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Investigation on activation in RAW264.7 macrophage cells and protection in cyclophosphamide-treated mice of *Pseudostellaria heterophylla* protein hydrolysate

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ABSTRACT

Our previous study has demonstrated that *Pseudostellaria heterophylla* protein hydrolysate (PPH) has immunomodulatory activity on murine spleen lymphocytes. The aim of this study was to investigate the excitation of PPH in RAW264.7 macrophage cells and the protective effect in cyclophosphamide (CTX)-treated mice. The results showed PPH of 50 μg/mL could stimulate macrophages resulting in significant promotions of nitric oxide (NO) production, endocytosis and reactive oxygen species formation. Meanwhile, enzyme-linked immunosorbent assay (ELISA) revealed that the levels of tumor necrosis factor-α and interleukin-10 were significantly upregulated by PPH. Furthermore, 50 mg/kg per day PPH restored the T lymphocyte proliferation and natural killer cell activity, and increased NO production and pinocytosis of peritoneal macrophages in CTX-treated mice. These findings indicate PPH plays a crucial role in RAW264.7 macrophage cells activation and in the protection against immunosuppression in CTX-treated mice and could be used as a potential immunostimulant agent.

1. Introduction

In recent years, greater attention has been paid for the immune system because of the increase of chronic illnesses and other unhealthy lifestyles. These undesirable factors lead to immunosuppression in the body, which is a state of temporary or permanent immunity dysfunction and it can make organism more sensitive to pathogens due to the damage of immune system. Therefore, it is urgent to search for an effective method to prevent and treat immunosuppressive diseases with a seemingly slow progress.

Many protein hydrolysates exhibit various physiological activities *in vivo* and *in vitro* (Santiago-Lopez et al., 2016; Yoshikawa, 2015). There were many reports on the immunomodulatory activity of enzymatic hydrolysates prepared from food protein, such as soy (Kong et al., 2008) and egg white proteins (Lozano-Ojalvo et al., 2016). Immunomodulatory peptides can enhance immune cell functions, measured as lymphocyte proliferation, antibody synthesis and cytokine secretion (Ahn et al., 2012; Hou et al., 2012; Lozano-Ojalvo et al., 2016; Wu et al., 2017). Moreover, immunomodulatory peptides derived from food possessed satisfying properties that include ease of absorption, nontoxicity and lack of immunogenicity compared with drug.

Pseudostellaria heterophylla is widely grown in Asia, such as Korea, Japan and China and has been described as a strengthening spleen drug, which has a beneficial effect for the body (Choi et al., 2017; Hu et al., 2013; Pang et al., 2011). In addition, *P. heterophylla* has mild toning effects on the body, leading to its widespread use in herbal medicines and it was authorized by the Ministry of Health, China, as one of "Chinese herbal medicine that can be used for the health foods" in recent years. *P. heterophylla* protein hydrolysates (PPH), containing 96.68% peptides whose molecular weight below 1000 Da, was prepared by enzymatic hydrolysis and gel filtration chromatography, which could promote the proliferation of lymphocyte (Yang et al., 2019). However, the effects of PPH on macrophage activation and cyclophosphamide (CTX)-induced mice were still unclear.

RAW264.7 macrophage cells are generally used to study the mechanisms of action governing the role of macrophages in immunity (Hartley et al., 2008) and inflammatory (Wallert et al., 2015; Xie et al., 2011), and they are ease of cell reproduction, high efficiency for DNA transfection and sensitivity to RNA interference. In this study, the immunomodulatory effects of PPH on RAW264.7 macrophage cells were determined by assessing nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), reactive oxygen species (ROS)

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production and endocytosis. To explore protective effects of the PPH against the CTX-induced immunosuppression in mice, the lymphocytes proliferation, natural killer (NK) cell activity, NO production and pinocytosis in peritoneal macrophages were measured. This work expected to provide an experimental foundation for further development of PPH as nutraceutical supplement to strengthen immune system in the human body.

2. Material and methods

2.1. Materials and reagents

P. heterophylla roots were acquired from a *P. heterophylla* planting base in Zherong (Fujian, China) which is one of the largest planting bases in China. *P. heterophylla* roots were clean and dried at 60 °C for 48 h before use. CTX, concanavalin (ConA) and lipopolysaccharide (LPS) were purchased from Yuanye Biotechnology Co. (Shanghai, China). Levamisole hydrochloride (LH) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Shanghai Macklin Biochemical Co. (Shanghai, China). TNF-α and IL-10 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Boster Biology Engineering Institute (Wuhan, China). Total RNA isolation kit and fluorescence real-time quantitative PCR premix (SYBR) were purchased from Tiangen Biotechnology Co. (Beijing, China). *TransScript®* One-Step gDNA Removal and cDNA Synthesis SuperMix was purchased from Transgen Biotechnology Co. (Beijing, China).

2.2. Evaluation of immunomodulatory effect of PPH on RAW264.7 macrophage cells

2.2.1. Cell culture and MTT assay

RAW264.7 macrophage cells were purchased from the BeNa Culture Collection (Kunshan, China). The cells were maintained in DMEM medium containing 10% heat inactivated fetal bovine serum (FBS), 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity of PPH was assessed by the MTT assay. In brief, RAW264.7 macrophage cells were seeded in 96-wells plates at a density of 2×10^6 cells/mL. After 24 h, the cells were treated with new medium or medium containing various concentrations of PPH (12.5, 25, 50, 100, 200 and 400 μg/mL). After another 24 h, MTT solution (5 mg/mL) was added and incubated for 4 h. Finally, the supernatant was discarded and 200 μL dimethyl sulfoxide (DMSO) was added to dissolve the purple crystals. The absorbance at 570 nm was measured using a microplate reader (SpectraMax iD3, Molecular Devices, Shanghai, China).

2.2.2. Determination of NO production

RAW264.7 macrophage cells were seeded in 96-wells plates at a density of 2×10^6 cells/mL. After 24 h, the cells were incubated with different concentrations of PPH (12.5, 25, 50 and 100 μg/mL) or 1 μg/mL LPS. After stimulation and treatment with PPH, the supernatant was collected and used to assay NO production. An aliquot of 50 μL of media was mixed with 50 μL Griess reagent I and 50 μL Griess reagent II at room temperature. 1–100 μM $NaNO_2$ was set as the standard curve. Then the absorbance was measured at 540 nm using a microplate reader. The NO concentrations of samples were calculated using a standard curve.

2.2.3. Analysis of endocytosis

Endocytosis of RAW264.7 macrophage cells was measured using Neutral Red assay and FITC-labelled dextran. The Neutral Red assay was measured according to previous report with modification (Dai et al., 2014). The prior stage culture of RAW264.7 macrophage cells was done as previously described. PPH with different concentration (12.5, 25, 50 and 100 μg/mL) as well as a blank control (phosphate-buffered saline, PBS) and positive control (LPS, 1 μg/mL) were added to a 96-wells plate and the plate was incubated at 37 °C for 24 h. Subsequently, the culture medium was discarded and 1 mg/mL neutral red dissolved in 0.9% sodium chloride was added to each well (100 μL per well). After further incubation for 30 min, the cells were washed with PBS three times to remove excess neutral red. Then the 100 μL lysis buffer (glacial acetic acid: ethanol = 1:1, v/v) was added to each well (100 μL per well) and the plate was incubated for 2 h at room temperature. Finally, the absorbance was measured at 540 nm using a microplate reader. The pinocytosis rate of RAW264.7 macrophage cells was calculated by the following equation:

$$Pinocytosis\ rate\ (\%) = \frac{Absorbance\ (sample)}{Absorbance\ (control)} \times 100 \quad (1)$$

The FITC-labelled dextran assay was measured according to the reported method with modification (Yu et al., 2014). RAW264.7 macrophage cells were seeded in 6-wells plates at 3×10^5 cells per well. After 24 h, the cells were incubated with different concentrations of PPH (12.5, 25, 50 and 100 μg/mL) or 1 μg/mL LPS. Subsequently, the supernatant was discarded and 1 mg/mL FITC-labelled dextran was added to each well. After further incubation for 1 h, cold PBS containing 2% FBS was added into the plate to terminate reaction. Then the cells were washed with PBS three times and resuspended in 500 μL PBS for analysis on flow cytometry (FACSAria III, Becton, Dickinson and Co., USA).

2.2.4. Determination of intracellular reactive oxygen species (ROS) formation

Intracellular ROS was measured by the fluorescence probe DCFH-DA, which was hydrolyzed to DCFH in cells. RAW264.7 macrophage cells were cultured as previously described. After incubation of PPH for 24 h, cells were harvested and washed with cold PBS thrice. Washed cells were further incubated with 10 mM DCFH-DA at 37 °C for 30 min in the dark. After that the cells were centrifuged at 1000 g for 20 min and washed with cold PBS twice. Finally, the cells were resuspended in 1 mL PBS for analysis of fluorescent intensity using fluorescence spectrophotometer (Fluoromax-4C-L, Horiba Instrument Co., USA).

2.2.5. Measurement of cytokines by ELISA

The prior stage culture of RAW264.7 macrophage cells was done as previously described. PPH with different concentration (12.5, 25, 50 and 100 μg/mL) as well as PBS and positive control (LPS, 1 μg/mL) were added to a 96-wells plate and the plate was incubated at 37 °C for 24 h. Subsequently, the supernatant was harvested and the levels of TNF-α and IL-10 were determined by ELISA kits, respectively.

2.3. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

RAW264.7 macrophage cells (2.88×10^5 cells per well) were treated with different concentrations of PPH (12.5, 25, 50 and 100 μg/mL) or 1 μg/mL LPS, accordingly. After the incubation for 24 h, cells were harvested for the preparation of total RNA using RNAprep Pure Cell kit. The total RNA was used for cDNA synthesis with *TransScript®* One-Step gDNA Removal and cDNA Synthesis SuperMix according to the manufacture's protocol. QPCR was performed for multiple cycles using a MyGo Real-Time PCR Detection System (MyGo Pro, IT-IS Life Science Ltd., North Yorkshire, UK) with the following program of denaturation at 95 °C for 15 min, annealing at 55 °C for 20 s, and elongation at 72 °C for 30 s and β-actin for normalization. qPCR was amplified using the following primers: iNOS, 5-CTCACCTACTTCCTGGACATTAC-3' (forward) and 5′-GCCTCCAATCTC TGCCTATC-3' (reverse); β-actin, 5′-GAGACCTTCAACCCAGCC-3' (forward) and 5′-AATGTCACGCACGATTTCCC-3' (reverse). The mRNA relative expressions were calculated by $2^{-\Delta\Delta Ct}$ method.

2.4. Protective effect of PPH on CTX-induced mice

2.4.1. Animal

Male ICR clean mice (20–24 g) were purchased from Wushi Animal (Minhou, China). Animals were housed in an air-conditioned house, with a temperature of 22 ± 2 °C, relative humidity of 50–60%, and 12 h/12 h light/dark cycle, free access to food and water. These conditions were kept for 1 week before the conduct of the experiments. The procedures involving animals were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals.

2.4.2. Dosage information

The mice were randomly divided into 5 groups composing of 8 mice each. From days 1–3, four groups of mice were given CTX at 80 mg/kg per day *via* intraperitoneal injection except normal control (NC) group. From days 4–10, the mice were administered as follows: model control (MC) group and NC group, physiological saline; low dosage PPH (LPPH) group, 50 mg/kg per day PPH; high dosage PPH (HPPH) group, 150 mg/kg per day PPH; LH group, 10 mg/kg per day LH, as positive control *via* intraperitoneal injection. Twenty-four hours after the last drug administration, the animals were weighed and then sacrificed by cervical dislocation, in line with the Commission for Animal Experimentation of Fujian Medical University (Fujian, China).

2.4.3. Peripheral white blood cell counts

Blood was collected on the day of sacrifice by retro-orbital bleed into tubes with EDTA-Na_2 as anticoagulant. White blood cell (WBC) was analyzed using Automated Hematology Analyzer (pocH-100iV Diff, Kobe, Japan).

2.4.4. Determination of stimulation index of spleen lymphocytes

Spleen collected under aseptic conditions was minced to obtain single spleen cell suspensions in RPMI 1640 medium. The cells were washed with PBS three times and adjusted to a density of 2×10^6 cells/mL in RPMI 1640 complete medium. Then 180 μL of spleen lymphocytes were seeded in a 96-wells plate, and stimulated with 20 μL LPS (1 μg/mL) or ConA (5 μg/mL) for 24 h. After incubation 20 μL of MTT solution (5 mg/mL) was added into each well then incubated for another 4 h. Then the supernatant was discarded and 200 μL DMSO was added per well to dissolve crystallization. Finally, the absorbance at 570 nm was measured a microplate reader.

2.4.5. Cytotoxicity assays of NK cell activity of spleen lymphocytes

Spleen lymphocytes were used as effector cells for NK cell cytotoxicity. HepG2 cells were used as the target cells. Briefly, effector cells were co-cultured with target cells at a ratio of effector to target cells of 50:1. The plates were then incubated for 20 h at 37 °C in 5% CO_2 atmosphere. Then MTT method was used to analyzed the NK cell cytotoxicity. NK cell cytotoxicity was calculated as the following equation:

$$NK\ \ cell\ activity\ \ (\%) = \frac{(OD_T - (OD_S - OD_E))}{OD_T} \times 100 \quad (2)$$

where OD_T is absorbance of target cells control, OD_S is absorbance of test samples, and OD_E is absorbance of effector cells control.

2.4.6. Preparation of peritoneal macrophages

The peritoneal macrophages were collected using the reported method (Li et al., 2017). Briefly, to collect peritoneal macrophages, each mouse received 5 mL cold sterile PBS and the peritoneal cavity was washed carefully. Peritoneal macrophages were aseptically collected by centrifugation at 1500 rpm, 4 °C for 5 min and resuspended in RPMI 1640 complete medium. After cultivated for 4 h, the non-adherent cells were discarded and the purified peritoneal macrophages were obtained. Before each experiment, cells were seeded in 96-wells plates at a density of 2×10^6 cells/mL. And the NO secretion and neutral red phagocytosis were measured using method described above.

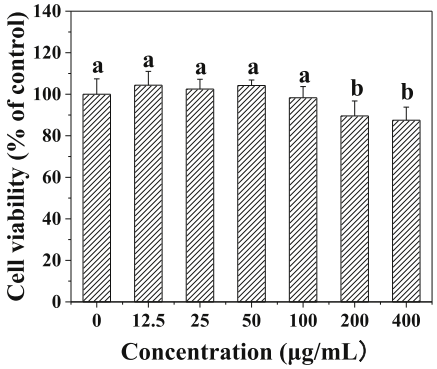

Fig. 1. Cytotoxicity of PPH in RAW264.7 macrophage cells. Data are presented as the mean ± SD (n = 3). Different lowercase letters represent significant differences ($p < 0.05$).

2.5. Statistical analysis

All results were expressed as means ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) with Duncan's multiple range test using IBM SPSS 17.0 software. A confidence level of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Cytotoxicity of PPH in RAW264.7 macrophage cells

Cytotoxicity of PPH was determined in RAW264.7 macrophage cells using the MTT assay to screen the safe concentration. As shown in Fig. 1, PPH did not show any cytotoxic effects on RAW264.7 macrophage cells at concentrations down to 100 μg/mL and then cell viabilities decreased with increasing concentration. Thus, the concentrations of 12.5–100 μg/mL of PPH were used to assess immunomodulatory effect of PPH in RAW264.7 macrophage cells.

3.2. Effect of PPH on NO secretion and iNOS mRNA expression in RAW264.7 macrophage cells

NO, which has multiple physiologic and pathophysiologic functions because of both a wide distribution of synthesis and diverse mechanisms of action, plays an important role in inflammation and immunity (Tripathi et al., 2007; Xu et al., 2012). Appropriate NO production is important for the immune system to attack xenobiotics. To detect the abilities of PPH and LPS to induce NO production in macrophages, RAW264.7 macrophage cells were stimulated with PPH (12.5–100 μg/mL) and LPS (1 μg/mL), respectively. As shown in Fig. 2A, compared with control, PPH promoted NO production in a dose-dependent manner in RAW264.7 macrophage cells. NO production of cells treated with 100 μg/mL PPH has no significant difference compared to cells treated with 1 μg/mL LPS. Consisted with our result, some reporters showed that polysaccharides from *Gracilaria lemaneiformis* also promote the production of NO (Ren et al., 2017).

NO generates from the amino acid L-arginine. NO synthesis is catalyzed by one of three isoforms of nitric oxide synthases (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), and iNOS is responsible for most NO synthesis (Lechner et al., 2005). Fig. 2B shows that 50 and 100 μg/mL PPH can significantly promote iNOS mRNA expression, which is consistent with the detected NO production. Therefore, PPH promotes NO production by up-regulating iNOS mRNA expression. Many previous studies have reported that immunomodulatory effect on RAW264.7 macrophage cells was related to NO production. Yang et al. reported that NO and iNOS mRNA production was significantly up-regulated with 200 and 400 μg/mL β-

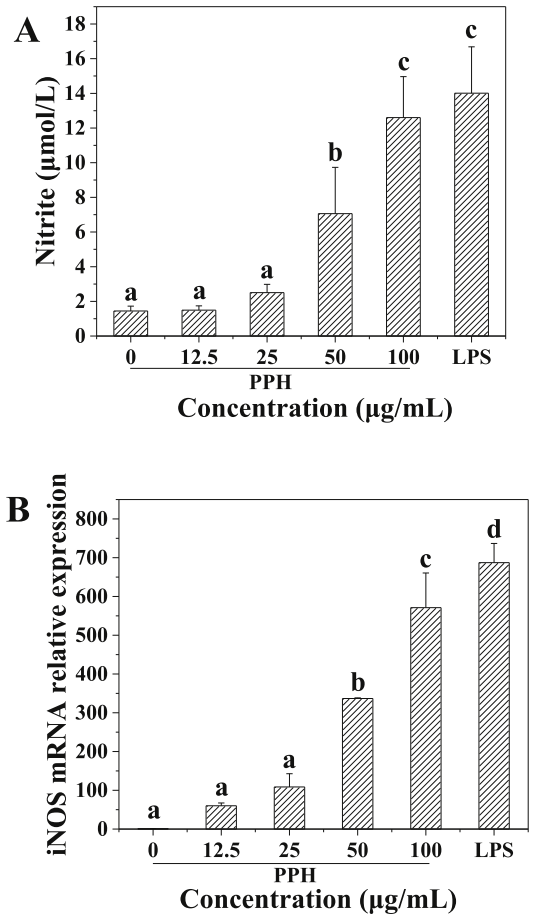

Fig. 2. (A) Effect of PPH on NO production in RAW264.7 macrophage cells. (B) Effect of PPH on the iNOS mRNA expression level in RAW264.7 macrophage cells. Data are presented as the mean ± SD (n = 3). Different lowercase letters represent significant differences ($p < 0.05$).

glucan from *Durvillaea Antarctica* (Yang et al., 2018). Compared with β-glucan, PPH could significantly increase NO production and iNOS mRNA expression at the concentration of 50 μg/mL.

3.3. Promotion effect of PPH on endocytosis in RAW264.7 macrophage cells

Macrophage endocytosis includes phagocytosis (of particles) and pinocytosis (of solutes) and is considered as part of immune responses to invading pathogens (Pratten and Lloyd, 1986). The rate of macrophage pinocytosis was assessed using neutral red assay and the phagocytosis of macrophage was determined by FITC-dextran. As shown in Fig. 3, the pinocytosis rate of RAW264.7 macrophage cells was significantly improved with only 25 μg/mL of PPH. PPH enhanced the pinocytotic activity of RAW264.7 macrophage cells in a dose-dependent manner and the pinocytosis rate of RAW264.7 macrophage cells treated with 100 μg/mL PPH has no significant difference compared to cells treated with 1 μg/mL LPS. Hence PPH of 25–100 μg/mL could activate macrophages pinocytotic activity.

The FITC-labelled dextran was employed to determine the phagocytosis of RAW264.7 macrophage cells. As shown in Fig. 4 and 12.5 μg/mL of PPH significantly improved the intracellular fluorescence intensity. This result indicated that 12.5 μg/mL of PPH could enhance phagocytosis activity of RAW264.7 macrophage cells. Moreover, the effect of 100 μg/mL PPH on phagocytosis activity was stronger than the effect of 1 μg/mL LPS. In summary, PPH could improve macrophage endocytosis activity of RAW264.7 macrophage cells.

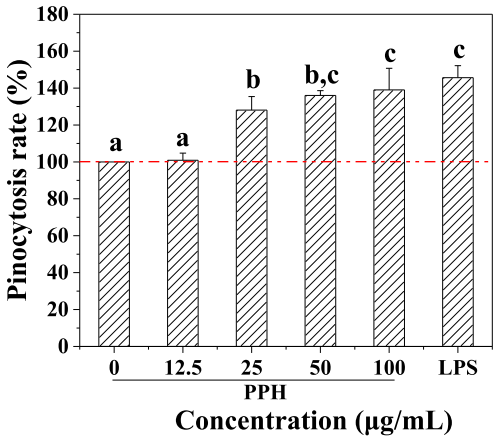

Fig. 3. Promotion effect of PPH on macrophage pinocytosis of neutral red in RAW264.7 macrophage cells. Data are presented as the mean ± SD (n = 3). Different lowercase letters represent significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

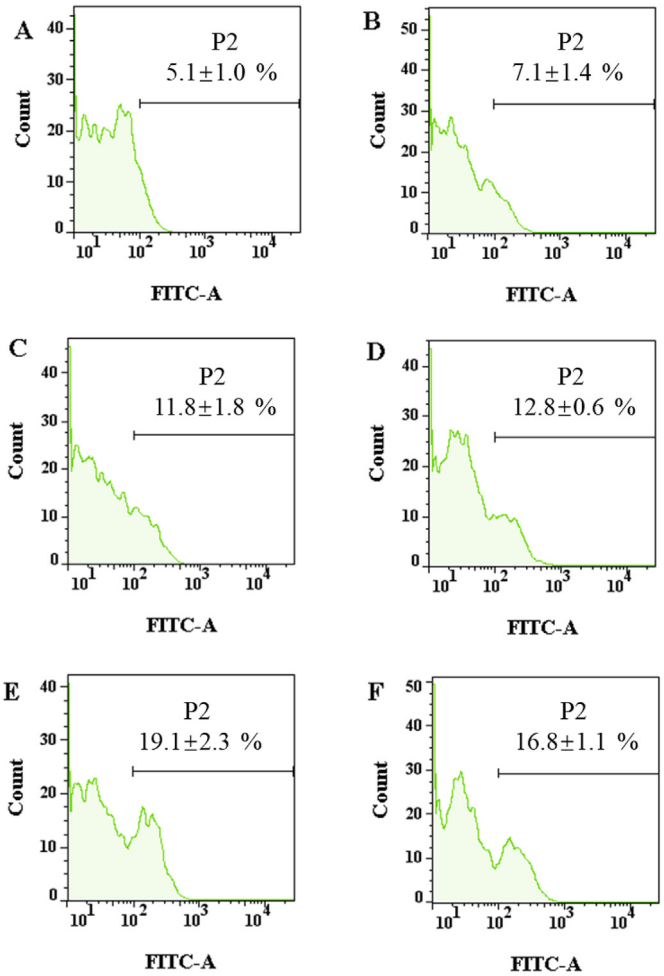

Fig. 4. Effect of PPH on FITC-dextran internalization by flow cytometry in RAW264.7 macrophage cells. RAW264.7 macrophage cells were treated with (A) PBS, (B)12.5 μg/mL PPH, (C)25 μg/mL PPH, (D)50 μg/mL PPH, (E)100 μg/mL PPH and (F)1 μg/mL LPS for 24 h. Cells were stained with FITC-dextran and fluorescence was determined by flow cytometry.

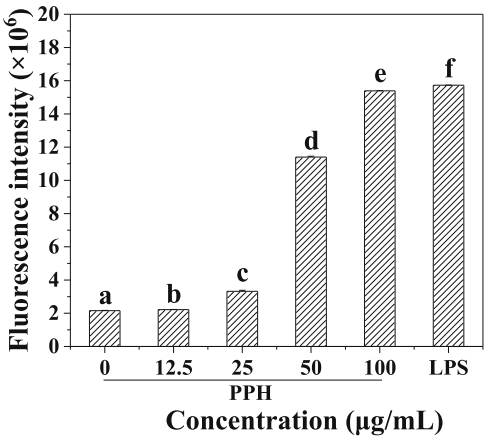

Fig. 5. Excitation of PPH on ROS generation in RAW264.7 macrophage cells. Data are presented as the mean ± SD (n = 3). Different lowercase letters represent significant differences ($p < 0.05$).

3.4. Excitation of PPH on ROS generation in RAW264.7 macrophage cells

ROS has been served as toxic by-products of metabolism, excessive accumulation of which could cause damage to organism. However, phagocytes such as neutrophils and macrophages produce ROS during phagocytosis or stimulation with a wide variety of agents to attack invading bacteria and serve as secondary messengers in signal transduction (Kohchi et al., 2009; Yang et al., 2018). ROS generation of RAW264.7 macrophage cells stimulated by PPH was measured by fluorescence probe. As is shown in Fig. 5, ROS production in RAW264.7 macrophage cells increased after PPH treatment. Previous studies were consistent with this result. A novel peptide, purified from wheat germ globulin, stimulated macrophages to produce ROS and TNF-α (Wu et al., 2017). *Ganoderma atrum* polysaccharide induced macrophages to produce ROS mediated by TLR4 (Yu et al., 2014). In summary, PPH induced RAW264.7 macrophage cells to produce ROS in a dose-dependent manner.

3.5. Effect of PPH on secretions of IL-10 and TNF-α in RAW264.7 macrophage cells

Cytokines are associated with the growth, differentiation and function of immune cells (Kubo et al., 2003). TNF-α and IL-10 are key cytokines in immunity, and they are necessary for proliferation and functioning of macrophages. Because of the pivotal role of cytokines, the potential for PPH to regulate the secretion of these mediators in RAW264.7 macrophage cells were examined. As shown in Table 1, the addition of PPH resulted in significant increase in IL-10 and TNF-α secretion levels. The TNF-α production of RAW264.7 macrophage cells treated with 100 μg/mL PPH has no significant difference compared to cells treated with 1 μg/mL LPS. These results further confirm that PPH can promote the activation of RAW264.7 macrophage cells. In conclusion, PPH could exert immunomodulatory effects on RAW264.7 macrophage cells to product inflammatory molecules which fight against pathogens. Furthermore, it suggested that PPH might use as a functional agent to enhance immunity of the body in immunosuppression condition.

Table 1
Effects of different concentrations (12.5, 25, 50 and 100 μg/mL) of PPH and 1 μg/mL LPS on secretions of IL-10 and TNF-α in RAW264.7 macrophage cells. Data are presented as the mean ± SD (n = 3). Different lowercase letters represent significant differences ($p < 0.05$).

	IL-10 (pg/mL)	TNF-α (pg/mL)
Blank	40.97 ± 1.70^{a}	1562.65 ± 217.06^{a}
12.5 μg/mL PPH	73.64 ± 7.19^{b}	1887.74 ± 28.78^{b}
25 μg/mL PPH	74.47 ± 9.49^{b}	1952.41 ± 41.42^{b}
50 μg/mL PPH	74.52 ± 4.09^{b}	1949.98 ± 57.65^{b}
100 μg/mL PPH	94.35 ± 4.70^{c}	1872.26 ± 12.20^{b}
1 μg/mL LPS	155.15 ± 5.82^{d}	1932.87 ± 0.68^{b}

Table 2
Effect of PPH on body weight in CTX-treated mice. Data are presented as the mean ± SD (n = 8). Different lowercase letters represent significant differences ($p < 0.05$).

Group	Initial weigh (g)	Weight of the fourth day (g)	Final weight (g)	Weight gain (g)
NC	22.28 ± 1.50^{a}	25.87 ± 1.02^{a}	29.93 ± 1.14^{a}	7.65
MC	22.24 ± 0.56^{a}	22.23 ± 0.99^{c}	27.30 ± 1.26^{c}	5.06
LPPH	22.87 ± 0.79^{a}	24.38 ± 0.77^{b}	29.98 ± 1.10^{b}	6.11
HPPH	23.05 ± 0.91^{a}	23.49 ± 2.34^{b,c}	28.41 ± 3.13^{a}	5.36
LH	20.90 ± 1.02^{a}	23.57 ± 1.07^{b,c}	28.10 ± 1.13^{b}	6.08

3.6. Effect of PPH on weight and WBC in CTX-treated mice

Daily intraperitoneal injection of PPH did not result in any mortality, which indicated that PPH did not have toxicity in the treated mice. As shown in Table 2, CTX could cause weight and hair loss in mice compared to NC group. However, after treatment LPPH, HPPH and LH groups reduced weight loss in final body weight, and the effect of HPPH group was weaker than LPPH and LH groups. The peripheral white blood cells (WBC) counts of the normal and experimental mice are shown in Table 3. 50, 150 mg/kg PPH and 10 mg/kg LH significantly increased ($p < 0.05$) the total peripheral white blood cells (WBC) toward the normal levels by resisting inflammation.

3.7. Effect of PPH on stimulation index of spleen lymphocytes induced by LPS and ConA in CTX-treated mice

As shown in Fig. 6A, MC group was found to significantly decrease ($p < 0.05$) the proliferation of spleen lymphocytes, compared with NC group. Combining with ConA, PPH significantly enhanced ($p < 0.05$) the proliferation of spleen lymphocytes. Stimulating with ConA, PPH at 50 mg/kg showed a stronger promotion in the proliferation of spleen lymphocytes than 10 mg/kg LH and 150 mg/kg PPH. But LPPH, HPPH and LH groups weren't found to restore the decrease of spleen lymphocytes proliferation combining with LPS. Previous studies have demonstrated that ConA is a mitogen able to stimulate mouse T lymphocytes and LPS is a potent mouse B lymphocytes mitogen which promotes expansion and immunoglobulin secretion (Lozano-Ojalvo et al., 2016; Xu et al., 2008). Our results suggest that PPH and LH could stimulate T lymphocytes but not B lymphocytes. This was consistent with our previous studies which showed that PPH promoted spleen lymphocyte proliferation induced by ConA not LPS *in vitro* (Fig. S1). Not in agreement with our results, the glycopeptide from *Paecilomyces sinensis* at 500 mg/kg showed a significant increase in the proliferation of splenocytes with both ConA and LPS (Zhu et al., 2016). However, PPH

Table 3
Effect of PPH on WBC in CTX-treated mice. Data are presented as the mean ± SD (n = 8). Different lowercase letters represent significant differences ($p < 0.05$).

Group	WBC (10^9/L)
NC	4.19 ± 0.72^{a}
MC	13.03 ± 6.91^{b}
LPPH	7.77 ± 1.96^{a}
HPPH	8.50 ± 2.61^{a,b}
LH	7.34 ± 2.23^{a}

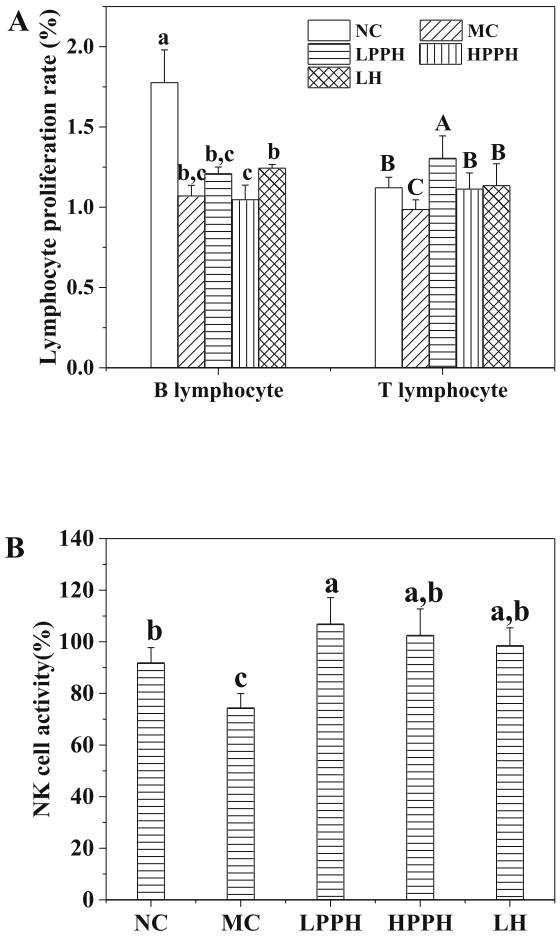

Fig. 6. Effects of PPH on (A) T lymphocyte, B lymphocyte proliferation and (B) NK cell activity in CTX-treated mice. Data are presented as the mean ± SD (n = 8). Different lowercase letters represent significant differences ($p < 0.05$).

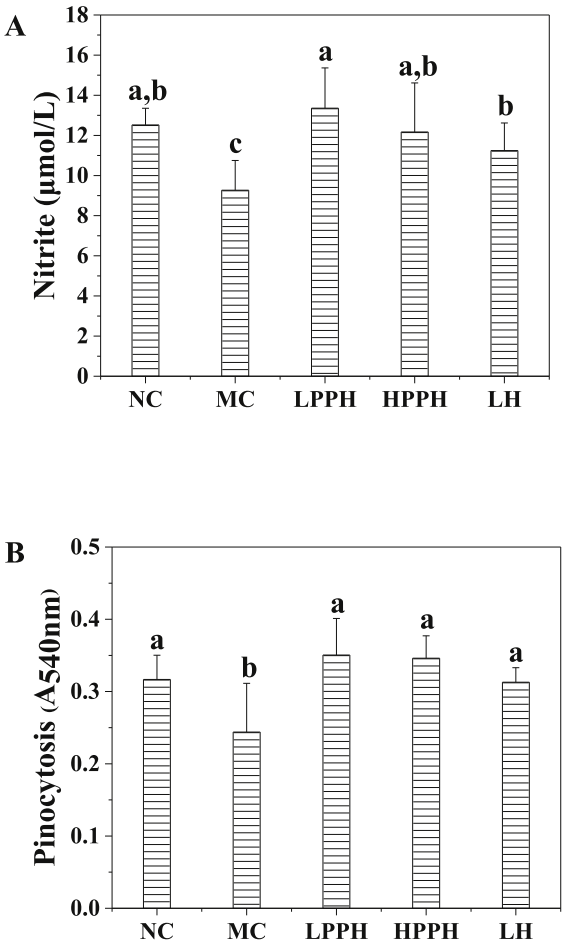

Fig. 7. Effects of PPH on (A) NO production and (B) phagocytosis in CTX-treated mice peritoneal macrophages. Data are presented as the mean ± SD (n = 8). Different lowercase letters represent significant differences ($p < 0.05$).

at 50 mg/kg could not only increase proliferation of T lymphocytes, but also show a higher level than NC group.

3.8. Excitation of PPH on NK cell activity in CTX-treated mice

NK cell is required to protect the human body against tumor cell. Therefore, the activity of NK cell against HepG2 cells was investigated. As shown in Fig. 6B, compared with NC group, NK cell activity of MC group was significantly decreased ($p < 0.05$), implying that the immunosuppressed model was successfully established by CTX. Treatment with 50 mg/kg, 150 mg/kg PPH and 10 mg/kg LH enhanced NK cell activity compared with MC group, suggesting that PPH and LH could improve the cell immune function in these mice. Furthermore, 50 mg/kg PPH showed a better recovery of NK cell activity compared with NC group.

3.9. Stimulations of PPH on NO secretion and pinocytosis of peritoneal macrophages in CTX-treated mice

Besides NK cell activity, peritoneal macrophage was another indispensable part of the innate immune system. To investigate the immunomodulatory effect of PPH *in vivo*, the NO secretion and pinocytosis of peritoneal macrophages were evaluated. As shown in Fig. 7, the NO secretion and pinocytosis were significantly decreased in MC group, relative to NC group. After treatment with PPH and LH, the NO secretion and pinocytosis restored to the normal levels. The results suggested that PPH could enhance the function of macrophages in CTX-treated mice. Consistent with our results, 80 mg/kg water-soluble polysaccharide extracted from highland barley could promote the proliferation and phagocytosis activity of macrophages in immunosuppressive mice (Han et al., 2019). In addition, yak bone hydrolysates could prevent immunosuppression by increasing innate immunity and adaptive immunity (Gao et al., 2019). Hence in our study the conceivable mechanism of immunomodulatory action of PPH in CTX-treated mice was shown in Fig. 8. It indicated that PPH enhanced the immunity of CTX-treated mice through strengthening T lymphocytes proliferation, NK cell activity and peritoneal macrophage activity.

It is interesting that phagocytes (neutrophils, monocytes and macrophages) are not only key participants in the innate immune response, but also play key roles in adaptive immunity. The activation of macrophages is benefit for initiating and propagating defensive reactions against pathogens. The present study showed that PPH significantly activated RAW264.7 macrophage cells *in vitro* and enhanced the phagocytosis of peritoneal macrophages *in vivo*, indicating that PPH could enhance the macrophage function of the CTX-treated mice. Furthermore, PPH has better immunoregulation effect in innate immunity *in vivo* than adaptive immunity.

4. Conclusions

Our results firstly demonstrated that *P. heterophylla* protein hydrolysate (PPH) showed immunomodulatory effect in RAW264.7 macrophage cells and protective effect in CTX-treated mice. *In vitro*, PPH elevated NO production and iNOS mRNA expression level in a dose-

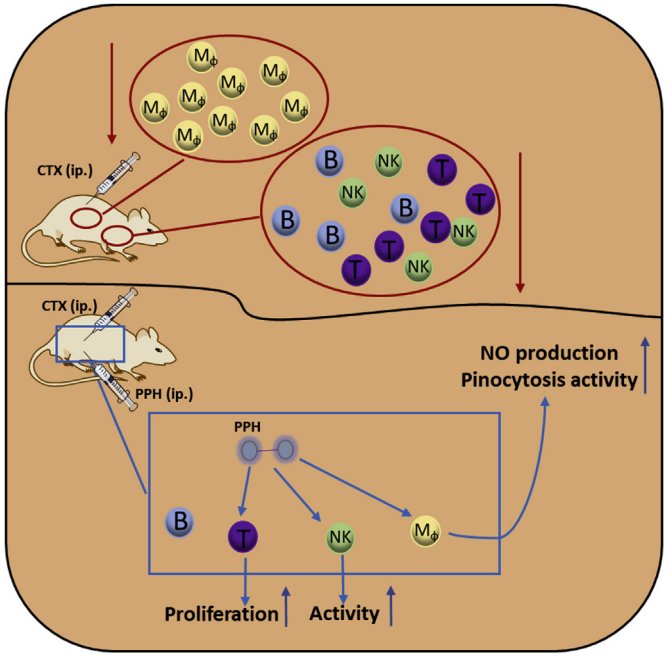

Fig. 8. Mechanism about immunomodulatory action of PPH in CTX-treated mice. M_{ϕ}, macrophages; T, T lymphocytes; B, B lymphocytes; NK, natural killer cells; CTX, cyclophosphamide; PPH, *Pseudostellaria heterophylla* peptide.

dependent manner in RAW264.7 macrophage cells. Furthermore, PPH could enhance both phagocytosis and pinocytosis of RAW264.7 macrophage cells as well as ROS generation and levels of IL-10 and TNF-α. *In vivo*, the proliferation of T lymphocyte and NK cell activity were elevated in CTX-treated mice after administrations of PPH. The significant increments in the NO production and pinocytosis in CTX-treated mice peritoneal macrophages by PPH treatment was found. These results suggested that PPH could activate macrophages and had a remarkable response of the innate immunity in the immunosuppressive mice model. This study may provide a basis to research on PPH as an immuno modulatory functional food.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110816.

Abbreviations

ConA	concanavalin
CTX	cyclophosphamide;
DMSO	dimethyl sulfoxide;
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
IL-10	interleukin-10
iNOS	inducible NOS
LPS	lipopolysaccharide;
LH	levamisole hydrochloride;
MC	model control
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide;
NC	normal control
NO	nitric oxide;
NK	natural killer
NOS	nitric oxide synthases
PBS	phosphate-buffered saline;
PPH	*Pseudostellaria heterophylla* protein hydrolysate ;
ROS	reactive oxygen species
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SD	standard deviation
TNF-α	tumor necrosis factor-α;
WBC	white blood cell

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