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Ginsenoside Rg3 improves cyclophosphamide-induced immunocompetence in Balb/c mice

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ABSTRACT

Ginsenoside Rg3 (Rg3), which comprises *Panax ginseng*, is commonly used to improve the immunocompetence of cancer patients undergoing chemotherapy. This study was designed to elucidate the immunoenhancement effects of Rg3 in immunosuppressed mice induced by cyclophosphamide (CTX) treatment. Balb/c mice were administered Rg3 intragastrically once daily for 19 consecutive days and were intraperitoneally administered CTX (80 mg/kg) on days 15–19. Weight and immune organ indices were recorded. Hematological tests and cytokines were assessed using ELISA. We measured the activity of LDH and ACP, performed pathological and immunohistochemical staining of immune organs, and evaluated cytokines and transcription factors using RT-PCR. Immunosuppressed mice showed weight loss, decreased thymus and spleen indices and severe pathological damage. CTX attenuated macrophage phagocytosis by decreasing activity of LDH and ACP and decreased the release of related immune factors, IgG, IL-2 and G-CSF. Subsequently, we observed T lymphocyte expression on the surface of the thymus and spleen, which inhibited T cell activity. Further mechanistic analysis showed that CTX decreased the expression of T-bet and IFN-γ and increased the expression of GATA-3 and IL-4 in the thymus and spleen, which affected the Th1/Th2 balance. However, Rg3 treatment reversed CTX-induced immunosuppression. In summary, all the results suggest that Rg3 has protective effects on CTX-induced immunosuppression, which could be partially related to macrophages, T cells and Th1/Th2 balance. Although deeper studies of its mechanism are needed, these findings support the hypothesis that Rg3 can improve the reduced immunocompetence after CTX injury.

1. Introduction

Cyclophosphamide (CTX) has been used in the clinic for > 50 years as a conventional alkylating drug and is extensively used in oncology as a chemotherapeutic agent. It is considered to be a first-line treatment for leukemia and solid tumors [1]. CTX inhibits the proliferation of cancer cells and has serious adverse effects on normal cells. Its use in chemotherapy can lead to significant morbidity and mortality, which is the main factor limiting its therapeutic effects in clinical chemotherapy. In recent years, the immunosuppression caused by chemotherapy has been given an increasing amount of attention. Although monotherapy regimens for cancer have yielded some success, there are significant limitations to response rates and therapy durations [2]. In the 1960s [3], combination therapy with two or more drugs was proposed, and has thus far been used in various cancer treatments to reduce adverse effects and enhance the chemotherapeutic effect [4–7]. Based on these limitations and on preclinical evidence for potential synergy between immunotherapy and other treatment modalities [8], there is now tremendous enthusiasm for combination strategies in cancer therapy. However, both individually and in combination, these therapies have been shown to have many limitations and drawbacks. For survival with cancer, traditional herbal medicines might play an important role [9].

Panax ginseng (*Panax ginseng* C. A. Mey) has been used as a traditional tonic for energizing the body and for longevity in China for thousands of years. At present, the research and clinical application of *Panax ginseng* (*P. ginseng*) are diverse and most noticeably focus on cancer treatment. *P. ginseng* apparently mitigates cancer through anti-inflammatory, antioxidant, and anti-apoptosis effects that influence

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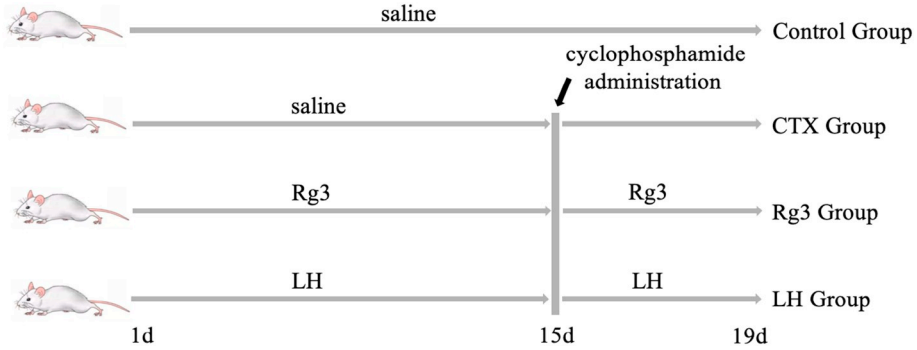

Fig. 1. Flow chart of the experiment procedure. Rg3 was administered intragastrically to mice, while the others were injected intraperitoneally. The samples were taken 24 h after the completion of administration on the 19th day.

gene expression, neurotransmission and immunosurveillance. Ginseng is a potential therapy to prevent autoimmune and allogenic immune disorders and has begun to receive more attention [10–12]. Low toxicity and positive studies on concomitant use with other chemotherapeutic agents are promising [13].

Pharmacological studies have proven that ginsenoside Rg3 (Rg3) has various biological activities, including enhancing immunity [14] and antioxidant and neuroprotective effects [15]. It can also attenuate ADM-induced cardiotoxicity to improve endothelial dysfunction by improving heart function, reverse vascular dysfunction [16], and reduce blood toxicity after chemotherapy. Some studies have shown that Rg3 has anti-tumor and immunomodulatory effects [14,17–19], and some have demonstrated that Rg3 is less toxic in vivo. Rg3 can effectively enhance cellular immune function in immunocompromised mice [20], which stimulates lymphocytes from the normal body to increase the number of T lymphocyte subsets of CD_4^+ cells in the body [21], and 20 (*R*)-Rg3 can also significantly enhanced both Th1 and Th2 immune responses to OVA in mice [22]. However, there is also a report that showed contrasting effects of Rg3 on Th1, including suppressed Th1 cell differentiation and diminished Th1 cell abundance in the mouse gut in vivo [23]. Immunosuppression induced by chemotherapy is not conducive to improved prognosis or quality of life, and it is necessary to further explore the effect and mechanism of Rg3 combined with chemotherapy on immune function.

In this present study, Rg3 was investigated for its immunocompetence activity in vivo. We evaluated the protective effects of Rg3 against immune injury by the chemotherapeutic agent CTX in BALB/c mice and explored its mechanism of action.

2. Materials and methods

2.1. Animal

Fifty male BALB/c mice (6–8 week) weighing 20 ± 2 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Certificate No. SCXK (Jing) 2016-0006, Beijing, China). The study was carried out in strict accordance with the recommendations of the Guidelines for the care and use of laboratory animals issued by the Ministry of Science and Technology of China. The agreement was approved by the Laboratory Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (Permit No. TCM-LAEC20170044).

2.2. Reagents and drugs

The 20(*R*)-ginsenoside Rg3 extracted from the root of *P. ginseng* was from Chengdu Man Site Bio-Technology Co. Ltd. (Chengdu, China), and the purity of the Rg3 was greater than or equal to 98%. CTX was purchased from Jiangsu Sheng Di Medicine Co. Ltd. Levamisole hydrochloride (LH is a known immunomodulatory agent) was obtained from the Shanxi Hong Bao Veterinary Medicine Co. Ltd. (Shanxi, China), and 0.9% NaCl was obtained from the Otsuka Pharmaceutical Co. Ltd. (Tokyo Metropolitan, Japan). Hydrogen peroxide (3%) was obtained from Sigma (H_2O_2, St Louis, USA). EDTA-2K was from Amresco Inc. (Ohio, USA), and 10% formalin was obtained from Shanghai Ha Ling Biotechnology Co. Ltd. (Shanghai, China).

2.3. Immunosuppressive model and drug administration

The mice were randomly divided into five groups: control group (Con), CTX group (80 mg/kg), 7.5 mg/kg Rg3 group (clinical equivalent dose), 15 mg/kg Rg3 group, and positive LH group (30 mg/kg). The mice in the control and CTX groups were treated with saline. The mice treated with Rg3 and LH groups were administered for 19 consecutive days. On the 15th day, the mice in all of the groups except the control group were administered 80 mg/kg CTX intraperitoneally for 5 days (Fig. 1). Each group included 10 animals.

2.4. Immune organ indices

The thymuses and spleens of 7 mice were cleaned and weighed with an electronic balance, and the following indices were calculated:

$$\text{Index (mg/10 g)} = \text{immune organ weight (mg)/body weight (g)} * 10$$

2.5. Hematological examination

EDTA-2K was used for adequate anticoagulation. Blood counts were performed using a hematology analyzer (MC-6200, Icubio Biotechnology, Shenzhen, China). The analysis included white (WBC) and red blood cell (RBC) counts, platelet count (PLT), lymphocyte percentage (LY%), neutrophil ratio (NE%), monocyte ratio (MO%), and mean hemoglobin concentration (MCHC).

2.6. Biochemical assay

The thymus and spleen organ were prepared into a 5% tissue homogenate. After determining the protein concentration of the homogenate (Beyotime Biotechnology, Shanghai, China), the activity was determined using lactate dehydrogenase (LDH) and acid phosphatase (ACP) kits (Nanjing Jiancheng Bioengineering Institute, Wuhan, China) following manufacturer instructions, and the absorbance value at 450 nm was read for statistical analysis.

2.7. Cytokine assay

Blood was taken from the canthus in mice and centrifuged at 4 °C for 3000 rpm × 10 min. Cytokines were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's

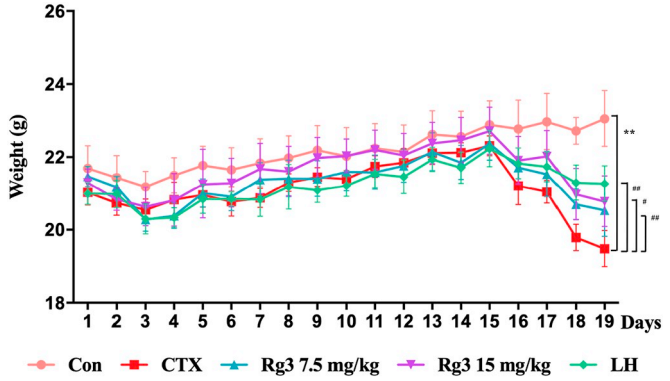

Fig. 2. Effects of Rg3 on the weight of immunosuppression mice. Weight was recorded from day 1 and administered accordingly. The data expressed as the mean ± SEM. $N = 10$ in each group. $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the CTX-induced group.

instructions (Uscn Business Co. Ltd. Wuhan, China). Immunoglobulin G (IgG) was determined by the competitive method, and interleukin-2 (IL-2) and granulocyte colony-stimulating factor (G-CSF) were determined by the double antibody method.

2.8. Pathology and immunohistochemistry

All organ tissues were fixed in 10% formalin for 72 h and embedded in paraffin with standard techniques. Then, 4 μm sections were cut and stained with hematoxylin-eosin (HE staining). The immunohistochemical detection of T lymphocyte subsets was performed with an SABC kit (Boster Biological Technology, Wuhan). Endogenous peroxidase was inactivated with 3% H_2O_2. Antigen retrieval was performed in hot water bath using sodium citrate buffer (Solarbio, Beijing, China) and EDTA-antigen repair solution (BBi Life Science, China). Sections were incubated with diluted anti-CD3 antibody (1:50; Abcam, Cambridge, UK), anti-CD8 antibody (1:200; Abcam, Cambridge, UK), and anti-CD4 antibody (1:900; Abcam, Cambridge, UK) overnight at 4 °C. Biotin-labeled secondary antibodies were added to the slides, and horseradish peroxidase (HRP)-labeled streptavidin was added. After staining with a DAB kit (Boster Biological Technology, Wuhan, China) and counterstaining with hematoxylin, the slides were recorded using a digital camera (Nikon Eclipse Ti-SR, Japan).

2.9. Quantification of target genes by real-time PCR

RNA was extracted from sorted cells with Trizol and reverse transcribed into cDNA with the GoScript Reverse Transcription System (Promega, Madison, USA). The cDNA was used in a qPCR reaction to assess the expression of T-Cell-Specific T-Box Transcription Factor T-bet (T-bet), trans-acting T-cell-specific transcription actor GATA-3 (GATA-3), interferon-γ (IFN-γ) and interleukin-4 (IL-4) in the thymus and spleen tissues. Real-time PCR was performed on an ABI 7500 System (Applied Biosystems, Foster City, USA) with Go Taq qPCR Master Mix (Promega, Madison, USA). The nucleotide sequences of the primers used were as follows: β-actin (Forward, CATC CGTA AAGA CCTC TATG CCAAC, Reverse, ATGG AGCC ACCG ATCC ACA), T-bet (Forward, CTGC CTAC CAGA ACGC AGA, Reverse, AAAC GGCT GGGA ACAG GA), GATA-3 (Forward, GGAT GTAA GTCG AGGC CCAAG, Reverse, TTGC AAAG GTAG TGCC CGGTA), IFN-γ (Forward, CGGC ACAG TCAT TGAA AGCC TA, Reverse, GTTG CTGA TGGC CTGA TTGTC), IL-4 (Forward, ACGG AGAT GGAT GTGC CAAAC, Reverse, AGCA CCTT GGAA GCCC TACA GA). The expression of related target genes was determined by the $2^{-\Delta\Delta CT}$ method.

2.10. Statistical analysis

Statistical analyses were performed with Statistical Package for Social Sciences (SPSS, Chicago, USA) software (Version 22.0). The experimental data were expressed as the mean ± standard error (X ± SEM). Differences in CD_3^+, CD_4^+ and CD_8^+ expression were analyzed by Image-pro Plus 6.0, then the mean Integral Optical Density (IOD) was measured, and the IOD values of slices in each group were calculated. One-way ANOVA was used to compare the differences among the groups, and the conventional $P < 0.05$ was the cut-off for significance.

3. Results

3.1. Effect of Rg3 on body weight and immune organ indices in immunosuppressed mice

By analyzing the body weight and organ indices, we found that there was an effect of Rg3 combined with CTX on immunosuppression. The representative body weight in the control group, the CTX group and the cotreatment group are presented in Fig. 2. As shown in Fig. 2, the weight in the CTX group began to drop linearly beginning at 16 d ($P < 0.01$), and the cotreatment group showed restoration of mouse weight to varying degrees ($P < 0.05$, $P < 0.01$).

As shown in Fig. 3, the thymus and spleen indices in CTX-treated mice receiving various treatment protocols were tested. The CTX group markedly reduced the thymus and spleen indices ($P < 0.01$). Cotreatment with either Rg3 (7.5, 15 mg/kg) or LH and CTX significantly increased the spleen and thymus indices ($P < 0.05$).

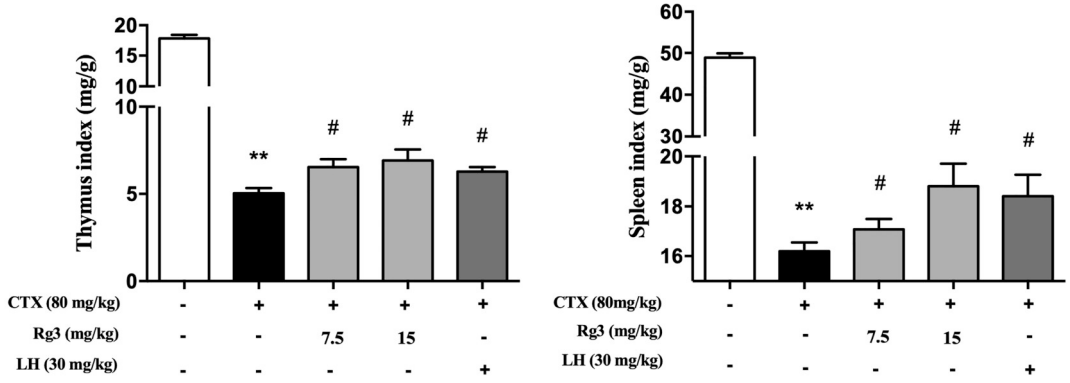

Fig. 3. Effects of Rg3 on the immune organ indices of immunosuppression mice. Mice were injected with 80 mg/kg CTX for 5 d, and Rg3 was continuously administered for 19 d to determine the wet weight of thymus and spleen. The data expressed as the mean ± SEM. N = 10 in each group. $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$ versus the CTX-induced group.

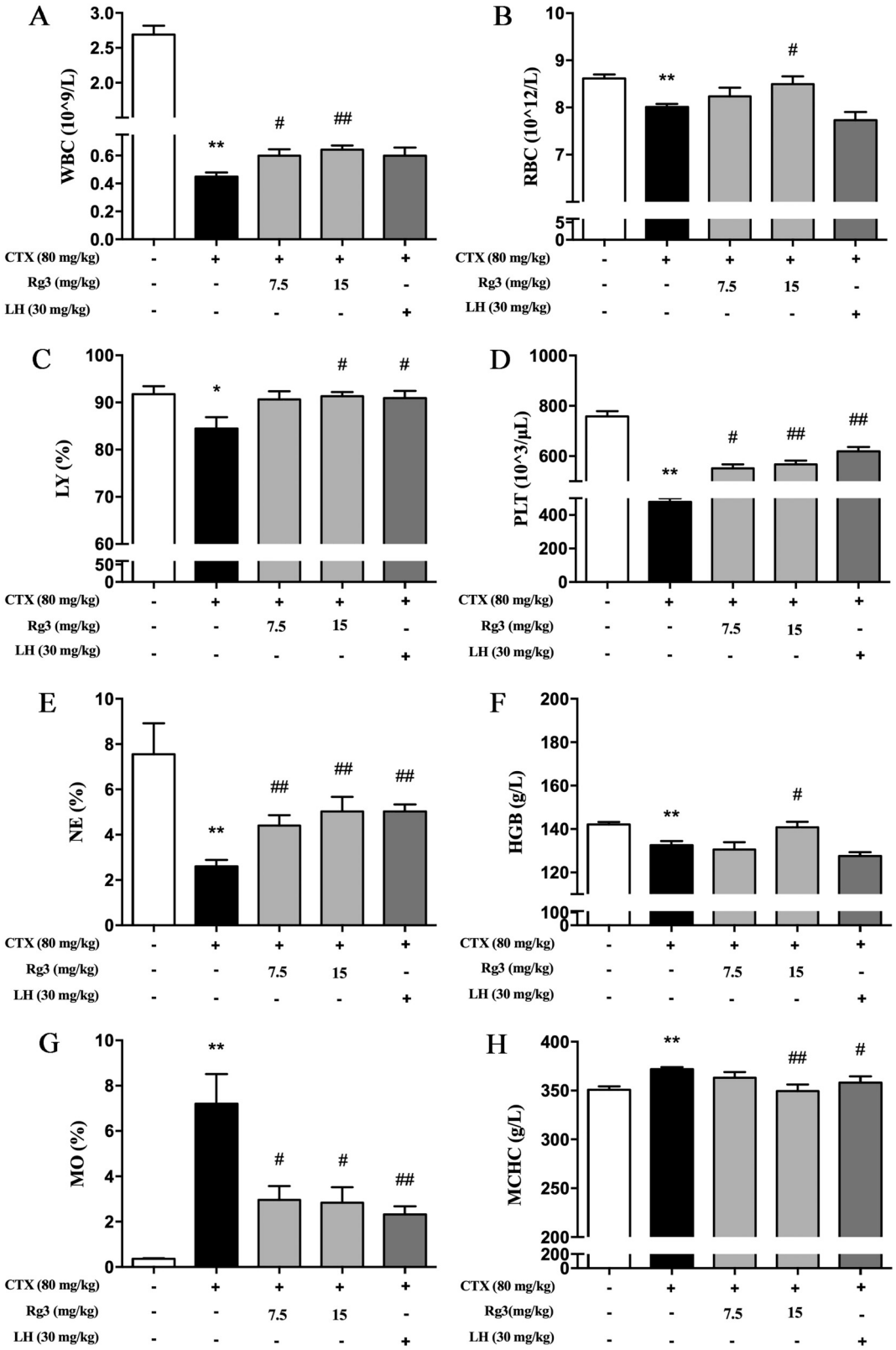

Fig. 4. Effects of Rg3 on hematological indices of immunosuppression mice. After 24 h from the last administration, the peripheral blood was taken for anticoagulation and blood count detection. (A) WBC, (B) RBC, (C) LY%, (D) PLT, (E) NE%, (F) HGB, (G) MO%, and (H) MCHC. The data are expressed as the mean ± SEM. N = 10 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the CTX-induced group.

3.2. Effect of Rg3 on hematological indices in immunosuppressed mice

The peripheral blood routine of each group was detected on 20 d, and the results are shown in Fig. 4A–H. Compared with the control group, the WBC, RBC, LY%, PLT, NE% and HGB were significantly decreased, and the MO% and MCHC values were significantly increased in the mice treated with CTX ($P < 0.05$, $P < 0.01$). Cotreatment with Rg3 (7.5 mg/kg, 15 mg/kg) significantly increased the WBC, PLT, and

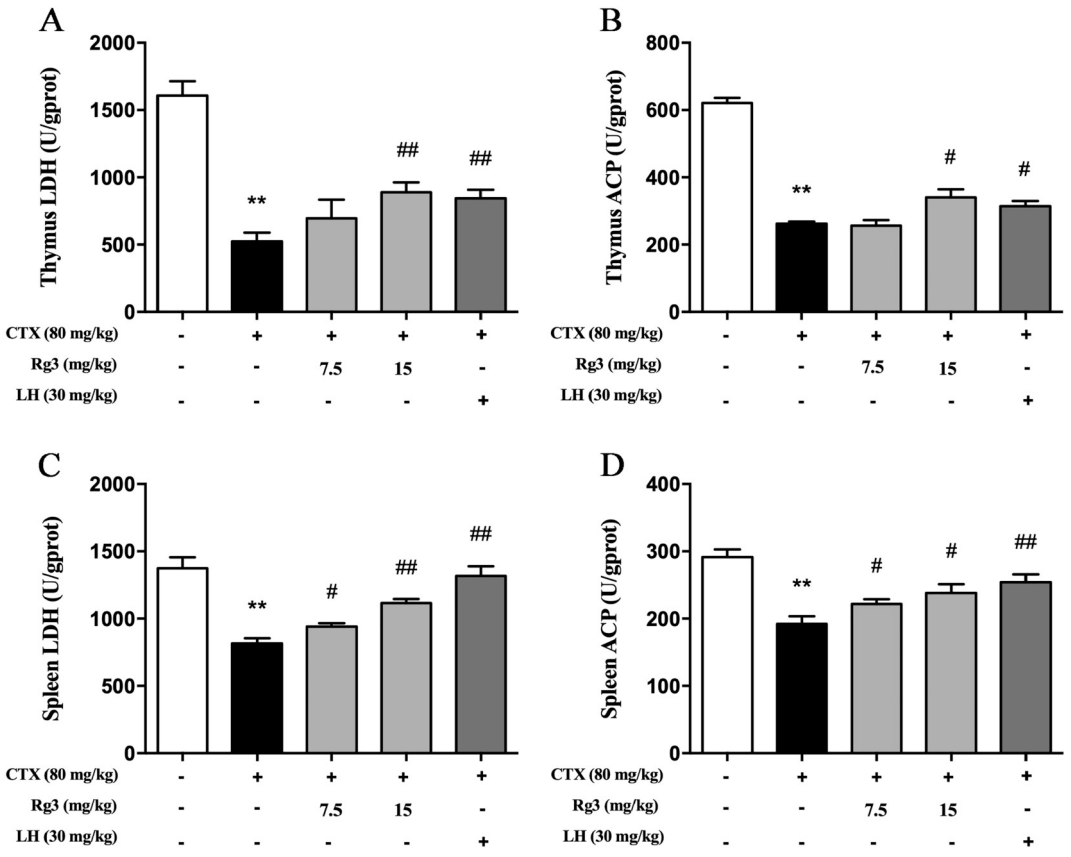

Fig. 5. Effects of Rg3 on the activities of LDH and ACP in immune organ of immunosuppression mice. The activities of LDH and ACP in thymus and spleen tissue homogenates were assayed after 24 h from the last administration in each group. (A) LDH in the thymus, (B) ACP in the thymus, (C) LDH in the spleen, and (D) ACP in the spleen. The data are expressed as the mean ± SEM. $N = 7$ in each group. $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the CTX-induced group.

NE% and decreased the MO% ($P < 0.05$, $P < 0.01$). Rg3 (15 mg/kg) significantly increased RBC, LY%, and HGB and decreased MCHC ($P < 0.05$, $P < 0.01$).

3.3. Effects of Rg3 on phagocytosis on macrophages in immunosuppressed mice

Phagocytosis by phagocytes was measured by detecting the LDH and ACP of the thymus and spleen. Compared with the control group, the activities of LDH and ACP in the thymus were significantly decreased in the mice treated with CTX ($P < 0.01$). Cotreatment with Rg3 (15 mg/kg) or LH and CTX significantly increased the activity of LDH and ACP ($P < 0.05$, $P < 0.01$) (Fig. 5A, B). The activities of LDH and ACP in the spleen were significantly decreased in mice treated with CTX ($P < 0.01$). Cotreatment with Rg3 (7.5 mg/kg, 15 mg/kg) or LH and CTX significantly increased the activity of LDH and ACP ($P < 0.05$, $P < 0.01$) (Fig. 5C, D).

3.4. Effect of Rg3 on cytokines in immunosuppressed mice

The immunomodulatory effects of cytokines were detected by measuring IgG, IL-2 and G-CSF in serum. As shown in Fig. 6, the release

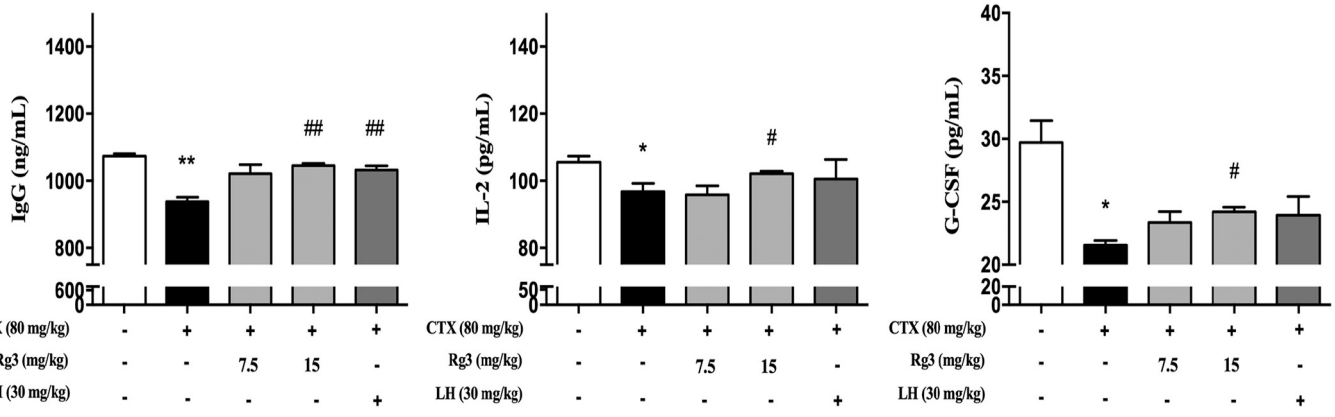

Fig. 6. Effects of Rg3 on the release of cytokines in immunosuppressed mice. The levels of cytokines in serum were quantified by enzyme-linked immunosorbent assay after the end of administration. The data are expressed as the mean ± SEM. N = 10 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the CTX-induced group.

of IgG, IL-2 and G-CSF was significantly decreased in the mice treated with CTX ($P < 0.05$, $P < 0.01$). Cotreatment with Rg3 (15 mg/kg) or LH and CTX significantly increased the release of IgG ($P < 0.01$). Rg3 (15 mg/kg) and CTX significantly increased IL-2 and G-CSF ($P < 0.05$), whereas cotreatment with Rg3 (7.5 mg/kg) or LH and CTX showed a trend of increased IL-2 and G-CSF, but it was not significant.

3.5. Effects of Rg3 on histopathological changes in immunosuppressed mice

Histological analysis of the thymus and spleen are shown in

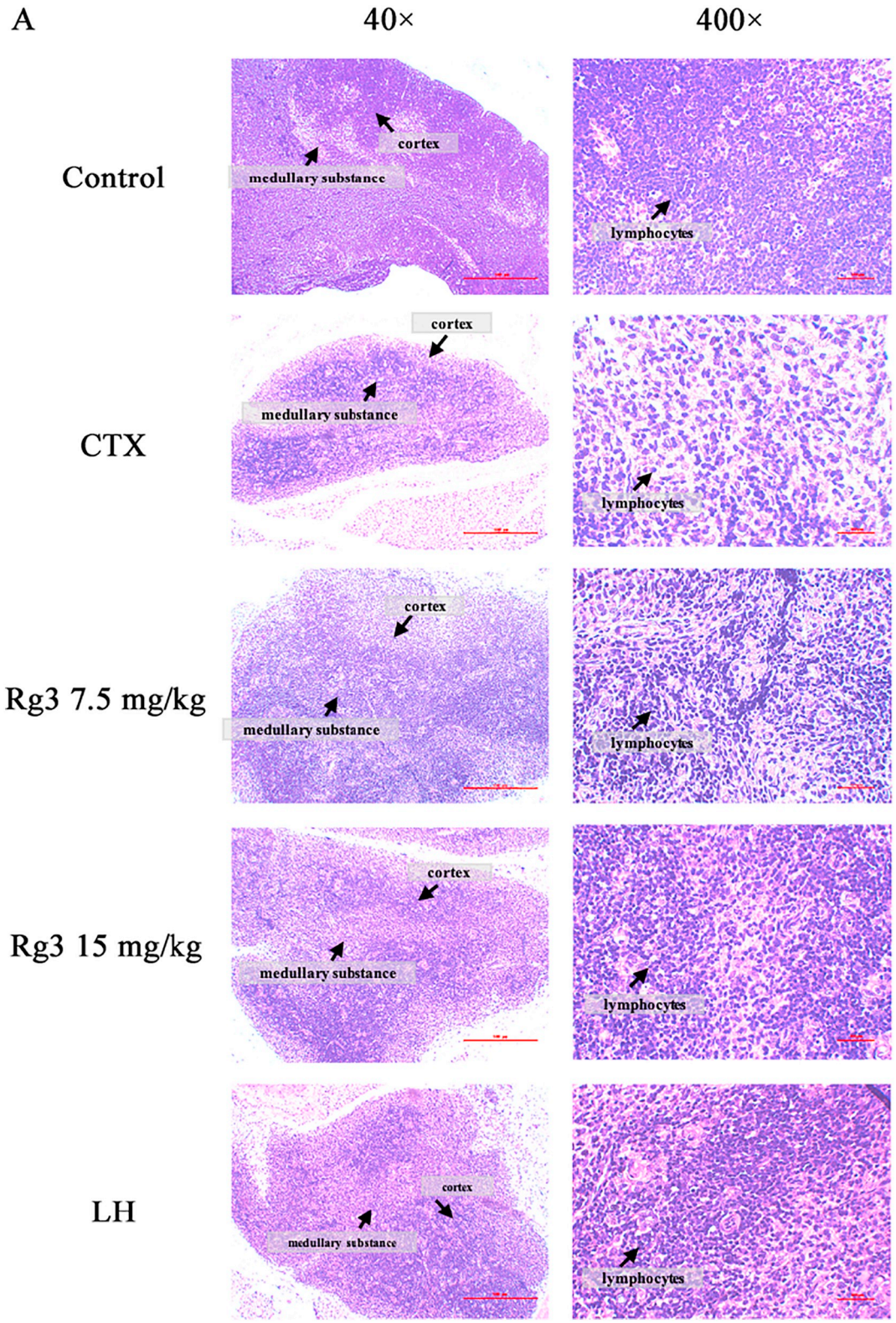

Fig. 7. Effect of Rg3 on HE staining of the thymus (A) and spleen (B). Mice were injected with 80 mg/kg CTX for 5 d, and Rg3 was continuously administered for 19 d. Mouse thymus and spleen tissues were embedded in paraffin and sectioned into 4 μm segments and then stained with baked slices and observed under microscope. $N = 3$ in each group (HE, 40×, 100×, 400×).

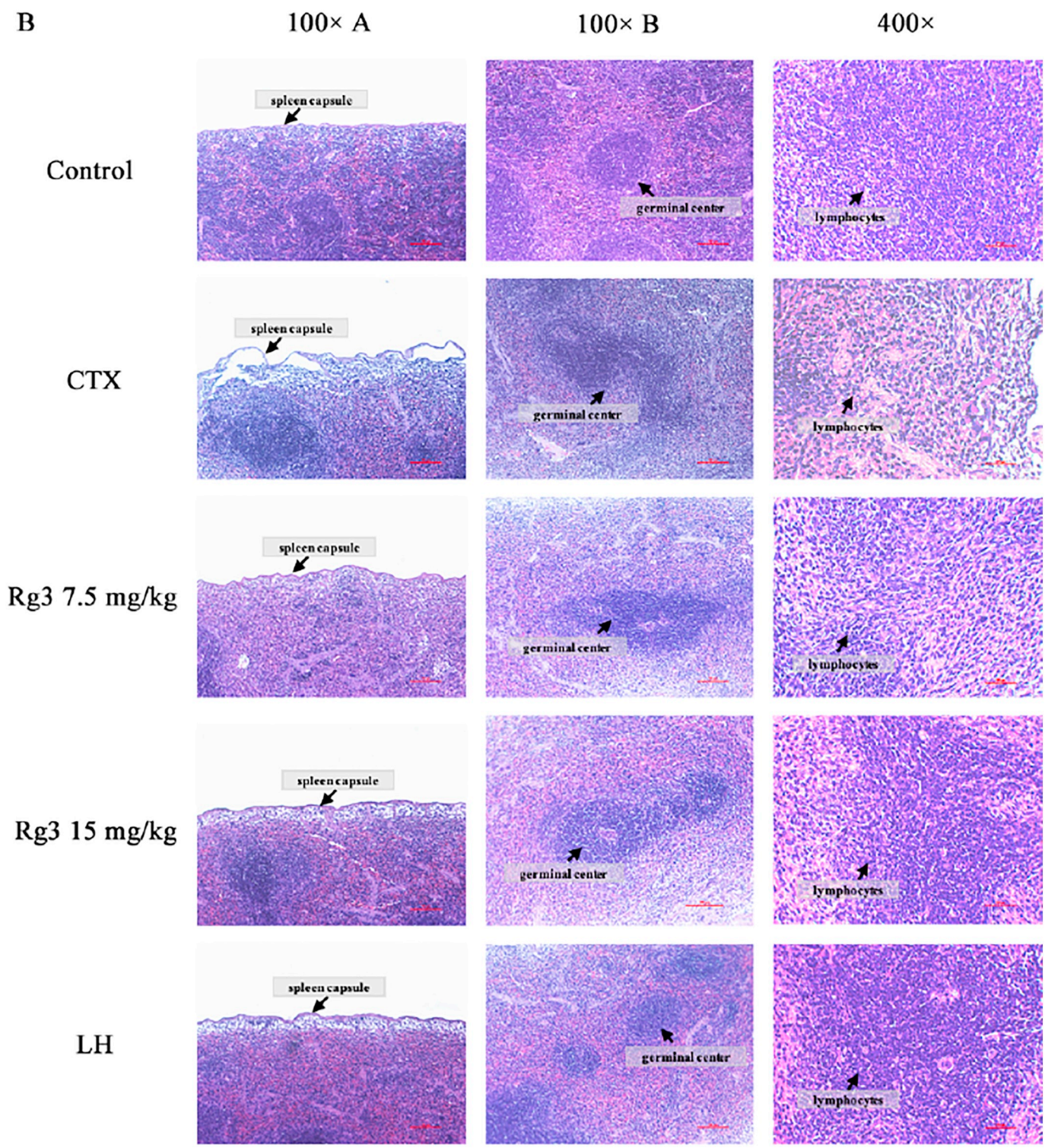

Fig. 7. (*continued*)

Fig. 7A–B. After HE staining, compared with the control group, the CTX group showed thymus gland atrophy, cortex thinning, medullary substance enlargement (Thymus 40×), and irregular, round lymphocytes (Thymus 400×). After treatment administration, in the Rg3 cotreatment (7.5 mg/kg or 15 mg/kg) or LH and CTX groups, the thymus lobules tended to be normal and showed clear demarcation of the cortex and medullary substances (Thymus 40×). The number of lymphocytes was significantly increased, and the lymphocytes tended to be round (Thymus 400×). This result indicated that Rg3 improved thymus damage from CTX in mice (Fig. 7A).

As shown in Fig. 7B, in the CTX group, the spleen capsule was destroyed (Spleen A 100×), the germinal center was scattered (Spleen B 100×), and the number of lymphocytes were decreased and sparse (Spleen 400×). After treatment administration, in the Rg3 cotreatment (7.5 mg/kg or 15 mg/kg) or LH and CTX groups, the spleen capsule tended to be intact (Spleen A 100×), and the germinal center converged (Spleen B 100×). The number of lymphocytes was significantly increased, and the lymphocytes were arranged tightly (Spleen 400×).

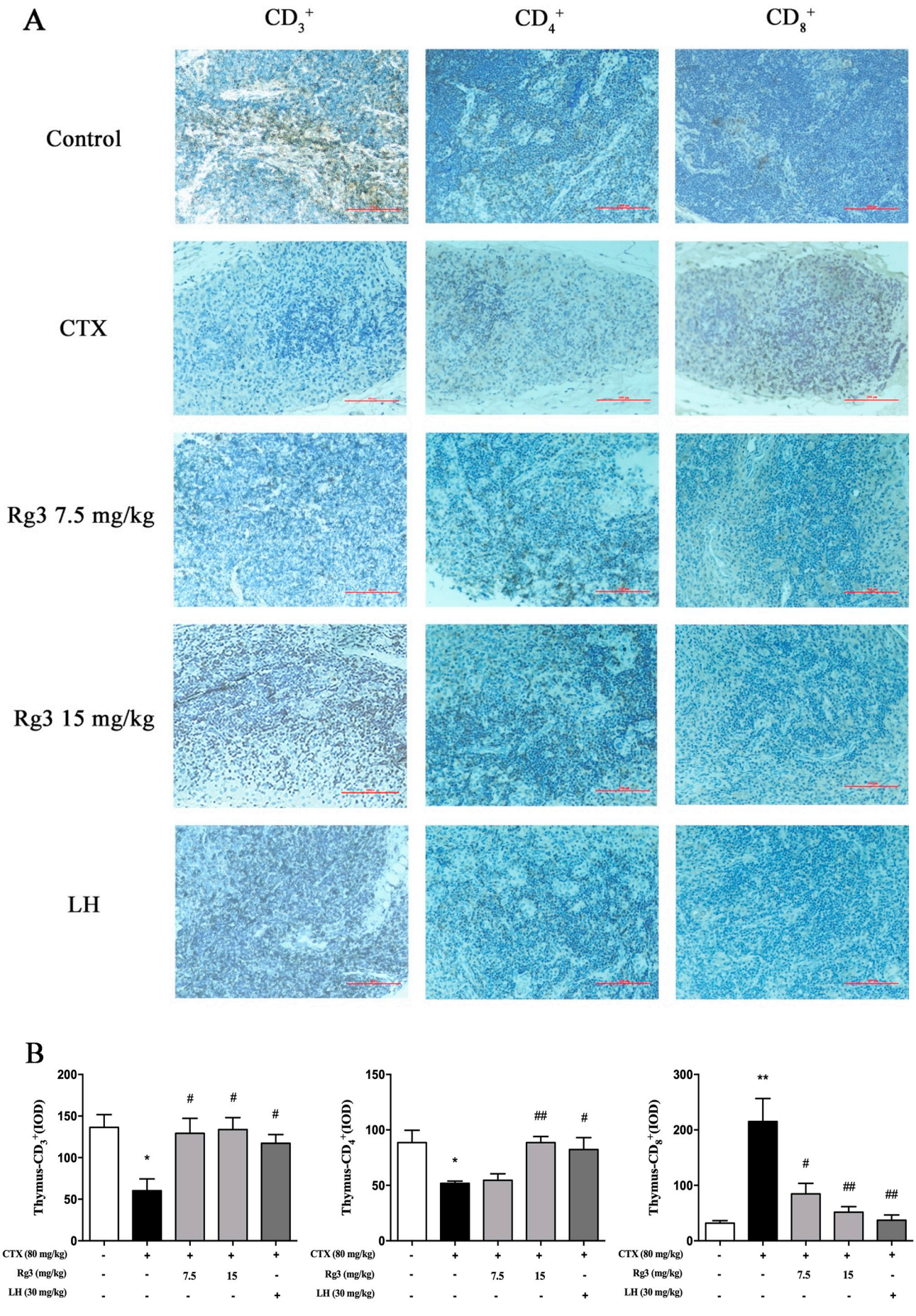

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Fig. 8. Effect of Rg3 on the expression and IOD values of CD_3^+, CD_4^+, and CD_8^+ in the thymus (A, B) and spleen (C, D). Mouse thymus and spleen tissues were embedded in paraffin and sectioned into 4 μm segments. The antigen was retrieved by hydration of baked slices. After overnight incubation with the primary antibody, the secondary antibody was added. Then, DAB coloration was performed, and hematoxylin was restained and observed under microscope (200×). Each group had 3 slices selected from 6 randomly selected visual fields on the immunohistochemical sections, and 10 × 20 images were collected and processed with image analysis software. The data are expressed as the mean ± SEM. $N = 6$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the CTX-induced group.

The result indicated that Rg3 improves mouse spleen damage induced by CTX.

3.6. Effects of Rg3 on T cell expression in immunosuppressed mice

Microscopically, the results of thymus and spleen immunohistochemistry showed that the expression levels of CD_3^+ and CD_4^+ on thymocyte and splenocyte surfaces in the CTX group were significantly lower than those of the control group, and the expression levels of CD_3^+ and CD_4^+ on thymocyte and splenocyte surfaces from Rg3 (7.5 mg/kg of 15 mg/kg) or LH and CTX mice was increased to some extent after treatment administration. Compared with the control group, the expression level of CD_8^+ on thymocyte and splenocyte surfaces of the CTX group was significantly higher than that of the control group, and the expression level of CD_8^+ on thymocyte and splenocyte surfaces from Rg3 (7.5 mg/kg or 15 mg/kg) or LH and CTX mice was reduced to some extent after treatment administration (Fig. 8A, C).

Integral optical density (IOD) values were used to quantify immunohistochemistry results. The IOD values for the thymus and spleen are shown in Fig. 8B, D, The IOD values for CD_3^+ and CD_4^+ in the thymus and spleen were significantly decreased in mice treated with CTX ($P < 0.05$, $P < 0.01$). CD_8^+ was significantly increased in mice treated with CTX ($P < 0.01$). Cotreatment with Rg3 (7.5 mg/kg, 15 mg/kg) or LH and CTX significantly increased CD_3^+ ($P < 0.05$, $P < 0.01$), whereas Rg3 (15 mg/kg) or LH and CTX significantly increased CD_4^+ ($P < 0.05$, $P < 0.01$) and Rg3 (7.5 mg/kg, 15 mg/kg), and LH and CTX treatment significantly decreased CD_8^+ ($P < 0.05$, $P < 0.01$).

3.7. Effect of Rg3 on Th1/Th2 balance in immunosuppressed mice

Cytokines and mRNA transcription factors expressed by thymocytes and splenocytes are shown in Fig. 9A–L. Compared with the control group, the expression of IFN-γ, the expression of transcription factor T-bet, and the ratios of IFN-γ/IL-4 and T-bet/GATA-3 were significantly decreased ($P < 0.05$, $P < 0.01$), and the expression of IL-4 and GATA-3 was significantly increased in mice treated with CTX ($P < 0.05$, $P < 0.01$). Cotreatment with Rg3 (15 mg/kg) or LH and CTX significantly increased the expression of IFN-γ, the expression of transcription factor T-bet, and the ratios of IFN-γ/IL-4 and T-bet/GATA-3 ($P < 0.05$, $P < 0.01$). Rg3 (15 mg/kg) or LH and CTX treatment significantly decreased the expression of IL-4 and GATA-3 ($P < 0.05$, $P < 0.01$).

4. Discussion

Tumors have been shown to be immunosuppressive [24,25], and some reports have shown that chemotherapy can reduce the infiltration of the content of leukocytes, T lymphocyte subsets and NK cells to reduce the immune function of patients and affect the cure rate of tumors. Cyclophosphamide is an immunosuppressant that has cytotoxicity toward all T cells at clinical doses for antitumor therapy [26]. The immune system plays an important role in anti-tumor defense. Therefore, it is urgent to find drugs that enhance the immune system in traditional medicine. The purpose of this study was to investigate the immunocompetence of Rg3 on CTX-induced immunosuppression in Balb/c mice.

Pharmacological studies have shown that ginsenoside Rg3 has a variety of biological activities, including enhancing immunity and enhancing the secretion of cytokines by activating T lymphocytes, which can help it to play an antitumor role and enhance patient immune function [15,27,28]. There are two types of ginsenoside Rg3, 20(*R*)-Rg3 and 20(*S*)-Rg3. It has been reported that ginsenoside Rg3 is stereospecific when stimulating the immune response, and 20(*R*)-Rg3 is more potent for treating cancers or other immune-mediated diseases clinically [19,22].

In the present study, we explored the regulatory effect of Rg3 on the immunocompetence of immunosuppressed mice and its possible mechanism. Some clinical reports have shown that Rg3 is administered orally in combination with chemotherapeutics. However, our results suggest that preadministration may have better therapeutic effect, which provides a scientific basis for the choice of clinical administration time.

Thymus and spleen indices are indicators of immunology, and the quality and organ morphology of the thymus and spleen can reflect nonspecific immunity of the body. Thymus and spleen atrophy and their indices were decreased in the CTX group. After treatment administration, Rg3 improved the morphology of the thymus and spleen and significantly increased the organ indices (Fig. 3).

Leukopenia (WBC), as the most common complication after chemotherapy, not only affects the therapeutic effect on tumors but also affects the quality of life for patients. WBC are thought to be concerned mainly with immune and defense mechanisms, whereas red blood cells (RBC) are an essential component of the immune system that mainly function as carriers of respiratory gases [29]. The number of WBC and RBC in peripheral blood can reflect the body's immune function. The neutrophil ratio (NE%) is an important mechanism for organisms to resist pathogen invasion. In this study, the peripheral blood counts in mice in which immunosuppression was induced by CTX either increased or decreased, but all were restored after Rg3 administration (Fig. 4A–H).

LDH and ACP are marker enzymes of macrophage activation and lysosomes [30,31]. Rg3 increases the activity of LDH and ACP in the thymuses and spleens of immunosuppressed mice treated by CTX (Fig. 5A–D) and then enhances the phagocytic function of macrophages. Therefore, Rg3 regulates the immune function of macrophages in immunosuppressed mice.

Cytokines are important in immune responses, and immunosuppressive agents, such as bacterial antigen lysates, significantly increase serum IgG levels in children with repeated infections [32,33]. IL-2 is mainly produced by activated T cells, which drives T-cell growth, enhances NK cytolytic activity and promotes the production of cytokines [34,35]. In previous reports, G-CSF stimulates changes in T cell function and regulates the balance of Th1/Th2 immune response by influencing cytokine production [36–38]. We measured the contents of the above three immune-related cytokines in the serum. The data indicated that Rg3 can maintain high levels of cytokines in the serum of immunosuppressed mice and play an immunomodulatory role (Fig. 6).

T lymphocytes are the central regulatory cells of the immune system [39]. CD_3^+, CD_4^+ and CD_8^+ are important indicators of the activity of T lymphocytes. Under normal circumstances, the total number of T cells is relatively stable. A change in T cell abundance is considered to be one of the main signs of immune dysfunction. The results of immunohistochemistry showed that the expression of CD_3^+, CD_4^+ and CD_8^+ on the surfaces of thymocytes and splenocytes and the IOD value were abnormally increased by CTX. After treatment administration, the

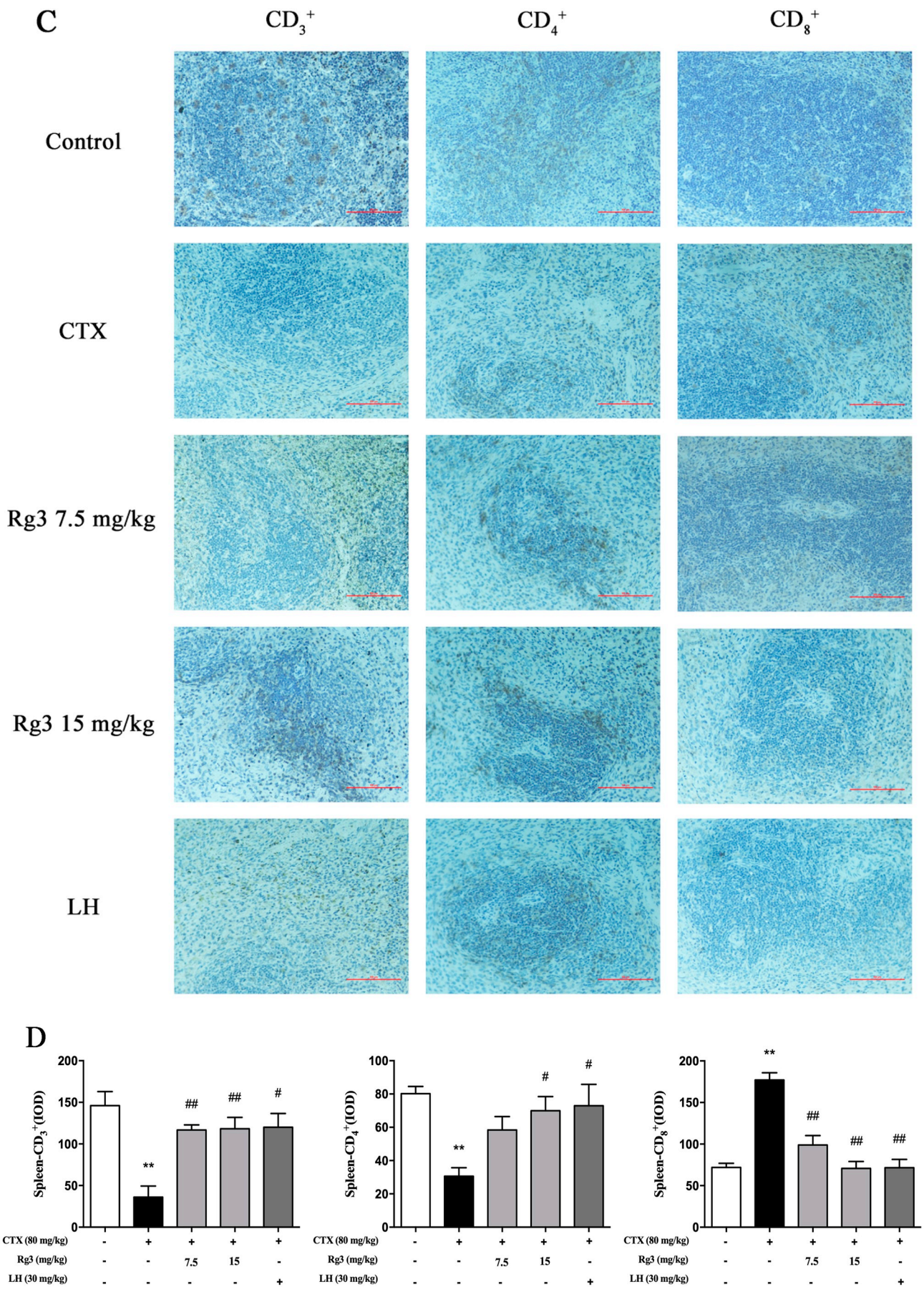

Fig. 8. (*continued*)

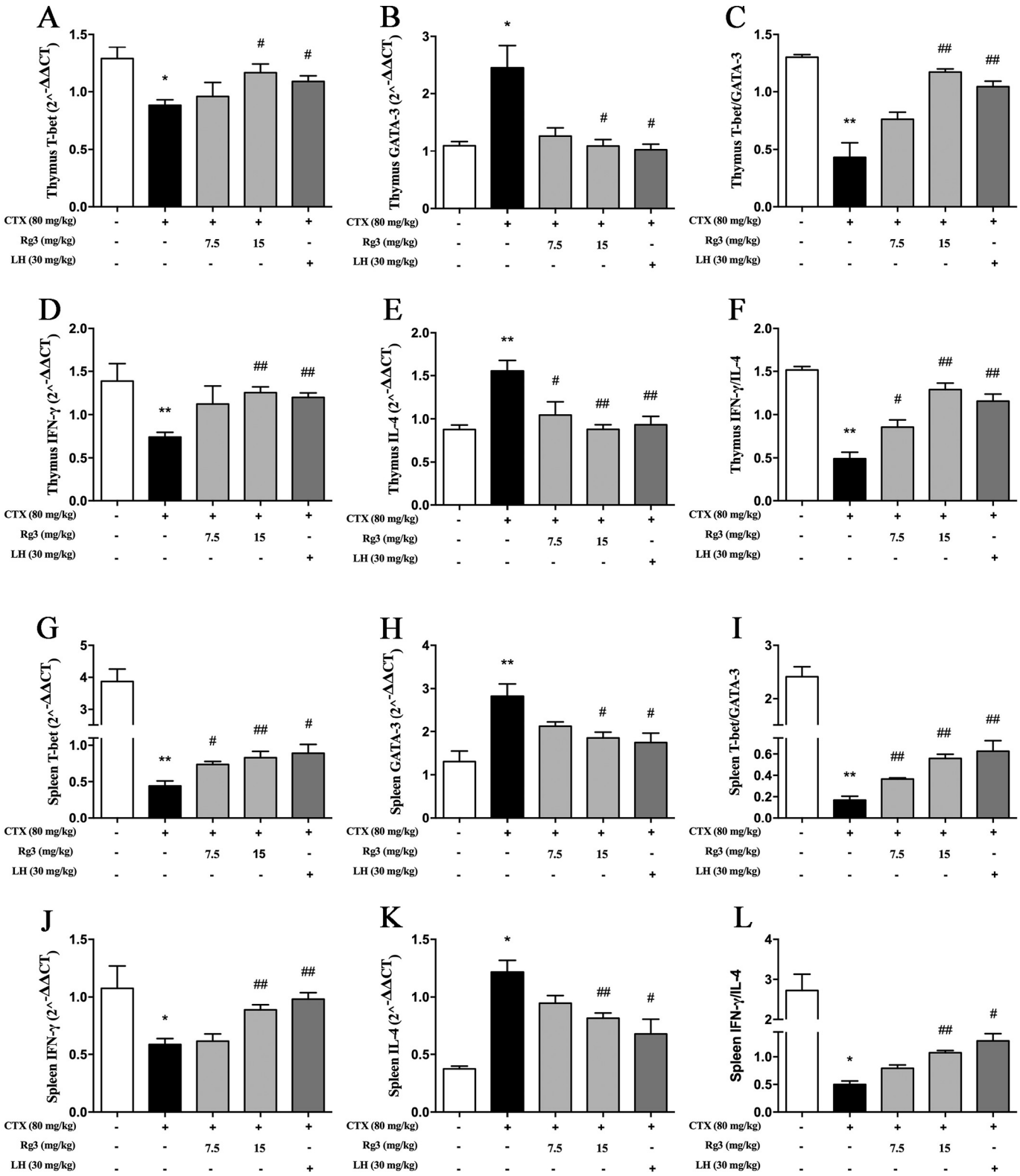

Fig. 9. Effects of Rg3 on expression levels in the thymus, (A) T-bet, (B) GATA-3, (C) T-bet/GATA-3, (D) IFN-γ, (E) IL-4, and (F) IFN-γ/IL-4, and spleen, (G) T-bet, (H) GATA-3, (I) T-bet/GATA-3, (J) IFN-γ, (K) IL-4, and (L) IFN-γ/IL-4 of immunosuppressed mice. The RNA was extracted from frozen tissue, the total RNA concentration was measured to obtain pure RNA, the cDNA was reverse transcribed and amplified, and the ΔΔCT value was calculated. The data are expressed as the mean ± SEM. N = 7 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the CTX-induced group.

abnormalities in each group recovered to varying degrees, indicating that Rg3 can improve the immune function of immunosuppressed mice (Fig. 8A–D).

In this study, we found that Rg3 has specificity in stimulating an immune response. Th1/Th2 subsets and their mutual balance play an important role in the regulation of the immune response. The cytokines and transcription factors produced by these cells can directly or indirectly regulate the immune response. They can not only determine the

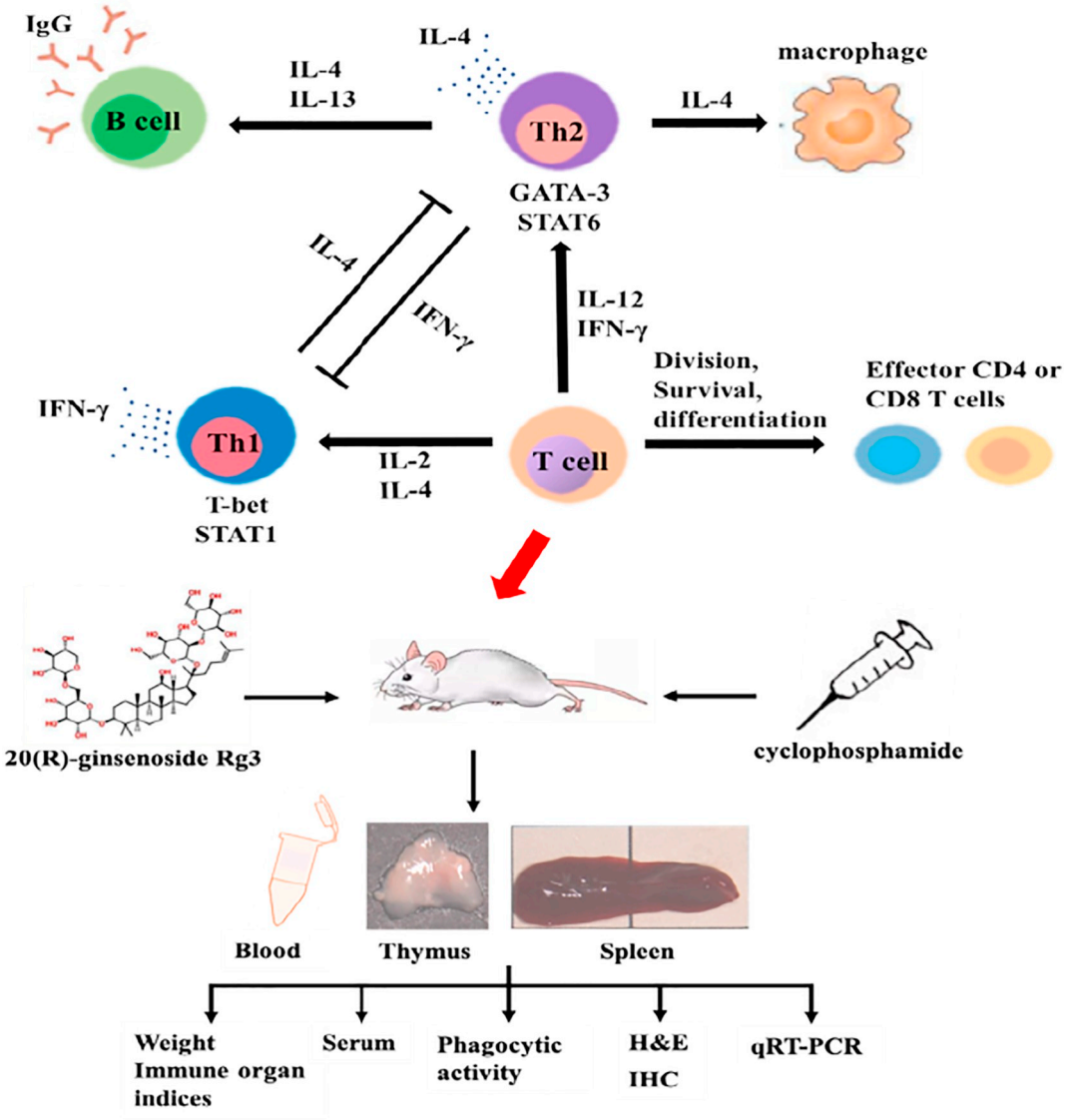

Fig. 10. The mechanism of this study.

function of the cell subsets but also participate in the activation and proliferation of the corresponding cell subsets and have mutually antagonistic functions such that Th1 and Th2 cells show functional mutual inhibition [40–42]. IFN-γ and IL-4 are specific cytokines of Th1 and Th2 cells, respectively, through which Th1 and Th2 cells control the activity of downstream effector cells [43]. IFN-γ activates antigen-presenting cells, upregulates transcription factor T-bet and promotes the differentiation of Th1 cells. IL-4 is a key regulatory factor in immune response, which can promote the differentiation of immature T cells into Th2 cells [44]. Specific transcription factors induce differentiation of T cell subsets during the differentiation of CD_4^+ T cells. Transcription factors, such as T-bet in Th1 cells and GATA-3 in Th2 cells, are considered major regulators [45]. Studies have shown that the transcription factors T-bet and GATA-3 are the key factors in determining the differentiation of Th0 cells into Th1 or Th2 cells. They can determine the stability of Th1/Th2 homeostasis, and there is interaction inhibition between them [46]. In the body's anti-tumor immune response, this interaction will cause an immunosuppressive state when Th1 drifts to Th2, and the body's anti-tumor immunity will also be seriously disturbed [47]. The detection of IFN-γ/IL-4 and T-bet/GATA-3 ratios can objectively reflect the balance of Th1/Th2 and the cytokine levels of Th1 and Th2, which could overcome the one-sidedness of a single cytokine's effect. The results showed that Rg3 regulates the mRNA expression of the above four cytokines and transcription factors from an abnormal state to a nearly normal state (Fig. 9A–L) such that the Th1/Th2 balance approached a normal level and the immunosuppressive effect of CTX in mice was improved.

5. Conclusions

In summary, as the main active ingredient of ginseng, ginsenoside Rg3 has potent immunomodulating properties and could reverse immunosuppression by enhancing immune organ development and phagocytosis of macrophages and by improving the expression of T lymphocyte subsets. Rg3 also regulates the cytokines and transcription factors that maintain the Th1/Th2 balance (Fig. 10). Therefore, we suggest that Rg3 has favorable research and development prospects.

We clearly decided to use color for any figures in printing.

Declarations of interest

None.

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Ethical standards

The study was carried out in strict accordance with the recommendations of the Guidelines for the care and use of laboratory animals issued by the Ministry of Science and Technology of China. The agreement was approved by the Laboratory Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (Permit No. TCM-LAEC20170044).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.04.003.

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