

Research Article

The Immunomodulatory Effects of Phellodendri Cortex Polysaccharides on Cyclophosphamide-Induced Immunosuppression in Mice

Yi Cheng,¹ Lu Liu,¹ Simei Mo,¹ Jianxiong Gao,¹ Hongjian Zhang,¹ Heli Zhang,¹ Chunsheng Zhang,¹ Xu Song,² Lixia Li,² and Zhe Geng¹

¹Department of Physical Education, Chengdu University of Information Technology, Chengdu, China

²College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China

Correspondence should be addressed to Zhe Geng; 121039959@qq.com

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Cyclophosphamide is a commonly used anticancer drug, and immunosuppression is one of the most common side effects. How to recover the immunological function is important for cyclophosphamide-treated patients. In the present study, Phellodendri Cortex polysaccharides (CPP) could enhance the proliferation of mouse spleen lymphocytes *in vitro*. The immunoregulatory function of CPP was then investigated in cyclophosphamide-induced immunosuppressed mice. In CPP-treated groups, mice were orally treated with CPP at doses of 1, 0.5, and 0.25 g/kg bodyweight from 1 to 11 d, respectively. The cyclophosphamide was administrated in CPP and cyclophosphamide groups from 12 to 14 d. In the cyclophosphamide and normal control groups, the mice received equal volume of saline from 1 to 14 d. The results showed that CPP (1 g/kg) could significantly increase the bodyweight of mice, even during cyclophosphamide treatment. The organ coefficients of the spleen and thymus were recovered by CPP treatment. CPP upregulated the contents of cytokines (IL-2, IL-6, IFN- γ , and TNF- α) in serum, which were downregulated by cyclophosphamide. The mRNA levels of these cytokines were also elevated by CPP treatment in the spleen. Cyclophosphamide upregulated the expressions of NF- κ B p65, TLR4, and MyD88, suggesting that the NF- κ B signaling pathway was activated by cyclophosphamide. After CPP treatment, it was recovered to normal level. These results indicated that CPP alleviated the cyclophosphamide-induced immunosuppression.

1. Introduction

Phellodendri Cortex (“Huangbo” in Chinese), a traditional Chinese herbal, is derived from *Phellodendron chinense* Schneid. or *Phellodendron amurense* Rupr. (family Rutaceae) and known as Phellodendri Cortex Chinensis (CPC) and Phellodendri Cortex Amurenensis (CPA), respectively [1]. Traditionally, it was used to clear heat, dry dampness, purge free, and remove toxicity [2]. Besides, modern pharmacological research studies indicated that Phellodendri Cortex exhibited various pharmacological activities, such as relaxing airway smooth muscle [2], inhibiting diabetes and gout [3], antioxidant [4], anti-inflammation [5–7], antiulcer [8], antibacteria, neuroprotective [9], antiviral, antidiarrhea [10],

bone growth-stimulation, and immune-stimulation activities [11, 12].

Cyclophosphamide, an alkylating agent, is widely used for cancer treatment, such as ovarian, lung, and breast cancers [13]. Cyclophosphamide can induce immunosuppression which is one of the most common side effects; thus, it is also used widely in organ transplantation and autoimmune disease [14]. The immunosuppression induced by cyclophosphamide or its metabolite could affect both cell-mediated and humoral immunity [15]. The immune system is closely relevant to host’s somatic functions, such as aging [16], mental state [17], inflammation [18], and various infections [19–21], which consists of different cell populations [16]. Once host suffers from infection or inflammation, the

immune system protects the host from external invaders or stimulations and altered or modified internal factors [22, 23]. Therefore, how to recover the immune function under immunosuppressive status is important for keeping healthy, especially for chemotherapeutically treated patients.

Previously, Phellodendri Cortex polysaccharides (CPP) exhibited potent antitumor activity against sarcoma [24]. The CPP fraction showed marked B-lymphocyte-stimulating activity [11]. However, few studies were conducted to evaluate immunoenhancement activity of CPP under immunosuppression. In the study, the immune-stimulation activity of CPP was investigated in immunosuppressive mice induced by cyclophosphamide for developing a new herbal-derived immunostimulant.

2. Materials and Methods

2.1. Extraction and Purification of Polysaccharides. The Phellodendri Cortex (batch number: 20200607) was purchased from Sichuan Zhongyong Pharmaceutical Co., Ltd. (Chengdu, China). The dried Phellodendri Cortex powder (200 g) was defatted by ethanol at 75°C for 6 h, and then, the defatted powder was decocted in distilled water for 3 times (1:10, g/v). The decoction was collected and concentrated, and then, ethanol was added (v/v, 1:3). The supernatant was removed by centrifugation, and the sediment (total polysaccharides) was collected. The crude polysaccharides were dissolved in hot water and deproteinized with Sevag's reagent, followed by dialysis against distilled water for 3 d to remove other impurities. Finally, CPP (8.27 g) was obtained by lyophilization, and the extraction rate was 4.14%. The content of CPP was 88.58%, which was detected by the phenol-sulfuric acid method. In the following tests in cells, the CPP was dissolved in phosphate-buffer saline (PBS) (4 mg/mL) and then diluted with culture medium. For the animal study, CPP was dissolved in saline.

2.2. Ethics Approval and Animals. The experimental protocol was approved by the National Institute of Ethics Committee at Chengdu University of Information Technology (approval number 2020-053). Four-week-old specific pathogen-free (SPF) KM mice (20 ± 2 g) were commercially provided by Chengdu Dossy Experimental Animals Co., Ltd. (license no. SCXK (Sichuan) 2015-030). The mice were fed normally with sterilized mice maintenance feed (Chengdu Dossy Experimental Animals Co., Ltd., China) at controlled temperature (23 ± 2°C), humidity of 55 ± 5%, and a 12 h light-dark cycle. After acclimatization for 7 d, the mice were used for the following study.

2.3. Preparation of Mice Splenic Lymphocytes. The normal and immunosuppressed (induced by cyclophosphamide) mice were sacrificed and sterilized in 75% (v/v) ethanol for 5 min and then dissected in clean bench. The spleen was separated and steeped in PBS. Cell strainers (Corning, USA) were used to grind spleens with rubber stopper syringes. The cell suspension was centrifuged at 1200 g at 4°C for 15 min, and the cell pellet was resuspended in PBS. Erythrocytes

were lysed by lysis buffer for 10 min, and then, the lysate was centrifuged at 1200 g at 4°C for 10 min. The cell pellet was resuspended with RPMI-1640 supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were kept at 37°C and 5% CO₂ for 4 h and then used for the following study.

2.4. Spleen Lymphocytes Cytotoxicity Assay. The cytotoxic effect of CPP on lymphocytes was measured by Cell Counting Kit-8 (CCK-8) assay. Cells were treated with different doses of CPP (400, 200, 100, and 50 µg/mL), respectively. After incubation at 37°C for 48 h, 10 µL of the CCK-8 solution (CA1210; Solarbio, Beijing) was added to each well. The plates were reincubated at 37°C for 30 min, followed by measurement of absorbance values at 450 nm using a microplate reader (Bio-Rad, USA). The cell viability was calculated as follows: cell viability = OD450 nm values of cells with CPP treatment ÷ OD450 nm values of control cells without treatment.

2.5. Spleen Lymphocytes Proliferation Assay. The proliferation promotion effect of CPP on lymphocytes was detected with CCK-8 assay. 100 µL splenocyte (5 × 10⁶ cell/mL) was added into each well. The cells were divided into 4 groups: CPP (200 µg/mL), LPS (2 µg/mL) + CPP (200 µg/mL), LPS (2 µg/mL), and control. The control group received an equal volume of culture medium. There were 8 repeats in each group, and the cells were cultured at 37°C with 5% CO₂ for 48 h. The CCK-8 solution (10 µL) was added into each well for another incubation for 4 h. The optical density (OD) of each well was measured at 450 nm. The cell viability = OD450 nm values of cells with treatment ÷ OD450 nm values of control cells.

2.6. Experimental Design. 50 mice (20 ± 2 g) were divided into 5 groups randomly (n = 10). The groups included the high dose of CPP (1 g/kg) group, medium dose of CPP (0.5 g/kg) group, low dose of CPP (0.25 g/kg) group, cyclophosphamide group, and control group. From 1 to 11 d, the CPP groups were orally administrated with CCP. The CY group and control group received equal volume of saline. From 12 to 14 d, the CCP groups and CY group received cyclophosphamide (20 mg/kg) by intraperitoneal injection; meantime, the mice in the CCP groups orally administrated with different doses of CCP and control group received equal volume of saline by gavage. The bodyweight variation of each mouse was recorded from 1 to 14 d. At 14 d, all mice were subject to euthanasia, and then, the spleen and thymus in each mouse were collected and weighed. The organ coefficient of each mouse was calculated according to the following formula: organ coefficient (mg/g) = organ weight/bodyweight.

2.7. Serum Cytokines Assay. Blood was sampled by eyeball extirpating in each group. After coagulation at room temperature for 30 min, the blood was centrifuged at 3000 g for 5 min. The serum in each group was collected for cytokines

assay (IL-2, IL-6, IFN- γ , and TNF- α) with ELIAS kits according to the manufacturer's instructions (Beijing Ger-sion Biotechnology Co., Ltd, China).

2.8. Transcriptional Levels of Target Genes Assay. The mRNA levels of cytokines (IL-2, IL-6, IFN- γ , and TNF- α) in the spleen were evaluated by real-time PCR (RT-PCR) assay. In brief, total RNA from the spleen in each group was extracted with RNAiso Plus (no. 9108, TaKaRa, China) according to the instructions of manufacturer. Equal amounts of RNA samples were reverse-transcribed into cDNA with RevertAid First Strand cDNA kit (no. K1622; Thermo ScientificTM) according to manufacturer's protocol. The RT-PCR was performed with SYBR Green Supermix kit (Bio-Rad, USA), and primers are given in Table 1. The PCR cycling was 3 min at 95°C and then 40 cycles of 10 s at 95°C, 30 s at 60°C, and finally 55°C for 5 s. In the end, the melting curve analysis was conducted. The difference in each sample was normalized with the expression of β -actin. Data analysis were performed by the Bio-Rad CFX Manager software (Bio-Rad, USA).

2.9. Western Blotting. The expressions of NF- κ B p65, TLR4, and MyD88 were detected by Western blotting. The total proteins of the spleen were extracted with a protein extraction kit (BOSTER, Wuhan, China). Total spleen lysates were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for 30 min with 80 V and 70 min with 120 V and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) for 90 min with 200 mA. The membranes were blocked with 5% BSA for 90 min (Solarbio, China) at room temperature, and then, proteins were stained using primary antibodies directed against TLR4 (CST, 14358S, 1:1000), MyD88 (CST, 4283S, 1:1000), p65 (CST, 8242T, 1:1000), and β -actin (Boster, China, 1:1000) at 4°C overnight. The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST) for 4 times and incubated with horseradish peroxidase-conjugated secondary antibody (Boster, China, 1:5000) for 1 h at room temperature, followed by washing for 4 times. Finally, the proteins were visualized using enzymatic chemiluminescence (ECL) reagents (Bio-Rad, USA) and normalized with the expression of β -actin. The ratios of protein band intensity were obtained with ImageJ software (Version 1.47, NIH, USA).

2.10. Statistical Analysis. All data were presented as mean \pm standard deviation (SD), whose statistical significance was compared by one-way analysis of variance (ANOVA) with Duncan's multiple range test in SPSS 19.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

3. Result

3.1. The Cytotoxicity of CPP. The cytotoxic effect of CPP on lymphocytes is shown in Figure 1. The CPP at the concentration of 400 μ g/mL inhibited the viability

of lymphocyte slightly, while CPP at concentrations of 200, 100, and 50 μ g/mL could promote the viability of lymphocyte.

3.2. The Promotion Effect of CPP on Lymphocyte Proliferation. As shown in Figure 2, compared with the control group, LPS treatment could significantly decrease the viability of lymphocytes isolated from both normal and immunosuppressed mice ($P < 0.05$), and CPP treatment (200 μ g/mL) upregulated the viability of lymphocytes to the normal level ($P < 0.05$). Without LPS stimulation, CPP treatment could significantly promote the lymphocyte proliferation from both normal and immunosuppressed mice in comparison with untreated lymphocytes ($P < 0.05$).

3.3. Bodyweight. Bodyweight of mice in each group were weighed and recorded every day. As shown in Figure 3, bodyweights of mice increased gradually in each group from 1 d to 11 d. In the CPP-H group (1 g/kg), the bodyweights of mice were higher than those in the other groups, suggesting that CPP could enhance the bodyweight. From 12 to 14 d, mice except the control group received cyclophosphamide, and then, the bodyweight showed a decrease trend, especially the bodyweights of mice in the CY group decreased significantly. The mice treated with CPP at a dose of 1 g/kg could maintain the bodyweight to the level of the control group during cyclophosphamide administration.

3.4. Organ Coefficient. The spleen and thymus are common immune organs, and the organ coefficients (the ratio of organ weight to bodyweight) reflect host's immune state to some extent. As shown in Figure 4, organ coefficients of the spleen and thymus in the CY group were significantly lower than those in the control group ($P < 0.05$). After CPP treatment (1 g/kg), the organ coefficients of the spleen and thymus were recovered to the level of the control group, which were significantly higher than that of the CY group ($P < 0.05$).

3.5. The Contents of Serum Cytokines. Cytokines are always relative to immunoreaction, which reflects host's immune status. Therefore, the contents of IL-2, IL-6, IFN- γ , and TNF- α in serum were detected by ELISA assay. As shown in Figure 5, cyclophosphamide significantly decreased the contents of IL-2, IL-6, and TNF- α in serum when compared with the control group ($P < 0.05$), while CPP treatment significantly increased the contents of IL-2, IL-6, TNF- α , and IFN- γ in comparison with the CY group ($P < 0.05$). The IFN- γ contents in the three CPP groups were significantly higher than that of the control group ($P < 0.05$).

3.6. The mRNA Levels of IL-2, IL-6, IFN- γ , and TNF- α in the Spleen. As shown in Figure 6, compared with the control group, cyclophosphamide treatment significantly increased the mRNA levels of IL-2, IL-6, IFN- γ , and TNF- α ($P < 0.05$).

TABLE 1: Primers.

Gene	Forward primer	Reverse primer
IL-2	ATTTGAGTGCCAATTCGATG	AGCCTTATGTGTTGTAAGCA
IL-6	AATGTCGAGGCTGTGCAGATTAGTAC	GGGTGGTGGCTTTGTCTGGATTG
IFN- γ	TCAGGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
TNF- α	GCACTGAGAGCATGATCCGAGAC	CGACCAGGAGGAAGGAGAAGAGG
β -Actin	GCGCTTTTGACTCAGGATTT	CCTGGGCCATTGAGAAATTA

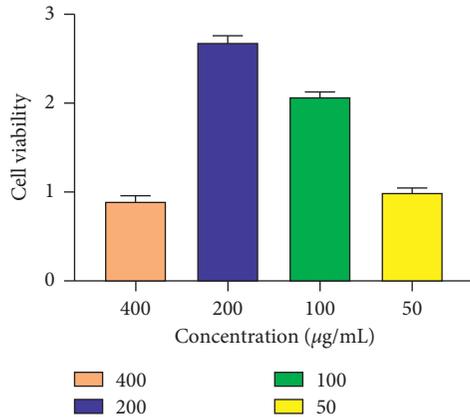


FIGURE 1: The cytotoxicity of CPP on lymphocyte. The lymphocytes from mice were treated with different doses of CPP (400, 200, 100, and 50 $\mu\text{g}/\text{mL}$) for 48 h. The cell viability was measured by CCK-8 assay and calculated as follows: cell viability = OD450 nm values of cells with CPP treatment \div OD450 nm values of control cells without treatment. The results are shown as mean \pm SD, $n = 6$.

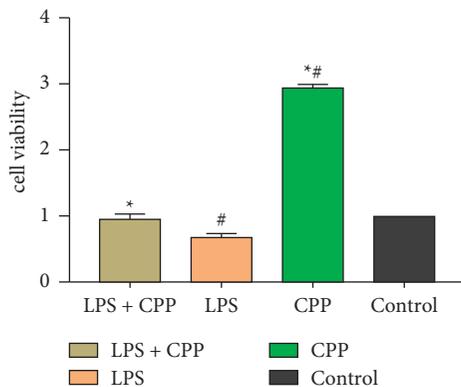


FIGURE 2: The proliferation promotion effect of CPP on lymphocytes. The lymphocytes were treated with LPS (2 $\mu\text{g}/\text{mL}$) in the presence or absence of CPP (200 $\mu\text{g}/\text{mL}$) for 48 h. The cell viability was measured by CCK-8 assay and calculated as follows: cell viability = OD450 nm values of cells with treatment \div OD450 nm values of control cells without treatment. The results are shown as means \pm SD, $n = 8$. * $P < 0.05$ vs. the LPS group. # $P < 0.05$ vs. the control group.

CPP treatment (1 and 0.5 g/kg) significantly increased the mRNA levels of IL-2, IL-6, IFN- γ , and TNF- α , when compared with the CY group ($P < 0.05$). These results suggested that CPP could promote the expressions of IL-2, IL-6, IFN- γ , and TNF- α in the spleen of cyclophosphamide-treated mice.

3.7. The Effect of CPP on NF- κ B and TLRs Signaling Pathways.

The NF- κ B and TLRs signaling pathways were measured via Western blotting assay. As shown in Figure 7, the expressions of TLR4, MyD88, and p65 were significantly enhanced by cyclophosphamide treatment when compared with the control group, suggesting that NF- κ B and TLRs signaling pathways were activated by cyclophosphamide. While CPP treatment alleviated this trend, especially in the CPP-H group, the expressions of p65, TLR4, and MyD88 signaling pathways were significantly inhibited ($P < 0.05$).

4. Discussion

Since 1950s, the pharmacological activities of polysaccharides have been explored gradually. Plant polysaccharides exhibited various functions, such as immunoregulatory [25], antioxidant [26, 27], antitumor [25, 28], anti-inflammation [29, 30], antiviral [31, 32], and antiradiation effects [33]. Many studies indicated that plant polysaccharides are relatively nontoxic without significant side effects [34]. For the clinical application, the immunoregulator is necessary, while toxicity and side effect of the immunoregulator are inevitable [34]; therefore, plant polysaccharides are the best candidate drugs to regulate immunity.

In the present study, the immunoregulation of CPP was first investigated in vitro and found that CPP could promote mouse lymphocyte proliferation. LPS is an endotoxin from Gram-negative bacteria that produces a variety of biological activities in human and other animal cells [35]. It was shown that LPS can induce an immune response in normal animal bodies, which can not only regulate the distribution of B lymphocytes in the spleen but also reduce the proliferation of T lymphocytes [36–38]. Our study found that CPP promotes spleen lymphocyte proliferation and inhibits the regulatory effect of LPS on lymphocyte proliferation. Besides, the polysaccharides from *Eupolyphaga Sinensis* Walker and sulfated Chinese yam polysaccharides could also promote splenic lymphocyte proliferation [39,40].

For further confirming immunomodulatory effects, an in vivo study was performed on cyclophosphamide-induced immunosuppression mice. Immunosuppression can be achieved via various ways, such as glucocorticoids which could make organism immunosuppression via decreased chemotaxis, vessel wall permeability, and antigen phagocytosis. Cytostatics, such as purine analogs including azathioprine, cyclophosphamide, and methotrexate, could also cause immunosuppression though inhibition of DNA synthesis or inosine monophosphate dehydrogenase to impair mitosis. The calcineurin inhibitors control the transcription of interleukins in T lymphocytes via binding to intracellular

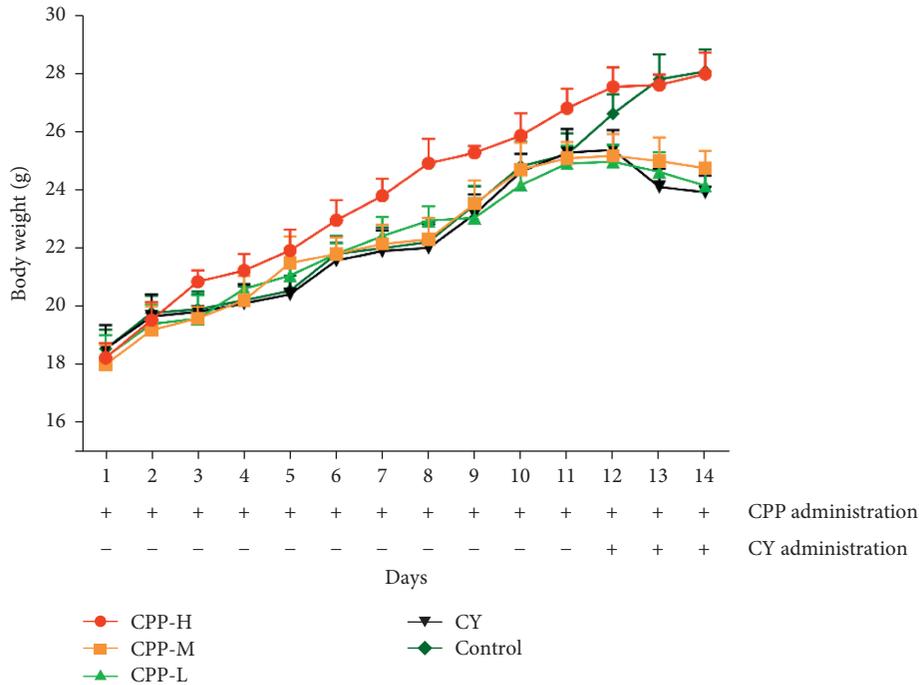


FIGURE 3: Bodyweights of mice in each group. The bodyweight of each mouse was recorded from 1 d to 14 d. CPP-H, CPP-M, and CPP-L represent the high (1 g/kg), medium (0.5 g/kg), and low (0.25 g/kg) doses of CPP groups. CY, cyclophosphamide. Control, the normal group without any treatment. The results are shown as mean ± SD, n = 10.

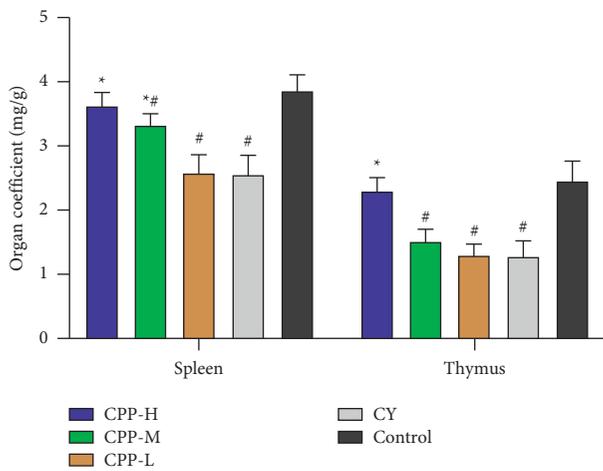


FIGURE 4: Organ coefficients of mice in each group. CPP-H, CPP-M, and CPP-L represent the high (1 g/kg), medium (0.5 g/kg), and low (0.25 g/kg) doses of CPP groups. CY, cyclophosphamide. Control, the normal group without any treatment. The results are shown as mean ± SD, n = 10. *P < 0.05 vs. the CY group. #P < 0.05 vs. the control group.

immunophilins to decrease IL-2 production and T cell proliferation, including cyclosporin A, tacrolimus, and mTOR inhibitors. The use of these immunosuppressors results in severe side effect for the organism. Therefore, the proper way to build the immunocompromised model was vital and essential for the present study. Considering that cyclophosphamide was widely used to build the immunocompromised mouse model and the methods are mature, in

this study, cyclophosphamide (20 mg/kg) was used as an immunosuppressor to build the immunocompromised mouse model. Changes in bodyweight and organ coefficients can reflect whether the body is in a pathological state [41]. After cyclophosphamide administration, the bodyweight and organ coefficients of the spleen and thymus decreased significantly, suggesting that the immunocompromised model was successively established. Previous studies have found that the cyclophosphamide-treated mice had a significantly reduced bodyweight and thymus and spleen coefficients [42, 43], which was consistent with our results.

Cytokines always reflects immune state to some extent [44]. Once immune system was damaged, the cytokines will be regulated immediately against intruder [45]. IL-2 regulates immunity via affecting and regulating T lymphocytes, which involves the whole life cycle of Th1 cells and Th2 cells. Besides, cyclophosphamide could cause Th1/Th2 bias [46]; thus, the level of IL-2 was detected in serum. Compared with the IL-2 level in the CY group, CPP significantly increased IL-2 contents. IL-6 involved the development and maturation of B cell to plasma cells and sustained antibody production [47–50]. IL-6 regulates inflammation and immune response as a mediator and warner [51]; therefore, IL-6 plays an important role in the immune system. In the present study, cyclophosphamide significantly decreased the IL-6 level, while CPP treatment upregulated the IL-6 level notably. IFN-γ plays an important role in immune response both in innate and acquired immune, especially in the early stage of immunity [52], which exhibited immunoregulation during blepharon conjunctivitis in brown Norway rats at the phase of induction [53]. Compared with the IFN-γ level in

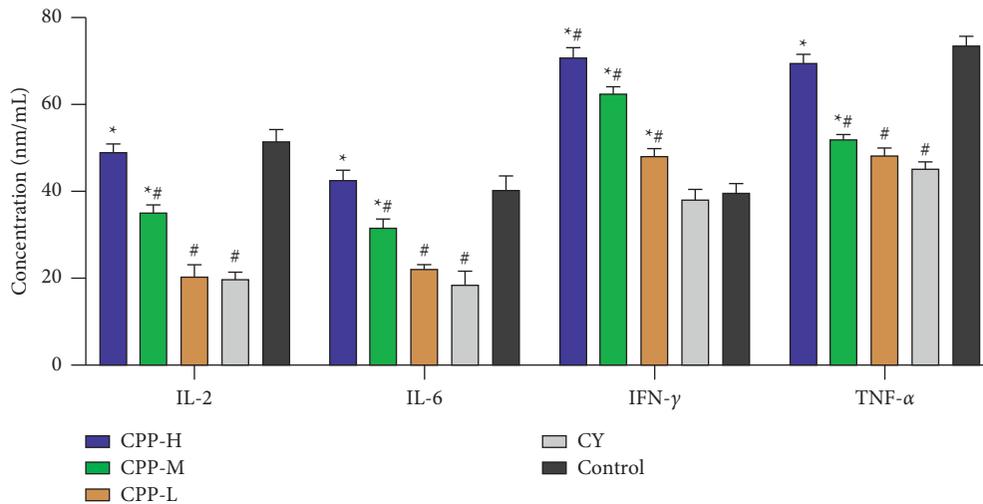


FIGURE 5: The contents of serum cytokines. CPP-H, CPP-M, and CPP-L represent the high (1 g/kg), medium (0.5 g/kg), and low (0.25 g/kg) doses of CPP groups. CY, cyclophosphamide group. Control, the normal group without any treatment. The results are shown as mean \pm SD, $n = 10$. * $P < 0.05$ vs. the CY group. # $P < 0.05$ vs. the control group.

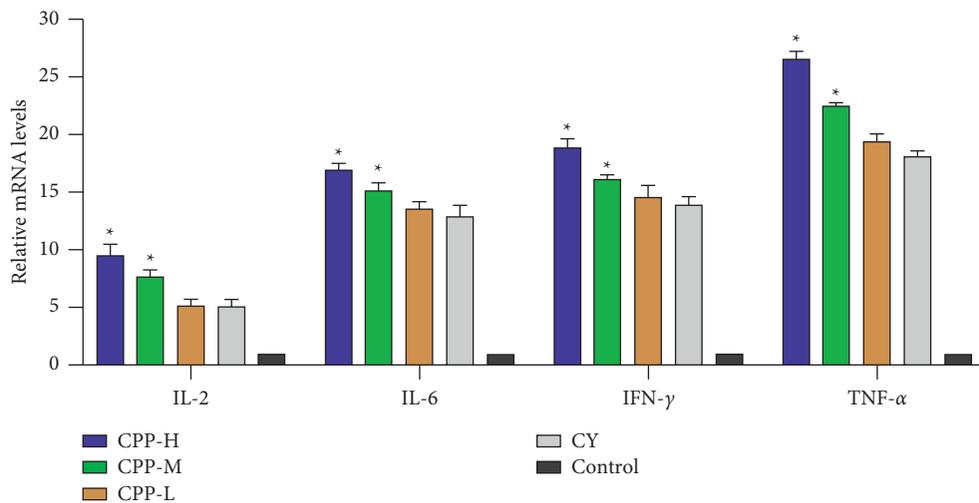


FIGURE 6: The mRNA levels of IL-2, IL-6, IFN- γ , and TNF- α in the spleen. CPP-H, CPP-M, and CPP-L represent the high (1 g/kg), medium (0.5 g/kg), and low (0.25 g/kg) doses of CPP groups. CY, cyclophosphamide group. Control, the normal group without any treatment. The results are shown as mean \pm SD, $n = 6$. * $P < 0.05$ vs. the CY group.

the control group, cyclophosphamide significantly decreased and then IFN- γ content, which was recovered by CPP treatment. TNF- α mediates innate immunity in the early stage of immune system damaged [54], and it also exhibited the ability to regulate leukocyte to mediate organism's immune state [45]; therefore, the level of TNF- α reflects immune state to some extent. The TNF- α content was decreased by cyclophosphamide which was also enhanced by CPP. In the present study, the levels of the test cytokines in serum were consistent with the mRNA levels in the spleen obtained by RT-PCR assay.

The NF- κ B signaling pathway is essential to rapid induction of expressions of acute-phase antimicrobial defense genes against pathogens, and the functions include regulating lymphoid organ development, B cell survival and maturation, and the differentiation of osteoclasts [55]. In the

present study, cyclophosphamide treatment significantly activated the NF- κ B signaling pathway, while CPP decreased the expression of NF- κ B p65. It indicated that CPP inhibited the immunosuppression induced by cyclophosphamide, which was consistent with the function of NF- κ B in the immune system [56]. Toll-like receptors (TLRs) inducing proinflammatory response is the first line of host defense against external invasion and protecting host [57, 58]. In all TLRs, TLR4 exhibited unique ability to identify pathogen-associated molecular patterns (PAMPs) from multiple types of pathogens [58]. In the present study, CY treatment activated the expression of TLR4 which was significantly decreased by CPP, indicating CPP exhibited potent anti-inflammation activity to protect host. MyD88, a common adaptor, was considered as an essential component for activation of all the TLRs in innate immunity [57]. Previous

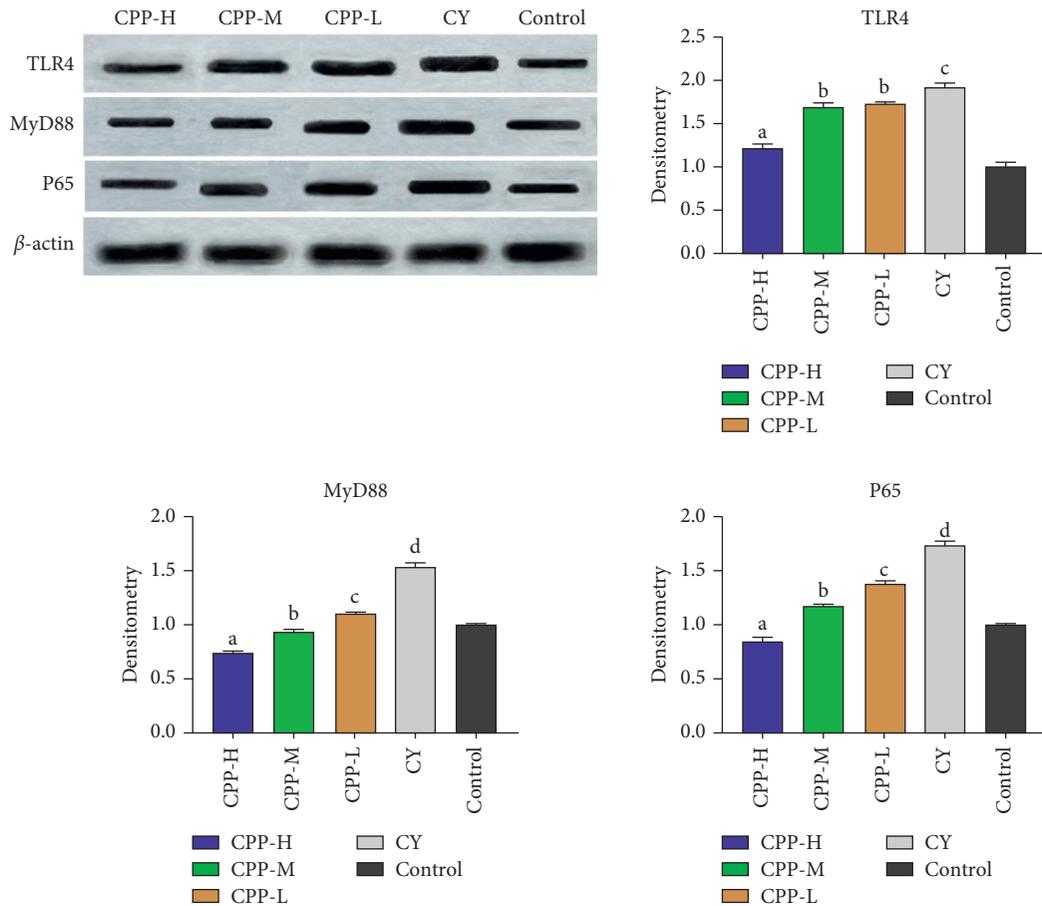


FIGURE 7: The effects of CPP on NF- κ B and TLRs signaling pathways in the spleen. CPP-H, CPP-M, and CPP-L represent the high (1 g/kg), medium (0.5 g/kg), and low (0.25 g/kg) doses of CPP groups. CY, cyclophosphamide group. Control, the normal group without any treatment. The results are shown as mean \pm SD, $n=3$. The different letters on a column differ significantly, $P < 0.05$.

studies indicated that there were few productions of inflammatory cytokines against TLR ligands in MyD88 knockout mice [59]. In this study, the expression of MyD88 was increased by cyclophosphamide, which was significantly inhibited by CPP. The variation of MyD88 was consistent with the change of the TLR4 level.

5. Conclusion

CPP showed potent immunoregulatory ability in immunosuppressive mice through regulating NF- κ B and TLRs signaling pathways. CPP exhibited the potential for treating immunosuppression, especially induced by cyclophosphamide.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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