Effect of *Momordica charantia* polysaccharide on immunomodulatory activity in mice

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Abstract. *Momordica charantia* polysaccharides (MCPs) have been reported to exert beneficial roles, such as disease healing, in medicine and pharmacy. However, little is known about their effects on immunomodulation. The present study aimed to explore the possible effects of *Momordica charantia* polysaccharide (MCP) on the immunomodulatory activity of mice lymphocytes. To this aim, male BALB/c mice aged 6-8 weeks were assigned to the following six experimental groups: i) Normal (NG); ii) model (MG); iii) positive (PG); iv) MCP low-dose (MLG); v) MCP medium-dose (MMG); and vi) MCP high-dose (MHG). An immunosuppressive model was established by the intraperitoneal injection of cyclophosphamide in all groups apart from NG. The NG and MG mice were fed with distilled water, whereas the PG mice were administered with levamisole and the MLG, MMG and MHG mice were fed on low, medium and high (100, 200 and 300 mg/kg, respectively) doses of MCP for 21 consecutive days. Subsequently, the mice underwent surgical procedure and were analysed using a range of procedures, including measurement of the thymus index (TI) and spleen index (SI), assessment of the lymphocyte proliferation rate and cell phagocytosis of peritoneal macrophages, lymphocyte proliferation, secretion and mRNA expression of cytokines IFN-γ, IL-6 and IL-12. The mice divided into six groups as mentioned above and treated for 7 days, in the first 6 days, except NG group, mice in each group were desiccated in the abdominal cavity and sensitized by 1% dinitrofluorobenzene (DNFB). On day 6, mice were sensitized with 20 μl DNFB/acetone/olive oil solution behind the right ear and in front of the right ear. Compared with those in the NG mice (not injected with 80 mg/kg cyclophosphamide), the TIs and SIs of the PG, MLG, MMG and MHG mice were increased. In addition, the inhibitory rate of ear swelling and the phagocytic activity of peritoneal macrophages in the PG, MLG, MMG and MHG mice were increased compared with those of MG. Furthermore, the lymphocyte proliferation rate, the secretion and relative mRNA expression levels of cytokines IFN-γ, IL-6 and IL-12 were significantly increased in the PG, MMG and MHG mice compared with those in the NG mice. The results from the present study suggest that treatment with MCP led to an upregulation of the organ indices of immunosuppressed mice, reduced their delayed allergic reaction indicated by the differential cytokine levels, improved the phagocytic activity of peritoneal macrophages, enhanced the proliferation rate of lymphocytes, increased the secretion and expression of IFN-γ, IL-6 and IL-12. Therefore, MCP may improve the immune function of the immunosuppressed mice.

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Introduction

Bitter melon (*Momordica charantia*) is a monoecious plant of the genus *Momordica* in the *Cucurbitaceae* family that is commonly utilized as both a medicine for lowering blood sugar, treating diabetes and alleviate the feeling of heat (1). It has been eaten as fresh fruit for thousands of years, although it can also be consumed sweetened with sugar. Although *Momordica charantia* naturally tastes bitter, it has both a high nutritional and medicinal value, because it has been documented to contain proteins, carbonic dioxide hydrate, phenolic acids, alkaloids, flavonoids, quinine, amino acids, saponin and fatty acids (1). *Momordica charantia* is also used as a herbal medicine in numerous countries such as Japan, India and countries in the southeast Asia, where the whole plant has been shown to possess potent pharmacological properties, especially within its seeds and fruits (1-3). In previous years, the functional components and health-associated benefits of *Momordica charantia* is gradually becoming understood, which may have wide-ranging prospects in terms of its clinical application. The active components of the plant are mainly comprised of the water extract, alcoholic extract, polysaccharide, saponin and *Momordica charantia* juice, protein and polypeptide (which may or may not be separated (1). In addition to exerting hypoglycaemic and lipid-lowering effects (2,3), they have been shown to possess antioxidant,

bacteriostatic and antitumour properties, which may assist in preventing obesity and improve metabolic syndromes, such as diabetes (4).

As a result of rapid developments in polysaccharide research, increasing focus is being placed on *Momordica charantia* polysaccharide (MCP) (1-4). Numerous biological functions and activities of MCP (such as antitumor, antidiabetic and antioxidant activities, immunomodulation and radioprotection) are beginning to garner the attention of the scientific community (5,6). Different components of MCP (1-7) have been previously extracted, separated and purified to examine the potential hypoglycaemic and antioxidant activities of MCP (1-7). Previous studies have shown that MCP can exert numerous physiological effects, such as improving immune function (5), antiviral (6) and antioxidant effects (7). In addition, it has been demonstrated to reduce blood sugar levels (8) and can act as an antitumor agent, for example in liver cancer, and overcome resistance mutations. Therefore, furthering the understanding of the functions and properties of MCP may have far-reaching implications in terms of its potential use in the medicinal and health care fields. However, although MCP is a bioactive substance with development value, several problems remain, such as discovering the target genes of MCP, unravelling the regulation of signaling pathways or conducting functional research in preclinical trials, and form the focus of the present study. since its underlying mechanism of action remains unclear.

Therefore, the present study aimed to investigate the mode of regulation and mechanism of MCP in immunosuppressed mice, in addition to identifying the potential role of MCP in terms of its application in health care. It is hoped that the present results provide valuable points of reference to further the research on MCP, given its wide applicability in food and medicine.

Materials and methods

Chemicals and reagents. Purified MCP (purity ≥90%) was obtained from Chenguang Biotech Group Co., Ltd. Methyl thiazole blue (MTT), trypan blue, RPMI-1640 medium, DMSO, trimethylol aminomethane, PBS and chloroform were obtained from Beijing Solarbio Science & Technology Co., Ltd. PrimerScript™ RT reagent Kit and TB Green™ Premix Ex Taq™ II (Tli RNase H Plus) kits were purchased from Takara Biotechnology Co., Ltd.

Mice. In total, 60 Specific pathogen-free BALB/c male mice (weighing 20±2 g; 6-8-week-old) were brought from the Academy of Life Sciences, Jinzhou Medical University (Jinzhou, China). Housed at a constant temperature of 25±2˚C and relative humidity of 50-70%, the mice were fed with food and water, and were permitted to feed themselves in a standard environment with a 12-h light/dark cycle. They were subjected to adaptive feeding for 1 week. These experiments were approved by the Ethics Committee of Jinzhou Medical University [Production License SCXY (Liao) 2019-0003; experimental animal ethics approval no. 2019014].

Animal experiments. A total of six groups of animals were prepared by randomly choosing 10 healthy mice for each group, namely the normal (NG), the model (MG), the positive (PG), the MCP low-dose (MLG), the MCP medium-dose (MMG) and the MCP high-dose (MHG) groups.

The animal protocols in the present study were identical to those performed in previous studies (9-19). Briefly, each group of mice was given an intragastric administration via gavage once a day for 21 days according to the following experimental design: From days 1 to 5, the mice all groups except for NG were intraperitoneally injected with 80 mg/kg cyclophosphamide (CY; by Jiangsu Hengrui Pharmaceutical Co., Ltd.), whereas NG and MG mice were given distilled water by intraperitoneal injection. From day 1, PG mice were given 200 mg/kg levamisole whereas the MLG, MMG and MHG mice were given MCP at different doses (100, 200 and 300 mg/kg, respectively; 0.1 ml/10 g per day). During the feeding process, the activity of the mice was observed daily. The body weight of the mice was also measured every day and the doses of drugs administered were adjusted according to changes in body weight.

Induction of delayed-type hypersensitivity (DTH) by dinitrofluorobenzene (DNFB). In the procedure used to determine the DTH, a sensitizer, such as DNFB, is injected into the abdominal cavity of mice before the ears of the mice are insulted with DNFB. Subsequently, the degree of swelling of the ears is measured. A total of five mice from each of the six different groups were treated for 7 days as follows: Before administration on the first day, the abdominal regions of mice in each group, except for those in the NG group, were depilated and 1% DNFB (Shanghai Jizhi Biochemical Technology Co., Ltd.)/acetone/olive oil solution was applied to the depilated region for sensitization. The next day, intensive application of the same solution was performed again On day 6 of the sensitization test, 20 μl DNFB/acetone/olive oil solution was used to sensitize the mice behind and in front of the right ear. After 24 h, the mice were sacrificed by dislocation of the cervical vertebrae before two ears of each mouse were removed using a puncher and weighed. DTH was expressed as the weight difference between the right and left ears.

Measurement of immune organ indices. After the mice had been sacrificed by cervical dislocation, their thymus and spleen were also dissected and rinsed in 0.9% normal saline. The surfaces of the thymus and spleen were dried with absorbent paper, after which their weights were measured using an analytical balance. The thymus index (TI) and spleen index (SI) were calculated (9) according to the following equations: TI=mean weight of thymus in the group/mean body weight in the group x10; SI=mean weight of spleen in the group/mean body weight in the group x10.

Histomorphological observations of thymus and spleen. The spleens and thymuses of the mice were dissected, washed with normal saline, fixed with neutral formalin fixative before paraffin sections were prepared. Tissues were then dehydrated, made transparent and wax-soaked. Before sealing, slices were embedded, made sticky and then baked, dewaxed, immersed in alcohol, dyed and dehydrated. Pathological sections were prepared and structural changes in each tissue were observed as previously reported (10). Specific details were as follows: Tissues (≤3 mm) were fixed in 10% neutral formalin for 24 h,

embedded in paraffin, and sliced at 3-5 μm. Subsequently, the sections were dewaxed in xylene, rehydrated using a series of ethanol (100, 95 and 80% for 10 min each), followed by washing in tap water and distilled water (for 1 min each). Staining was performed with hematoxylin for 4 min followed by washing with tap water for 2 min, differentiation with 1% hydrochloric acid ethanol for 20 sec, washing with tap water for 2 min, incubation with 6.1% diluted ammonia water for 30 sec, within with tap water 2 min and distilled water for 1 min. Eosin staining was subsequently performed for 90 sec, followed by dehydration with 80, 95 and 100% ethanol. The sections were washed with xylene and sealed with neutral gum. After staining, the pathological changes of the tissue were observed under a light microscope.

Isolation and culture of mice peritoneal macrophages. After the mice were sacrificed by dislocation of the cervical vertebrae, they were soaked in 75% ethanol at room temperature for 3 min for disinfection. A total of 5 ml pre-cooled PBS was then injected into the abdominal cavities before they were gently massaged for ~2 min. After allowing the abdomen to rest for 5 min, the peritoneal fluid was extracted, centrifuged at ~670.8 x g and 4˚C for 5 min before the supernatant was discarded. The pellet was subsequently diluted in erythrocyte lysate (Red Blood Cell Lysis Buffer; Beijing Solarbio Science & Technology Co., Ltd.), allowed to stand and then centrifuged at ~670.8 x g and 4˚C for 5 min, before the supernatant was again discarded. The cells were suspended in RPMI-1640 complete culture medium [with 10% FBS (Zhejiang Tianhang Biotechnology Co., Ltd.)] for counting before adjusting to the required concentration of $5x10^6$ cells/ml. Subsequently, the cells were incubated in an incubator at 37˚C with 5% CO_2 for 18 h until complete adherence had occurred. The supernatant was subsequently removed and discarded, where non-adherent cells were removed. Fresh RPMI-1640 complete medium was added to obtain the purified peritoneal macrophages (11,12).

Lymphocyte suspension preparation. After the mice had been sacrificed by cervical dislocation, the mice were clamped with tweezers and soaked in 75% alcohol for 1-2 min for disinfection purposes. The spleens were extracted on a sterile clean bench, placed into a Petri dish with PBS for 1-2 min at 4˚C, transferred to RPMI-1640 medium and ground through a 200-mesh screen with the end of a syringe pull rod. The filtrate was then sucked into a centrifuge tube before being centrifuged at ~377.32 x g and 4˚C for 5 min, after which the supernatant was discarded and 3 ml erythrocyte lysis solution (Red Blood Cell Lysis Buffer) was added. The mixture was allowed to stand for 5 min at 4˚C, after which it was re-centrifuged at ~377.32 x g at 4˚C for 5 min and the supernatant was discarded again. This step was repeated until the erythrocytes were completely lysed. The precipitated cells were resuspended in RPMI-1640 complete culture medium (with 10% FBS) and filtered using a 70-μm cell strainer into another centrifuge tube. After trypan blue staining, cell viability was adjusted to >95% and RPMI-1640 complete culture medium was added until the cell density reached $5x10^6$ cells/ml, thereby forming the lymphocyte suspension (13). After the preparation of lymphocyte suspension, the cells were cultured for 48 h and were observed to be in good condition.

Lymphocyte proliferation assay. Lymphocyte suspension (200 μl; ~100-300 cells) was inoculated into a 96-well cell culture plate, cultured at 37˚C with 5% CO_2 for 48 h and subsequently 20 μl MTT (5 mg/ml) solution was added in each well. After 4 h (37˚C) of continuous culture, the supernatant was discarded and 150 μl DMSO was added to each well. Finally, the absorbance at 570 nm (Abs_{570}) was measured using a microplate reader and the proliferation rate of the mouse spleen lymphocytes was calculated (14,15) according to the following equation: Spleen lymphocyte proliferation rate (%)=(Abs_{570} experimental group)/(Abs_{570} control group) x100.

Determination of cell phagocytosis of peritoneal macrophages. Mice peritoneal macrophages were collected before the cell concentration was adjusted to $2x10^6$ cells/ml in RPMI-1640 complete medium to ensure that the concentration of the macrophage suspension from each mouse was consistent. The macrophage suspension was then added into 96-well plates and the plates were cultured in an incubator at 37˚C with 5% CO_2 for 3 h. After observing the adherent cells under a microscope, the culture medium was removed and the cells were washed twice with PBS pre-heated to 37˚C to remove the nonadherent cells. Subsequently, 200 μl 0.1% neutral red dye (cat. no. G1310; Beijing Solarbio Science & Technology Co., Ltd.) was added to each well and the culture was allowed to continue for 3 h (13-15). After washing the cells three times with preheated PBS to remove excess neutral red, 100 μl cell lysis solution [comprising absolute ethanol/glacial acetic acid, 1:1 (v/v)] was added to each well followed by mixing. The plates were incubated overnight at 4˚C before absorbance at 540 nm was read using a microplate reader.

Cytokine assay. The lymphocyte suspension was inoculated into 24-well plates and 2 ml lymphocyte suspension (~$1x10^4$ cells) was used for each well. After 48 h of culture at 37˚C with 5% CO_2, the cells were centrifuged at ~377.32 x g and 4˚C for 10 min and the supernatant was collected. The concentrations of IFN-γ (cat. no. ml002277), IL-6 (cat. no. ml063159) and IL-12 (cat. no. ml037868;) secreted by the lymphocytes in the supernatant were measured using ELISA. These procedures were performed following the protocols of the mouse cytokine kit (all from Shanghai Enzyme-linked Biotechnology Co. Ltd.). The absorbance values were measured at a wavelength of 450 nm using a microplate reader and a standard calibration curve was generated to calculate the concentrations of the secreted compounds in pg/ml (16).

Reverse transcription-quantitative PCR (RT-qPCR). The lymphocyte suspension was inoculated on a 24-well cell culture plate and cultured at 37˚C with 5% CO_2 for 48 h. Subsequently, the cells were collected, centrifuged at 377.32 x g at 4˚C for 5 min and the supernatant was discarded, before washing the cells twice with PBS and TRIzol® (Thermo Fisher Scientific, Inc.) was added to completely lyse the cells. Total RNA was extracted according to the protocols of the TRIzol kit and the ratio between the optical density measured at 260 and 280 nm was used to assess the RNA purity (17).

Table I. Forward and reverse primers used for reverse transcription-quantitative PCR.

Primer	Sequence	Accession no.
IFN-γF	5'-CGGCACAGTCATTGAAAGCCTA-3'	K00083
IFN-γR	5'GTTGCTGATGGCCTGATTGTC-3'	
IL-6F	5'-CCACTTCACAAGTCGGAGGCTTA-3'	X54542
IL-6R	5'-CCAGTTTGGTAGCATCCATCATTTC-3'	
IL-12F	5'-TTCATAAGAGTCAGGTGGTCTTGG-3'	NM_008351
IL-12R	5'-CCTTTGGGGAGATGAGATGTG-3'	
β-actin F	5'-CATCCGTAAAGACCTCTATGCCAAC-3'	NM_007393
β-actin R	5'-ATGGAGCCACCGATCCACA-3'	

F, forward; R, reverse.

Following precisely the protocols of the PrimerScript™ RT reagent Kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Inc.), RNA was reverse-transcribed into cDNA using the temperature protocols of 37˚C for 15 min and 85˚C for 5 sec. qPCR was subsequently performed using the TB Green™ Premix Ex Taq™ II kit, using the following thermocycling conditions: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The primer pairs used for qPCR (Table I) were designed the mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene β-actin (18).

Reverse transcription-PCR. Using a 24-well cell culture plate, 1 ml lymphocyte suspension (~$2x10^4$ cells) was added into each well incubated at 37˚C for 48 h. Total RNA of lymphocytes was extracted using the TRIzol method according to the manufacturer's protocol. A protein/nucleic acid analyzer (NanoDrop One; Thermo Fisher Scientific, Inc.) was used to detect the RNA concentration and purity, before the total RNA concentration was adjusted to 400-600 ng/μl range. Reverse transcription was performed according to the instructions of PrimeScript™ RT Reagent Kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Inc.), which was 37˚C for 15 min followed by 85˚C 5 sec. The products of qPCR (described in the previous paragraph) were used for 2% agarose gel electrophoresis. The following reagents were used: DL 1000DNA Marker Takara Japan (cat. no. 3591Q; Takara Bio, Inc.); gel stain 10,000X (Beijing TransGen Biotech Co., Ltd.).

Statistical analysis. Statistical analysis was performed using SPSS 21.0 software (IBM Corp.) and the experimental data are shown as the mean ± standard deviation. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of MCP on organ indices in mice. Compared with those in the NG mice, the thymus and spleen indices of mice in the MG group were significantly decreased (P<0.05; Table II), suggesting that the immune function of the mice was significantly inhibited. Compared with those MG mice, the TI was significantly increased in the PG, MHG, MMG and MLG groups (P<0.05; Table II), whereas the SI was increased significantly in the PG, MMG and MHG groups (P<0.05; Table II). The SI of mice in MLG was also significantly increased compared with that in the MG group (P<0.05). These data suggest that the immune organ indices of the immunosuppressed mice increased in response to MCP in a dose-dependent manner, although it remained lower compared with that of the NG mice.

Table II. Effects of MCP on immune organ index of immunosuppressed mice.

Group	Thymus index, mg/g	Spleen index, mg/g
Normal saline	1.58±0.01	5.70±0.27
Model	0.67±0.14[a]	0.85±0.01[a]
Positive	1.49±0.11[a]	3.90±0.34[a]
MCP low-dose	0.90±0.14[b]	1.39±0.34
MCP medium-dose	1.14±0.20[c]	2.35±0.78[b]
MCP high-dose	1.31±0.14[d,e]	4.73±0.30[b]

Different lower-case letters showed statistical significance (P<0.05). MCP, *Momordica charantia* polysaccharide. [a]P<0.05 vs. normal saline; [b]P<0.05 vs. model; [c]P<0.05 vs. MCP low-dose; [d]P<0.05 vs. positive; [e]P<0.05 vs. MCP medium-dose.

Histomorphological observation of the thymus and spleen. The spleen tissue of MG mice was found to be markedly smaller compared with that of NG mice, with lower density, uneven distribution of lymphocytes and unclear boundaries between the red and the white medulla (Fig. 1B). Lymphocytes in the spleen tissue of mice in the MHG and PG groups were densely but unevenly distributed, with deep staining (Fig. 1C and F). The distribution of lymphocytes in the red medulla region of the spleen tissue from the MLG and MMG mice were loose with no evident damage found in the spleen tissues of mice in either group (Fig. 1D and E). These observations suggest that MCP can promote the development of spleen in mice with CY-induced hypoimmunity, thereby improving the immunity of mice (Fig. 1).

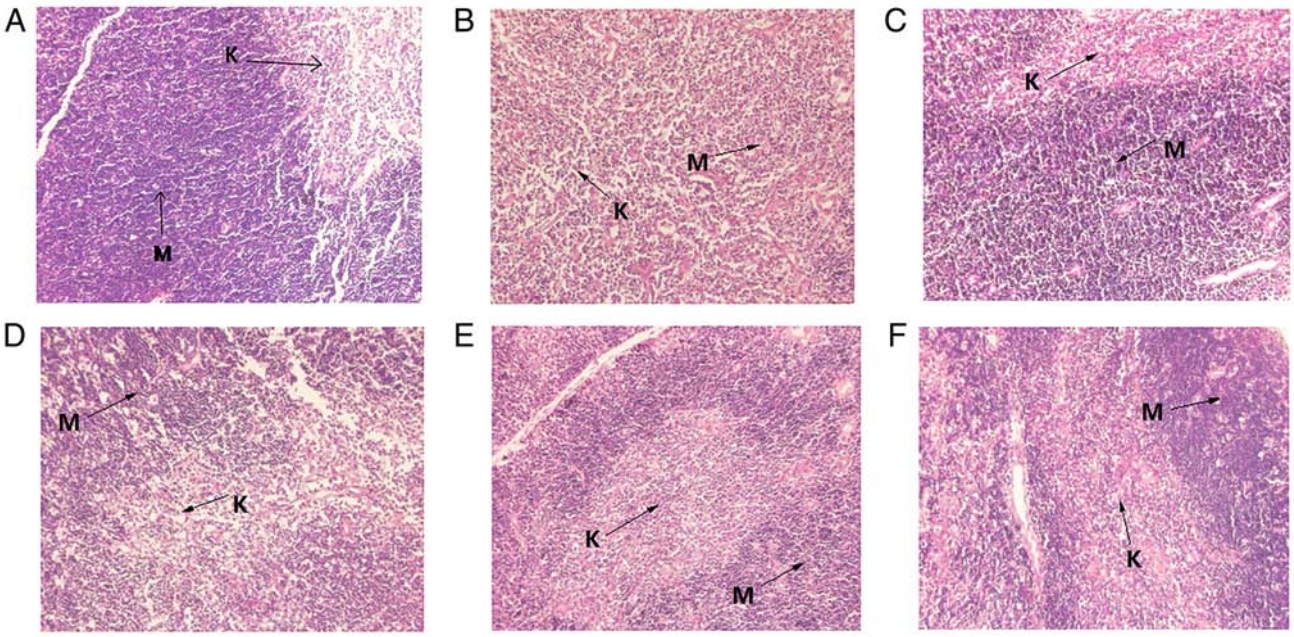

Figure 1. Effects of MCP on histomorphological changes in the spleen of mice. Representative images of H&E staining of (A) normal, (B) model, (C) positive, (D) MCP low-dose (100 mg/kg), (E) MCP medium-dose (200 mg/kg) and (F) MCP high-dose (300 mg/kg) groups. Magnification, x20. MPC, *Momordica charantia* polysaccharide; K, white medulla; M, red medulla.

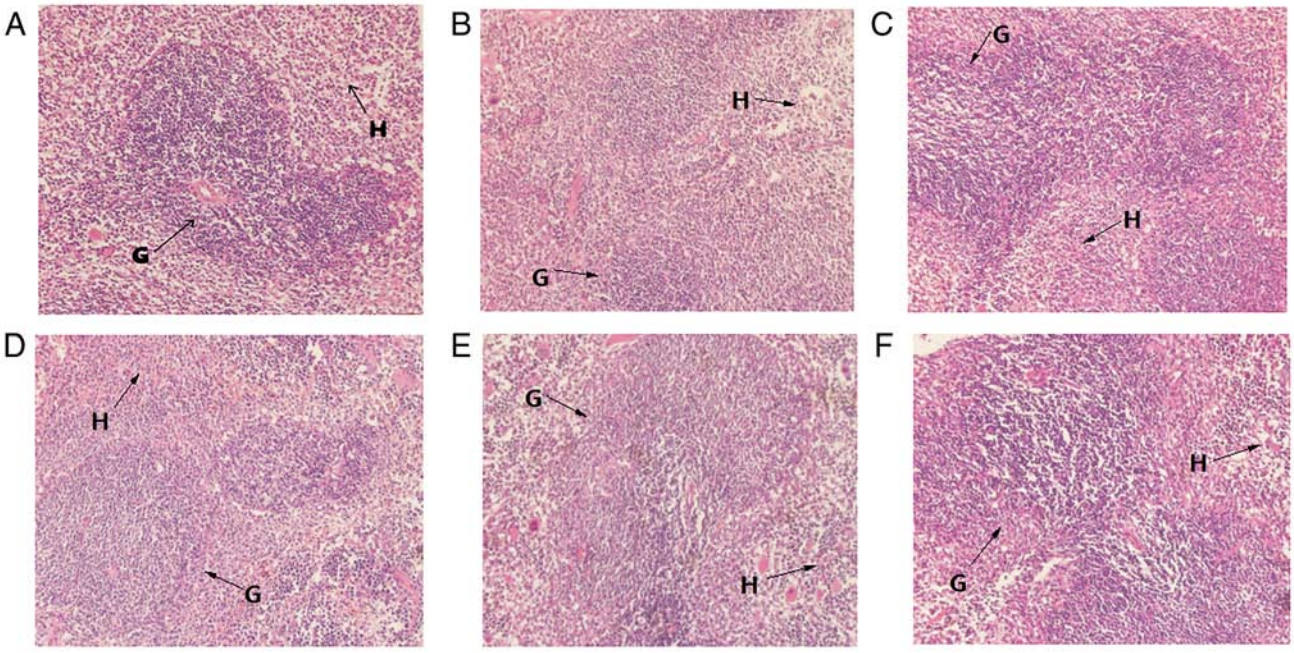

Figure 2. Effects of MCP on histomorphological changes in the thymus of mice. Representative images of H&E staining of (A) normal, (B) model, (C) positive, (D) MCP low-dose (100 mg/kg), (E) MCP medium-dose (200 mg/kg) and (F) MCP high-dose (300 mg/kg) groups. Magnification, x20. MPC, *Momordica charantia* polysaccharide; H, cortex; G, medulla.

The number of lymphocytes in the thymus tissue of MG mice was decreased compared with that of NG mice, where the boundary between the medulla and cortex was not clear (Fig. 2B). In the PG, MMG and MHG mice, the cortical lymphocytes were dense, evenly distributed and deeply stained, where medullary lymphocytes were loosely distributed, and the blood vessels and reticular cells were clear (Fig. 2C, E and F). These observations suggest that MCP can enhance the development of lymphocytes. The lymphocyte distribution in the tissues of MLG mice was loose and uneven, which indicated that MCP was able to repair injuries sustained to the thymus of immunocompromised mice (Fig. 2D).

Effect of MCP on DTH in mice. The degree of DTH (Table III) in the MG mice was significantly decreased compared with that in the NG mice ($P<0.05$). The DTH in PG mice and those

Table III. Effect of MPC on splenic lymphocyte proliferation, DTH and phagocytosis of macrophages in immunosuppressive mice.

Group	DTH, mg	Lymphocyte proliferation index	Phagocytic capacity of macrophages
Normal saline	5.35±0.09	0.87±0.06	0.73±0.04
Model	2.19±0.65[a]	0.44±0.02[a]	0.42±0.01[a]
Positive	4.15±0.27[b]	0.79±0.06[b]	0.65±0.02[b]
MCP low-dose	2.85±0.35	0.49±0.04[c]	0.47±0.04[d]
MCP medium-dose	3.54±0.17[b]	0.57±0.05[b,d]	0.56±0.08[e]
MCP high-dose	4.76±0.33[b]	0.69±0.06[e]	0.61±0.07[b]

Different lower-case letters show statistical significance (P<0.05). DTH, delayed-type hypersensitivity; MCP, *Momordica charantia* polysaccharide. [a]P<0.05 vs. normal saline; [b]P<0.05 vs. model; [c]P<0.05 vs. MCP medium-dose; [d]P<0.05 vs. positive; [e]P<0.05 vs. MCP low-dose.

Table IV. Effect of MPC on the secretion of IFN-γ, IL-6 and IL-12 by lymphocytes.

Group	IFN-γ, pg/ml	IL-6, pg/ml	IL-12, pg/ml
Normal saline	693.81±0.02	112.13±0.03	104.96±1.51
Model	506.88±0.03[a]	77.31±0.03[a]	78.66±0.03[a]
Positive	615.15±0.04[b]	104.42±0.05[c]	99.24±0.02[c]
MCP low-dose	559.87±0.00[d]	84.40±0.04[d]	86.22±0.05[e]
MCP medium-dose	585.83±0.03[e]	92.53±0.02[e]	92.88±0.06[b]
MCP high-dose	647.08±0.00[c]	99.23±0.03[b]	97.08±0.01[c]

Different lower-case letters show statistical significance (P<0.05). MCP, *Momordica charantia* polysaccharide. [a]P<0.05 vs. normal saline; [b]P<0.05 vs. MCP low-dose; [c]P<0.05 vs. model; [d]P<0.05 vs. MCP medium-dose; [e]P<0.05 vs. positive.

administered with MCP at concentrations of 200 or 300 mg/kg (MMG and MHG) was significantly increased compared with that in MG mice (P<0.05). The difference in DTH resulting from treatment with MCP at a concentration of 100 mg/kg (MLG) was smaller compared with that in the MMG and MHG groups, but remained significant compared with that in the MG group (P<0.05).

Effect of MCP on splenic lymphocyte proliferation in immunosuppressive mice. The effect of MCP on the proliferation of splenic lymphocytes show that CY treatment in MG mice significantly reduced lymphocyte proliferation compared with that in NG mice (P<0.05; Table III). The proliferation of splenic lymphocytes in the MCP treatment groups (MLG, MMG and MHG) appeared to be dose-dependent (Table III). The proliferation of splenic lymphocytes in the PG, MMG and MHG mice was significantly increased compared with that in the MG mice (P<0.05; Table III). These data suggest that MCP can effectively promote the proliferation of spleen lymphocytes in immunosuppressive mice.

Effect of MCP on cell phagocytosis of mice macrophages. As shown in Table III, the phagocytic activity of peritoneal macrophages in MG mice was significantly decreased compared with that in NG mice (P<0.05), suggesting that CY treatment reduced the activity of phagocytotic macrophages in mice. The phagocytic activity of peritoneal macrophages in the PG, MMG and MHG mice was significantly higher compared with that in the MG mice (P<0.05; the activity of phagocytotic macrophages). By contrast, cell phagocytosis of mice in the MLG group was also markedly enhanced compared with that of MG mice, although these changes were found to be not significant.

Effect of MCP on the lymphokine content in splenic lymphocytes of mice. The effects of MCP on the levels of the cytokines IFN-γ, IL-6 and IL-12 in immunosuppressed mice are shown in Table IV. In MG mice, the levels of the helper T (Th) cell type 1 cytokines IL-12 and IFN-γ and the Th2 cytokine IL-6 were significantly lower compared with those in the NG mice (P<0.05). Compared with those in the MG mice, the levels of IL-12 and IFN-γ in the PG, MLG, MMG and MHG mice were significantly higher (P<0.05; Table IV). The levels of IL-6 in MMG and MHG mice were significantly increased (P<0.05), whereas those in MLG mice was also significantly increased but to a lesser extent (P<0.05; Table IV), compared with those in the MG group. As the MCP dose increased, the levels of secreted IFN-γ, IL-6 and IL-12 were also increased.

Effects of MCP on the mRNA expression levels of cytokines in splenic lymphocytes. The present study next examined the mRNA expression levels of IFN-γ, IL-6 and IL-12 after MCP was administered at different concentrations (Fig. 3). The mRNA expression levels of IFN-γ, IL-6 and IL-12 in the spleen

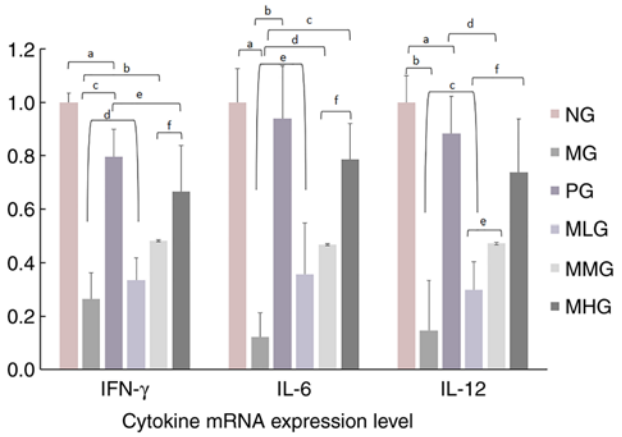

Figure 3. Effect of MCP treatment on the mRNA expression level of IFN-γ, IL-6 and IL-12. Different lower-case letters showed statistical significance (P<0.05). MCP, *Momordica charantia* polysaccharide; NG, normal saline group; MG, model group; PG, positive group; MLG, MCP low-dose group; MMG, MCP medium-dose group; MHG, MCP high-dose group.

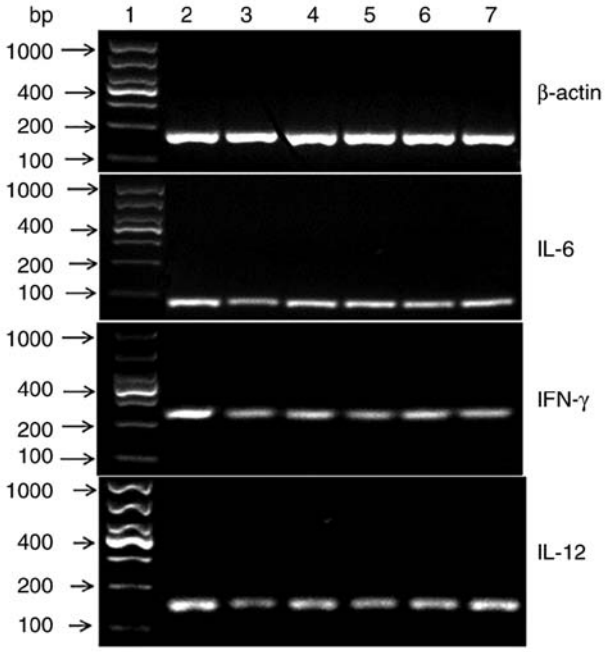

Figure 4. Results of RT-PCR reaction for the expression of IFN-γ, IL-6 and IL-12 mRNA. A representative image of a gel electrophoresis used to visualize the results of RT-PCR measuring the expression of IFN-γ, IL-6 and IL-12 mRNA in the six experimental groups. MCP, *Momordica charantia* polysaccharide; NG, normal saline group; MG, model group; PG, positive group; MLG, MCP low-dose group; MMG, MCP medium-dose group; MHG, MCP high-dose group; RT, reverse transcription; 1, Marker; 2, NG; 3, MG; 4, PG; 5, MLG; 6, MMG; 7, MHG.

lymphocytes of MG mice were significantly lower compared with those in the spleen lymphocytes of NG mice (P<0.05). Fig. 4 also shows that the expression levels of the cytokines IFN-γ, IL-6 and IL-12 in the spleens of MG mice were markedly lower compared with those in the NG group. After treatment with different concentrations of MCP, the mRNA expression levels of IFN-γ, IL-6 and IL-12 were observed to increase by varying degrees. The mRNA expression levels of IFN-γ, IL-6 and IL-12 in the PG and MCP dosage groups were significantly higher compared with those in MG mice (P<0.05; Fig. 3). In addition, the mRNA expression levels of IFN-γ, IL-6 and IL-12 showed a certain dose-effect relationship with the dose of MCP used. Fig. 5 shows the results of the cell viability test measured with lymphocytes and macrophages at 570 nm. Although the results obtained with the MHG and PG mice were comparable, they did not fully return to normal levels. Finally, the cell viability of CY-immunosuppressed mice was found to be increased in the MLG mice. Compared with that in the NG mice, the lymphocyte viability of mice in the MG group was significantly decreased (P<0.05; Table II). Compared with the MG mice, the lymphocyte viability was significantly increased in the PG, MHG, MMG and MLG groups (P<0.05; Table II). These data suggested that the lymphocyte viability of the immunosuppressed mice increased in response to MCP in a dose-dependent manner, although it remained lower compared with that of the NG mice (Fig. 5A). Compared with that in the NG group, the macrophage viability of mice in the MG group was significantly decreased (P<0.05; Table II). Compared with the MG mice, the macrophage viability was significantly increased in the PG, MHG, MMG and MLG groups (P<0.05; Table II). These data suggested that the macrophage viability of the immunosuppressed mice increased in response to MCP in a dose-dependent manner (Fig. 5B).

Discussion

CY is a potent alkylating agent that is widely used in clinical chemotherapy (19,20). It has been previously shown that it can inhibit humoral and cellular immune responses of animals, resulting in immunosuppression (19). The dose and time of CY administration are key factors that can determine whether the model of hypoimmunity can be successfully established (20). In the present study, normal mice were injected intraperitoneally with CY at a concentration of 80 mg/kg from days 1 to 5, rendering this a suitable method for establishing a long-term immunosuppressive model.

The main function of the immune system is to identify non-self substances for removal from the body (21,22). All cells that perform this function belong to the immune system (21,22). Immune organs include bone marrow, thymus, spleen and lymph nodes, whereas immune cells include lymphocytes, monocytes, macrophages and natural killer (NK) cells (21-23). In addition, immune molecules include antibodies, complement proteins and cytokines (23).

The spleen and thymus are crucial immune organs of the body, since they form sites where immune cells proliferate (24). Activity in the immune organs can directly regulate downstream immune function and the ability to resist various diseases, which can be measured using indices of immune organs. It has been shown that polysaccharides can significantly increase the index of immune organs and inhibit their atrophy in mice (9,24-26). In the present study, the organ indices of MG mice was significantly lowered compared with that of NG mice, suggesting that CY can significantly reduce the weight of the thymus and spleen of mice. This also suggests that the immunosuppressive mice model was successfully established. Compared with that in the MG mice, the organ indices of mice in each dose group of MCP (MLG, MMG and MHG) was significantly higher, suggesting that MCP treatment

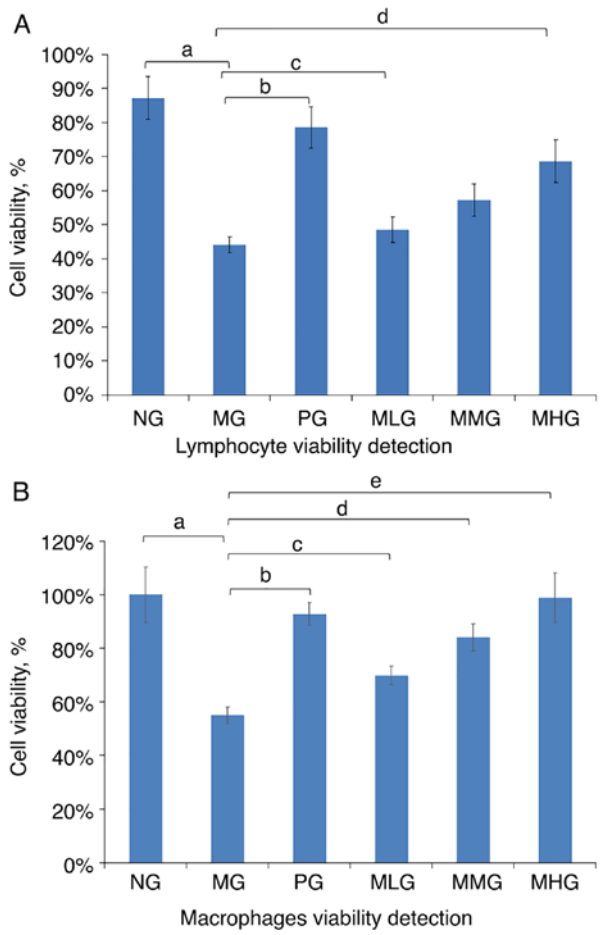

Figure 5. Cell viability assay. (A) Lymphocyte and (B) macrophage viability. Different lower-case letters showed statistical significance (P<0.05). MCP, MCP, *Momordica charantia* polysaccharide; NG, normal saline group; MG, model group; PG, positive group; MLG, MCP low-dose group; MMG, MCP medium-dose group; MHG, MCP high-dose group.

could correct atrophy of the thymus and spleen caused by CY. However, compared with NG mice, differences remain in the MCP treatment groups, where the detrimental effects of CY could not be completely reversed. This finding was consistent with the results obtained by Chao *et al* (25), who studied the immunological effects of *Cheonggukjang* polysaccharides and those of Mei *et al* (9), who studied the protective effects of chitosan oligosaccharide on CY-induced immunosuppression in mice.

The measurement of DTH reflects the strength of cellular immunity (26). Therefore, the degree of DHT can be used as an indicator of the health of cellular immune function in mice. A previous study reported that treatment with *Chaenomeles speciose* polysaccharides (CSP) can support a possible role of CSP in assisting the cell-mediated immune response in the spleen (27). The present results showed that the degree of DTH in the immunosuppressed mice was improved following MCP treatment, suggesting that MCP was able to effectively enhance DTH.

Immune function can be divided into specific immunity and non-specific immunity (28,29). Lymphocytes can reflect the state of specific immunity, whereas the ability of phagocytosis by macrophages may be applied to reflect the strength of non-specific immunity (28). Peritoneal macrophages have a potent capacity to engulf foreign bodies and fulfil an important role in immune surveillance (28). By contrast, lymphocytes form the core components of the immune system and serve as one of the most important immune cell types in the body (28). The proliferation and differentiation of lymphocytes are the most important stage of the immune response (28). Since the spleen contains a large number of lymphocytes, splenic lymphocyte proliferation tests provide an important method to test the vitality of lymphocytes (28). A previous study on the effects of *Rehmannia glutinosa* polysaccharide (RGP) on the immune function in mice showed that the administration of RGP increased the population of sheep red blood cells, induced DTH and led to an increase in the spleen and thymus indices (29). In addition, treatment with RGP led to increases in both splenic lymphocyte proliferation and in the level of peritoneal macrophage phagocytosis in a dose-dependent manner. These results indicated that RGP can enhance the cellular immune response in mice (29). The present study also demonstrated that the proliferation rate of spleen lymphocytes in immunosuppressed MG mice was significantly decreased. Compared with that in the MG mice, the proliferation index of spleen lymphocytes in mice treated with different concentrations of MCP was increased significantly, suggesting that MCP could effectively stimulate the proliferation of spleen lymphocytes in mice whilst enhancing the specific immune function of immunosuppressed mice.

Another previously published study (30) evaluated the possible immune activity of *Cordyceps militaris* polysaccharides (CMP) *in vivo* and found that the administration of CMP was able to overcome CY-induced immunosuppression, led to an increase in the spleen and thymus indices and enhanced both the spleen lymphocyte activity and macrophage function. In addition, Chen *et al* (31) previously evaluated the regulatory effects of *Potentilla anserina* polysaccharide (PAP) by using mice peritoneal macrophages and CY induction as an immunosuppressive model. They demonstrated that PAP could upregulate phagocytosis by phagocytes and also cause spleen cell proliferation, leading to increases in both the TI, SI, serum IL-10 and IFN-γ levels of immunosuppressive mice, thereby exerting an immunoregulatory role (31). In the present study, the level of phagocytosis by macrophages in MG mice was shown to be significantly reduced, whereas the phagocytosis index of immunosuppressed mice was significantly increased after treatment with MCP (MLG, MMG and MHG) and in the PG mice. Therefore, these observations suggest that MCP can effectively enhance the level of phagocytosis by macrophages and the non-specific immune function of immunosuppressed mice.

Cytokines are proteins or small-molecule polypeptides synthesized by stimulating immune cells (such as mononuclear phagocytes, T, B and NK cells) and certain non-immune cells (such as vascular endothelial cells, epidermal cells and fibroblasts), which can transmit information among cells to regulate immune regulation (32). The profile of cytokines reflects the state of both the immune system and the nutritional metabolic status of the body (32,33). Th cells can be mainly divided into the Th1 and Th2 cell subsets according to their different cytokine production types and biological functions (32,33). Th1 cell subsets mainly secrete inflammatory cytokines, such as IL-2, IFN-γ and IL-12, which mediate cytotoxicity and delay hypersensitivity inflammation, participate in the cellular immune

response and have an important role in resisting intracellular pathogen infections (32,33). By contrast, Th2 cells mainly secrete anti-inflammatory cytokines, such as IL-4, IL-5, IL-6 and IL-10, which can promote B cells into differentiating into plasma cells and produce antibodies (32,33). Th2 responses are typically associated with humoral immunity (32,33). It was previously reported that *Astragalus* polysaccharide (APS) liposome (APSL) could significantly promote lymphocyte proliferation, enhance the antibody titer and promote the secretion of IFN-γ, IL-2, IL-4 and IL-10 (32,33). In addition, medium dosage was shown to possess optimal efficacy, suggesting that APSL could significantly improve the adjuvanticity and drug action of APS (34,35).

In the present study, it was found that the levels of the Th1-type cytokines, IL-12 and IFN-γ, in addition to the Th2 cytokine IL-6 secreted by spleen lymphocytes, were decreased significantly in MG mice. By contrast, the levels of IFN-γ, IL-6 and IL-12 secreted in the MCP treatment groups were increased in a dose-dependent manner. These results suggest that MCP can promote lymphocyte proliferation, increase cytokine levels and enhance cellular immunity in immunosuppressed mice, implying that MCP exerts an immunomodulatory role.

Interleukins comprise a group of cytokines that can transmit information among immune cells. IL-6 is a pleiotropic cytokine, which can participate in the immune defence of the body (35,36). IL-6 can induce lymphocyte proliferation and the production of IL-12 and TFN-α, thereby mediating antiviral effects (36). IL-4 is a cytokine that is produced by mononuclear macrophages and endothelial cells (36,37). IL-4 has been reported to be involved in a series of processes, such as inflammation, the immune response and stress (36). In addition, it can participate in the occurrence and development of diseases, such as breast cancer and asthma (37). IFN-γ is a highly active and multifunctional cytokine that is secreted by Th1 cells, which can enhance both the activity of Th1 cells, the overall cellular immune function and antiviral effects (38). Furthermore, it can inhibit the proliferation of Th2 cells, thereby inhibiting humoral immune function (38). IL-12 is another type of cytokine and mainly targets T-cells, NK cells and bone marrow progenitor cells (39,40). IL-12 can promote the proliferation of activated T and NK cells, enhance their cytotoxicity and promote the production of IFN-γ and TNF-β (39,40). IL12 serves an important role in the balance of the Th1/Th2 response (39,40). IL-12 can induce Th0 cells into differentiating into Th1 cells, thereby exerting an important role in cell-mediated immunity (39,40). A previous study showed that polysaccharides from *Physalis alkekengi* can improve the state of immunity by stimulating the expression of IL-4 and IFN-γ mRNA in chicken spleen lymphocytes (41). Wang *et al* (42) demonstrated that polysaccharide isolated from *Kadasura marmorata* can improve the immune activity of the body by promoting the secretion of IL-2, IFN-γ and TNF-α by spleen lymphocytes *in vitro*.

In the present study, RT-qPCR was used to determine the relative expression levels of IFN-γ, IL-6 and IL-12 in the mouse spleen cells. The results showed that the mRNA expression levels of IFN-γ, IL-6 and IL-12 in the spleen lymphocytes of MG mice were significantly decreased. Upon treating the cells with different concentrations of MCP, the mRNA expression levels of IFN-γ, IL-6 and IL-12 were all significantly increased in a dose-dependent manner. Based on these results, it is possible that MCP can promote the secretion of both Th1 and Th2 cytokines by mice spleen lymphocytes and increase their mRNA expression levels, thereby fulfilling a dual immunomodulatory role.

In conclusion, to investigate the role of MCP on the immunological activity of mice, six groups of samples were organized by assigning 10 male BALB/c mice aged 6-8-week-old into each group. An immunosuppressive model was established by the intraperitoneal injection of CY in all groups, except for NG. The different groups of mice were then administered distilled water, levamisole (200 mg/kg) and different doses of MCP (100, 200 or 300 mg/kg). The organ index was measured, the tissue sections of immune organs were imaged, the degree of mouse ear swelling model was established, the delayed allergic reaction was measured, the phagocytic function of the peritoneal macrophages was measured using the neutral red phagocytosis test, lymphocyte proliferation was measured using the MTT method, the spleen lymphocyte factors of IFN-γ, IL-6 and IL-12 were measured by the ELISA method and the mRNA expression levels of cytokineassociated genes were analysed using RT-qPCR.

Collectively, the present results showed that MCP could enhance immune function and improve the TI and SI in immunosuppressed mice. MCP could also repair the damage caused by CY to the immune organs. In summary, MCP was shown to enhance the DTH and cell phagocytosis by abdominal macrophages, in addition to stimulating the proliferation of splenic lymphocytes, induce the secretion of the cytokines IL-6, IFN-γ and IL-12 and upregulate their mRNA expression levels. The present study demonstrated, through the establishment of an immunosuppressed mice model, that the daily intake of MCP can lead to a reversal of the decline of immune function in mice treated with CY. This may provide a basis for the further research and development of MCP as an effective immunopotentiator.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AA and YY conceived and designed the experiments. AA performed the animal experiments. AB, YY and XJ performed the experiments. AA, YY and JSL analyzed the data. AA and

YY confirm the authenticity of all the raw data. AA wrote the first manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Jinzhou Medical University Ethics Committee [production license SCXY(Liao) 2019-0003; approval no. 2019014].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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