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journal homepage: www.elsevier.com/locate/intimpEffect of recombinant *Trichinella spiralis* cysteine proteinase inhibitor on TNBS-induced experimental inflammatory bowel disease in mice[☆]Jingyun Xu, Mingxu Liu, Pengcheng Yu, Lijia Wu, Yixin Lu^{*}

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic autoimmune disease with a high recurrence rate. Ulcerative colitis (UC) and Crohn's disease (CD) are two types of IBD. At present, parasite-derived cysteine protease inhibitors have received extensive attention from researchers, and experiments have confirmed that these protease have an effect on certain autoimmune diseases. So we conducted experiments to investigate the effect of *Trichinella spiralis* cysteine protease inhibitors on TNBS-induced mouse CD models. In this experiment, 72 male BALB/c mice aged 6–8 weeks were randomly divided into two groups: prevention group and therapy group. The mice were sacrificed and harvested on the 7th day after the model was established to measure the changes of various indicators of colitis.

The comparison of the TsCystatin + TNBS group with the PBS + TNBS group showed that the DAI score, MPO activity, and colonic macroscopic and microscopic damage significantly reduced, IFN- γ significantly decreased, IL-4 expression increased, and NF- κ B expression decreased. The percentage of CD4 + CD25 + Foxp3 + Treg and CD8 + CD28 – Treg in spleen, and the proportion of CD4 + /CD8 + Treg cells decreased. In the therapy group, we found no significant difference between the TNBS + PBS group and TNBS + TsCystatin group.

Treatment with TsCystatin exerted a good intervention effect on the TNBS-induced mouse CD model. TsCystatin possibly induced a Th2-type immune response in the body, which balanced the Th1-type immune response induced by TNBS administration, thereby relieving colitis.

1. Introduction

Inflammatory bowel disease (IBD) is the most common and most serious form of autoimmune disease that occurs in the intestine. The pathogenesis of IBD is complicated, and its etiology remains unclear; IBD includes ulcerative colitis (UC) and Crohn's disease (CD) [1–3]. Epidemiological studies have shown that IBD is common in developed countries and is rare in less developed countries where helminth infections are common [4]. T cells constitute an important part of many immune responses, including those associated with IBD and intestinal nematode infections. In different T helper (Th) subsets, CD is mainly associated with the Th1-type immune response, and many nematode infections produce strong Th2 responses. The cross-regulation between Th1 and Th2 cells suggests that the Th2 response produced by the nematode may prevent or reduce the effects of Th1-mediated diseases. Recent clinical studies have provided evidence that pig whipworm egg therapy is effective in the treatment of CD and UC [5,6]. However,

ingesting live parasites or eggs as a treatment may not be accepted by most patients. Therefore, the new research strategy aims to use parasite products as a substitute for parasitic infection to treat IBD.

Cysteine protease inhibitor (CPI or cystatin) is a inhibitor reversible binding cysteine protease that specifically inhibits the activity of cysteine proteases such as cathepsin and papain, and there are evolutionary similarities in structure and function, forming a superfamily [7]. In addition to its unique cysteine protease inhibitory activity, the cystatin of parasitic nematode has a regulatory effect on the host immune response [8,9], including the intervention of antigen presentation process and T cell response, regulation of cytokine production, stimulation of IFN- γ activation of macrophage secretion of NO [10]. Therefore, the cystatin of parasitic nematode plays an important role in parasites escaping host immune responses and adapting to parasitic life. At present, the screening of worm-derived immunomodulatory molecules has received great attention from researchers; studies have confirmed the importance of this class of worm-derived

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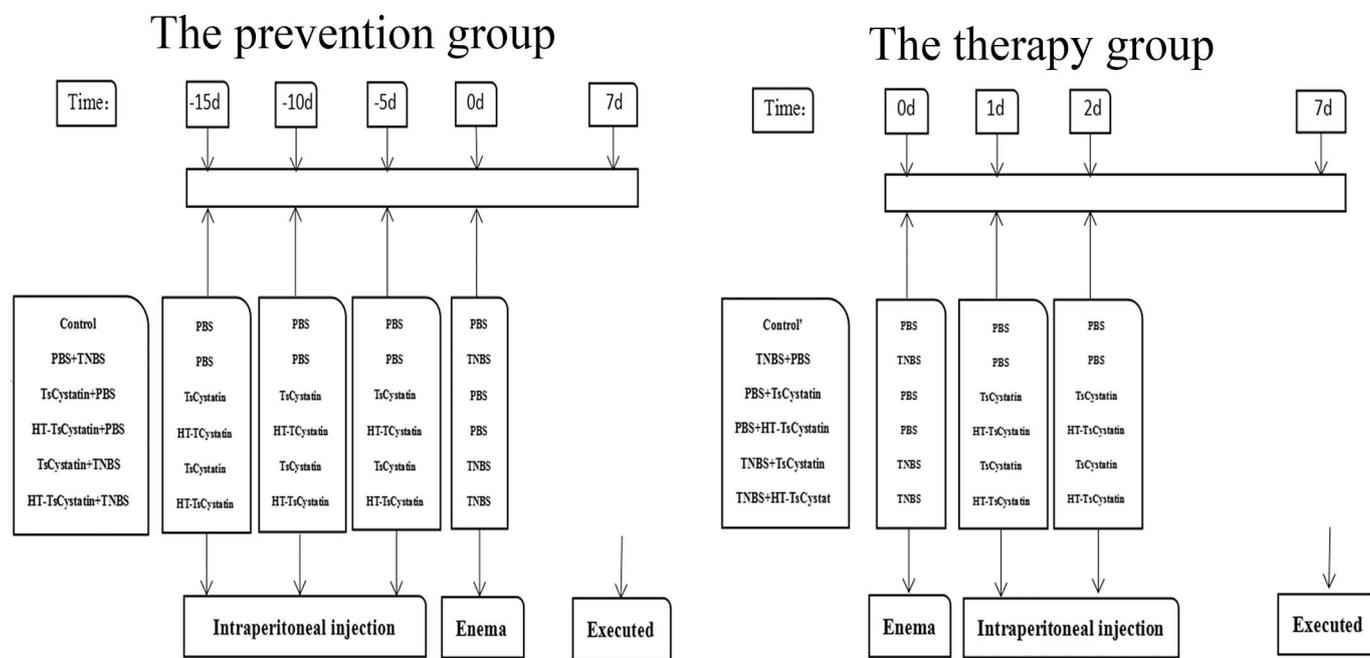


Fig. 1. A scheme to clarify the exact moments of immune with TsCystatin/PBS and TNBS/PBS administration.

immunomodulatory molecules [11,12]. Experiments on worm-derived cystatins have demonstrated that these proteins have a good effect on certain autoimmune diseases [13]. Pre-infection of *Trichinella spiralis* can induce a predominant Th2 response in mice and significantly reduce intestinal inflammation in IBD has been confirmed by experiments [14]. In this experiment, the expression levels of Th1 and Th2 cytokines, the expression of NF-κB, and the percentage of CD4 + CD25 + Foxp3 + Treg cells, CD8 + CD28- Treg were detected to explore whether mice treated with TsCystatin can alleviate TNBS-induced colitis and elucidate the underlying mechanism. This study provides a novel idea for the treatment of IBD.

2. Methods

2.1. Animals

Six- to eight-week-old male Balb/c mice (SPF) were purchased from the Animal Center of Harbin Medical University. They were allowed to adapt to the feeding environment for 4 days before the experiment and given free access to food and water. All procedures were in strict accordance with the guidelines of the Chinese Institute of Health on the care and use of experimental animals. The number of animals used was minimized, and reduced their suffering.

2.2. Preparation of TsCystatin recombinant protein

The laboratory designed primers based on *Trichinella spiralis* cystatin-B (Tsp_03420) mRNA (accession number: XM_003379718.1) published on NCBI, and specifically amplified the cystatin gene by RT-PCR. The positive expression plasmid of pET-28a-TsCystatin was amplified and expressed in the best expression conditions. The recombinant proteins expressed in *Escherichia coli* were separated by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). The gel band containing the target protein stained with 0.25 mol/L KCl was cut, and the gel was crushed. An appropriate amount of PBS was added to the gel, which was frozen and thawed repeatedly and centrifuged at high speed to obtain purified protein. The purified protein was identified by SDS-PAGE, and a single and

consistent protein band was obtained, which proved that the prepared protein was purified target protein. The target protein was obtained and frozen at -80 °C, and the protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology).

2.3. Experimental grouping

The experimental mice were randomly divided into two groups: prevention group and therapy group. The prevention group was divided into six subgroups: (1) control group, which was treated with 50 μg of PBS by intraperitoneal injection (i.p.) three times at 5 day intervals before given 100 μL of PBS intrarectally; (2) PBS + TNBS group, in which 50 μg of PBS was injected intraperitoneally three times at 5 day intervals before colitis was induced; (3) TsCystatin + PBS group, in which 50 μg of TsCystatin was i.p. three times at 5 day intervals before 100 μL of PBS was administered intrarectally; (4) HT-TsCystatin + PBS group, treated with 50 μg HT-TsCystatin which boiled 10 min by i.p. three times at 5d intervals before intrarectal injected with 100ul PBS; (5) TsCystatin + TNBS group, in which 50 μg of TsCystatin was injected intraperitoneally three times at 5 day intervals before colitis was induced, and (6) HT-TsCystatin + TNBS group, treated with 50 μg HT-TsCystatin which boiled 10 min by i.p. three times at 5 d intervals before introduction of colitis; The therapy group was also divided into six subgroups: (7) control' group, which was treated with 50 μg of PBS by i.p. at 24 and 48 h after intrarectal injection with 100 μL of PBS; (8) TNBS + PBS group, which was treated with 50 μg of PBS by i.p. at 24 and 48 h after TNBS administration; (9) PBS + TsCystatin group, which was treated with 50 μg of TsCystatin by i.p. at 24 and 48 h after intrarectal injection of 100 μL of PBS; (10) PBS + HT-TsCystatin group, treated with 50 μg HT-TsCystatin which boiled 10 min by i.p. 24 h and 48 h after 100ul PBS intrarectal injection; (11) TNBS + TsCystatin group, which was treated with 50 μg of TsCystatin by i.p. at 24 and 48 h after TNBS administration; (12) TNBS + HT-TsCystatin group, treated with 50 μg HT-TsCystatin which boiled 10 min by i.p. 24 h and 48 h after TNBS administration. Each group comprised six or more mice. All the mice were sacrificed at day 7 after the model was established and tested for various indexes. The experimental flow chart is shown in Fig. 1. The body weight of the mice in all groups was determined daily.

2.4. Modeling method

Stallmach and others [15] established the TNBS-induced colitis model approach. At 24 h after fasting, the mice in each group were anesthetized via intraperitoneal injection with 3% sodium pentobarbital. A 1.0 mm-thin catheter was inserted into the colon. The mice were rapidly injected with 100 µL of 2.5 mg of TNBS solution (50 µL of 5% [m/v] TNBS dissolved in 50 µL of 50% ethanol). After the injection, the anus of the mice was pinched, and the mice were inverted for 3–4 min by grabbing the tail. The mice were then replaced in the cages with free access to food and water. This colitis animal model promotes the Th1-type immune response similar to human CD. Inflammation was determined based on the following parameters: clinical disease activity, macroscopic and microscopic inflammation score, and myeloperoxidase (MPO) activity in the colon tissue. The grades were conveyed by three investigators blinded for all the groups.

2.5. Disease activity index (DAI) evaluation

The mice were observed daily for changes in mental status, activity, hair luster, appetite, and defecation (e.g. whether stool contained blood, stool pattern, and defecation frequency). Each group of mice was scored for DAI according to international standards: DAI = (weight loss score + stool performance score + blood stool score)/3 (Table 1). The benzydine method was used to detect fecal occult blood.

2.6. MPO activity assay

The degree of colonic inflammation was investigated by MPO activity assay. MPO activity was measured using the MPO assay kit (Alpha Biotech). Colon tissue was weighed, cut into fine pieces, and added with 150–250 µL of RIPA lysate per 20 mg of tissue. The samples were homogenized using a glass homogenizer. After lysis completion, the samples were centrifuged at 10,000–14,000 ×g for 3–5 min to obtain the supernatant. The protein concentration was examined using a Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology). Follow up was conducted according to the MPO assay kit's instructions. MPO activity of the supernatants was determined and expressed as units per gram of total protein (U/g). Data were obtained from three separate experiments.

2.7. Macroscopic and microscopic assessments of colon injury

After the mice were sacrificed using cervical dislocation, the abdominal wall was opened and the intestine was exposed. The entire segment of the colon from the rectum to the cecocolic junction was removed and rinsed thoroughly with normal saline. The isolated colon was examined for macroscopic damage. Scores were obtained by using the following damage scoring system [16]: 0: no damage; 1: localized hyperemia without ulcers; 2: ulceration without hyperemia or bowel wall thickening; 3: ulceration with inflammation at one site; 4: ulcer and inflammation at two or more sites; 5: major sites of inflammation and ulceration extending > 1 cm along the length of colon; and 6–10: when an area of damage extended > 2 cm along the length of colon, the score was increased by one for each additional centimeter of involvement. Subsequently, the colon specimens were fixed in 10%

Table 1
Disease activity index score parameters (DAI).

Weight loss (%)	Stool performance	Bloody stool	Index
0–1	Normal	None	0
1–5	Soft and shaped	Between	1
5–10	Loose	Fecal occult blood	2
10–15	Between	Between	3
> 15	Diarrhea	Defecate haemorrhage	4

paraformaldehyde for hours and embedded into paraffin sections. Hematoxylin and eosin staining was performed. The pathological sections of the colon were observed under an optical microscope. The criteria of Wallace and Keenan (16) were used to assess histological damage: 0: intact tissue construction with no apparent damage; 1: damage limited to surface epithelium; 2: localized ulcer confined to mucosa; 3: focal, transmural inflammation, and ulceration; 4: extensive transmural ulceration and inflammation adjacent to normal mucosa; and 5: extensive transmural ulceration and inflammation involving the entire section. Data were from three separate experiments, and the scores were assigned by three observers without knowing the state of the mice.

2.8. ELISA detection of IL-4 and IFN-γ expression in colon tissues

Colon tissues were cut and weighed. The lysate was added, and the samples were homogenized using a glass homogenizer. After lysis completion, the samples were centrifuged at 10,000–14,000 ×g for 3–5 min to obtain the supernatant. The ELISA kit (Bioss) manual was followed for cytokine detection. The amount of cytokines in the tissues was expressed per milligram of tissue protein. Data were from three separate experiments.

2.9. Western blot of NF-κB p65 expression in colon tissues

Colon tissues were cut and weighed. The lysate was added, and the samples were homogenized using a glass homogenizer. After lysis completion, the samples were centrifuged at 10000–14000 ×g for 3–5 min to obtain the supernatant. And the protein concentration was examined using a Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology). Approximately 50 µg of colon homogenate proteins was boiled with 5 × SDS-PAGE sample loading buffer and separated using 12% polyacrylamide gel electrophoresis. The proteins were blotted onto a nitrocellulose filter membrane. The membrane was placed into blocking buffer (5% nonfat milk) for 2 h at room temperature. Subsequently, the membrane was incubated with anti-NF-κB and anti-β-actin (1:4000 diluted in blocking buffer, Bioss) at 4 °C overnight. After washing using PBST, the membrane was incubated with a peroxidase-conjugated secondary antibody, which was diluted in 5% nonfat milk (1:5000) on a shaker for 1 h at room temperature. After washing, ultrasensitive ECL chemiluminescence reagent (Sangon Biotech) was dropped to the membrane, and the membrane was exposed using exposure equipment. The bands were quantified via densitometry and analyzed with Image J software.

2.10. Expression of CD4 + CD25 + Foxp3 + Treg, CD8 + CD28 – Treg in spleen by flow cytometry (FCM)

In the preparation of single cell suspensions of splenic lymphocytes, each sample was divided into two tubes with 1 × 10⁶ cells per tube. The cells of one tube were resuspended in PBS and incubated for 30 min in the dark with FITC rat anti-mouse CD4 and APC rat anti-mouse CD25 (Sungene Biotech). The resuspended cells were added with a fixation/permeabilization solution (Invitrogen, USA), incubated for 30–60 min, and washed again. After cell resuspension, PE rat anti-mouse Foxp3 (Sungene Biotech) was added. The solution was incubated for 20 min, washed, and resuspended again for FCM. Another tube was resuspended in PBS; added with PE anti-mouse CD8, and APC anti-mouse CD28 (Sungene Biotech); and incubated in the dark at 4 °C for 30 min. The solution was washed and resuspended for examination by FCM. Data were from three separate experiments.

2.11. Statistical analysis

All results were expressed as the mean ± standard error. Data were evaluated using one-way ANOVA analysis, two-way ANOVA and SPSS 13.0 software. Multiple comparison between the groups was performed

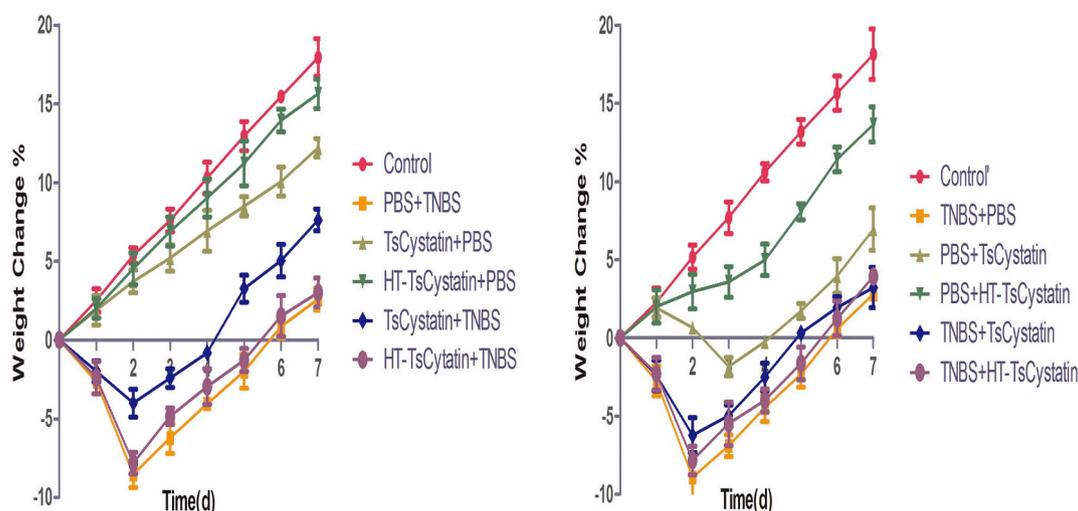


Fig. 2. Changes in weight of the mice is expressed as the difference between the weight on the day and the initial weight. The weight of the mice in PBS + TNBS and TNBS + PBS groups decreased until day 3 and in TsCystatin + TNBS and TNBS + TsCystatin groups decreased until day 2. Afterward, they slowly regained. Data are shown as mean \pm SD of 3 mice per group.

using the Tukey post-hoc test. $P < 0.05$ was considered statistically significant. All statistical analysis were performed using GraphPad Prism software.

3. Results

3.1. Survival rate

The survival rate of mice in the PBS + TNBS and TNBS + PBS group were only 55.56% and 66.67%, which were significantly lower than the survival rate of the TsCystatin+TNBS (88.89%; $P < 0.05$) and TNBS + TsCystatin group (86.67%; $P < 0.05$). Moreover, the survival rate of mice in the other groups were 100%.

3.2. Weight changes

The day of TNBS modeling was experimental day 0. The weight change of the mice was recorded daily until the experiment was finished. Results are shown in Fig. 2. Among the protection subgroups, the body weight of mice in the PBS + TNBS group continued to decline, decreased to the lowest value in the 2nd day, and fell to $-8.53\% \pm 0.83\%$. The body weight gradually increased and increased to $2.59\% \pm 0.70\%$ on the 7th day. Compared with the PBS + TNBS group, weight loss of mice in the TsCystatin+TNBS group was relatively slow ($P < 0.001$), fell to $-4.01\% \pm 0.89\%$ on the 2nd day, and gradually recovered and reached to $7.62\% \pm 0.69\%$ on the 7th day, which was higher than that of the PBS + TNBS group ($P > 0.05$). The change of the HT-TsCystatin + TNBS group was similar to that of PBS + TNBS group, however the degree of decrease was slightly lower than that of PBS + TNBS group ($P > 0.05$) and significantly higher than that of TsCystatin + TNBS group ($P < 0.001$). The control group and the TsCystatin+PBS group demonstrated an increasing state of weight, but the increase in the TsCystatin + PBS group was significantly lower than that of the control group ($P < 0.001$) from the 3rd day and higher than that of the PBS + TNBS and TsCystatin+TNBS groups ($P < 0.001$). However, among the therapy subgroups, the body weight changes in the TNBS + PBS, TNBS + TsCystatin, TNBS + HT-TsCystatin groups were significantly lower than those in the control' group ($P < 0.001$). The TNBS+PBS, TNBS + Tscystatin, TNBS + HT-TsCystatin groups fell to $-8.91\% \pm 1.12\%$, $-6.21\% \pm 1.12\%$ and $-7.82\% \pm 0.91\%$ on the second day respectively, and then reached to $2.83\% \pm 0.88\%$, $3.22\% \pm 1.29\%$ and $3.93\% \pm 0.42\%$ respectively on the 7th day. No significant difference in the trend

between the three groups was observed ($P > 0.05$). And PBS + TsCystatin group due to immune proteins, the weight declined in the first 3 days, reached a minimum of $-1.83\% \pm 0.59\%$ on the 3rd day, gradually recovered, and reached to $6.96\% \pm 1.36\%$ on the 7th day. The TNBS+HT-TsCystatin group was gradually increased to $13.66\% \pm 1.12\%$ on the 7th day, and the change tendency were similar with the control' group ($P > 0.05$).

3.3. DAI score

At 24 h after modeling, the mice in the PBS + TNBS and TNBS + PBS groups showed various degrees of apathetic behavior, delayed activity, loss of appetite, rough hair, diarrhea, bloody stools or fecal occult blood, weight loss, and other clinical manifestations. On the 2nd day, these symptoms were most evident. The symptoms of the TsCystatin + TNBS (2.33 ± 0.30) and TNBS + TsCystatin groups (2.67 ± 0.30) were significantly relieved compared with those of the PBS + TNBS (3.67 ± 0.18) and TNBS+PBS (3.33 ± 0.18) groups ($P < 0.001$). Those of the HT-TsCystatin + TNBS group (2.33 ± 0.30) and TNBS+HT-TsCystatin (2.67 ± 0.30) were similar with the PBS + TNBS and TNBS + PBS group ($P > 0.05$). The above symptoms began to improve on the 3rd day, but the mice in the TsCystatin + TNBS and TNBS + TsCystatin groups still recovered significantly ($P < 0.001$) compared with the PBS + TNBS and TNBS + PBS groups. No significant difference was found between the groups from the 5th day ($P > 0.05$). Both the TsCystatin + PBS and PBS + TsCystatin groups showed low DAI scores, and the DAI values at different time points are shown in Fig. 3.

3.4. MPO activity

Among the protection subgroups, MPO activity in the PBS + TNBS (1.40 ± 0.07 ; $P < 0.001$), TsCystatin + PBS (0.45 ± 0.07 ; $P < 0.05$), HT-TsCystatin + PBS (0.36 ± 0.04 ; $P < 0.05$) and TsCystatin + TNBS (0.70 ± 0.09 ; $P < 0.01$), HT-TsCystatin + TNBS groups (1.14 ± 0.11 , $P < 0.001$) was significantly higher than that in the control group (0.27 ± 0.06). MPO activity in the TsCystatin + TNBS group was significantly lower than that in the PBS + TNBS group ($P < 0.001$) and significantly higher than that in the TsCystatin + PBS group ($P < 0.05$); There was difference between HT-TsCystatin + TNBS (1.15 ± 0.13) and PBS + TNBS groups ($P < 0.05$). And the TsCystatin + TNBS group were significantly lower than that of the HT-TsCystatin+TNBS group ($P < 0.01$). The same

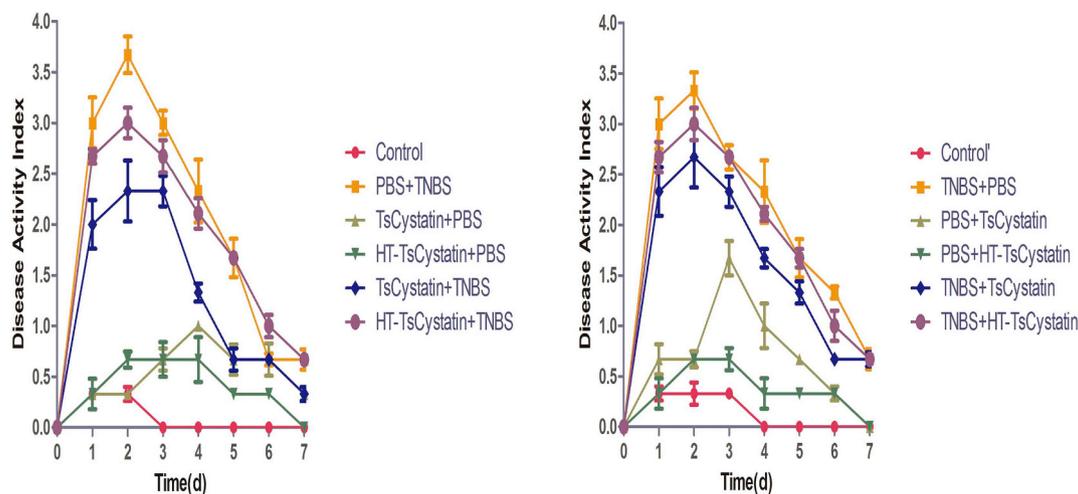


Fig. 3. DAI assessment of TsCystatin + TNBS group was lower than that of PBS + TNBS group. The same result was seen compared the TNBS + TsCystatin with TNBS+PBS group. Data are shown as mean ± SD of 3 mice per group.

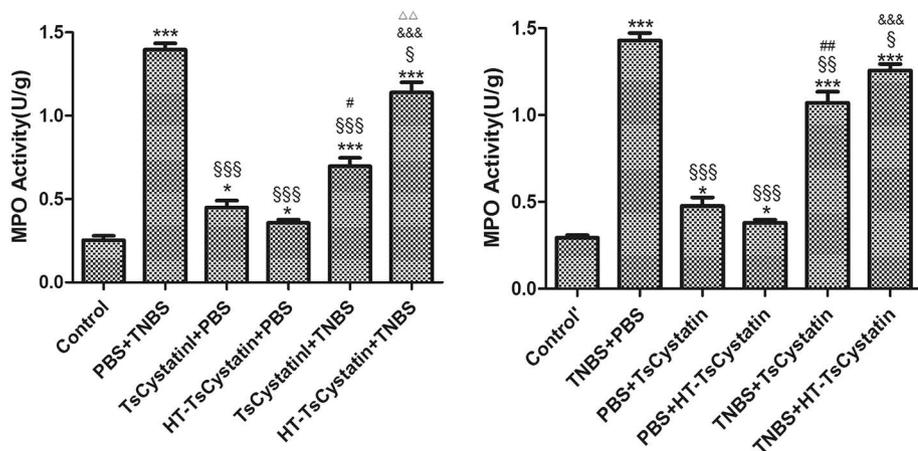


Fig. 4. MPO activity in all groups. Data are shown as mean ± SD of 3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Control and Control' group; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ versus PBS + TNBS and TNBS + PBS group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ for TsCystatin + PBS vs HT-TsCystatin + PBS, TsCystatin + TNBS and PBS + TsCystatin vs PBS + HT-TsCystatin, TNBS + TsCystatin; & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$ for HT-TsCystatin + TNBS vs HT-TsCystatin + PBS and TNBS + HT-TsCystatin vs PBS + HT-TsCystatin; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ for HT-TsCystatin + TNBS vs TsCystatin + TNBS and TNBS + HT-TsCystatin vs TNBS + TsCystatin. The results of doing two-way ANOVA showed that there was no interaction between the two factors of enema with TNBS or PBS and intraperitoneal injection with recombinant protein or PBS.

result was found in the therapy subgroups. MPO activity in the TNBS + PBS (1.43 ± 0.07 ; $P < 0.001$), PBS + TsCystatin (0.48 ± 0.09 ; $P < 0.05$), PBS + HT-TsCystatin (0.38 ± 0.03 ; $P < 0.05$) and TNBS + TsCystatin (1.07 ± 0.01 ; $P < 0.001$), TNBS + HT-TsCystatin groups (1.26 ± 0.07 , $P < 0.001$) were significantly higher than that in the control' group (0.29 ± 0.03). MPO activity in the TNBS + TsCystatin group was significantly lower than that in the TNBS + PBS group ($P < 0.01$) and significantly higher than that in the PBS + TsCystatin group ($P < 0.01$). We also found that MPO activity of the TsCystatin + TNBS group was significantly lower than that of the TNBS + TsCystatin group ($P < 0.01$). The results were shown in Fig. 4.

3.5. Colonic pathological changes

(1) Macroscopic score of colonic damage

On the 7th day after the TNBS-induced colitis model was established, macroscopic injury of the colon showed intestinal wall edema, congestion, and stenosis, forming ulceration. In severe cases, transmural and adhesion to the surrounding tissues were observed. Mucosal and submucosal segmental hyperemia and edema occurred (Fig. 5). The injury score of the PBS + TNBS and TNBS + PBS groups were 6.89 ± 0.19 and 6.67 ± 0.34 , respectively. And the HT-TsCystatin + TNBS (5.89 ± 0.51), TNBS + HT-TsCystatin (6.22 ± 0.19) were lower than PBS + TNBS ($P < 0.05$) and TNBS + PBS group ($P > 0.05$). The TsCystatin + TNBS (4.33 ± 0.34) and

TNBS + TsCystatin groups (5.45 ± 0.39) had only mild congestion and edema of the colon and occasional small ulcers. Compared with the PBS + TNBS and TNBS + PBS groups, the TsCystatin + TNBS and TNBS + TsCystatin groups significantly improved ($P < 0.05$). The TsCystatin + PBS and PBS + TsCystatin groups only showed very slight intestinal injury, and their injury scores were 2.22 ± 0.19 and 2.00 ± 0.33 , respectively, which were significantly lower than those of the other groups ($P < 0.001$).

(2) Microscopic score of colonic damage

On the 7th day after the TNBS-induced colitis model was established, inflammatory reaction symptoms, necrosis and shedding of intestinal epithelial cells, shortening and fusion of small intestine villi, and infiltration of crypts were observed. Simultaneously, a large number of lymphocytes and neutrophils and varying numbers of eosinophils infiltrated into the mucosa and submucosa. The mucosal glands were not aligned or damaged (Fig. 6), and the damage scores of the PBS + TNBS and TNBS + PBS groups were 4.22 ± 0.19 and 4.33 ± 0.34 , respectively. And the HT-TsCystatin + TNBS (3.44 ± 0.20), TNBS + HT-TsCystatin (3.78 ± 0.20) were lightly lower than PBS + TNBS ($P < 0.01$) and TNBS + PBS group ($P > 0.05$). The lesions in the TsCystatin + TNBS and TNBS + TsCystatin groups were confined to the mucosal layer. The infiltration of inflammatory cells was weakened, and edema was slight. The damage scores of the TsCystatin + TNBS and TNBS + TsCystatin groups were

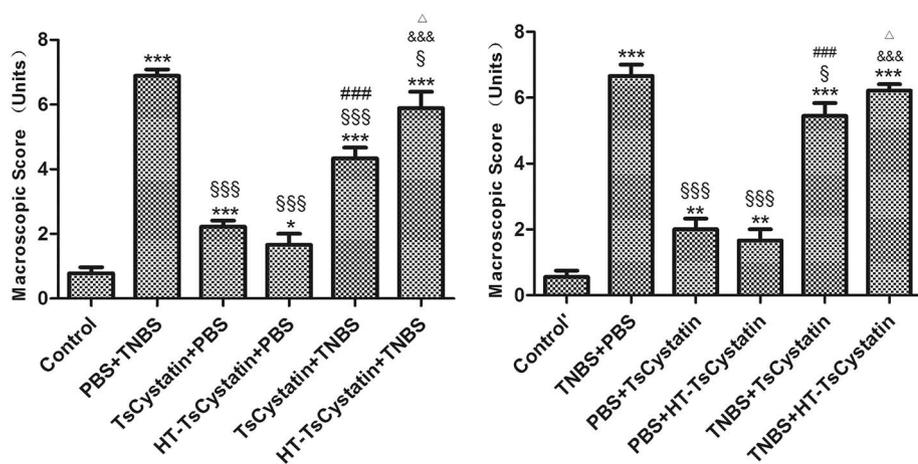


Fig. 5. Macroscopic scores of the colons of twelve groups. Data are shown as mean ± SD of 3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Control and Control' group; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ versus PBS + TNBS and TNBS + PBS group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ for TsCystatin + PBS vs HT-TsCystatin + PBS, TsCystatin + TNBS and PBS + TsCystatin vs PBS + HT-TsCystatin, TNBS + TsCystatin; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ for HT-TsCystatin + TNBS vs HT-TsCystatin + PBS and TNBS + HT-TsCystatin vs PBS + HT-TsCystatin; △ $P < 0.05$, △△ $P < 0.01$, △△△ $P < 0.001$ for HT-TsCystatin + TNBS vs TsCystatin + TNBS and TNBS + HT-TsCystatin vs TNBS + TsCystatin. There was no interaction between the two factors of enema with TNBS or PBS and intraperitoneal injection with recombinant protein or PBS by doing two-way ANOVA.

2.56 ± 0.20 and 3.45 ± 0.39, respectively. The microscopic lesions of the colon significantly decreased compared with the PBS + TNBS ($P < 0.001$) and TNBS + PBS groups ($P < 0.05$). The microscopic scores of the TsCystatin + PBS and PBS + TsCystatin groups were 1.56 ± 0.20 and 1.22 ± 0.39, respectively, and only minor damage was present. HT-TsCystatin + PBS and PBS + HT-TsCystatin groups also showed minimal changes in intestinal damage (Fig. 7).

3.6. IL-4 and IFN-γ changes in the colon of mice

ELISA results of the prevention subgroups showed that the IL-4 (Fig. 8(a)) in PBS + TNBS (186.05 ± 1.73; $P < 0.05$), TsCystatin + PBS (457.00 ± 29.46; $P < 0.001$), HT-TsCystatin + PBS (175.33 ± 5.72; $P > 0.05$), TsCystatin + TNBS (247.33 ± 6.11; $P < 0.01$) and HT-TsCystatin + TNBS groups (206.03 ± 5.94; $P < 0.05$) were all higher than the control group (140.11 ± 25.06). Moreover, compared with the PBS + TNBS group, IL-4 expressions in the HT-TsCystatin + TNBS ($P < 0.05$), TsCystatin + TNBS ($P < 0.001$) groups significantly increased. And there was significantly difference between the HT-TsCystatin + TNBS and TsCystatin + TNBS groups ($P < 0.001$). Similarly, among the therapy subgroups, the

TNBS + PBS (156.33 ± 10.26; $P < 0.05$), PBS + TsCystatin (247.67 ± 16.26; $P < 0.001$), PBS + HT-TsCystatin (143.27 ± 9.00; $P > 0.05$), TNBS + TsCystatin (187.33 ± 25.42; $P < 0.05$) and TNBS + HT-TsCystatin (170.03 ± 5.48; $P < 0.01$) groups were all higher than the control' group (128.00 ± 12.36). The TNBS + TsCystatin group was higher than the TNBS + PBS group ($P > 0.05$) but lower than the PBS + TsCystatin group ($P < 0.05$). Regardless of the prevention group or the therapy group, the expression level of IFN-γ (Fig. 8(b)) in the other subgroups was higher than that in the control and control' groups. Among the prevention subgroups, the TsCystatin + PBS (619.00 ± 29.46; $P < 0.01$) and TsCystatin + TNBS groups (766.33 ± 14.19; $P < 0.05$) were significantly lower than the PBS + TNBS group (902.67 ± 61.61). And the HT-TsCystatin + TNBS (878.95 ± 29.63) was not different from the PBS + TNBS group ($P < 0.05$). Similarly, among the therapy subgroups, the PBS + TsCystatin (627.33 ± 29.20; $P < 0.001$) and TNBS + TsCystatin groups (735.00 ± 37.80; $P < 0.01$) were lower than the TNBS + PBS group (474.67 ± 4.73). And there was significantly difference between the TNBS + TsCystatin (735.00 ± 37.80) and TNBS + HT-TsCystatin (880.83 ± 24.94) groups ($P < 0.01$).

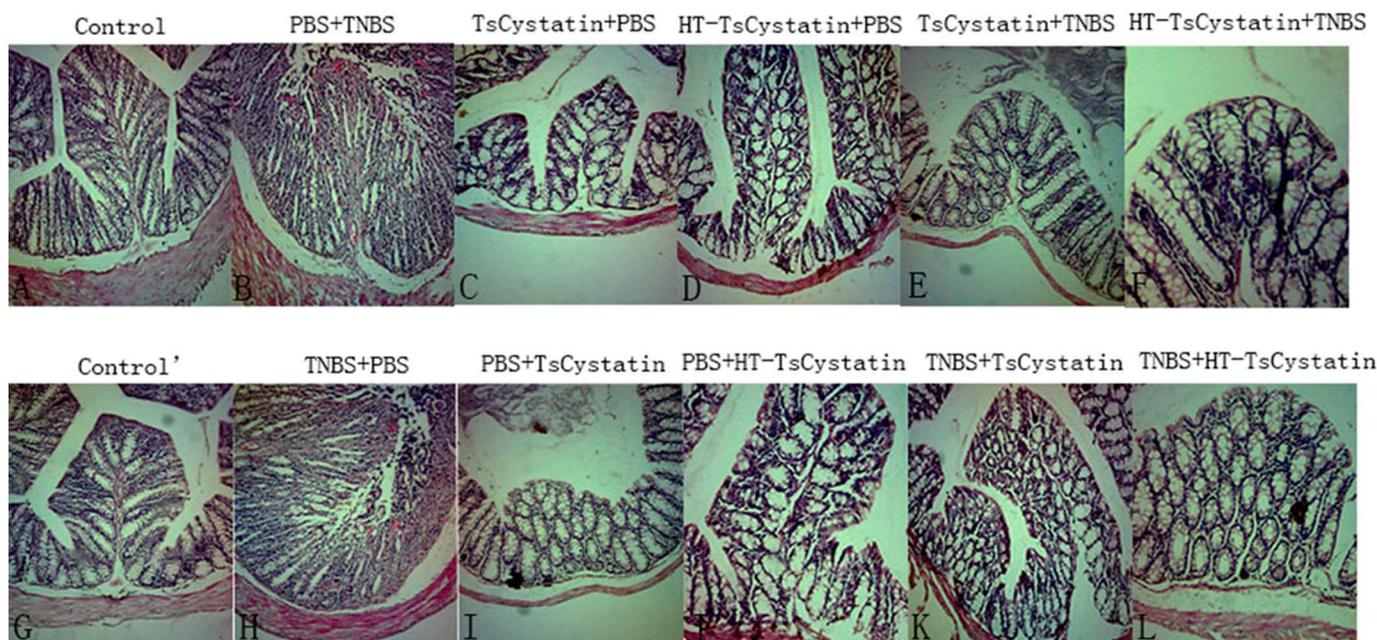


Fig. 6. Light micrograph of HE-stained colonic section of eight groups. Scale bar represents 200 μm.

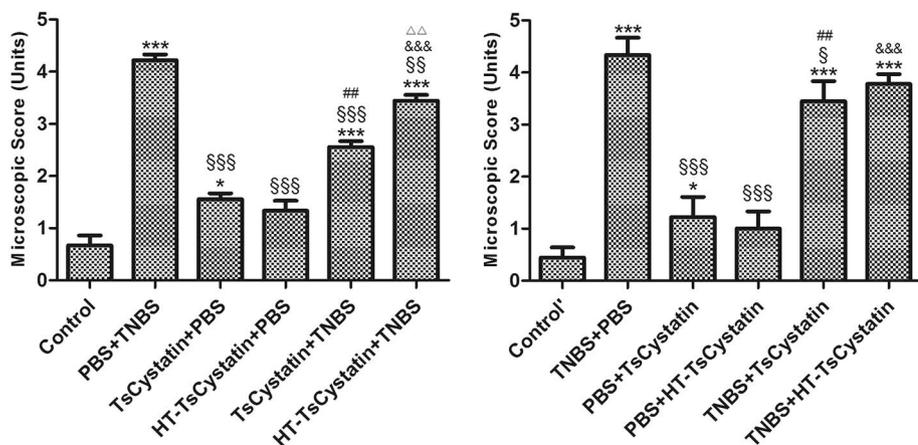


Fig. 7. Microscopic scores of the colons of twelve groups. Data are shown as mean ± SD of 3 mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus Control and Control' group; §*P* < 0.05, §§*P* < 0.01, §§§*P* < 0.001 versus PBS + TNBS and TNBS + PBS group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 for TsCystatin + PBS vs HT-TsCystatin + PBS, TsCystatin + TNBS and PBS + TsCystatin vs PBS + HT-TsCystatin, TNBS + TsCystatin; &*P* < 0.05, &&*P* < 0.01, &&&*P* < 0.001 for HT-TsCystatin + TNBS vs HT-TsCystatin + PBS and TNBS + HT-TsCystatin vs PBS + HT-TsCystatin; △*P* < 0.05, △△*P* < 0.01, △△△*P* < 0.001 for HT-TsCystatin + TNBS vs TsCystatin + TNBS and TNBS + HT-TsCystatin vs TNBS + TsCystatin. There was no interaction between the two factors of enema with TNBS or PBS and intraperitoneal injection with recombinant protein or PBS.

or PBS.

3.7. NF-κB p65 expression changes in colon tissues

Among the prevention subgroups, the expression of NF-κB in the PBS + TNBS group was significantly higher than that in the other groups (*P* < 0.001), whereas NF-κB expression in the TsCystatin + TNBS group was lower than that in the PBS + TNBS and TsCystatin + PBS groups (*P* < 0.001). And there was significantly difference between the TsCystatin + TNBS and HT-TsCystatin + TNBS

groups (*P* < 0.05). Among the therapy subgroups, NF-κB expression in the TNBS+PBS group was also higher than that in the control, PBS + TsCystatin, PBS + HT-TsCystatin groups (*P* < 0.001) and TNBS + TsCystatin, TNBS + HT-TsCystatin (*P* < 0.05), but NF-κB expression in the TNBS+TsCystatin group was lower than that in the TNBS + PBS group (*P* < 0.05) and higher than that in the PBS + TsCystatin group (*P* < 0.05). The results were shown in Fig. 9(a) and (b).

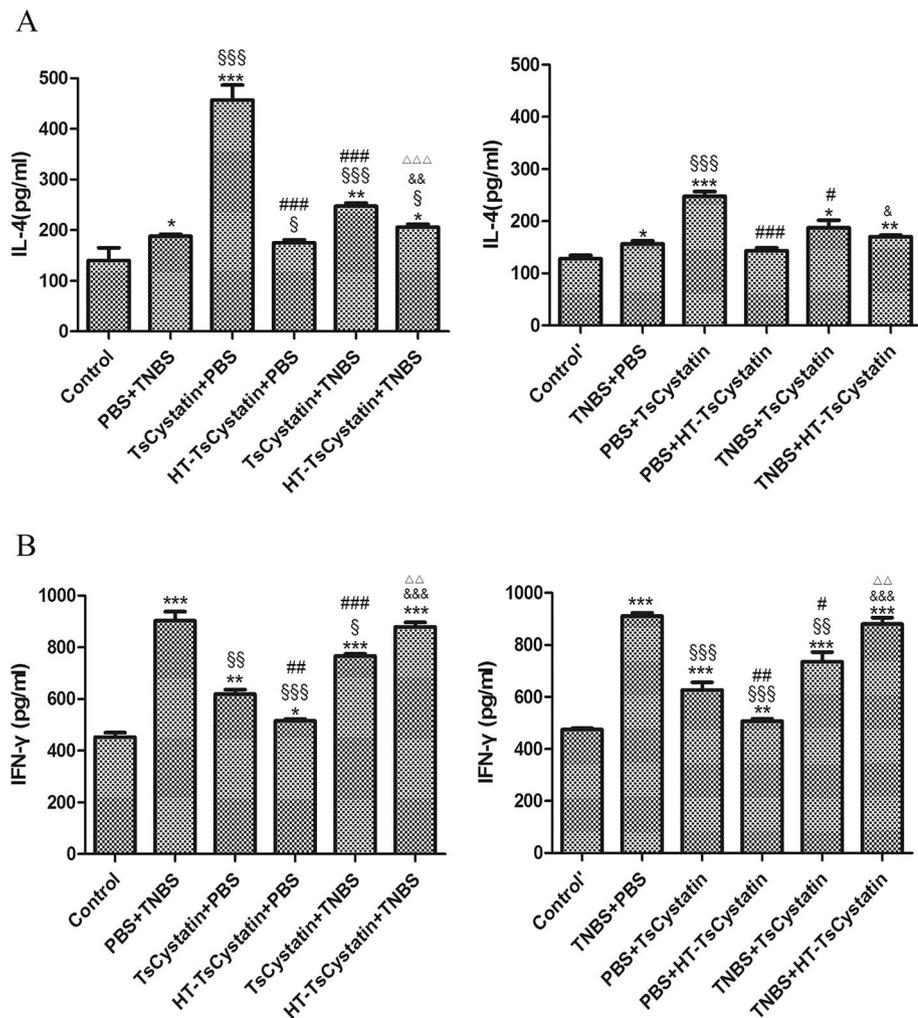


Fig. 8. IL-4 concentration in the colon homogenate supernatants was tested by using ELISA (a). IL-4 level decreased in the PBS + TNBS and TNBS+PBS group and increased in TsCystatin + TNBS and TNBS + TsCystatin group. IFN-γ concentration in the colon homogenate supernatants was tested (b). IFN-γ concentration increased in PBS + TNBS and TNBS + PBS groups and decreased in TsCystatin + TNBS and TNBS + TsCystatin groups. Data are shown as mean ± SD of 3 mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus Control and Control' group; §*P* < 0.05, §§*P* < 0.01, §§§*P* < 0.001 versus PBS + TNBS and TNBS + PBS group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 for TsCystatin + PBS vs HT-TsCystatin + PBS, TsCystatin + TNBS and PBS + TsCystatin vs PBS + HT-TsCystatin, TNBS + TsCystatin; &*P* < 0.05, &&*P* < 0.01, &&&*P* < 0.001 for HT-TsCystatin + TNBS vs HT-TsCystatin + PBS and TNBS + HT-TsCystatin vs PBS + HT-TsCystatin; △*P* < 0.05, △△*P* < 0.01, △△△*P* < 0.001 for HT-TsCystatin + TNBS vs TsCystatin + TNBS and TNBS + HT-TsCystatin vs TNBS + TsCystatin. There was no interaction between the two factors of enema with TNBS or PBS and intraperitoneal injection with recombinant protein or PBS.

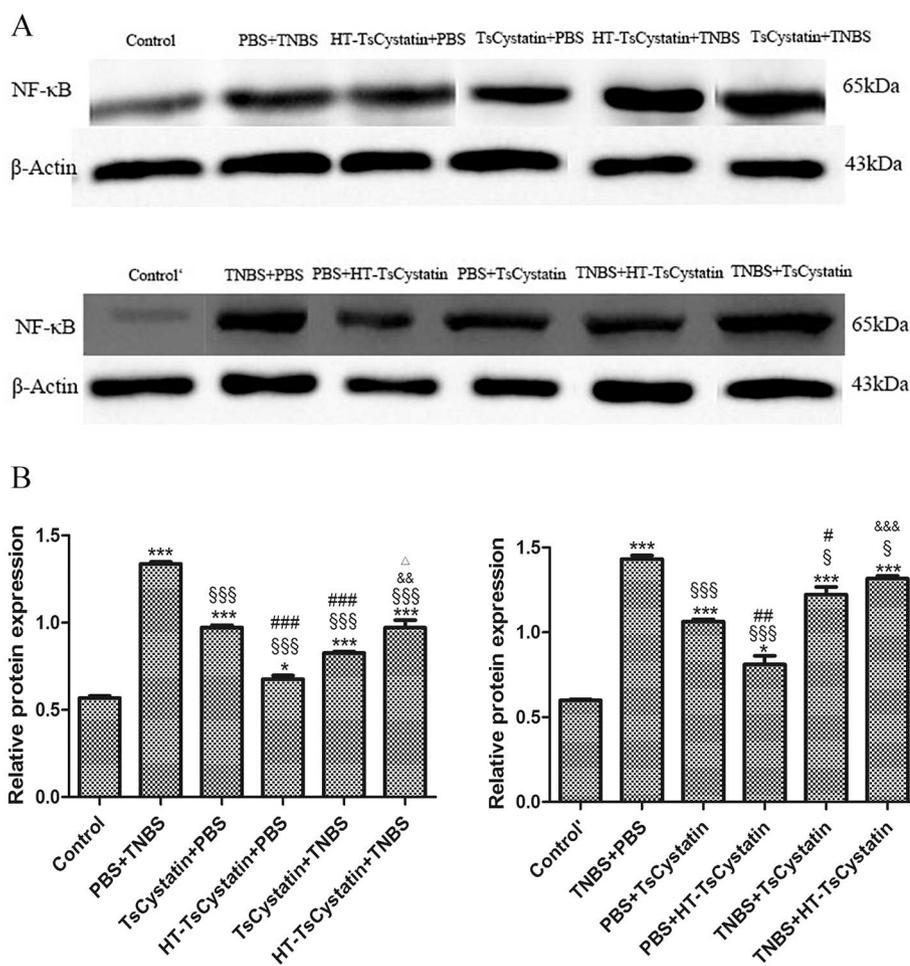


Fig. 9. The representative gel of the Western Blotting were shown in (a), and the graph of the quantified band density were also shown in (b). The expression of NF-κB in colon tissues of PBS + TNBS group significantly higher than TsCystatin + TNBS group. The similar result was found in the therapy large group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus Control and Control' group; §*P* < 0.05, §§*P* < 0.01, §§§*P* < 0.001 versus PBS + TNBS and TNBS + PBS group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 for TsCystatin + PBS vs HT-TsCystatin + PBS, TsCystatin + TNBS and PBS + TsCystatin vs PBS + HT-TsCystatin, TNBS + TsCystatin; &*P* < 0.05, &&*P* < 0.01, &&&*P* < 0.001 for HT-TsCystatin + TNBS vs HT-TsCystatin + PBS and TNBS + HT-TsCystatin vs PBS + HT-TsCystatin; Δ*P* < 0.05, ΔΔ*P* < 0.01, ΔΔΔ*P* < 0.001 for HT-TsCystatin + TNBS vs TsCystatin + TNBS and TNBS + HT-TsCystatin vs TNBS + TsCystatin. There was no interaction between the two factors of enema with TNBS or PBS and intraperitoneal injection with recombinant protein or PBS.

3.8. Changes in expression of CD4 + CD25 + Foxp3 + Treg and CD8 + CD28 – Treg in the spleen

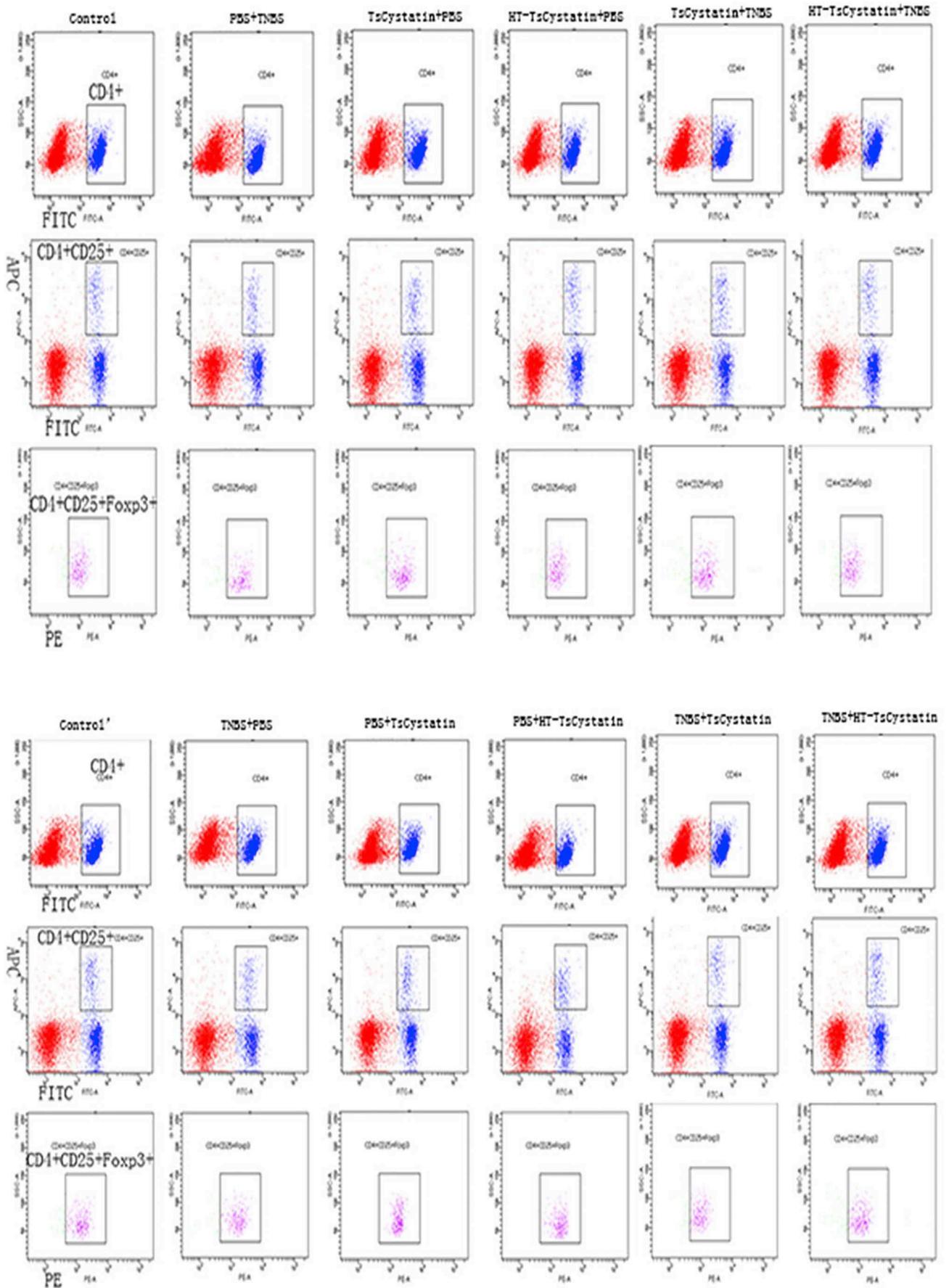
The mice were sacrificed on the 7th day after TNBS modeling. The spleen of each group was used to prepare the cell suspension. The expression levels of CD4 + CD25 + Foxp3 + Treg (Fig. 10(a)) and CD8 + CD28 – Treg (Fig. 10(b)) in the spleens of each group were detected by FCM. The ratio of CD4 + /CD8 + T cells (Fig. 11(c)) in each group was calculated. Among the prevention subgroups, the ratios of CD4 + /CD8 + T cells of the TsCystatin + PBS (1.71 ± 0.20) and PBS + TNBS groups (2.19 ± 0.15) were lower than that in the TsCystatin + TNBS group (1.90 ± 0.08; *P* < 0.05). And the TsCystatin + TNBS was significant different from the PBS + TNBS and HT-TsCystatin + TNBS groups. However, no significant difference was found between each group and the control group (1.97 ± 0.11; *P* > 0.05). Among the therapy subgroups, no significant difference was found compared the TNBS + TsCystatin (2.10 ± 0.09), TNBS + HT-TsCystatin (2.21 ± 0.11) with TNBS + PBS group (2.22 ± 0.13), and there was no difference between the TNBS + TsCystatin and TNBS + HT-TsCystatin groups (*P* < 0.05). Among the prevention subgroup, the percentages of CD4 + CD25 + Foxp3 + Treg in the CD4 + Treg cells (Fig.11(a)) in the PBS + TNBS (12.34 ± 0.62; *P* < 0.05), TsCystatin + PBS (17.30 ± 0.68; *P* < 0.001), TsCystatin + TNBS (14.52 ± 0.17; *P* < 0.05) significantly differed from control groups (13.94 ± 0.23). Moreover, the TsCystatin + TNBS group exhibited a higher percentage than the PBS + TNBS group (*P* < 0.001) but a lower percentage than the TsCystatin + PBS group (*P* < 0.01). The HT-TsCystatin + TNBS group (12.87 ± 0.77) was higher than the PBS + TNBS (*P* > 0.05) and lower than the TsCystatin + TNBS group (*P* < 0.05). Among the therapy subgroups, the control' group

(13.85 ± 0.19) significantly differed from the other groups. The TNBS + TsCystatin group (13.15 ± 0.15) was lower than the PBS + TsCystatin group (15.14 ± 0.31; *P* < 0.001) but not significantly different compared with the TNBS + PBS group (12.15 ± 0.82; *P* > 0.05). And there was no difference between the TNBS + TsCystatin and TNBS + HT-TsCystatin group (12.48 ± 0.45). Regarding the percentage of CD8 + CD28 – Treg cells in the total lymphocyte cells in the gate (Fig. 11(b)), the difference compared the other group with control group in the prevention subgroups was not obvious. Only the TsCystatin + TNBS (13.51 ± 1.24), TsCystatin + PBS (13.59 ± 0.39) and HT-TsCystatin + PBS group (12.84 ± 0.30) were significantly higher than the PBS + TNBS group (10.95 ± 0.95; *P* < 0.05). Meanwhile, among the therapy subgroups, the TNBS + TsCystatin group (11.47 ± 0.81) was not significantly different compared with the TNBS + PBS group (11.51 ± 0.47; *P* > 0.05) but significantly lower than the PBS + TsCystatin group (14.96 ± 0.90; *P* < 0.01). And compared the TNBS + HT-TsCystatin with the TNBS + PBS and TNBS + TsCystatin groups, there was no significant difference.

4. Discussion

TNBS is an organic chemical reagent, which is a hapten substance. After ethanol burns the colonic mucosa, it enters the mucosa and combines with the lysine ε-amino group of the tissue protein to form a complete antigen, which in turn causes an inflammatory reaction and a series of immune responses. The TNBS-ethanol model can be observed successfully on the second day after modeling. It is also a well-recognized CD model at home and abroad and the most widely used experimental CD model. The model is simple in modeling, reproducible, with outstanding inflammation symptom and low price, so it is suitable

A



(caption on next page)

Fig. 10. Demonstration of the gating strategy for the flow cytometric analysis of mouse CD4 + CD25 + Foxp3 + Treg (a), CD8 + CD28- Treg (b) from spleens. In this experiment a single cell suspension was prepared from the spleen of each group of mouse and stained with CD4 (FITC), CD25 (APC) and Foxp3 (PE), CD8 (PE), CD28 (APC) based on surface and intracellular staining protocols, respectively. Data were collected with FACSDiva flow cytometer and analyzed. Lymphocytes are identified by their scatter properties (FSC-A × SSC-A plot).

for immunological research of IBD. TNBS-induced experimental colitis in mice is a characteristic transmural colon inflammation that causes a Th1-type immune response and can be considered a model of CD. Worms and worm-derived products can inhibit the development of IBDs [17–20] mainly through downregulation of Th1 and Th17 immune responses. In this experiment, the role of TsCystatin in the mouse IBD model was initially studied.

Compared with the mice in the PBS + TNBS and TNBS + PBS groups, the mice in the two subgroups treated with TsCystatin demonstrated less intestinal damage, lower disease activity index, lower MPO activity, and significantly improved intestinal inflammation symptoms. TsCystatin also proved to be effective in alleviating TNBS-induced colitis.

The high level of prokaryotic expression of recombinant proteins forms inclusion bodies in *E. coli*. Gao et al. [21] established a novel and highly efficient method for purifying proteins by KCl staining and purifying proteins is antigenic and induces immune responses in animals. Therefore, this method was used to prepare and purify recombinant proteins. Since protein was expressed in *E. coli*, *E. coli*-expressed proteins nearly always have high levels of endotoxin, so a control protein which is inactive but expressed in a similar system should be used to show that the target proteins rather than contaminants therein mediate the effects. Therefore, the recombinant

protein was boiled for 10 min to inactivate the protein. Meanwhile obvious symptoms were showed 24 h after the colitis model was established. Therefore, the mice were treated with the protein 24 h after the modeling and boosted once after 48 h.

Many studies on nematodes have found that cystatin is a key molecule in protein degradation, antigen presentation, apoptosis, protein processing, inflammation, and cancer development [22–26]. Cystatin can increase the production of IL-10 in the animal body and IL-10 is an inhibitory cytokine that inhibits the synthesis of Th1 cytokines and cell growth and differentiation, thereby downregulating the proliferation of non-specific and specific antigen-stimulated T cells [10].

ELISA results showed that treatment with TsCystatin significantly reduced the expression of IFN- γ in TNBS-induced mice, and the expression of IL-4 significantly increased. However, in the therapy subgroups, no significant difference was found in the changes in IL-4 between the TNBS + TsCystatin and TNBS + PBS groups. Meanwhile, the level of IL-4 expression in the TsCystatin + PBS group was curiously much higher than that in the PBS + TsCystatin group. This may be due to the fact that the number and doses of protein in the therapy subgroups did not cause the body to produce a high level of immune response. Thus, TsCystatin shifted the CD-induced mucosal immune response from a Th1-type to a Th2-type response, reducing the intensity of the Th1-type immune response, decreasing the expression of pro-

B

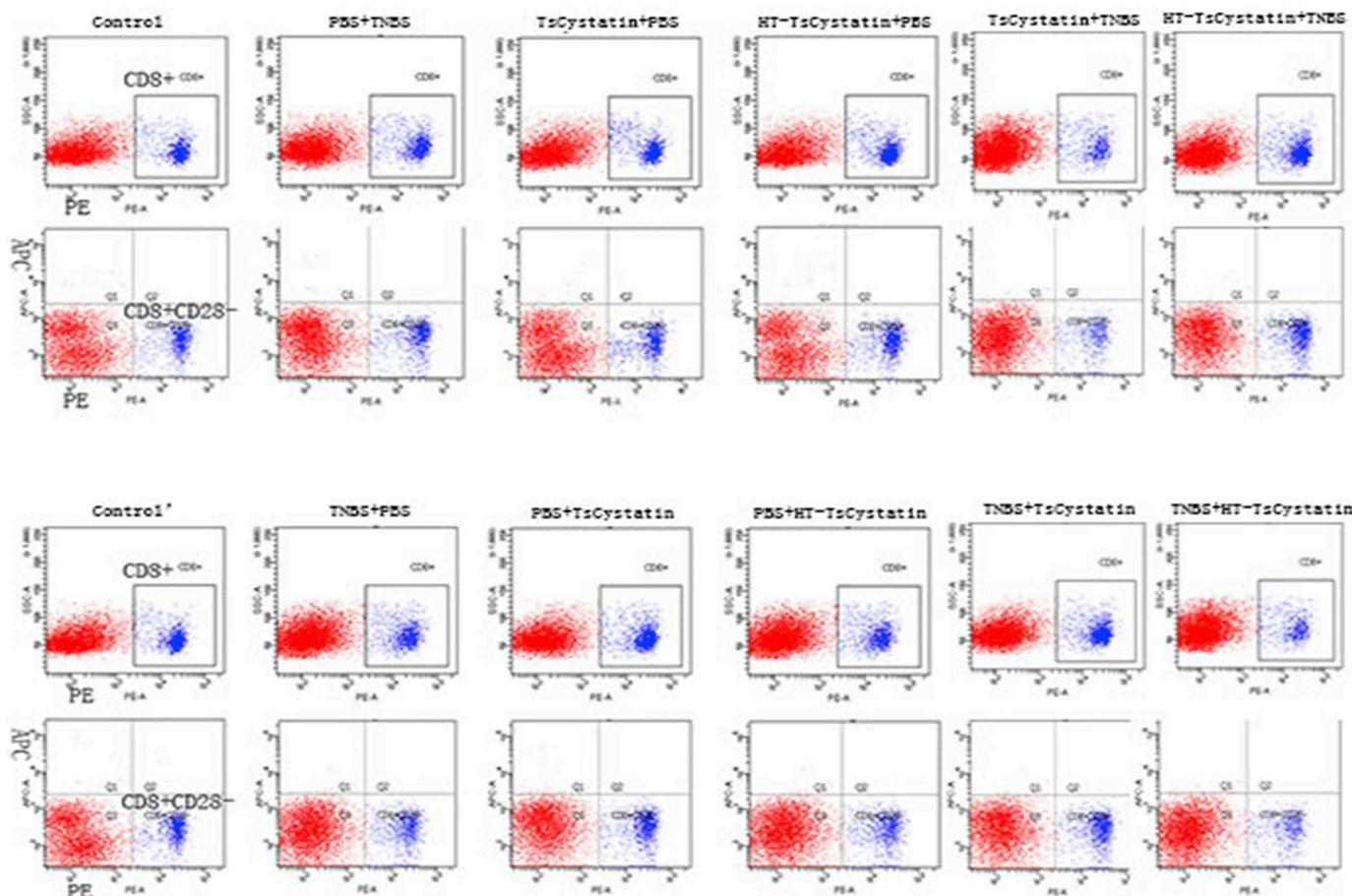


Fig. 10. (continued)

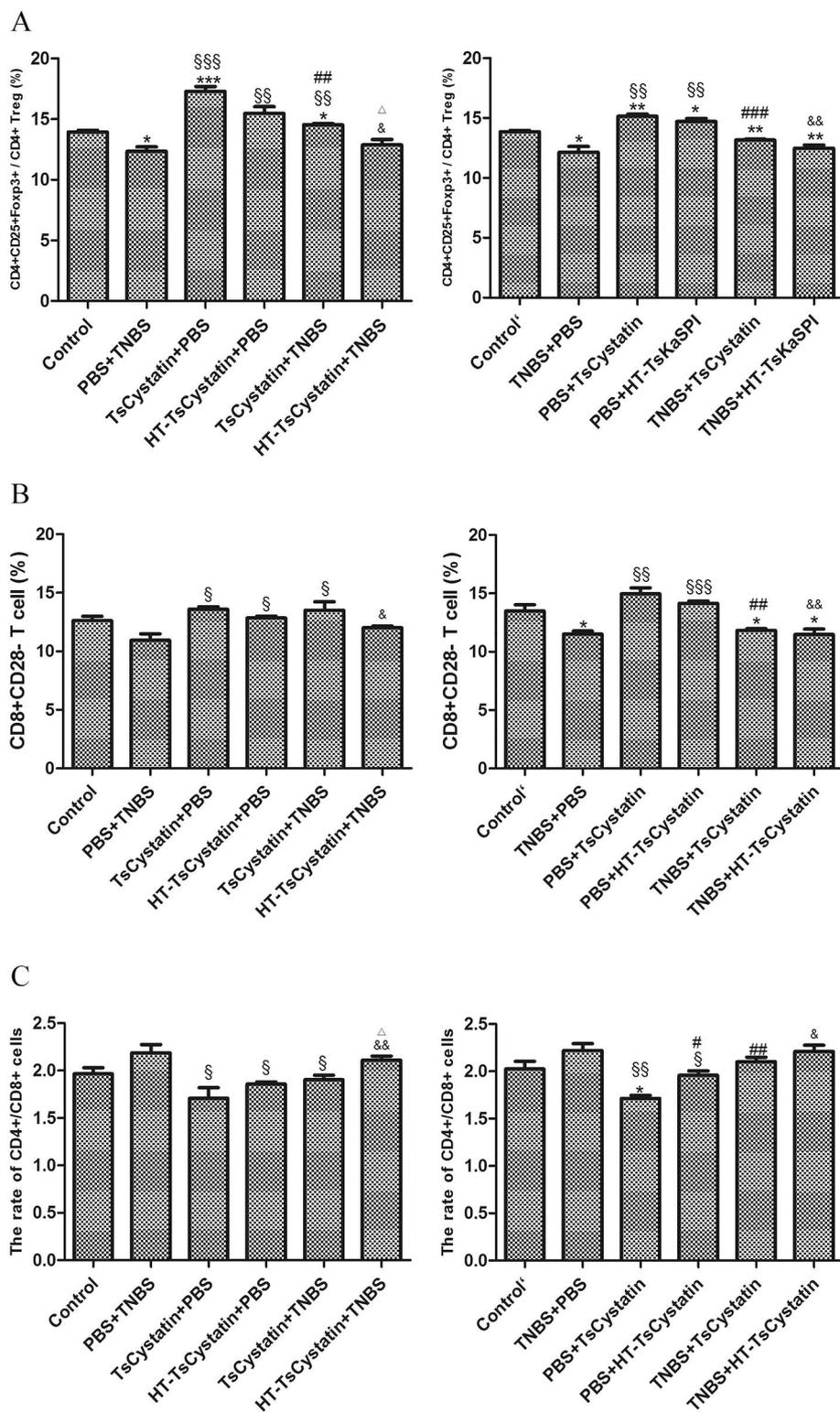


Fig. 11. The percentage of CD4+CD25+Foxp3+ Treg cells in CD4+ T lymphocytes (a), the percentage of CD8+CD28- Treg cells in the total cell number in the Gate (b) and the rate of CD4+/CD8+ Treg cells (c) in spleens of twelve groups. Data are shown as mean \pm SD of 3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Control and Control' group; \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ versus PBS + TNBS and TNBS + PBS group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ for TsCystatin + PBS vs HT-TsCystatin + PBS, TsCystatin + TNBS and PBS + TsCystatin vs PBS + HT-TsCystatin, TNBS + TsCystatin; & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$ for HT-TsCystatin + TNBS vs HT-TsCystatin + PBS and TNBS + HT-TsCystatin vs PBS + HT-TsCystatin; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ for HT-TsCystatin + TNBS vs TsCystatin + TNBS and TNBS + HT-TsCystatin vs TNBS + TsCystatin. The two-way results showed that there was no interaction between the two factors of enema with TNBS or PBS and intraperitoneal injection with recombinant protein or PBS.

inflammatory cytokines, and increasing the expression of anti-inflammatory cytokines. The effect of relieving intestinal inflammation was consistent with the expected experimental results.

NF- κ B plays an important role in the disturbance of the intestinal mucosal immune response and the signal transduction of intestinal inflammation in IBD [27–30]. Activated NF- κ B promotes the expression of many pro-inflammatory cytokines and increases the recruitment of inflammatory cells. The Western blot results of this experiment showed that the expression of NF- κ B in the colon tissue of the PBS + TNBS and

TNBS + PBS groups was significantly higher than that of the other groups, indicating that overactivation of NF- κ B aggravated intestinal inflammatory symptoms. Moreover, the expression of NF- κ B in the TsCystatin + TNBS and TNBS + TsCystatin groups was significantly higher than that in the PBS + TNBS and TNBS + PBS groups, but the difference in the prevention groups was more obvious than that in the therapy groups. Treatment with TsCystatin could reduce the secretion of pro-inflammatory cytokines and reduce intestinal inflammation by reducing the activation of NF- κ B.

The occurrence and development of IBD are also associated with numerous intestinal mucosal immune regulatory cells. T lymphocytes are an important class of immunocompetent cells that are classified into CD4+ (TH) and CD8+ (TS/TC) subpopulations depending on their function. The subpopulations are interconnected, promote each other, and restrict each other. The dynamic balance of the CD4+/CD8+ ratio determines the state of immune regulation and immunity, and a decrease in the ratio of CD4+/CD8+ marks the inhibition of immune function [31]. Barriga [32] proposed that the excretion and secretion protein of *T. spiralis* can induce the production of IL-2 [33]. IL-2 stimulates the proliferation and differentiation of CD8+ cells, thereby decreasing the ratio of CD4+/CD8+ and suppressing immune function in the host. A decrease in the ratio of CD4+/CD8+ may have a mitigating effect on autoimmune diseases. The experimental results showed that treatment with TsCystatin decreased the ratio of CD4+/CD8+ and inhibited the increase in the CD4+/CD8+ ratio caused by induced colitis. However, the differences between the subgroups were not significant. Therefore, the change in the CD4+/CD8+ ratio may not be the main mechanism of TsCystatin's effect on IBD.

CD4+ T cells can differentiate into four types of cells with different functions under varying cytokines and environmental effects: Th1, Th2, CD4+CD25+ regulatory T cells (Treg), and Th17 cells. Among them, the role of Treg cells in IBD has attracted increasing attention. Treg is a subset of T cells with immunomodulatory functions that inhibit the activation and function of other immune cells and participate in the regulation of the body's own immune system [34,35]. Foxp3 is a core transcription factor that regulates the development and differentiation of Treg cells. Only CD4+CD25+ Tregs that express Foxp3 have immunomodulatory effects [36,37]. The results of this experiment showed that the percentages of spleen CD4+CD25+Foxp3+ Treg in CD4+ Treg cells in the PBS + TNBS group and TNBS+PBS group were significantly lower than those of the control group and control' group. Moreover, the percentage of CD4+CD25+Foxp3+ Treg cells in the TsCystatin + TNBS group was significantly higher than that in the PBS + TNBS group, but no significant difference was noted between the TNBS + TsCystatin group and TNBS + PBS group. The percentage of CD4+CD25+Foxp3+ cells in the TsCystatin + PBS group was lower than that of the PBS + TsCystatin group, which could be due to the fact that treated protein at 24 and 48 h after the establishment of the colitis model did not produce sufficient stimulation to the immune system of the body.

Based on whether the expression of CD28 on CD8+ T cells, CD8+ cells can be divided into two groups: CD8+CD28+ (specific killer T cells, Tc) and CD8+CD28- (immunosuppressive T cells, Ts). CD8+ T cells differentiate into Ts and Tc under the action of cytokines secreted by CD4+ T cells and Tc cell subpopulations exert a specific killing effect on target cells infected with the virus, Ts cell subpopulations have immunosuppressive effects and inhibit the production of antibodies, cell proliferation induced by allogeneic antibodies, and the production of cytokines by CD4+ T cells, thereby inhibiting the toxicity of Tc cells [38]. The experimental results showed that the induction of colitis reduced the percentage of CD8+CD28- cells, whereas the cells treatment with protein increased the percentage of CD8+CD28- cells. Thus, TsCystatin exerted a mitigating effect on IBD.

TsCystatin has been shown to relieve IBD. In this experiment, the protective and therapeutic effects of TsCystatin in a mouse IBD model were initially studied. For autoimmune diseases with complex and diverse mechanisms, we need to conduct in-depth studies on the mechanism of action of TsCystatin in all aspects. Such work is conducive to the development of new and effective helminth-derived proteins/drugs.

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Competing interests

The authors declare that they have no competing interests.

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