Original Research

Immune regulation effect of lienal polypeptides extract in Lewis lung carcinoma-bearing mice treated with cyclophosphamide

Yan-Ping Wu[1,2], Jie Deng[1,2], Shu-Hua Ouyang[1,2], Zhong-Fu Mao[1,2], Guo-En Wang[1,2], Hiroshi Kurihara[1,2], Rong-Rong He[1,2] and Yi-Fang Li[1,2]

[1]Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou 510632, China; [2]Anti-Stress and Health Research Center, College of Pharmacy, Jinan University, Guangzhou 510632, China

Corresponding authors: Yi-Fang Li. Email: liyifang706@jnu.edu.cn; Rong-Rong He. Email: rongronghe@jnu.edu.cn

Impact statement

The immunomodulatory activities of polypeptides extracted from animal immune organs have incurred people's interests since a long time ago. In this study, we investigated the immune regulation effects of a polypeptide mixture extracted from health calf spleen (lienal polypeptide [LP]) in Lewis lung carcinoma-bearing mice treated with cyclophosphamide (CTX). Liquid chromatography–electrospray ionization-coupled with tandem mass spectrometry-based peptidomics and bioinformatics analysis unraveled the main polypeptides in LP and further confirmed that LP is mainly associated with immune regulating pathway, especially in tumor cell phagocytosis-related pathway. Our study for the first time revealed that polypeptides from spleen can relieve the immunosuppression induced by CTX and is a beneficial supplement in cancer therapy.

Abstract

Polypeptides extracted from animal immune organs have been proved to exert immunomodulatory activities in previous reports. However, relative experimental data regarding the influence of a polypeptide mixture extracted from healthy calf spleen (lienal polypeptide [LP]) on the immune function in tumor therapy are limited, and the components in LP remain unclear. In the present study, the immune regulatory effect of LP was investigated in normal mice and Lewis lung carcinoma (LLC)-bearing mice treated with cyclophosphamide (CTX). The components of LP were identified by liquid chromatography–electrospray ionization-coupled with tandem mass spectrometry (LC–MS/MS) analysis and bioinformatic analysis. In LLC-bearing mice, LP showed a synergic antitumor effect with CTX, whereas LP alone did not present direct antitumor activity. Further, LP was found to enhance immune organ indexes, splenocyte number, and T lymphocyte subsets in normal mice and LLC-bearing mice treated with CTX. The decline of white blood cell and platelet counts, splenocyte proliferation activity, and peritoneal macrophage phagocytic function caused by CTX were also significantly suppressed by LP treatment in LLC-bearing mice. Notably, LP treatment significantly decreased the expression of phagocytosis-related proteins including CD47/signal regulatory protein α/Src homology phosphatase-1 in the tumor tissue of LLC-bearing mice treated with CTX. LC-MS/MS-based peptidomics unraveled the main polypeptides in LP with a length from 8 to 25 amino acids. Bioinformatics analysis further confirmed the possibility of LP to regulate immunity, especially in phagocytosis-related pathway. Our above findings indicated that LP can relieve the immunosuppression induced by chemotherapy and is a beneficial supplement in cancer therapy.

Keywords: Lienal polypeptides, cyclophosphamide, immunomodulation, antitumor, peptidomics, phagocytosis

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Introduction

As for cancer treatment, chemotherapy is a main therapeutic modality in clinic treatment, although there has been a rapid development of immunotherapy and targeted therapy in recent years. However, traditional chemotherapeutic drugs when treating tumour cells also have cytotoxicity for healthy cells, especially immune cells, which seriously influences the quality of life of patients.[1] cyclophosphamide (CTX), widely applied among chemotherapeutic drugs, contains a preferable therapeutic index in oncotherapy.[2] The antitumor mechanism of CTX is to kill the tumor cells through inhibiting the synthesis of DNA, which also leads to extensive side effects, such as leukopenia, bone marrow suppression, and immune suppression.[3–5]

Therefore, the immune system is in a damaged condition during the chemotherapy. It reminders that immunomodulation is needed to lessen the side effects induced by CTX and improve the antitumor effect.

The immunomodulation activities of polypeptides extracted from animal immune organs have incurred people's interests since a long time ago. Hormone thymopoietin with 49-amino acid sequence was isolated from the thymus of calf, and thymopentin (TP5), a 5-amino acid polypeptide (Arg–Lys–Asp–Val–Tyr), was later identified as its active center and widely used as an immunomodulator in clinic.[6] Clemente *et al.*[7] reported that TP5 activated CD_4^+ and CD_8^+ lymphoid cells in melanoma patients. Lau *et al.*[8] found that splenocytes from LLC-bearing C57BL/6 mice treated with TP-5 and P815-bearing DBA mice could enhance cytotoxic activity against tumor cells. Furthermore, Taylor *et al.*[9] declared that the combination of TP5 and 5-FU increased T lymphocyte functional parameters and produced a reduction in tumor growth and lung metastases in mice bearing PAN2 tumors comparing with 5-FU treatment alone. Subsequently, thymosin α1, a 28-amino acid polypeptide, also originally identified from calf thymus, presented semblable immunocompetences to TP5. Thymosin α1 also has been reported to promote T-cells, dendritic cells, and antibodies response, and enhance the phagocytic activity of macrophages.[10] Moreover, thymosin α1-thymopentin syncretic peptide also showed favourable activity in enhancing the splenocyte proliferation and the phagocytosis of macrophage in mice.[11] Thymosin α1-thymopentin fusion polypeptide was also reported to recover the thymus and spleen index, $CD_4^+CD_8^+$ thymocyte population in hydrocortisone-induced immunosuppressive mice, and had a synergistic antitumor activity with CTX in C57BL/6 mice bearing B16 melanoma cells.[12] Nevertheless, few studies have paid attention to the active polypeptides from spleen, another important immune organ.

LP is a polypeptide mixture extracted from healthy calf spleen and is widely used in immune-modulating therapy in China (State Food and Drug Administration Approval No. H22026497). However, relative experimental data regarding the influence of LP on the immune regulation in tumor therapy are limited, and the components in LP remain unclear. Hence, the immune regulation effect of LP was studied in normal mice and LLC-bearing mice treated with CTX. Meanwhile, peptidomics and bioinformatic analysis was conducted to identify active components in LP by liquid chromatography–electrospray ionization-coupled with tandem mass spectrometry (LC-MS/MS).

Materials and methods

Drugs

LP (Batch No. 20130405) was given by Jilin Fengsheng Pharmaceutical Co., Ltd (Jilin, China). TP5 (Batch No. 14040062) was purchased from Wuhan Hualong Bio-Pharmaceutical Co., Ltd (Wuhan, China). CTX (Batch No. 13112825) was bought from Jiangsu Hengrui Pharmaceutical Co., Ltd (Jiangsu, China). Anti-CD_3-PE (mouse), anti-CD_4-FITC (mouse), and anti-CD_8-FITC (mouse) were bought from eBioscience (San Diego, CA, USA). Lipopolysaccharide (LPS) and concanavalin A were bought from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent microsphere was bought from Invitrogen (Carlsbad, CA, USA). Anti-CD47 antibody and Anti-Src homology phosphatase-1 (SHP-1) antibody were brought from Abcam (Cambridge, MA, USA), Anti-signal regulatory protein α (SIRPα) antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), β-actin was bought from FuDe Biological Technology Co., Ltd (Hangzhou, China).

Animals and treatments

Male C57BL/6 mice (aged approximately seven weeks) were purchased from Guangdong Medical Laboratory Animal Center (Permission No. SCXK 2013–0002). All experiments were performed and overseen by the Animal Care and Use Committee of Jinan University with approval No. 20150310001 and followed by the 7th edition Guide of "National Institute of Health for the Care and Use of Laboratory Animals."

For the sake of studying immunomodulatory function of LP in normal mice, all mice were assigned into five groups (n = 7): control, CTX (200 mg/kg), CTX+ LP-L (8 mg/kg, low dosage of LP), CTX + LP-H (16 mg/kg, high dosage of LP), CTX + TP5 (2 mg/kg). The doses of LP and TP5 were calculated based on their doses used in clinic, and they were administered to mice once daily lasted for seven days by intraperitoneal injection. Other groups administered the same volume of physiological saline. On the seventh day, CTX was administrated to mice by intraperitoneal injection. Twenty-four hours after CTX treatment, all mice were weighted and sacrificed with diethyl ether to obtain spleen and thymus tissues.

Another experiment was conducted to study the immunomodulatory function of LP in LLC-bearing mice, which was established by subcutaneously inoculating LLC cells. LLC-bearing mice were randomly distributed into eight groups (n = 9): control, CTX (20 mg/kg), CTX + LP-L (8 mg/kg, low dosage of LP), CTX + LP-H (16 mg/kg, high dosage of LP), CTX + TP5 (2 mg/kg), LP-L, LP-H, TP5. The dose of CTX (20 mg/kg) was chosen according to previous reports.[13–16] On the sixth day after subcutaneous inoculation of LLC cells, CTX, LP, and TP5 were administered, respectively, to mice once daily by intraperitoneal injection lasted for 15 days. Body weight of each mouse was monitored every day. Tumor volumes were monitored twice a week and analyzed with the formula: $\text{Volume}(mm^3)=0.5\times(\text{width}(mm))\times(\text{width}(mm))\times(\text{length}(mm))$. On the last day, LLC-bearing mice were sacrificed with diethyl ether; the spleen, thymus, and tumor were isolated and weighed. Tumor inhibition and Q value were calculated, respectively, through the following formulas. E_{a+b} is the inhibition rate of drug combination, and E_a or E_b is the inhibition rate of drug alone.

$$\text{Tumor inhibition} = \left(\frac{\text{tumor weight}_{\text{control group}} - \text{tumor weight}_{\text{experiment group}}}{\text{tumor weight}_{\text{control group}}}\right) \times 100\%$$

$$Q\,\text{values} = \frac{E_{a+b}}{(E_a + E_b - E_a \times E_b)}$$

Immune organ indexes analysis

After separating and weighting the thymus and spleen tissue, the index of spleen and thymus were expressed by the ratio of tissue weight (g)/body weight (g), respectively.

Splenocytes and thymus cell preparation

The spleen or thymus dispersed into single cell by a grinder with cold phosphate buffered saline (PBS). The total thymus and spleen cell counts were determined in each sample by a cell counter (Erma, Tokyo, Japan). After separating the debris with a centrifugation at 210 *g* for 5 min, lysing the erythrocytes, as well as washing cells with cold PBS, the cells were suspended by cold complete RPMI-1640 medium. Subsequently, the number of splenocytes was counted by cell counter (Erma, Tokyo, Japan).

Measurement of T lymphocyte subsets in the spleen

Splenocytes were treated with double-staining combinations (1 μL of anti-mouse CD_3 conjugated with PE and 0.5 μL of anti-mouse CD_4 conjugated with FITC, or 1 μL of anti-mouse CD_3 conjugated with PE and 0.5 μL of anti-mouse CD_8 conjugated with FITC). After incubating at room temperature in dark lasted for 20 min, washing with cold PBS, splenocytes were resuspended and analyzed using flow cytometry (Beckman, Brea, CA, USA).

Splenic T lymphocyte proliferation and B lymphocyte proliferation analysis

After sacrificing with diethyl ether, mice were sterilized and then splenocytes were obtained under aseptic environment and prepared as method mentioned above. Suspending in complete RPMI-1640 medium, the spleen lymphocytes were adjusted to 5×10^5 cells/mL. Then, 100 μL of spleen lymphocyte suspension was added in 96-well plates, 100 μL of Con A, LPS, or medium was added into the well, respectively, used as the mitogen of T lymphocyte, the mitogen of B lymphocyte, or control. After culturing in the incubator for 72 h, lymphocyte proliferation was performed by methyl thiazolyl tetrazolium (MTT) assay and analyzed following the formula

$$\text{Lymphocyte proliferation}\ (\%) = \left(\frac{A_{570,\text{experiment}} - A_{570,\text{control}}}{A_{570,\text{control}}}\right) \times 100\%$$

White blood cells (WBC) and platelets (PLT) in peripheral blood analysis

Blood sample gathered from the mouse was mixed with the EDTA·2Na anticoagulant solution (5 μL). The number of WBC and PLT in peripheral blood was analyzed through a hemocytometer (Sysmex XS-800i, Kobe, Japan).

Phagocytic function analysis

The assay of peritoneal macrophage phagocytic function is according to the method described previously.[17,18] After sacrificing with diethyl ether, mice were sterilized using 75% ethanol for 5 min. Mice were injected with 0.9% normal saline (7 mL) through enterocoelia, and the abdominal cavity was massaged for 2 min. Then the peritoneal fluid was obtained, centrifuged for 5 min at 210 *g* and washed with PBS twice. Next, macrophages were suspended by RPMI-1640 medium, adjusted to 1×10^6 cells/mL, placed in 24-well flat bottomed microplate, and cultured in the incubator for 3 h. After the supernatant was thrown away, the macrophages were washed by PBS, added with fluorescence-labeled latex beads (1:15) opsonized by bovine serum albumin (BSA), and continually incubated in incubator for 40 min. After the incubation, macrophages were harvested and analyzed using the flow cytometer (Beckman, Brea, CA, USA).

Polypeptide omics identification

The extraction of peptides was conducted by the previous method.[19] The peptides from samples were freeze to dry power by vacuum freeze drier (ScanVac, Lynge, Denmark) and reconstituted by 200 μL of tetraethylammonium bromide (TEAB) and 800 μL of dithiothreitol (DTT, 10 mM) dissolved in acetone, and incubated in 2 h. Subsequently, the peptides were centrifuged at 5000 *g* (15°C, 20 min), and the supernatant was discarded. In order to break the proteins' disulfide bonds, the peptides were reconstituted and mixed with 800 μL of DTT (10 mM) dissolved in acetone and incubated at 56°C for 45 min. After centrifugating at 5000 *g* (15°C, 20 min) again, the supernatant was discarded, left the peptides as dry as possible, and added 200 μL of TEAB to dissolve the peptides. Next, the purified peptides were gained through Strata-X C18 pillar (Phenomenex, Torrance, CA, USA) for three times, washed twice with formic acid (0.1%) and acetonitrile (5%), eluted once with formic acid (0.1%) and acetonitrile (80%), and then dried by vacuum concentration meter. The dried peptides were reconstituted with 20 μL of TEAB (0.5 M). LC-MS/MS analysis was conducted with a triple time-of-fight, 5600 plus system as previous research.[20]

For the MS/MS analysis, the MS/MS file data from LC-MS/MS system were sent to the ProteinPilot Software version 4.5 (AB SCIEX). The abundance of peptides was quantified by label-free through Skyline Software version 2.5, and the high abundant peptides were submitted to the programs of ToxinPred and CellPPD for the prediction of toxin and cell penetrating properties.[21–23] Besides, bioinformatic analysis of peptides was also performed as described previously.[24] The peptides were further assigned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Western blot analysis

Tumor tissue in LLC-bearing mice was lysed for Western blot analysis by RIPA buffer. Proteins (80 μg) were separated by 8% SDS-PAGE gel and transferred onto the polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked in 5% defatted milk and then incubated with primary antibodies (CD47, SIRPα, SHP-1, or β-actin) overnight at 4°C. Incubating with horseradish peroxidase-conjugated secondary antibodies, bound proteins were visualized by enhanced chemiluminescence (MultiSciences Biotech, Hangzhou, China) and detected using Tanon, 5200 Multi (Tanon, Shanghai, China). Blots were performed in triplicate and bands density was quantified by Quantity One software (Bio-Rad, Hercules, CA).

Statistical analysis

Results were presented as mean ± standard error of mean (SEM). Values of $P < 0.05$ were represented as the significant difference in statistic under one-way analysis of variance and a Tukey *post hoc* test through the software of GraphPad Prism 6. The original data of LC-MS/MS were against Bos Taurus Uniprot database (update at March 2016) using ProteinPilot software version 4.5 (AB SCIEX). Peptides were considered as identified if the confidence was larger than 0.95, which means at least 95% confidence was correct.

Results

LP enhanced the antitumor activity of LLC-bearing mice treated with CTX

The synergic antitumor effect of LP in CTX-treated mice was explored by the LLC-bearing mice model. Body weight and tumor volume of mice were monitored for 15 days. We found that body weight and tumor volume were remarkably reduced in CTX (20 mg/kg, i.p.) group while LP (8 and 16 mg/kg, i.p.) and TP5 (2 mg/kg, i.p.) alone treatment groups have no significant changes in comparing with the control group (Figure 1(a) and (b)). Intriguingly, tumor volume of "CTX + LP-L" ($P < 0.001$) and "CTX + LP-H" ($P < 0.05$) treatment groups had a greater decrease than CTX group. These data reminded us that LP might enhance the antitumor effects of CTX.

Subsequently, tumors were isolated, weighted, and photographed to calculate tumor inhibition rate at the end of this experiment. In contrast with the control group (Figure 1(c), (d), and (e)), tumor weight was also significantly declined in CTX ($P < 0.05$) with tumor inhibition rate at 46.06% while LP-L, LP-H, and TP5 alone treatment had no significant differences on tumor weight with tumor inhibition rate at 32.08, 31.29, 35.23%, respectively. Of note, when compared with the CTX group, tumor weight was significantly decreased in "CTX + LP-L" ($P < 0.001$), "CTX + LP-H" ($P < 0.01$), and "CTX + TP5" ($P < 0.05$) groups showing a higher tumor inhibition rate at 92.49, 83.88, and 74.25%, respectively, and the Q value of these treatments was 1.56, 1.37, and 1.21, respectively. It is considered a synergistic interaction between two drugs, when Q value was more than 1.15. These results further confirmed that LP treatment alone has not possessed the direct antitumor effect, but it has a synergic antitumor effect with CTX.

LP increased the immune organ indexes and T lymphocyte subsets in normal mice and LLC-bearing mice treated with CTX

With the purpose of determining whether the synergistic antitumor activity of LP treated with CTX was associated with the immune regulatory effect of LP, we further determined the immune organ indexes and T lymphocyte subsets in normal mice and LLC-bearing mice. In the normal mice, we first conducted a preliminary experiment to find out an appropriate dose of CTX to establish immunosuppression model in mice. Our results showed that compared to the control group, different CTX treatments (150 mg/kg, one day; 200 mg/kg, one day; 80 mg/kg, three days; 100 mg/kg, three days) all significantly induced an immunosuppression in normal mice in varying degree, reflected by the changes of spleen and thymus index, splenocyte number, T lymphocyte subsets, and WBC counts (Supplementary Figure S1). However, 80 or 100 mg/kg for three days of CTX caused strong adverse effects, including loss of weight and appetite and slowed behavior. Therefore, we chose the dose of CTX (200 mg/kg) to investigate the protective effect of LP. Results showed that CTX (200 mg/kg, i.p.) treatment significantly reduced the spleen and thymus index (Figure 2(a) and (b), $P < 0.05$), and the count of splenocytes (Figure 2(c), $P < 0.05$). In contrast, LP (16 mg/kg, i.p.) treatment significantly recovered these immune organ indexes (Figure 2(a) and (b), $P < 0.05$), and the splenocytes number in CTX-treated mice (Figure 2(c), $P < 0.05$). TP5 (2 mg/kg, i.p.) treatment only recovered thymus index (Figure 2(b)) ($P < 0.05$), but decreased the spleen index and the splenocytes number in CTX-treated mice (Figure 2(a) and (c), $P < 0.05$). What's more, $CD_3^+CD_4^+$ subset and $CD_3^+CD_8^+$ subset of lymphocytes in the spleen analyzed through flow cytometry showed that CTX treatment remarkably lowered the number of $CD_3^+CD_4^+$ and $CD_3^+CD_8^+$ T lymphocytes (Figure 2(d) and (e), $P < 0.01$), and decreased ratio of $CD_3^+CD_4^+/CD_3^+CD_8^+$ (Figure 2(f), $P < 0.01$). However, these indexes were significantly recovered by LP (16 mg/kg, i.p.) treatment ($P < 0.05$).

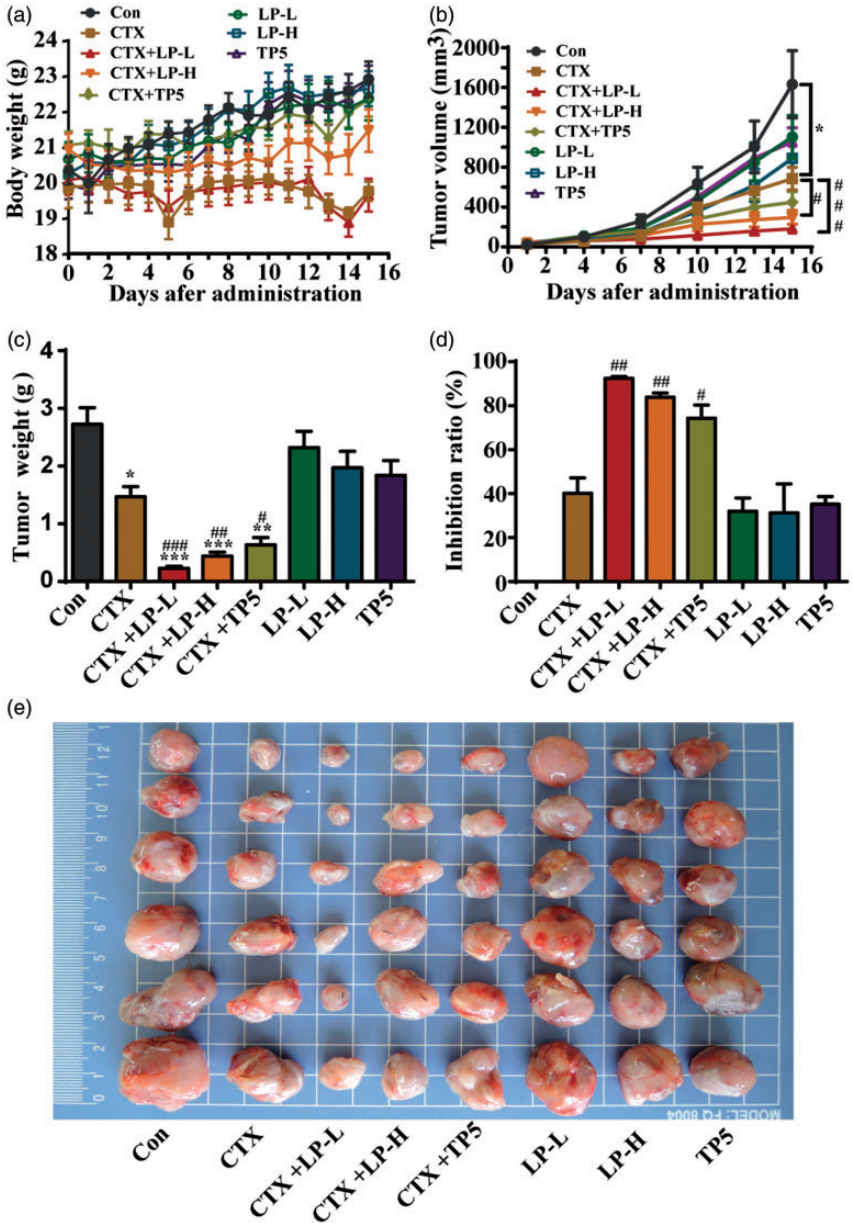


Figure 1. Effects of LP on the antitumor activity of LLC-bearing mice treated with CTX. CTX, LP, and TP5 were, respectively, administered to mice by intraperitoneal injection once daily from the sixth day after subcutaneous inoculation of LLC cells and lasted for 15 days. (a) Body weight and (b) tumor volume in LLC-bearing mice were measured; (c) tumor weight on 16th day, (d) tumor inhibition ratio of different treatment groups, and (e) excised solid tumors were shown. Results were presented as mean ± SEM. The data were analyzed by ANOVA and a Tukey *post hoc* test using GraphPad Prism 6.$^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ versus control group, $^{###}P < 0.001$, $^{##}P < 0.01$, and $^{#}P < 0.05$ versus CTX group were considered statistically significant. ANOVA: analysis of variance; CTX: cyclophosphamide; LLC: Lewis lung carcinoma; LP: lienal polypeptide; SEM: standard error of mean. (A color version of this figure is available in the online journal.)

Next, we found that CTX (20 mg/kg, i.p.) treatment presented a notable decline in spleen and thymus index (Figure 3(a) and (b), $P < 0.001$) in LLC-bearing mice. In contrast, these indexes were significantly recovered by LP (8 and 16 mg/kg, i.p.) and TP5 (2 mg/kg, i.p.) treatment ($P < 0.05$). Besides, splenocytes number was significantly reduced in CTX group ($P < 0.001$), but recovered by LP and TP5 treatment ($P < 0.05$). Furthermore, CTX also diminished evidently the number of $CD_3^+CD_4^+$ and $CD_3^+CD_8^+$ T lymphocytes (Figure 3(d) and (e), $P < 0.001$), and increased their ratio (Figure 3(f), $P < 0.05$) in the spleen of LLC-bearing mice. Nevertheless, the changes were significantly restored by LP and TP5 treatment ($P < 0.05$). LP (8 and 16 mg/kg, i.p.) and TP5 (2 mg/kg, i.p.) alone treatment had no significant difference excepting for increasing the number of $CD_3^+CD_4^+$ lymphocytes in LLC-bearing mice in contrast with the control group (Figure 3(a) to (f)). These effects reflected that LP could relieve the decrease of immune index and the damage of spleen lymphocyte induced by CTX in normal mice and LLC-bearing mice.

LP promoted the spleen lymphocyte proliferation of LLC-bearing mice treated with CTX

The proliferative responses of splenic B lymphocyte cells and splenic T lymphocyte cells, stimulated by Con A or LPS

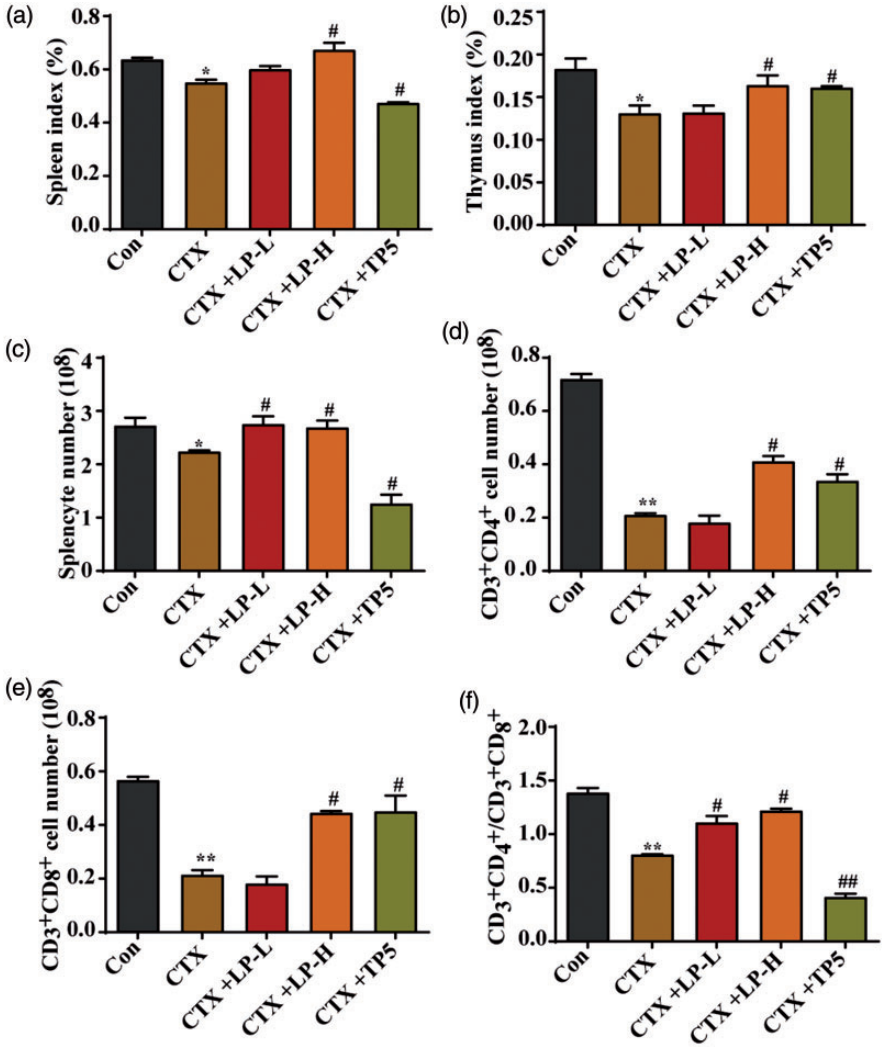


Figure 2. Effects of LP on the immune organ indexes and T lymphocyte subsets in normal mice treated with CTX. LP and TP5 were given to mice continuing for seven days by intraperitoneal injection once daily, CTX treatment was administrated by intraperitoneal injection on day 7 inducing immunosuppression. (a) Spleen index and (b) thymus index on eighth day in CTX-treated mice were calculated; the splenocytes number (c) in CTX-treated mice were measured by blood cell counter; (d) and (e) the number of spleen lymphocyte subset CD_3^+ CD_4^+ cell and CD_3^+ CD_8^+ cell and (f) the ratio of $CD_3^+CD_4^+/CD_3^+CD_8^+$ in CTX-treated mice were measured on eighth day by flow cytometer. Results were presented as mean ± SEM. The data were analyzed by ANOVA and a Tukey *post hoc* test using GraphPad Prism 6. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control group, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ versus CTX group were considered statistically significant. ANOVA: analysis of variance; CTX: cyclophosphamide; LP: lienal polypeptide; SEM: standard error of mean. (A color version of this figure is available in the online journal.)

in vitro, respectively, were determined to study the immunomodulatory effect of LP in LLC-bearing mice. CTX (20 mg/kg, i.p.) treatment significantly decreased the proliferative responses of splenic B lymphocytes (Figure 4(a), $P < 0.05$) and splenic T lymphocytes (Figure 4(b), $P < 0.01$), while LP (8 and 16 mg/kg, i.p.) and TP5 (2 mg/kg, i.p.) alone treatment had no significant influence. In contrast, LP-L, LP-M, and TP5 remarkably regained the proliferation of splenic B and T lymphocytes in LLC-bearing mice treated with CTX (Figure 4(a) and (b), $P < 0.05$).

LP increased WBC and PLT counts of LLC-bearing mice treated with CTX

WBC and PLT counts are considered as the major clinical index directly reflecting the damage of chemotherapy. Therefore, the WBC and PLT counts were analyzed by a hemocytometer and the results are shown in Figure 5(a) and (b). Our data showed that CTX (20 mg/kg, i.p.) treatment markedly decreased the peripheral WBC ($P < 0.01$) and PLT ($P < 0.05$) counts of LLC-bearing mice in comparing with control group. However, WBC counts were notably recovered in "CTX + LP-L" ($P < 0.05$) and "CTX + LP-H" ($P < 0.05$) group in LLC-bearing mice treated with CTX. Besides, LP-L ($P < 0.05$), LP-H ($P < 0.01$), and TP5 ($P < 0.05$) treatment significantly regained the number of PLT in LLC-bearing mice treated without CTX or with CTX. These results indicated that LP might contribute to decreasing leukopenia in LLC-bearing mice treated with CTX.

LP promoted peritoneal macrophages phagocytic function in LLC-bearing mice treated with CTX

Phagocytosis capacity of peritoneal macrophages of mice was determined through flow cytometry after incubating with BSA-opsonized fluorescent microsphere (2 μm)

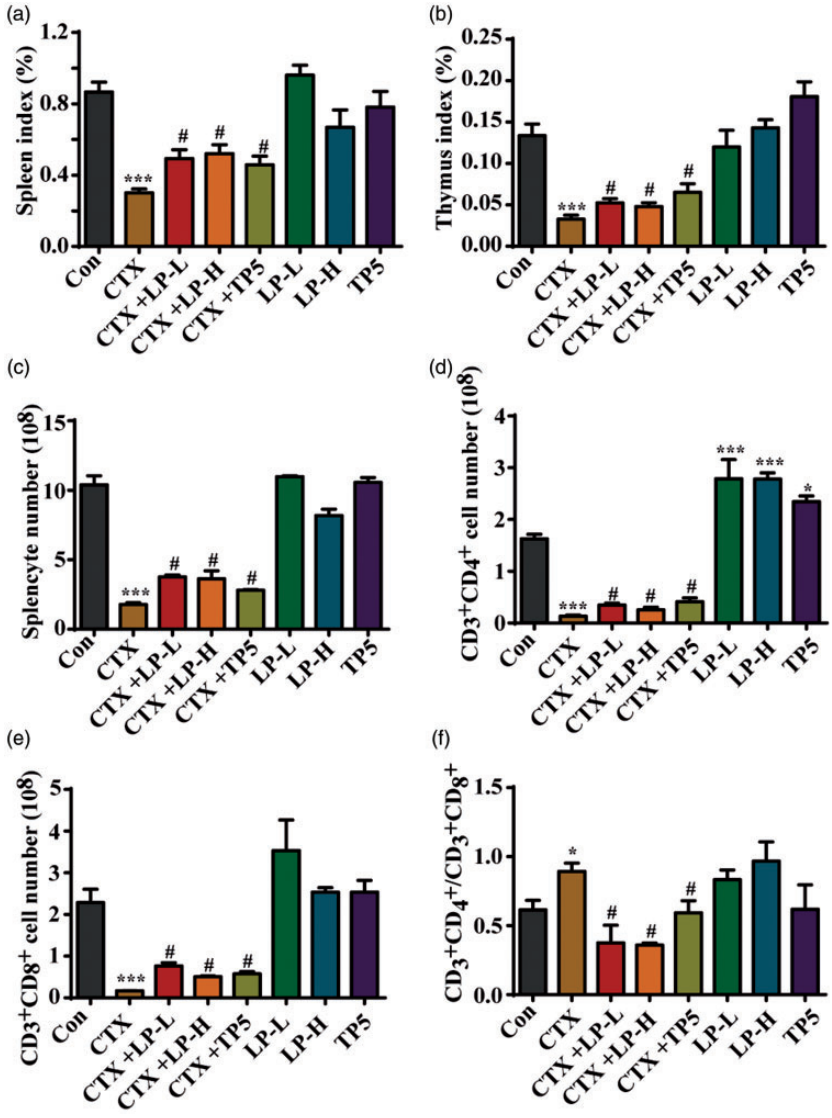


Figure 3. Effects of LP on immune indexes and T lymphocyte subsets in LLC-bearing mice treated with CTX. CTX, LP, and TP5 were, respectively, administered to mice by intraperitoneal injection once daily from the sixth day after subcutaneous inoculation of LLC cells and lasted for 15 days. (a) Thymus index and (b) spleen index in LLC-bearing mice were measured; (c) splenocytes number in LLC-bearing mice were measured by blood cell counting chamber; (d) and (e) the number of spleen lymphocyte subset $CD_3^+CD_4^+$ and $CD_3^+CD_8^+$, and (f) the ratio of $CD_3^+CD_4^+/CD_3^+CD_8^+$ in LLC-bearing mice were measured by flow cytometer. Results were presented as mean ± SEM. The data were analyzed by ANOVA and a Tukey *post hoc* test using GraphPad Prism 6. ***$P < 0.001$ and *$P < 0.05$ versus control group, ###$P < 0.001$ and #$P < 0.05$ versus CTX group were considered statistically significant. ANOVA: analysis of variance; CTX: cyclophosphamide; LLC: Lewis lung carcinoma; LP: lienal polypeptide; SEM: standard error of mean. (A color version of this figure is available in the online journal.)

(Figure 6). In contrast with control group, the phagocytosis percentage of peritoneal macrophages in LLC-bearing mice was inhibited markedly by CTX (20 mg/kg, i.p., $P < 0.01$), which was markedly recovered by LP-L ($P < 0.05$), LP-H ($P < 0.01$), and TP5 ($P < 0.001$) administration. Of note, LP-L (8 mg/kg, i.p., $P < 0.001$), LP-H (16 mg/kg, i.p., $P < 0.01$), and TP5 (2 mg/kg, i.p., $P < 0.001$) groups could elevate the phagocytosis ratio in LLC-bearing mice treated without CTX as well.

Polypeptide omics identification of LP

The components of LP were identified by LC-MS/MS-based peptidomics, and the major function of peptides in LP was analyzed by bioinformatics analysis. The result of peptides identification was according to the filter criteria (Conf > 95). The peptide mass tolerance was ranging from −0.05 to +0.05 Da (Figure 7(a)), which indicated that the result of peptides identification had a high accuracy, and most of peptides length distributed in the range of 8–25 (Figure 7(b)). Moreover, the functions of the identified peptides were further analyzed using the KEGG database and they were found to be enriched in regulating actin cytoskeleton (7.98%), phagosome (7.63%), Fc gamma R-mediated phagocytosis (3.07%), pathways in cancer (2.38%), NK cell-mediated cytotoxicity (2.19%), and T-cell receptor signaling pathway (0.82%) (Figure 7(c)). These findings were coincidence with the influence of LP on immunity, especially phagocytosis-related pathway.

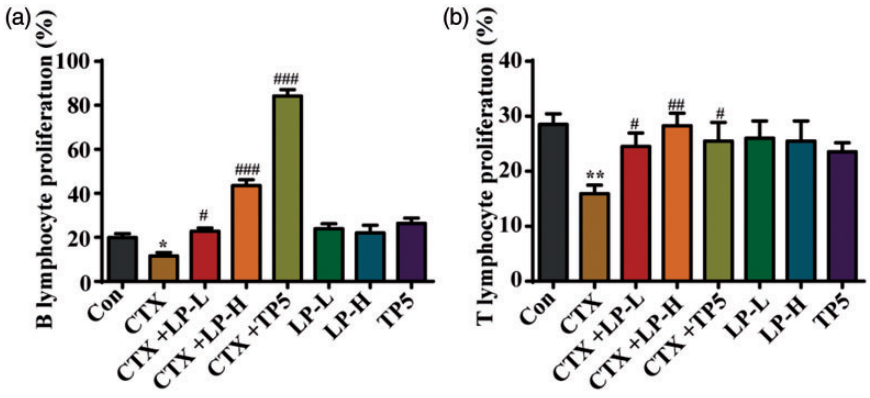


Figure 4. Effects of LP on spleen lymphocyte proliferation in LLC-bearing mice treated with CTX. CTX, LP, and TP5 were, respectively, administered to mice by intraperitoneal injection once daily from the sixth day after subcutaneous inoculation of LLC cells and lasted for 15 days. (a) Splenic B lymphocyte cells and (b) splenic T lymphocyte cells were measured by MTT assay on 16th day. Results were presented as mean ± SEM. The data were analyzed by ANOVA and a Tukey *post hoc* test using GraphPad Prism 6. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control group, $^{###}P < 0.001$, $^{##}P < 0.01$, and $^{#}P < 0.05$ versus CTX group were considered statistically significant. ANOVA: analysis of variance; CTX: cyclophosphamide; LLC: Lewis lung carcinoma; LP: lienal polypeptide; MTT: methyl thiazolyl terazolium; SEM: standard error of mean. (A color version of this figure is available in the online journal.)

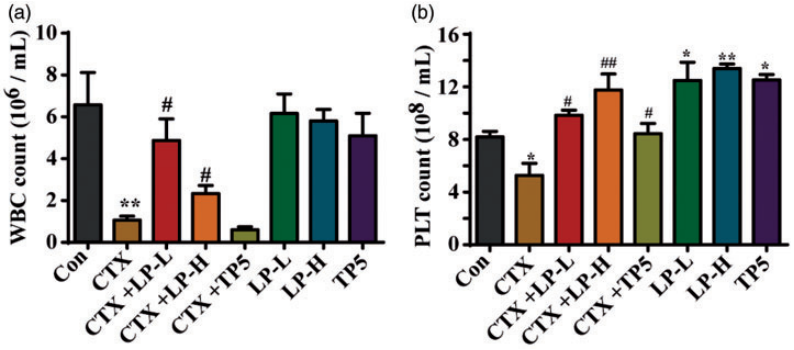


Figure 5. Effects of LP on peripheral WBC and PLT counts in LLC-bearing mice treated with CTX. CTX, LP, and TP5 were, respectively, administered to mice by intraperitoneal injection once daily from the sixth day after subcutaneous inoculation of LLC cells and lasted for 15 days. (a) WBC count and (b) PLT count were measured on 16th day by a hemocytometer. Results were presented as mean ± SEM. The data were analyzed by ANOVA and a Tukey *post hoc* test using GraphPad Prism 6. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control group , $^{##}P < 0.01$ and $^{#}P < 0.05$ versus CTX group were considered statistically significant. ANOVA: analysis of variance; CTX: cyclophosphamide; LLC: Lewis lung carcinoma; LP: lienal polypeptide; PLT: platelet; SEM: standard error of mean; WBC: white blood cell. (A color version of this figure is available in the online journal.)

The abundance of peptides was quantified by label-free method, and the specific sequences and the molecular weight (Mw) of the peptides with high abundance were shown in Figure 7(d). The mass spectrums of six high abundant peptides were shown in Supplementary Figure S2(a) and 2(b). The sequences of these peptides were LDLAGRLTDYL (Mw: 1364.4999), LHVDPENFKLLGNVL (Mw: 1707.9662), VDEVGGEALGRLL (Mw: 1327.4830), DQPTIDKIFQDLD (Mw: 1547.6613), NEALAALLREGESL (Mw: 1485.6383), and FNSGKVDIVAINDPFID (Mw: 1864.0591). Then, these peptides were submitted to the programs of ToxinPred and CellPPD for the prediction of toxin and the cell penetrating properties, and we found that all of the peptides were non-toxin and non-cell penetrating properties.

LP enhanced the expressions of phagocytosis-related proteins in tumor tissues of LLC-bearing mice treated with CTX

It has been revealed that "don't eat me signal" plays a dominant role during antitumor treatment by inhibiting the phagocytosis of macrophage.[25,26] CD47, an important "don't eat me signal," can interact with SIRPα expressed on the surface of macrophage, resulting in the phosphorylation of immuno-receptor tyrosine-based inhibition motifs (ITIM) sequences, and inducing the recruitment of SHP-1. The recruitment of SHP-1 then inhibits the phosphorylation of ITAM sequences of FcγR, leading to the failure of Syck protease recruitment to the phosphorylated ITAM sequences. This action finally suppresses the Src/PI3K/Rac signal pathway, and thus inhibits the polymerization of actin and the phagocytosis of macrophage.[25,27] To further understand the molecular mechanisms underlying the protective effect of LP on tumor cell phagocytosis, the expression of CD47/SIRPα/SHP-1 proteins in the tumor tissues of LLC-bearing mice was detected by Western blot method. As shown in Figure 8(a) to (d), the expression of CD47/SIRPα/SHP-1 proteins in LLC-bearing mice was obviously increased by CTX treatment (20 mg/kg, i.p., $P < 0.05$ and $P < 0.01$) when compared with the control group. In contrast, LP-L (8 mg/kg, i.p.), LP-H (16 mg/kg, i.p., $P < 0.05$), and TP5

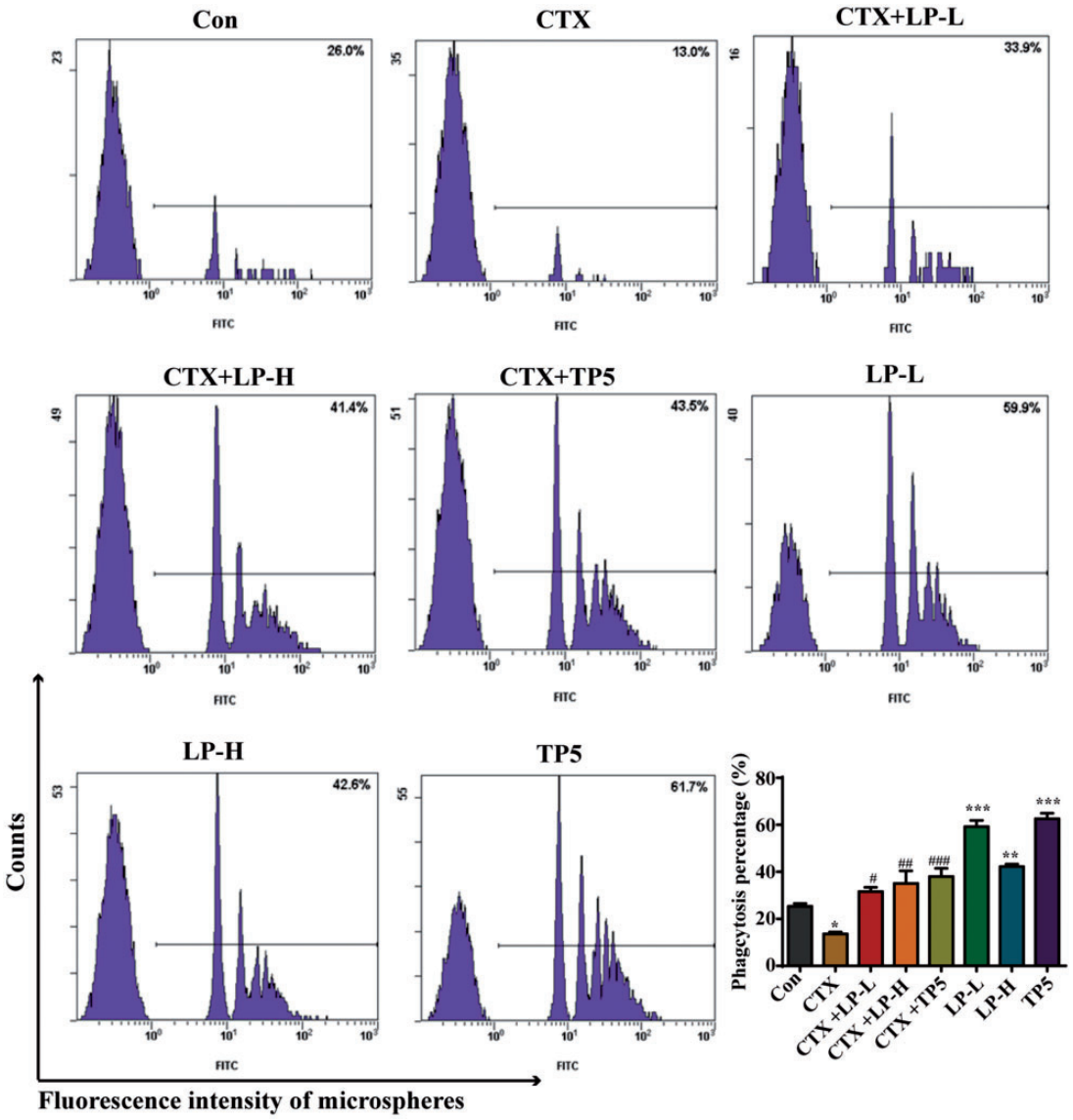


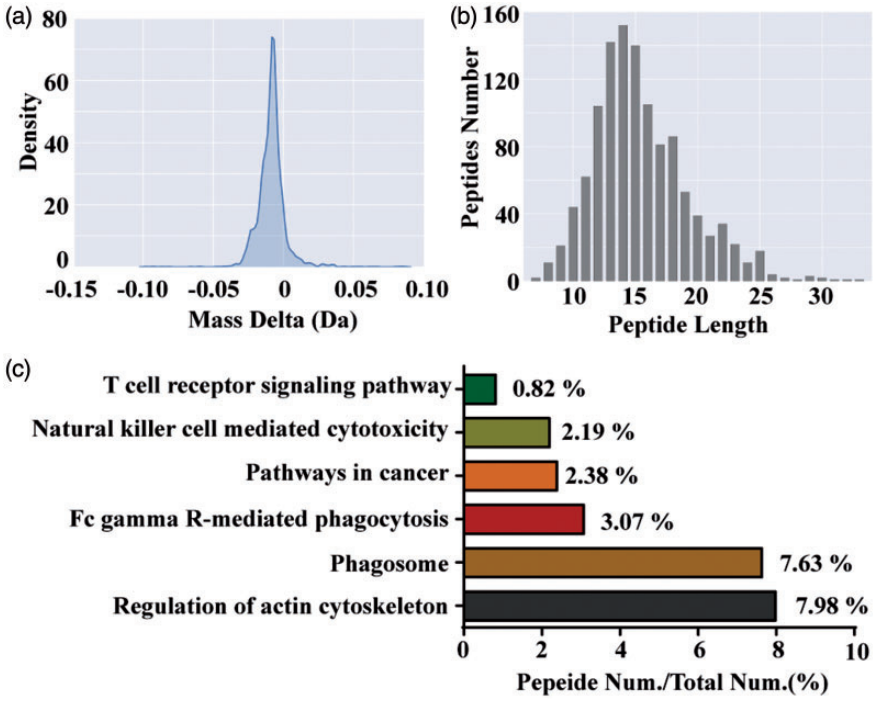
Figure 6. Effects of LP on peritoneal macrophages phagocytic function in LLC-bearing mice treated with CTX. CTX, LP, and TP5 were, respectively, administered to mice by intraperitoneal injection once daily from the sixth day after subcutaneous inoculation of LLC cells and lasted for 15 days. Phagocytic capability of peritoneal macrophages was determined using flow cytometer by swallowing the BSA-incubated fluorescence latex beads. Results were presented as mean ± SEM. The data were analyzed by ANOVA and a Tukey *post hoc* test using GraphPad Prism 6. Compared to control group, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus control group, $^{###}P < 0.001$, $^{##}P < 0.01$ and $^{#}P < 0.05$ versus CTX group were considered statistically significant. ANOVA: analysis of variance; CTX: cyclophosphamide; LLC: Lewis lung carcinoma; LP: lienal polypeptide; SEM: standard error of mean. (A color version of this figure is available in the online journal.)

(2 mg/kg, i.p., $P < 0.05$) treatment remarkably decreased the expression of CD47/SIRPα/SHP-1 (Figure 8).

Discussion

CTX is widely applied in treating various tumors. Nonetheless, its clinic usage has a severe restriction for its poisonousness and damage to immune system. Hence, it is necessary to develop an adjuvant therapy to reduce undesirable side effects of CTX.[28] It has been reported that animal-derived polypeptides possess immunomodulatory effects and have synergistic antitumor activities in animal and clinic.[7,10,12] The present study demonstrated LP treatment exerted desirably synergistic antitumor effects with CTX in LLC-bearing mice, although LP alone did not reduce the tumor inhibition rate. This actin of LP can be explained by its peotective effect against CTX-caused immunosuppression in LLC-bearing mice.

The thymus and spleen are important primary and secondary lymphoid organs, respectively. The spleen mainly generates the mature lymphocytes, including the Th type lymphocytes ($CD_3^+CD_4^+$) and Tc type lymphocytes ($CD_3^+CD_8^+$), and the ratio of $CD_3^+CD_4^+/CD_3^+CD_8^+$ is an indicator of immune dysfunction. In consistent with previous researches,[14,15,29,30] we also found that CTX caused spleen atrophy, perturbed the balance of $CD_3^+CD_4^+/CD_3^+CD_8^+$, and suppressed lymphocyte proliferation response in normal or LLC-bearing mice, but LP



(d)

Pepetides	Molecular weight
LDLAGRDLTDYL	1364.4999
LHVDPENFKLLGNVL	1707.9662
VDEVGGEALGRLL	1327.4830
DQPTIDKIFQDLD	1547.6613
NEALAALLREGESL	1485.6383
FNSGKVDIVAINDPFID	1864.0591

Figure 7. The polypeptide omics identification of LP. (a) The peptide mass tolerance and (b) the peptides length distribute were analyzed by LC-MS/MS. (c) The main signal pathway was analyzed by bioinformatics analysis. (d) The sequence and molecular weight of high abundant peptides were quantified by label-free method. A: alanine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; LC-MS/MS: liquid chromatography–electrospray ionization-coupled with tandem mass spectrometry; LP: lienal polypeptide; N: asparaginate; P: proline; Q: glutanine; R: arginine; S: serine; T: threonine; V: valine; Y: tyrosine. (A color version of this figure is available in the online journal.)

treatment remarkably reversed these changes caused by CTX.

WBC and PLT originate in the bone marrow and circulate throughout the bloodstream. The decrease of peripheral WBC and PLT counts reflects the myelosuppression in cancer patients after chemotherapy. Our result that CTX markedly diminished the WBC and PLT counts is consistent with previous reports.[13,31] More significantly, the administration of LP remarkably regained the WBC and PLT counts reduced by CTX in LLC-bearing mice, which indicated that LP might be a preferable regulator for decreasing the myelosuppression induced by CTX.

Macrophages are also important immune cells taking part in non-specific immune defense of host to defense against the invading cells including tumor cells.[32,33] The dying tumor cell eliminated by macrophage is a crucial step in chemotherapy.[34] The tumor cells treated with chemotherapeutic drugs can induce the exposure of "eat-me signal" calreticulin which can be recognized by CD91 expressed on macrophages,[35] thus activating the small G-protein and enhancing the recombination of actin to initiate the phagocytosis of macrophages.[36,37] Meanwhile, the "don't eat me signal," namely CD47/SIRPα/SHP-1 pathway, also plays a dominant role during antitumor treatment by inhibiting the phagocytosis of macrophage.[25,26] Thus, it can be inferred that drugs or chemicals regulating phagocytosis could elevate the chemotherapy efficacy. In this study, we found that LP treatment could recover peritoneal macrophage phagocytic activity in LLC-bearing mice treated with CTX. Furthermore, the bioinformatic analysis also indicated that that some polypeptides are involved in the phagocytosis-related activities such as regulation of actin cytoskeleton, phagosome, and FcγR-mediated phagocytosis. What's more, LP treatment could decrease the proteins expressions of CD47/SIRPα/SHP-1. These findings pointed out that the synergic antitumor effect of LP with

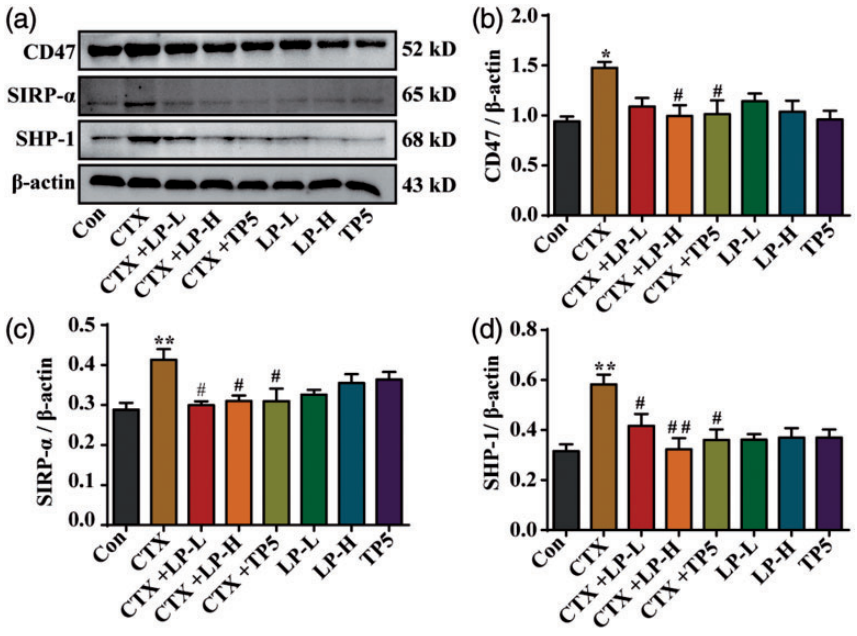


Figure 8. LP enhanced the protein expressions of CD47/SIRPα/SHP-1 in tumor tissues of LLC-bearing mice. CTX, LP, and TP5 were, respectively, administered to mice by intraperitoneal injection once daily from the sixth day after subcutaneous inoculation of LLC cells and lasted for 15 days. (a) The protein expressions of CD47/SIRPα/SHP-1 in tumor tissue were analyzed by Western blot and (b–d) their corresponding densities were quantified. Results were presented as mean $\pm$ SEM. The data were analyzed by ANOVA and a Tukey *post hoc* test using GraphPad Prism 6. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control group, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ versus CTX group were considered statistically significant. ANOVA: analysis of variance; CTX: cyclophosphamide; LLC: Lewis lung carcinoma; LP: lienal polypeptide; SEM: standard error of mean; SHP-1: Src homology phosphatase-1; SIRPα: signal regulatory protein α. (A color version of this figure is available in the online journal.)

CTX partially related with its capacity to enhance the tumor cell phagocytosis. Nevertheless, how does LP regulate this "don't eat-me signal" pathway and which polypeptide accounts for this effect of LP required further investigation.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. YPW, YFL conceived and designed the experiments; YPW, JD, SHO, ZFM, and GEW performed the experiments; HK, RRH, and YFL administered and supervised project, acquired funding; YPW wrote the original manuscript; YFL and RRH reviewed and edited the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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