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Toosendanin alleviates dextran sulfate sodium-induced colitis by inhibiting M1 macrophage polarization and regulating NLRP3 inflammasome and Nrf2/HO-1 signaling

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ABSTRACT

Toosendanin (TSN), a triterpenoid extracted from the bark of fruit of Melia toosendan Sieb et Zucc, has been proven to have various biological activities including anti-inflammatory activity. But its effects on experimental colitis remain unreported. Herein, we investigated the role and potential mechanisms of TSN in dextran sulfate sodium (DSS) induced colitis in mice. The results showed that, TSN reduced colitis-associated disease activity index (DAI), shortened colon length, and weakened the pathological damage of the colon tissues in murine colitis models. Further studies disclosed that, TSN inhibited the secretion of proinflammatory cytokines and oxidative stress, and suppressed M1 macrophage polarization and the activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome, but upregulated HO-1/Nrf2 expression in murine colitis. In addition, TSN maintained intestinal barrier by regulating zonula occludens-1 (ZO-1) and occludin expression. In conclusion, our findings demonstrated that, TSN alleviates DSS-induced experimental colitis by inhibiting M1 macrophage polarization and regulating NLRP3 inflammasome and Nrf2/HO-1 signaling, and may provide a novel Chinese patent medicine for the treatment of murine colitis.

1. Introduction

Ulcerative colitis (UC) is an idiopathic intestinal inflammatory disease, and its incidences is increasing worldwide [1,2]. Currently, traditional therapeutic strategies including sulfonazine, glucocorticoids and immunosuppressants have been used for treating UC, but their costly expenses and side effects limit their extensive use [3]. Therefore, identification of safe and effective traditional Chinese medicine for treating UC is urgently needed.

Previous studies showed that, DSS-induced UC is driven by intestinal macrophages, which can release proinflammatory cytokines and chemokines, induce migration of neutrophils and lead to tissue damage [4]. NLR family pyrin domain containing 3 (NLRP3) inflammasome consists of NLRP3 scaffold, the adaptor apoptosis-associated speck-like protein containing a CARD domain (ASC), and caspase-1 and causes the activation of macrophages [5,6]. Activation of NLRP3 inflammasome results in the activation of caspase-1 and release of mature IL-1β [7,8]. The polymorphism of NLRP3 is associated with an increased risk in patients with UC [4,9], but suppression of NLRP3 ameliorates experimental colitis in colonic macrophages [10]. In contrast, the proinflammatory effects induced by LPS-stimulated macrophages can be decreased by NFE-related factor 2 (Nrf2) and antioxidant enzyme heme oxygenase-1 (HO-1) [11–14].

Toosendanin (TSN) is extracted from the bark or fruits of Melia toosendan Sieb et Zucc, which is colorless and acicular and exhibits the analgesic, insecticidal and anti-inflammatory activities [15]. It is also used as a digestive tract-parasiticide, an agricultural insecticide, a selective presynaptic blocker and an effective antibotulismic agent in China, induces differentiation and cell apoptosis and suppresses cell proliferation in multiple malignancies [16]. Our previous study showed that, TSN has an antitumor role in gastrointestinal tumors [15]. However, the effect of TSN on DSS-induced colitis is still unknown. Herein, we found that, TSN alleviated DSS-induced colitis not only by inhibiting M1 macrophage polarization and the activation of NLRP3 inflammasome but also by regulating Nrf2/HO-1 signaling and maintaining the intestinal integrity, which might provide a novel strategy for the

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therapeutic of murine colitis.

2. Materials and methods

2.1. Chemicals and reagents

TSN (purity ≥98%) was obtained from MedChemExpress (Shanghai, China). DSS (36000–50,000 Da) was obtained from Yeasen (Shanghai, China). An enzyme-linked immunosorbent assay (ELISA) kit was purchased from eBioscience (San Diego, CA, USA). All antibodies used in our study were obtained from Abcam.

2.2. Animals and experimental colitis models

Forty-eight male C57BL/6 mice weighing 20–22 g were offered by the animal center of our hospital. They were housed in cages under a controlled temperature of 22 ± 1 °C and 12 h light-dark cycles, provided by standard laboratory chow and water ad libitum and allowed to acclimatize for at least one week.

Animals were randomly divided into four groups: control group (n = 12), DSS group (n = 12), DSS + TSN 0.5 mg/kg group (n = 12), DSS + TSN 1 mg/kg group (n = 12). The experimental colitis model was established by replacing their drinking water with 2.5% DSS dissolved in water for 7 days. For the DSS + TSN 0.5 mg/kg and DSS + TSN 1 mg/kg groups, mice in the treatment groups received intraperitoneal injections of 0.5 mg/kg or 1 mg/kg TSN in sterile saline once a day, respectively, while the mice in normal control group and DSS group were given saline. Mice were sacrificed 24 h after the last injection of TSN and the whole blood was obtained from the heart. The entire colon was quickly removed from the cecum to the anus. The blood samples were stored at −20 °C until use. Colon tissue samples were obtained, the colon length was measured, a portion of the tissue was used for flow cytometry and a portion was immediately stored at −80 °C. The other portions were fixed in 4% paraformaldehyde for histopathological examination. The animal experiment was performed in accordance with the Ethical and Research Committee of our hospital.

2.3. Disease activity index (DAI) scores

The DAI scores were calculated according to the previous reports [17,18]. Briefly, DAI scores were counted depending on the average score of body weight loss, feces consistency and fecal blood test scores (Table. 1).

2.4. Myeloperoxidase (MPO) measurement

The measurement of MPO activity in colonic tissue was conducted according to the previous protocol [19]. Briefly, the colonic tissue was weighed about 100 mg, and homogenized with PBS [1:9 (w/v)]. The detection of MPO was performed by using a commercial kit (Biovision, San Francisco, USA) following the guidelines of manufacturers.

2.5. ELISA assay and SOD, GSH and MDA detection

The serum concentrations of TNF-α, IL-1β, IL-6 were assayed by ELISA (eBioscience, San Diego, USA) in each group according to the instructions of manufacturers. Superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) in colon tissues in each group were examined according to the instructions of manufacturers (Jiancheng Bioengineering, Nanjing, China).

Table 1
Disease activity index (DAI) scoring system.

DAI score	Weight loss(%)	Stool consistency	Occult/gross bleeding
0	None	Normal	Normal
1	1–5		
2	5–10	Loose stools	Hemoccult positive
3	10–20		
4	> 20	Diarrhea	Gross bleeding

2.6. Isolation of colon lamina propria mononuclear cells (LPMCs)

According to the previous report [14], the colon tissues were cut into small pieces, and these pieces were incubated in a pre-digestion solution 1 × Hank's balanced salt solution (HBSS) containing 5mmM EDTA (Gibco, California, USA) and 1 mM DTT (Roche, Shanghai, China) at 37 °C for 30 min in order to remove the epithelial cells. After being filtered with a 100 μm cell strainer, the remaining pieces were then incubated with RPMI medium containing 0.05% collagenase D (Roche) and 0.05% DNase I (Roche) for 30 min with gentle shaking. The cell suspensions were passed through 70 μm cell strainer and were collected for subsequent flow cytometry analysis.

2.7. Flow cytometry analysis

LPMCs were stained with the following antibodies: CD11b allophycocyanin (e-Bioscience, San Diego, CA, USA), CD11c phycoerythrin (e-Bioscience), anti-F4/80 fluorescein isothiocyanate (e-Bioscience), CD206 perodinin-chlorophyll-protein/Cy5.5 (BD Biosciences, San Jose, CA, USA). CD11b + CD11c + cells were considered M1 macrophages. F4/80 + CD206 + cells were considered as M2 macrophages.

2.8. qRT-PCR analysis

Total RNA was extracted from colonic tissue using TRIzol reagent (Invitrogen, CA, USA), and then cDNA was generated using a first-strand cDNA synthesis kit (Takara, Tokyo, Japan). qRT-PCR analysis was performed using a SYBR Green PCR kit (Accurate Biotechnology, Hunan, China) with the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Shanghai, China). The amplification reaction conditions were defined as follows: 95 °C for 30 s., 95 °C for 5 s. and 60 °C for 30 s. This procedure was repeated for 40 cycles. Relative mRNA expression was calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method. GAPDH was used as the control gene. The primers of TNF-α, IL-1β, IL-6 and GAPDH were listed as follows: TNFα forward, 5′-CCA AAGGGATGAGA AGTTCC-3′ and reverse, 5′-CTCCACTTG GTGGTTTG CTA-3′; IL-1β forward, 5′-TTCAGGCAGGCAGTATCA-3′ and reverse, 5′-GTCA CAACCAGCAGGTTA-3′; IL-6 forward, 5′-CCCGGAGGAGACTT CAG-3′ and reverse, 5′-CAGATTGCCATT GCACAAC-3′; and GAPDH forward, 5′-TGTGTCCGTCGTGGATCTGA-3′ and reverse, 5′-CCTGCTT CACCACCTTCTTGA-3′.

2.9. Western blot analysis

The protein levels of NLRP3, caspase-1, ASC, IL-1β, Nrf2, HO-1, ZO-1 and occludin in colonic tissue in each group were determined by western blot analyses. Briefly, the colonic tissues were homogenized in an ice-cold lysis buffer containing a cocktail of protease inhibitors. The supernatant fluid of the lysate was collected by centrifugation (4 °C, 12,000 rpm, 15 min). After electrophoresis, the proteins were electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA, USA). The membrane was then rinsed with a blocking solution of 5% nonfat milk for 1 h and incubated overnight at 4 °C with primary antibodies (anti-NLRP3, Abcam, USA, ab232401; anti-caspase-1, Abcam, USA, ab1872; anti-ASC, Abcam, USA, ab175449; anti-IL-1β, Abcam, USA, ab9722; anti-Nrf2, Abcam, USA, ab137550; anti-HO-1, Abcam, USA, ab13243; anti-ZO-1, Abcam, USA, ab96587; anti-occludin, Abcam, USA, ab167161). The proteins were visualized using an enhanced chemiluminescence (ECL) system (Thermo Scientific).

Table 2
Histological scoring system.

Score	Inflammation	Mucosal damage	Crypt damage	Range of lesions (%)
0	None	None	None	0
1	Mile	Mucous layer	1/3	1–25
2	Moderate	Submucoas	2/3	26–50
3	Severe	Muscularis and serosa	100%	51–75
4	–	–	100% + epithelium loss	76–100

2.10. Histopathological examination

Colonic tissue samples were incised and fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The grading of histological damage was evaluated as previously described [6] (Table. 2).

2.11. Immunohistochemistry (IHC)

IHC analysis was performed to examine the protein expression of TNF-α, IL-1β and IL-6 in colonic tissue samples in each group according to our previous report [18]. Briefly, colon tissue slides were deparaffinized, rehydrated, and subjected to antigen retrieval with 10 mM sodium citrate buffer (pH 6.0, at 90 °C for 30 min). Then, they were blocked and incubated with antibodies. The slides were preincubated with 0.04% bovine serum albumin (BSA) to block nonspecific binding. Subsequently, the slides were incubated with primary polyclonal antibodies (Absin Bioscience, Shanghai, China) at a dilution of 1:200 overnight at 4 °C. After washing with PBS, the slides were incubated with a secondary antibody (KeyGen Biotech, Nanjing, China) at room temperature for 1 h. Finally, the sections were stained with a 3,3-diaminobenzidine (DAB) solution and counterstained with hematoxylin. Images were visualized under a microscope at a magnification of 200 × (Olympus, Tokyo, Japan).

2.12. Immunofluorescence staining

According to the previous report [14], paraffin-embedded colon sections were incubated with primary antibodies (anti-F4/80, 1:100, Abcam, Cambridge, MA, USA; anti-NLRP3, 1:50, Abcam, Cambridge, MA, USA; anti-HO-1, 1:200, Abcam, Cambridge, MA, USA) diluted in 1% donkey serum at 4 °C overnight, and the nuclei were stained with DAPI for 10 min at room temperature.

2.13. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The significance of differences in histopathological scores was assessed by the Kruskal-Wallis test. Other continuous data were analyzed by Student's *t*-test and factorial design ANOVA. All statistical analyses were carried out using GraphPad Prism 7 (La Jolla, CA, USA) with statistical significance set at $P < 0.05$.

3. Results

3.1. TSN ameliorated DSS-induced colitis

A DSS-induced colitis model was used to assess the effects of TSN on experimental murine colitis. We found that, DSS markedly shortened the colon length as compared with the control group, while this effect was reversed by 0.5 mg/kg or 1 mg/kg TSN in experimental colitis (Fig. 1A). Likewise, the body weight was lowered by DSS as compared with the normal control group, but this effect was also reversed by 0.5 mg/kg or 1 mg/kg TSN as compared with DSS group in murine colitis (Fig. 1B). In contrast, the DAI was increased by DSS as compared with the normal control group, but this effect was reduced by 0.5 mg/kg or 1 mg/kg TSN as compared with DSS group in murine colitis (Fig. 1C). Meanwhile, we also estimated the weight of spleen and found that, the spleen weight was elevated by DSS as compared with the normal control group, but this effect was reversed by 0.5 mg/kg or 1 mg/kg TSN as compared with DSS group in murine colitis (Fig. 1D).

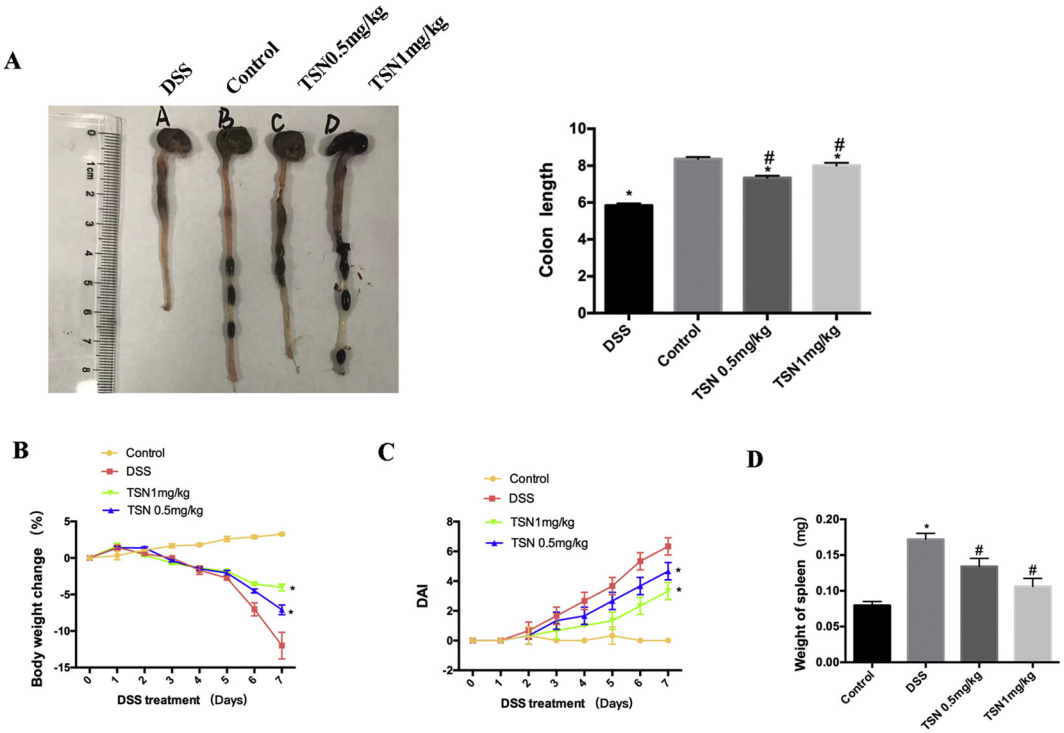

Fig. 1. TSN protects against DSS-induced colitis in mice. A. Representative photographs of colon and colon length. B. Effect of TSN on body weight. C. The scoring criteria for DAI. D. Effect of TSN on spleen weight. Results were expressed as mean ± SD. *$P < 0.05$ vs. the normal control group. #$P < 0.05$ vs. the DSS group.

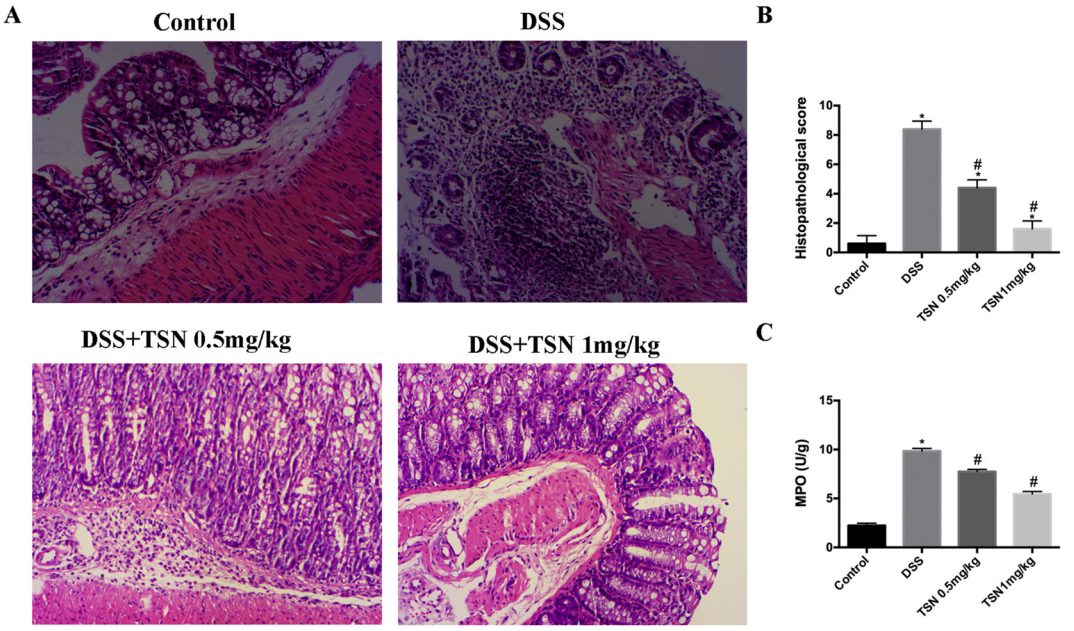

Fig. 2. Effect of TSN on histopathological changes of mice in DSS-induced colitits. A Histological analysis. B. Histological score. C. The expression of MPO in each group. *$P < 0.05$ vs. the normal control group. # $P < 0.05$ vs. the DSS group.

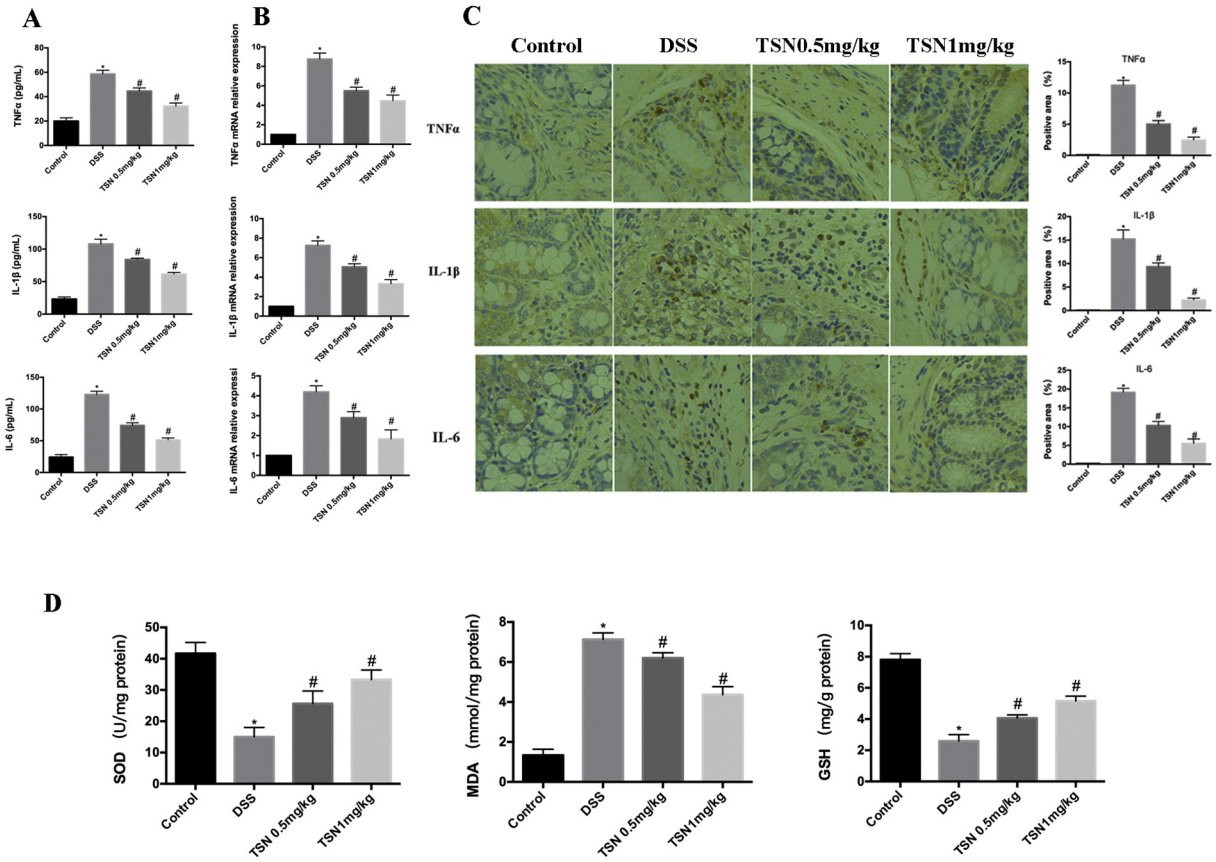

Fig. 3. TSN inhibited the expression of proinflammatory cytokines in DSS-induced UC and improved oxidative stress. A. The protein levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6) in serum were tested by ELISA. B. The mRNA expression levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6) in colonic tissue were tested by qTR-PCR. C. The protein expression levels of prionflammatory cytokines (TNF-α, IL-1β, IL-6) in colonic tissue were texted by IHC. D. MDA, SOD and GSH, the markers of oxidative stress were examined. *$P < 0.05$ vs. the normal control group. # $P < 0.05$ vs. the DSS group.

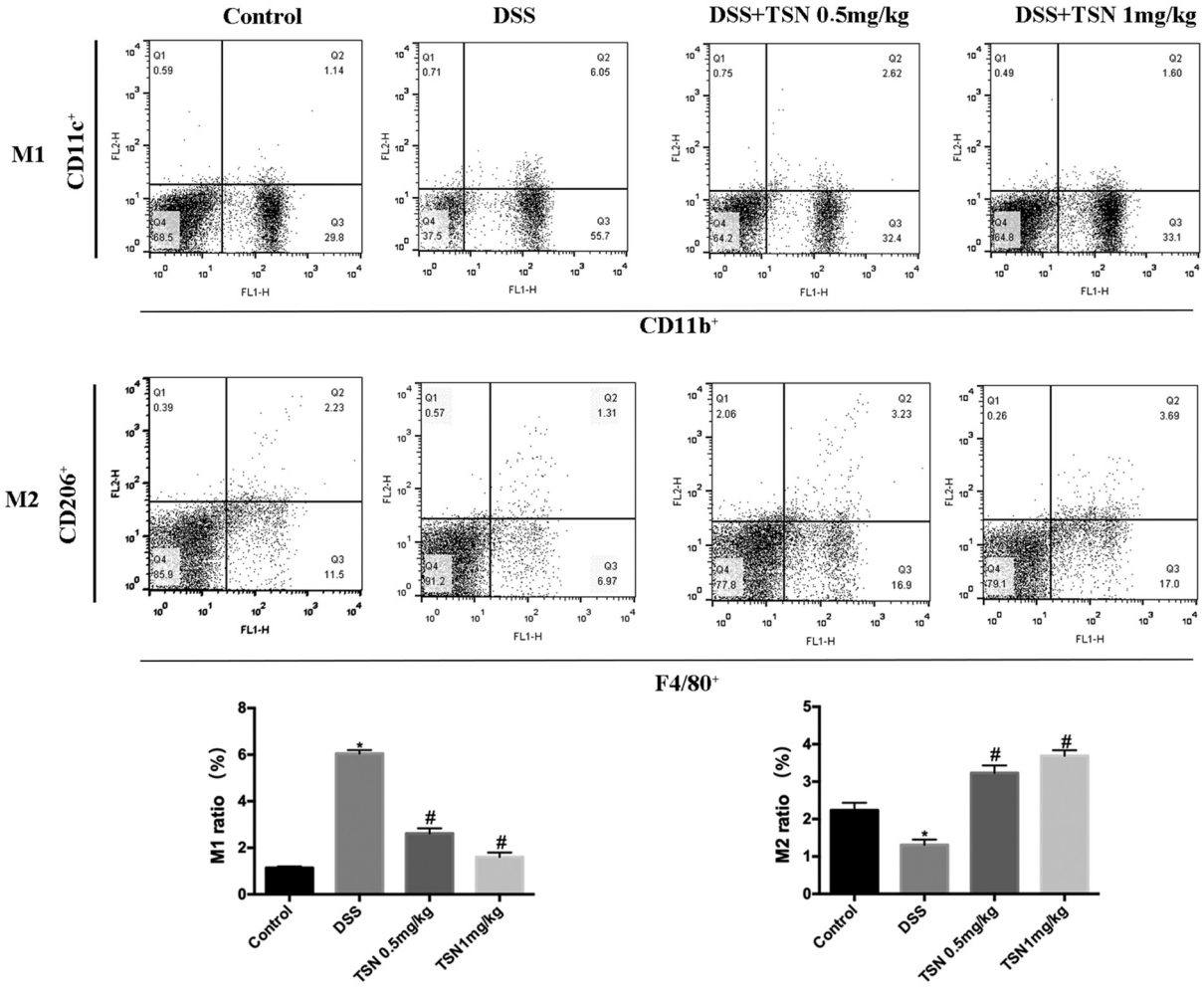

Fig. 4. TSN decreased the ratio of M1 macrophages and increased the ratio of M2 macrophages. The mean percentage of $CD11b^+CD11c^+$ (M1 phenotype) and $F4/80^+CD206^+$ (M2 phenotypes) in each group. *$P < 0.05$ vs. the normal control group. # $P < 0.05$ vs. the DSS group.

3.2. TSN reduced colonic tissue damage in DSS-induced colitis

As shown in Fig. 2A, we found that, DSS resulted in extensive colonic tissue injury, including inflammatory cell infiltration, crypt damage, and focal formation as compared with the normal control group, but these results could be reversed by 0.5 mg/kg or 1 mg/kg TSN in murine colitis (Fig. 2B). MPO is an enzyme that can reflect the inflammation degree of colonic tissue in colitis [19,20]. We also found that, MPO activity was tremendously increased by DSS as compared with the normal control group, while this effect could be reversed by 0.5 mg/kg or 1 mg/kg TSN in murine colitis (Fig. 2C).

3.3. TSN suppressed the secretion of proinflammatory mediators and improved oxidative stress in DSS-induced colitis

It is known that, TNF-α, IL-1β and IL-6 as the proinflammatory mediators are implicated in the pathogenesis of colitis [18]. Herein, we found that, the expression levels of TNF-α, IL-1β and IL-6, indicated by qRT-PCR analysis were increased by DSS as compared with the normal control group, but this effect could be reversed by 0.5 mg/kg or 1 mg/kg TSN in murine colitis (Fig. 3A). The similar results for the serum and protein levels of TNF-α, IL-1β and IL-6 were also confirmed by ELISA (Fig. 3B) and IHC analysis (Fig. 3C).

Whether MDA, SOD and GSH, the markers of oxidative stress were affected by TSN was evaluated. We found that, the serum levels of SOD and GSH were downregulated, but those of MDA were upregulated by DSS, while TSN treatment could increase SOD and GSH levels but decrease MDA levels as compared with DSS group in experimental colitis (Fig. 3D).

3.4. TSN suppressed M1 macrophage polarization in DSS-induced colitis

Previous studies showed that, macrophages act critical roles in the progression of colitis [4]. We next investigated the effects of TSN on macrophage polarization in DSS-induced colitis, and found that, the ratio of M1 macrophages ($CD11b^+CD11c^+$) was increased but that of M2 macrophages ($F4/80^+CD206^+$) was decreased in DSS group as compared with the normal control group, while TSN treatment reduced the ratio of M1 macrophages but increased that of M2 macrophages in colitis as compared with the DSS group (Fig. 4).

3.5. TSN inhibited the activation of NLRP3 inflammasome and regulated the Nrf2/HO-1 signaling in DSS-induced colitis

Previous studies showed that NLRP3 inflammasome was involved in DSS-induced colitis [17,21,22]. We further evaluated the effects of TSN on the activity of NLRP3 inflammasome in DSS-induced colitis, and found that the protein expression of NLRP3, ASC, Caspase-1 and IL-1β was increased by DSS as compared with the normal control group, but these changes could be reversed by 0.5 mg/kg or 1 mg/kg TSN in murine colitis (Fig. 5A, B).

The loss or interruption of Nrf2/HO-1 signaling enhances the

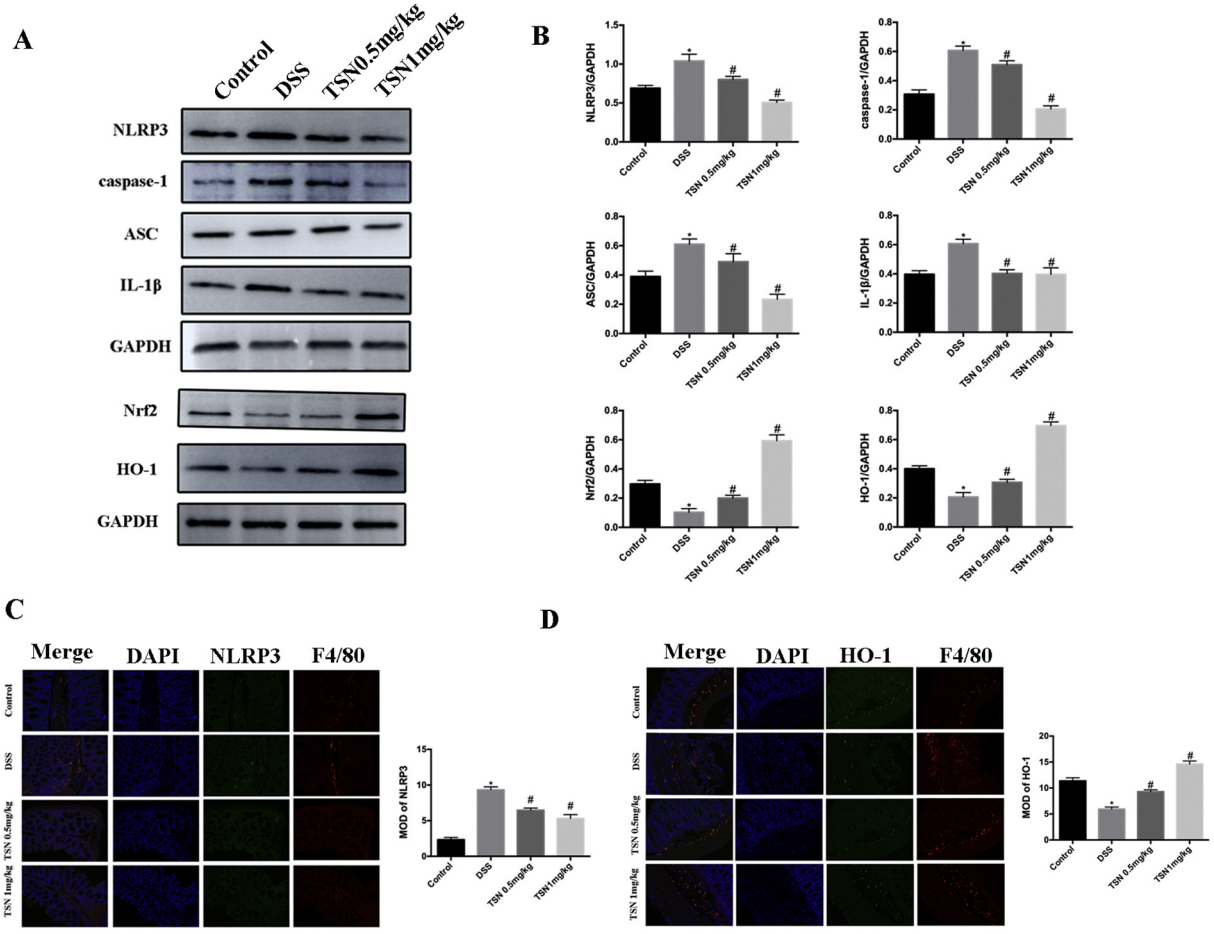

Fig. 5. TSN suppressed the activation of NLRP3 inflammasome and promoted Nrf2/HO-1 expression in colonic tissue and macrophages. The protein expression levels of NLRP3 inflammasome and Nrf2/HO-1 (A, B) in colonic tissue were texted by western blot and normalized to GAPDH. C, D. Double labelling with NLRP3 (green) and F4/80 (red), HO-1 (green) and F4/80 (red) in different group, respectively. Nuclei were stained with DAPI (blue). *$P < 0.05$ vs. the normal control group. # $P < 0.05$ vs. the DSS group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

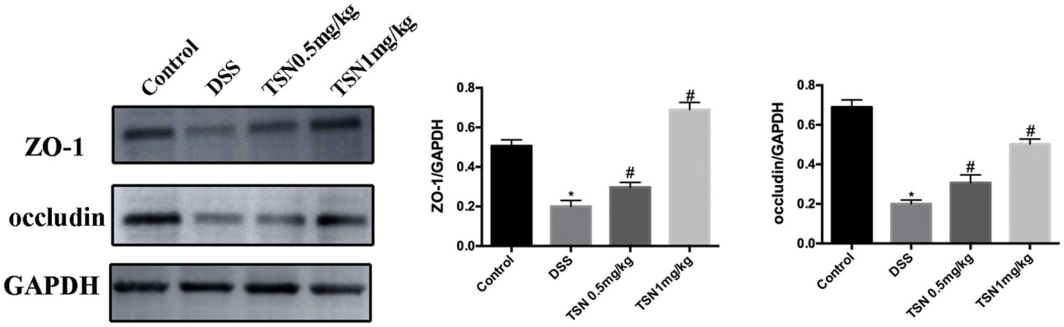

Fig. 6. TSN promoted the cell tight junction in DSS-induced colitis. The expression of ZO-1 and occludin in colonic tissue were texted by western blot and normalized to GAPDH. *$P < 0.05$ vs. the normal control group. # $P < 0.05$ vs. the DSS group.

sensitivity to oxidative or electrophilic stresses and subsequently tissue damage [23]. We then examined the protein expression of Nrf2 and HO-1 in DSS-induced colitis, and found that Nrf2 and HO-1 expression was decreased by DSS as compared with the normal control group, but TSN treatment increased their expression levels as compared with the DSS group (Fig. 5A, B).

Moreover, the expression of NLRP3 and HO-1 in the colonic macrophages was investigated by immunofluorescence double staining, indicating that, NLRP3 activation was increased, but HO-1 expression was decreased in F4/80$^+$ macrophages derived from colonic tissue in DSS group as compared with the normal control group, while 0.5 mg/kg or 1 mg/kg TSN administration inhibited the activation of NLRP3, but upregulated HO-1 expression as compared with the DSS group in DSS-induced colitis. (Fig. 5C, D).

3.6. TSN promoted the cell tight junction in DSS-induced colitis

Epithelial TJ proteins including ZO-1 and occludin are most important components of intestinal epithelial barrier, and their abnormal expression increases the permeability of intestinal epithelial barrier, causing pathogenic antigens into the lamina propria of mucosa and blood circulation and triggering inflammation and immune response

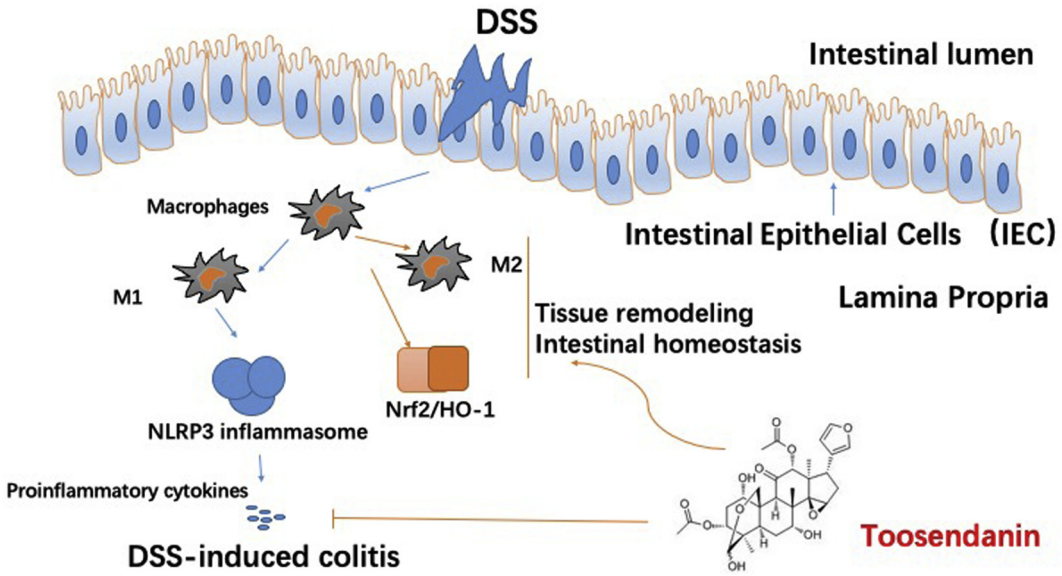

Fig. 7. Schematic representation of the protective mechanism of TSN in DSS- induced colitis. TSN alleviates DSS-induced colitis by inhibiting macrophage M1 polarization and NLRP3 inflammasome, and regulating Nrf2/HO-1 signaling.

[24]. Herein, we found that, the expression of ZO-1 and occludin was decreased in DSS group as compared with the normal control group in colonic tissues, whereas their expression levels were increased by 0.5 mg/kg or 1 mg/kg TSN administration as compared with the DSS group in DSS-induced colitis (Fig. 6).

4. Discussion

Ulcerative colitis (UC) is a complicated inflammatory bowel disease that seriously affects the patients' life quality and increases the risk of colon cancer [25]. DSS induced experimental colitis is a well-established model that can be used to understand the pathogenesis of UC [26]. Previous studies showed that, TSN exerts various biological activities such as anti-inflammatory and anti-tumor activities [15,27]. Herein, we found that, TSN administration could decrease shortened colon length, the body weight loss, and DAI scores, and attenuate DSS-induce colon pathological damage, indicating that TSN might be an effective therapeutic strategy for treatment of colitis.

Increasing evidence shows that, the inflammatory response is determined by the imbalance between pro- and anti-inflammatory cytokines [28]. TNF-α, IL-1β and IL-6 as key proinflammatory cytokines are closely related to the initiation of UC [29–31]. Herein, we found that, TSN treatment could ameliorate the tissue and serum levels of TNF-α, IL-1β and IL-6 in DSS-induced colitis. In addition, excessive inflammation activates the oxidative stress via the generation of reactive oxygen and nitrogen species (RONS) during the development of UC [32]. In our study, we assessed the serum levels of SOD, MDA and GSH, three major markers of oxidative stress, and found that, TSN administration reduced the serum levels of MDA, but increased those of SOD and GSH in DSS-induced colitis. These results indicated that, TSN had the protective effects in DSS-induced colitis via improving the inflammatory responses and oxidative stress.

It has been well established that, M1 macrophages can contribute to DSS-induced colitis by activating proinflammatory cytokines, while M2 macrophages maintain tissue remodeling and intestinal homeostasis [33,34]. Herein, we investigated the effects of TSN on macrophage polarization, and found that, TSN could inhibit M1 macrophage polarization, but promote M2 macrophage polarization in DSS-induced colitis.

NLRP3 inflammasome is a large multimeric protein complex [19]. The activation of NLRP3 promotes colon inflammation and DSS-induced UC [22,23,35]. In addition, Nrf2, a key transcription factor, facilitates the antioxidant response via the synthesis of HO-1, and protects DSS-induced colitis [36,37]. HO-1 in macrophages possesses anti-inflammatory potential and attenuates murine colitis [38]. Herein, we assessed the expression of NLRP3, ASC, casepase-1, Nrf2 and HO-1 in colonic tissues and the distribution of NLRP3 and HO-1 in colonic macrophages, and found that, TSN inhibited the activation of NLRP3 inflammasome and promoted the Nrf2/HO-1 signaling in DSS-induced colitis.

Intestinal epithelium is an important barrier to maintain its permeability and avoid intestinal inflammation. The abnormal expression of TJ proteins ZO-1 and occludin contributes to the destruction of intestinal barrier in UC patients [39]. Herein, we found that, TSN increases the expression of ZO-1 and occludin in colonic tissues in DSS-induced colitis, indicating that, TSN promotes the cell tight junction in DSS-induced colitis.

5. Conclusion

Our findings demonstrated, for the first time, that TSN alleviated DSS-induced colitis not only by inhibiting M1 macrophage polarization and the activation of NLRP3 inflammasome but also by regulating Nrf2/HO-1 signaling and maintaining the intestinal integrity (Fig. 7), and may provide a novel Chinese patent medicine for the treatment of murine colitis.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgment

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