Anemoside B4 ameliorates TNBS-induced colitis involved suppressing the S100A9/NF-κB signaling pathway

Yong Zhang
Soochow University
Zhengxia Zha
Soochow University
Wenhua Shen
Soochow University
Dan Li
Soochow University
Naixin Kang
Soochow University
Zhong Chen
Soochow University
Yanli Liu
Soochow University
Guoqiang Xu
Soochow University
Qiongming Xu (xuqiongming@suda.edu.cn)
Soochow University

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Abstract

Background

Despite the increased morbidity of ulcerative colitis (UC) in the developing countries, available treatments remain unsatisfactory. Therefore, it is urgent to discover more effective therapeutic strategies. *Pulsatilla chinensis* was widely used for the treatment of inflamed intestinal diseases including UC for thousands of years in China. However, it is unclear which compound in *P. chinensis* is responsible for the therapeutic effect. Our previous study reported that anemoside B4, the most abundant triterpenoid saponin isolated from *P. chinensis*, exerts anti-inflammatory and antioxidant effects.

Methods

Here, we used the 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis rat model to evaluate the therapeutic effect of anemoside B4. Blood samples of colitis rat were collected for hematology analysis. The effects of anemoside B4 on inflammation-associated mediators were investigated by Enzyme-linked immunosorbent assay (ELISA) and hematoxylin and eosin staining (HE) staining. Cell proliferation or apoptosis was measured by immunofluorescence technique. The mechanisms of anemoside B4 was investigated using label-free quantitative proteomics. The level of proteins was quantified by western blotting. mRNA expression was quantified by quantitative real-time RT-PCR.

Results

The results showed that anemoside B4 ameliorated TNBS-induced colitis symptoms, including tissue damage, inflammatory cell infiltration, and pro-inflammatory cytokine production, apoptosis and slowed proliferation in the colon. Quantitative proteomic analyses discovered that 56 proteins were significantly altered by anemoside B4 in the TNBS-induced rats. These proteins were mainly clustered in tricarboxylic acid cycle (TCA) cycle and respiratory electron transport chain. Among the altered proteins, S100A9 is one of the most significantly downregulated proteins and associated with NF-κB and MAPK signaling pathways in the pathogenesis of UC. Further experiments revealed that anemoside B4 suppresses the expression of S100A9 and its downstream genes including TLR4, NF-κB, and p-JNK in colon. *In vitro*, S100A9 protein could active NF-κB signaling pathway in human intestinal epithelial Caco-2 cells. However, anemoside B4 could inhibit the NF-κB signaling pathway induced by S100A9 protein. Besides, it also inhibited active of NF-κB signaling pathway stimulated by LPS or IL-6.

Conclusions

Our results demonstrate that anemoside B4 prevents TNBS-induced colitis involved inhibiting the NF-κB signaling pathway through inactivating S100A9 suggesting that anemoside B4 is a promising therapeutic

candidate for colitis.

Background

Inflammatory bowel disease (IBD) is a prevailing worldwide disease, especially in developing countries and its incidence has increased significantly in the past two decades [1]. The two main forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD), which are characterized by abdominal pain, diarrhea, bowel obstruction, weight loss, and related immune disorders. Both of them are incurable and are usually diagnosed at a young age with a significant morbidity [2].

It is currently accepted that multifactor, such as microbial flora, dysregulation of the immune response, genetic susceptibility, and environmental factors, involve in IBD, although it's precise etiology remains elusive. In particular, a pronounced imbalance in the activation of pro-inflammatory and anti-inflammatory signaling pathways in the gut is thought to be an important contributor to the development and progression of IBD. S100A9 is an intracellular calcium-binding protein belonging to the S100 family, which was originally discovered as an immunogenic protein expressed and secreted by neutrophils [3]. Besides neutrophils and monocytes, S100A8 and S100A9 can also be induced in keratinocytes and epithelial cells under inflammatory conditions [4, 5]. Growing evidence shows that S100A9 has a dual role in the inflammation response which is strongly upregulated in trauma, infections, heat, stress, and many other inflammations. S100A9 frequently interacts S100A8 to form a heterodimer (called calprotectin) and exhibits its biological functions [6]. Extracellular S100A8/A9 functions by binding to different receptors, including pattern recognition receptors, like the receptor for advanced glycation end products (RAGE) [7] and toll-like receptor 4 (TLR4) [8]. Then, the downstream signal of TLR4 is activated through the generation of the adaptor molecules containing the toll/interleukin-1 receptor (TIR) domains, such as TRIF-related adaptor molecule (TRAM)/toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF) and myeloid differentiation factor 88 (MyD88), which causes the translocation of NF-κB and/or IRF3 into the nucleus [9]. RAGE promoter contains NF-κB-binding sites and activates NF-κB or interacts high-mobility group box 1 (HMGB1), leading to the intracellular activation of NF-κB [10]. S100A8/A9 is the proposed functional form in lesions associated with neutrophil influx, displaying antimicrobial activities against a variety of micro-organisms [11, 12]. S100A9 induces various inflammatory responses, such as cytokine secretion, chemotaxis, cell viability, and caspase-dependent or independent cell death [13, 14].

S100A9 is also upregulated in acute or chronic local inflammatory diseases. It is found that lungs of patients with airway inflammation including cystic fibrosis, asthma, acute respiratory distress disorder, and chronic obstructive pulmonary disease have a high expression of S100A8/A9 [15]. Calprotectin complex of S100A8/A9 is present at high levels in the serum and corresponding intestinal tissues during inflammation and has been established as a valuable biomarker for a number of colonic inflammatory conditions [16]. Several papers reported that S100A9 expression in myeloid cells is essential for the development of colon tumors, which activates the downstream genes promoting the occurrence, growth and metastasis of colon-associated tumors [8]. A recent study found that dextran sulfate sodium sulfate (DSS)-induced colitis can be significantly improved by administration of a neutralizing anti-S100A9

antibody protein, accompanied with that the cellular infiltrate of innate immunity cells and production of pro-inflammatory cytokines is also significantly reduced. In addition, the inflammatory response, tumor cell proliferation and immune cell infiltration in colon tissue are suppressed in the mouse experimental model of azoxymethane (AOM)/DSS-induced colitis-associated cancer [17]. Therefore, it is highly likely that S100A9 may be a therapeutic target for colitis and colitis-associated cancer.

Now clinical treatments for UC include several anti-inflammatory drugs, such as sulfasalazine, glucocorticoids, nonsteroid anti-inflammatory agents, inhibitors of pro-inflammatory pathways, tumor necrosis factor (TNF)-α, gut-homing α4β7 integrin, interleukin (IL)-12/IL-23, and Janus kinases [18]. However, these drugs are less effective for some patients and also frequently cause severe side effects, including opportunistic infections and malignancies [19]. Therefore, no effective and safe clinical treatment strategy has been discovered for all IBD patients.

At present, the use of complementary and alternative medicine, including acupuncture, homeopathy and herbal medicines, for the treatment of patients with IBD is increasing worldwide. It is estimated that up to 70% of patients with IBD in North America and Europe were treated with complementary and alternative medicine [20, 21]. In China, several Chinese herbs are often used in the treatment of IBD, such as *Pulsatilla chinensis* (Bunge) Regel and Pulsatilla decoction, which were mainly used to treat UC in the traditional Chinese medicine for thousands of years [22]. Pulsatilla decoction possesses a variety of pharmacological effects, and the active ingredients from these herbal plants and has shown hepatic protective, antiinflammatory, antibacterial, antitumor and antioxidant effects [23]. Several studies have reported that Pulsatilla decoction could alleviate the release of inflammatory factors, such as IL-17, IL-1β, TNF-α in the serum of IBD patients, reduce syndromes and symptom scores, and suppress the activation of the NF-κB signaling pathway [24].

As the most important herb in Pulsatilla decoction, *P. chinensis* was widely used for the treatment of amoebic dysentery and malignant dysentery in China for two thousand years, which exhibits “blood-cooling” and detoxification activities [25]. Accumulating evidence has demonstrated that *P. chinensis* and its ingredients have antioxidant, anti-inflammatory and antitumor effects. Our previous study reported that anemoside B4, the most abundant triterpenoid saponin isolated from *P. chinensis*, decreases inflammation and oxidant injury induced by cisplatin. It ameliorates LPS-induced kidney and lung damage, inhibits LPS-induced NF-κB activation *in vivo* [26, 27]. However, it is unclear whether anemoside B4 exhibits effective roles in treating IBD and it might be the molecular basis for the clinical effect of *P. chinensis*.

In the present study, we investigated the therapeutic effect of anemoside B4, on 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis rats. We firstly found that anemoside B4 exerts anti-inflammatory and anti-apoptotic properties through downregulating colonic inflammatory cytokine levels, and inhibiting colonic epithelium apoptosis. Furthermore, quantitative proteomics analyses indicated that S100A9-dependent pathway is involved in the therapeutic effects of anemoside B4. Our data suggest that anemoside B4 has the potential ability for the further therapy of colitis.

Methods

Chemicals and reagents

TNBS and lipopolysaccharide (LPS) were supplied by Sigma-Aldrich, Germany (Cat. no. P2297 and L6529-1MG). Mesalazine (Cat. no. 89-57-6) was purchased from Ethypharm Pharmaceutical Co Ltd (Shanghai, China). Hunan recombinant S100A9 protein was purchased from Abcam (Cat. no. ab95909). Rat IL-1β, IL-6, and TNF-α ELISA kits were supplied by MultiSciences (Hangzhou, China). PCR primers (Supplementary Table S1) for quantitative real-time PCR analysis were synthesized by GENEWIZ (Suzhou, China). The primary antibodies for p38 (Cat. no. 8960) (1:2000), p-p38 (Cat. no. 4511) (1:1000), JNK (Cat. no. 9252) (1:1000), p-JNK (Cat. no. 9255) (1:2000), ERK1/2 (Cat. no. 4695) (1:2000), p-ERK1/2 (Cat. no. 4370) (1:1000), NF-κB p65 (Cat. no. 8242) (1:2000), p-NF-*κ*B p65 (Cat. no. 3033) (1:1000), Bcl-2 (Cat. no. 3498) (1:1000), cleaved-caspase 3 (Cat. no. 9664) (1:1000), and S100A9 (Cat. no. 73425) (1:1000) were purchased from Cell Signaling Technology (Danvers, USA), and those for Bax (Cat. no. sc-20067) (1:2000), p53 (Cat. no. sc-126) (1:1000), cleaved-PRAP (Cat. no. sc-74470) (1:1000), IL-6 (Cat. no. sc-57315) (1:1000), TLR4 (Cat. no. sc-293072) (1:1000), NOD2 (Cat. no. sc-56168) (1:1000), and GAPDH (Cat. no. sc-365062) (1:2000) were purchased from Santa Cruz Biotechnology Co., Ltd (Santa Cruz, USA). Caco-2 cell (PN 5) lines were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), and routinely maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco, USA). Anemoside B4 (20 g, purity more than 99%) was isolated from the roots of *P. chinensis* and its structure was determined as previous described [26].

Experimental animals

SD rats (male, 220–250 g) were obtained from the Experimental Animal Center of Soochow University. All animals were given free access to water and food with air filtration (22 ± 2 °C, 12-h light/12-h dark). All animal experiments were conducted in accordance with the procedure approved by the Ethical Review Committee for Laboratory Animal Welfare of the Soochow University.

Induction of transfer colitis and experimental design

All animals were divided into five groups (n = 7 per group): vehicle group, TNBS + saline group, TNBS + anemoside B4 group (5 mg/kg), TNBS + anemoside B4 group (10 mg/kg), and TNBS + mesalazine group (200 mg/kg). All rats were fasted for 24 h and weighed before injection, and then were anesthetized by injecting chloral hydrateintraperitoneally (300 mg/kg). In the vehicle group, 40% ethanol solution was slowly instilled into the colon of anesthetized rats, while for the other four groups, TNBS solution (80 mg/kg in 40% ethanol v/v) was instilled. Rats in the vehicle group and TNBS + saline group were intraperitoneally injected with saline, and those in the TNBS + anemoside B4 groups were intraperitoneally injected with anemoside B4, while those in the TNBS + mesalazine group were treated with mesalazine by gavage.

Assessment of colonic damage

During the experiment, rats were weighed every day and disease activity index (DAI) was recorded for seven days after the induction of colitis according to previous reference [28]. The DAI is the combined score of weight loss compared to initial weight, stool consistency, and bleeding. In brief, No weight loss was registered as 0, weight loss of 1% to 5% from baseline was assigned 1 point, weight loss of 6% to 10% from baseline was assigned 2 points, weight loss of 11% to 20% from baseline was assigned 3 points and weight loss of more than 20% from baseline was assigned 4 points. Stool consistency scores were 0 (normal, well-formed pellets), 2 (loose stool, pasty and semi-formed stools that did not adhere to the anus), and 4 (diarrhea, liquid stools that adhered to the anus). Bleeding scores were 0 (no blood), 2 (Hemoccult positive and visual pellet bleeding), and 4 (gross bleeding, blood around anus).On the 8th day, rats were sacrificed by cervical dislocation, the colons were harvested and rinsed with saline. Colon length was recorded as an indirect marker of inflammation. Myeloperoxidase (MPO) activity was measured to assess inflammation. Colon samples were fixed in 4% paraformaldehyde for histological examination and immunofluorescence staining. The remaining colon samples were kept at − 80 °C for further assessment of proteomics and enzyme-linked immunosorbent assay (ELISA), qPCR, and Western blotting analyses (n = 3).

Histopathological analysis of the rectums

For histological assessments, sections of the rectums were stained with HE stains. Light microscopy (CX31 research microscope, Olympus Optical Co., Ltd., Tokyo, Japan) and panoramic viewer camera system were used for examining, scanning, and analyzing the sections. Histological score was measured by inflammation severity: 0 (normal colonic mucosal), 1 (crypt damage less than 1/3), 2 (crypt damage less than 1/3 − 2/3), 3 (mucosal erosion), 4 (muscosal erosion or ulcer with significant infiltration of inflammatory cells) according to reference [29].

Label-free quantitative proteomics

On the 7th day after rats were treatment with TNBS (80 mg/kg), colon tissues from the rats in TNBS + saline group (n = 3) and TNBS + anemoside B4 group (10 mg/kg) (n = 3) were extracted, which then were lysed in a HEPES buffer (pH 8.0) containing 8 M urea to obtain total protein. Disulfide bonds in these proteins were reduced with 2 mM dithiothreitol at 37 °C for 45 min. The free thiols were further alkylated with 8 mM chloroacetamide for 1 h at room temperature. The excess chloroacetamide was then quenched by adding 2 mM dithiothreitol for 45 min. After acetone precipitation, proteins were resuspended in a HEPES buffer containing 8 M urea and incubated with the sequencing grade Lys-C (TaKaRa Bio, Japan) for 4 h at 37 °C. The samples were diluted with 10 mM HEPES (pH 8.0) four times and continued for digestion with MS-grade trypsin (TaKaRa Bio, Japan) at 37 °C for 20 h. Next, the peptides were desalted with C_{18} Zip-tips (Merck Millipore, Massachusetts, USA), which were then separated by capillary high performance liquid chromatography and analyzed in an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA). Data analysis was performed using MaxQuant, and proteins with FDR ≤ 0.01 were considered as positive identification. The independent sample *t*-test in IBM SPSS software (Ver 19) was used to calculate the *P*-values for the

identified proteins. The proteins with fold change > 1.50 or < 0.67 and $P < 0.05$ are considered as significantly differentially expressed proteins.

Western blot analysis

Western bolt analysis was performed as previously described [27]. In brief, proteins were extracted in RIPA lysis buffer, separated by SDS-PAGE, and transferred into PVDF membranes. The membranes were incubated with different primary antibodies and again with the peroxidase (HRP) conjugated goat secondary antibodies (Beyotime, Haimen, Jiangsu, China). Then, proteins were exposed to HRP substrate reagent (Millipore Corporation, Billerica, USA) and signals were detected with the ChemiDoc XRS Imager (Bio-Rad, USA).

Quantitative real-time RT-PCR

According to the manufacturer's instructions, total RNA was extracted with TRIzol reagent (Ambion, USA) and quantified by a NanoDrop ND-2000 spectrophotometer (Thermo Fisher). After quality detection, the total RNA samples (1000 ng per sample) were reverse-transcribed using the revertaid 1st cDNA synth kit (Thermo Scientific). Standard curves were constructed with the CFX96™ real-time PCR detection system (Bio-Rad). SsoAdvanced™Universal SYBR® Green (Bio-Rad) was used for qPCR detection. The cycling conditions for the qPCR were as follows: 95 °C for 3 min, followed by 37 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The relative expression levels of the genes were calculated with the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) value. Rat S100A9 primer: 5, GGCACGAGCTCCTTAGCTTT, 3, TCTCCCTCTTCAGAAAATTTGGC; rat GAPDH primer: 5, AGTGCCAGCCTCGTCTCATA, 3, GATGGTGATGGGTTTCCCGT.

ELISA

Inflammatory cytokines including TNF-α, interleukin-6 (IL-6), and interleukin-1β (IL-1β) in the colon tissue samples were assayed using ELISA kits according to the manufacturer's instructions.

Ethynyl-2'-Deoxyuridine (EdU) cell proliferation assay

Cell proliferation was detected by the BeyoClick™EdU-488 Kit (Beyotime, Haimen, Jiangsu, China). Briefly, 4 h before sacrifice, rats were injected intraperitoneally with 5 mg/kg EdU and then the colon was collected and rinsed with saline. Colon samples were then processed using the BeyoClick™ EdU-488 kit according to the manufacturer's instructions. The EdU positive cells were detected with a confocal laser scanning microscope (LSM 710, ZEISS, Germany).

Apoptosis detection assay

The Situ TUNEL Apoptosis Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to detect the apoptotic cells according to the manufacturer's instructions. The stained samples were measured under a confocal laser scanning microscope.

Immunofluorescence assay

The expressions of CD11b (Novus, USA) and S100A9 in colon tissues were detected by immunofluorescence staining. The colon sections were infiltrated with 0.5% Triton X-100 in PBS, blocked with 3% BSA, incubated overnight with either S100A9 or CD11b antibodies, and further incubated with a secondary antibody for 1 h. The immunofluorescence was detected by a confocal laser scanning microscope.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured in the colon. The colon was homogenized in 1:10 (w/v) of 50 mM phosphate buffer (pH 6.0) using a Polytron homogenizer. The homogenate was centrifuged at 12,000 rpm for 10 min. Then,MPO activity was detected by myeloperoxidase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Cell line experiments

Human intestinal epithelial Caco-2 cells were incubated in 5% CO_2 (95% relative humidity) at 37 °C, stimulated by 1 μg/ml LPS or S100A9 and treated with anemoside B4 after 1 h. At 6 h after the stimulation, total protein samples were extracted from the cells.

Data and statistical analysis

Data were expressed as the mean ± SD. Statistics were analyzed by one-way ANOVA and Tukey's multiple comparisons test using SPSS 16.0 statistical analysis software except indicated. $P < 0.05$ was considered as statistically significant.

Results

Anemoside B4 ameliorated TNBS-induced colitis in rats

The TNBS hapten, given as one or two enemas with ethanol as a carrier to disrupt epithelial integrity, induces acute inflammation and colitis in the mouse with Crohn's colitis-like transmural tissue damage [30]. In this study, TNBS-induced severe acute colitis rat model was used to determine the pharmacological activity of anemoside B4. The doses of anemoside B4 were chosen on the basis of our preliminary tests of LPS-induced inflammation and acetic acid-induced analgesia [27]. Mesalazine was orally administrated to rats with the same dose as the positive control [31].

In the model group, we observed IBD-like colitis, diarrhoea and bloody mucopurulent stools. The average body weight of the rats in the model group declined one day after TNBS administration. Compared with the mock-treated group, TNBS-induced rats showed a significantly higher DAI score and body weight loss. In TNBS-induced severe acute colitis model, both anemoside B4 and mesalazine inhibited body weight loss during disease progression (Fig. 1A). TNBS induced a significant increase in DAI score, an important marker of IBD. In contrast, anemoside B4 ameliorated TNBS-induced DAI score in a dose-dependent manner (Fig. 1B). TNBS treatment induced a significant decrease in colon length. Furthermore, treatment with anemoside B4 ameliorated TNBS-induced colon length shortening in a dose-dependent manner and

mesalazine was slightly less effective (Fig. 1C and D). These results demonstrate that inhibited effects of anemoside B4 on colitis is slightly more effective than mesalazine.

Anemoside B4 suppressed TNBS-induced inflammation

HE staining showed that the colon tissues of the rats in TNBS-induced groups had pathological structures, including obvious infiltration of inflammatory cells, decreased number of intestinal villus, epithelial cell damage and apoptosis. There were mark difference of pathological score between normal group and TNBS group. However, anemoside B4 relieved all pathological changes and score in a dose-dependent manner (Fig. 2B).

TNBS-induced model of acute colitis maintains many pathological features of human colitis, especially increasing type I inflammatory response and helper T cell 1 (Th1)-related cytokines, such as TNF-α or interferon-γ (IFN-γ). During inflammation, monocytes are recruited to inflamed sites and lymphoid tissues, where macrophage differentiation plays an important role during the beginning and resolution of inflammatory processes [32]. Therefore, we measured the effect of anemoside B4 on immune cell infiltration and inflammatory cytokine expression in rats with TNBS-induced acute colitis. Compared with the rats in vehicle group, those exposed to TNBS had significantly ($P < 0.05$) increased levels of colonic proinflammatory cytokines and chemokines, including IL-6, TNF-α, and IL-1β. However, anemoside B4 significantly reduced the levels of these colonic pro-inflammatory cytokines (Fig. 2C-E).

Anemoside B4 suppressed TNBS-induced proliferation and apoptosis

Next, we sought to the effects of anemoside B4 on the proliferation and apoptosis induced by TNBS. The EdU *in vivo* imaging kit was used to assess cell proliferation. 5-EdU was injected intraperitoneally 5 h before the rats were sacrificed, the colons were then harvested and operated according to the manufacturer's instruction. Normal colonic epithelial cells have regenerative proliferation and stained with green. More EdU stained spots were observed in vehicle rats. These results demonstrated TNBS decreased the ability of regenerative proliferation. However, anemoside B4 partially restored the ability of regenerative proliferation (Fig. 3).

It has been previously reported that TNBS-induced colitis are associated with apoptosis of the colonic epithelial cells and its toxicology is thought to be related to the induction of apoptosis and the destruction of the intestinal mucosal barrier [33, 34]. In IBD, high levels of apoptosis have been observed in the intestinal epithelium of patients [35]. In the intracellular machinery of apoptosis, the Bcl-2 family proteins involving anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) members mainly control the intrinsic pathway. The up-regulation of Bax, down-regulation of Bcl-2 and cleavage of caspase 3 are all mediated by p53 activation, which plays critical roles in the induction of apoptosis [36]. The increased number of apoptotic epithelial cells and enhanced Bax but attenuated Bcl-2 signaling during active colitis may lead to a defective epithelial barrier and result in pathogenic microorganism infiltration.

In the present study, TUNEL assay was carried out to detect apoptotic cells. More TUNEL stained spots were observed in the colon tissue obtained from TNBS-induced colitis rats. Anemoside B4 treatment significantly decreased the TUNEL stained spots. Further, Western blotting was used to detect apoptosis and related protein expression levels, respectively. Consistent with previous reports, we found that the TNBS group had markedly up-regulated Bax, p53 and cleaved caspase 3 but down-regulated Bcl-2 in the colon. In contrast, anemoside B4 significantly increased Bcl-2/Bax ratio and inhibited cleaved caspase3, p53 protein levels in a dose dependent manner, suggesting that anemoside B4 could reduce colitis apoptosis in colon sample (Fig. 4). These observations indicated that anemoside B4 was obviously effective in mitigating TNBS-induced acute colitis in rats.

Anemoside B4 inhibited NF-κB activation induced by LPS in colonic epithelial cells

The above experiment data showed that anemoside B4 had anti-inflammatory activity and inhibits inflammatory cytokine secretion induced by colitis. It is well known that NF-κB plays a critical role in inflammation involved various human diseases including IBD and in animal disease models. Activated NF-κB pathway promotes the expression of various pro-inflammatory genes and influences the course of mucosal inflammation [37].

In order to explore the mechanism of anti-inflammatory activity of anemoside B4, we investigated the effect of anemoside B4 on the activation of the NF-κB signaling pathway, as it has been documented to be related to inflammation. As expected, the level of p-NF-κB/p65 protein is increased upon LPS treatment, while it was significantly reduced by anemoside B4 (Fig. 5). In addition, LPS activated NF-κB upstream signaling pathway, including p-p38, p-JNK, and p-ERK in colonic epithelial cells, while these levels were similarly reduced by anemoside B4.

Proteomic identification of the differentially regulated proteins by anemoside B4

The above studies showed that 10 mg/kg anemoside B4 remarkably diminished the TNBS-induced colitis and the therapeutic effect was comparable to that of mesalazine. Therefore, in the following experiments, anemoside B4 was used at the dose of 10 mg/kg.

To further investigate the mechanisms of the therapeutic effect of anemoside B4 on TNBS-induced colitis, we performed a label-free quantitative proteomic analysis to identify the differentially expressed proteins in the colon samples from TNBS-induced group and anemoside B4-treated group.

In total, we identified 604 proteins and quantified 591 proteins, whose ratios were shown in the volcano plot. Among the quantified proteins, 56 protein groups with fold change > 1.50 or < 0.67 and $P < 0.05$ were marked with red (upregulation) or green (downregulation) dots in the anemoside B4-treated group compared with the TNBS-induced group (Fig. 6A).

Next, the online STRING database was used to analyze the biological processes which these 56 proteins participated in. The result showed that the differentially regulated proteins were mainly clustered in the tricarboxylic acid cycle (TCA) and respiratory electron transport chain. S100A9 protein is one of the proteins that are mostly affected by anemoside B4 (Fig. 6A, B).

Western blotting and qPCR analysis revealed that both the protein and mRNA levels of S100A9 were significantly increased in colon tissues from rats treated by TNBS and these increases could be attenuated by the further treatment with anemoside B4 (Fig. 6C, D).

Anemoside B4 suppresses S100A9/TLR4/NF-κB downstream signaling pathway in vivo

S100A9 combines with S100A8 to form heterodimers of S100A8/S100A9, which bind to TLR4, then downstream activation of JNK and NF-κB occurs through MyD88-induced activation in a TLR4 dependent manner, thereby inflammation is promoted. Therefore, we examined the activation of S100A9/TLR4/NF-κB signaling pathway in colonic homogenate to explore the further molecular evidence of the anti-inflammatory effects of anemoside B4. As shown in Fig. 7, TNBS induction significantly increased the expression of TLR4 protein in rat colon, and which was significantly reduced by the treatment of anemoside B4 at a dose of 10 mg/kg. Then we detected the expressions of some key proteins involved in the downstream pathway of TLR4, such as p-NF-κBp65, p-JNK, and effector molecules IL-6, NOD2, which was significantly increased in rat colons after TNBS challenge, while anemoside B4 could reduce these increased expressions. These results demonstrated that anemoside B4 could inhibit the TNBS-induced activation of S100A9/ TLR4/NF-kB pathway in rat colons.

Anemoside B4 inhibited the recombinant S100A9 protein-stimulated downstream signaling pathways in colonic epithelial cells

Recombinant S100A9 protein stimulated the expressions of its downstream target genes including TNF-α and IL-1β in macrophages, while anti-S100A9 antibody significantly blocked this effect [17]. To evaluate whether S100A9 is a key factor in the effect of anemoside B4, we used S100A9 recombinant protein treated colonic epithelial Caco-2 cells. The cell lysis was collected 24 h after cells were treated with S100A9 protein. As was shown in Fig. 8, S100A9 activated phosphorylation of NF-κB/p65. In contrast, anemoside B4 inhibited this phosphorylation activation induced by S100A9. An important upstream component of NF-κB is MAPK signaling pathways which comprise of p38, JNK and ERK1/2. MAPK activation also induces phosphorylation activation of NF-κB/p65[38]. In our study, S100A9 increased the phosphorylation of p38, ERK1/2 and JNK. While, treatment with anemoside B4 ameliorated this increase (Fig. 8). Hence, we could convincingly propose that anemoside B4 mediated its protective role by regulating S100A9 signaling pathway.

Anemoside B4 inhibited the recruitment of inflammatory cells to colon

The recruitment of inflammatory cells to the site of infection or inflammation represents an important process in IBD. Neutrophils are often the first immune cells recruited to the site of inflammation and play a key role in combatting microbial invasion [39] and then monocytes and macrophage are also recruited to the inflammation site.

To evaluate the effect of anemoside B4 on inflammatory infiltration in a murine model of intestinal inflammation, a fluorescence labelled antibody against murine CD11b was used to measure inflammatory recruitment and infiltration into the murine intestine. Infiltration of inflammation cells including neutrophils, monocyte and macrophage into the intestinal wall of rat colitis was measured after induction of colitis by immunofluorescence staining for CD11b. Compared to non-colitic control rats, colitis animals showed a significantly elevated number of CD11b positive cells in the intestinal wall (Fig. 9A). By contrast, anemoside B4 significantly decreased the number of positive cells in colon tissue of a TNBS-induced colitis.

In inflammation tissue, intestinal epithelial cells can secret S100A9 protein induced by inflammation, neutrophils, monocyte and macrophage could also secret S100A9 protein. Therefore, we measured the localization of S100A9. The data showed that there were more S100A9 positive cells in colon tissue from colitic rats than from vehicle rats. In contrast, anemoside B4 significantly decreased S100A9 positive cells in colon tissue. It is interesting that S100A9 localization is consistent with the location of CD11b positive cells. Additionally, the concentration of MPO, which reflects both neutrophil numbers and inflammatory activity, was greatly elevated in colonic tissue. By contrast, anemoside B4 significantly decreased the concentration of MPO in colon tissue (Fig. 9B). These data indicated that anemoside B4 significantly inhibited inflammation cell recruitment.

Discussion

In this paper, we reported for the first time, to our knowledge, the *in vitro* and *in vivo* therapeutic effect of anemoside B4 on colitis involving in its anti-inflammatory and anti-apoptotic activities. Anemoside B4 ameliorated TNBS-induced colonic tissue apoptosis, inflammatory cell infiltration, and pro-inflammatory cytokine production in the colon. Our previous study showed that anemoside B4 has low toxicity without influencing body weight and function of hepatitis and kidney in mice [26]. Taken together, these studies indicated that anemoside B4 might provide therapeutic potential for IBD.

Although the pathogenesis of IBD is still unclear, inflammation is thought to be a key factor that regulates the pathophysiological process of IBD. Inflammation is a beneficial response to tissue injury and infection and plays an essential role in the restoration of homeostasis. However, sustained or unregulated inflammation can promote pathophysiological process of IBD. IBD is a chronic inflammatory disease of the gastrointestinal tract characterized by breakdown of the epithelial barrier and disruption of intestinal

homeostasis. Emerging experimental and clinical data has indicated that pro-inflammatory cytokines, such as TNF-α, IFN-α and IL-6, play crucial roles in colitis pathogenesis [40, 41]. The high levels of pro-inflammatory cytokines induced tissue damage and cell apoptosis. In this study, anemoside B4 exerted its anti-inflammatory effect by inhibiting the secretion of pro-inflammatory factors such as IL-1β, IL-6 and TNF-α in colon. Furthermore, anemoside B4 attenuated the pathological structure of colitis, including increased number of intestinal villus and attenuated epithelial cell damage induced by TNBS.

Moreover, as an indicator of the severity of colitis, high DAI represents the pathological states of diseased animals, and a shortened colon is generally considered to be a marker of inflammation, which was also observed in rats treated with TNBS [42] and was significantly reduced by treatment with anemoside B4. These results suggested that anemoside B4 diminished TNBS-induced inflammation.

Recently, studies demonstrated that dysregulation of apoptosis in colonic mucosa is involved in the pathogenesis of IBD. Numerous studies have identified that apoptosis in intestinal epithelial cells is driven by increased cytokine activity such as TNF, IL, and interferon family members [43]. Increased apoptosis of intestinal epithelial cells has been observed at colitis inflammatory sites, which can disrupt intestinal mucosal integrity and barrier function eventually leading to inflammation [33]. Elucidating the molecular mechanism how to efficiently decrease colonic epithelial cell apoptosis and how to repair mucosal tissues has become the focus of therapy of UC [44]. The increased number of apoptotic epithelial cells induced by enhanced Bax but attenuated Bcl-2 during active UC may lead to a defective epithelial barrier and result in pathogenic microorganism infiltration [35, 45]. Our results indicated that colitis rats had more apoptotic cells, evidenced by the increased Bax and cleaved caspase3 but reduced anti-apoptosis protein Bcl-2. Treatment with anemoside B4 decreased the number of apoptotic cells and moderated the expression levels of these apoptosisrelated proteins. Thus, anemoside B4 might protect the integrity of the intestinal barrier by inhibiting apoptosis of intestinal epithelial cells. The results above clearly demonstrated that anemoside B4 attenuated TNBS-induced colitis by inhibiting inflammation and protecting the integrity of the intestinal barrier.

In order to elucidate the mechanism by which anemoside B4 attenuates colitis, we performed a label-free quantitative proteomic study to identify its target protein and 56 potential proteins regulated by anemoside B4 were identified. Among them, S100A9 might play a vital role in the regulation of the biological effect of anemoside B4.

S100A8 and S100A9 have been emerged heavily as an important pro-inflammatory mediator and have been reported to be a useful biomarker in IBD [46]. S100A8/A9 stimulated all members of the MAPK cascades, including p38, ERK, and JNK families, and promotes NF-κB activation through TLR4 or RAGE receptor [47]. In intestinal inflammation, S100A9 is an effector molecule that enhances TLR signaling and recruits granulocytes. Thus, the blockade of this molecule could ameliorate disease severity in TNBS-induced colitis. S100A9 may be a therapeutic target for colitis [17]. In present study, anemoside B4 reduced the activation of S100A9/TLR4/NF-*κ*B signaling pathway *in vitro* and *in vivo*. Anemoside B4 (10 mg/kg) significantly decreased TLR4 expression and its downstream pathways, including the

phosphorylation of NF-κB/p65, JNK, and expression of effector molecule IL-6. Further, experiments with colonic epithelial Caco-2 cells treated with S100A9 recombinant protein found S100A9 activated NF-κB/p65. An important upstream component of NF-κB is MAPK signaling pathways which comprises of p38, JNK and ERK1/2. MAPK activation also induces NF-κB/p65 activation. Anemoside B4 inhibited S100A9/TLR4/NF-κB signaling pathway stimulated by the recombinant S100A9 protein.

Apart from the known S100A9 functions under inflammatory conditions, little has been shown on the expression mechanism of S100A9 in intestinal epithelial cells, which is important for intestinal homeostasis. Our results showed that anemoside B4 inhibited S100A9 pathway effectively in intestinal epithelial cells. S100A8/S100A9 can activate RAGE and TLR4, which also results in the activation of NF-κB and the upregulation of CC-chemokine ligand 2 (CCL2) and CC-chemokine ligand 5 (CCL5), involving in cellular inflammatory responses [48].

S100A8 and S100A9 are expressed primarily in neutrophils and monocytes and induced under inflammatory conditions in keratinocytes and epithelial cells [49]. S100A8 and S100A9 comprise ~ 40% of the cytosolic protein of neutrophils and their intracellular expression has been associated with calcium sensing [50]. Infiltration of neutrophils into colonic mucosa has been associated with the severity of IBD. The presence of neutrophils in the intestinal mucosa constitutes a prominent inflammatory component of active UC. S100A8/A9 are not only synthesized and secreted by myeloid cells, but also myeloid cells have binding sites for S100A8/A9 and activate intracellular signaling that promote their migration [51]. Upon certain inflammatory stimuli, neutrophils form networks of extracellular fibers called neutrophil extracellular traps (NETs). Apart from their antimicrobial function, the pathogenic role of NETs in several inflammatory and thrombotic diseases has been highlighted in many studies [52, 53]. There is evidence to support that neutrophils express and release a variety of proteins through NETs, which are associated with certain phenotypes of diseases and determined by the disease-specific inflammatory environment. Altogether neutrophils are often the first type of immune cells recruited to the site of inflammation and play a key role in combatting microbial invasion. When these pathways become uncontrolled, extensive tissue damage and development of chronic disease might occur. The role of neutrophils in the pathogenesis of IBD remains controversial and possibly differs between UC and CD and no therapeutic strategies exist to selectively target neutrophils [54]. Monitoring neutrophil trafficking to the site of inflammation would be helpful for understanding their contribution to the pathogenesis of disease and furthermore, this would help to visualize and assess the anti-inflammatory effects of new drugs.

MPO activity is a marker of infiltration of neutrophil. In the present study, we measured the activities of MPO in the colon tissues of rats in each group. MPO is present in primary granules for medially during neutrophil maturation and is liberated in copious amounts by activated neutrophils to amplify the inflammatory response by inducing oxidative modification of proteins and lipids. Its activity is linearly correlated to neutrophil infiltration in inflamed tissue [55]. Confirmatory, colonic MPO levels, correlating with the number of neutrophils were significantly increased in colon tissue obtained from colitic rats versus non-colitic animals. Thus, we measured MPO, the peroxidase abundantly expressed in neutrophil granulocytes. A higher level of MPO activity was observed in the TNBS-induced group, suggesting

neutrophil accumulation [56, 57]. When compared with that of the TNBS-induced group, treatment with anemoside B4 significantly suppressed the elevated MPO activity. Further, our immunofluorescence experiment found that S100A9 was distributed on the surface of colon. TNBS induced myeloid cells to migrate into damaged colon mucosa. S100A9 shared the same location in tissue. These data demonstrated that anemoside B4 inhibited the myeloid cell migration and S100A9 expression. Based on MPO experiment, our data indicated that anemoside B4 inhibited neutrophil accumulation.

Conclusion

In summary, our study demonstrated that anemoside B4 exhibited significant protective effects on TNBS induced colitis via suppressing the S100A9/NF-κB signaling pathway (Fig. 10).

Abbreviations

AOM, azoxymethane; CD, Crohn's disease; CCL2, CC-chemokine ligand 2; CCL5, CC-chemokine ligand 5; DAI, Disease activity index; DSS, dextran sulfate sodium; Edu, ethynyl-2'-deoxyuridine; HMGB1, interact high-mobility group box 1; IBD, inflammatory bowel disease; IFN-γ, Interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; MAPK, mitogen-activated protein kinases; MPO, myeloperoxidase; MyD88, myeloid differentiation factor 88; NETs, neutrophil extracellular traps; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RAGE, receptor for advanced glycation end products; TCA, tricarboxylic acid cycle; Th1, helper T cell 1; TIR, Toll/interleukin-1 receptor; TLR4, toll-like receptor 4; TNBS, 2, 4, 6-trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor; TRAM, TRIF-related adaptor molecule; TRIF, Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UC, ulcerative colitis.

Declarations

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study was established according to the ethical guidelines and approved by the Ethics Committee on Laboratory Animal Management of Soochow University.

Consent for publication

We declare that the Publisher has the Author's permission to publish the relevant contribution.

Competing interests

The authors declare no competing conflict of interests.

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Authors' contributions

XQ, LY and XG designed the research. ZY, ZZX, SWH and LD conducted the experiments. ZY, LY and XQ wrote the manuscript. ZY, LY, KN, CZ, XQ, XG revised the manuscript. All authors read and approved the final manuscript.

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Figures

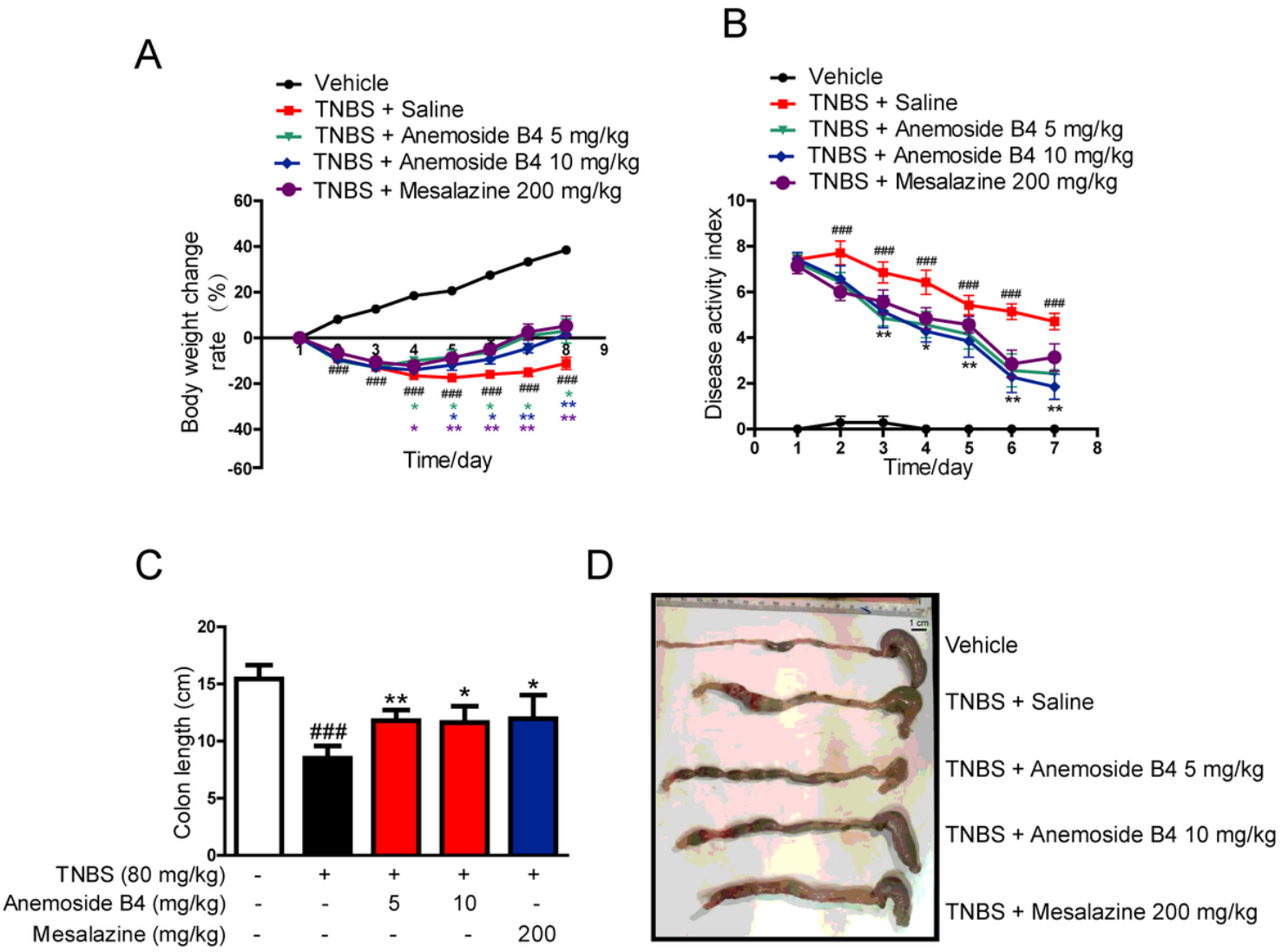

Figure 1

Anemoside B4 ameliorates TNBS-induced colitis in rats. Rats were orally administrated with 80 mg/kg TNBS for one day and then were continuously injected i.p anemosdie B4 at the doses of 5 and 10 mg/kg twice per day for 7 days. Mesalazine was administrated consecutively i.g. at the doses of 200 mg/kg for 7 days. Rats were sacrificed on the 8th day after colitis induction. (A) Body weight of each group was recorded during the progression; (B) DAI of each group during the disease process; (C) Colon length of rats from different groups; (D) Representative images of colons at the end of the experiment. Scale bar, 100 μm. Results in (A)-(C) are presented as mean ± SD (n=7). Two-way ANOVA followed by Tukey's multiple comparison test was used to analyze statistical significance. ###P < 0.001, vs. vehicle group, **P < 0.01, ***P < 0.001 vs. TNBS+saline group.

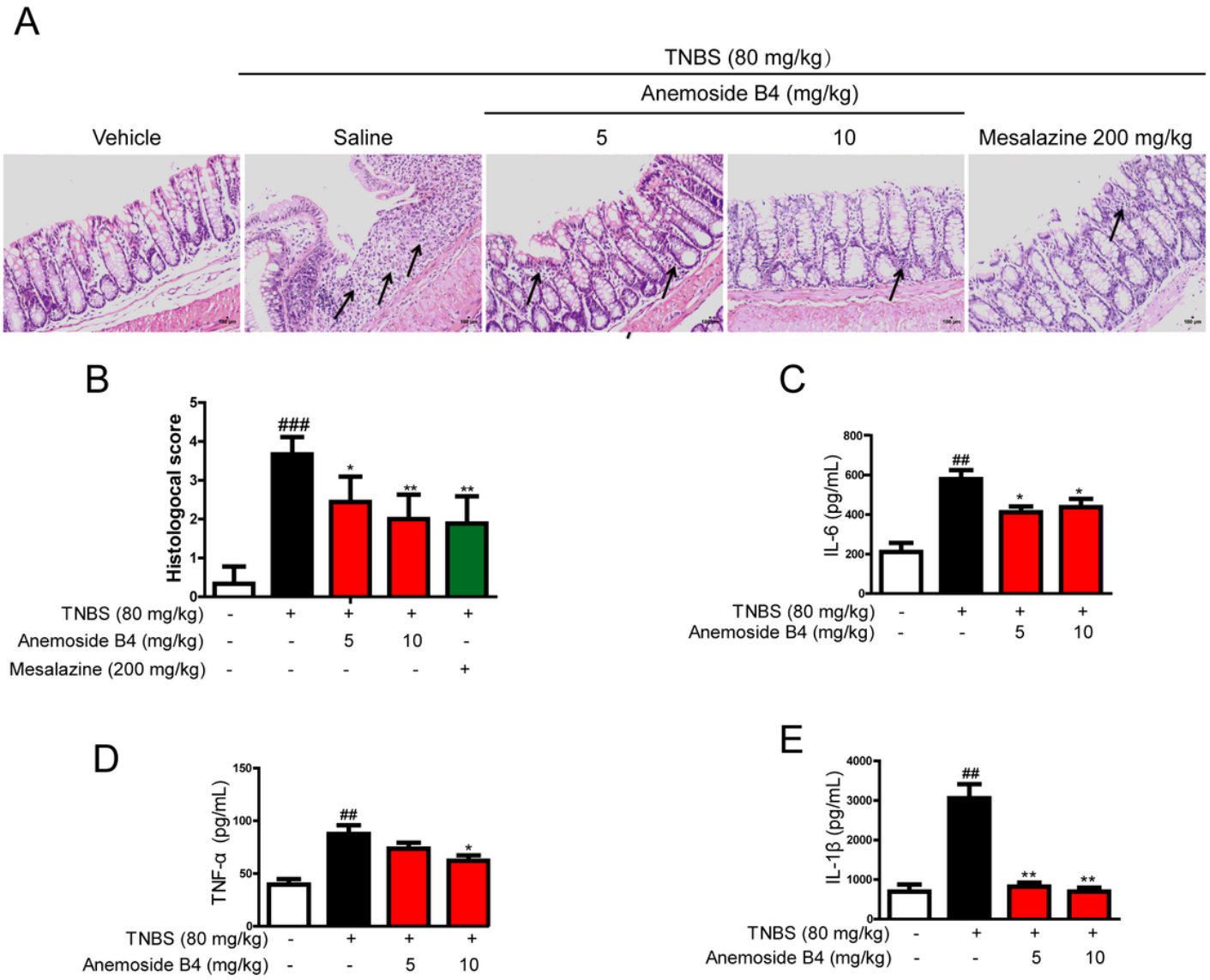

Figure 2

Anemoside B4 ameliorates inflammatory response of TNBS-induced colitis in rats. (A) Serial sections of colon tissues were stained with hematoxylin and eosin (HE). Black arrows indicated the inflammatory cells. Colon histology was examined by HE staining of paraffin-embedded sections in vehicle and TNBS-treated rats on the 8th day after TNBS administration. Scale bars: 100 mm. (B) IL-6, (C) TNF-α, and (D) IL-1β were measured by ELISA. Data are shown as mean ± SD (n = 3). ###P < 0.001, vs. vehicle group. *P < 0.05 vs. TNBS group.

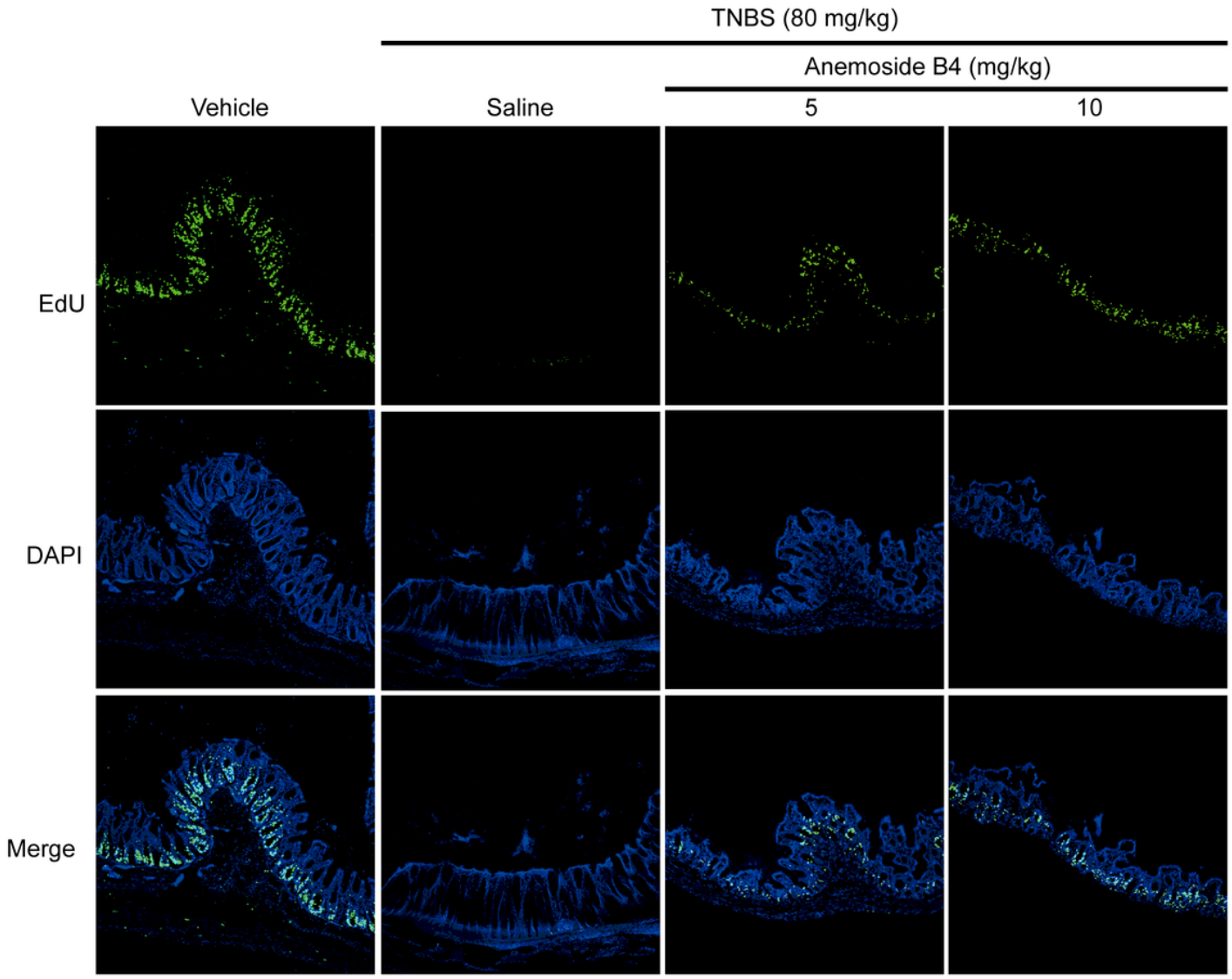

Figure 3

Effect of anemoside B4 on the proliferation of intestine epithelial cells of the TNBS-induced rat colitis. Sections of colon tissue (n = 3) were immunostained with Edu and observed by confocal laser-scanning microscope.

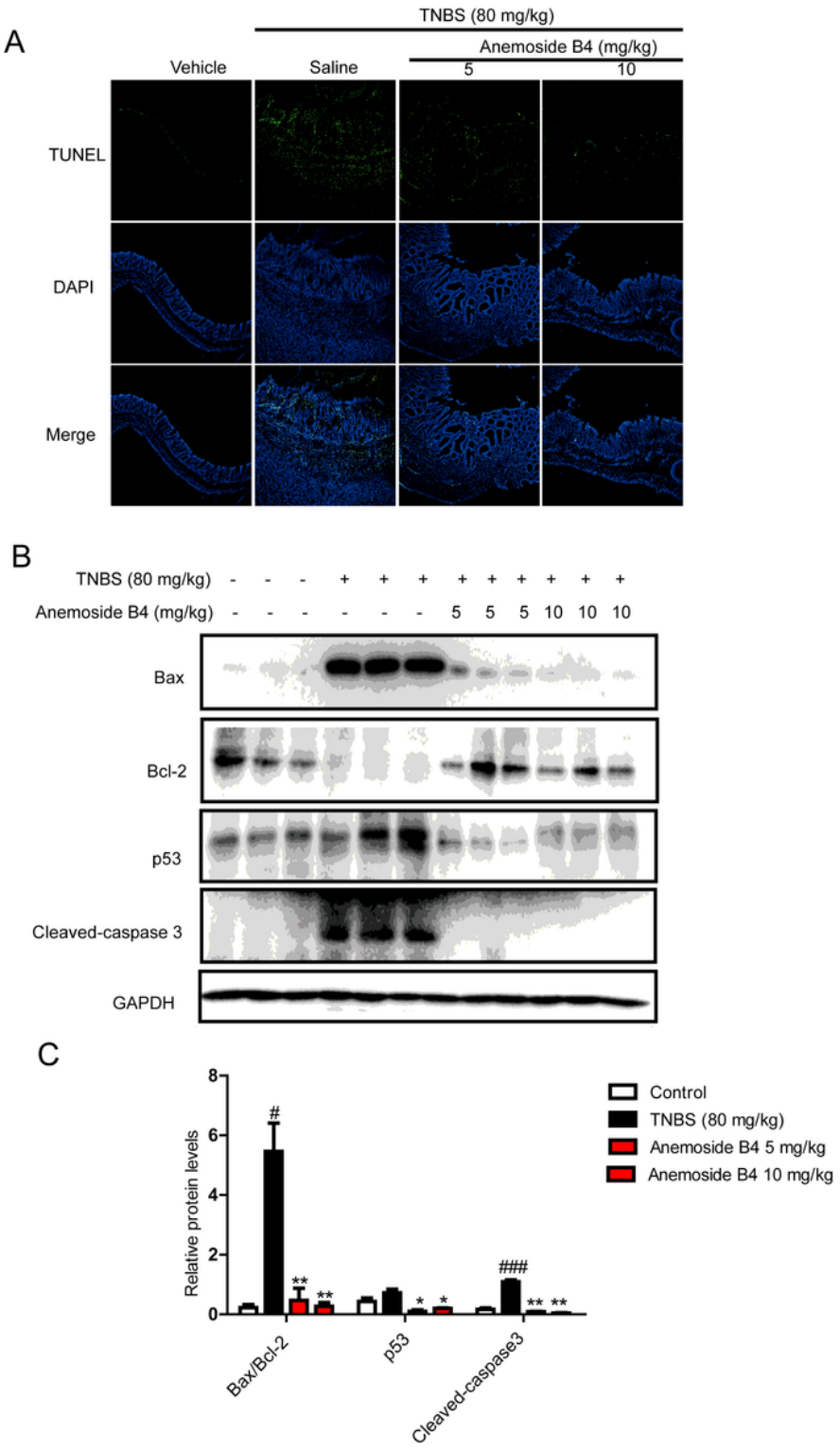

Figure 4

Effect of anemoside B4 on the apoptosis of intestine epithelial cells of the TNBS-induced rat colitis. (A) Sections of colon tissue were immunostained with TUNEL and observed by confocal laser-scanning microscope (n = 3). (B) p53, Bcl-2, Bax and cleaved caspase 3 was measured by western blotting (n = 3).

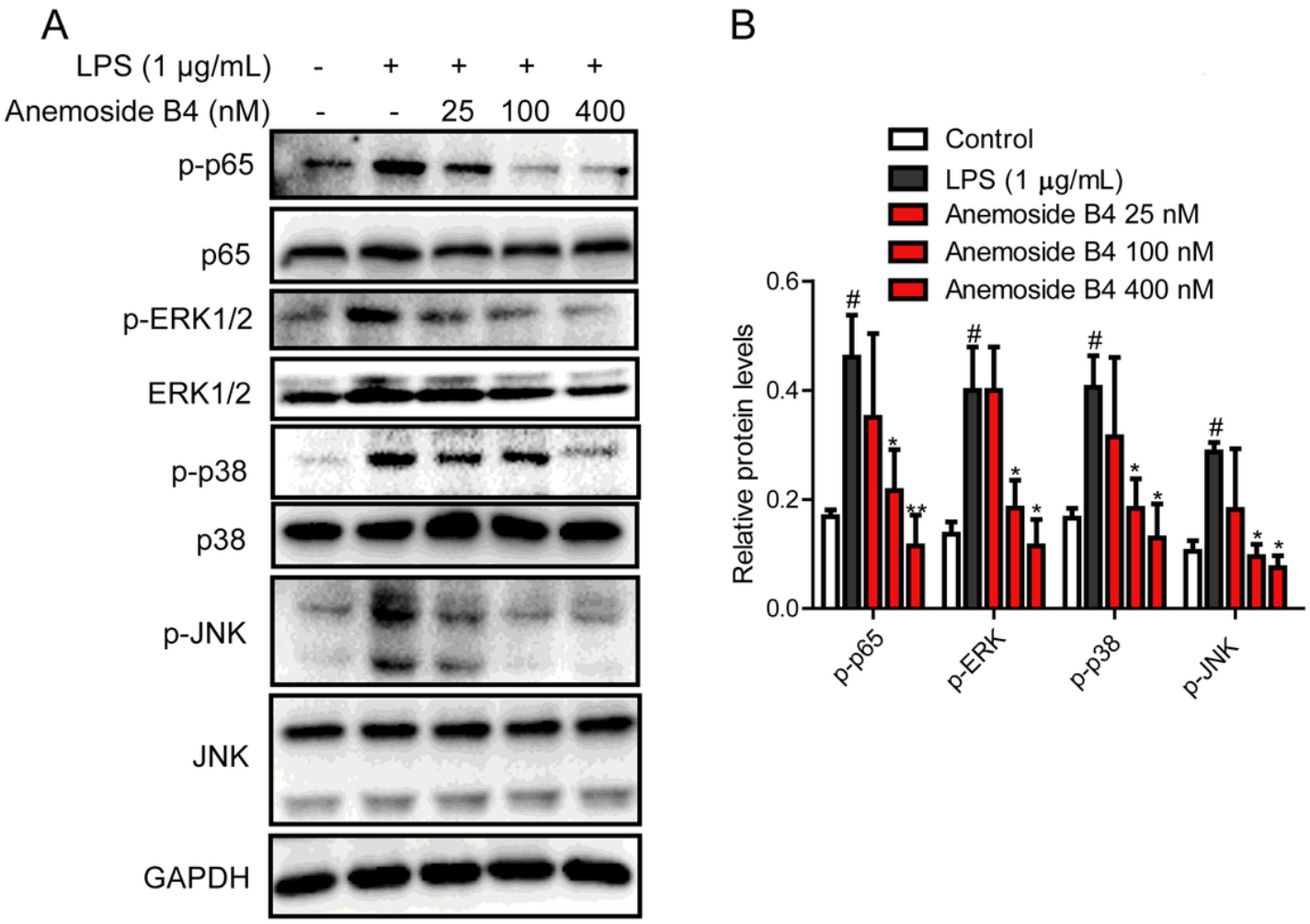

Figure 5

Anemoside B4 inhibits LPS-induced NF-κB activation in Caco-2 cells. Caco-2 cells were treated with anemoside B4 as indicated concentration and LPS (1 μg/ml) for 60 min. Cells were harvested and total cell extracts were prepared. Phosphorylated-ERK, phosphorylated-JNK, phosphorylated-p38 and phosphorylated-NF-κB/p65 subunit were detected by Western blot analysis. Total ERK, JNK, p38, p65 and GAPDH were used as internal standards. Each experiment was repeated for three times.

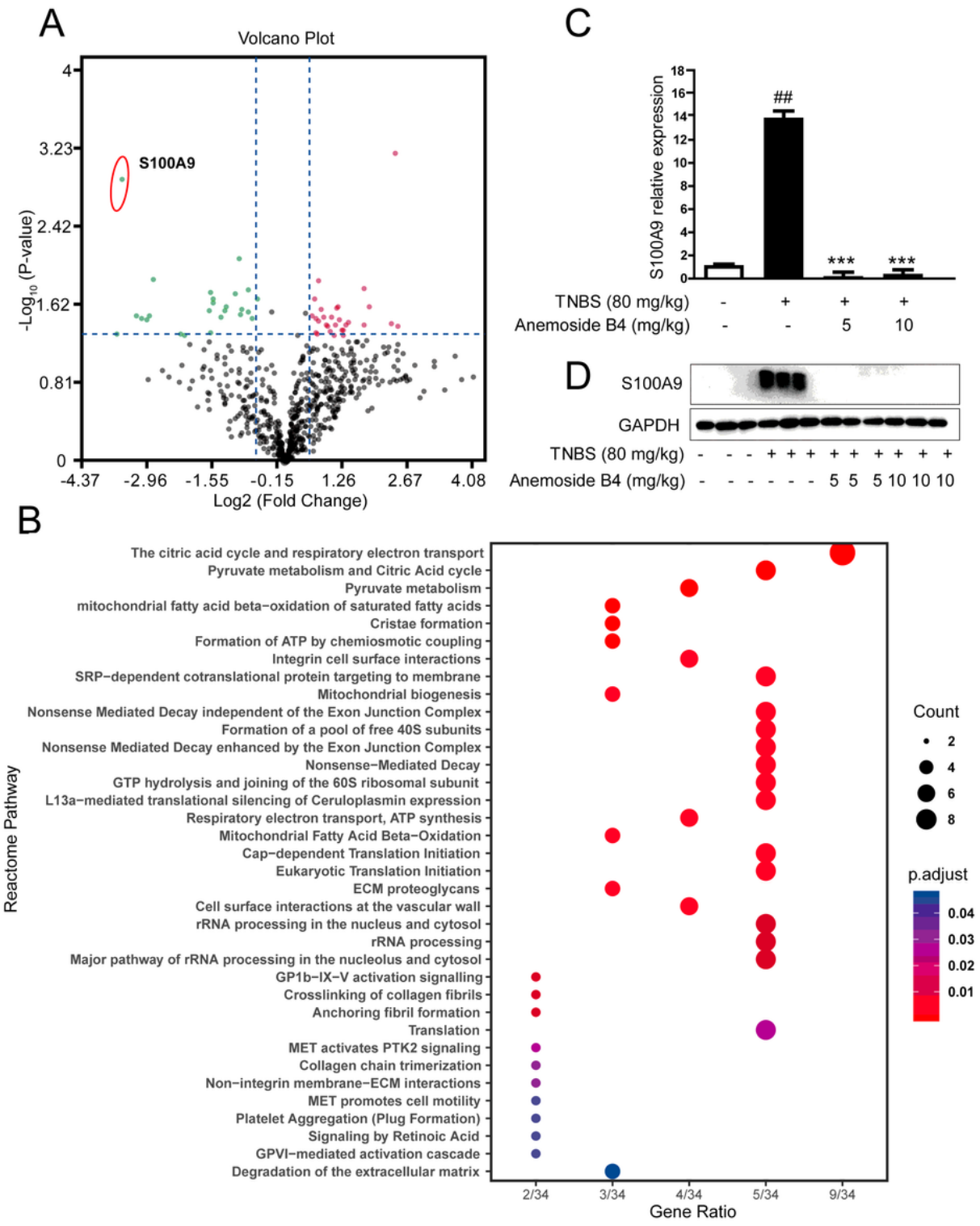

Figure 6

Quantitative proteomic analysis identifies the differentially regulated proteins by anemoside B4 upon TNBS-induced colitis colon tissue from rats. Rats were orally administrated with 80 mg/kg TNBS. Anemoside B4 was continuously injected i.p at the doses of 5 and 10 mg/kg twice a day for 8 days. Rats were sacrificed on day 8 after colitis induction. (A) The volcano plot for the MS identified 604 proteins in colon tissue. Each point showed the log2 (Fold change) in the x axis versus their corresponding -log10 (p-

value) in the y axis. The filter boundaries were set so that a fold change above 1.5, or a fold change below 0.67, were considered significant, and a p-value of 0.05 was used as the cutoff value. The horizontal dotted line indicates the cutoff p-value of 0.05 and all points above this line have $p < 0.05$. The black points indicate 591 quantified unaltered proteins. The green points and red points (26) indicated the down-regulated and (30) up-regulated proteins, respectively. The arrow indicated the proteins used for the biochemical validation. (B) Reactive pathway analysis discovers that S100A9 is associated with protein processing in neutrophil degranulation. (C) Quantification of S100A9 mRNA expression. (D) Western blotting analysis of S100A9 protein level. Data are shown as mean ± SD (n = 3). ##P < 0.001, vs. vehicle group; ***P < 0.001 vs. TNBS group.

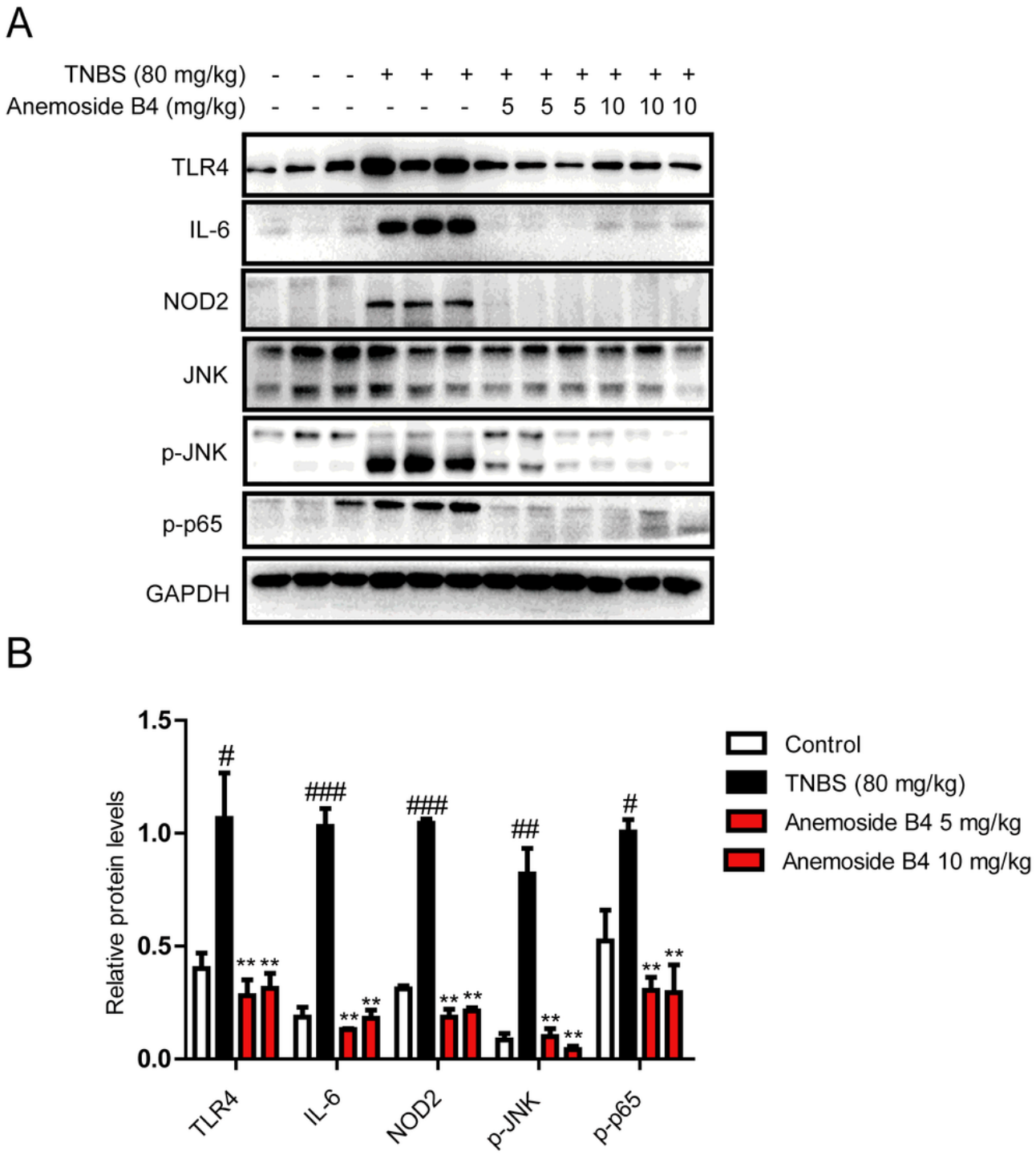

Figure 7

Effect of anemoside B4 on S100A9 signaling pathway in colon tissues. Rats were orally administrated with 80 mg/kg TNBS. Anemoside B4 was continuously injected i.p. at the doses of 5 and 10 mg/kg twice a day for 8 days. Rats were sacrificed on day 8 after colitis induction. Proteins in the downstream or upstream of S100A9 signaling pathway were detected by western blotting (n = 3).

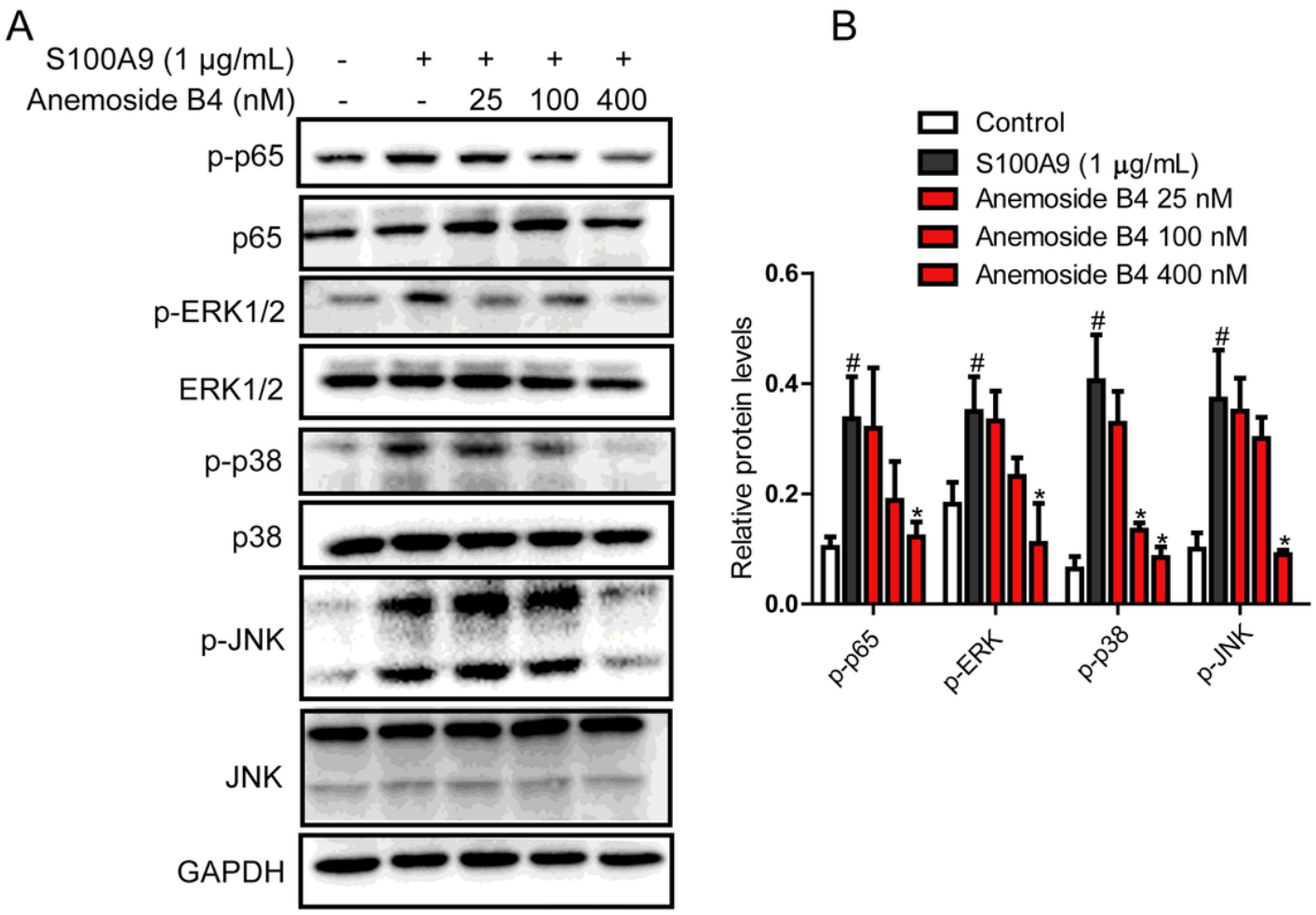

Figure 8

Anemoside B4 attenuates the effect of S100A9 recombinant protein on the phosphorylation and activation of NF-κB and MAPKs in Caco-2 cells. Human Caco-2 cells were treated with S100A9 (1 μg/mL) for 1 h, and cell lysates were collected. Phosphorylation of p65, ERK1/2, JNK1/2 and p38 MAPKs and total proteins were analyzed by western blotting. Each experiment was repeated for three times.

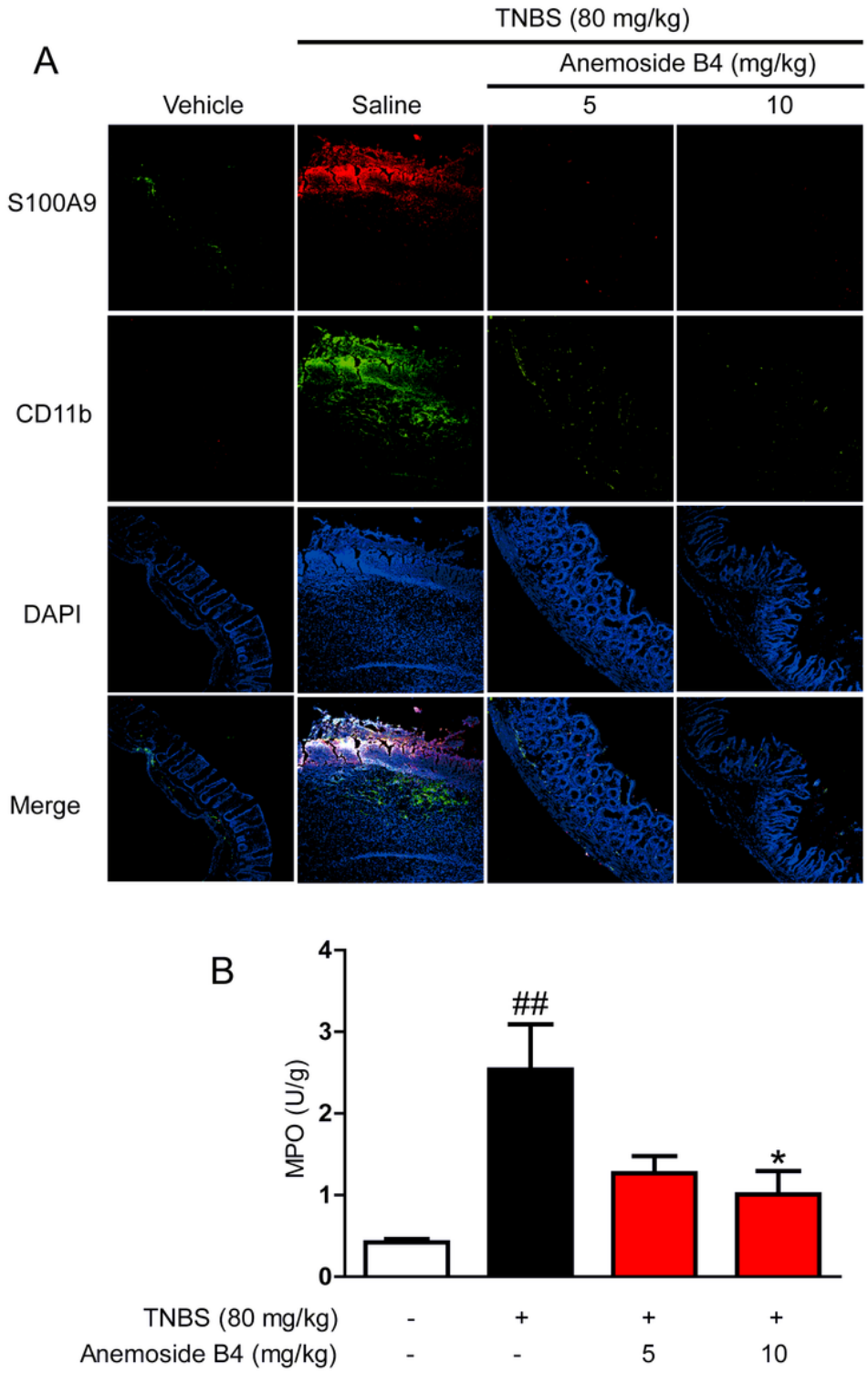

Figure 9

Assessment of intestinal neutrophil infiltration. Infiltration of neutrophils into the intestinal wall was determined in TNBS-colitic rats and healthy controls. (A) Sections of colon tissue were immunostained with S100A9 (red) and anti CD11b-FITC (green) and observed by confocal laser-scanning microscope. Shown are data from one experiment (n = 3 per group). (B) MPO in colon tissues (per 100 mg) was determined by ELISA. Data are shown as mean ± SD (n = 3).