple range test was used to determine the difference among CPPS, $sCPPS_5$, and control groups. Significant differences between means were considered at $p < 0.05$.

Results and Discussion

Infrared Spectroscopy Characteristics of $sCPPS_5$

The FT-IR spectra of CPPS and $sCPPS_5$ in the ranges of 4,000 to 400 cm^{-1} are illustrated in Fig. 1. In the spectra of CPPS and $sCPPS_5$, the band in the region of 3,600–3,200 cm^{-1} corresponds to the hydroxyl stretching vibration The bands attributed to C-O-C stretching vibrations appeared at about 1,400–1,000 cm^{-1}, and the bands attributed to C=O stretching vibrations appeared at 1,621 cm^{-1}. This amalgamation indicates that the CPPS and $sCPPS_5$ were polysaccharides (Figs. 1A and 1B). As compared with the spectrogram of CPPS, the FT-IR spectroscopy of $sCPPS_5$ presented one characteristic absorption band at 894 cm^{-1} describing an asymmetrical Se=O stretching vibration, and another characteristic absorption band at 668 cm^{-1} describing an asymmetrical Se-O-C stretching vibration (Fig. 1B), which signified that $sCPPS_5$ was successfully modified by selenylation.

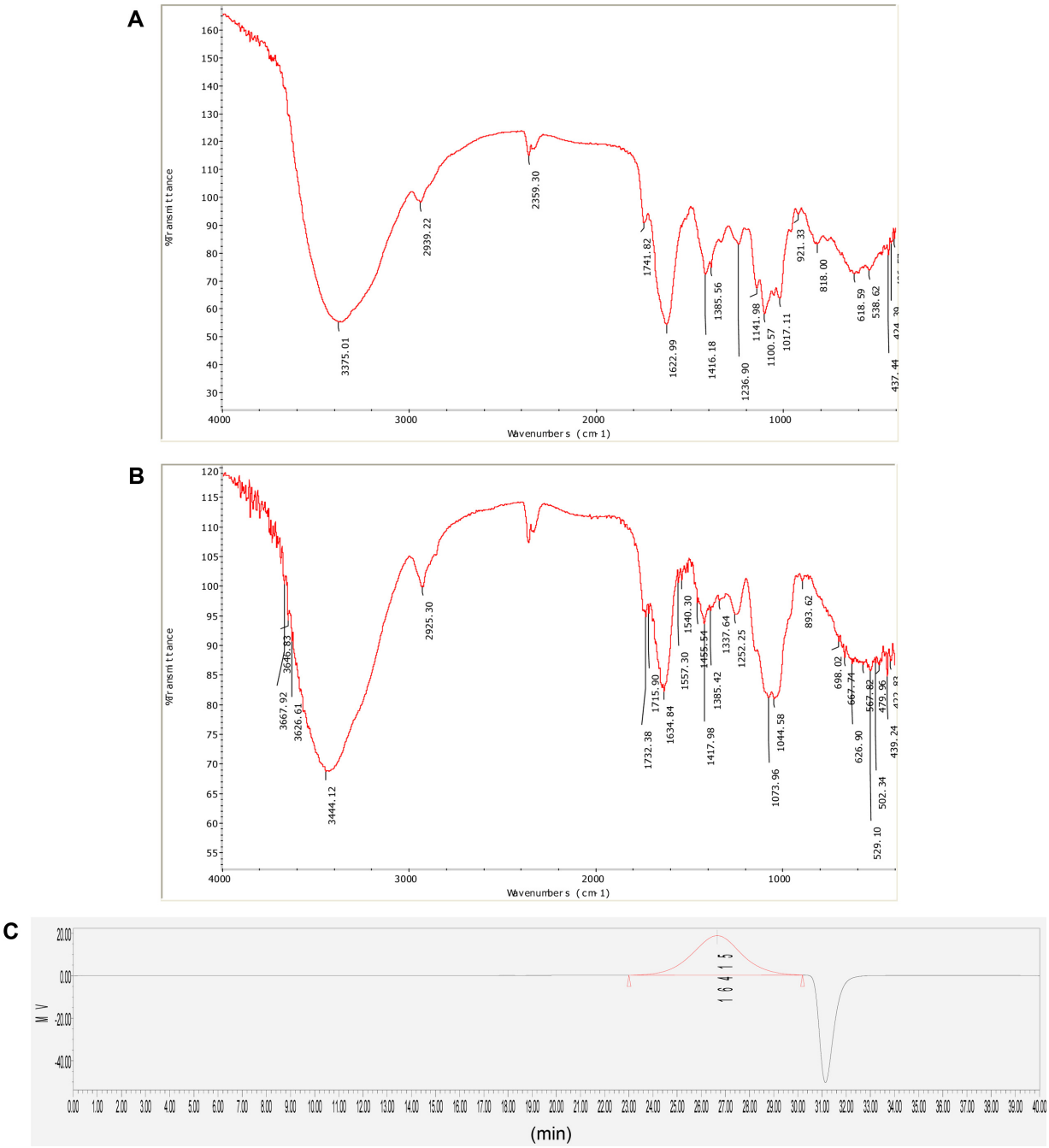

Fig. 1. Infrared spectrum of CPPS (**A**) and $sCPPS_5$ (**B**) and the M_W of the CPPS5 and sCPPS5 by HPLC-GPC analysis (**C**).

Chemical Analysis

The M_W was determined through HPLC-GPC (Fig. 1). The M_W of $sCPPS_5$ was approximately 16.1 kDa. This result indicates that $sCPPS_5$ was successfully selenylated.

Effects of CPPS and $sCPPS_5$ on the Viability of Macrophages

The relative survival rates of all groups are illustrated in Fig. 2. The relative survival rate in the CPPS at 6.25–800 μg/ml, and $sCPPS_5$ at 6.25–100 μg/ml groups wAs not significantly larger than that of the cell control group ($p > 0.05$). However, the relative survival rates in the $sCPPS_5$ at 200–800 μg/ml groups were significantly smaller than that of the cell control group ($p < 0.05$). To avoid the cytotoxicity of test sample, CPPS and $sCPPS_5$ at the indicated concentrations

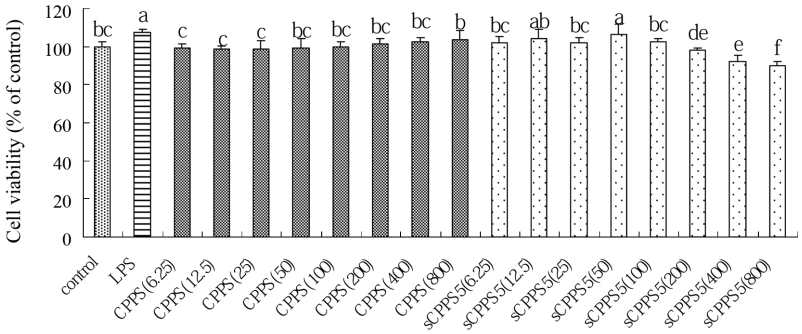

Fig. 2. Relative survival rate of every group.
Bars without the same superscripts (a–f) differ significantly ($p < 0.05$).

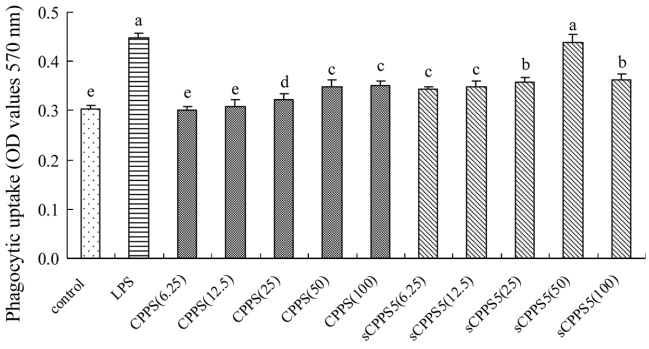

Fig. 3. Phagocytic uptake of every group.
Bars without the same superscripts (a–e) differ significantly ($p < 0.05$).

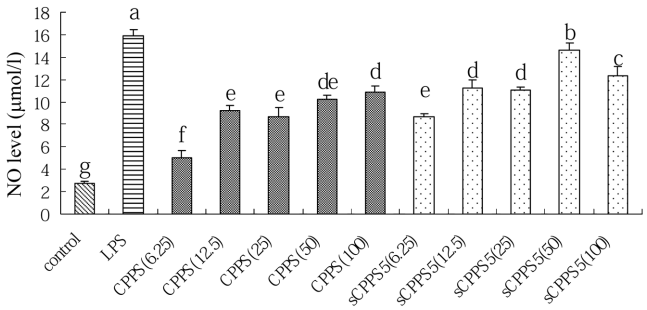

Fig. 4. NO production of every group.
Bars without the same superscripts (a–g) differ significantly ($p < 0.05$).

of 6.25, 12.5, 25, 50, 100 μg/ml were selected to conduct immune-enhancing activity assays.

sCPPS$_5$ Enhances Phagocytic Uptake Activity

The effects of CPPS and sCPPS$_5$ on phagocytosis of neural red are illustrated in Fig. 3. The A_{570} values in the LPS, CPPS at 25–100 μg/ml, and sCPPS$_5$ at 6.25–100 μg/ml groups were significantly larger than that of the cell control group ($p < 0.05$). The A_{570} values in the sCPPS$_5$ at 25–100 μg/ml group was significantly larger than those in the CPPS at 6.25–100 μg/ml group ($p < 0.05$). Macrophage is the largest phagocytic cell in organisms and can devour pathogens and microbes [7]. Phagocytosis of macrophages is very important to evaluating the innnate immune function of an organism. In the present study, sCPPS$_5$ enhanced the phagocytic uptake capacity of RAW264.7 macrophages, indicating that selenylation modification could improve the immune-enhancing activity of CPPS on the nonspecific immune response. Gao *et al.* [5] also proved that selenizing angelica polysaccharide and selenizing garlic polysaccharide could significantly enhance the phagocytosis activity of the peritoneal macrophages in vitro as compared with angelica and garlic polysaccharide.

CPPS$_5$ Induces the Production of NO in Macrophages

The NO production in all groups is illustrated in Fig. 4. The NO production in the LPS, CPPS at 6.25–100 μg/ml, and sCPPS$_5$ at 6.25–100 μg/ml groups was significantly larger than that of the cell control group ($p < 0.05$). The NO production in the sCPPS$_5$ at 50–100 μg/ml group was significantly larger than those in the CPPS at 6.25–100 μg/ml group ($p < 0.05$). NO is a kind of biological substance that acts as a cytotoxic agent and modulates the immune response [10]. Activated macrophages release a large number of NO that can contribute to killing pathogens, parasites, and tumor cells [13]. In the present study, sCPPS$_5$ was shown to increase NO production. The result indicated that selenylation modification could significantly increase the NO production of RAW264.7 in vitro as compared with CPPS.

sCPPS$_5$ Enhances Secretion of TNF-α and IL-6 in Macrophages

The TNF-α concentrations in all groups are illustrated in Fig. 5. TNF-α concentrations in the LPS, CPPS at 12.5–

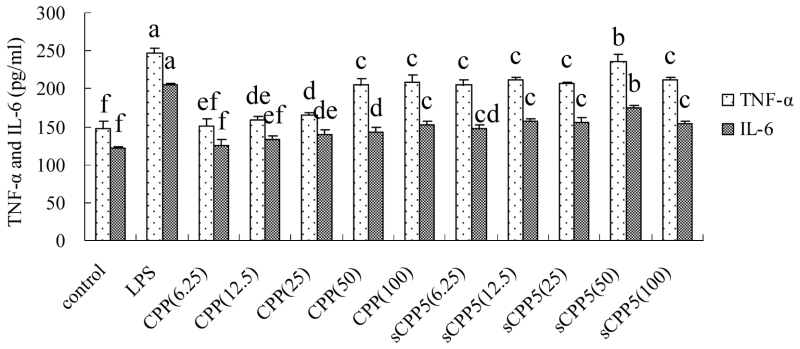

Fig. 5. TNF-α and IL-6 concentrations of every group.
Bars without the same superscripts (a–g) differ significantly ($p < 0.05$).

100 μg/ml, and $sCPPS_5$ at 6.25–100 μg/ml groups were significantly larger than that of the cell control group ($p < 0.05$). The TNF-α concentrations in the $sCPPS_5$ at 50 μg/ml group was significantly larger than those in the CPPS at 6.25–100 μg/ml group ($p < 0.05$).

The IL-6 concentrations in all groups are illustrated in Fig. 5. IL-6 concentrations in the LPS, CPPS at 25–100 μg/ml, and $sCPPS_5$ at 6.25–100 μg/ml groups were significantly larger than that of the cell control group ($p < 0.05$). The IL-6 concentrations in the $sCPPS_5$ at 50 μg/ml groups were significantly larger than those in the CPPS at 6.25–100 μg/ml group ($p < 0.05$).

TNF-α has many biological activities and plays a crucial role in immunoregulation [21]. Activated macrophages can kill invading pathogens and microbes by secretion of TNF-α. The experimental results showed that the concentrations of TNF-α in the $sCPPS_5$ group were significantly higher than those of the non-selenizing CPPS group. It indicated that the selenylation modification could significantly promote the secretion of TNF-α in vitro as compared with CPPS.

IL-6 is one of the pro-inflammatory cytokines and could actively participate in immune response, such as activate lymphocytes, promote the generation of antibody, and so on [14]. The experimental results showed that the concentrations of IL-6 in the $sCPPS_5$ group were significantly higher than those of all non-selenizing CPPS groups. Therefore, these indicated that the selenylation modification could significantly promote the secretion of IL-6 as compared

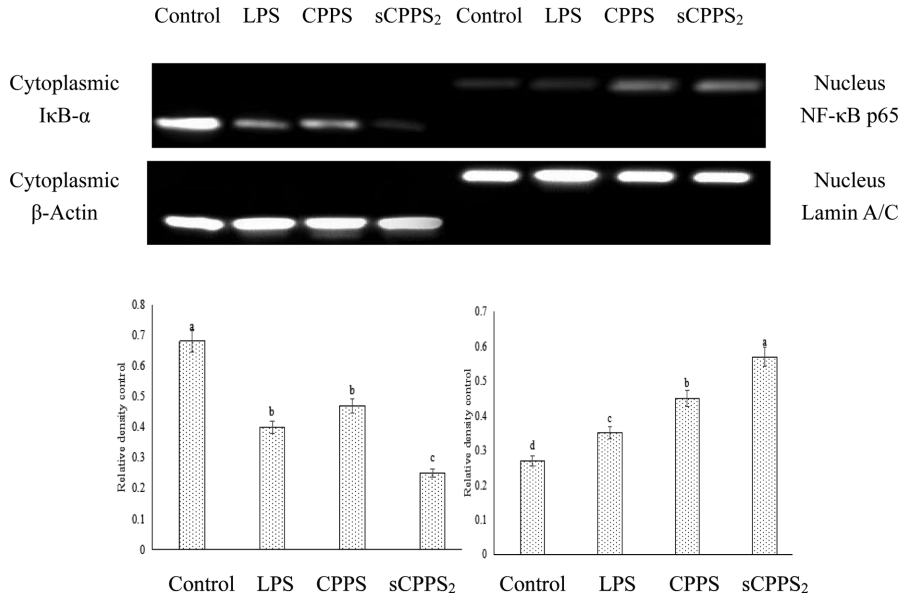

Fig. 6. Effects of CPPS and $sCPPS_5$ on the IκB-α and NF-κB protein expression levels of RAW264.7 cells.
Bars without the same superscripts (a–d) differ significantly ($p < 0.05$).

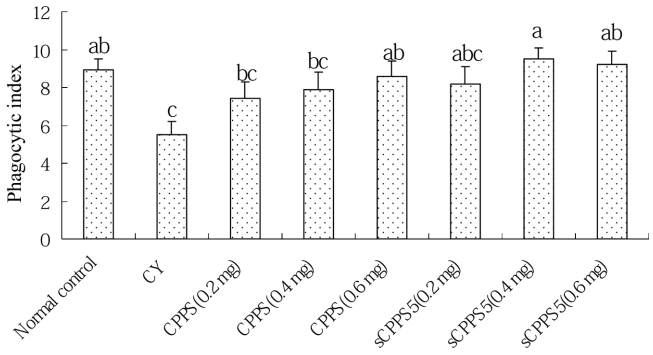

Fig. 7. Phagocytic index of every group.
Bars without the same superscripts (a–c) differ significantly ($p < 0.05$).

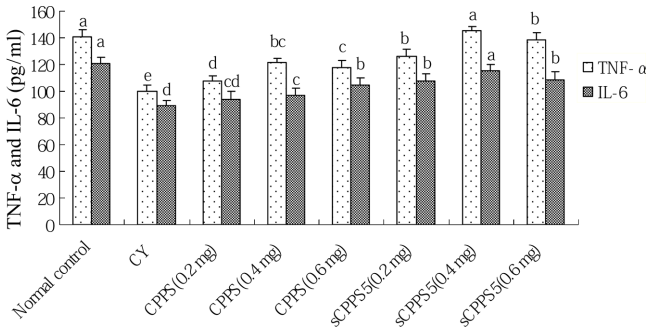

Fig. 8. Cytokine concentrations of every group.
Bars without the same superscripts (a–e) differ significantly ($p < 0.05$).

with CPPS. Many researchers also confirmed that Chinese herbal medicines could significantly improve the secretion of cytokines in vitro after selenylation modification [17].

NF-κB Pathway

NF-κB is an important transcription factor for regulating pro-inflammatory mediators in activated macrophages. Activation of NF-κB occurs following the phosphorylation, ubiquitination, and proteolytic degradation of IκB-α [1]. The signals originated from TLR are known to activate NF-κB. To investigate whether the NF-κB signaling pathway is activated, RAW264.7 cells were treated with CPPS and $sCPPS_5$ for 30 min, and then the cytoplasmic level of IκB-α and nuclear level of NF-κB p65 subunit were analyzed by western blot assay. As shown in Fig. 6, CPPS and $sCPPS_5$ induced IκB-α degradation in the cytosol and the translocation of NF-κB p65 subunit into the nucleus of RAW264.7 cells. The cytoplasmic level of IκB-α of the $sCPPS_5$ group was significantly lower than the CPPS group ($p < 0.05$), but was significant higher than the CPPS group in nuclear level of NF-κB p65 ($p < 0.05$). These results suggested that the activity of $sCPPS_5$ was dependent on the activation of NF-κB signaling pathways. For the first time, we have established the regulation of multiple TLRs and their associated signaling genes following activation of RAW264.7 cells by $sCPPS_5$. These data further expand current knowledge on how $sCPPS_5$ acts as a potent adjuvant and antitumor agent with immunomodulatory activity.

Effects of $sCPPS_5$ on Phagocytic Index in the CY-Treated Mice

The phagocytic index in all groups are illustrated in Fig. 7. The phagocytic indexes in the normal control, CPPS at 0.6 mg/ml, and $sCPPS_5$ at 0.4–0.6 mg/ml groups were significantly larger than that of the CY group ($p < 0.05$). The phagocytic index in the $sCPPS_5$ at 0.4 mg/ml group was significantly larger than those of the CPPS at 0.2–0.4 mg/ml group ($p < 0.05$), but there were no significant differences between the $sCPPS_5$ at 0.4 mg/ml group and normal control group ($p > 0.05$). The results showed that $sCPPS_5$ could significantly improve the phagocytic activity of macrophages in vivo as compared with CPPS, which demonstrated that the immuno-enhancement effect of CPPS was significantly improved after selenylation modification of the polysaccharide.

Changes of Serum TNF-α and IL-6 Concentrations in the CY-Treated Mice

The serum cytokine concentrations of every group are illustrated in Fig. 8. The serum TNF-α concentrations in the normal control, CPPS at 0.2–0.6 mg/ml, and $sCPPS_5$ at 0.2–0.6 mg/ml groups were significantly larger than that of the CY group ($p < 0.05$). The serum TNF-α concentrations in the $sCPPS_5$ at 0.2–0.6 mg/ml group were significantly larger than those of the CPPS at 0.2 and 0.6 mg/ml group ($p < 0.05$). The serum TNF-α concentrations in the $sCPPS_5$ at 0.4 mg/ml group were significantly larger than those of the CPPS at 0.2–0.6 mg/ml group ($p < 0.05$), but there were no significant differences between the $sCPPS_5$ at 0.4–0.6 mg/ml group and normal control group ($p > 0.05$).

The serum IL-6 concentrations in the normal control, CPPS at 0.4–0.6 mg/ml, and $sCPPS_5$ at 0.2–0.6 mg/ml groups were significantly larger than that of the CY group ($p < 0.05$). The serum IL-6 concentrations in the $sCPPS_5$ at 0.2–0.6 mg/ml group were significantly larger than those of the CPPS at 0.2–0.4 mg/ml group ($p < 0.05$). The serum IL-6 concentrations in the $sCPPS_5$ at 0.4 mg/ml group were significantly larger than those of the CPPS at 0.2–0.6 mg/ml group ($p < 0.05$), but there were no significant differences between the $sCPPS_5$ at 0.4 mg/ml group and normal control group ($p > 0.05$).

In vivo, these indicate that the selenylation modification could significantly promote the secretion of TNF-α and IL-6 as compared with CPPS. It is analogous to the results of our previous study in vitro. The reasons may be that the selenylation modification is a widely used method to enhance the biological activities, especially in antioxidant and immune enhancement [18, 19]. Many other researchers also proved that the efficacy of polysaccharides by reforming their structure was significantly better than non-reformed drugs [20].

Acknowledgments

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