Cryptotanshinone alleviates DSS-induced colitis in mouse by regulating the balance of Treg/Th17 cells and M1 macrophage activation

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ABSTRACT

Inflammatory bowel disease (IBD) including ulcerative colitis (UC) and Crohn's disease has become a global disease in the 21st century, with increasing incidence rates in almost every industrialized country. Previous studies have suggested that the traditional Chinese medicine herb, cryptotanshinone (CTN), a major liposoluble extract of *Salvia miltiorrhiza*, alleviates the symptoms of experimental colitis *in vitro* and *in vivo*. However, the mechanisms underlying the protective effects of CTN against IBD remain exclusive. The present study found that CTN reversed lipopolysaccharide-induced inflammation in human colon epithelial cells (HIEC-6) by inhibiting the NF-κB pathway. In addition, CTN alleviated dextran sulfate sodium (DSS)-induced inflammatory bowel disease in mice by regulating the balance of TH17/Treg cells. CTN also exerted its role by inhibiting the polarization of M1 macrophages in mice with DSS-induced colitis. Of note, the effects of CTN on these immune cells may be mediated *via* changes in the levels of TNF-α and IL-6 directly in mice. Taken together, these findings may provide new insight regarding the therapeutic potential of CTN for UC.

Key words: cryptotanshinone; inflammatory bowel disease; regulatory T cells; macrophage; STAT3.

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Introduction

Inflammatory bowel disease (IBD),[1] including ulcerative colitis (UC)[2] and Crohn's disease,[3] has become a global disease in the 21st century, with increasing incidence rates in almost every industrialized country.[4] It is characterized by a dysregulated mucosal immune response to the intestinal microbiota in genetically susceptible individuals.[4] Common symptoms of IBD include chronic diarrhea, abdominal pain, rectal bleeding and fatigue.[1] Traditional drug treatments for IBD, such as 5-aminosalicylates (5-ASA) and steroids, are recommended for the induction and maintenance of remission in mild to moderate disease.[5] Systemic corticosteroids and immunosuppressants are used for the treatment of moderate to severe ulcerative colitis,[2] however, there is currently no high-quality evidence available to support their use in Crohn's disease.[5] In addition to the uncertain efficacy of traditional therapies, clinicians should also be aware of potential severe adverse reactions, such as drug-induced fever, rashes and even neurotoxicity.[6] High recurrence rates and contraindications also limit the use of conventional therapies. Regulatory T-cells (Tregs), also known as suppressor T-cells in the early stage, are a subset of T-cells characterized by the expression of Foxp3, CD25 and CD4.[7] The thymus is the main site for the development of Tregs, and these cells are one of the key factors in maintaining immune tolerance in the body.[7] Tregs exert immunosuppressive effects and can inhibit the immune responses of other immune cells, serving as the primary controller of self-tolerance.[2] As part of $CD4^+$ cells, Tregs work in concert with other conventional helper T-cell subsets, such as Th1, Th2, Tfh, Th17 and Th22.[8] Therefore, in the event that Tregs are absent or function abnormally, this can lead to the occurrence of autoimmune diseases in the body.[8] Th1 and Th2 were the first two subsets discovered . Subsequently, new $CD4^+$ helper T subsets were found, namely Th17 cells, named for secreting the IL-17A cytokine; Th17 cells play a role in the host defense against extracellular pathogens, particularly at mucosal and epithelial barriers; however, their abnormal activation is associated with various autoimmune diseases.[9] In addition, Th17 cells are able to produce inflammatory factors, including interleukin (IL)-17A, IL-21 and IL-2. The imbalance of Tregs and Th17 cells can contribute to the pathogenesis of IBD.[9]

Cryptotanshinone (CTN), a major liposoluble extract of *Salvia miltiorrhiza*, is an orange needle-like crystal, which belongs to the diterpene quinone class of compounds.[10] The majority of these components have a tricyclic or tetracyclic structure of ortho-quinone or para-quinone on the skeleton, which endows them with various activities such as antitumor, antioxidant, anti-inflammatory and anti-bacterial properties.[10] Previous studies have demonstrated that CTN can be used in the treatment of UC; however, the mechanisms underlying the anti-inflammatory effects of CTN remain to be fully elucidated.[11,12]

In the present study, it was found that CTN alleviates acute ulcerative colitis (UC) by decreasing Th17 cells and inhibiting M1 macrophage polarization in mice with dextran sulfate sodium (DSS)-induced colitis. The data presented herein suggest that CTN might serve as a promising therapeutic agent for UC.

Materials and Methods

Cells and cell culture

The human colon epithelial cell line (HIEC-6) was purchased from the American Type Culture Collection (ATCC). DMEM:F12 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific., Inc.) was used for HIEC-6 cell culture, and the cells were incubated at 37°C with 5% CO_2. CTN was purchased from MedChemExpress (MCE; Monmouth Junction, NJ, USA).

CCK-8 assay

HIEC-6 cells were seeded in 96-well plates (10,000 cells per well) and cultured overnight in an incubator at 37°C. The cells were then incubated with various concentrations of lipopolysaccharide (LPS; 5, 10, 25, 50 or 100 µg/mL, MCE) for 24 h.[13] Subsequently, 10 mL CCK-8 solution were added to each well and incubated for a further 4 h at 37°C. The absorbance values of each well were measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). The HIEC-6 cells were then treated with LPS at 20 µg/mL for 24 h, in order to mimic UC *in vitro*.

EdU staining

HIEC-6 cell proliferation was measured using an EdU detection kit (Beyotime Institute of Biotechnology, Haimen, China). The cells were fixed in 4% paraformaldehyde for 2 h at room-temperature (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) and then stained with EdU-488 solution for 1 h at 37°C in the dark. The cells were then incubated with DAPI for 15 min at room-temperature (Beyotime Institute of Biotechnology). Subsequently, three random fields were selected and the EdU-positive cells were observed and counted using a fluorescence microscope (Eclipse Ci-L, Nikon Corporation, 200× magnification). The EdU positive cells in three random images per section were scored and quantified.

RT-qPCR

Total RNA was extracted from human colon epithelial cells using a total RNA extraction kit (Thermo Fisher Scientific, Inc.). A total of 1 µg RNA was reverse transcribed into cDNA using a EntiLink™ 1st Strand cDNA Synthesis kit (Wuhan ELK Biotechnology, Wuhan, China). The cDNA was then mixed with EnTurbo™ SYBR-Green PCR SuperMix (Wuhan ELK Biotechnology) and detected using real-time PCR (QuantStudio 6 Flex; Life Technologies, Carlsbad, CA, USA). The sequences of the primers were: IL-6 (sense, 5'-CTTCTTGGGACTGATGCTGGT-3' and antisense, 5'-CACAACTCTTTTCTCATTTCCACG-3'); TNF-α (sense, 5'-GATCTCAAAGACAACCAACTAGTGG-3' and antisense, 5'-GAGGTTGACTTTCTCCTGGTATGAG-3'); GAPDH (sense, 5'-TGAAGGGTGGAGCCAAAAG-3' and antisense, 5'-AGTCTTCTGGGTGGCAGTGAT-3'). Target genes were normalized to their internal control (GAPDH) and quantified using the $2^{-\Delta\Delta Cq}$ method.

Western blot analysis

Cells were collected and lysed using immunoprecipitation assay (RIPA) buffer (MilliporeSigma, Burlington, USA) containing 1% Complete Protease Inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich). The concentrations of proteins were determined using the BCA assay (Pierce Biotechnology, Inc., Rockford, IL, USA). A total of 30 mg protein was separated using SDS-PAGE and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 □m: MilliporeSigma). The membrane was then blocked with 5% skimmed milk for 1 h, and sequentially incubated with the primary and the corresponding HRP conjugated secondary antibody solutions (1:3,000, Cat. 32460; Thermo Fisher Scientific, Inc.) overnight at 4°C and 1 h at room temperature, respectively. Finally, ECL detection reagents were used to detect target proteins on X-ray film. The primary antibodies used were as follows: phospho-NF-κB p65 (1:1,000, ab76302; Abcam,

Cambridge, UK), NF-κB p65 (1:1,000, ab16502; Abcam), phospho-STAT3 (1:1,000, ab76315; Abcam), STAT3 (1:1,000, ab109085; Abcam), GAPDH (1:1,000, ab181602; Abcam).

TUNEL assay

HIEC-6 cell apoptosis was detected using a TUNEL kit (Wuhan Servicebio Technology Co., Ltd.). Briefly, the cells were fixed in 4% paraformaldehyde for 2 h at room temperature. Subsequently, 0.2% Triton X-100 (2 min at room temperature) was used for cell membrane penetration. The cells were then stained with TdT-488 labeling solution for 1 h at 37°C and the nuclei were stained with 0.1 μg/ml DAPI for 30 min. Finally, three random fields were selected and the TUNEL-positive cells were observed and counted using a fluorescence microscope (Eclipse Ci-L, Nikon Corporation, 200× magnification). The TUNEL positive cells in three random images per section were scored and quantified.

Animals

Female C57BL/6 mice, aged 7-8 weeks at the beginning of the experiments, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were bred in a specific pathogen-free (SPF) environment at 22±2°C and a humidity of 55±10% on a 12-h light/dark cycle. Animal study protocols were performed according to the Institutional Animal Ethics Committee of Bestcell (authorization no. 2025-08-06A).

DSS-induced colitis in mice

Mice were treated 3% DSS (w/v; 36,000-50,000 M.W.; MP Biomedicals, Irvine, CA, USA) in water for a continuous period of 7 days to induce colitis. Moreover, mice were treated with CTN (50 mg/kg/day) orally *via* gavage for 7 days from the beginning of DSS administration.[14] The stool consistency, fecal blood and weight loss of each mouse were assessed daily. The DAI score was calculated as the mean of the three parameters.[15]

H&E staining

Colon tissues were washed with PBS and fixed in 4% paraformaldehyde for 48 h at room-temperature. The colon tissues were then embedded in paraffin, sectioned (4-μm-thick) followed by staining with hematoxylin for 10 min. The sections were then rehydrated in alcohol at concentrations of 90 and 70% for 15 min each at room temperature. Subsequently, the sections were incubated with eosin staining solution for 10 min at room temperature. Images were observed using a light microscope (CX31, Olympus Corporation, 200× magnification).

Immunohistochemistry

Four-μm-thick paraffin sections were heated in a 60°C oven. Next, samples were deparaffinized, rehydrated and boiled in 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave oven for 10 min for antigen retrieval. Then, samples were blocked with 0.3% hydrogen peroxide for 15 min at room temperature. Then, the sections were incubated with 10% normal goat serum for 60 min and probed with primary antibodies CD86 (1:200, ab119857; Abcam) and p-STAT3 (1:200, ab76315; Abcam) at 4°C overnight. After that, the samples were incubated with a secondary antibody (1:200) for 30 min at 37°C. The secondary antibody was purchased form Wuhan Servicebio (Wuhan, China). The sections were stained with 3,3'-diaminodbenzidine solution (Wuhan Servicebio). Finally, the images were captured using a light microscope CX31, Olympus Corporation, 200× magnification). The intensity of staining and proportion of positive cells in three random images per section were scored by the experienced pathologist. Negative control samples were prepared by excluding the primary antibody and replacing it with PBS.

Flow cytometric detection of Treg cells

Flow cytometry was used to detect the Tregs, Th17 and macrophage cells. Firstly, peripheral blood or spleen cells were incubated with anti-CD16/32 (BioLegend, San Diego, CA, USA) for 20 min for Fc receptor blocking. Leukocytes were stained with anti-CD4 and anti-Foxp3 antibodies for Treg identification. For Th17 cell analysis, leukocytes were stimulated with a Cocktail kit (Tonbo Biosciences, San Diego, CA, USA) containing phorbol ester, phorbol 12-myristate 13-acetate, ionomycin, brefeldin A and monensin for 6 h. The lymphocytes were then incubated with anti-CD4 and anti-IL-17 antibodies. The percentages of $CD4^+$, $Foxp3^+$ and $IL\text{-}17^+$ cells were measured using flow cytometry (BD). The data were analyzed using FlowJo V.7.6.2 software.

ELISA

Mouse IL-6 (Wuhan ;Biotechnology), mouse TNF-α (Wuhan Elk Biotechnology), nitric oxide (NO, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) mouse TNF-α (Wuhan Elk Biotechnology) and mouse TGF-β (Wuhan Elk Biotechnology) detection kits were used to detect the IL-6, TNF-α, NO, IL-10 and TGF-β levels in the supernatant of HIEC-6 cells. All ELISAs were conducted according to the manufacturers' instructions and the results were analyzed using a microplate reader (Thermo Fisher Scientific, Inc.).

Statistical analysis

GraphPad Prism 8.0 (Dotmatics, Boston, MA, USA) was used for data analysis, and results are presented as the mean ± SD. An unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) was used for statistical analyses. *p*-values <0.05 were considered to indicate statistically significant differences and were defined as follows: $*p<0.05$, $**p<0.01$.

Results

CTN reverses LPS-induced inflammation in HIEC-6 cells

First, to mimic inflammatory bowel disease *in vitro,* HIEC-6 cells were treated with various concentrations of LPS (5, 10, 25, 50 or 100 μg/mL) for 24 h. The data presented in Figure 1A suggest that 25, 50 or 100 μg/mL LPS significantly inhibited HIEC-6 cell viability. Of note, 50 μg/mL LPS induced ~50% cell growth inhibition; thus, 50 μg/mL LPS was selected for use in the subsequent experiments. Subsequently, the present study examined the protect effect of CTN on LPS-treated HIEC-6 cells. The results of EdU assay indicated that LPS notably decreased the number of EdU-positive cells, while 5 or 10 μM CTN significantly revered this phenomenon (Figure 1B). NF-κB (p65) has been reported to play a crucial role in intestinal inflammation.[16] In the present study, the upregulation of p-p65 induced by LPS in HIEC-6 cells was revered by treatment with 5 or 10 μM CTN (Figure 1C), indicating that LPS-induced inflammation was prevented by CTN. In addition, LPS increased the levels of inflammatory cytokines, including IL-6 and TNF-α in the cell supernatant; this effect was significantly reversed by treatment with 5 or 10 μM CTN (Figure 1D). These results indicate that CTN is able to reverse LPS-induced inflammation in HIEC-6 cells.

CTN reverses the LPS-induced apoptosis of HIEC-6 cells

The present study then examined whether CTN can protect HIEC-6 cells against LPS-induced apoptosis, using TUNEL assay. The results of TUNEL staining showed that LPS increased the number of TUNEL-positive cells to ~30%, while the numbers in the control group were ~3% (Figure 2A). Either 5 or 10 μM CTN treatment was able to reverse the increase in the number of TUNEL-positive HIEC-6 cells induced by LPS (Figure 2A). Additionally, the LPS-induced upregulation in the NO level in the supernatant of HIEC-6 cells was significantly reversed by treatment with 5 or 10 μM CTN (Figure 2B). Notably, LPS decreased the secretion of the anti-inflammatory factors, IL-10 and TGF-β, in the supernatant of the cells, while these phenomena were reversed by treatment with 5 or 10 μM CTN (Figure 2C). All these results demonstrated that CTN was able to reverse the LPS-induced apoptosis and the increase of anti-inflammatory factors (IL-10 and TGF-β) in HIEC-6 cells.

CTN alleviates DSS-induced UC in mice

To explore the effects of CTN on the progression of UC, a UC model was established using C57BL/6 mice administered a 3% DSS solution. The results of the animal experiments indicated that the body weights of the mice and the DAI scores in the DSS groups were reduced compared to those in the control group; however, these changes were significantly attenuated by treatment with 50 mg/kg CTN (Figure 3 A,B). In addition, the colon length of the mice in the DSS group was markedly reduced compared with the control group (Figure 3C). By contrast, treatment with 50 mg/kg CTN notably increased the colon length of mice with DSS-induced UC (Figure 3C). Moreover, the results of H&E staining indicated that the colons of the mice in the control group were normal, displaying an unblemished mucosal structure without inflammatory cell infiltration. By contrast, DSS led to the development of crypt lesions and the marked infiltration of inflammatory cells (Figure 3 D,E). These phenomena were notably revered by treatment with CTN (Figure 3 D,E). On the whole, these results suggest that CTN reduces the colonic pathological damage induced by DSS in mice with UC. Furthermore, DSS notably increased the concentrations of pro-inflammatory cytokines in the serum of DSS mice compared with the control group (Figure 3 F,G). Consistently, treatment with 50 mg/kg CTN significantly reduced the DSS-induced upregulation in the levels of these cytokines in the serum samples of mice (Figure 3 F,G). Moreover, compared with the control group, the levels of the anti-inflammatory factors, IL-10 and TGF-β, were notably reduced in the serum samples of mice with DSS-induced colitis. However, 50 mg/kg CTN significantly enhanced the levels of these proteins compared with the DSS group (Figure 3 H,I). All these data demonstrated that treatment with 50 mg/kg CTN effectively alleviated DSS-induced UC in mice.

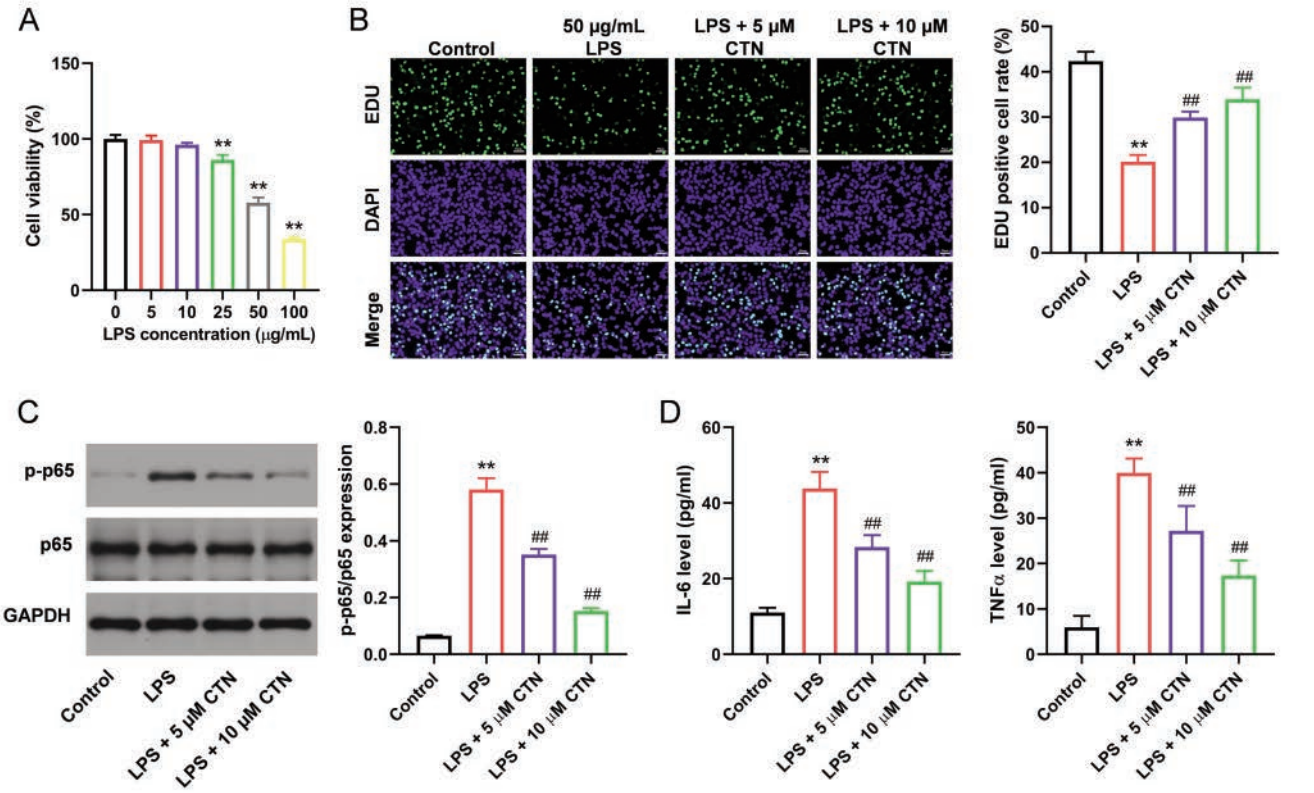

Figure 1. CTN reversed LPS-induced inflammation in HIEC-6 cells. **A**) HIEC-6 cells were treated with different concentrations of LPS (5, 10, 25, 50 or 100 μg/mL) for 24 h and the cell viability was detected with CCK8 assay. **B**) HIEC-6 cells were treated with 50 μg/mL LPS, 50 μg/mL LPS+ 5 μM CTN or 50 μg/mL LPS+ 10 μM CTN for 24 h and cell proliferation was measured with EdU staining; 200× magnification; scale bar: 50 μm. **C**) The protein expression of p-p65 and p65 in HIEC-6 cells was detected using with Western blot. **D**) The inflammation cytokines including IL-6 and TNFα level in cell supernatant was determined with ELISA assay. n=3; $^{**}p<0.05$ compared to control group, $^{\#\#}p<0.05$ comparing with LPS group.

CTN restores the Treg/Th17 balance in DSS-treated mice

Subsequently, the present study investigated the mechanisms underlying the protective effects of CTN against UC in mice. As aforementioned, an imbalance in Tregs and Th17 cells is able to induce UC. The results of flow cytometric analysis suggested that compared with the control group, DSS notably increased the $CD4^+IL\text{-}17^+$ T cell rate in the peripheral blood and spleen cells of mice (Figure 4A). By contrast, treatment with 50 mg/kg CTN reversed the upregulation in the $CD4^+IL\text{-}17^+$ T cell rate in mice with DSS-induced UC (Figure 4A). Additionally, DSS notably decreased the $CD4^+Foxp3^+$ T-cell rate in the peripheral blood and spleen cells of mice, whereas these phenomena were reversed by treatment with 50 mg/kg CTN (Figure 4B). Consistently, the levels of the inflammatory factors, IL-6 and TNF-α, were notably reduced by the treatment of CTN in the colon tissues of mice with DSS-induced UC. However, treatment with 50 mg/kg CTN significantly enhanced the levels of these proteins relative to the DSS group (Figure 4 C,D). These results indicate that CTN was able to restore the Treg/Th17 balance in mice with DSS-induced UC.

CTN inhibits M1 macrophage polarization in mice with DSS-induced UC

A previous study demonstrated that macrophages exhibit unique phenotypic and functional differences when responding to various inflammatory stimuli, presenting as M1/M2 polarized macrophages.[17] Thereby, the number of macrophages in the spleen cells of mice was determined using flow cytometric analysis. The results suggested that DSS notably increased the $CD11b^+F4/80^+$ T cell rate compared with the control group (Figure 5A). By contrast, treatment with 50 mg/kg CTN reversed the increase in the $CD11b^+F4/80^+$ T-cell rate in mice with DSS-induced UC (Figure 5A). Macrophages can differentiate into pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes in response to different stimuli.[18] The results of RT-qPCR revealed that DSS significantly increased the level of CD86 (a specific marker of M1 macrophages), while it decreased the level of CD208 (a specific marker of M2 macrophages) in the colon tissues of mice (Figure 5 B,C). As was expected, treatment with 50 mg/kg CTN reversed the changes in the levels of CD86 and CD208 in mice with DSS-induced UC (Figure 5 B,C). Moreover, the upregulation of p-p65 and p-STAT3 induced by DSS in the colon tissues of mice was reversed by treatment with CTN, indicating that DSS-induced inflammation was prevented by CTN (Figure 5D). The results of IHC confirmed that the upregulation of macrophage cells (CD86) and p-STAT3 induced by DSS in the colon tissues of mice was reversed by treatment with CTN as well (Figure 6 A,B). These results indicated that CTN inhibited M1 macrophage polarization in mice with DSS-induced UC and that the NF-κB p65/STAT3 pathway may be involved in the regulatory effects of CTN on macrophage polarization.

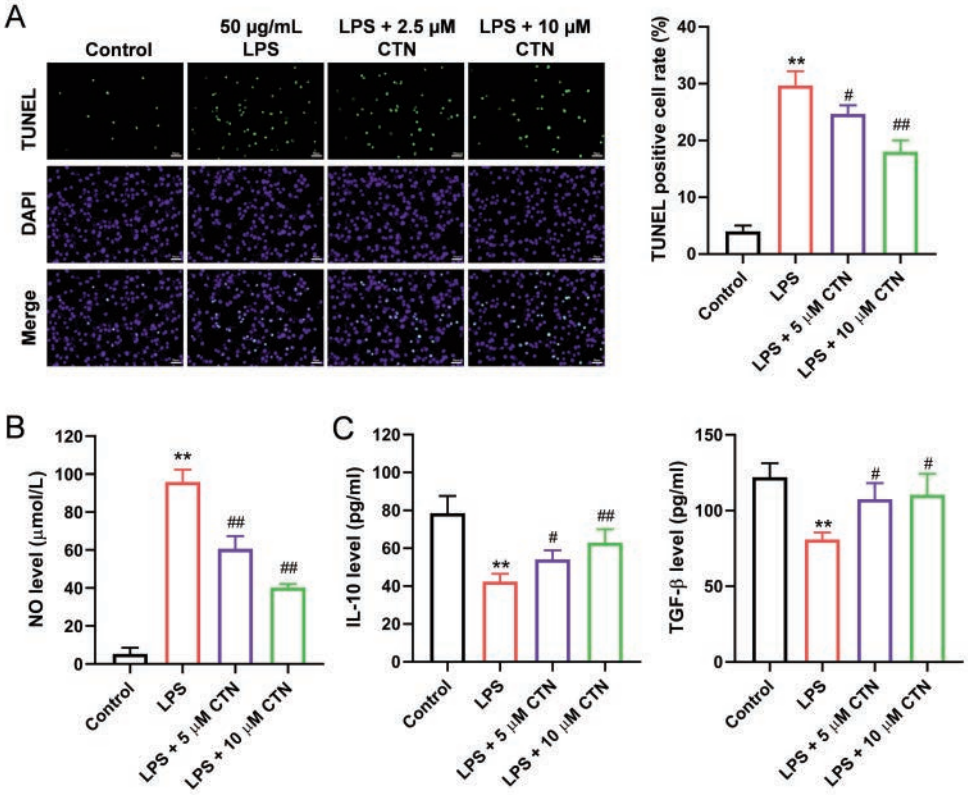

Figure 2. CTN reversed LPS-induced apoptosis in HIEC-6 cells. **A**) HIEC-6 cells were treated with 50 μg/mL LPS, 50 μg/mL LPS+ 5 μM CTN or 50 μg/mL LPS+ 10 μM CTN for 24 h and cell apoptosis was measured with TUNEL staining; 200× magnification; scale bar: 50 μm. **B,C**) The levels of NO, IL-10 and TGF-β in the supernatant of cells were detected with ELISA kit. n=3; $^{**}p<0.05$ compared to control group, $^{\#\#}p<0.05$ compared to LPS group.

Discussion

IBD is a chronic and recurrent inflammatory condition of the intestines, with symptoms including acute abdominal pain, chronic diarrhea and weight loss. The underlying mechanisms of this disease remain unclear, and the existing treatment strategies have failed to meet the expectations of patients.[19]

A number of Chinese traditional medicines have been reported to exert therapeutic effects against IBD.[20] CTN, a major liposoluble extract of *Salvia miltiorrhiza* has previously been reported to exert anti-inflammatory effects on numerous diseases, including chronic obstructive pulmonary disease, hyperoxia induced lung injury and osteoarthritis.[21-23] Additionally, it has been shown that CTN exerts a therapeutic effect on DSS-induced UC by inhibiting intestinal inflammation.[24] The present study also indicated that CTN alleviated the DAI, downregulated the levels of TNF-α and IL-6 and increased the levels of IL-10 and TGF-β in mice with DSS-induced colitis. Although these findings demonstrate that CTN is able to alleviate the symptoms of UC in mice, the target cells in the intestine through which CTN exerts its effects have not yet been fully elucidated.

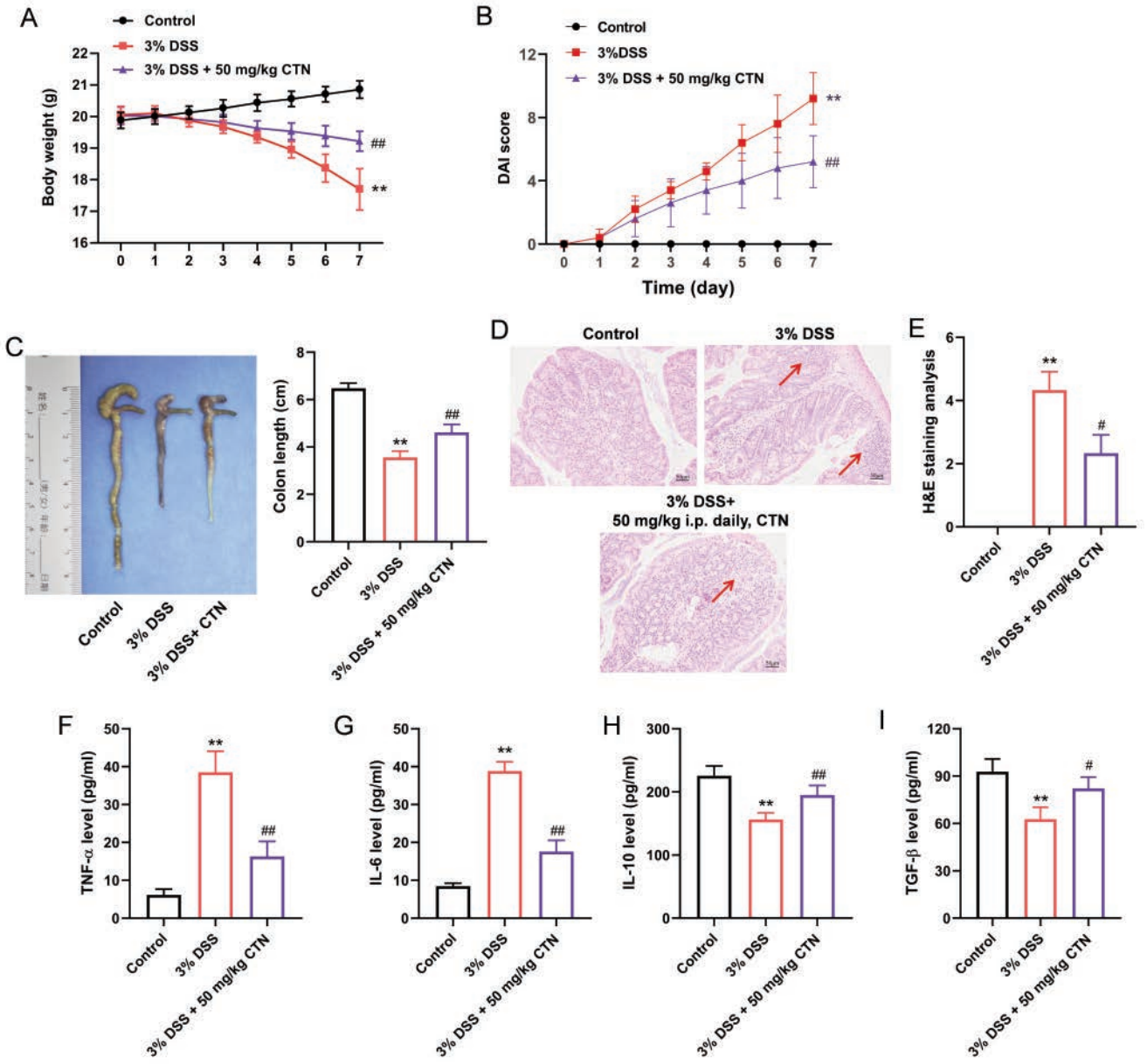

Figure 3. CTN alleviates DSS-induced inflammatory bowel disease in mice. **A**) The body weight of mouse in each group was monitor daily. **B**) DAI score of mice in each group was recorded in the end of animal study. **C**) The representative images of the colon in each group, and the length of the colon in each group was recorded. **D,E**) The pathological changes of mice colon were analyzed with H&E staining; 200× magnification; scale bar: 50 μm. Inflammation cells were marked with red arrow. **F-I**) ELISA kits were used to determine TNF-α IL-6, IL-10 and TGF-β levels in the serum of mice. n=3, $^{**}p<0.01$ *vs* control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* DSS group.

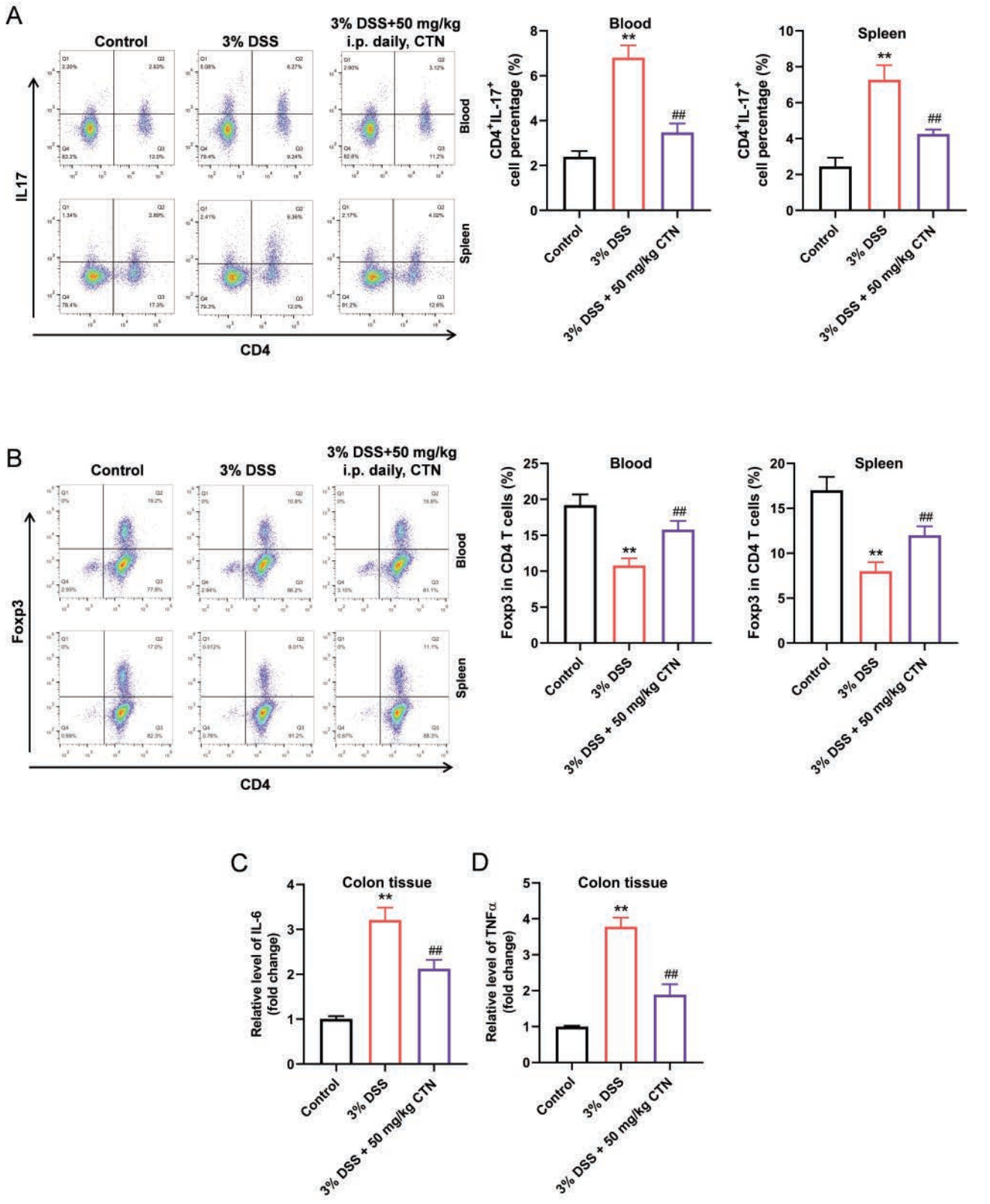

Figure 4. CTN restored the Treg/Th17 balance in DSS-treated mouse. **A**) The $CD4^+Th17^+$ T cells in the peripheral blood and spleen cells of mice were detected with flow cytometry in the end of animal study. **B**) The $CD4^+Foxp3^+$ T cells in peripheral blood and spleen cells of mice were detected with flow cytometry in the end of animal study. **C,D**) ELISA kits were used to determine TNF-α and IL-6 levels in the colon tissue of mice. n=3, $^{**}p<0.01$ *vs* control group; $^{\#\#}p<0.01$ *vs* DSS group.

As a CD4+ T-cell subset, Th17 cells play a pro-inflammatory role and exacerbate the intestinal inflammatory response.[9] A previous study demonstrated that Th17 cells play a vital role during the pathogenesis of IBD; compared with healthy control groups, there is Th17 cell infiltration in the intestinal mucosa of patients with IBD, and the specific cytokine, IL-17, secreted by Th17 cells is increased.[25] Compared with Th17 cells, Tregs not only can prevent the occurrence of autoimmune diseases, but can control the intestinal inflammation; the level of Tregs is decreased in the peripheral blood of mice administered DSS.[26] Consistent with these findings, the present study confirmed that DSS notably increased the $CD4^+IL\text{-}17^+$ T-cell rate in the peripheral blood and spleen cells of mice. By contrast, CTN reversed the upregulation in the $CD4^+IL\text{-}17^+$ T-cell rate and the decrease in the $CD4^+Foxp3^+$ T-cell rate in mice with DSS-induced UC. This indicates that CTN is able to regulate the balance of Th17/Treg cells in mice administered DSS; thus, CTN may be a novel therapeutic agent for UC.

Cytokines, including TGF-β, IL-2, IL-6, IL-15, IL-18 and IL-23 have been reported to be involved in regulating the balance of Th17 cells and Tregs.[27] TGF-β is able to promote the development of Tregs *via* acting on naive $CD4^+$ T-cells, while IL-6 induces Th17-specific genes, including IL-17 and IL-23 receptor (IL-23R) *via* phosphorylating STAT3, and induces the upregulation of Th17 cells.[27,28] STAT3 activation promotes Th17 cell differentiation and upregulation of Th17 cells, and this contributes to the pathogenesis of IBD.[29] Thereby, it was hypothesized that CTN increased TGF-β

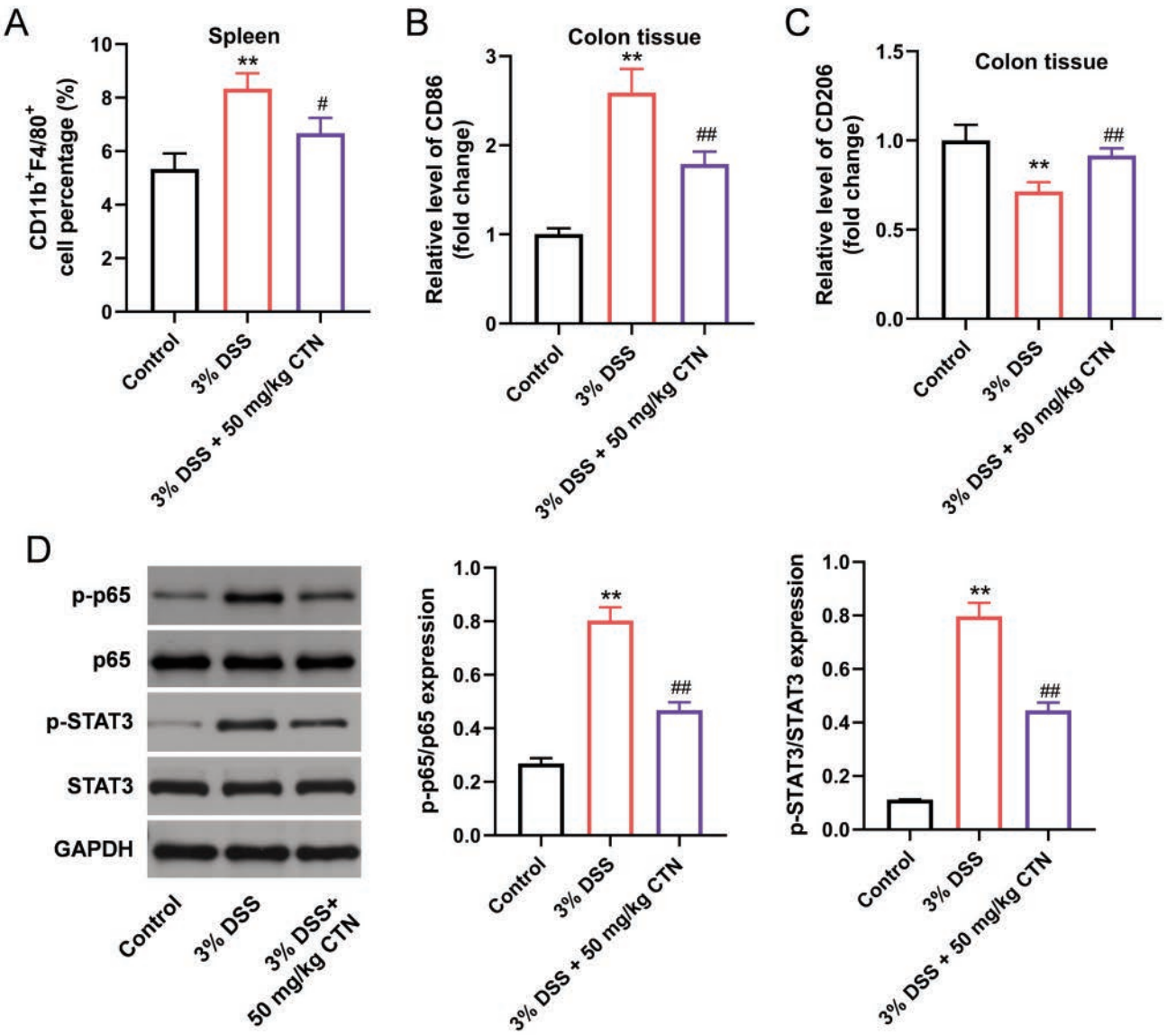

Figure 5. CTN inhibited M1 macrophage polarization in DSS-treated mouse. **A**) The CD11b+F4/80+ cells in the spleen cells of mice were detected with flow cytometry in the end of animal study. **B,C**) The colon tissues of mouse were collected. The gene expressions of CD86 and CD206 in colon tissue were detected using with RT-qPCR. **D**) The protein expressions of p-p65, p65, p-STAT3 and STAT3 in the colon tissues of mouse were detected by Western blot. n=3, $^{**}p<0.01$ *vs* control group; $^{\#}p<0.05$ *vs* DSS group, $^{\#\#}p<0.01$ *vs* DSS group.

and decreased IL-6 in mice administered DSS *via* inhibiting the phosphorylating STAT3, further regulating the balance of TH17/Tregs and playing a vital anti-inflammatory role in UC.

It is well known that M1 macrophages mainly exert pro-inflammatory effects by inducing IL-6 and TNFα, while M2 macrophages mainly exert anti-inflammatory effects by inducing the production of IL-10 and TGF-β.[30] The present study indicated that CTN improved M2 polarization by preventing M1 differentiation. These results suggest that CTN may regulate the balance of M1 and M2 macrophage phenotypes in mice administered DSS by modulating the secretion of cytokines, including IL-6, TNFα, IL-10 and TGF-β. However, the relationship between TH17/Tregs and M1 macrophages remains unclear and this is the limitation of current study. It is possible that both pathway play important roles during the protective effects of CTN.

In conclusion, the present study indicates that CTN may play a protective role in DSS-induced colitis *via* regulating the balance of TH17/Tregs in mice. It was also revealed that CTN exerted its protective effects by inhibiting the polarization of M1 macrophages in mice with DSS-induced colitis. This highlights the novelty of the present study. In addition, the effects of CTN on the changes of these immune cells may be mediated by changes in the levels of TNF-α and IL-6 directly in mice. Taken together, these findings may provide new insight regarding the therapeutic potential of CTN in UC.

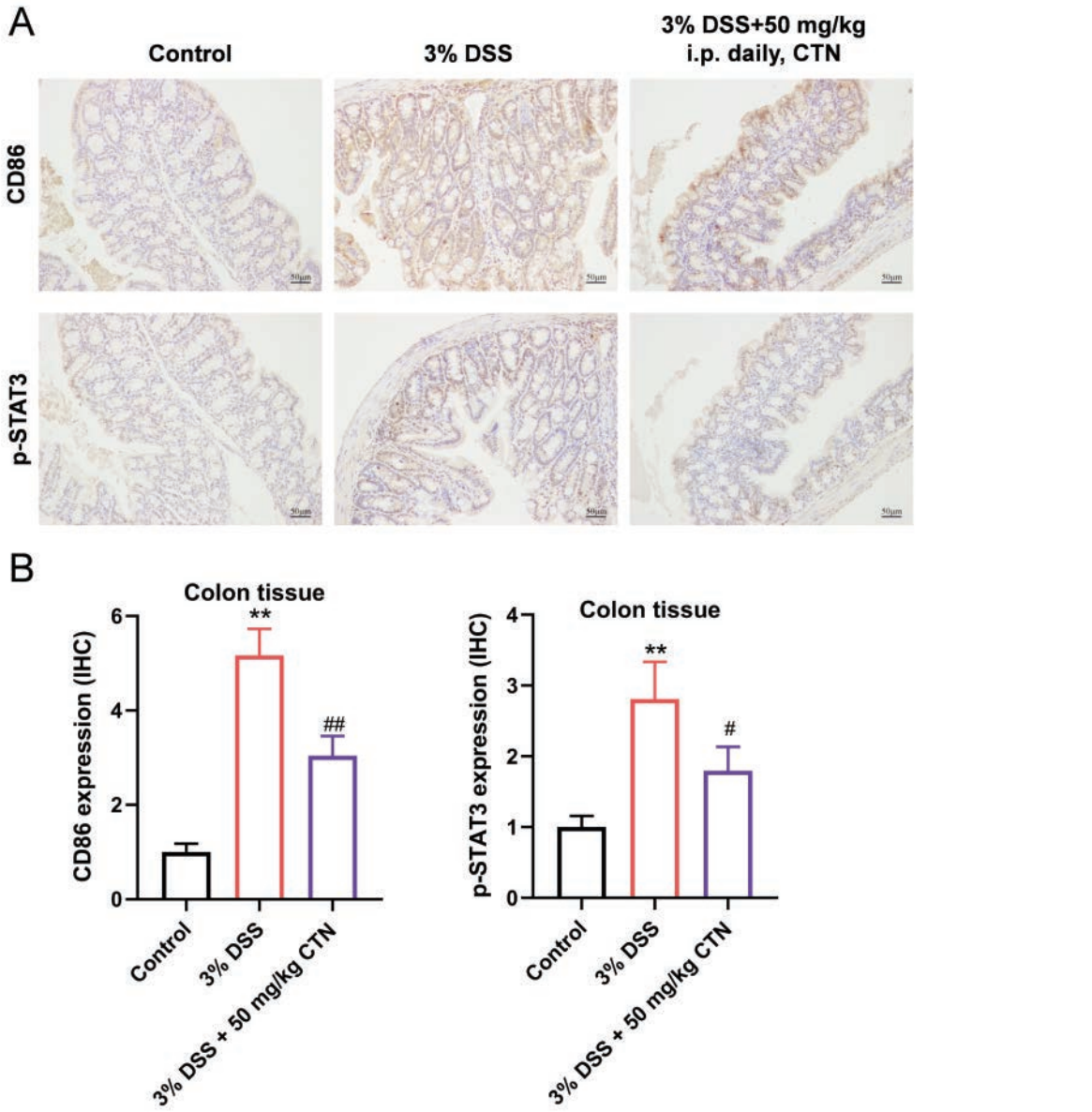

Figure 6. The upregulation of macrophage cells (CD86) and p-STAT3 induced by DSS in the colon tissues of mice was reversed by treatment with CTN. **A,B**) The protein expressions of CD86 and p-STAT3 in colon tissue were detected by immunohistochemistry; 200× magnification; scale bar: 50 μm; $^{**}p<0.01$ *vs* control group; $^{\#}p<0.05$ *vs* DSS group, $^{\#\#}p<0.01$ *vs* DSS group.

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