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Anemoside B4 alleviates DSS-induced colitis by inhibiting CD1d-dependent NLRP3 inflammasome activation in macrophages

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Abstract

Background and Purpose: Abnormal activation of the NLRP3 inflammasome in macrophages is closely associated with Ulcerative colitis (UC), and targeting the NLRP3 inflammasome has been proposed as a potential therapeutic approach, but the underlying mechanism by which it regulates intestinal inflammation remains unclear. Anemoside B4 (AB4) has anti-inflammatory activity, but whether it alleviates UC by inhibiting the activation of NLRP3 inflammasome remains unclear. More importantly, the molecular targets of AB4 remain unknown. Experimental Approach: We explored the role of AB4 in the development of dextran sodium sulfate (DSS)-induced colitis in wild-type (WT) mice and its effect on NLRP3 inflammasome. We isolated intestinal macrophages and epithelial cells, and validated them in DSS-induced NLRP3-deficient (NLRP3-/-) mice. The target and molecular mechanism of AB4 were identified in LPS-induced macrophages in vitro and DSS-induced macrophage-specific CD1d depletion (CD1d-/-) mice in vivo. Key Results: This study showed that AB4 had a strong anti-inflammatory effect DSS-induced colitis in WT mice, whereas the protective effects were lost in NLRP3-/- mice. AB4 inhibited the activation of NLRP3 inflammasome in colonic macrophages without affecting intestinal epithelial cells. Mechanistically, AB4 might target CD1d thus reducing the AKT-STAT1-PRDX1-NF-ϰB signaling pathway, eventually inhibiting the activation of NLRP3 inflammasome. Macrophage-specific CD1d depletion had been shown to reverse the protective effect of AB4. Conclusions and Implications: Our data showed that AB4 attenuated DSS-induced colitis by inhibiting CD1d-dependent NLRP3 inflammasome activation in macrophages. Therefore, as a natural product with high safety index, AB4 might be considered a promising candidate drug for the treatment of colitis.

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Abstract

Background and Purpose: Abnormal activation of the NLRP3 inflammasome in macrophages is closely associated with Ulcerative colitis (UC), and targeting the NLRP3 inflammasome has been proposed as a potential therapeutic approach, but the underlying mechanism by which it regulates intestinal inflammation remains unclear. Anemoside B4 (AB4) has anti-inflammatory activity, but whether it alleviates UC by inhibiting the activation of NLRP3 inflammasome remains unclear. More importantly, the molecular targets of AB4 remain unknown.

Experimental Approach: We explored the role of AB4 in the development of dextran sodium sulfate (DSS)-induced colitis in wild-type (WT) mice and its effect on NLRP3 inflammasome. Next, we isolated intestinal macrophages and epithelial cells, and validated them in DSS-induced NLRP3-deficient ($NLRP3^{-/-}$) mice. The target and molecular mechanism of AB4 were identified in lipopolysaccharides (LPS)-induced macrophages in vitro and DSS-induced macrophage-specific CD1d depletion ($CD1d^{-/-}$) mice in vivo.

Key Results: This study showed that AB4 had a strong anti-inflammatory effect DSS-induced colitis in WT mice, whereas the protective effects were lost in $NLRP3^{-/-}$mice. Interestingly, AB4 inhibited the activation of NLRP3 inflammasome in colonic macrophages without affecting intestinal epithelial cells. Mechanistically, AB4 might target CD1d thus reducing the AKT-STAT1-PRDX1-NF-κB signaling pathway, eventually inhibiting the activation of NLRP3 inflammasome. Macrophage-specific CD1d depletion had been shown to reverse the protective effect of AB4.

Conclusions and Implications: Our data showed that AB4 attenuated DSS-induced colitis by inhibiting CD1d-dependent NLRP3 inflammasome activation in macrophages. Therefore, as a natural product with high safety index, AB4 might be considered a promising candidate drug for the treatment of colitis.

Introduction

Ulcerative colitis (UC) is a type of nonspecific inflammatory bowel disease (IBD), which starts from the rectum and extends continuously to proximal segments of the colon (Ge, Li, Gong, & Zhu, 2018). With the rapid development of industrialization and modernization, the global incidence of UC has also been rising continuously. The main pathological lesions are mucosal ulcers, and the typical clinical symptoms are diarrhea, rectal bleeding, and weight loss. Repeated episodes of UC increase the cumulative risk of colorectal cancer (CRC) by 18-20%, which undoubtedly brings serious mental burden and psychological pressure to UC patients and seriously affects their normal life (Ge et al., 2018; Torres et al., 2021). Although the exact cause of UC is uncertain at present, the activation of the mucosal immune system and the subsequent pathological cytokines play roles in the generation of UC (Ge et al., 2018; Torres et al., 2021). At present, UC patients can only receive long-term immunosuppressive and anti-inflammatory treatment, such as glucocorticoids, immune-suppressants, biological agents, and 5-aminosalicylic acid (5-ASA), as well as even require surgery, which is limited due to more side effects or high recurrence rate (de Lange & Barrett, 2015; Kaplan, 2015). In this case, it is urgently needed to develop highly effective drugs with fewer side effects, long-term control ability of inflammation development, and stabilization of intestinal microenvironment.

Macrophages are abundant in colon samples from UC patients and animal models, which play an essential function in the occurrence, development and resolution of inflammation (Moreira Lopes, Mosser, & Goncalves, 2020). Macrophages can respond to the damage-associated molecular patterns (DAMPs) and the pathogen-associated molecular patterns (PAMPs), enhance the recruitment, and activate other innate and adaptive immune cells to amplify intestinal inflammation (de Lange & Barrett, 2015; Moreira Lopes et

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al., 2020). The NLR family pyrin domain containing 3 (NLRP3) inflammasome is a multiprotein complex consisting of NLRP3, the apoptosis-associated peck-like protein with CARD domain (ASC), and Caspase-1. NLRP3 is a well-studied inflammasome, and numerous types of research have revealed that the NLRP3 activation of macrophages plays an important role in mediating UC inflammatory response (Hirota et al., 2011; Moreira Lopes et al., 2020; Song et al., 2021; Zaki, Lamkanfi, & Kanneganti, 2011). Activation of the NLRP3 inflammasome is mediated by two key steps: Priming and assembling. (Hirota et al., 2011; Moreira Lopes et al., 2020; Song et al., 2021; Zaki et al., 2011). The priming step is mediated by activation of nuclear factor kappa-B (NF-κB) signaling to up-regulate the transcription of inflammasome-related proteins (NLRP3, pro-interleukin (IL)-1β and IL-18). The assembling signal is induced by various triggers, such as adenosine 5'-triphosphate (ATP), potassium (K^+) efflux, mitochondrial reactive oxygen species (mtROS), or lysosomal destabilization/rupture, induces the assembly of NLRP3 inflammasome (Hirota et al., 2011; Song et al., 2021; Zaki et al., 2011). Activation of NLRP3 inflammasome promotes the cleavage of Caspase-1 as well as the maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18 (Hirota et al., 2011; Moreira Lopes et al., 2020; Song et al., 2021; Zaki et al., 2011). Many studies have confirmed that the inhibition of NLRP3-mediated IL-1β and IL-18 production in macrophages improves dextran sulfate sodium (DSS)-induced inflammation (Bauer et al., 2010; Hirota et al., 2011; Sun et al., 2015). Toward this end, searching for drug candidates targeting NLRP3 inflammasome activation is an effective anti-inflammatory strategy for the potential treatment of UC.

Natural products provide a new source of compounds for the treatment of UC due to their abundant resources, definite efficacy, few side effects, and low price (Gu, 2018; Newman & Cragg, 2020).*Pulsatilla*decoction (Bai-Tou-Weng-Tang, BTWT) is a classic Chinese herbal formula for the treatment of intestinal bacterial diseases in humans (Gu, 2018). In recent years, many basic studies have verified the anti-colitis efficacy of BTWT (Canxing, Xinlong, Jian, Yue, & Xiaobo, 2017; Gu, 2018; Xiaomei et al., 2018). However, the active ingredient of BTWT's anti-colitis activity remains uncertain, which limits the discovery of its biological mechanisms, and hinders the further translation of BTWT into standard clinical application. Anemoside B4 (AB4) is a main natural saponin component isolated from the root of *Pulsatilla Chinensis,*which can be used as a quality control index (Li, Zou, Han, Deng, & Weinshilboum, 2020). Recently, AB4 has been shown to possess antibacterial, anti-diarrhea, anti-inflammatory, anti-endotoxin, anti-tumor, and immunomodulatory (Han et al., 2022; L. He et al., 2019; Li et al., 2020; Ma et al., 2020; Yuan et al., 2020; Zhang et al., 2021). Therefore, we hypothesized that AB4 might be a main component of BTWT anti-colitis. It has been previously shown that AB4 protects against colitis by regulating inflammatory responses such as NF-κB, IL-6, and tumor necrosis factor (TNF)-α or gut microbiota (Han et al., 2022; Ma et al., 2020; Zhang et al., 2021). However, whether AB4 alleviates UC by inhibiting the activation of NLRP3 inflammasome remains unclear. What's more, the molecular target of AB4 remains unknown. In this study, we verified AB4's protective effect on DSS-induced colitis. The mechanistic study highlighted that AB4 inhibited NLRP3 inflammasome activation by targeting macrophage CD1d to regulate AKT-STAT1-PRDX1-NF-κB signaling, thereby attenuating DSS-induced colitis.

Methods

Chemicals and reagents

AB4 (C59H96O26, MW: 1221.38, >98% purity), AA3 (C41H66O12, MW: 750.96, >98% purity), and 23-HA (C30H48O4, MW: 472.7, >98% purity) were purchased from Sichuan weikeqi-biotech (Chengdu, China). 5-ASA was purchased from Ipsen Pharma (Houdan, France). Dulbecco's modified Eagle medium (DMEM), RPMI-1640, Fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco (Grand Island, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Tokyo, Japan). Lipopolysaccharides (LPS), adenosine triphosphate (ATP), phorbol myristate acetate (PMA), and Nigericin (Nig) were purchased from Sigma-Aldrich (St. Louis, USA). Dextran sulfate sodium (DSS, molecular weight 36-50kDa) was purchased from MP Biomedicals (Solon, USA). Recombinant Murine M-CSF was purchased from PEPROTECH (Rocky Hill, USA).

Cells culture and treatments

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Human monocyte cell line THP-1 was obtained from the Cell Bank of the Chinese Academic of Sciences (Shanghai, China). THP-1 cells were cultured in 1640 supplemented with 2-mercaptoethanol (0.5mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS in an atmosphere of 5.0% CO_2 at 37 °C. THP-1 cells can be induced to differentiate into macrophages by 0.5 mM PMA for 3h. Bone marrow-derived macrophages (BMDMs) were cultured in a complete DMEM medium supplemented with 10% FBS and 30ng/ml M-CSF (Cui et al., 2020). THP-1 cells and BMDMs were treated with LPS (1μg/ml; 6 h) in the absence or presence of AB4. In order to activate NLRP3 inflammasome, BMDMs and THP-1 cells were first treated by LPS (1μg/ml; 6 h), and cells were further co-treated with ATP (5 mM; 30 min), or nigericin (10 μM; 30 min), respectively. In some experiments in this paper, cells were first treated with the NF-κB inhibitor JSH-23 (25 μM; 10 h; #B1645, APExBIO Technology LLC, USA), the AKT inhibitor MK220 (20 μM; 10 h; #SF2712, Beyotime, Shanghai, China), the AKT agonist SC79 (20 μM; 12h; #SML0749, Sigma-Aldrich, Louis, USA), cells were further co-treated with LPS (1μg/ml; 6 h). Cell lysates were extracted for qPCR or Western blot, and supernatants were collected for ELISA to detect the release of related cytokines.

Cell viability

Assess the viability of cells by using the CCK-8 assay. Briefly, THP-1 cells or BMDMs were plated overnight in 96-well plates at a cell density of $1x10^5$ cells/well. Cells were treated with various concentrations of AB4, AA3, and 23-HA (50, 100, 200μM) for 24 h, with Nacl or 0.1% DMSO as control. All samples were then incubated with 90 μl fresh medium and 10 μl CCK-8 at 37°C for 2h. Absorbance was measured with a microplate reader at 450 nm. Experiments were performed independently three times.

Animals

C57BL/6 mice (male, 20-22g, 6-8 weeks) were purchased from Dalian Medical University (Dalian, China). NLRP3-deficient ($NLRP3^{-/-}$; #0017970) mice (male, 20-22g, 6-8 weeks) and macrophage-specific CD1d deficient ($CD1d^{-/-}$; #008881) mice (male, 20-22g, 6-8 weeks) were obtained from the Third Military Medical University (Department of Immunology, Chongqing, China). All mice were housed at a temperature of 20-25 and a relative humidity of 55%-65%, and free diet and water. All animal experiments were approved by the Animal Welfare and Ethics Committee of Dalian University (no. SCAV-EXPANIM).

DSS-induced colitis and design of drug treatment

C57BL/6 mice were fed with 3.0% (*w/v*) DSS in drinking water for 7 days to induce acute colitis. In order to explore the impact of AB4 on colitis, the mice were randomly divided into 5 groups (n =6 in each group): Normal group, DSS group, 5-ASA (200 mg/kg) group, AB4 pretreatment group (5 mg/kg), and AB4 treatment group (5 mg/kg). To further confirm the protective effect of AB4, the mice were randomly divided into 6 groups (n =8 in each group): Normal group, DSS group, AB4 (5, 10, and 15mg/kg) groups, and AB4 (15mg/kg) alone group.

To further confirmed that the protective effect of AB4 in DSS-induced colitis is dependent on the intervention of NLRP3 inflammasome, WT and $NLRP3^{-/-}$ mice were randomly divided into 6 groups (n=8 in each group): WT normal group, WT+DSS group, WT+DSS+AB4 (15mg/kg) group, $NLRP3^{-/-}$ normal group, $NLRP3^{-/-}$+DSS group, $NLRP3^{-/-}$+DSS+AB4 (15mg/kg) group.

To investigate whether the protective effect of AB4 against colitis depends on the CD1d signaling pathway, the WT and $CD1d^{-/-}$ mice were randomly divided into 6 groups (n=8 in each group): WT normal group; WT+DSS group; WT+DSS+AB4 (15mg/kg) group; $CD1d^{-/-}$ normal group; $CD1d^{-/-}$+DSS group; $CD1d^{-/-}$+DSS+AB4 (15mg/kg) group. Observe and record the changes in body weight, blood in the stool, and diarrhea every day, and use a complete system to calculate the DAI score(Cui et al., 2020; Lv et al., 2021).

Spleen index

On the 7th day of modeling, the mice were sacrificed and the spleens of each group were taken. Spleen index= (Spleen weight (mg)/ Body weight (g)) x10.

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FITC-dextran intestine-blood barrier tests

To analyze the permeability of the epithelial barrier in mice, on the 7th day, mice in each group were deprived of water and fasted for 4 h, and then each mouse was gavaged with FITC-dextran (0.6 mg/g; Sigma-Aldrich). The blood was collected after 4 h, and the content of FITC-dextran in serum was measured with a fluorescence spectrophotometer setup with an emission wavelength of 490 nm and an excitation wavelength of 520 nm.

Histological analysis and histopathological scores

On the 7th day of modeling, the mice were sacrificed and the colons were taken. The colons were carefully rinsed with PBS solution, and the same parts of each colon were soaked and fixed with 4% paraformaldehyde. Conventional paraffin-embedded sections were stained with hematoxylin and eosin (H&E staining). The histopathological scores were determined using a well-established system (Cui et al., 2020; Lv et al., 2021).

Immunofluorescence of colon issues.

Paraformaldehyde-fixed colon tissues were embedded in paraffin for analysis of $F4/80^{+}$ cell infiltration. Sections were washed three times with PBS and then exposed to 3.0% H_2O_2 for 1h to block endogenous peroxidase activity. Subsequently, the sections were blocked with 3% BSA for 30min at room temperature and then incubated at 4degC overnight with anti-F4/80 (#GB11027, 1:500 per mouse, Servicebio). The next day, sections were placed in PBS (PH7.4) and washed 3 times on a decolorizing shaker for 5min each time. The corresponding secondary antibody was added and incubated for 50min at room temperature and away from light. Fluorescence microscopy to analyze the results (Zeiss Axioplan 2).

Enzyme-linked immunosorbent assay (ELISA)

Assays were performed according to the manufacturer's protocol (CUSABIO BIOTECH, Wuhan, China), using mouse IL-1β, IL-18, IL-6, TNF-α, iNOS ELISA kits to detect supernatants of BMDMs culture, mouse serum, or colon tissue homogenate, and using human IL-1β, IL-18 ELISA kits to detect the supernatant of THP-1 cells culture.

Quantitative Real-time Polymerase Chain Reaction (qPCR)

The expression of mRNA encoding for indicated genes in THP-1cells or BMDMs was quantified by qPCR with the SYBR® Premix Ex Taq (#RR820A, Takara). Table 1 showed the main primers sequence used in this experiment. The relative expression of target gene was calculated by the $2^{-C\ t}$ method.

Table 1 Primer sequences

Primer	Sequence (5'-3')	
GAPDH	Forward	CCCACTCCTCCACCTTTGAC
	Reverse	TGTTGCTGTAGCCAAATTCGTT
IL-1β	Forward	CAGGCAGGCAGTATCACTCATTG
	Reverse	CGTCACACACCAGCAGGTTATC
NLRP3	Forward	CCTGACCCAAACCCACCAGT
	Reverse	TTCTTTCGGATGAGGCTGCTTA
Caspase-1	Forward	AAGAACAGAACAAAAGAAGATGGA
	Reverse	ACCCTCGGAGAAAGATGTTGAAA
ASC	Forward	GGATCCCACCCCACCCTAA
	Reverse	CTCGAGTCAGCAGGCAGGAATAG
IL-18	Forward	TGAAGTAAGAGGACTGGCTGTGA
	Reverse	ATCTTGTTGTGTCCTGGAACACG
IL-6	Forward	GACTGATGCTGGTGACAACC
	Reverse	AGACAGGTCTGTTGGGAGTG
TNF-α	Forward	CTCATGCACCACCATCAAGG
	Reverse	ACCTGACCACTCTCCCTTTG

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Primer	Sequence (5'-3')	
IL-10	Forward	AGCCTTATCGGAAATGATCCAGT
	Reverse	GGCCTTGTAGACACCTTGGT

Western blots

Protein expression was detected by Western blots in THP-1 cells, BMDMs, colonic macrophages, colonic epithelial cells, or colon tissues. Briefly, cells or tissues were lysed using RIPA lysis buffer (#P0013B, Beyotime Biotechnology). The cell lysates were centrifuged at 12000 rpm/min for 15 min, and the supernatant was mixed with 5xSDS sample buffer (#P0015L, Beyotime Biotechnology). After boiling, each group of samples was separated by electrophoresis and transferred to PVDF membrane (#FFP39, Beyotime Biotechnology). The membranes were probed with the appropriate antibodies and then detected using Western Blotting Substrate (#180-501, Tanon). The antibodies used are as follows: anti-actin-β (#AF7018, Affinity), anti-GAPDH (#AF7021, Affinity), anti-AKT (#AF6216, Affinity), anti–p-AKT (#AF0016, Affinity), anti-STAT1 (#AF6300, Affinity), anti-p-STAT1 (#AF3300, Affinity), anti-PRDX1 (#DF6652, Affinity), anti-CD1d (#ab215445, Abcam), anti-IL-18 (#ab71495, Abcam), anti-IL-1β (#ab234437, Abcam), anti-ZO1 (#ab216880, Abcam), anti-Claudin1 (#ab180158, Abcam), anti-Occludin (#ab222691, Abcam),anti-PCNA (#13100, CST), anti-p-IκBα (#2859, CST), anti-IκBα (#4812, CST), anti-p-p65 (#3033, CST), anti-p65 (#WL01273b, Wanleibio), anti-ASC (#WL02462, Wanleibio),anti-IL-22 (#WL04441, Wanleibio), anti-IL-10(#sc-365858, Santa Cruz Biotechnology), anti-Caspase-1 (#AG-20B-0042, AdipoGen), and anti-NLRP3 (#AG-20B-0014-C100, AdipoGen). The primary antibody dilution was 1:1000 ˜ 1:2000.

Isolation of colonic macrophages from mice

Colonic macrophages were prepared as previously described (Zhao et al., 2019). First, the whole colons of mice in each group were collected and washed several times with HBSS. Then, the colonic tissue was cut into small pieces of 0.5cm and added to predigestion solution (containing 1mM DTT, 5mM EDTA, and 5% FBS) to remove epithelial cells and mucus. Then centrifuge at 1200rpm for 5min. The supernatant was aspirated and the remaining colon fragments were cultured with 8 times the volume of digestive fluid containing 1mg/ml collagenase VIII, 0.2mg/ml DNaseI, 1mg/ml DispaseII, and 10% FBS. It was digested at 37°C for 90 minutes. The samples were filtered, and resuspended in 40% and 80% fractions of the Percoll solution. After centrifugation at 2000rpm for 20 min, live cells in the middle layer were collected and the colonic macrophages were classified using Anti-F4/80 MicroBeads UltraPure, mouse (#130-110-443, Miltenyi Biotec).

Isolation of intestine epithelial cells from mice

Intestine epithelial cells were prepared as previously described[23]. Briefly, precooled PBS (containing 5% penicillin, and streptomycin) was used to wash the colons of each group of mice, and ophthalmic shears were used to remove the Pyle's node, fat, and mesentery on the surface of the small intestine, and the tissue was cut to the size of 1cm. Incubate with 1 mM DTT and 3 mM EDTA at room temperature for 1h without shaking. Cells were washed with PBS by centrifuging and then were solubilized in cell lysis buffer containing 1% Triton X-100, 1mM EDTA, 1 mM EGTA, 10 mM Tris (pH 7.4), 150 mM NaCl, and protease, and phosphatase inhibitor cocktail (Solarbio).

Statistical analysis

Data shown in this study were obtained in at least three independent experiments. All results represent mean ± SD Statistical analysis was performed with GraphPad Prism 8.0 (San Diego, CA), and the differences among multiple groups were evaluated by one-way ANOVA test, and the survival data of in vivo experiments were analyzed by the log-rank test of the curve. $P < 0.05$ was considered statistically significant.

Results

AB4 ameliorates DSS-induced colitis symptoms

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In order to seek the *Pulsatilla saponins* with anti-inflammatory activities in vitro, we screened a series of *saponins* AB4, Anemoside A3 (AA3), and 23-hydroxy botulinic acid (23-HA) (Fig. 1A) and tested their inhibitory activity against IL-1β in LPS-challenged differentiated THP-1 cells. Interestingly, AB4 showed the highest inhibitory activity against IL-1β (Fig. 1B and C). In parallel, the cell viability assay showed that AB4 was not cytotoxic at concentrations below 200μM (Supporting Information Fig. S1A-C). Therefore, we hypothesized that AB4 might be the main component of BTWT against colitis.

DSS-induced colitis is known to be a widely accepted model with clinical symptoms similar to human UC, including diarrhea, rectal bleeding and weight loss (Chassaing, Aitken, Malleshappa, & Vijay-Kumar, 2014; Clapper, Cooper, & Chang, 2007). To evaluate the effect of AB4 on colitis in mice, C57BL/6 (wild-type; WT) mice were challenged with 3.0% DSS for 7 days and then administered with AB4 (5 mg/kg) daily for 7 days or 14 days, as well as the 5-ASA (200 mg/kg) being the positive control (Supporting Information Fig. S2A). AB4 markedly decreased the disease activity indices characterized by diarrhea, bleeding, and weight loss compared to the DSS group (Fig. S2B and S2C). Decreased disease severity was also accompanied by a reduction of colon shortening, which was ameliorated by both AB4 treatment and pretreatment. There was no significant difference between AB4 (5mg/kg) alone group and the normal group (Fig. S2D). Notably, we found that the AB4 pretreatment group was more effective than the AB4 treatment group and the 5-ASA group (Fig. S2B-D). Therefore, we continued to investigate the effect of AB4 pretreatment on DSS-induced colitis in mice. We found AB4 (5, 10, and 15 mg/kg) markedly decreased the disease activity indices characterized by body weight loss, diarrhea, and bleeding in a dose-dependent manner compared with the DSS group (Fig. 1D and E). Colonic shortening (Fig. 1F), and splenomegaly (Fig. 1G) caused by the DSS challenge were also improved at the given doses. There was no significant difference between AB4 (15mg/kg) alone group and the normal group. At the same time, the survival experiment showed that AB4 improved the survival rate of mice compared with the DSS group (Fig. 1H). These data suggested that AB4 successfully ameliorated DSS-induced colitis in mice.

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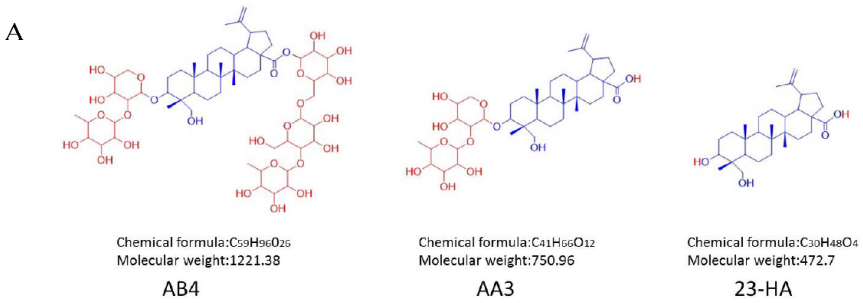

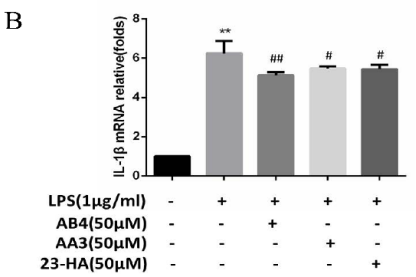

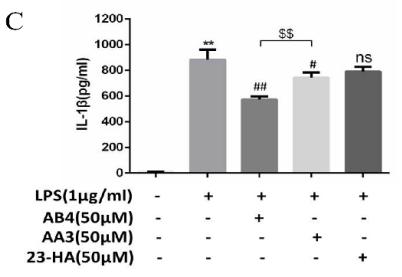

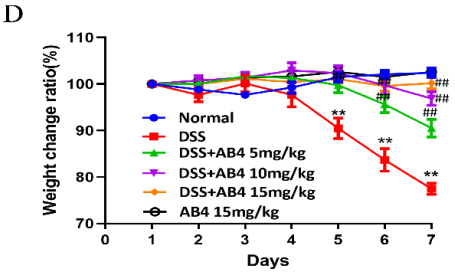

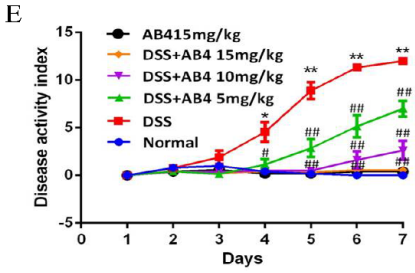

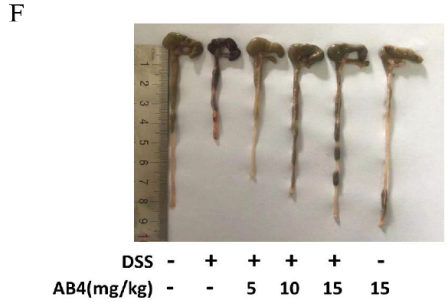

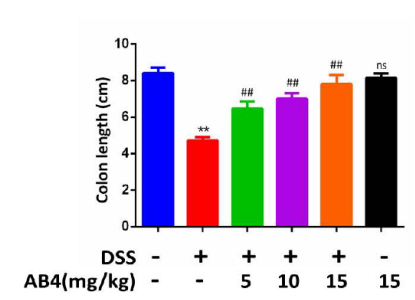

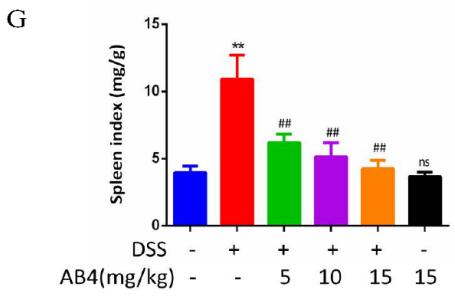

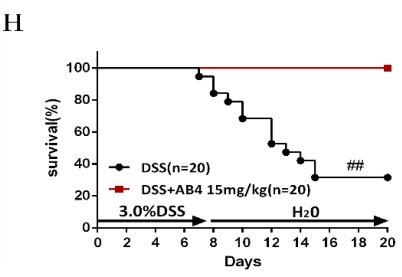

Figure 1. AB4 ameliorates DSS-induced colitis symptoms.(**A**) Chemical structure of AB4, AA3, and 23-HA. (**B-C**) THP-1 cells (pretreated with 500 nM PMA for 3 h) were pretreated with AB4, AA3, and 23-HA (50μM) for 4h, and then cultured with LPS (1μg/ml) for 6h. (**B**) The mRNA expression of IL-1β was analyzed by qPCR. (**C**) Released IL-1β in the supernatant was analyzed by ELISA. Data are presented as mean ± SD. $^{**}P < 0.01$ *vs.* Control group;$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and ns, not significantly different *vs.* LPS group. $^{\$\$}P < 0.01$ LPS+AB4*vs.* LPS+AA3. C57BL/6 mice were intraperitoneally injected with AB4 (5, 10, and 15mg/kg) for 7 days, and then given 3.0% DSS in drinking water for 7 days, during which AB4 (5, 10, and 15mg/kg) was continuously injected intraperitoneally (n = 8 per group). At the end of the experiment, the mice were sacrificed, and the colons and the spleens were collected. (**D**) The body weight change, (**E**) the disease activity index (DAI), (**F**) the colon length, (**G**) the spleen index, and (**H**) the survival rate were measured. Data are presented as mean ± SD. $^{**}P < 0.01$and ns, not significantly different *vs.* Normal group;$^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* DSS group.

AB4 attenuates DSS-induced colon injury

The increased permeability in the intestinal epithelium is an important indicator that the mechanical barrier function of the intestinal mucosa is impaired (Sommer et al., 2021). Next, we further evaluated the protective effect of AB4 on DSS-induced colitis. FITC-dextran assay of intestinal permeability in mice showed that the diffusion of FITC-dextran across the epithelium was significantly lower in AB4 administration mice (Fig. 2A). This supported the conclusion that AB4 reduced DSS-induced intestinal mucosal injury in mice. Intestinal barrier function is maintained by tight junction proteins, such as Occludin, Claudin-1, and ZO-1 (Sommer et al., 2021). Compared with the DSS group, AB4 (5, 10, and 15mg/kg) significantly enhanced the expression of Occludin, Claudin-1, and ZO-1 proteins (Fig. 2B), which was consistent with the FITC-dextran results. Hematoxylin and eosin (H&E) staining indicated that AB4 (5, 10, and 15 mg/kg) markedly alleviated mucosal damage, infiltration of inflammatory cells, and loss of crypts (Fig. 2C). AB4 decreased histological colon damage score compared to the DSS group (Fig. 2C). Consistently, AB4 (5, 10, and 15 mg/kg) significantly abolished the distribution of $F4/80^{+}$ macrophages in colonic lamina propria (Fig. 2D). Thus, AB4 attenuated the severity of DSS-induced colonic injury in mice.

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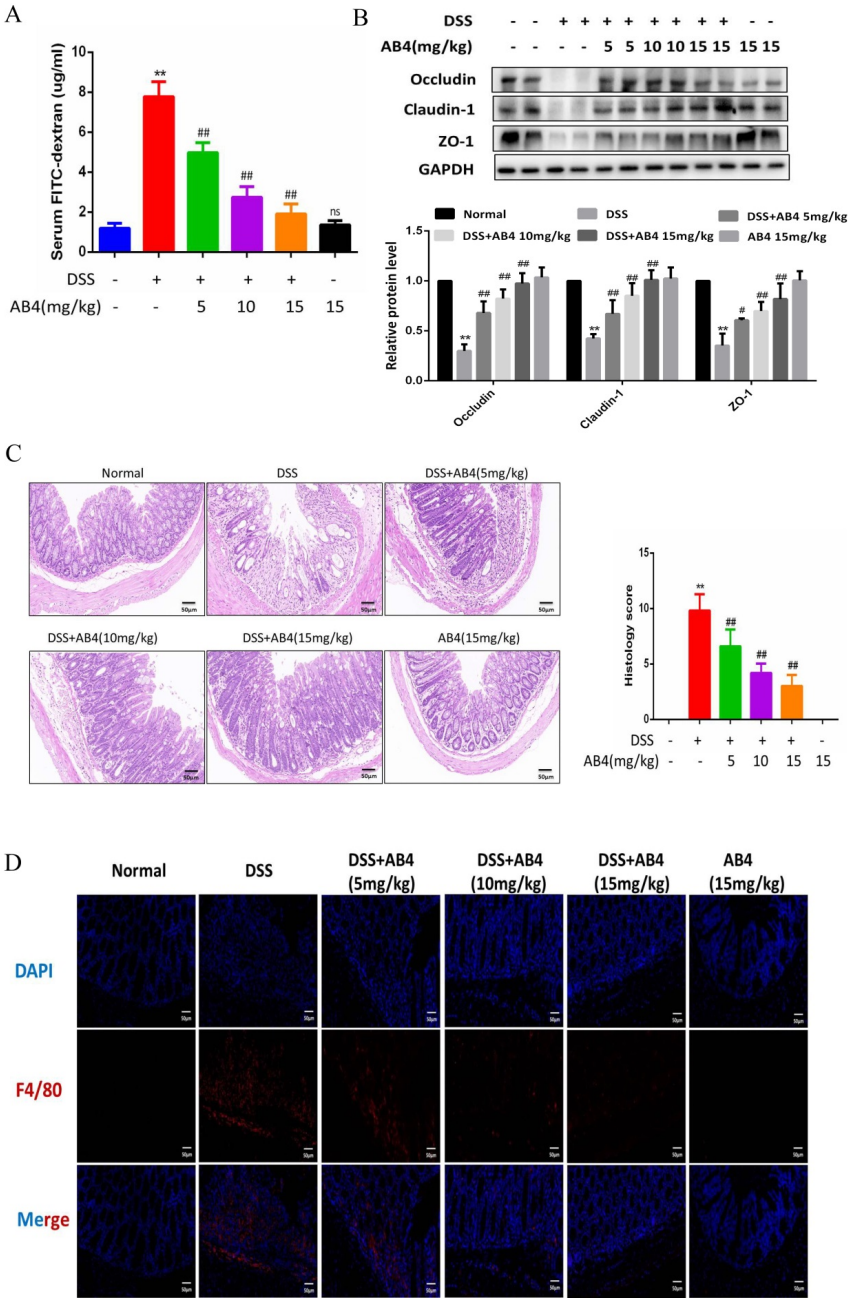

Figure 2. AB4 attenuates DSS-induced colon injury. Mice were pretreated with AB4 (5, 10, and 15mg/kg) for 7 days, followed by DSS-induced colitis, the serum, and the colons were collected on day 7. (**A**) Measurement of serum permeability tracer FITC-dextran. (**B**) The protein expression of Occludin, Claudin-1 and ZO-1 was detected by Western Blot. (**C**) H&E staining analysis of histopathological changes (left) and semi-quantitative scoring of histopathology (right) and the images was taken at 200x magnification (scale bar: 50μm). (**D**) The infiltration of F4/80^{+} macrophages in colonic tissues were detected by immunofluorescence, and the images were taken at 200x magnification (scale bar: 50μm). Data are presented as mean ± SD. $^{**}P < 0.01$ *vs.* Normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* DSS group.

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AB4 specifically inhibits NLRP3 inflammasome activation in colonic macrophages

As previously reported, increased production of inflammatory cytokines in serum and colon is an important hallmark of DSS-induced colitis (de Lange & Barrett, 2015; Moreira Lopes et al., 2020). We examined the regulation of AB4 on the secretion of inflammatory cytokines in DSS-induced colitis. Indeed, AB4 (5, 10, and 15mg/kg) inhibited the secretion of pro-inflammatory cytokines secretion in the serum after the DSS challenge, such as IL-1β, IL-18, IL-6, inducible NOS (iNOS) and TNF-α (Fig. 3A). As an important component of innate immunity, NLRP3 inflammasome plays an important role in the development of UC, and is the main and key source of inflammatory cytokines IL-1β and IL-18. Targeting NLRP3 inflammasome has been shown to have a definite therapeutic effect (Hirota et al., 2011; Moreira Lopes et al., 2020; Song et al., 2021; Zaki et al., 2011). To investigate the regulatory role of AB4 on the NLRP3 inflammasome in DSS-induced colitis, we evaluated both mRNA and protein levels of related cytokines in collected colons. AB4 (5, 10, and 15mg/kg) exhibited significant inhibition on protein expression of NLRP3, ASC, Caspase-1 p20, IL-1β p17, and IL-18 in the colons of colitis mice (Fig. 3B). In parallel, AB4 significantly decreased the mRNA levels of NLRP3, Caspase-1, IL-1β, IL-18, IL-6 and TNF-α (Fig. 3C). IL-10 is a typical anti-inflammatory cytokine, and both IL-22 and IL-10 seem to maintain the integrity of the colonic epithelium (Huber et al., 2012; Q. Wu et al., 2021). We observed that AB4 enhanced the expression of IL-10 and IL-22 proteins in colonic homogenates of DSS-induced colitis, and enhanced the mRNA level of IL-10 (Fig. 3B and C). In agreement, we found that the colonic tissues from AB4 administration mice expressed high levels of proliferative cell nuclear antigen (PCNA) (Fig. 3B). Therefore, we hypothesized that AB4 might inhibit the expression of NLRP3 inflammasome and the release of inflammatory cytokines, thereby ameliorating impaired intestinal barrier function and alleviating DSS-induced colitis. To determine whether AB4-inactivated NLRP3 inflammasome was derived from macrophages or intestinal epithelial cells, we isolated these two types of cell lines from different groups of mice. Interestingly, Western Blot and ELISA results showed that AB4 significantly inhibited the protein expression of NLRP3, Caspase-1 p20, IL-1β and IL-18 in colonic macrophages (Fig. 3D), but did not affect the expression in intestinal epithelial cells (Fig. 3E).

To further confirmed that the relief of AB4 from DSS-induced colitis depended on the intervention of NLRP3 inflammasome, we verified it in DSS-induced NLRP3-knockout ($NLRP3^{-/-}$) mice and WT mice. DSS-induced $NLRP3^{-/-}$mice exhibited considerably less weight loss, lower DAI score (Fig. 4A and B), and longer colons presentation (Fig. 4C) as compared to WT mice, supporting a critical role of NLRP3 in the development of colitis. However, it was worth noting that the protective effect of AB4 on the DSS challenge was lost in $NLRP3^{-/-}$ mice (Fig. 4A-C). H&E staining showed that the epithelial damage of WT mice colon tissue was more severe and crypt loss than $NLRP3^{-/-}$mice and AB4 improved the damage and crypt loss of colon tissue in WT mice but had no significant effect on $NLRP3^{-/-}$ mice (Fig. 4D). These data suggested that inhibition of NLRP3 inflammasome activation might be one of the main mechanisms by which AB4 attenuated DSS-induced inflammatory injury in colitis.

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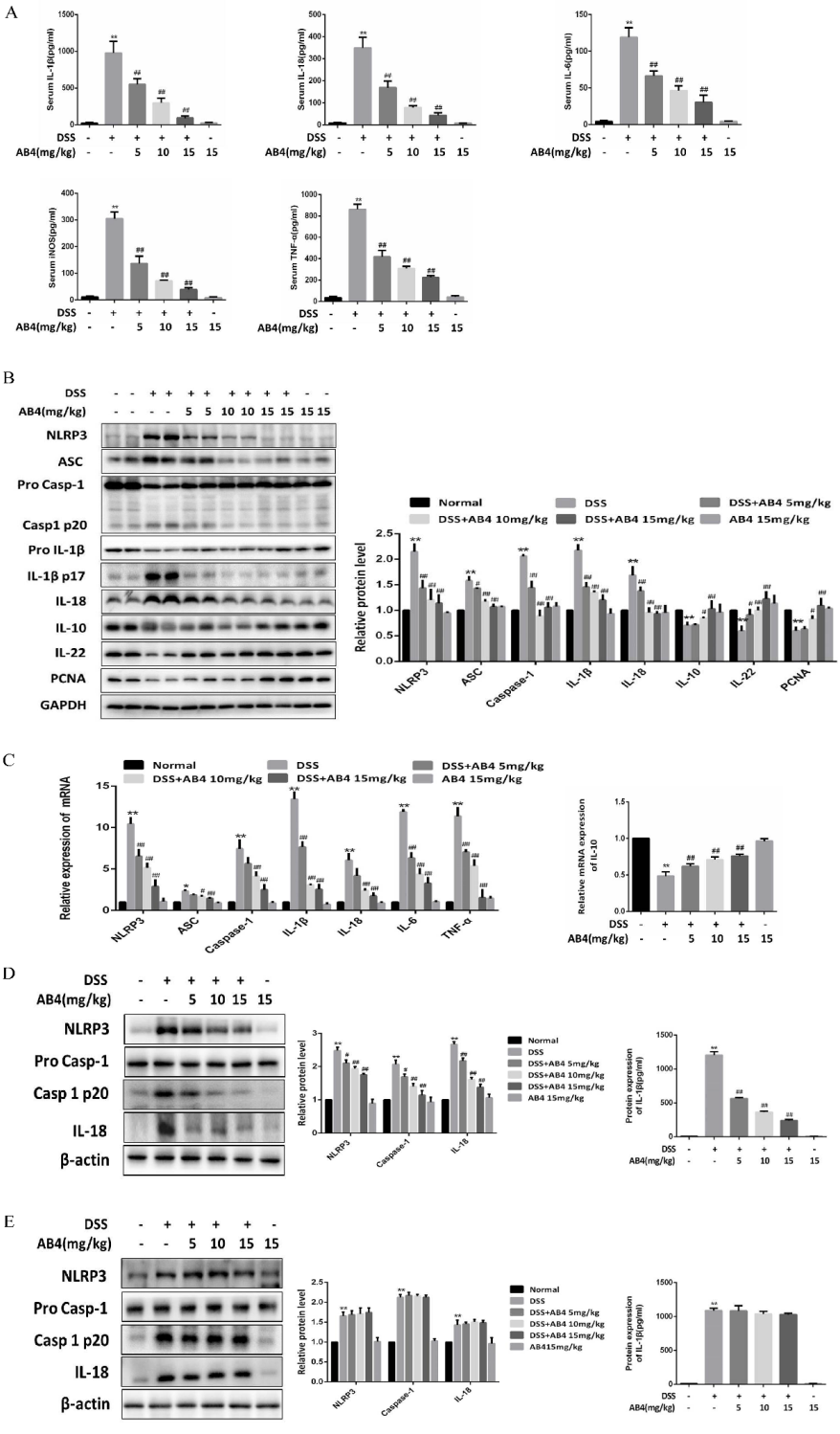

Figure 3. AB4 specifically inhibits NLRP3 inflammasome activation in colonic macrophages. (**A**) Production of IL-1β, IL-18, IL-6, iNOS, and TNF-α in the serum of DSS-induced colitis mice was determined by ELISA. (**B**) The indicated proteins of colonic homogenate-related factors were determined

by Western Blot. (**C**) The mRNA levels of colonic homogenate-related factors were determined by qPCR. Western Blot was used to detect NLRP3, Caspase-1, and IL-18 protein expression in colon macrophages (**D**) and epithelial cells (**E**), and ELISA was used to detect IL-1β protein expression. Data are presented as mean ± SD. $^{**}P < 0.01$*vs.* Normal group; $^{\#}P < 0.05, ^{\#\#}P < 0.01$ *vs.* DSS group.

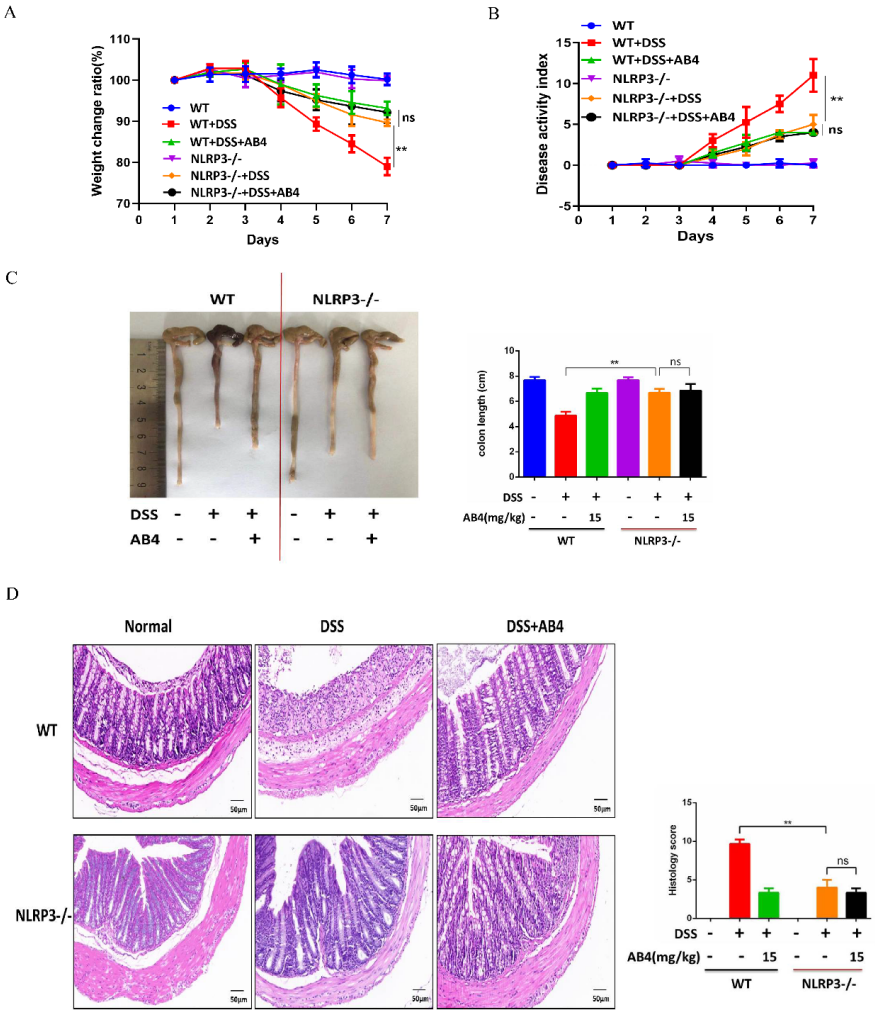

Figure 4. NLRP3 is critical for protection against DSS-induced injury by AB4. NLRP3$^{-/-}$ mice and WT mice were intraperitoneally injected with AB4 (15mg/kg) for 7 days, and then given 3.0% DSS in drinking water for 7 days, during which AB4 (15mg/kg) was continuously injected intraperitoneally (n=8 per group). At the end of the experiment, the mice were sacrificed, and the colons were collected. (**A**) The body weight change, (**B**) the disease activity index (DAI), and (**C**) the colon length was measured. (**D**) H&E staining analysis of histopathological changes (left) and semi-quantitative scoring of histopathology (right) and the images was taken at 200x magnification (scale bar: 50μm). Data are presented as mean ± SD. $^{**}P < 0.01$ NLRP3$^{-/-}$+DSS*vs.* WT+DSS; ns, not significantly different NLRP3$^{-/-}$+DSS+AB4 *vs.*NLRP3$^{-/-}$+DSS.

AB4 inhibits the activation of NLRP3 inflammasome in vitro

Next, we examined whether AB4 could regulate the activation of NLRP3 inflammasome in macrophages in vitro. BMDMs were pretreated with AB4 (50, 100, and 200μM) for 4h, and then treated with LPS (1μg/ml) for 6h. Meanwhile, CCK-8 results showed that 50-200μM AB4 had no toxic effects on BMDMs

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(Supporting Information Fig. S3). The mRNA expression of related components in NLRP3 inflammasome was analyzed by qPCR. As shown in Fig. 5A, AB4 (50, 100, and 200μM) significantly inhibited the mRNA expressions of NLRP3, IL-1β, and IL-18 in LPS-primed BMDMs. By contrast, the expression of ASC and Caspase-1 were unaffected by AB4. Moreover, AB4 (50, 100, and 200μM) could significantly inhibit the protein expression of NLRP3, proIL-1β, and IL-18 (Fig. 5B). These data suggested that AB4 might partially regulate NLRP3 inflammasome activation by inhibiting the transcription of NLRP3, pro-IL-1β and IL-18 genes in macrophages. The constituent proteins of the NLRP3 inflammasome are widely considered to be the rate-limiting point regulating inflammasome activation (Song et al., 2021). Western Blot confirmed that AB4 (50, 100, and 200μM) significantly inhibited the protein expressions of NLRP3, Caspase-1 P20, IL-1β p17 and IL-18 in LPS-primed BMDMs (Fig. 5C) and differentiated THP-1 cells (Fig. 5D) in response to ATP (5mM;30min) or nigericin (Nig;10μM;30min), suggesting inactivation of NLRP3 inflammasome by AB4. Consistently, ELISA results also confirmed that IL-1β and IL-18 in the supernatants were suppressed by AB4 in LPS-primed BMDMs and differentiated THP-1 cells in response to nigericin (Fig. 5E). These data suggested that AB4 might also affect the NLRP3 inflammasome assembly stage, namely reducing NLRP3-mediated Caspase-1 activation and inhibiting macrophage IL-1β and IL-18 secretion. Taken together, AB4 inhibited the activation of NLRP3 inflammasome in vitro.

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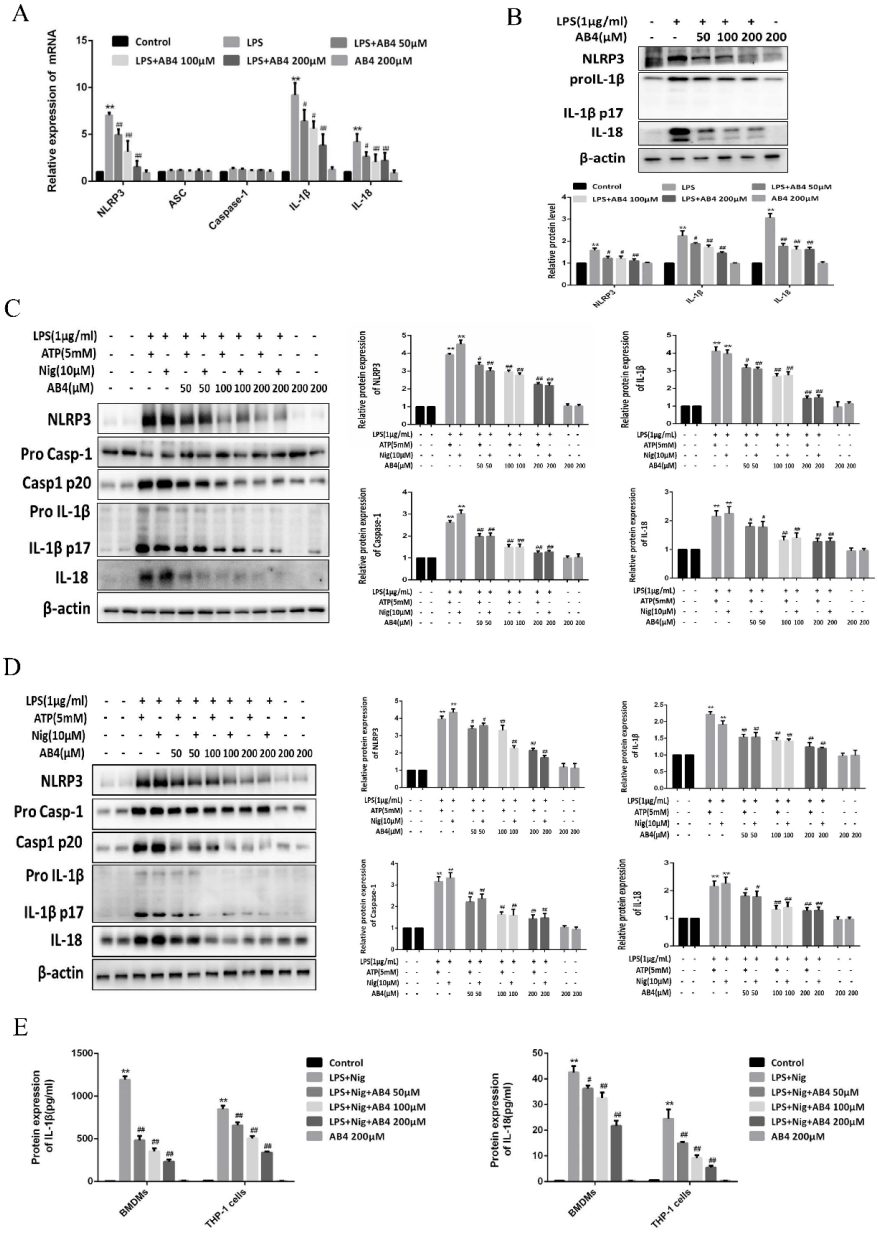

Figure 5. AB4 inhibits the activation of NLRP3 inflammasome in vitro. (**A-B**) BMDMs were pretreated with AB4 (50, 100, and 200μM) for 4h, and then cultured with LPS (1μg/ml) for 6h. The mRNA expression of related components in NLRP3 inflammasome was analyzed by qPCR (**A**). The protein expression of related components in NLRP3 inflammasome was analyzed by Western Blot (**B**). Data are presented as mean ± SD. ***P < 0.01 vs.* Control group;*#P < 0.05, ##P < 0.01 vs.* LPS group. (**C-E**) BMDMs and differentiated THP-1 cells were pretreated with AB4 (50, 100, and 200μM) for 4h, and then cultured with LPS(1μg/ml) for 6h, followed by incubation with ATP (5 mM) for 30min, nigericin (10 μM) for 30min. The protein expressions of NLRP3, Caspase-1, IL-1β, and IL-18 were determined by Western Blot in BMDMs (**C**) and THP-1 cells (**D**); the production of IL-1β and IL-18 was analyzed by ELISA (**E**). Data are presented as mean ± SD. ***P <0.01 vs.* Control group; *#P< 0.05* , *##P <0.01 vs.* LPS+ATP or LPS+Nig group.

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AB4 inactivates AKT-STAT1-PRDX1-NF-κB signaling in macrophages

Subsequently, we explored how AB4 regulated NLRP3 inflammasome activation. NF-κB is a key activator of inflammation, which primes the activation of NLRP3 inflammasome by promoting the transcription of NLRP3, IL-1β, IL-18 (Schreiber, Nikolaus, & Hampe, 1998). First, we assessed the effects of AB4 on NF-κB signaling in vitro. the results showed AB4 (50, 100, and 200μM) significantly decreased the protein expression of NF-κB p65 phosphorylation and nuclear factor κB (IκBα) phosphorylation in LPS-challenged BMDMs (Fig. 6A) and differentiated THP-1 cells (Fig. 6B), indicating an inhibitory action of AB4 on NF-κB signaling. Meanwhile, inhibition of NF-κB P65 and IκBα phosphorylation with NF-κB inhibitor JSH-23 (25μM;10h) also attenuated LPS-challenged up-regulation of NLRP3, proIL-1β, and IL-18, both at protein (Fig. 6C) and mRNA (Fig. 6D) levels in BMDMs, which is synergistic with AB4. These data indicated that the classical NF-κB signaling pathway mediated AB4-dependent inhibition of NLRP3, IL-1β, and IL-18. TLR4 can recognize the downstream transcription factor signals initiated by LPS and cause the transcriptional expression of inflammatory genes (Sheng et al., 2021). However, our result showed that AB4 was not associated with TLR4-involved activation of NLRP3 inflammasome signaling (Supporting Information Fig. S4).

Next, we explored the direct signaling events of NF-κB inactivation by AB4. Peroxiredoxin 1 (PRDX1), a protein capable of promoting NF-κB activation by inducing IκBα phosphorylation, is considered a competitive molecule for the transcriptional control of inflammatory genes (Cui et al., 2020; Ishii et al., 1993).Western Blot confirmed that AB4 (50, 100, and 200μM) significantly reduced LPS-challenged PRDX1 protein expression in BMDMs (Fig. 6A) and differentiated THP-1 cells (Fig. 6B). Studies had shown that LPS-dependent PRDX1 expression was mediated by Protein kinase B (AKT)/signal transducer and activator of transcription 1(STAT1) signaling (Cui et al., 2020). In agreement, AB4 (50, 100, and 200μM) significantly reduced phosphorylation of AKT and STAT1 in LPS-challenged BMDMs (Fig. 6A) and differentiated THP-1 cells (Fig. 6B). Western Blot confirmed that AKT inhibitor MK2206 (20μM; 10h) effectively inhibited LPS-challenged AKT and STAT1 phosphorylation, resulting in the reduction of PRDX1 expression, phosphorylation of IκBα and P65, and the down-regulation of NLRP3, proIL-1β, and IL-18 (Fig. 6E). By contrast, AKT agonist SC79 (20μM; 12h) could reverse the inhibitory effect of AB4 on LPS-challenged p-AKT, p-STAT1, PRDX1, p-P65, p-IκBα, NLRP3, proIL-1β and IL-18 proteins in BMDMs (Fig. 6F). In keeping with this, we also demonstrated that AB4 (5, 10, and 15mg/kg) could reduce the expression of p-AKT/AKT, p-STAT1/STAT1, PRDX1, p-P65/P65, p-IκBα/IκBα proteins in colonic homogenates of 3.0% DSS-induced WT mice (Fig. 6G). These data suggested that AB4 might inhibit NLRP3 inflammasome activation by inactivating NF-κB by inhibiting AKT/STAT1-mediated PRDX1 expression.

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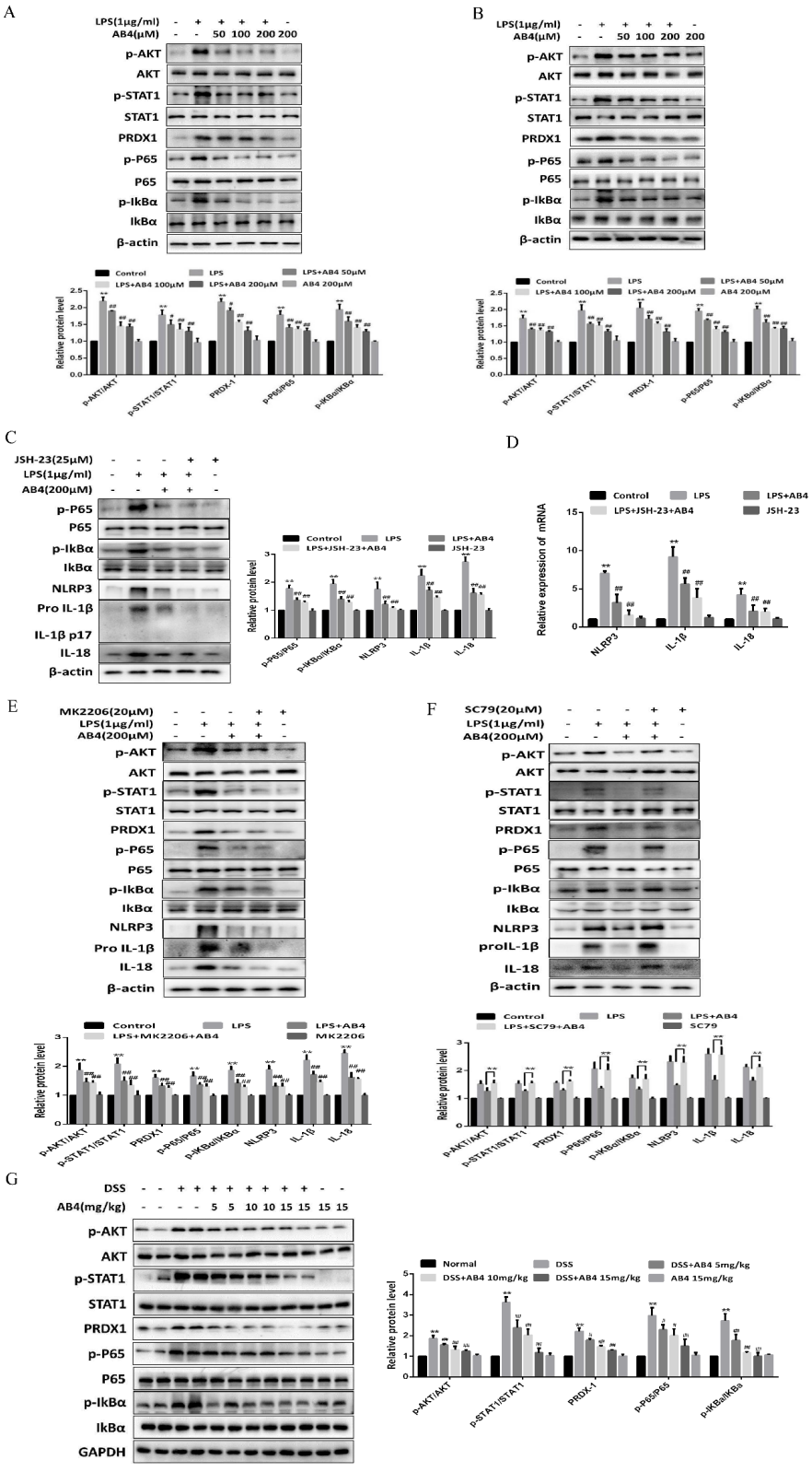

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Φιγυρε 6. AB4 ιναστιατες AKT-ΣTAT1-ΠΡΔΞ1-NΦ-κB σιγναλινγ ιν μασροπηαγες. BMDMs (**A**) and differentiated THP-1 cells (**B**) were pretreated with AB4 (50, 100, and 200μM) for 4h, and then cultured with LPS (1μg/ml) for 6h. The protein of the indicated molecule was detected by Western Blot. BMDMs were pretreated with AB4 (200μM) or NF-κB inhibitor JSH-23 (25μM) for 4h and then cultured with LPS (1μg/ml) for 6h. The protein of the indicated molecule was detected by Western Blot (**C**), and the mRNA expression of NLRP3, IL-1β, and IL-18 was analyzed by qPCR (**D**). (**E**) BMDMs were pretreated with AB4 (200μM) or AKT inhibitor MK2206 (20μM) for 4h and then cultured with LPS (1μg/ml) for 6h. The protein of the indicated molecule was detected by Western Blot. Data are presented as mean ± SD. $^{**}P < 0.01$ *vs.* Control group;$^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* LPS group. (**F**) BMDMs were pretreated with AB4 (200μM) for 4h or AKT agonist SC79 (20μM) for 6h and then cultured with LPS (1μg/ml) for 6h. The protein of the indicated molecule was detected by Western Blot. Data are presented as mean ± SD. $^{**}P < 0.01$ LPS+SC79+AB4 *vs.* LPS+AB4 group. (**G**) WT mice were subjected to DSS-induced colitis. The protein expression of the indicated molecule in colons was detected by Western Blot. Data are presented as mean ± SD. $^{**}P < 0.01$ *vs.*Normal group; $^{\#}P < 0.05$,$^{\#\#}P < 0.01$ *vs.* DSS group.

AB4 inhibited the activation of NLRP3 inflammasome through targeting CD1d

CD1d molecule has the role of antigen presentation, and several studies have confirmed that CD1d-related immune pathways have important effects on UC (Fuss et al., 2004; Huang et al., 2016; Lee et al., 2019; Mizoguchi, Mizoguchi, Takedatsu, Blumberg, & Bhan, 2002; Olszak et al., 2014). One of our recent works showed that macrophage CD1d could inhibit NLRP3 inflammasome expression during inflammation (Cui et al., 2020). To further investigate the mechanism by which AB4 alleviated the progression of colitis, we next investigated whether AB4 inhibited NLRP3 inflammasome expression in macrophages through CD1d signaling. In 3.0% DSS-induced WT mice colitis, compared with the control group, the expression level of CD1d protein in the colon tissue of the DSS group was significantly decreased. Compared with the DSS group, AB4 (5, 10, and 15mg/kg) significantly enhanced CD1d protein (Supporting Information Fig. S5) expression in colonic homogenates of mice. Strikingly, AB4 enhanced CD1d protein expression in colonic macrophages (Fig. 7A). To investigate whether the protective effect of AB4 on colitis depended on the CD1d signaling pathway, we constructed 3% DSS-induced colitis in macrophage-specific CD1d-knockout ($CD1d^{-/-}$) mice and WT mice. Inflammation index values (i.e., body weight loss, DAI score, and colon length) demonstrated that $CD1d^{-/-}$ mice were more susceptible to DSS-induced colitis compared with WT mice (Fig. 7B-D), supporting a critical role of macrophage CD1d in the disease development. However, macrophage-specific CD1d depletion reversed the protective effects of AB4 (15mg/kg) on body weight loss, DAI score, and colon shortening in DSS-induced colitis (Fig. 7B-D). H&E staining and $F4/80^{+}$ immunofluorescent revealed macrophage-specific CD1d depletion abolished AB4's protective effects both in the colonic morphometry (Fig. 7E) and macrophagic observation (Fig. 7F). Western Blot and ELISA results confirmed that macrophage-specific CD1d depletion reversed the inhibition of the protein expression of p-AKT/AKT, p-STAT1/STAT1, PRDX1, p-P65/P65, p-IκBα/IκBα, NLRP3, ASC, Caspase-1 p20, IL-1β, and IL-18 by AB4 (Fig. 7G and H). Collectively, these data suggested that AB4 might target CD1d thus reducing the AKT-STAT1-PRDX1-NF-κB signaling pathway, eventually inhibiting the activation of NLRP3 inflammasome and ameliorating DSS-induced colitis in mice.

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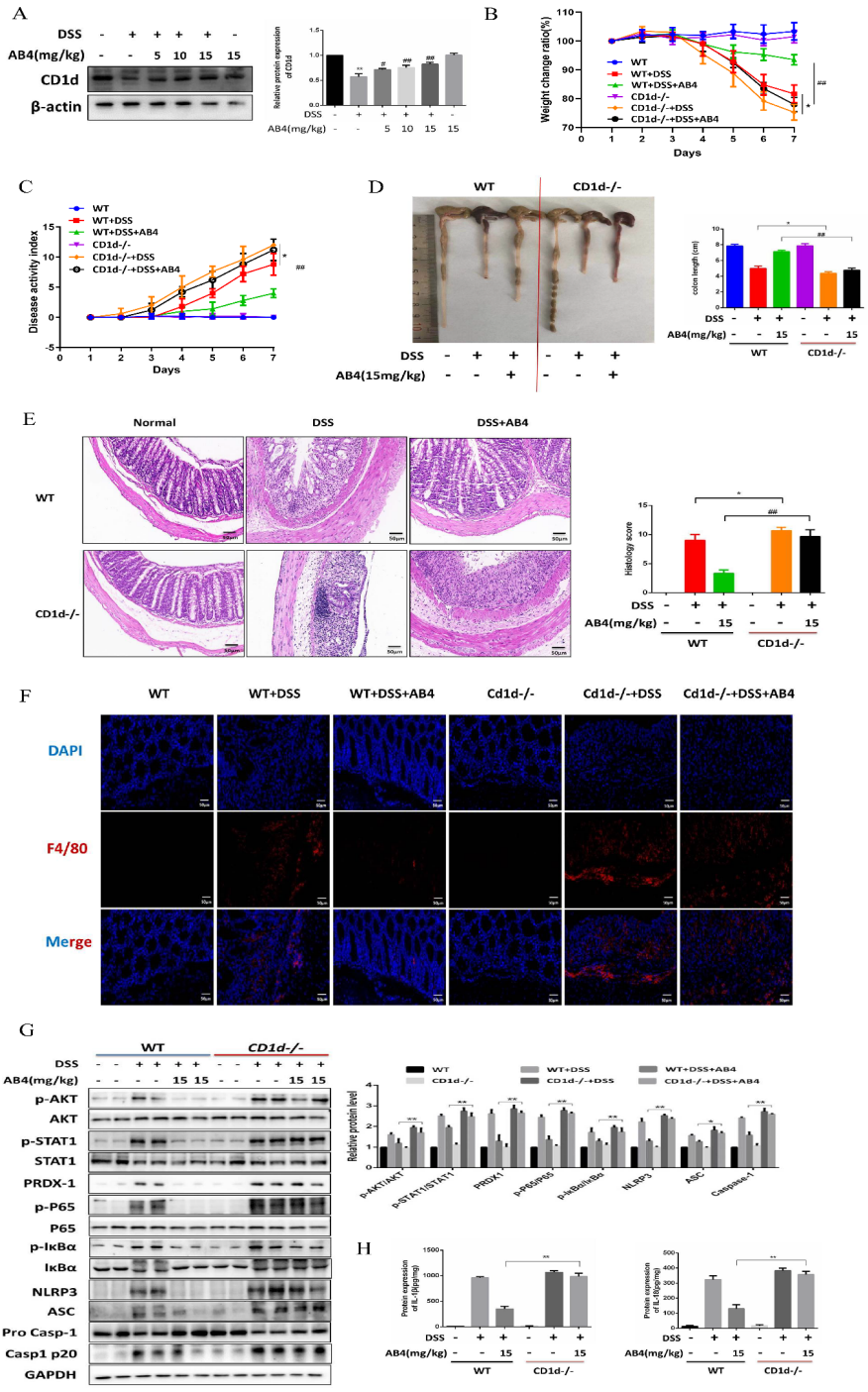
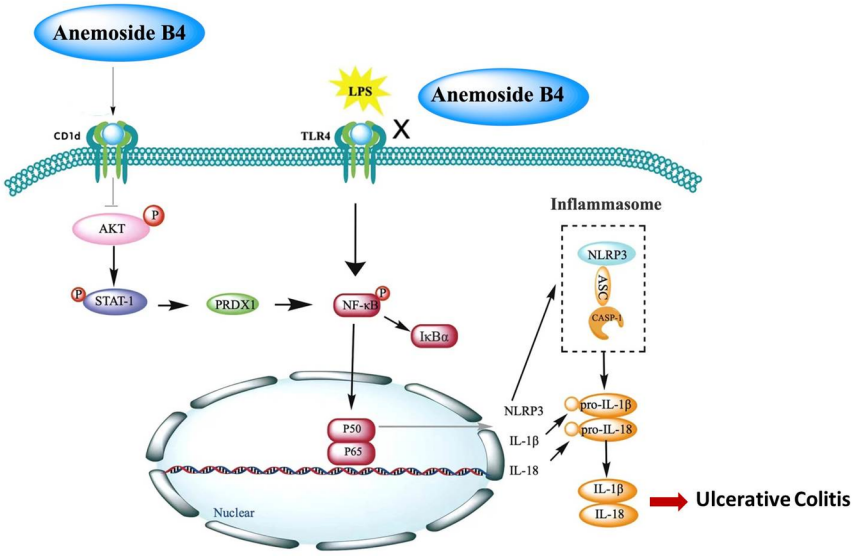


Figure 7. AB4 inhibits the activation of NLRP3 inflammasome through targeting CD1d. WT mice were subjected to DSS-induced colitis. The protein expression of CD1d in colonic macrophages (**A**) was detected by Western Blot. Data are presented as mean ± SD. $^{**}P < 0.01$ *vs.* Normal group;$^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* DSS group. CD1d$^{-/-}$ mice and WT mice were intraperitoneally injected with AB4 (15mg/kg) for 7 days, and then given 3.0% DSS in drinking water for 7 days, during which AB4 (15mg/kg) was continuously injected intraperitoneally (n=8 per group). At the end of the experiment, the mice were

sacrificed, and the colons were collected. (**B**) The body weight change, (**C**) the disease activity index (DAI), and (**D**) the colon length was measured. (**E**) H&E staining analysis of histopathological changes (left) and semi-quantitative scoring of histopathology (right) and the images was taken at 200x magnification (scale bar: 50μm). (**F**) The infiltration of F4/80^{+} macrophages in colonic tissue was determined by using an immunofluorescence assay, and the images were taken at 200x magnification (scale bar: 50μm). Data are presented as mean ± SD. $^{*}P < 0.05$CD1d$^{-/-}$ +DSS *vs.* WT+DSS;$^{\#\#}P < 0.01$CD1d$^{-/-}$ +DSS+ AB4 *vs.* WT+DSS+ AB4 group. (**G**) The protein of the indicated molecule was detected by Western Blot. (**H**) The production of IL-1β and IL-18 in colons was analyzed by ELISA. Data are presented as mean ± SD.$^{\#\#}P <$ *0.01*CD1d$^{-/-}$ +DSS+ AB4 *vs.* WT+DSS+ AB4 group.



Φιγυρε 8. Ανεμοσιδε Β4 ταργετς μαςροπηαγες ̈Δ1δ το ρεδυςε ΑΚΤ-ΣΤΑΤ1-ΠΡΔΞ1-ΝΦ-κΒ σιγναλινγ ςασςαδε, εεντυαλλψ ινηιβιτινγ τηε αςτιατιον οφ ΝΛΡΠ3 ινφλαμμα-σομε ανδ αμελιορατινγ ΔΣΣ-ινδυςεδ ςολιτις

Discussion

In this study, we demonstrated that AB4 of the three *Pulsatilla saponins* AB4, AA3, and 23-HA had the strongest inhibition rate of IL-1β secretion in macrophages. To assess the anti-inflammatory activity of AB4, we constructed DSS-induced colitis. We notably found that the AB4 pretreatment group was more effective than the AB4 treatment group and the 5-ASA positive group. AB4 reduced the severity of colitis in WT mice by inhibiting the activation of NLRP3 inflammasome and promoting the balance of inflammatory factors and repair of intestinal epithelial damage. In contrast, it lost its ability to alleviate DSS-induced colitis in NLRP3$^{-/-}$ mice. Then, we were surprised to find that AB4 inhibited NLRP3 inflammasome activation in colonic macrophages, but not in intestinal epithelial cells. Mechanistically, AB4 might target CD1d thus reducing the AKT-STAT1-PRDX1-NF-κB signaling pathway, eventually inhibiting the activation of NLRP3 inflammasome. Macrophage-specific CD1d depletion had been shown to reverse the protective effect of AB4. Together these data indicated that AB4 attenuated DSS-induced colitis by inhibiting CD1d-dependent NLRP3 inflammasome activation in macrophages.

Ulcerative colitis (UC) is a chronic, non-specific, non-infectious, inflammatory intestinal disease mediated by abnormal immunity caused by multiple etiological factors (de Lange & Barrett, 2015; Ge et al., 2018; Kaplan, 2015; Torres et al., 2021). UC has become a huge burden to human life due to its repeated course of the disease, difficult to cure and easy to cause cancer (de Lange & Barrett, 2015; Ge et al., 2018; Kaplan,

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2015; Torres et al., 2021). During the novel Corona Virus Disease 2019 (COVID-2019) pandemic, the clinical management of UC has always been an area of high concern for patients and physicians around the world. UC patients have changed their potential immune response, which may make them more susceptible to infection (Peng et al., 2020). The standard treatment for patients with UC is to receive long-term immunosuppressive and anti-inflammatory therapy, however, this can lead to severe side effects that limit long-term use (de Lange & Barrett, 2015). Therefore, there is an urgent need for drugs with high efficacy, low cost and few side effects.

Pulsatilla decoction (Bai-Tou-Weng-Tang, BTWT) is a famous Chinese medicine prescription for intestinal diseases caused by inflammation. The main component of BTWT is*Pulsatilla chinensis* (Gu, 2018). It contains a large number of triterpenoids saponins and is considered to be its main active ingredient (Li et al., 2020; Ma et al., 2020; Zhang et al., 2021). Among them, the contents of AB4, AA3, and 23-HA are higher (Ip et al., 2017; Wang, Lin, Lin, Yao, & Zhang, 2019; Yao et al., 2018; Ye & Ji, 2012). In order to further clarify the main active ingredients of BTWT anti-colitis efficacy. In this study, we demonstrated that AB4 of the three *Pulsatilla saponins* AB4, AA3, and 23-HA had the strongest inhibition rate of IL-1β secretion in macrophages. Recent studies had shown that AB4 reduced the levels of inflammatory cytokines IL-1β, IL-6, TNF-α, or NF-ϰB to alleviate colitis (Ma et al., 2020; Zhang et al., 2021). To evalute the anti-inflammatory activity of AB4, we investigated the role of AB4 in 3.0%DSS-induced colitis. The data showed that AB4 (5mg/kg) could attenuate the severity of colitis in WT mice. Notably, we first found that the AB4 pretreatment group was more effective than the AB4 treatment group and 5-ASA positive group. Interestingly, we found that AB4 (5, 10, and 15mg/kg) had a significant dose-dependent effect in reducing the severity of colitis. Considering drug safety, we found no significant difference between AB4 (15mg/kg) alone group and the normal group. In addition, studies had shown that the median lethal dose (LD50) of AB4 after intravenous injection in mice was 3.36g/kg (Qin et al., 2019), and the intravenous infusion dose was 2.5g/kg for 14 consecutive days, with no significant toxic changes such as body weight, liver, and kidney function of mice were detected (L. He et al., 2019). These data indicated that AB4 had a relatively high safety index and no adverse reactions had been detected to date. Therefore, as a natural product with high safety index, AB4 might be a promising drug candidate for the treatment of colitis. But the exact molecular mechanism remains unclear.

Activation of the NLRP3 inflammasome plays an important role in mediating the inflammatory response in UC (Qu et al., 2021; Zmora, Levy, Pevsner-Fishcer, & Elinav, 2017). Increasing evidence confirms that blocking NLRP3 inflammasome activation in macrophages is a novel strategy to block inflammatory and immune responses (Lv et al., 2021; Zhao et al., 2019). In this paper, we found that the protective effect of AB4 on DSS-induced colitis in mice was attributable to the inhibition of NLRP3 inflammasome activation and subsequent stimulation of colon epithelial cell proliferation, local IL-22 and IL-10 expression. At the same time, we verified DSS-induced colitis in NLRP3$^{-/-}$and WT mice, and we found that AB4 lost its protective effect in NLRP3$^{-/-}$ mice. This confirmed that the protection of AB4 against DSS-induced colitis depended on the intervention of NLRP3 inflammasome. However, it had been demonstrated that activation of the NLRP3 inflammasome in intestinal epithelial cells led to the secretion of IL-18 and contributed to ameliorating intestinal epithelial barrier dysfunction (Dupaul-Chicoine et al., 2010). Dupaul-Chicoine showed administration of exogenous recombinant IL-18 could improve the inflammatory symptoms of DSS-induced colitis, and the colitis was more severe in NLRP3$^{-/-}$ mice than in WT mice (Dupaul-Chicoine et al., 2010). Thus, drugs that selectively inhibit NLRP3 inflammasome activation in colonic macrophages but not intestinal epithelial cells have the potential to treat colitis. Our data strongly suggested that AB4 inhibited the activation of NLRP3 inflammasome in colonic macrophages, but not in intestinal epithelial cells. Therefore, we first found that AB4 selectively inhibited the activation of NLRP3 inflammasome in colonic macrophages to attenuate DSS-induced colitis.

Although various stages of signaling involved in NLRP3 inflammasome activation have been studied, NLRP3 expression is considered to be an important factor in its associated inflammatory mechanisms (Hirota et al., 2011; Moreira Lopes et al., 2020; Song et al., 2021; Zaki et al., 2011). Many regulatory mechanisms had been shown to inhibit NLRP3 inflammasome signaling, the most classic being the activation of the NF-ϰB

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signaling pathway (Guo et al., 2020; Schreiber et al., 1998). NF-κB plays a key role in the pathogenesis of colon immune cell infiltration in UC patients and experimental colitis models (Guo et al., 2020). Our study confirmed that AB4 significantly inhibited the NF-κB signaling pathway, showing down-regulated expression of p-P65/P65 and p-IκB/IκB, which is synergistic with NF-κB inhibitor JSH-23. TLR4 can recognize LPS-activated downstream transcription factor signals and induce transcription expression of inflammatory genes (Sheng et al., 2021). Notably, we found that AB4 was not associated with the activation of the NLRP3 inflammasome signaling pathway involved in TLR4. However, this contradicts previous research (Zhang et al., 2021). The difference in conclusions might be caused by the difference in administration concentration, action time, mouse background, and intestinal microbe. PRDX1 is a peroxidase reductase that plays an important regulatory role in reactive oxygen species scavenging, cell proliferation, differentiation, apoptosis, and inflammation (Y. He et al., 2019). A recent study showed that PRDX1 expression was increased in DSS-induced colitis, and silencing PRDX1 expression inhibited DSS-induced inflammation and apoptosis, thereby ameliorating colonic injury in rats (N. Wu et al., 2021). Hansen demonstrated that extracellular PRDX1 promoted the activation of NF-κB by inducing the phosphorylation of IκBα (Hansen, Moriarty-Craige, & Jones, 2007). In this study, we found that AB4 inhibited PRDX1 protein expression in LPS-challenged macrophages. AKT signaling pathway is involved in the regulation and release of pro-inflammatory cytokines, plays an important role in the occurrence and development of UC, and can mediate the expression of LPS-induced PRDX1 (Bast, Fischer, Erttmann, & Walther, 2010; Q. Wu et al., 2021). Considering the inhibitory effect of AB4 on PRDX1, we further investigated its effect on AKT signaling. Western Blot confirmed that AB4 inhibited AKT/STAT1 signaling pathway synergically with AKT inhibitor MK2206. Moreover, AKT agonist SC79 reversed the inhibitory effect of AB4 on the AKT-STAT1-PRDX1-NF-κB-NLRP3 signaling pathway. Similarly, in vivo studies also confirmed that AB4 inhibited NLRP3 inflammasome activation through the AKT-STAT1-PRDX1-NF-κB signaling pathway.

The molecule CD1d has the antigen-presenting effect and is a member of the glycoprotein CD1 family. The homology between human CD1d and mouse CD1d1 is more than 95% (Ishii et al., 1993; Olszak et al., 2014; Sheng et al., 2021). Multiple studies have confirmed that CD1d-related immune pathways have an important effect on UC (Fuss et al., 2004; Huang et al., 2016; Lee et al., 2019; Mizoguchi et al., 2002; Olszak et al., 2014). It had been reported that $CD1d^{-/-}$ mice were more sensitive to DSS-induced colitis, CD1d expressed in colonic intestinal epithelial cells of mice binds to the exogenous glycolipid ligand α-galactothenamide (α-GalCer) and induced the activation of NKT cells in a CD1d restrictive manner, thus alleviating DSS-induced colitis (Saubermann et al., 2000). Moreover, CD1d could transmit interactive signals that trigger CD1d expressing intestinal epithelial cells to produce anti-inflammatory cytokine IL-10 and heat shock protein 110 (HSP110) to relieve DSS-induced colitis in mice (Olszak et al., 2014). Targeting this mechanism may help improve the treatment of UC and prevent colitis-related colorectal cancer. A recent work had demonstrated that CD1d1 negatively regulated the expression of NLRP3 inflammasome (Cui et al., 2020). Therefore, we hypothesized that AB4 might play a protective role in colitis by regulating the NLRP3 inflammasome through the CD1d signaling pathway. First, we performed molecular dynamics simulations to assess the stability of AB4 and CD1d. The results showed that AB4 was successfully fitted to the catalytic domain and formed multiple interactions with the internal residues of CD1d (data not shown). Next, we constructed 3.0% DSS-induced colitis in WT mice and found that AB4 significantly enhanced the expression of CD1d protein in colon tissue and colon macrophages. Surprisingly, macrophage-specific CD1d depletion had been shown to reverse the protective effects of AB4 on the NLRP3 inflammasome and DSS-induced colitis, which validated that the CD1d-dependent NLRP3 axis was a preferential signaling pathway. These results demonstrated for the first time that AB4 might trigger the endogenous negative signaling of CD1d, inhibiting the expression of NLRP3 inflammasomes through the AKT-STAT1-PRDX1-NF-κB signaling cascade, and alleviating DSS-induced colitis in mice. As CD1d is an MHC-like transmembrane protein, it contains 336 amino acids and 10 amino acids in the cytoplasmic tail. Therefore, whether AB4 directly targets CD1d or indirectly targets CD1d, as well as the specific amino acid sites are worth further investigation.

In summary, our study confirmed that AB4 alleviated inflammatory damage in DSS-induced colitis by reducing the expression of inflammatory factors and improving intestinal barrier damage. Notably, we found

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for the first time that CD1d might be a therapeutic target for AB4. AB4 targets macrophages CD1d thus to reduce AKT-STAT1-PRDX1-NF-ϰB signaling cascade, eventually inhibiting the activation of NLRP3 inflammasome and ameliorating DSS-induced colitis (Fig. 8). As the main active component of BTWT, AB4 lays the groundwork for a better understanding of BTWT's clinical efficacy from an active ingredient perspective, and also lays a theoretical foundation for the future systematic study of the corresponding mechanism of action of BTWT. More importantly, new resources have been provided for the treatment of UC. However, the comprehensive safety evaluation and treatment optimization of AB4 in the clinical application is worthy of further study.

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Author contributions

Jiao Li and Pan Li contributed to the conceptualization and writing of the manuscript. Shuo Yuan contributed to the methodology of the manuscript. Jia-Chen Xue contributed to the software of the manuscript. Huan Meng and Xiao-Ting Hou contributed to the data curation of the manuscript. Qing-Gao Zhang, Bi-Hu Gao and Xu-De Wang contributed to the manuscript revision, and decision to submit for publication. Qing-Gao Zhang, Bi-Hu Gao and Xu-De Wang contributed to reference analysis (corresponding authors). Jiao Li and Pan Li contributed equally to this work (co-first authors).

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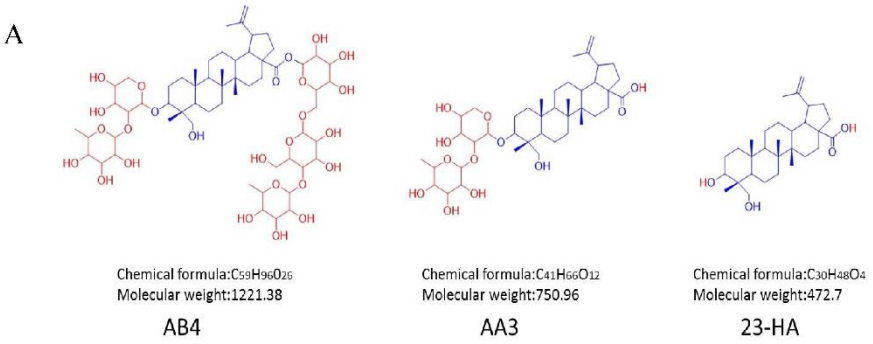
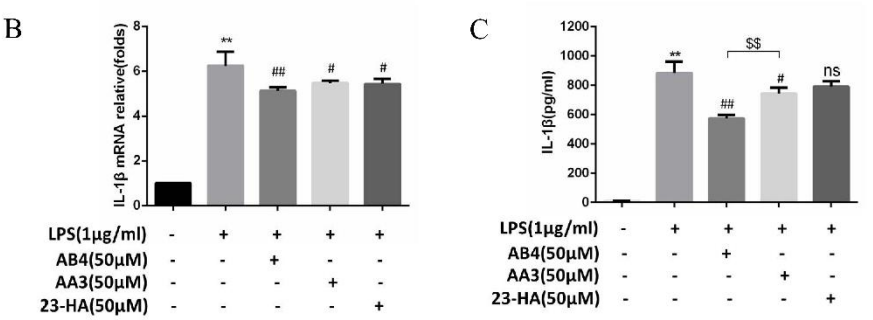
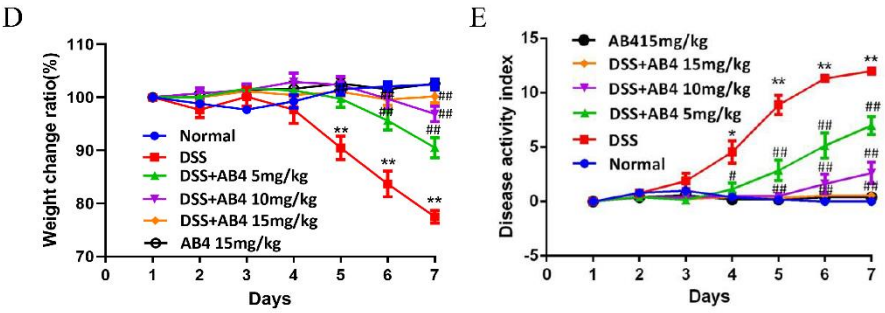
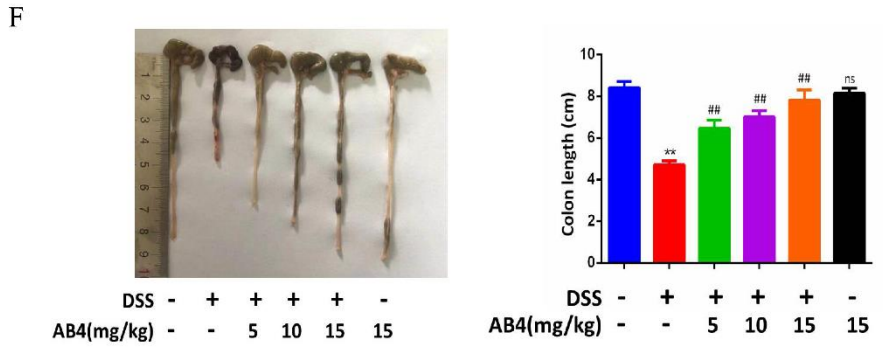
Figure.1


A
Chemical formula:C59H96O26
Molecular weight:1221.38
AB4
Chemical formula:C41H66O12
Molecular weight:750.96
AA3
Chemical formula:C30H48O4
Molecular weight:472.7
23-HA


B
IL-1β mRNA relative(folds)
LPS(1μg/ml)
AB4(50μM)
AA3(50μM)
23-HA(50μM)
C
IL-1β(pg/ml)
LPS(1μg/ml)
AB4(50μM)
AA3(50μM)
23-HA(50μM)


D
Weight change ratio(%)
Normal
DSS
DSS+AB4 5mg/kg
DSS+AB4 10mg/kg
DSS+AB4 15mg/kg
AB4 15mg/kg
Days
E
Disease activity index
AB415mg/kg
DSS+AB4 15mg/kg
DSS+AB4 10mg/kg
DSS+AB4 5mg/kg
DSS
Normal
Days


F
DSS
AB4(mg/kg)
Colon length (cm)
DSS
AB4(mg/kg)

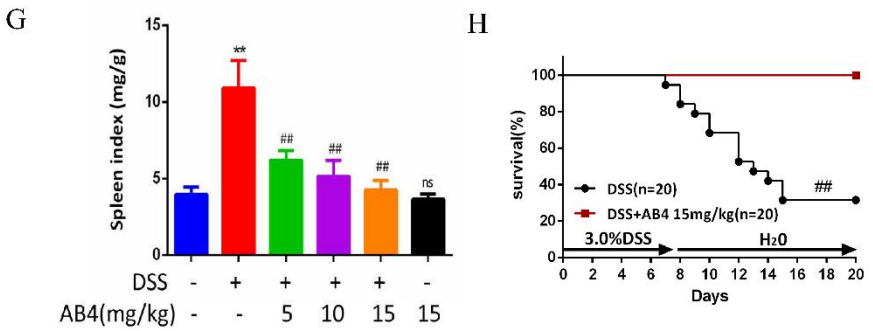

G
Spleen index (mg/g)
DSS
AB4(mg/kg)
H
survival(%)
DSS(n=20)
DSS+AB4 15mg/kg(n=20)
3.0%DSS
H2O
Days

Figure.2

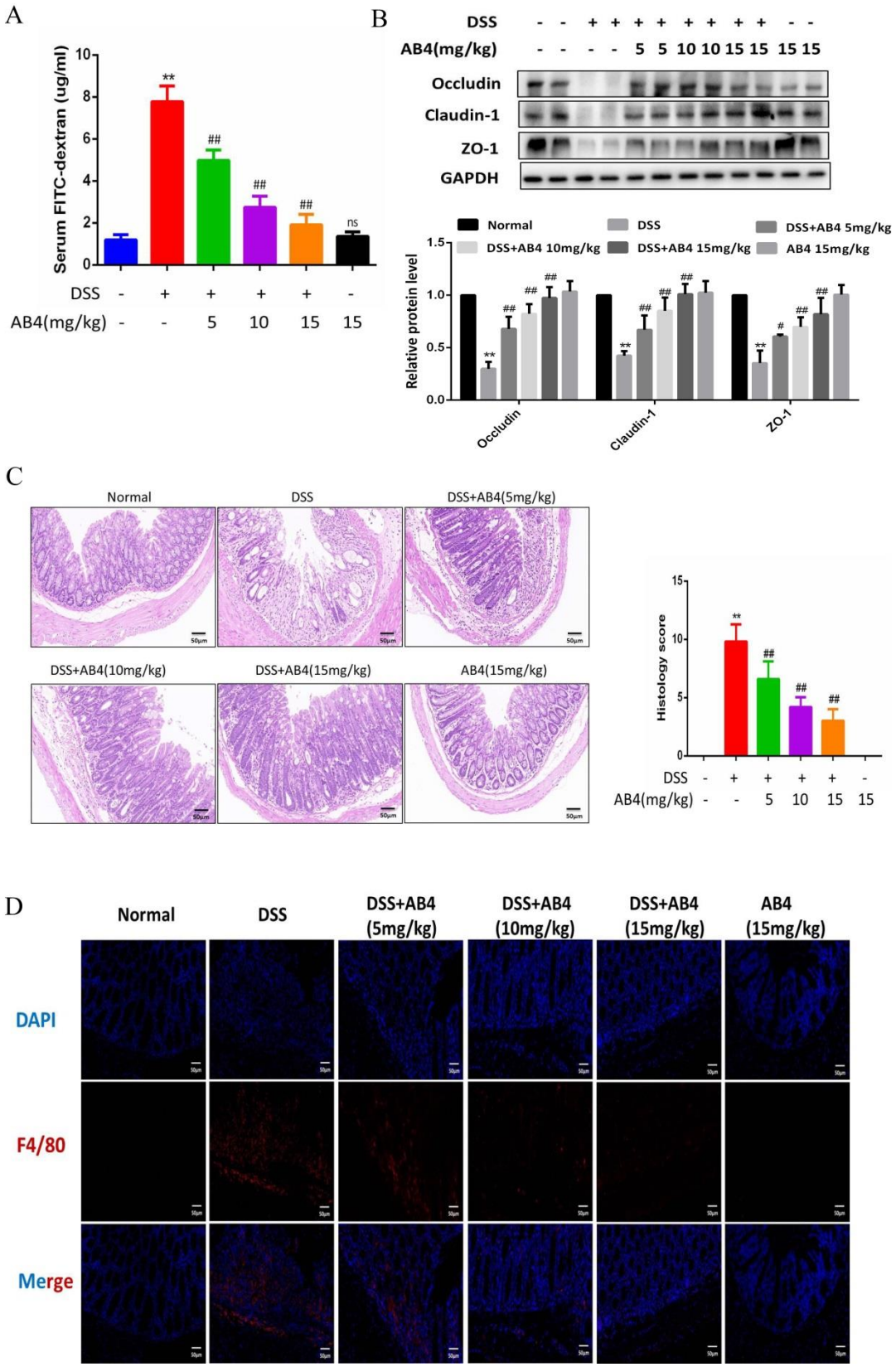

A
Serum FITC-dextran (ug/ml)
DSS
AB4(mg/kg)
B
DSS
AB4(mg/kg)
Occludin
Claudin-1
ZO-1
GAPDH
Normal
DSS
DSS+AB4 5mg/kg
DSS+AB4 10mg/kg
DSS+AB4 15mg/kg
AB4 15mg/kg
Relative protein level
Occludin
Claudin-1
ZO-1
C
Normal
DSS
DSS+AB4(5mg/kg)
DSS+AB4(10mg/kg)
DSS+AB4(15mg/kg)
AB4(15mg/kg)
Histology score
DSS
AB4(mg/kg)
D
Normal
DSS
DSS+AB4 (5mg/kg)
DSS+AB4 (10mg/kg)
DSS+AB4 (15mg/kg)
AB4 (15mg/kg)
DAPI
F4/80
Merge

Figure.3

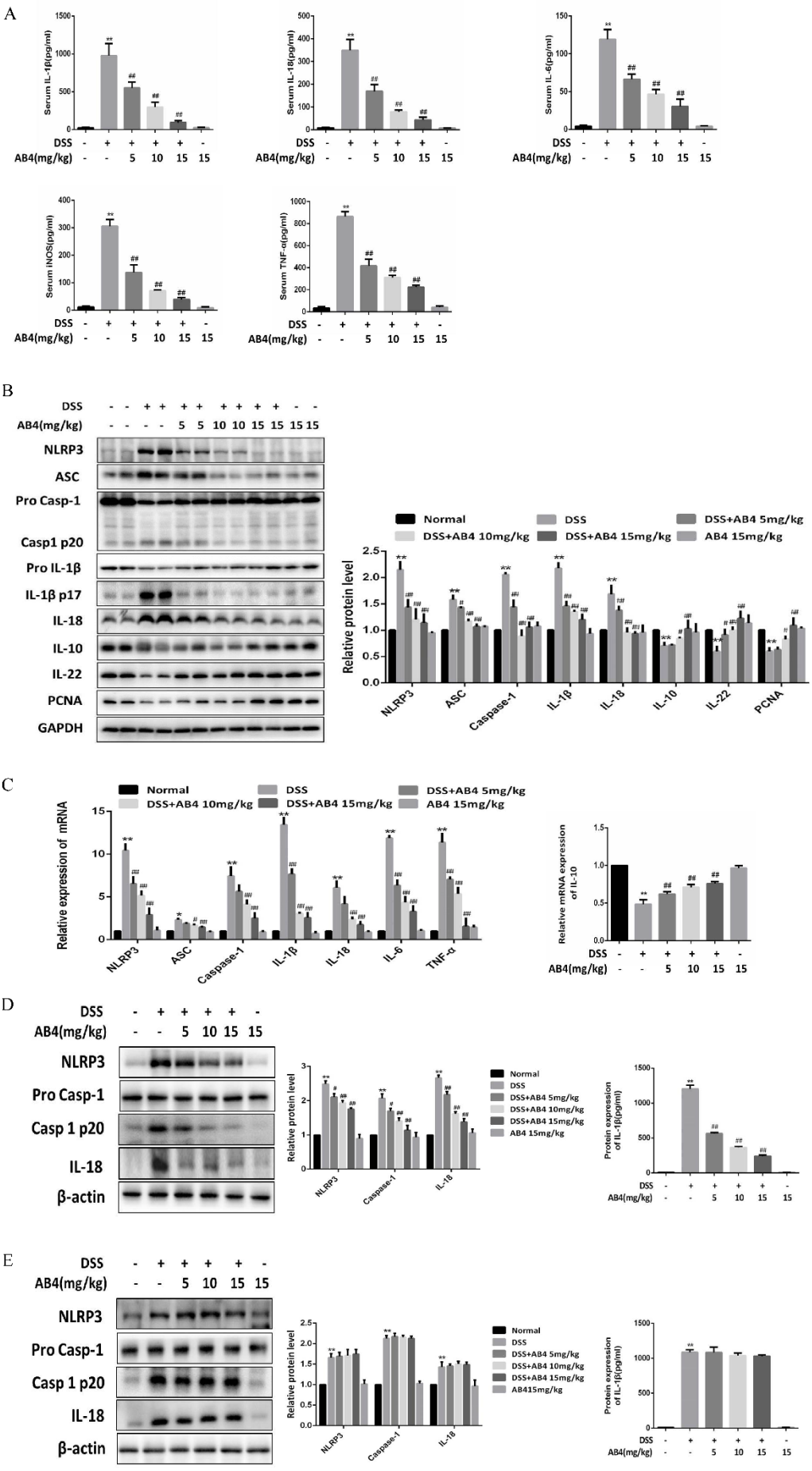

A
Serum IL-1β(pg/ml)
Serum IL-18(pg/ml)
Serum IL-6(pg/ml)
Serum iNOS(pg/ml)
Serum TNF-α(pg/ml)
DSS
AB4(mg/kg)
B
NLRP3
ASC
Pro Casp-1
Casp1 p20
Pro IL-1β
IL-1β p17
IL-18
IL-10
IL-22
PCNA
GAPDH
Normal
DSS
DSS+AB4 5mg/kg
DSS+AB4 10mg/kg
DSS+AB4 15mg/kg
AB4 15mg/kg
Relative protein level
C
Relative expression of mRNA
IL-6
TNF-α
Relative mRNA expression of IL-10
D
Casp 1 p20
IL-18
β-actin
Protein expression of IL-1β(pg/ml)
E

Figure.4

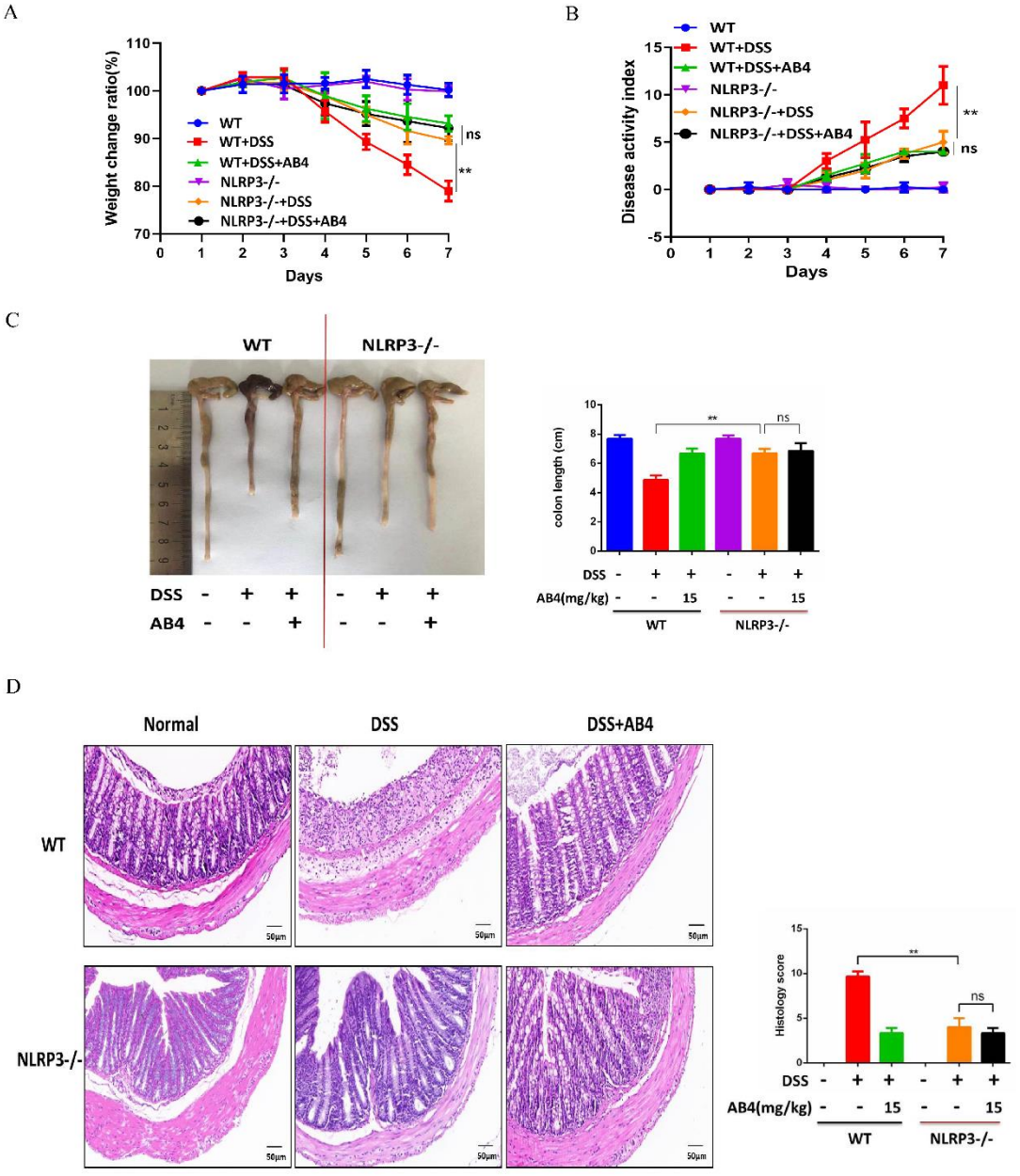

A
Weight change ratio(%)
Days
WT
WT+DSS
WT+DSS+AB4
NLRP3-/-
NLRP3-/-+DSS
NLRP3-/-+DSS+AB4
ns
**
B
Disease activity index
Days
C
WT
NLRP3-/-
DSS
AB4
colon length (cm)
AB4(mg/kg)
D
Normal
DSS
DSS+AB4
WT
NLRP3-/-
Histology score

Figure.5

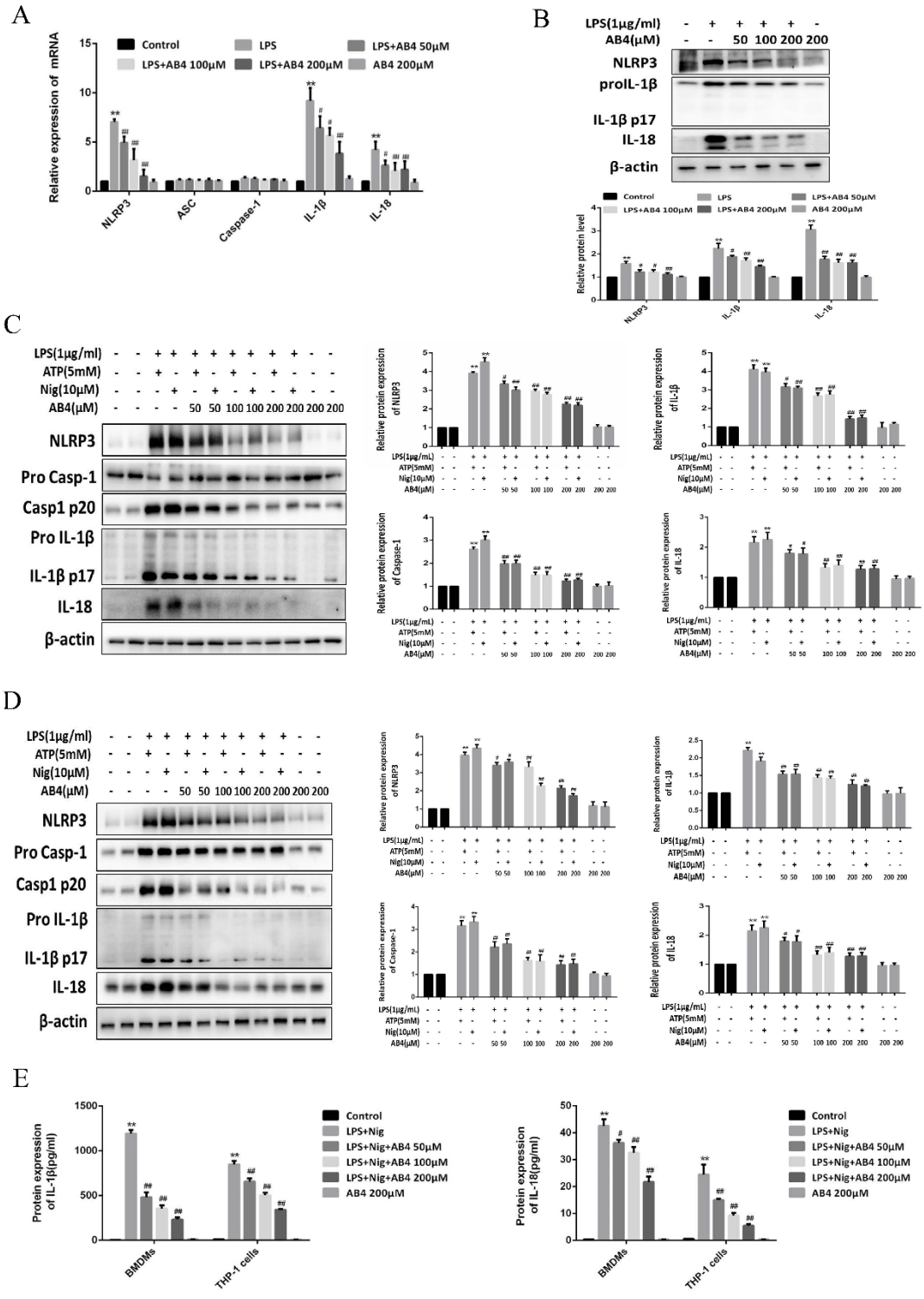

A
Relative expression of mRNA
Control
LPS
LPS+AB4 50μM
LPS+AB4 100μM
LPS+AB4 200μM
AB4 200μM
NLRP3
ASC
Caspase-1
IL-1β
IL-18
B
LPS(1μg/ml)
AB4(μM)
50 100 200 200
NLRP3
proIL-1β
IL-1β p17
IL-18
β-actin
Relative protein level
C
LPS(1μg/ml)
ATP(5mM)
Nig(10μM)
AB4(μM)
50 50 100 100 200 200 200 200
NLRP3
Pro Casp-1
Casp1 p20
Pro IL-1β
IL-1β p17
IL-18
β-actin
Relative protein expression of NLRP3
Relative protein expression of IL-1β
Relative protein expression of Caspase-1
Relative protein expression of IL-18
D
LPS(1μg/ml)
ATP(5mM)
Nig(10μM)
AB4(μM)
50 50 100 100 200 200 200 200
NLRP3
Pro Casp-1
Casp1 p20
Pro IL-1β
IL-1β p17
IL-18
β-actin
Relative protein expression of NLRP3
Relative protein expression of IL-1β
Relative protein expression of Caspase-1
Relative protein expression of IL-18
E
Protein expression of IL-1β(pg/ml)
Control
LPS+Nig
LPS+Nig+AB4 50μM
LPS+Nig+AB4 100μM
LPS+Nig+AB4 200μM
AB4 200μM
BMDMs
THP-1 cells
Protein expression of IL-18(pg/ml)
Control
LPS+Nig
LPS+Nig+AB4 50μM
LPS+Nig+AB4 100μM
LPS+Nig+AB4 200μM
AB4 200μM
BMDMs
THP-1 cells

Figure.6

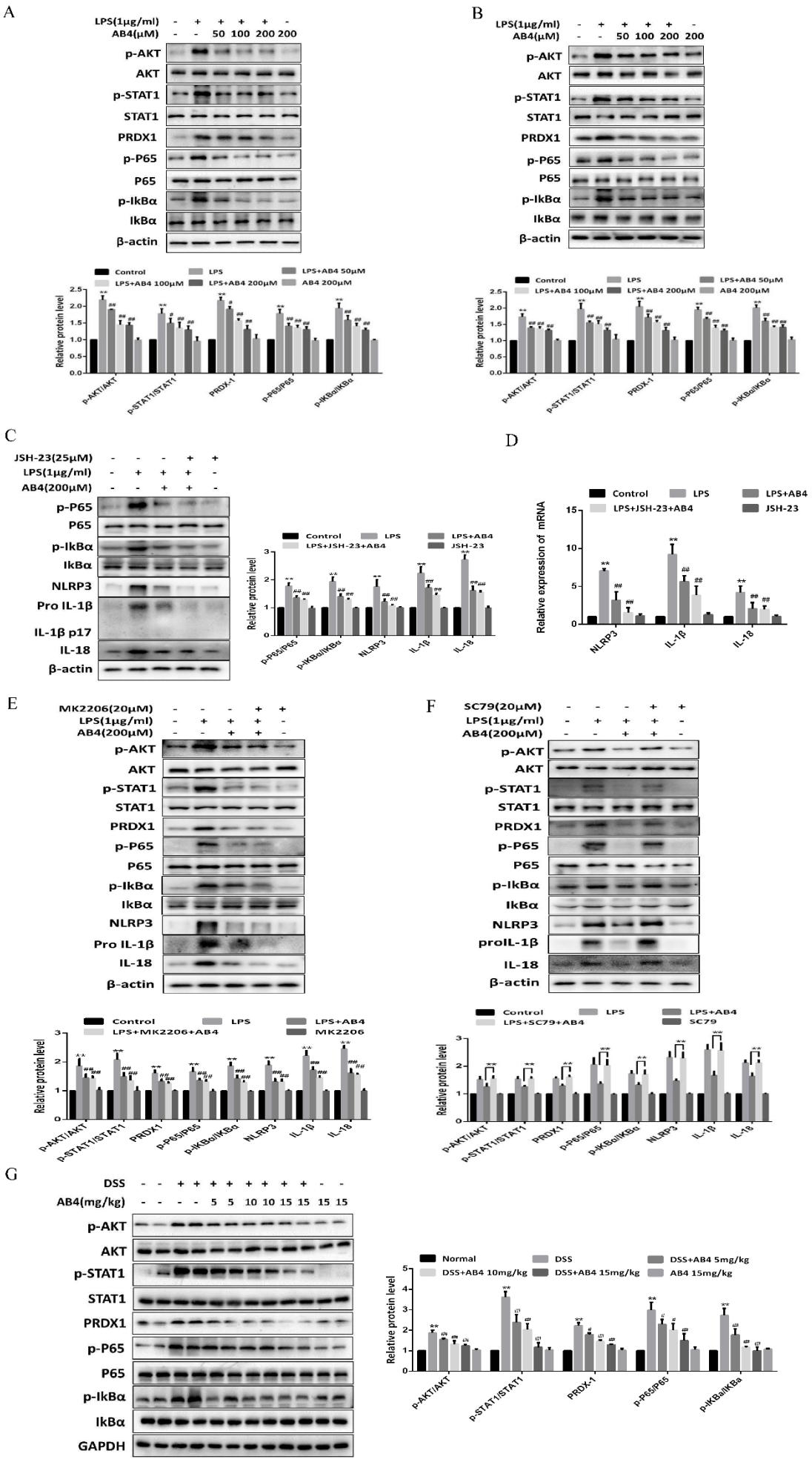

A
LPS(1μg/ml)
AB4(μM)
50 100 200 200
p-AKT
AKT
p-STAT1
STAT1
PRDX1
p-P65
P65
p-IkBα
IkBα
β-actin
Control
LPS
LPS+AB4 50μM
LPS+AB4 100μM
LPS+AB4 200μM
AB4 200μM
Relative protein level
p-AKT/AKT
p-STAT1/STAT1
PRDX-1
p-P65/P65
p-IKBα/IKBα
B
C
JSH-23(25μM)
LPS(1μg/ml)
AB4(200μM)
NLRP3
Pro IL-1β
IL-1β p17
IL-18
LPS+JSH-23+AB4
JSH-23
D
Relative expression of mRNA
NLRP3
IL-1β
IL-18
E
MK2206(20μM)
LPS+MK2206+AB4
MK2206
F
SC79(20μM)
LPS+SC79+AB4
SC79
proIL-1β
G
DSS
AB4(mg/kg)
GAPDH
Normal
DSS
DSS+AB4 5mg/kg
DSS+AB4 10mg/kg
DSS+AB4 15mg/kg
AB4 15mg/kg

Figure.7

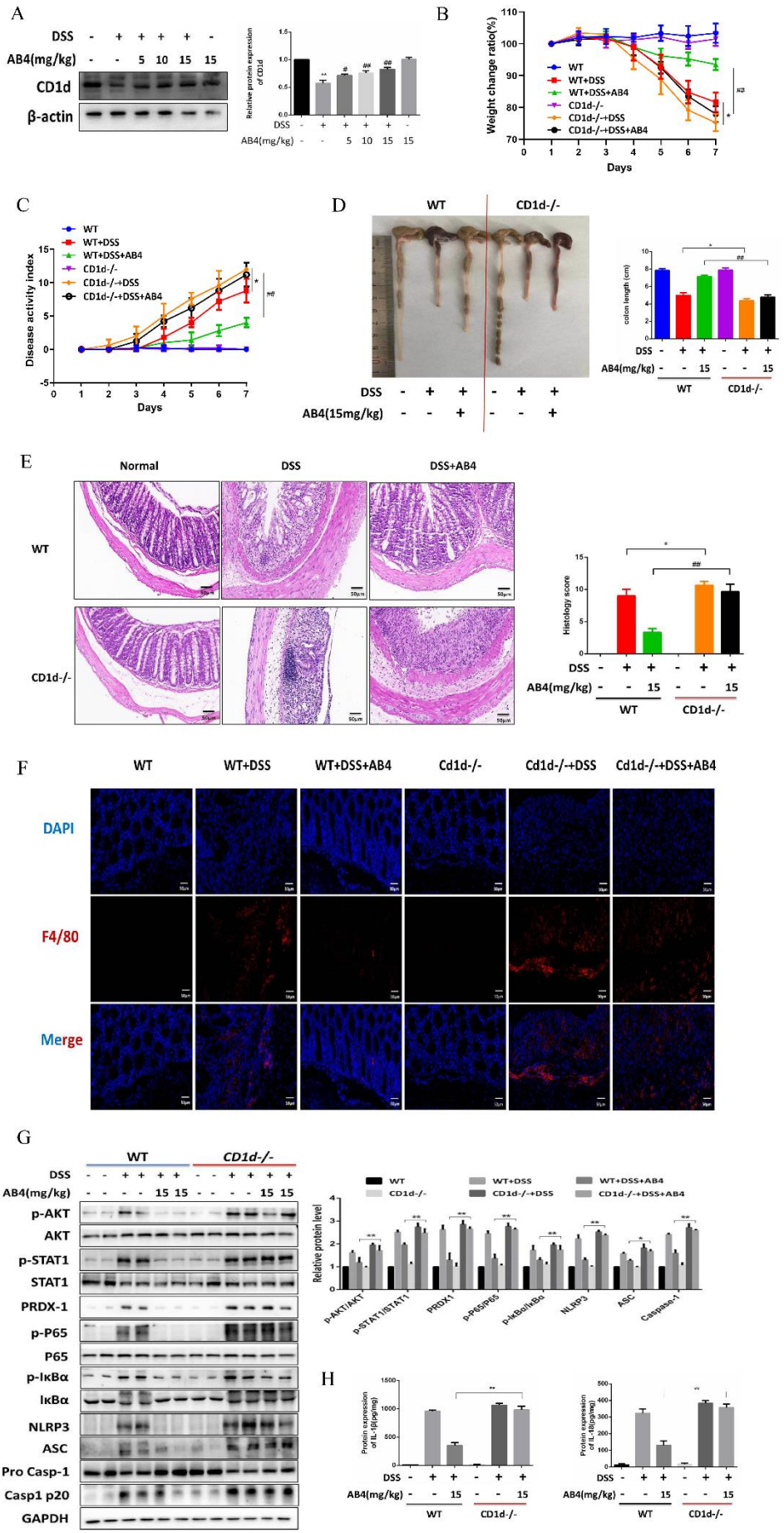
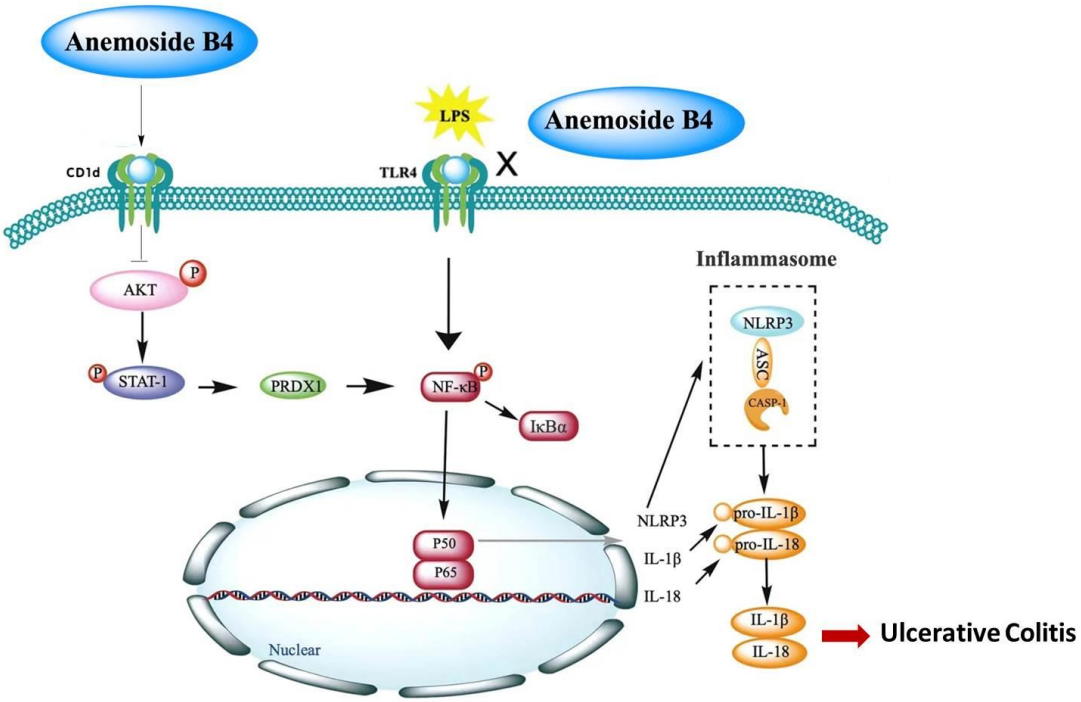

A
DSS
AB4(mg/kg)
CD1d
β-actin
Relative protein expression of CD1d
B
Weight change ratio(%)
WT
WT+DSS
WT+DSS+AB4
CD1d-/-
CD1d-/-+DSS
CD1d-/-+DSS+AB4
Days
C
Disease activity index
Days
D
WT
CD1d-/-
DSS
AB4(15mg/kg)
colon length (cm)
E
Normal
DSS
DSS+AB4
WT
CD1d-/-
Histology score
F
WT
WT+DSS
WT+DSS+AB4
Cd1d-/-
Cd1d-/-+DSS
Cd1d-/-+DSS+AB4
DAPI
F4/80
Merge
G
WT
CD1d-/-
p-AKT
AKT
p-STAT1
STAT1
PRDX-1
p-P65
P65
p-IκBα
IκBα
NLRP3
ASC
Pro Casp-1
Casp1 p20
GAPDH
Relative protein level
p-AKT/AKT
p-STAT1/STAT1
PRDX1
p-P65/P65
p-IκBα/IκBα
NLRP3
ASC
Caspase-1
H

Figure 8


Anemoside B4
LPS
Anemoside B4
CD1d
TLR4
X
AKT
P
STAT-1
PRDX1
NF-κB
IκBα
Inflammasome
NLRP3
ASC
CASP-1
P50
P65
Nuclear
NLRP3
IL-1β
IL-18
pro-IL-1β
pro-IL-18
IL-1β
IL-18
Ulcerative Colitis