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Original Article

Synergistic and Toxicity-reducing Effects of *Periplaneta americana* Extract CII-3 Combined with CTX on H22 Tumor Bearing Mice

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ABSTRACT

Background: Cyclophosphamide (CTX) is widely used in tumor treatment, but its clinical therapeutic effect is not ideal due to many side effects. **Materials and Methods:** In the present study, we researched the synergistic and attenuating effects of CII-3 combined with CTX and their underlying mechanism in H22 tumor-bearing mice. Firstly, we established an H22 tumor-bearing mice model, and the body weight, tumor weight, and survival time were recorded. Secondly, HE staining of tumor tissue was performed, and the related organ index, NK cell killing activity, peripheral blood cells, and bone marrow nucleated cells were measured. Moreover, the changes in IL-6 and IFN-1β in serum were detected by ELISA. Finally, RT-qPCR and Western Blot were performed to detect the expressions of TLR4, TLR9 and NF-κB in tumor tissue. **Results:** The treatment of CII-3 combined with CTX could increase life extension rate of H22 tumor-bearing mice, reduce tumor weight. Additionally, it could inhibit tumor cell proliferation, increase thymus and spleen index, enhance the activity of T cells in spleen, promote the killing activity of NK cells, and had a certain ameliorate effect on the reduction of WBC, Neut, LYM and bone marrow nucleated cells caused by CTX. And the expression of mRNA and the protein of TLR4, TLR9 and NF-κB in tumor mass of H22 tumor-bearing mice were down-regulated. **Conclusion:** There were synergistic and attenuating effects of CTX combined with CII-3 in the treatment of tumor and the effects might be mediated by the TLR4/NF-κB and TLR9/NF-κB signaling pathways.

Keywords Anti-tumor, Cyclophosphamide, Immune depression, *Periplaneta americana*, Synergism and attenuation.

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INTRODUCTION

Primary liver cancer is the sixth most commonly occurring cancer in the world and the third largest contributor to cancer mortality. The highest incidence rates of liver cancer in the world are in Asia and Africa. Approximately 75% of liver cancer occurs in Asia, with China accounting for more than 50% of the world's burden.[1,2] At present, there are three main therapeutic methods for malignant tumors in clinical practice: surgical treatment, radiotherapy and chemotherapy. Although surgical resection is the first choice for the treatment of malignant tumors, chemotherapy is the main treatment for postoperative recurrent tumors, metastatic tumors and patients who are not suitable for surgical treatment. Although chemical drugs have a good killing effect on most tumor cells, patients have to face many side effects brought by chemotherapy drugs while using chemotherapy drugs. Take Cyclophosphamide (CTX), the most widely used drug in clinic, as an example,[3] the main mechanism of its killing effect on tumor cells is to inhibit the growth and reproduction of cells by cross-linking with the DNA of cells through alkylation.[4] However, its low cell selective led to the killing of tumor cells at the same time, also caused damage to normal cells, causing cell toxicity, especially in view of the rapid proliferation of the cells, produce the side effects, such as the immunosuppression, bone marrow suppression in the body's immune organ damage, participate in the immune cells decreased, the number of Red Blood Cells (RBCs), White Blood Cells (WBCs) and platelets derived from bone marrow hematopoietic stem cells decreased in peripheral blood.[5,6] Many side effects can lead to the decrease of treatment effect and the prolongation of treatment time. Therefore, how to reduce the cytotoxicity caused by chemotherapy drugs has become an urgent problem to be solved in clinical practice.[7]

Traditional Chinese Medicine (TCM) has unique advantages as an adjuvant drug in chemotherapy of tumor.[8] Studies have shown that *Astragalus polysaccharide* plays an important role in

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the process of tumor treatment, reducing the generation of side effects, and improving the biological effect of chemotherapy drugs to a certain extent.[9-16] It can improve the therapeutic effect of chemotherapy drugs and the quality of life of patients, and reduce the medical cost. The *Periplaneta americana*, commonly known as cockroach, is the largest insect of the cockroach family. Its earliest medicinal history dates back to Shennong Materia Medica.[16] Modern pharmacological studies have shown that *Periplaneta americana* has a protective effect on the liver and stomach, can promote wound healing, improve the body's immunity, and has antiviral and anti-tumor activities.[17-21] In the previous study, our research group extracted the effective anti-tumor active ingredient CII-3 from *Periplaneta americana*. The main active ingredient of CII-3 is small molecule peptide, and the content of total peptide was determined by Folin-method according to the national pharmacopoeia. Compared with TCM injection, CII-3 has a wide safety range and will not cause obvious adverse reactions at the dose of routine administration. When the dose is more than 25 times of the dose of routine clinical administration, adverse reactions in the central nervous system and digestive system will occur. Studies have shown that CII-3 combined with cisplatin can enhance the inhibitory effect of cisplatin on the proliferation of tumor cells in mice, significantly prolong the life of mice and improve the tumor inhibition rate, which proves that CII-3 has a synergistic effect on the treatment of tumor with cisplatin.[22] At the same time, the CII-3 combined with cisplatin can be used for relieving side effects of liver injury caused by cisplatin therapy, which can significantly improve the liver, spleen, lung and thymus index, increase the number of white blood cells in peripheral blood in mice, and promote the lymphocyte proliferation, killing activity of NK cells, shows that the combination of cisplatin with CII-3 lead to less toxic and side effects on tumor treatment. Previous studies have shown that CII-3 combined with CTX can significantly enhance the anti-tumor effect of CTX and reduce tumor weight in the treatment of tumors.[23] In addition, CII-3 combined with CTX can significantly reduce the toxicity of CTX in tumor treatment. The potential mechanism may be related to the TAK1/NF-κB signaling pathway. However, whether the mechanism of the synergistic and attenuated effect of CII-3 on the treatment of tumor is related to other signaling pathways has not yet been clarified. In this essay, our aim is to clarify the synergistic and attenuating effects of CII-3 combined with CTX and their underlying mechanism in H22 tumor-bearing mice.

MATERIALS AND METHODS

Experimental cells and animals

SPF BALB/c mice (20±2 g) aged from 6 to 8 weeks, half male and half female (200 in total), were purchased from Slike Jingda Experimental Animal Co., Ltd., (Hunan, China, license number: SCXK (Hunan) 2016-0002). H22 hepatoma cells were preserved in this experiment. The animal experimental research was approved by the Animal Research Ethics Committee of Dali University.

Preparation of experimental reagents

Preparation of CTX injection

In the ultra-clean worktable (Suzhou Antai Air Technology Co., Ltd.,) take 40 mg CTX powder for injection (Jiangsu Shengdi Pharmaceutical Co., Ltd.,) and mix with 50 mL Normal Saline (NS) (Guizhou Tiandi Pharmaceutical Co., Ltd.,). Take this powder as high concentration and dilute it with NS equal times to 80, 40, 20 mg/kg.

Preparation of *Periplaneta americana* extract CII-3

0.25 g of CII-3 (provided by Zhengchun He, Dali University) was accurately weighed and dissolved in 50 mL of NS to obtain 100 mg/kg of CII-3 suspension.

Cell culture and animal modeling

The frozen H22 mouse hepatoma cell lines were cultured for routine resuscitation and counted. The cells were inoculated intraperitoneally in BALB/c mice with a cell density of 2×10^6/mL and 0.2 mL/mouse for 7 d. The cervical vertebra was dislocated and killed. The ascites was disinfected in the abdomen under sterile conditions and diluted into $2\text{-}3\times10^6$/mL. A solid tumor model of H22 tumor-bearing mice was established.

Grouping and administration of experimental animals

Two hundred BALB/c mice (half male and half female) were taken at 22-25°C and 50-80% humidity. The solid tumor model of H22 tumor-bearing mice was established after feeding for 3 d. After modeling, the mice were randomly divided into 10 groups with 20 mice in each group (Table 1). The survival time of 1-10 mice in each group was observed to calculate the life extension rate. Tumor mass was taken from mice 11-20 and reconstructed to make pathological sections of tumor mass. Meanwhile, organs were stripped to calculate organ index. 24 hr after modeling, drug administration was started, among which, the blank group and model group were intragastrically given normal saline. The single administration group was given CII-3 by gavage or different doses of CTX by intraperitoneal injection. In the combined administration group, CII-3 was given by intragastric administration and CTX was injected intraperitoneally half an hour later. The mice were weighed every other day and given medicine continuously for 15 d. The daily survival status of the mice was observed. After 15 d, various indexes were detected.

Determination of body weight gain rate, tumor weight and tumor inhibition rate

24 hr after the last administration, the subcutaneous tumor tissue was removed, and the residual blood was rinsed with NS

Table 1: Grouping and administration of Lewis tumor-bearing mice.

Groups	Route of administration	Dosage (mL/ 10 g/d)
Blank	Intragastric administration	0.2
Model	Intragastric administration	0.2
Positive (1250 mg/kg Shenqi fuzheng injection+20 mg/kg CTX)	Intraperitoneal injection	0.1+0.15
CII-3 (100 mg/kg)	Intragastric administration	0.2
20 mg/kg CTX	Intraperitoneal injection	0.1
20 mg/kg CTX+100 mg/kg CII-3	Intraperitoneal injection + Intragastric administration	0.1+0.2
40 mg/kg CTX	Intraperitoneal injection	0.1
40 mg/kg CTX+100 mg/kg CII-3	Intraperitoneal injection + Intragastric administration	0.1+0.2
80 mg/kg CTX	Intraperitoneal injection	0.1
80 mg/kg CTX+100 mg/kg CII-3	Intraperitoneal injection + Intragastric administration	0.1+0.2

and dried with filter paper. The tumor weight was weighed and recorded. The body weight gain rate and tumor inhibition rate of each group were calculated by the formula, and the Q value was calculated according to the King's formula to evaluate the efficacy of the combination drug. The calculation formula is as follows: *body weight gain rate(%)=*$(W_r - W_i)/W_i$**100%*, where W_r stands for the body weight of mice after removing the tumor tissue, and W_i stands for the initial body weight of mice; *tumor inhibition rate(%)=* $(W_C - W_a)/W_a$**100%*, where W_C stands for the average weight of the control group and W_a is the average weight of the administration groups; $Q = E_{AB}/[E_A + (1 - E_A)*E_B]$, where E_{AB} is the combined tumor inhibition rate of the two drugs, E_A and E_B indicate the tumor inhibition rate of the signal drug. According to the results, it represented the antagonism of the two drugs when the Q value was lower than 0.85; it represented the synergy of two drugs when the Q value was higher than 1.15; when the Q value was between 0.85 and 1.15, represented the two drugs had additive effects.

Determination of life extension rate

The body weight of mice was weighed and recorded on alternate days to describe the changes in body weight of mice in each group during administration and to observe the daily survival status of mice, and to calculate the corresponding life extension rate. Calculation formula: *life prolongation rate (%) =* $(D_a - D_c)/D_c$**100%*, where D_a stands for the mean living days of the administration groups, and D_c stands for the mean living days of the control group.

HE staining

24 hr after the last administration, blood was collected from the orbit, and then the mice were killed by cervical dislocation. Tumor blocks were completely removed, and adipose tissue, calcified tissue and blood clots were removed. Tumor blocks with a size of about 1.0 cm × 1.0 cm × 0.3 cm were cut off and soaked in 10% formaldehyde solution for 24 hr for fixation, washing and dehydration. The slices were embedded in paraffin. The slices (thickness was 4 μm) were expanded in 30%-40% ethanol and water at 38°C in sequence. The histopathological changes of tumor were observed by HE staining.

Proliferative activity of splenic lymphocytes in mice

Splenic lymphocyte suspension (2×10^6/mL) was inoculated with 1640 medium containing 10% fetal bovine serum (200 μL/ well), and 2 μL of Con A medium containing 0.5 mg/mL (the final concentration was 5 μg/mL) was added as the stimulation well. At the same time, a control hole without ConA was set, and 6 duplicate holes were set for the stimulation hole and the control hole respectively. Put the mixture in an incubator at 37°C, 5% CO_2 for 48 hr, then add MTT (Beijing Solebo Technology Co., Ltd.,) 5 mg/mL solution 20 μL for further incubation for 4 hr, centrifuge at 2000 r/min for 10 min, absorb and discard the supernatant, add DMSO 150 μL to each well, fully oscillate and dissolve for 10 min. OD value at 490 nm was detected by enzyme plate analyzer and calculate Stimulation Index (SI). The calculation formula is as follows: *SI = OD value of stimulation hole/OD value of control hole.*

Preparation of splenic lymphocyte suspension in mice

24 hr after the last administration, the mice were killed by cervical dislocation and placed in 75% alcohol. After 5 min, the spleens of the mice were separated under sterile conditions and the excess tissues were removed. The spleen was prepared into cell suspension and the cell concentration was adjusted.

Determination of killing activity of NK cells

Spleen lymphocytes (2×10^6/mL) were prepared with 1640 medium containing 10% fetal bovine serum, and YAC-1 cells (target cells) in logarithmic growth phase were selected at 4×10^4/mL as target cells. The ratio of effector cells to target cells was 50:1. Add 100 µL of effector cells/ well, then add 100 µL of target cells in logarithmic growth phase, and inoculate into 96-well plate. At the same time, set effector cells, target cells and blank control, each set 3 double Wells. The killing activity of NK cells was detected by MTT assay, and the formula was as follows: *killing activity of NK cells (%) = (1- OD value of the administration groups)/OD value of the control group*100%*.

Detection of splenic lymphocyte subsets in mice

Splenic lymphocyte suspension (1×10^6/mL) containing 1640 medium containing 10% FBS was added into the flow detection tube at 100 µL/ tube. The blank group was set without any antibody, and the other groups were added FITC Hamster Anti-Mouse CD3e, PE Rat Anti-Mouse CD4, Per-C*p* Anti-Mouse CD8a, And FITC Hamster Anti-Mouse CD3e, PE Rat Anti-Mouse CD4 and Per-CP anti-mouse CD8a mixed antibodies respectively. After shaking and mixing, the mixture was hidden from light at 4°C for 30 min. The mixture was washed with PBS and centrifuged twice (400 g, 2 min), discard the supernatant, then add 500 µL PBS rinse solution to resuspend the cells, and detect by flow cytometry. Cell-Quest analysis was also used, and the results were expressed as positive percentage of cells.

Determination of peripheral blood images

24 hr after the last administration, 20 µL blood was collected from the orbital venous plexus, and the number of peripheral White Blood Cells (WBC), Neutrophils (Neut), Platelets (PLT), Lymphocytes (LYM) and Red Blood Cells (RBC) were measured by a whole blood analyzer.

Determination of organ index

24 hr after the last administration, the heart, liver, spleen, lung, kidney and thymus were extirpated, the residual blood was cleaned with NS, and then dried with filter paper for weighing records. The organ index of each group was calculated by the formula. The calculation formula is as follows: *organ index=organ weight (mg)/body weight (g)*.

Determination of bone marrow nucleated cells

24 hr after the last administration, the mice were killed by dislocation, and the femur on both sides of the mice was removed. The bone marrow cavity was rinsed with RPMI-1640 medium, and all the bone marrow was rinsed to the centrifuge tube. Finally, the bone marrow nucleated cell suspension was obtained with RPMI-1640 medium, and the cells were counted under a microscope.

Double anti-body sandwich ELISA detects the changes of IL-6 and IFN-1β in serum

24 hr after the last administration, 0.4-0.8 mL of blood was extracted from the eyes of the mice. After resting at 4°C for 15 min, the blood was placed in a low temperature frozen high-speed centrifuge at 3000 r/min for 15 min, and the supernatant was extracted. Serum levels of IL-6 and IFN-1β were measured using an Enzyme-Linked Immunosorbent Assay Kit (Eptec, USA).

mRNA expression analysis of TLR4, TLR9 and NF-κB in tumor tissues

The primers for β-actin, TLR4, TLR9 and NF-κB mRNA were designed with software. The sequences are shown in Table 2 (synthesized by Nanjing Kingsley Biotechnology Co., Ltd.,). RNA was extracted from tumor tissues pre-stored in liquid nitrogen, and the total RNA of the samples was reverse-transcripted into cDNA using RT Supermix (Nanjing Novizon Biotechnology Co., Ltd.,) which was then amplified by real-time quantitative polymerase chain reaction (RT-qPCR) using the synthesized cDNA. The cycling conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec and 72°C for 5 min. RT-qPCR analysis was performed with the CFX96™ Real-Time System.

The annealing temperature of each primer was β-actin (55°C), TLR4 (54°C), TLR9 (54°C), NF-κB (55°C).

Table 2: Primers used for reverse transcription-quantitative polymerase chain reaction.

Genes		Primers (5′ to 3′)	Bases	Sequencelength (bp)
β-actin	Forward	GGCTGTATTCCCCTCCATCG	20	154
	Reverse	CCAGTTGGTAACAATGCCATGT	22	154
TLR4	Forward	ATGGCATGGCTTACACCACC	20	129
	Reverse	GAGGCCAATTTTGTCTCCACA	21	129
TLR9	Forward	ATGGTTCTCCGTCGAAGGACT	21	118
	Reverse	GAGGCTTCAGCTCACAGGG	19	118
NF-κB	Forward	ATGGCAGACGATGATCCCTAC	21	167
	Reverse	CGGAATCGAAATCCCCTCTGTT	22	167

Table 3: Effects of CII-3 combined with CTX on tumor weight, tumor inhibition rate and Q value in H22 tumor-bearing mice ($x \pm s$, n=10).

Group	Tumor inhibition rate (%)	Q value
Blank	—	—
Model	—	—
Positive	44.12	—
CII-3	30.58	—
CTX 20 mg/kg	38.65	—
CII-3+CTX 20 mg/kg	44.93	1.85
CTX 40 mg/kg	49.53	—
CII-3+CTX 40 mg/kg	56.93	2.46
CTX 80 mg/kg	52.11	—
CII-3+CTX 80 mg/kg	62.10	3.02

Western blot analysis detects the expression of related proteins in tissues

Tumor tissue was extracted and lysed with precooled RIPA lysate (Beijing Solaibo Technology Co., Ltd., Beijing, China). The supernatant was taken and BCA kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to determine the protein concentration in the total proteolytic solution. Take 20 μL protein sample and use sodium dodecyl sulfate -- polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology, Shanghai, China), the protein was transferred to PVDF membrane (Millipore Company, USA), put into 5% skimmed milk powder, shake the table, and sealed at room temperature for 2 hr. The membrane was diluted with primary antibody diluent and incubated in an incubator with primary antibody at room temperature for 2 hr. After cleaning the membrane with TBST, the membrane was diluted with secondary antibody diluent, and the membrane was incubated in the incubator box with secondary antibody at room temperature for 2 hr. The membrane was cleaned with TBST. The ECL luminescent solution (Milliore Company, USA) was evenly dropped onto the membrane, and incubated for 5 min in the dark. The color was developed, and photos were taken and recorded. Image J analysis software was used to quantitatively analyze the gray values of protein bands. The mean value was taken as the gray value and the relative content of the target protein = target protein density/β-actin density.

Statistical analysis

The experimental results data were imported into the SPSS statistical software, results were expressed as mean ± Standard Deviation (SD), and statistical differences were determined by one-way analysis of variance. When $p < 0.05$ was statistically significant.

RESULTS

Synergistic effect of CII-3 combined with CTX on H22 tumor-bearing mice

Histopathologic changes of tumor tissues show CII-3 combined with CTX could promote the inhibitory effect of tumor cell proliferation

After inoculation with H22 tumor cells, the growth rate of tumor masses was accelerated. Compared with the model group, the above phenomena were reduced in the administration group. As shown in Figure 1, the tumor cells in the model group were in good condition, irregular shape, large volume, diffuse distribution and unclear to each other. The cell nuclei were of different colors, sizes and shapes, and the blood supply of the tumor tissue was abundant, suggesting that the tumor cells were in good condition and vigorous growth. In the positive group, the tumor tissue was loose, the cell number was significantly reduced, the cell arrangement was irregular, and some nuclei were condensed. In CTX group, there was a large area of necrosis. The tumor tissue was loose, the number of cells was significantly reduced, and the cells were arranged irregularly. There were still scattered tumor cells of different sizes, and the nuclei were concentrated and deeply stained. CII-3 combined with CTX (20 mg/kg) showed that some tumor cells were round and uniform in size, and a large number of tumor cells were disintegrated and granulated, with obvious edema in the stroma and a few vacuoles. CII-3 combined with CTX (40 mg/kg) showed patchy necrotic tumor cells with clear contours, varying cell sizes, interstitial edema, sporadic inflammatory cell infiltration and vacuoles. CII-3 combined with CTX (80 mg/kg) showed tumor cells of different sizes, different shapes, irregular arrangement, partial nuclear concentration, interstitial hemorrhage and edema, and a large number of fat vesicles. It suggested that CII-3 combined with CTX (20, 40, 80 mg/kg) groups could increase the inhibitory effect of tumor cell proliferation.

CII-3 combined with CTX could reduce tumor weight, promote tumor inhibition rate

As shown in Figure 2 and Table 3, compared with the model group, the tumor inhibition rate of CII-3 group to H22 tumor-bearing mice was 30.58%, while the tumor inhibition rate of CII-3 combined with CTX (20, 40, 80 mg/kg) groups could reach 44.93%, 56.93%, 62.10%, and the decrease of tumor mass was statistically significant ($p < 0.01$). However, the tumor inhibition rate of CTX alone group with the same dose was lower than that of CII-3 combined CTX group. These results indicated that CII-3 combined with CTX could significantly inhibit tumor growth in H22 tumor-bearing mice. The anti-tumor rates of CTX group and CII-3 group were 38.65%, 49.53%, 52.11% and

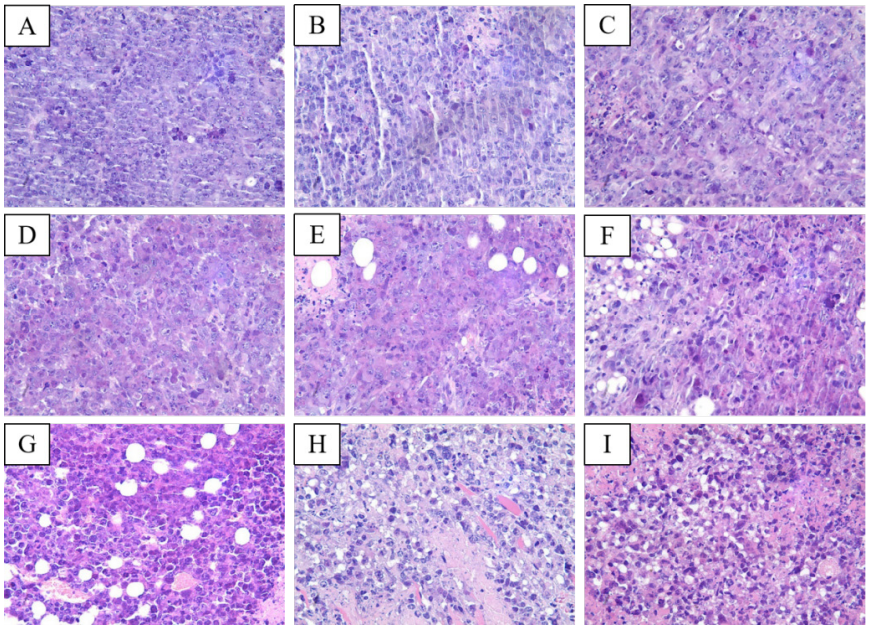

Figure 1: Histopathological changes in tissues of mice. (A) Model group; (B) CII-3 group; (C) Shenqi Fuzheng Injection+CTX (20mg/kg); (D) CTX (20mg/kg) group; (E) CTX (40mg/kg) group; (F) CTX (80mg/kg); (G) CII-3+CTX (20mg/kg) group; (H) CII-3+CTX (40mg/kg) group; (I) CII-3+CTX (80mg/kg) g roup.

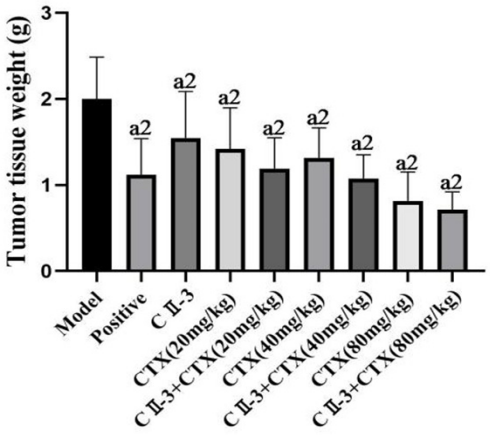

Figure 2: CII-3 combined with CTX in the treatment of tumor weight in H22 tumor-bearing mice. Compared with model group, $^{a1}p < 0.05$, $^{a2}p < 0.01$.

30.58%, respectively. The anti-tumor rate of CII-3 combined with CTX (80 mg/kg) group was 62.10%, which was significantly higher than that of CII-3 group ($p < 0.05$). The Q values of CII-3 combined with (20, 40, 80 mg/kg) CTX group were 1.85, 2.46, 3.02 (Q > 1.5), respectively, suggesting that the anti-tumor effect was enhanced and had synergistic effect.

CII-3 combined with CTX could promote the proliferation activity of T cells and killing activity of NK cells, reduce the level of IL-6 and IFN-β in the serum

As shown in Figure 3A, compared with the blank group, the T cell activity of CTX group was decreased, and the difference was significant ($p < 0.05$ or $p < 0.01$); Compared with model group, the T cell activity of CII-3 combined with CTX (20, 40, 80 mg/kg) was increased, and significantly higher than that of CTX group with the same dose ($p < 0.05$ or $p < 0.01$). The proliferation ability of lymphocytes in the positive group was similar to that in the CII-3 group. As shown in Figure 3B, compared with the blank group, the killing activity of NK cells in CTX group was decreased, and the difference was significant ($p < 0.01$). Compared with model group, the killing activity of NK cells increased in CII-3 combined with CTX, and was superior to that in CTX alone group with the same dose ($p > 0.05$). The killing activity of NK cells in the positive group was higher than that in the CII-3 group. As shown in Figure 3C, compared with the blank group, the serum IL-6 content in the model group was significantly lower than that in the blank group ($p < 0.05$). Compared with model group, all treatment groups could significantly reduce the serum IL-6 content of mice except CII-3, CTX (20 mg/kg) and CTX (40 mg/kg) groups, with statistical significance ($p < 0.05$ or $p < 0.01$) The effect of CII-3 combined with CTX (40 mg/kg) group was significantly lower than that of CTX alone. As shown in Figure 3D, compared with blank group, except CTX (80 mg/kg) and CII-3 combined CTX (80 mg/kg) groups, the serum IFN-β content of mice in other treatment groups decreased, with statistical significance ($p < 0.05$ or $p < 0.01$). Compared with model group, serum IFN-β content in CTX (80 mg/kg) and CII-3 combined CTX (80 mg/kg) groups was increased, which was close to the blank group, with statistical significance ($p < 0.05$ or $p < 0.01$). It suggested that CII-3 combined with CTX group could enhance the proliferation activity of T cells in spleen, promote

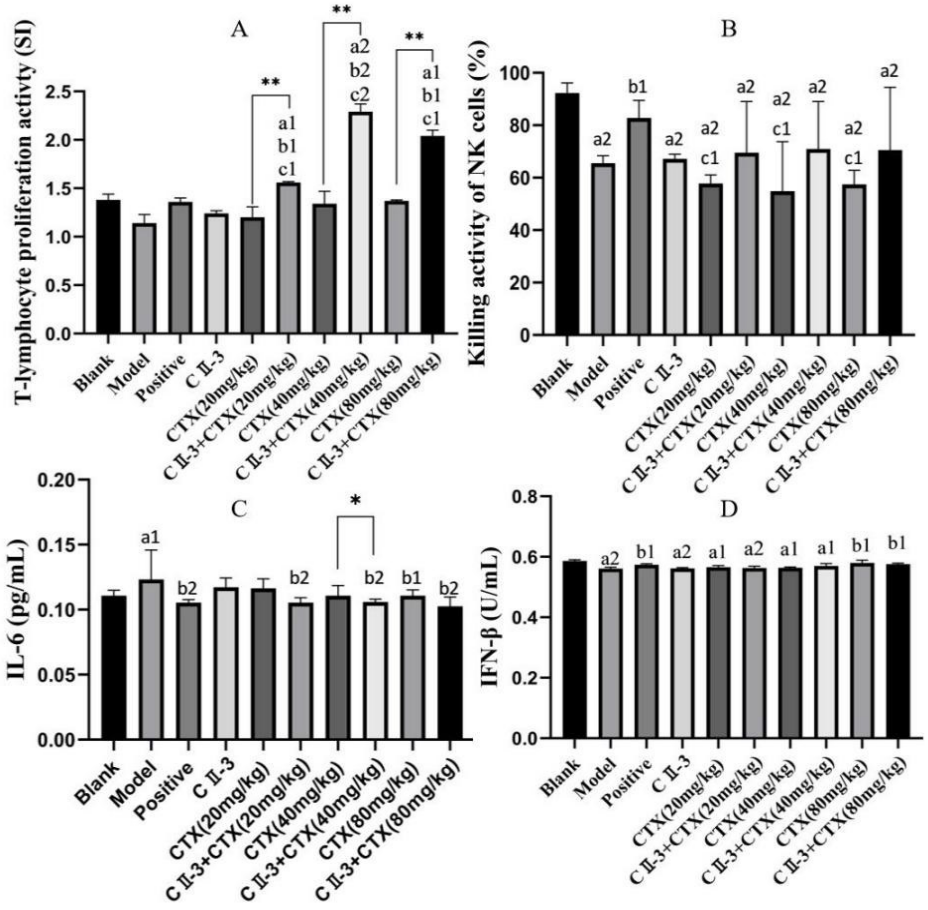

Figure 3: (A) CII-3 combined with CTX on T cell proliferation in H22 tumor-bearing mice. (B) CII-3 combined with CTX on the killing activity of NK cells in H22 tumor-bearing mice. (C) Effects of CII-3 combined with CTX on serum IL-6 and (D) IFN-β in H22 tumor-bearing mice. Compared with blank group, a1$p < 0.05$, a2$p < 0.01$; compared with model group, b1$p < 0.05$, b2$p < 0.01$; compared with positive group, c1$p < 0.05$, c2$p < 0.01$; *$p < 0.05$, **$p < 0.01$.

killing activity of NK cells and improve the body's immunity and reduce the secretion of inflammatory factors.

CII-3 combined with CTX could enhance the activity of spleen T cells

As shown in Figure 4, the lymphocytes in each group were all clustered, and the proportion of $CD4^+$ cells and $CD3^+$ cells were higher. Compared with blank group, $CD3^+$, $CD4^+$ and $CD8^+$ in lymphocytes of model group were significantly decreased. $CD3^+$ was significantly increased in CII-3 combined with CTX group ($p < 0.01$). Compared with the model group, $CD3^+$ and $CD4^+$ were increased in CII-3 combined with CTX (40 and 80 mg/kg) groups, and the ratio of $CD4^+/CD8^+$ was higher than that in the model group, with statistical significance ($p < 0.05$). Compared with the positive group, $CD3^+$ in lymphocytes of CII-3 group was higher than that of the positive group. These results indicated that CII-3 combined with CTX (40 and 80 mg/kg) could protect the immune function of spleen in mice, enhance the activity of spleen T cells, and make them more effectively participate in the anti-tumor immune response.

Attenuated effect of CII-3 combined with CTX on H22 tumor-bearing mice

CII-3 combined with CTX could increase life prolongation rate, body weight gain rate, organ indexes of mice

As shown in Figure 5A, compared with model group, except CII-3 group and CTX (20 mg/kg) group, the life prolongation rate of all the combined administration groups was increase, with statistical significance ($p < 0.05$); although there was no significant difference between different groups ($p > 0.05$), the life extension rate of mice in the CII-3 combined CTX group was higher than that in the same dose of CTX alone group and the CII-3 group, and the life extension rate of mice in the CII-3 combined CTX (80 mg/kg) group was 73.37%. As shown in Figure 5B, compared with the blank group, the body weight proliferation rate of CTX

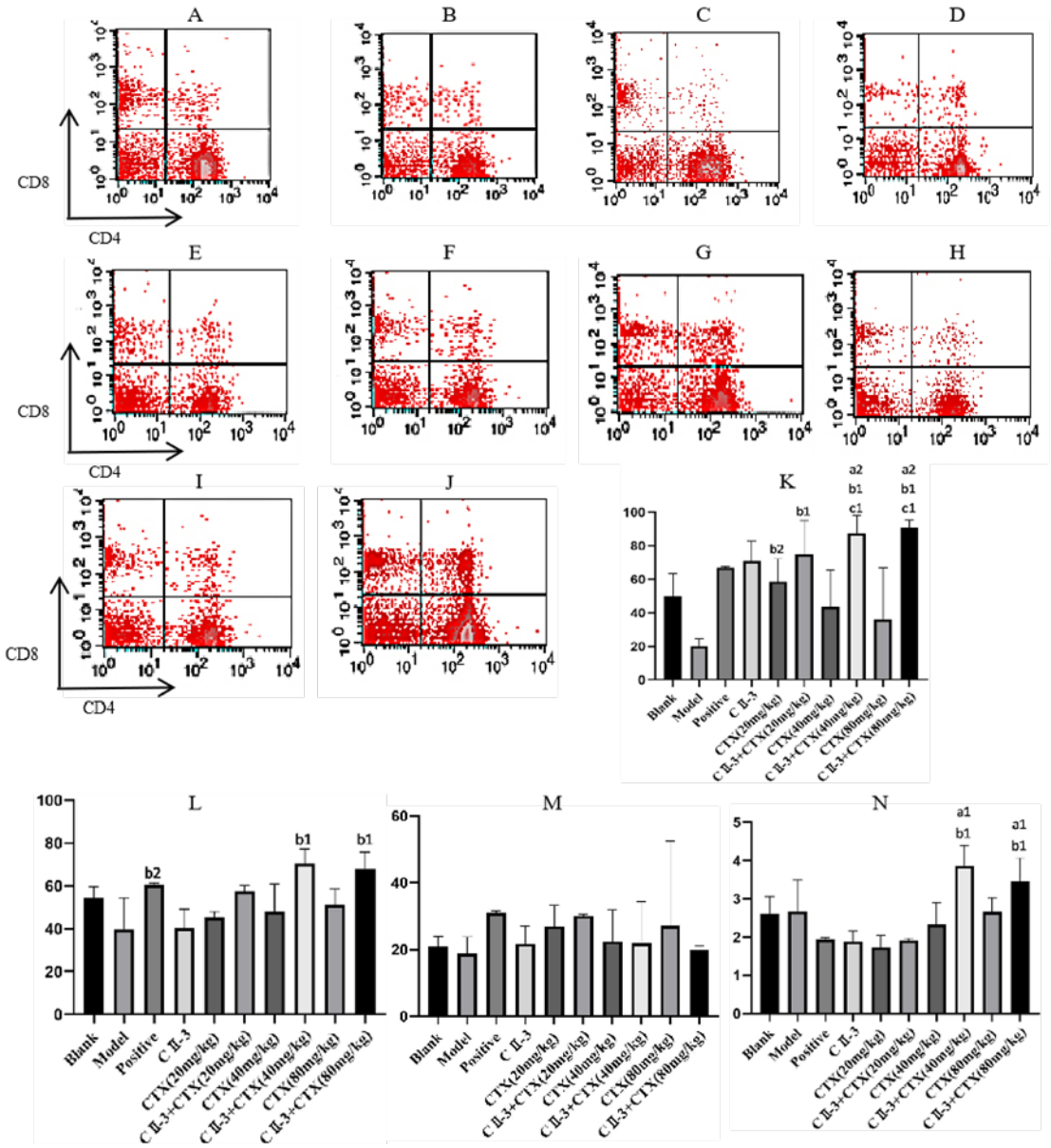

Figure 4: Test of splenic lymphocyte subsets in tumorbearing mice. (A) Blank group; (B) Model group; (C) CII-3 group; (D) CTX (20 mg/kg) group; (E) CTX (40 mg/kg) group; (F) CTX (80 mg/kg); (G) CII-3+CTX (20 mg/kg) group; (H) CII-3-3+CTX (40 mg/kg) group; (I) CII-3+CTX (80 mg/kg) group; (J) Shenqi Fuzheng Injection+CTX (20 mg/kg) group. Analysis of splenic lymphocyte subsets in tumorbearing mice. (K) CD3+; (L) CD4+; (M) CD8+; (N) CD4+/CD8+. Compared with blank group, a1p < 0.05, a2p < 0.01; compared with model group,b1p < 0.05, b2p < 0.01; compared with positive group, c1p < 0.05, c2p < 0.01.

alone group decreased significantly ($p < 0.05$ or $p < 0.01$), except for combined CTX (80 mg/kg), the body weight gain rate of mice in CII-3 combined CTX group was close to that in blank group and model group, and the body weight gain rate of mice in CII-3 group and CII-3 combined CTX (20, 40 mg/kg) group could significantly increase the body weight gain rate of mice, with statistical significance ($p < 0.01$), and was better than the same dose of CTX alone group compared with the positive group, the body weight gain rate of CII-3 group was higher than the positive group, which was statistically significant ($p < 0.05$). As shown in Figure 5C, compared with the blank group, CTX alone could cause obvious thymus atrophy and lower organ index ($p < 0.05$), while CII-3 combined with CTX group increased the organ index of thymus ($p < 0.05$); compared with the model group, CII-3 combined with CTX group and CTX alone group could induce a decrease in the index of thymus and spleen of mice ($p < 0.05$), in which CII-3 combined with CTX group was superior to the same dose of CTX alone group. As shown in Figure 5D, the index of thymus and spleen in CII-3 combined with CTX group was better than that in the same dose of CTX alone group. The CII-3 combined with CTX group (40, 80 mg/kg) and CTX group (40, 80 mg/kg) showed significant difference ($p < 0.01$). The thymus and spleen indices of the positive group were significantly lower than those of the CII-3 group ($p < 0.05$). It suggested that CII-3

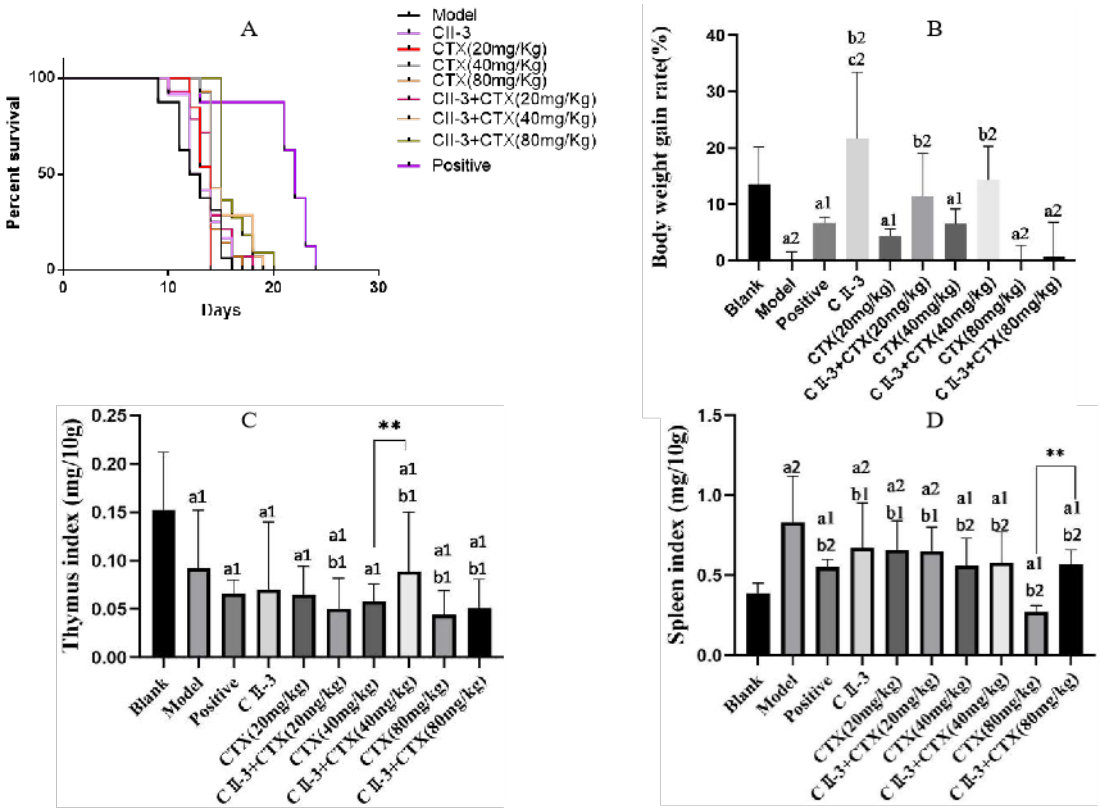

Figure 5: (A) CII-3 combined with CTX on the survival time of mice. (B) CII-3 combined with CTX on the body mass gain rate of H22 tumor-bearing mice; (C) CII-3 combined with CTX on thymus and (D) Spleen index of H22 tumor-bearing mice. Compared with blank group, a1$p < 0.05$, a2$p < 0.01$; compared with model group, b1$p < 0.05$, b2$p < 0.01$; compared with positive group, c1$p < 0.05$, c2$p < 0.01$; *$p < 0.05$, **$p < 0.01$.

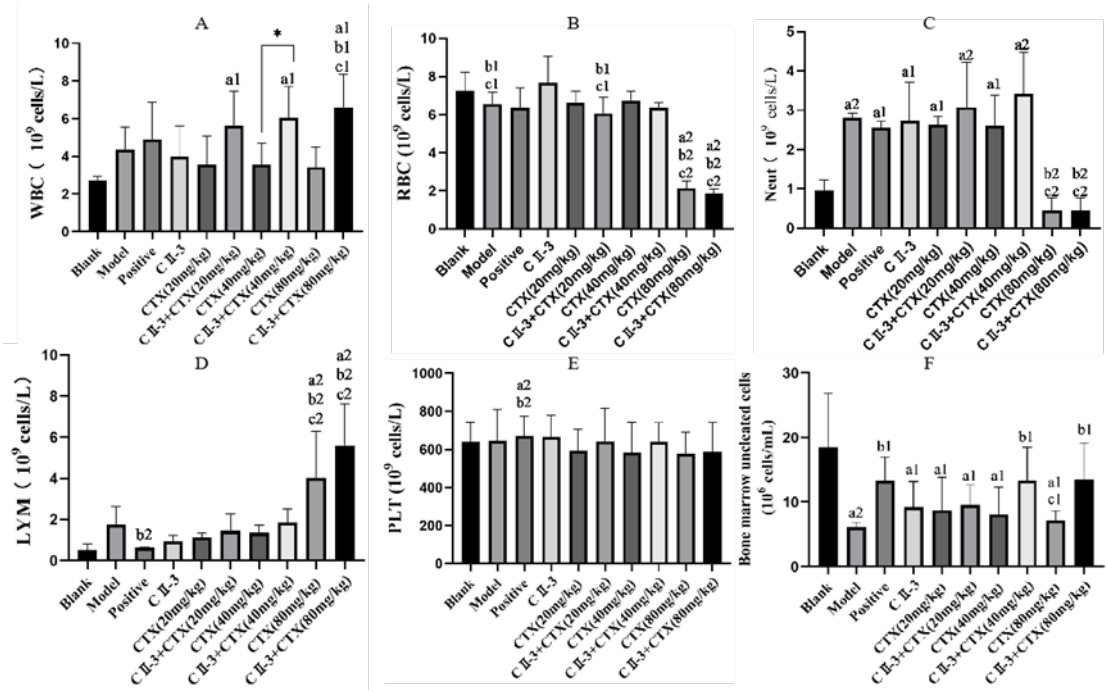

Figure 6: CII-3 combined with CTX on peripheral blood and hematopoietic function of H22 tumor-bearing mice. (A) White blood cells; (B) Red blood cells; (C) Neutrophils; (D) Lymphocytes; (E) Platelets; (F) Bone marrow nucleated cells. Compared with blank group, a1$p < 0.05$, a2$p < 0.01$; compared with model group,b1$p < 0.05$, b2$p < 0.01$; compared with positive group, c1$p < 0.05$, c2$p < 0.01$; *$p < 0.05$, **$p < 0.01$.

combined with CTX group could increase the life prolongation rate of mice, increase the body weight of H22 tumor-bearing mice, and improve the organ index of immune organs, protect and restore the function of thymus and spleen in mice

CII-3 combined with CTX had ameliorate effect on the reduction of WBC, Neut, LYM and Nucleate cells in the bone marrow caused by CTX

As shown in Figure 6, the results of peripheral blood cell count showed that the number of White Blood Cells (WBC) (Figure 6A), Red Blood Cells (RBC) (Figure 6B), Neutrophils (Neut) (Figure 6C), Lymphocytes (LYM) (Figure 6D) and Platelets (PLT) (Figure 6E) in peripheral blood of mice treated with CTX alone decreased. CII-3 combined with CTX (20, 40, 80 mg/kg) increased the amount of WBC and Neut in peripheral blood to different degrees, with significant differences ($p < 0.05$ or $p < 0.01$). Compared with the positive group, the quantities of WBC, NEUT, LYM and PLC in CII-3 group were all increased and higher than those in the positive group. The number of WBC in CII-3 combined with CTX (80 mg/kg) group was significantly higher than that in the positive group ($p < 0.05$). As shown in Figure 6F, compared with the blank group, the number of bone marrow nucleated cells in CTX alone group decreased significantly ($p < 0.05$). Compared with model group, the number of bone marrow nucleated cells in CII-3 combined CTX group was increased in all the other groups except CII-3 combined CTX (20 mg/kg) group, with significant difference ($p < 0.05$), and was better than the same dose of CTX alone group ($p > 0.05$). Compared with the positive group, the number of bone marrow nucleated cells in CII-3 group was close to the positive group. It suggested that CII-3 combined with CTX group had ameliorate effect on the reduction of WBC, Neut and LYM caused by CTX, could improve the hematopoietic function of mice.

The investigation of the synergistic and attenuatory mechanism of CII-3 combined with CTX in the treatment of H22 tumor-bearing mice based on TLRS / NF-κB signaling pathway

The mRNA expression of TLR4, TLR9 and NF-κB was down-regulated in CII-3 combined with CTX group

As shown in Figure 7, compared with model group, CII-3 combined with CTX (20, 40, 80 mg/kg) groups significantly decreased the expression of target gene mRNA ($p < 0.05$). Compared with CTX administration group, the combination group was better than CTX administration group, and the expression of TLR4 mRNA in CII-3 combined with CTX (20, 40, 80 mg/kg) group was significantly down-regulated compared with CTX (20, 40, 80 mg/kg) group ($p < 0.05$), the expression of TLR9 mRNA in CII-3 combined with CTX (40 mg/kg) group was significantly down-regulated than that in CTX ($p < 0.05$), the mRNA expression of NF-κB in CII-3 combined with CTX 20 and 40 mg/kg group was significantly down-regulated compared with that in CTX ($p < 0.05$). It suggested that mRNA expression of TLR4, TLR9 and NF-κB was down-regulated in CII-3 combined with CTX group.

The proteins expression of TLR4, TLR9 and NF-κB in CII-3 combined CTX group was down-regulated

As shown in Figure 8, compared with the model group, the expression of TLR4 protein in tumor tissues of CII-3 combined with CTX group was significantly down-regulated, and the difference was statistically significant ($p < 0.01$). The protein expression levels of TLR9 and NF-κB in tumor tissues of CII-3 combined with CTX (40 and 80 mg/Kg) groups were significantly down-regulated, and the differences were statistically significant ($p < 0.01$). Compared with CTX administration group, the combination group was better than CTX administration group, and the TLR4 protein expression level of CII-3 combined with CTX (20, 40 mg/kg) group was lower than that of CTX (20, 40 mg/kg) group, the difference was statistically significant ($p < 0.01$), the protein expression levels of TLR9 and NF-κB in CII-3 combined with CTX (40 and 80 mg/kg) group were lower than those in CTX (40 and 80 mg/kg) group, and the differences were statistically significant ($p < 0.05$). It suggested that the expression of TLR4, TLR9 and NF-κB in CII-3 combined CTX group was down-regulated.

DISCUSSION

Chemotherapy is an important method for the treatment of malignant tumors. However, its serious adverse reactions greatly limit the clinical therapeutic effect. In order to reduce the side effects of chemotherapeutic drugs, some drugs are often used in combination to reduce the toxicity of chemotherapeutic drugs and enhance the therapeutic effect.[24,25]

In recent years, the research on the mechanism of the killing effect of drugs on tumor cells mainly focuses on the effects of drugs on the proliferation, differentiation and apoptosis of tumor cells, as well as the control of oncogenes and tumor suppressor genes, and immune regulation.[26] As pathogen-associated molecular pattern recognition receptors of innate immunity, Toll-Like Receptors (TLRs) participate in the process of tumor genesis and development. Explore the relationship between TLRs and inflammation-associated tumors has become one of the hot trends in tumor immunology research. At present, studies[27] have shown that natural medicinal polysaccharides have immunomodulatory activity, and this effect is related to TLRs signal transduction process. As a membrane-recognizing receptor protein, TLRs monitor a variety of pathogen-associated molecular patterns and tumor-associated immune systems. TLRS/NF-κB is an important signaling pathway that mediates the mutual expression between inflammatory factors and cells, and plays an important role in the process of tumor development. Dying tumor cells are

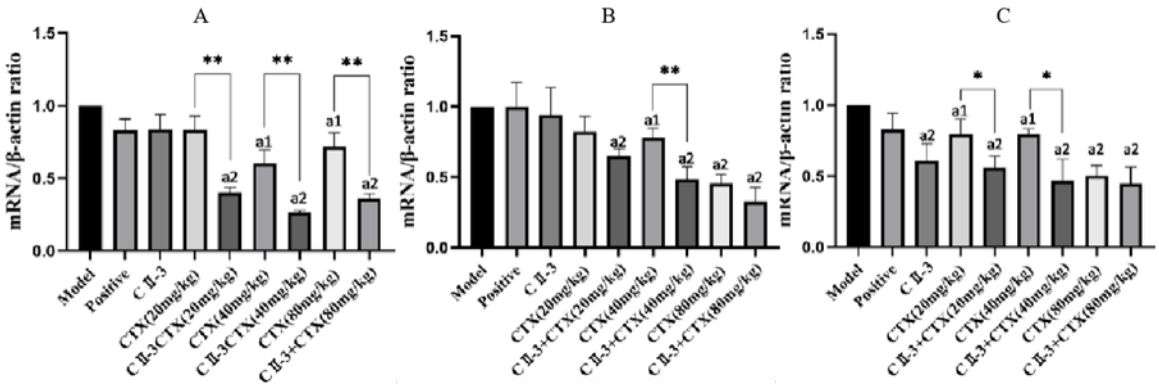

Figure 7: The result of RT-qPCR detection of mRNA expression of (A) TLR4, (B) TLR9 and (C) NF-κB in tumor tissues. Compared with blank group, $^{a1}p < 0.05$, $^{a2}p < 0.01$; $^{*}p < 0.05$, $^{**}p < 0.01$.

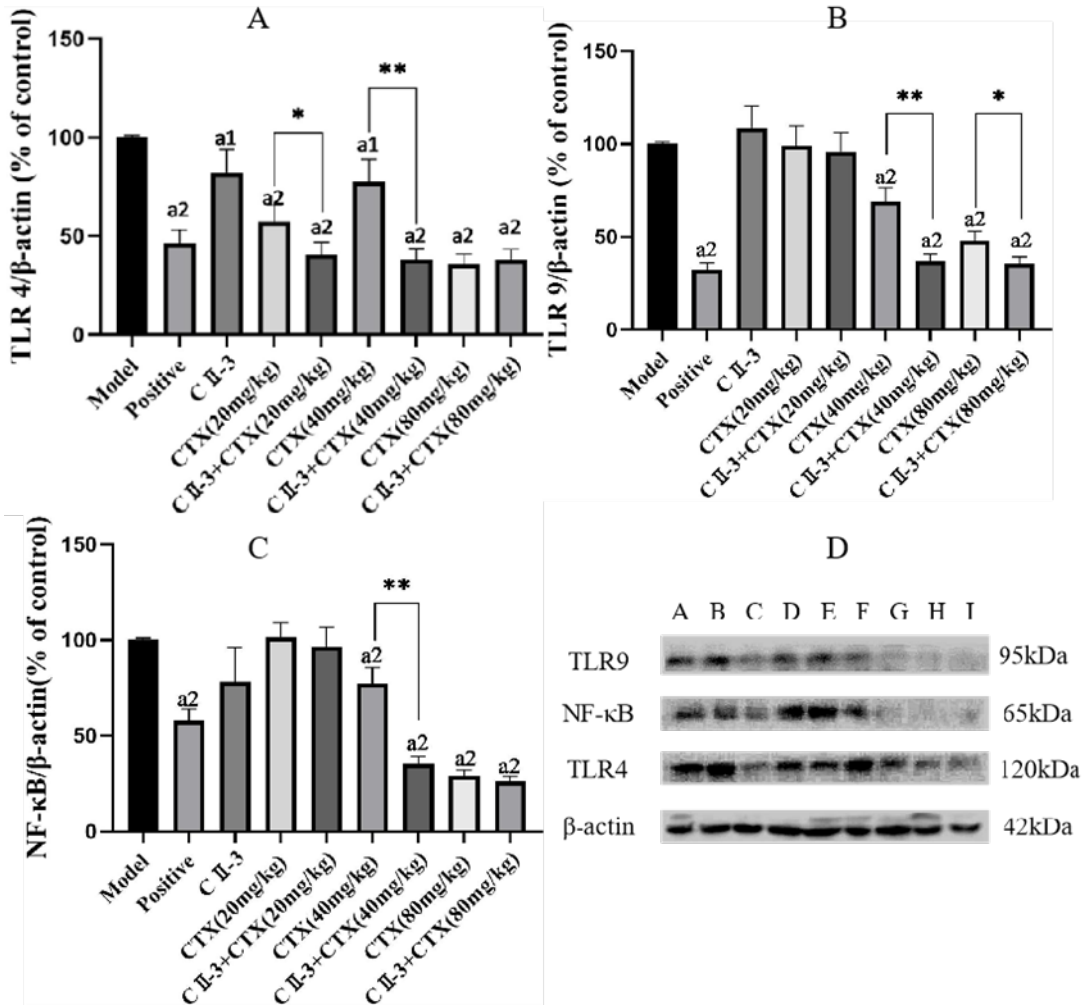

Figure 8: Western Blot detection of the protein expressions of (A) TLR4, (B) TLR9 and (C) NF-κB in tumor mass of H22 tumor-bearing mice treated with CII-3 combined with CTX. Compared with blank group, $^{a1}p < 0.05$, $^{a2}p < 0.01$; $^{*}p < 0.05$, $^{**}p < 0.01$.

able to generate antigenic interactions in a TLR4 and MyD88 dependent manner that can be presented to dendritic cells to trigger an effective immune response, the study showed.[28] This process is mainly mediated by TRIF, which activates NF-κB and regulates the expression of several important cytokines, adhesion molecules and chemokines, promotes tumor cell proliferation, inhibits tumor cell apoptosis, and plays a role in the occurrence and development of tumors. TLR4 has been shown to be involved in the genesis and development of tumor cells.[29-31] TLR4 performs signal transduction through MyD88-independent pathway,

and TLR4 expression is upregulated in liver cancer patients and promotes the occurrence and development of tumors. The specific mechanism needs to be further studied. TLR9 is expressed in a variety of cells and it signaling is dependent on MyD88, ultimately inducing the production of related inflammatory cytokines and the expression of type I IFN (IFN-A and IFN-B) genes, promoting the Th1 immune response[32,33] and Cytotoxic T Lymphocyte (CTL) response,[34] thereby generating a tumor suppressive microenvironment. A large number of recent studies indicate TLR4 expression in HCC tissue specimens, at the meantime further investigation of the signaling of TLR4 induced by LPS involves HCC invasion and metastasis, suggesting that the mechanism may be achieved by the direct activation of NF-κB signaling by TLR4. Combined with the above findings, we conjecture that the mechanism of synergistic and attenuatory effect of CII-3 on CTX in tumor treatment may be achieved by the inhibition of TLR4, TLR9, NF-κB mRNA, and the corresponding proteins expression in the TLR4/NF-κB and TLR9/NF-κB signaling pathways.

In the previous experiments, it has been confirmed that the extract of *Periplaneta americana* CII-3 has a synergistic and attenuated effect on the chemotherapy of H22 tumor-bearing mice.[22,23] The main purpose of this study is to clarify the synergistic and attenuated mechanism of CTX combined with CII-3 in the treatment of tumor. The results showed that CTX combined with CII-3 could enhance the inhibitory effect of tumor cell proliferation, significantly improve the survival time of tumor-bearing mice after CTX treatment, enhance the body mass of mice, and improve the tumor inhibition rate, indicating that CTX combined with CII-3 has a therapeutic synergistic effect in the treatment of tumor-bearing mice. At the same time, CTX combined with CII-3 can improve the spleen and thymus atrophy caused by CTX treatment, and improve the immunosuppression and myelosuppression caused by CTX treatment, suggesting that CII-3 can reduce the toxic and side effects caused by CTX treatment. It was proved that TLR4, TLR9 and NF-κB mRNA and related proteins were expressed in H22 tumor-bearing mice, and compared with the positive group and model group, CTX combined with CII-3 could decrease the expressions of TLR4, TLR9 and NF-κB related genes and proteins in H22 tumor-bearing mice. These results suggested that there are synergistic and attenuated effects of CTX combined with CII-3 on H22 tumor-bearing mice, which might be due to the inhibition of the expressions of TLR4, TLR9 and NF-κB related genes and proteins in the TLR4/NF-κB and TLR9/NF-κB signaling pathways.

However, studies[35] have shown that TLRs have a bidirectional effect on tumor genesis and progression. TLRS agonists can enhance the immune response of immune cells such as T cells and B cells. On the other hand, activation of TLRs can lead to the secretion of inflammatory factors and promote the formation of tumor microenvironment. The existence of tumor microenvironment promotes the proliferation, migration and immune escape of tumor cells, thus promoting the occurrence and development of tumor. How to hold the balance and inhibit the development of tumor through TLRS pathway is an urgent problem to be solved.

CONCLUSION

Our study demonstrated that CTX combined with CII-3 can enhance the therapeutic effect of CTX and reduce the toxic and side effects caused by treatment, and the mechanism may be the inhibition of the expression of TLR4, TLR9 and NF-κB related genes and proteins in the TLR4/NF-κB and TLR9/NF-κB signaling pathways. This study is helpful for the development of new drugs and the improvement of clinical chemotherapy effect.

FUNDING SOURCE

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CTX: Cyclophosphamide; **RBCs:** Red blood cells; **WBCs:** White blood cells; **TCM:** Traditional Chinese medicine; **Neut:** Neutrophils; **PLT:** Platelets; **LYM:** Lymphocytes; **SD:** Standard deviation; **RT-qPCR:** Real-time quantitative polymerase chain reaction; **TLRs:** Toll-like receptors; **CTL:** Cytotoxic T lymphocyte.

SUMMARY

We found out that CTX combined with CII-3 can enhance the therapeutic effect of CTX and reduce the toxic and side effects caused by treatment, and in this article, our aim is to explain the mechanism of the synergistic and attenuated effects of CII-3 combined with CTX in the treatment of H22 tumour-bearing mice. It turned out the effects might be mediated by the TLR4/NF-κB and TLR9/NF-κB signaling pathways.

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