The Institute of Medicine[1], Qiqihar Medical College, Qiqihar; Postdoctoral Research Center[2], Harbin Medical University, Harbin, China

Immunostimulatory activities of a low molecular weight antitumoral polysaccharide isolated from *Agaricus blazei* Murill (LMPAB) in Sarcoma 180 ascitic tumor-bearing mice

YING-CAI NIU [1], JI-CHENG LIU[1,2], XUE-MEI ZHAO[1], FU-QIN SU[1], HONG-XIA CUI[1]

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Ji-Cheng Liu, 249 Heping Road, Fula'erji District, The Institute of Medicine, Qiqihar Medical College, Qiqihar, 161042, China
qyybliu@126.com

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LMPAB is a linear β-(1–3)-glucan we isolated from polysaccharide extract of *Agaricus blazei* Murill (AbM). Effects of LMPAB on splenic natural killer (NK) cell activity, splenocyte proliferation, index of spleen and thymus, IFN-γ expression in spleen and the concentration of IL-12, IL-18 and TNF-α in serum of S180 ascitic tumor-bearing mice were detected. The results showed that intraperitoneal injection of LMPAB (100 mg · kg^{-1} · d^{-1}) significantly increased the thymus index. LMPAB augmented splenic NK cell activity in a dose-dependent manner (50–200 mg · kg^{-1} · d^{-1}). The concanavalin A (3 μg/ml) stimulated splenocyte proliferation was significantly enhanced by LMPAB at dosages of 50, 100 or 200 mg · kg^{-1} · d^{-1}. Further studies showed that LMPAB (50, 100 or 200 mg · kg^{-1} · d^{-1}, 14d) significantly increased the production of IL-12, TNF-α, IL-18 and the expression IFN-γ as determined by ELISA and immunohistochemistry, respectively. These results clearly indicate that the anti-tumor effects of LMPAB are closely associated with up-regulation of activity of NK cells, expression of IFN-γ in spleen and the systemic level of IL-12, IL-18 and TNF-α in tumor-bearing mice.

1. Introduction

Mushrooms have been considered as an important functional food and a source for the development of drugs. Mushroom extracts are commonly described as having immunological, hypocholesterlemic, antiviral, antibacterial, anti-carcinogenic and antiparasitic activities (Manzi et al. 1999; Mattila et al. 2000; Wasser and Weis 1999). An interesting example is *Agaricus blazei* Murill. (AbM), a member of the Bacidiomycetes family, located in a mountain region near Sao Paulo of Brazil and now produced on an industrial scale in some countries like China, Japan and Brazil. AbM has been traditionally used as a medicine against a variety of diseases, including diabetes, arteriosclerosis, hepatitis and cancer (Takaku et al. 2001; Huang 1997).

AbM is considered to have strong antitumoral and immunoactive effects (Kawamura et al. 2005; Kuo et al. 2002; Nakajima et al. 2002; Shimizu et al. 2002). Chemical components of AbM, including steroids, lectins, and various polysaccharides (Takaku et al. 2001; Ohno et al. 2001), have been widely studied. It has been reported that the aquoneus extract of AbM has antitumor activity in tumor-bearing mice. From the water soluble residues of AbM, a protein-(1–6)-β-D-glucan complex, was isolated. This protein-polysaccharide complex is characterized by developing immunomodulatory activities in fibrosarcoma bearing mice and inhibiting the growth of sarcoma 180 (S180) implanted in mice (Ebina and Fujimiya 1998), possibly due to immunological mechanisms involving the action of various immunocompetent cells (Ebina et al. 1998; Fujimiya et al. 1999). Polysaccharides, particularly rich in ABM, have shown strong efficacy in treating and preventing cancers. Fujimiya et al. (1998) reported that AbM fractions containing (1–4)-α-D-glucan and (1–6)-β-D-glucan components showed antitumoral activity against Meth A fibrosarcoma through the activation of specific tumor-infiltrating lymphocytes (TIL).

AbM has been found to be rich in immunomodulating polysaccharides such as β-glucans. Recently, we successfully isolated a low molecule polysaccharide from polysaccharide extract of AbM (LMPAB), which is a linear β-(1–3)-glucan (data submitted for publication). LMPAB is a homogeneous pure polysaccharide composed of only glucose, whose molecular weight was 48,000 KDa. In a previous study, we reported that LMPAB inhibited the proliferation of leukemia cell line K562, the growth of vascular endothelial cell and invasion and metastasis of BEL-7402 cells (Data submitted for publication). It is believed that polysaccharides from mushrooms do not attack cancer cells directly, but exert their antitumor effects by activating different immune responses in the host (Wasser 2002). To clarify the mechanisms of antitumoral activities of LMPAB, we investigated the effects of LMPAB on cellular immune responses. In this study, we demonstrated that LMPAB enhanced the splenic natural killer (NK) cell activity, splenocyte proliferation, indexes of spleen and thymus, and antitumor cytokines production in tumor-bearing mice.

Table 1: Effect of LMPAB on spleen index and thymus index in S180 tumor bearing mice

Group	Dosage ($mg \cdot kg^{-1} \cdot d^{-1}$)	Index of spleen (mg/10g, Mean ± SD)	Index of thymus (mg/10g, Mean ± SD)
Normal control (n = 12)	0	87.18 ± 12.92	21.89 ± 6.89
Model control (n = 12)	0	59.02 ± 22.54‡	17.78 ± 4.23‡
FUP (n = 12)	5	61.15 ± 16.12‡	14.58 ± 4.53‡
AbMP (n = 12)	1000	61.15 ± 23.46‡	19.67 ± 5.30∫
LMPAB (low dose, n = 12)	50	70.16 ± 19.81†	20.71 ± 3.15§
LMPAB (middle dose, n = 12)	100	85.34 ± 12.28**§	22.80 ± 4.40*§
LMPAB (high dose, n = 12)	200	84.58 ± 18.83**§	20.26 ± 3.81§

FUP: fungus umbellatus polysaccharides; AbMP: *Agaricus blazei* Murill. polysaccharides.
†, P< 0.05, ‡, P < 0.01 versus the normal control group;
*, P < 0.05, **, P < 0.01 versus the model control group; and
∫, P < 0.05, §, P < 0.01 versus FUP group

2. Investigations and results

2.1. Indexes of spleen and thymus

Table 1 shows the changes in the thymus and spleen indexes in each group on the 14th day after the tumor was implanted. Intraperitoneal injection of LMPAB significantly increased the thymus and spleen indexes (both $P < 0.05$) compared to the model control group, while administration of fungus umbellatus polysaccharide did not show any effect on the indexes.

2.2. Effect of LMPAB on NK cell activity

LMPAB was administered i.p. daily in mice for 14 days, and the NK activity was examined one day after the final dose using the splenic lymphocytes as the effector cells and YAC-1 cells as the target cells. It was demonstrated that LMPAB significantly enhanced the splenic NK cell activity in a dose-dependent manner (Table 2), indicating that LMPAB activates NK cells *in vivo*.

Table 2: Effect of LMPAB on NK cell activity in S180 tumor bearing mice

Group	Dosage ($mg \cdot kg^{-1} \cdot d^{-1}$)	NK cell activity (%) (mean ± SD)
Normal control (n = 12)	0	36.32 ± 5.92
Model control (n = 12)	0	23.10 ± 3.12‡
FUP (n = 12)	5	27.13 ± 4.99
AbMP (n = 12)	1000	38.48 ± 7.91**∫
LMPAB (low dose, n = 12)	50	32.27 ± 3.06**∫
LMPAB (middle dose, n = 12)	100	37.35 ± 5.64**§
LMPAB (high dose, n = 12)	200	39.12 ± 3.84**§

FUP: fungus umbellatus polysaccharides; AbMP: *Agaricus blazei* Murill. polysaccharides.
‡, P < 0.01 versus the normal control group;
*, P < 0.05, **, P < 0.01 versus the model group; and
∫, P < 0.05, §, P < 0.01 versus FUP group

2.3. Effect of LMPAB on splenocyte proliferation

To understand the immunomodulatory activity of LMPAB, we investigated its effect on the proliferation of splenocytes. It was observed that the Con-A (3 μg/ml) stimulated splenocyte proliferation was significantly enhanced by LMPAB at doses of 5, 10 or 20 mg/kg (Table 3).

2.4. Effect of LMPAB on the expression level of IFN-γ in mice spleen

LMPAB treatment increased the expression of IFN-γ in the spleen of tumor-bearing mice. In FUP and AbMP treated groups, IFN-γ was elevated from its model level (1.746 ± 0.266) to 2.327 ± 0.170 and $2.823 \pm 0.0.316$ (Table 4). But the IFN-γ expression level in the LMPAB-treated group was not as high as fungus umbellatus polysaccharides and AbM polysaccharides control group.

Table 4: Effect of LMPAB on spleen IFN-γ expression in S180 tumor bearing mice

Group	Dosage ($mg \cdot kg^{-1} \cdot d^{-1}$)	Index of IFN-γ expression ($\times 10^3$) (mean ± SD)
Model control (n = 6)	0	1.746 ± 0.266
FUP (n = 6)	5	2.327 ± 0.170**
AbMP (n = 6)	1000	2.823 ± 0.316**§
LMPAB (low dose, n = 6)	50	1.818 ± 0.222§
LMPAB (middle dose, n = 6)	100	1.971 ± 0.130§
LMPAB (high dose, n = 6)	200	2.230 ± 0.068**

FUP: fungus umbellatus polysaccharides; AbMP: *Agaricus blazei* Murill. polysaccharides.
**, P < 0.01 versus the model control group;
§, P < 0.01 versus FUP group

Table 3: Effect of LMPAB on Con A-stimulated splenocyte proliferation

Group	Con A ($\mu g \cdot ml^{-1}$)	Absorbance (570 nm) (Mean ± SD)	Average stimulation index
Normal control (n = 12)	0	0.331 ± 0.020	–
Model control (n = 12)	3	0.462 ± 0.067‡	1.40
FUP (5 $\mu g \cdot ml^{-1}$, n = 12)	3	0.498 ± 0.032‡	1.50
AbMP (1000 $\mu g \cdot ml^{-1}$, n = 12)	3	0.579 ± 0.033‡**§	1.75
LMPAB (50, $\mu g \cdot ml^{-1}$, n = 12)	3	0.541 ± 0.036‡**	1.64
LMPAB (100, $\mu g \cdot ml^{-1}$, n = 12)	3	0.557 ± 0.037‡**	1.68
LMPAB (200, $\mu g \cdot ml^{-1}$, n = 12)	3	0.515 ± 0.028‡*	1.55

FUP: fungus umbellatus polysaccharides; AbMP: Agaricus blazei Murill. Polysaccharides.
†, P< 0.05, †, P < 0.01 versus control group;
*, P < 0.05, **, P <0.01 versus model group; and
†, P < 0.05, †, P <0.01 versus FUP group

Table 5: Effect of LMPAB on serum IL-12, IL-18 and TNF-α concentration in S180 tumor bearing mice

Group	Dosage ($mg \cdot kg^{-1} \cdot d^{-1}$)	IL-12 ($pg \cdot ml^{-1}$) (mean ± S.D)	IL-18 ($pg \cdot ml^{-1}$) (mean ± S.D)	TNF-α ($pg \cdot ml^{-1}$) (mean ± SD)
Normal control (n = 12)	0	122.10 ± 17.00	163.92 ± 29.02	131.65 ± 38.66
Model control (n = 12)	0	90.39 ± 11.79‡	125.93 ± 10.05†	173.35 ± 18.88‡
FUP (n = 12)	5	130.48 ± 7.43**	158.53 ± 21.86**	174.59 ± 9.30‡
AbMP (n = 12)	1000	124.51 ± 16.98**	207.62 ± 21.9†**§	187.62 ± 19.91‡
LMPAB (low dose, n = 12)	50	101.94 ± 6.68§	163.76 ± 9.54**	173.23 ± 14.01‡
LMPAB (middle dose, n = 12)	100	155.34 ± 18.58‡*	170.92 ± 15.22**	192.73 ± 28.12‡*
LMPAB (high dose, n = 12)	200	148.68 ± 16.01†*	207.71 ± 27.96†**	199.08 ± 20.40‡*

†, $P < 0.05$, ‡, $P < 0.01$ versus the normal control group;
*, $P < 0.05$, **, $P < 0.01$ versus the model control group; and
∫, $P < 0.05$, §, $P < 0.01$ versus FUP group

2.5. Effect of LMPAB on serum levels of IL-12, IL-18 and TNF-α in tumor bearing mice

On the 14th day, the level of TNF-α in tumor bearing mice without any treatment was highly elevated, compared with the level in the normal control group (173.35 ± 18.88 pg/ml vs. 131.65 ± 38.66 pg/ml ($P < 0.0001$). The level was significantly increased to 199.08 ± 20.40 pg/ml in tumor bearing mice with treatment of LMPAB ($200 mg \cdot kg^{-1} \cdot d^{-1}$) ($P < 0.05$) (Table 5). The levels of IL-12 and IL-18 were decreased in tumor bearing mice without any treatment, compared with the levels in normal control mice (Table 5). However, the treatment of LMPAB significantly increased the levels of IL-12 and IL-18 ($P < 0.0001$) in a dose dependent fashion (from 50 to 200 $mg \cdot kg^{-1} \cdot d^{-1}$) in tumor bearing mice (Table 5).

3. Discussion

Extensive studies on Basidiomycetes have shown that mushrooms including AbM has strong antitumor effects (Wasser and Weis 1999; Jong et al. 1989). Glucans, which are an integral part of the cell wall of mushrooms, exert their antitumoral effect, probably mainly by stimulating monocytes and macrophages of the innate immune system (Bøgwald et al. 1984). The hot water extract of AbM has potent antitumoral activity in sarcoma 180-bearing mice (Kawagishi et al. 1990; Fujimiya et al. 1998), and the antitumoral substance has been postulated to be a mixture of (1, 3)-, (1–4)- and (1–6)-D-glucans.

Previously, we isolated an antitumor β-(1–3)-glucan, LMPAB, from the polysaccharides extract of AbM (unpublished data). We observed its antitumoral activities of LMPAB via both *in vitro* and *in vivo* pharmacological models (unpublished data). LMPAB had an inhibitory effect against metastasis of cells of a human liver tumor cell line, Bel-7402, *in vitro*. LMPAB also inhibited B16 melanoma metastasis in mice (unpublished data). Furthermore, inhibitory effects of LMPAB on the tumor growth and metastasis were accompanied by the inhibition of tumor-induced neovascularization (unpublished data).

A large number of plants used in traditional medicines have been shown to possess nonspecific stimulating activities on immune responses (Sriwanthana and Chavalittumrong 2001). It is believed that polysaccharides from mushrooms do not attack cancer cells directly, but exert their antitumoral effects by activating different immune responses in the host. Therefore, the main objective of this study was to characterize the immunomodulatory activities of LMPAB. As a first step, the effect of LMPAB on mouse thymus index, spleen index and splenocyte proliferation were dertermined in tumor-bearing mice. LMPAB was found to significantly increase the thymus index, spleen index and viability of spleen cells, which demonstrates that LMPAB, derived from the polysaccharide extract of AbM, possesses a potent immunostimulant activity.

Activation of lymphocytes and NK cells, as well as the release of some cytokines is of great significance in the effective control of tumor progression and metastasis. Studies in humans have found a positive correlation between levels of NK activity and resistance to malignancy (Taketomi et al. 1998; Coca et al. 1997). Animal studies have also indicated that NK cells are particularly important in controlling metastatic development, and NK cells are sources of potent immunostimulatory cytokines such as TNF-α and IFN-γ (Robertson and Ritz 2000). IL-12 is an antigen presenting cell-derived cytokine that stimulates T cells and NK cells to secrete IFN-γ and augments the proliferation and cytolytic activity of these cells (Gately et al. 1998). IFN-γ is a proinflammatory cytokine with pleiotropic functions (Schroder et al. 2004). A role for IFN-γ in the host defense against tumors has been identified (Blankenstein and Qin 2003). IFN-γ was initially considered to be produced solely by cells of lymphoid origin, particularly NK cells and T cells. Munder et al. (1998) described that bone marrow-derived macrophages could be stimulated with IL-12 and IL-18 to produce IFN-γ. TNF-α is one of the cytokines released by activated macrophages, NK cells and T-lymphocytes (Mocellin et al. 2005). An adequate dose of TNF-α can induce death receptor-mediated apoptosis of tumors (Leist and Jaattela 2001). Our results showed that treatment with LMPAB enhanced the splenic NK cell activity and the release of IL-12, IL-18, IFN-γ and TNF-α in S180 tumor bearing mice in a dose-dependent manner, indicating that the inhibition on tumor growth and metastasis by LMPAB is at least partly attributed to its potent immunostimulatory activities.

In conclusion, LMPAB derived from AbM is an active polysaccharide that exerts a variety of immunostimulatory activities including up-regulation of activity of NK cells, splenocyte expression of IFN-γ and serum levels of IL-12, IL-18 and TNF-α, which are believed to contribute to its antitumoral effects such as inhibition of tumor growth, neovascularization and metastasis.

4. Experimental

4.1. Reagents

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Unless otherwise stated, all other reagents were obtained from Sigma (St. Louis, MO, USA). Highly specific ELISA Kits for IL-12, IL-18, TNF-α and IFN-γ were purchased from Pierce Biotechnology.

AbM (Zhejiang Borui Pharmaceuticals Co., Ltd, Qingyuan, China) was used for the isolation of LMPAB as previously described (Liu and Sun 2006). The molecular weight and purity of LMPAB was 48,000 kDa and

98.6%, respectively, as determined by high-performance liquid chromatography-gel permeation chromatography and gel chromatography (Sephadex G-100). Sugar composition analysis showed that the polysaccharide was composed of β-(1−3)-glucan. The contamination of proteins was excluded according to the absorption at 260 and 280 nm respectively. LMPAB was dissolved in PBS (pH 7.2) at a concentration of 3 g/L prior to each experiment.

4.2. Animals, cell culture and cancer cell line transplantation

Pathogen-free 6 weeks old female/male BALB/c mice were purchased from the Beijing Vital River Laboratories (Beijing, China). The animals were maintained in our animal laboratory with SPF standards. The animal room was kept on a 12 h light/dark cycle at 20 ± 2 °C and 50% humidity. The animals were provided with sterilized food and water. They were applied to our experiment after they had been fed for one week since they were purchased.

Then, 10^6 cells of mouse malignant S180 cells, which were provided by Institute of Cancer Research, Heilongjiang Cancer Hospital, Harbin, and passaged three times in the abdominal cavity of the mice, were inoculated i.p. into 72 BALB/c mice in a volume of 0.1 ml. These model animals were randomly divided into 6 groups with 12 (6 males and 6 females) in each group. These included three groups treated with LMPAB at different dosages (50, 100, or 200 $mg \cdot kg^{-1} \cdot d^{-1}$), one group treated with fungus umbellatus polysaccharides (FUP; 5 mg/kg), one group treated with *Agaricus blazei* Murill. polysaccharides (AbMP; 1000 mg/kg), and one model control group treated with 0.9% normal saline. LMPAB, FUP, AbMP or normal saline was given *i.p.* daily for 14 consecutive days starting on the day of S180 inoculation. In addition, 12 mice (6 males and 6 females) without S180 inoculation and treatment served as normal controls.

The study protocol was approved by the Ethics Committee of Qiqihar Medical college. Cells of a mouse leukemia cell line, YAC-1, were cultured in RPMI1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in a 5% CO_2 humidified incubator.

4.3. Thymus index and spleen index

On the 14th day after treatment, all mice were weighted and sacrificed as described above, and the thymus gland and spleen from each mouse were excised and weighed to calculate the thymus index and spleen index. Both indexes were calculated as (organ weight/body weight) × 1000.

4.4. Preparation of effector cells for the NK cell activity assay

NK cell activity was evaluated 24 h after the last dose of LMPAB, PUP, AbMP or normal saline when BALB/c mice were sacrificed to obtain spleens. Single-cell suspension, obtained from the spleens, was collected and passed through a stainless-steel mesh net. Resultant mononuclear cells were isolated from the cell suspension by Ficoll-Hypaque (Sigma) gradient separation, washed and resuspended in enriched RPMI 1640 culture medium supplemented with 10% FBS. To remove adherent cells, the cell suspension was placed in a 150-mm tissue culture dish and incubated at 37 °C under 5% CO_2 for 90 min. Non-adherent cells were then harvested by gentle pipetting, and the cells were washed and the cell concentration was adjusted to 5×10^6 cells/ml (Justo et al. 2003).

4.5. Determination of natural killer cell activity by the MTT assay

The assay for cytotoxicity was determined by modification of the technique described previously (Flick and Gifford 1984). Briefly, isolated splenocytes (10^5 cells per well) were plated in a 96-well culture plate and co-incubated with YAC-1 cells (10^4 cells per well; an initial effector: target cell ratio of 10 : 1) in the presence of samples for 48 h at 37°C in a 5% CO_2 incubator. Cell density was then assessed by incubating the cells with 25 μg/ml MTT for another 4 h. Formazan produced was dissolved in DMSO and the optical density of each well was determined at 570 nm using a microplate reader. Cytolytic activity was expressed as the percentage of tumor cytotoxicity, where cytotoxicity (%) ={1- [O.D. of (effector cells + target cells)-O.D. of effector cells]/O.D. of target cells} × 100.

4.6. Determination of splenocyte proliferation by the MTT assay

BALB/c mice were sacrificed to obtain spleens. The abdominal cavities were opened and the spleens were removed under aseptic conditions. Spleen collected under aseptic conditions in Hank's buffered salt solution was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380 g at 4 °C for 5 min), the pelleted cells were washed three times with PBS and resuspended in complete RPMI 1640 medium supplemented with 10% FBS. Cell number was counted with a haemocytometer by the trypan blue dye exclusion technique. To evaluate the effect of LMPAB on the proliferation of splenic lymphocytes, spleen cell suspension (1×10^7 cell/ml) was pipetted into 96 well plates (200 μl/well) and cultured at 37 °C for 44 h in a humid saturated atmosphere containing 5% CO_2 in the presence of Con-A (3 μg/ml). After 44 h, 10 μl of MTT solution (5 mg/ml) were added to each well and incubated for 4 h. The plates were centrifuged (1,400 × g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100 μl of a DMSO working solution (192 μl DMSO with 8 μl 1 M HCl) was added and incubated for 10 min, and the absorbance was evaluated at 570 nm with a reference at 630 nm in a microplate reader.

4.7. Determination of IFN-γ expression in mouse spleens by immunohistochemistry

Small pieces of spleen tissue were fixed in 4% buffered, freshly prepared paraformaldehyde, embedded in paraffin, and stained for routine histology (i.e. hematoxylin-eosin staining). To detect IFN-γ, spleen specimens were stained for IFN-γ by using a monoclonal antibody (Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked with 0.1% NaN_3/0.3% (vol/vol) H_2O_2 dissolved in 5 mL dimethylformamide and 95 mL acetate buffer (pH 4.9, 50 mmol/L) and 0.03% (vol/vol) H_2O_2. As secondary antibody, horseradish peroxidase-conjugated rabbit antimouse immunoglobulin G (Dako) was used. Finally, slides were stained for horseradish peroxidase activity by incubating the sections with 5 mg/mL 3-amino-9-ethyl carbazole (Sigma) and 0.03% H_2O_2. Incubation without the primary antibody was performed as a control for the background staining. Five different fields in each 6 spleen section at magnification ×400 were analyzed by a digital medical image analysis system (Motic Med 6.0, Motic Med. CMIAS, Beijing, China). The rate of the positive staining for IFN-γ was expressed as expression index (EI) in 30 randomly selected fields. The intensity of the IFN-γ was graded as follows: 0 point for non-staining, 1 point for lower staining, 2 points for even staining, and 3 points for stronger staining. The average of each score of each group represented the IFN-γ EI, resulting in scores ranging from 0 to 3. Histological evaluation was performed by a pathologist who was blind to the pharmacological characteristics of the drugs and their postoperative outcomes.

4.8. Determination of serum IL-12, IL-18 and TNF-α levels by enzyme-linked immunosorbent assays

The levels of IL-12, IL-18 and TNF-α in the serum of mice were determined by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's recommended protocol.

4.9. Statistical analysis

Results are reported as means ± standard deviation (SD). ANOVA was used to evaluate the differences among multiple groups. The data were analyzed by SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). A P of less than 0.05 was considered as statistically significant.

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