Polysaccharides extracted from the roots of Bupleurum chinense DC modulates macrophage functions

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[ABSTRACT] The present study aimed to investigate the effects of polysaccharides extracted from Bupleurum chinense DC (BCPs) on macrophage functions. In the in vivo experiment, 1 mL of 5% sodium thioglycollate was injected into the abdomen of the mice on Day 0 and macrophages were harvested on Day 4. The macrophages were cultured in plates and treated with different concentrations of BCPs and stimuli. Effects of BCPs on macrophage functions were assessed by chemotaxis assay, phagocytosis assay and Enzyme-Linked Immunosorbent Assay (ELISA). Our results showed the enhanced chemotaxis, phagocytosis and secretion of nitric oxide (NO) and inflammatory cytokines by macrophages when treated with BCPs. However, when chemotaxis and phagocytosis were up-regulated by complement components or opsonized particles, BCPs inhibited these effects. Also, the NO production induced by lipopolysaccharides (LPS) was suppressed by BCPs mildly. Moreover, BCPs had an inhibitory effect on the [Ca²⁺]i elevation of macrophages. These results suggested that BCPs exerted modulatory effects on macrophage functions, which may contribute to developing novel approaches to treating inflammatory diseases.

[KEY WORDS] Bupleurum chinense DC; Polysaccharides; Macrophage functions; [Ca²⁺]i

Introduction

Bupleurum spp. is a traditional Chinese medicine with proven anti-inflammatory, antipyretic, analgesic and immunomodulatory activities [1-3]. Previous researchers have extracted polysaccharides from Bupleurum smithii, which have proven effective against autoimmune disease [4-5] such as systemic lupus erythematosus [2]. Compared with polysaccharides extracted from Bupleurum smithii (BPs), polysaccharides extracted from Bupleurum chinense DC (BCPs) have distinct compositional differences [6-7], while both were found having high anti-complementary activity and inhibiting inflammation in animal models [8-9]. In a previous study, we have observed that BCPs attenuate inflammatory cells aggregation in the bronchoalveolar lavage fluid and prevent TNF-α and NO over-production. BCPs also significantly attenuate lung injury with improved lung morphology in LPS-induced acute lung injury mice, in which macrophages play an important role [8]. Inflammation is a complex process mediated by a variety of signaling molecules and cells. When inflammation happens, monocytes/macrophages and neutrophils accumulate at infection sites to kill pathogens. Macrophages are major inflammatory and immune system cells. Activated macrophages are present in inflamed tissues and produce a variety of pro-inflammatory mediators, such as cytokines [interleukin 1(IL-1), tumor necrosis factor α (TNF-α), chemotactic factors, adherence factors (monocyte chemo-attractant protein 1 (MCP-1), IL-8), and cytotoxic mediators (nitric oxide (NO), reactive oxygen species (ROS))] [10-11]. Macrophages also clear pathogens and apoptotic cells by phagocytosis, which is...
mediated by receptors on the membrane\textsuperscript{[12]}.

Free Ca\textsuperscript{2+} in immune cells help modulate inflammatory response\textsuperscript{[13]}. In macrophages, low free cytosolic Ca\textsuperscript{2+} is found to play a regulatory role in inducible nitrous oxide synthase (iNOS) reaction\textsuperscript{[14]}. Lipopolysaccharide (LPS) treatment has proven to increase intracellular calcium concentration ([Ca\textsuperscript{2+}]) which is related to the release of the cytokine, TNF-\(\alpha\)\textsuperscript{[15]}. Blocking calcium influx could modulate both LPS and interferon-\(\gamma\) (IFN-\(\gamma\)) inflammatory responses, including pro-inflammatory cytokines secretion and phagocytic activity\textsuperscript{[16]}.

Presently, the mechanisms of BCPs’ function in inflammation remain unclear. Based on the above-mentioned studies and macrophage roles in inflammatory diseases, we investigated the effects of BCPs on macrophage functions, including chemotaxis, phagocytosis, cytokine production and intracellular Ca\textsuperscript{2+} fluctuation.

**Materials and Methods**

**Animals**

Male Balb/c mice and Kunming mice (6-8-weeks-old) were purchased from Slac Laboratory Animal Corporation (Shanghai, China) and housed under specific pathogen free (SPF) conditions, with a 12 h/12 h light/dark cycle, while accessing to tap water and food ad libitum. All the experimental protocols described in the present study were approved by the Animal Ethics Committee of the School of Pharmacy, Fudan University (approval Number: 2013-53, approved on 15\textsuperscript{th} Oct., 2013).

**Isolation and purification of BCPs**

*Bupleurum chinense* DC polysaccharides (BCPs) were isolated at the pharmaceutical chemistry laboratory of Dr. Chen Dao-Feng (Fudan University, Shanghai, China). Identification of *Bupleurum chinense* DC and methods for isolating polysaccharides have been previously reported\textsuperscript{[3]}. Briefly, the dried roots were ground into fine particles and defatted using 95\% EtOH, and the residues were then dried in the shade and extracted with hot water. The supernatant was extensively dialyzed against running water and precipitated by adding 4 volumes of 95\% EtOH. After centrifugation, the precipitate was washed with anhydrous EtOH and then lyophilized to produce the crude polysaccharide (BCPs).

**Chemicals and Reagents**

LPS (from *Escherichia coli* O111: B4), fluorescein isothiocyanate-conjugated *Staphylococcus aureus* (FITC-conjugated *S. aureus*), and dexamethasone (DEX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3 AM ester was provided by Invitrogen (Carlsbad, CA, USA). Fetal calf serum (FCS) was purchased from Gibco Inc (CA, USA). ELISA assay kits for mouse TNF-\(\alpha\), IL-6, IL-1\(\beta\), and IL-12 were purchased from Boehringer Ingelheim (Shanghai, China). Recombinant human complement components C3a and C5a were purchased from R&D Corp. (Minneapolis, MN, USA). RPMI 1640 medium was purchased from Hyclone (Logan, UT, USA). Complement-activated product was prepared by our lab and added into cell culture plates according to protocols below.

Preparation of complement-activated product (CAP) began with washing fresh sheep blood cells in sterile saline and centrifuging them at 800 g. The procedure was repeated thrice to obtain packed sheep red blood cells, subsequently diluting these with sterile saline (\(V/V\)) to 2\% sheep red blood cells (2%-SRBC). Next, equal volumes 2%-SRBC and rabbit anti-SRBC IgG (1 : 1000 dilution) were mixed, then incubated in a water bath at 37 °C for 30 min. Once centrifuged at 734 g and 4 °C for 10 min, the sediment was mixed with serum from guinea pigs, which was bathed at 37 °C for 30 min, so that the red blood cells completely dissolved. After centrifuging again under the same conditions, the supernatants, now considered as complement-activated product, were collected and stored at −20 °C until experiments.

**Macrophage Isolation and Culture**

Peritoneal macrophages were isolated as previously described, with minor modifications\textsuperscript{[17]}. Briefly, 1 mL of 5\% sodium thioglycollate was injected into to the abdomen of the BALB/c mice on Day 0. On Day 4, the animals were euthanized. About 5 mL of cold RPMI 1640 serum-free medium, containing 100 U·mL\textsuperscript{−1} of penicillin and 100 μg·mL\textsuperscript{−1} of streptomycin, was injected into the abdomen. The reacted RPMI 1640 medium was then drawn back and centrifuged at 80 g for 10 min. After discarding the supernatant, the cells were re-suspended and plated on 48-well plates at a density of \(1 \times 10^6\) cells per well in RPMI 1640 medium with 10\% (\(V/V\)) FCS. Once cultured for 2 h in moist air at 37 °C with and in 5\% CO\textsubscript{2}, the cells were washed twice with the medium and then reserved for the following experiments. In all experiments, macrophages incubated in RPMI 1640 medium with 10\% (\(V/V\)) FCS alone were considered the control group, while those treated with CAP, C3a, C5a, and LPS were considered the model group. Those cells treated with DEX were used as the positive control.

RAW264.7 macrophage cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium with 10\% (\(V/V\)) fetal bovine serum (FBS), 100 U·mL\textsuperscript{−1} penicillin, and 100 μg·mL\textsuperscript{−1} streptomycin, at 37 °C in a humidified, 5\% CO\textsubscript{2} incubator. The cells were then used for the intracellular calcium assay.

**Assay on macrophages chemotaxis**

Chemotaxis assay was conducted using trans-well migration assay\textsuperscript{[18]}, with some modifications. For the test investigating the effects of BCPs on chemotaxis, macrophages were plated at a density of \(1 \times 10^6\) cells per well in the upper compartment. BCPs (100 μL) at final concentrations of 10, 20, 40, and 80 μg·mL\textsuperscript{−1} or 100 μL of DEX (10 μmol·L\textsuperscript{−1}) was added to the lower compartment. Then the trans-well plate was incubated in a humidified, 5\% CO\textsubscript{2} incubator for 4 h, whereupon the chambers were washed twice with PBS to remove non-migrated macrophages. Chemo-attracted macrophages on the lower surface of the microporous membrane were fixed by exposure to 3\% glutaraldehyde for 30 min at room tempera-
ture, stained with Wright-Giemsa pigment, and finally observed by light microscopy at 400× magnification. 10 views were photographed in each well and the number of cells per view was counted.

For the test that explored the effects of BCPs on chemotaxis under complement components stimulation, macrophages were plated at a density of 1 × 10^6 cells per well in the upper compartment. After chemotactic factor (100 μL of CAP, 500 ng·mL⁻¹ of C3a, or 50 ng·mL⁻¹ of C5a) had been added to the lower compartment, either 100 μL of DEX (10 μmol·L⁻¹), or 100 μL of BCPs at final concentrations of 10, 20, 40, and 80 μg·mL⁻¹ was also added. The subsequent steps were exactly the same as mentioned above.

**Assay of macrophage phagocytosis**

The phagocytic experiments conducted were similar to those reported previously [2], for which macrophages were plated at a density of 2 × 10^5 cells per well in 48-well culture plates and incubated for 1 h. Then, the wells were washed thrice to remove non-adhering cells, while the adherent cells were reserved for further study.

**Phagocytosis assay of IgG-opsonized sheep red blood cells (SRBCs)**

IgG-opsonized SRBCs (IgG-SRBCs) were prepared by incubating sheep red blood cells with a 1 : 4 000 dilution of rabbit anti-SRBC IgG for 30 min at 37 °C. Centrifuging the mixture at 734 g and 4 °C for 10 min preceded retention and re-suspension, sediment to be used as IgG-opsonized SRBC.

After 100 μL of IgG-opsonized SRBCs was added to the wells, either 100 μL of DEX (10 μmol·L⁻¹) or 100 μL of BCPs at final concentrations of 10, 20, 40, and 80 μg·mL⁻¹, was added gently to the 24-well plates and co-incubated with macrophages for another 30 min. Then, the 24-well plates were washed with PBS to remove non-engulfed SRBCs. Finally, macrophages were fixed for 50 min with 99.9% methanol and stained with Wright-Giemsa pigment.

The phagocytic index and phagocytic rate were defined as follows: phagocytic rate = number of macrophages that engulfed SRBCs out of 100 macrophages, phagocytic index = number of internalized SRBCs in 100 macrophages. **Phagocytosis assay of CRBCs treated with/without complement activation product (CAP)**

CRBCs were washed thrice with saline and diluted at 1: 50 with 10% FCS-1640 to form 2% CRBCs, which was added to the 48-well plates. Then 100 μL of DEX (10 μmol·L⁻¹) or 100 μL of BCPs at final concentrations of 10, 20, 40, and 80 μg·mL⁻¹, was added gently to the 48-well plates and co-incubated with macrophages for 1 h. After, the plates were washed thoroughly to remove non-engulfed CRBCs, and then macrophages were fixed with 99.9% methanol and marked with Wright-Giemsa staining.

In the assay of phagocytosis of CRBCs opsonized with CAP, 2% CRBCs, prepared as mentioned above, were next exposed to complement-activated product V/V = 1: 1) at 30 °C for 30 min. After centrifugation, the CAP-treated CRBCs were collected and re-suspended in 10% FCS-1640 (V/V = 1: 50). Either 100 μL of DEX (10 μmol·L⁻¹) or 100 μL of BCPs at final concentrations of 10, 20, 40, and 80 μg·mL⁻¹, was added gently to the 48-well plates, to which CAP-treated CRBCs (200 μL per well) had been previously added, and then co-incubated with macrophages for 1 h. After, the plates were washed thoroughly to remove non-engulfed CRBCs, and then macrophages were fixed with 99.9% methanol and marked with Wright-Giemsa staining.

Phagocytosis was assessed under light microscopy at 400× magnification. Calculation of phagocytic index and phagocytic rate was similar: phagocytic rate = number of macrophages that engulfed CRBCs out of 100 macrophages, phagocytic index = number of internalized CRBCs out of 100 macrophages. **Phagocytosis assay of FITC-Staphylococcus aureus (FITC- S. aureus) treated with/without CAP**

The phagocytosis was assayed by detecting ingestion of FITC- S. aureus particles, similar to our previous study with FITC-E. coli [3]. Either 100 μL of DEX (10 μmol·L⁻¹) or 100 μL of BCPs at concentrations equal to the previous 3 tests, with 20 μL of FITC-S. aureus (5×10⁶ FITC-S.aureus·mL⁻¹), were added to the cell cultures (FITC-S. aureus: cells = 50 : 1) and co-incubated with macrophages for 30 min. The 48-well plates were next washed to remove non-engulfed FITC- S. aureus, and then cells were fixed with 99.9% methanol and observed under a fluorescence microscope. The fluorescent intracellular area and integral optical density (IOD) were calculated by the software image-J (National Institutes of Health, Bethesda, MD, USA).

For the phagocytosis assay of FITC-S. aureus opsonized with CAP, extra 100 μL of CAP was added to the cell cultures before co-incubating. The other steps were exactly the same as mentioned above.

**Determination of Nitric Oxide (NO) and Cytokines**

Macrophages were plated at a density of 1 × 10⁶ cells per well into 48-well culture plates and incubated for 24 h with different concentrations of BCPs or BCPs combined with 1 μg·mL⁻¹ of LPS. Next the supernatants were collected and stored at −80 °C for measurement of NO and cytokines. **Determination of nitric oxide (NO)**

Determination was performed based on the method published by Igor A. Schepetkin [19], in which nitrite ion concentration was used as a NO production indicator and NaNO₂ served as a standard substance. Briefly, the supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. Next, the concentration of nitric oxide was measured at 540 nm on a microplate reader. **Determination of cytokines**

Using specific murine ELISA kits (Boatman Corp., Shanghai, China), following the manufacturer’s instructions, levels of TNF-α, IL-6, IL-1β, and IL-12p40 were measured. **Intracellular calcium assay**

The method for measuring [Ca²⁺] in macrophages was in
detailed earlier report [20]. Briefly, the RAW264.7 macrophages were scraped from culture flasks and adjusted to a density of $2 \times 10^5$ cells·mL$^{-1}$ in RPMI 1640 medium. Then the cells were plated on 96-culture plates and allowed to adhere for 24 h at 37 °C in moist air containing 5% CO$_2$. The non-adherent cells were removed by washing twice with RPMI 1640. Fluo-3 AM loading solution was added to the plates to adjust the fluo-3 AM concentration to 1 µmol·L$^{-1}$, then the plates were incubated at 37 °C in moist air with 5% CO$_2$ for 45 min. After washing the plates with RPMI 1640 twice, different concentrations of BCPs were added to the plates. 12 min later, Ca$^{2+}$ fluorescence intensity was measured every 60 s by fluorescence spectrophotometer. The excitation and emission wavelengths were set at 488 nm and 526 nm, respectively. During the experiment, the 96-culture plates were kept constant at 37 °C.

To investigate whether BCPs functions are related to [Ca$^{2+}$], EGTA (ethylene glycol tetraacetic acid) was used to chelate the extracellular calcium. After washing the plates with RPMI 1640 twice, BCPs (80 µg·mL$^{-1}$) and EGTA were added to the plates, with a final EGTA concentration of 0.5 mmol·L$^{-1}$. Finally, Ca$^{2+}$ fluorescence intensity was measured as described above.

Statistical Analysis
All the data are expressed as means ± SD. To perform statistical analysis, one-way analysis of variance (ANOVA) was used. Statistical significance was accepted at $P < 0.05$.

Results
Effects of BCPs on chemotaxis of macrophages
It has been proven that the complement system and its products have an important influence on the activation of the innate immune system [21]. Thus, we investigated the effects of BCPs on macrophage chemotaxis, with and without complement-activated product (CAP), C3a, and C5a.

Effects of BCPs on chemotaxis
As shown in Fig. 1A, chemotactic cell number increased significantly when macrophages were treated with BCPs at 40 and 80 µg·mL$^{-1}$, compared with the control group ($P < 0.05$). DEX had an inhibitory effect on it, but did not reach the level of statistical significance.

Effects of BCPs on chemotaxis induced by CAP
Chemotaxis of macrophages was significantly enhanced by CAP ($P < 0.001$), compared to the control group. Both BCPs (20 µg·mL$^{-1}$ and above) and DEX (10 µmol·L$^{-1}$) significantly suppressed that increase ($P < 0.01$; Fig. 1B).

![Fig. 1 Effects of BCPs on chemotaxis with/without CAP, C5a, and C3a. The data are expressed as means ± SD, n = 3 for each group. A: effect of BCPs on chemotaxis. B: effects of BCPs on CAP-induced chemotaxis. C: effects of BCPs on C5a-induced chemotaxis. D: effects of BCPs on C3a-induced chemotaxis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control group, $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ compared with CAP, C5a or C3a stimulated model group, tested by ANOVA](image)
Effects of BCPs on chemotaxis induced by C5a

The data shown in Fig. 1C demonstrated that chemotactic cell number increased significantly when cells were treated with C5a (50 ng·mL⁻¹; P < 0.001). BCPs at 40 μg·mL⁻¹ and 80 μg·mL⁻¹, as well as DEX, significantly suppressed the increase of cell number (P < 0.01).

Effects of BCPs on chemotaxis induced by C3a

Chemotactic cell number increased significantly when macrophages were stimulated with C3a (500 ng·mL⁻¹). However, BCPs and DEX did not decrease chemotactic cell number as induced by C3a (500 ng·mL⁻¹; Fig. 1D).

Effects of BCPs on phagocytosis

Phagocytosis of pathogens by macrophages initiates the innate immune response. Macrophages have evolved a restricted number of phagocytic receptors to recognize pathogens and opsonized particles, which is critical for the uptake and degradation of infectious agents in inflammation [22]. Therefore, the effect of BCPs on phagocytosis by macrophage was investigated.

Effect of BCPs on phagocytosis of IgG-SRBCs

Compared with control group, BCPs increased phagocytosis of IgG-SRBCs (P < 0.01). In contrast, DEX showed a significantly inhibitory effect on phagocytic index and a mild effect on the phagocytic rate (Figs 2A and 2B).

Effect of BCPs on phagocytosis of CRBCs

BCPs promoted phagocytic index and phagocytic rate of CRBCs, when compared with the control group (P < 0.05), but DEX showed a mild inhibitory effect on it.

Both phagocytic rate and phagocytic index also increased in the CAP group. In this case, both BCPs and DEX had significant decreasing effects on phagocytic index (Figs. 3A and 3B), as compared with CAP.
Effects of BCPs on phagocytosis of FITC- S. aureus

As shown in Figs. 3C and 3D, area of fluorescence increased significantly when macrophages were treated with 80 μg·mL⁻¹ BCPs, compared to the control group. However, BCPs just promoted moderate increase of fluorescence intensity.

Fluorescence area and index increased significantly when cells were treated with FITC-S. aureus and CAP. A considerable decrease of both fluorescence area and density was observed in BCPs and DEX groups as compared with the CAP group (P < 0.001).

Effects of BCPs on NO and cytokine production

Cytokines and inflammatory mediator secreted by macrophage actively intervene in inflammation regulation, such as activating and differentiating T cells, stirring up the adaptive immune response, and also have an important role in the pathogenesis [23]. Suppressing the over secretion of cytokines will be helpful in inflammatory diseases. Thus, we investigated the effect of BCPs on secretion of cytokines and inflammatory mediator.

NO in the medium was significantly increased by BCPs (at 80 μg·mL⁻¹), compared to the control group, as well as by treatment with 1 μg·mL⁻¹ of LPS (Fig 4A). The increase induced by LPS was significantly inhibited by BCPs and DEX. However, the effect of BCPs was much weaker than DEX (P < 0.05).

TNF-α, IL-1β, IL-6, IL-12p40, and IL-10 are important inflammation related factors. BCPs increased the production of TNF-α, IL-6, IL-12p40, and IL-10 significantly, as compared with the control group (P < 0.05; Figs. 4B, and 4D-4F). Macrophages generated only a lower level of IL-1β and BCPs had no significantly effect on it (Fig 4C).

Compared with the control group, all these cytokines were increased significantly by LPS stimulation (P < 0.001). The elevation of TNF-α, IL-1β, and IL-10 was suppressed significantly by BCPs, while the effects on TNF-α and IL-1β were mild (Figs. 4B-4D). However, IL-6 and IL-12p40 were not influenced significantly by BCPs (Figs. 4E and 4F).

Fig 4. Effects of BCPs on peritoneal macrophages NO and cytokine production. The data are expressed as means ± SD., n = 3 for each group. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with the control group, * P < 0.05, ** P < 0.01, *** P < 0.001 compared with LPS-stimulated model group, tested by ANOVA.
**Effects of BCPs on [Ca^{2+}] in macrophages**

The levels of [Ca^{2+}] rose gradually when the cells were exposed to UV radiation continually. 80 μg·mL^{-1} of BCPs inhibited elevation of [Ca^{2+}], compared to both the cells and EGTA groups, the [Ca^{2+}] level in the BCPs group (at 80 μg·mL^{-1}) was less after 30 min.

To determine whether BCPs inhibit the influx of intracellular or extracellular Ca^{2+}, extracellular Ca^{2+} was chelated by EGTA. Compared to both the cells and EGTA groups, the [Ca^{2+}] level in the BCPs group was significantly lower.

**Discussion**

**Bupleurum**, a Chinese traditional herb, has been used to treat various diseases for hundreds of years. Polysaccharides fractionated from roots of *Bupleurum chinense* DC present antioxidant activity and hepatoprotective effect in vivo, and anti-complementary activity in vitro. Considering that tissue macrophages involved in many inflammatory diseases, we hypothesized that BCPs may influence macrophage behavior so as to ameliorate these diseases.

Macrophages differentiate from circulating peripheral blood mononuclear cells and are present in virtually all tissues. They play an important role in maintaining homeostasis and have at least three major functions: antigen presentation, phagocytosis and immunomodulation through production of biologically active molecules. When macrophages are exposed to endogenous danger signals, the responses of macrophages in host defense consist of the following steps: recruitment to the infected site, recognition of pathogens, phagocytosis and destruction of ingested microbes.

Macrophage accumulation at local sites is therefore obviously important for host defense against foreign bodies. The recruitment of macrophages to tissue sites is stimulated by a number of factors, including lymphocyte-derived chemotactic factors and complement factors. Some factors derived from complement system, which is a part of the immune system and enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells. The complement system consists of a large number of distinct plasma proteins that can recognize potential threats and then undergo triggered-enzyme cascade to produce a response of sufficient proportions to neutralize the inflammation. Researches also have proven that complement components C3a and C5a play a critical role in the modulation of immune system, by interacting and activating immune cells via C3a receptor and C5a receptor, respectively. Both are chemoattractant molecules, and allow basophils, macrophages, neutrophils, and lymphocytes recruitment at the inflammatory site.

In the present study, we found that BCPs not only promoted chemotaxis of macrophages, but also inhibited enhanced chemotaxis as stimulated by CAP (mixture of complement components after activation), C3a and C5a. Part of the results was consistent with our previous *in vivo* study. BCPs decreased the total number of inflammatory cells in LPS-induced acute lung injury mice. While our research further suggested that BCPs had a modulatory effect on chemotaxis, BCPs showed better inhibitory effect on chemotaxis induced by C5a than it did for C3a, which might be partly due to the number and affinities of their respective receptors. Another reason for uneven effect may be that C3a is a specific chemotactic mediator which selectively influences eosinophil adhesion, but not transmigration.

As an important special phagocyte, macrophages express a broad array of receptors that mediate recognition and internalization when they play their scavenger role in the clearance of foreign materials. In terms of phagocytosis, as related to three kinds of particles studied in our research, Fcγ receptor is involved in the phagocytosis of IgG-SRBC, CRBC has notable positive correlation with expression of toll-like receptor 4 and phagocytosis of *S. aureus* is related to the mannose receptor. Our research showed that BCPs alone augmented the phagocytosis of macrophages, while inhibiting it when macrophages were treated with opsonized particles. These results indicated that BCPs might have similar influences on the receptors mentioned above.

Cytokines are essential molecules involved in the differentiation, maturation and activation of immune cells, and thus have a significant influence on the inflammatory response.
Over-activated macrophages release a great amount of different cytokines which bring about tissue injury \[39\]. NO is another substance secreted by macrophages, which is a critical mediator modulating a variety of biological functions, and whose excessive production leads to a range of important pathologies \[40\]. Polysaccharides have been proven to modulate the production of inflammatory mediators \[41\]. As observed in our research, BCPs showed a modulatory effect on the production of NO and some cytokines. TLR-4 is the ligand of LPS \[42\], and previous studies have shown that polysaccharides extracted from *Bupleurum smithii* (BPs) attenuate LPS-stimulated inflammatory response by modulating the TLR-4 signaling pathway in macrophages \[3\]. The preceding fact reminds us that TLR-4 signaling pathway may be a potential target for BCPs, but it is not the only one.

Although *Bupleurum chinense* DC and *Bupleurum smithii* belong to the same genus, BCPs and BPs, extracted from these plants respectively, do not have the same actions. BCPs significantly increased production of cytokines, but BPs did not. When macrophages were treated with LPS, BPs had a potent inhibitory effect on TNF-\(\alpha\), IL-6, IL-1\(\beta\), and IL-12p40 production, while it promoted IL-10 production \[3\]. Contrastingly, BCPs showed the inhibitory effect on IL-10. IL-10 is a potent anti-inflammatory cytokine \[43\], which acts at various stages of the immune response in a coordinated way that efficiently restrains the inflammatory response. It affects the functions of monocytes, macrophages or other immune cells, including phagocytosis, presentation of antigen. It also inhibits the production of pro-inflammatory cytokines and chemokines by immune cells such as macrophages. Hence, IL-10 limits the immune response during infection and prevents the immune system damage to the host \[44\]. In the present study, the secretion of IL-10 was inhibited. The reason might be that BCPs directly suppressed inflammation through down-regulating the chemotaxis and phagocytosis of microorganisms, leading to the decrease of cytokines secretion. Besides, the inhibition of IL-10 secretion might account for the weak action of BCPs on pro-inflammatory cytokine suppression. Overall, BCPs showed a modulatory effect on production of cytokines by macrophages, which is different from the inhibitory effects of BPs. The differences of effects on cytokine production between BCPs and BPs might be related to the different influences on the phenotypic switching mechanism in macrophage polarization \[45\], which is needed to be explored in the future.

Intracellular Ca\(^{2+}\) coordinates a variety of cellular functions, including gene expression, cell cycles, differentiation, migration and tolerance \[46\]. In the present study, we found that BCPs (80 \(\mu\)g mL\(^{-1}\)) inhibited the elevation of intracellular Ca\(^{2+}\), compared to the control cell group. Furthermore, the effects of BCPs persisted when extracellular Ca\(^{2+}\) was chelated with EGTA. Previous study has shown that UV-irradiation can damage macrophages, leading to increased extracellular Ca\(^{2+}\). An increase in extracellular Ca\(^{2+}\) is accompanied by an increase in the number of damaged cells \[47\]. This implied that BCPs might protect macrophages from damage due to over-activation by stimulus, such as LPS, while helping maintain normal macrophage function by inhibiting the over release of intracellular Ca\(^{2+}\). Further study is needed to investigate the potential mechanisms of this phenomenon.

By removing or deactivating mediators and inflammatory effector cells, inhibition of inflammation contributes to the repair of damaged tissues \[28\]. BCPs exposure resulted in enhanced phagocytosis, chemotaxis and secretion of macrophage cytokines, all of which indicated that BCPs might contribute to maintaining the homeostasis under normal conditions. Once the functions of macrophages were significantly enhanced by stimulus, BCPs acted to suppress them, which suggests that BCPs may be able to suppress the over-activation of macrophages, attenuating tissue injury, such as LPS-induced acute lung injury \[48\]. In general, BCPs showed bilateral regulatory effects on the functions of macrophages. The stimulus substances used in the present study were LPS, chemotactic factors, and opsonized particles, which interacted with various receptors and triggered different signal pathways. BCPs suppressed the actions of all the named stimuli, suggesting that BCPs may modulate those pathways in common.

In conclusion, we demonstrated that BCPs modulated cytokine secretion, phagocytosis, and chemotaxis by macrophages under both non-inflammatory and inflammatory conditions. We also found that BCPs had a protective effect on macrophages by inhibiting the release of intracellular Ca\(^{2+}\) under UV-irradiation. The effectual mechanisms of BCPs on macrophages call for further study, yet these findings suggested that BCPs may provide new therapeutic approaches to treating inflammatory diseases.

References


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The research work of Prof. ZHANG is on immunopharmacology. He has been working on pharmacology effects of polysaccharide extracted from Traditional Chinese Medicine. Studies revealed that polysaccharides might enhance the function of macrophage but suppress it when cells were over activated. Recently, he focus on the use of polysaccharides in the treatment of influenza and lung injury. Papers about these researches have been published.