Original Article

Sirtuin 3 ameliorates inflammatory bowel disease via inhibiting intestinal inflammation and oxidative stress

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Sirtuin 3 involved in development of various diseases, but its role in inflammatory bowel disease is still unknown. We used inflammatory bowel disease biopsies, colitis animal model, and *vitro* cells RAW264.7 to study the role of Sirtuin 3 in the pathophysiology of inflammatory bowel disease. Sirtuin 3 negatively correlated with intestinal TNF-α. Sirt3 was less pronounced in pediatric and adult inflammatory bowel disease patients compared with corresponding control group. Sirtuin 3 activator Honokiol suppressed dextran sulfate sodium induced colonic manifestations, while Sirt3 inhibitor caused opposite results. Honokiol inhibited colonic oxidative stress by and reduced intestinal permeability. Honokiol repressed inflammatory response by reducing macrophage infiltration, pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 levels, and inhibiting activation of NF-κB p65 in the colitis mice. However, Sirt3 inhibitor amplified colonic oxidative stress and inflammatory response. In *vitro* study, Sirt3 inhibitor or siRNA Sirtuin 3 activated NF-κB p65 and enhanced TNF-α, IL-1β, and IL-6 secretion from LPS stimulated RAW264.7, while Honokiol remarkably attenuated these pro-inflammatory cytokines secretion. Finally, knockdown of Sirt3 in Caco-2 cells enhanced TNF-α induced intestinal barrier integrity injury. Sirtuin 3 negatively regulates inflammatory bowel disease progression via reducing colonic inflammation and oxidative stress. Sirtuin 3 is a promising therapeutic target in clinical application for inflammatory bowel disease therapy.

***Key Words*: colitis, inflammatory bowel disease, honokiol, sirtuin 3, 3-TYP**

Ulcerative colitis (UC) and Crohn's disease (CD), refers to the inflammatory bowel diseases (IBD), are chronic and non-specific immune-mediated gastrointestinal disease, in which usually develops at the sites of ileum, rectum, and colon.[1] Currently, it is estimated more than two million North Americans live with IBD, and that population is forecasted to be approaching four million by 2030. In 2018, the direct medical costs in Canada was conservatively estimated at $1.28 billion, roughly $4,371 per person per year.[2,3] The incidence of IBD is steadily increasing in newly industrialized regions in Africa, Asia, and South America.[2,3] Clinical presentation of IBD presents a wide range of symptoms and signs that can be specific to the gastrointestinal tract, and others that are non-specific, which can be related to extraintestinal manifestations. IBD is a long-lasting disease and often does not recover completely. The chronic disease significantly affects quality of life. Nowadays, there is no specific cure for IBD and pharmacological approaches aim to induce and maintain the patient in remission. The long-term use of conventional medical treatment 5-aminosalicylic acid drugs, glucocorticoids and immunosuppressants caused a variety of adverse reactions, and reduced the quality of life of patients.[4] Therefore, identifying a potential therapeutic target for IBD has become a priority in the recent research.

Although the cause of IBD remains unknown, studies have provided evidence that pathogenesis of IBD is associated with genetic susceptibility of the host, intestinal microbiota, other environmental factors, immunological abnormalities, and oxidative stress disruption.[1] Several types of immune cells have been shown to contribute to IBD pathogenesis. Neutrophils perpetuate intestinal inflammation through impairment of epithelial barrier function and release of multiple inflammatory mediators,[5] and macrophages[6] produce inflammatory cytokines amplifies and maintains the chronic inflammation in IBD by promoting transcription of other pro-inflammatory cytokines, up-regulating adhesion molecules in the endothelium. Moreover, oxidative stress, an imbalance between the production and elimination of ROS as well as ROS related regulator, not only occurs in the injury intestinal mucosa but also extends into the deeper layers of the intestinal wall to push the development of IBD.[7]

Sirtuins (Sirt) is a family of proteins that act predominantly as nicotinamide adenine dinucleotide-dependent deacetylases, causing post-translational modifications in target proteins to regulate their function.[8] Sirt comprises seven members, named from Sirt1 to Sirt7. Sirt locate in the different subcellular organelles based on their type, and regulate metabolic enzyme activity, oxidative phosphorylation, mitochondrial dynamics, and anti-inflammatory activities, affecting the process of cell physiological activity, such as proliferation, differentiation, migration, autophagy, apoptosis, etc.[8,9] Therefore, Sirt has been extensively implicated in human diseases, including malignant tumors, inflammatory and autoimmune diseases, coronary artery disease, respiratory system diseases, and intestinal diseases, etc.[9] Sirt2 and Sirt6 are down-regulated in IBD and their deletion promotes inflammatory response by regulating NF-κB activation, which highlights their protective roles.[10,11] Moreover, there is a significant down-regulation in mRNA and protein expression of Sirt1 in patients suffering from IBD, and a decrease in Sirt1 with an increase in age has been shown to aggravate colitis and cause other impairments in a mouse model.[12] However, the role of Sirt3 in IBD has rarely been investigated., and its function remain to be revealed. In present study, we investigated the role

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of Sirt3 in development of IBD using clinical specimen, colitis animal model as well as in *vitro* cells.

Materials and Methods

Patients and samples. Pediatric IBD patients who underwent colonoscopy or balloon-assisted enteroscopy at Anhui Provincial Children's Hospital between November 2021 and November 2022 with samples obtained on the day endoscopy were considered eligible. Moreover, the adult IBD patients were enrolled from the Third Affiliated Hospital of Anhui Medical University between September and November 2023 in the same way, and the tissues were collected as well. The clinical disease activity for UC patients was evaluated using the Mayo subscores for stool frequency and rectal bleeding. The clinical disease activity for CD patients was evaluated using the Crohn's disease activity index (CDAI), with clinical remission defined as a CDAI<150. The exclusion criteria was having had a colostomy and failure to achieve full endoscopic observation for the patient's lesions. Mucosal biopsies were taken from inflamed areas of 26 CD pediatric patients, and 29 pediatric UC patients with undergoing colonoscopy for a clinically active disease. Colon biopsies without abnormalities from otherwise healthy pediatric patients undergoing screening colonoscopies were used as controls (four boys and three girls, mean age: 7.57 years). Moreover, the biopsies were also collected from 15 CD adult patients and 15 UC adult patients with undergoing colonoscopy for a clinically active disease, and meanwhile the biopsies from controlled health colon tissues (three male and three female, mean age: 50.20 years) were collected as well. Demographics and clinical characteristics of IBD patients used in presented study were showed in Supporting materials (Table 1 and 2). The biopsies were collected at the time of initial diagnosis, and all enrolled patients didn't receive any drug in the past three months. Informed consent was obtained from all patients and study protocol was approved by the ethics committee of the Anhui Provincial Children's Hospital (number: EYLL-2021-025) and Third Affiliated Hospital of Anhui Medical University (number: 20230915), respectively. All procedures involving human participants were performed in accordance with the ethical standards described in the 1964 Declaration of Helsinki and its later amendments.

Quantitative real-time PCR. Mucosal biopsies were used to extract RNA by TRIzol® Reagent (Invitrogen, Carlsbad, CA), and following that the RNA was reverse transcripted into cDNA using commercial kit (Invitrogen) based on the manufacturer's instructions. qRT-PCR was performed using an ABI 7000 Prism Step One plus detection system (Life Technologies, Grand Island, NY). The primers used in the current study: for Sirt3, Forward 5'-CGTTGTGAAGCCCGACATTG-3', Reverse 5'-CGTTGTGAAGCCCGAC ATTG-3'; for TNF-α, Forward 5'-CTTCTGCCTGCTGCACTTTG-3', Reverse 5'-GTC ACTCGG GGTTCGAGAAG-3'; for GAPDH, Forward 5'-TGT TGCCATCAATGACCCCTT', Reverse 5'-CT CCACGACGTA CTCAGCG-3'.

Animals and IBD model induction. All procedures involved in the present animal experiment were approved by the Animal Experiment Ethics Committee of Anhui medical University (number: 20211502), and performed in accordance with ethical standards described in the guidelines of laboratory animal use and care of the European Community (EEC Directive of 1986; 86/609/EEC). Male BALB/c mice (8 weeks old, male) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (China). All animals received tap water and food *ad libitum* and were placed in the room with standard conditions (22–25°C; humidity, 45–60%; 12–12 h light/dark cycle). The mice were randomly divided into five groups, including normal control (NC) group, dextran sodium sulfate (DSS) group, Sirt3 activator Honokiol (HNK, 5.0 mg/kg, purity>98%, Aladdin Biochemical Technology Co., Ltd., Shanghai, China) group, Sirt3 inhibitor 3-(1H-1,2,3-triazol-4-yl) pyridine (3-TYP, 50 mg/kg, purity>98%, Aladdin Biochemical Technology Co., Ltd.) group, and positive group Infliximab (IFX, 5.0 mg/kg, MedChemExpress LLC, Shanghai, China), and each group has eight mice. To induce colitis, the mice in each group (excluded NC group) received DSS (2.5%, w/w; MW of DSS, 36,000 to 50,000 Da; MP Biomedicals, LLC, Santa Ana, CA) in drinking water, while those in the NC group only received distilled water *ad libitum*.

One week after colitis induction, the mice in the NC group and DSS group received 0.9% saline via intraperitoneal injection (i.p.) daily, HNK and 3-TYP groups received drugs by i.p. once per day, and IFX group received treatment by i.p. once for three days. The whole therapy lasted for one week. Disease activity index (DAI) score was used to assess the severity of colitis. DAI

Table 1. Demographics and clinical characteristics of paediatric patients with inflammatory bowel disease

	CD (*n* = 26)	UC (*n* = 29)
Sex (male/female)	14/12	15/14
Median age, years	8.23	8.07
Ileal biopsies (*n*)	7	
Colonic biopsies (*n*)	19	29
CD location		
L1 Ileal (*n*)	7	
L2 Colonic (*n*)	7	
L3 Ileocolonic (*n*)	12	
CD behaviour		
B1 Non-stricturing, non-penetrating (*n*)	18	
B2 Stricturing (*n*)	6	
B3 Penetrating (*n*)	2	
UC extent		
E1 Proctitis (*n*)		5
E2 Left-sided colitis (*n*)		6
E3 Extensive colitis (*n*)		12
E4 Total colitis (*n*)		6

Table 2. Demographics and clinical characteristics of adult patients with inflammatory bowel disease

	CD (*n* = 15)	UC (*n* = 15)
Sex (male/female)	9/6	7/8
Median age, years	49.1	48.6
Ileal biopsies (*n*)	4	
Colonic biopsies (*n*)	11	15
CD location		
L1 Ileal (*n*)	3	
L2 Colonic (*n*)	5	
L3 Ileocolonic (*n*)	7	
CD behaviour		
B1 Non-stricturing, non-penetrating (*n*)	11	
B2 Stricturing (*n*)	3	
B3 Penetrating (*n*)	1	
UC extent		
E1 Proctitis (*n*)		1
E2 Left-sided colitis (*n*)		4
E3 Extensive colitis (*n*)		7
E4 Total colitis (*n*)		3

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score was determined using the following Calculation formula: DAI = (Weight loss + stool condition + gross bleeding)/3. Therefore, to achieve this, body weight, stool condition, and gross bleeding of the mice were recorded.

Histological and immunohistochemical analysis. Lamina propria colon tissues were removed from the mice and fixed in the 10% formalin and sectioned in 4 μm slides in thickness. The sections were conventionally stained with hematoxylin and eosin. Histological score was determined. Moreover, for the immunohistochemical analysis, sections were placed in PBS containing 0.1% protease. These sections were placed in PBS supplemented with 3% H_2O_2 to prevent endogenous peroxidase activity. After blocking with 2% serum in PBS for 1 h, the specimens were incubated with the primary antibody of CD11c+ (Abcam, Cambridge, MA) overnight at 4°C. On the following day, they were incubated with the appropriate secondary antibody for 30 min at room temperature. The sections were then counterstained with diaminobenzidine and then were observed with optical microscopy (Olympus, Tokyo, Japan).

Measurement of oxidative stress markers. Colon samples were cut into small pieces and homogenized in assay buffer on ice. The levels of SOD, GSH, and MDA were measured using commercial kits from Jiancheng Bioengineering Research Institute (Nanjing, China), and the detection was performed based on the manufacturer's instruction.

Intestinal permeability assay. Fluorescein isothiocyanate dextran (FITC-dextran) was used to measure intestinal permeability of mice. In brief, the animals were deprived of food and water for 4 h and then orally administered with FITC-dextran with (4 kDa, 50 mg/100 g, Sigma-Aldrich, St. Louis, MO). After 5 h, serum of blood was collected, and the fluorescence intensity was measured at wavelengths of 480 nm and 520 nm emission wavelengths (VersaAmax microplate reader, Molecular Devices).

Measurement of inflammatory response markers. Colons from mice of each group were homogenized with lysis buffer to extract total protein. The concentration of total protein was determined by the bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The levels of TNF-α, IL-1β, and IL-6 in colon homogenates and cell culture supernatants were quantified by ELISA kits according to the manufacturer's protocol (R&D systems, Minneapolis, MN).

Cells culture and Sirt3 siRNA transfection. Murine macrophage cell line RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and the human epithelial colorectal adenocarcinoma cell line Caco-2 was from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Both of the cells were cultured in DMEM, containing 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured in a humidified incubator with 5% CO_2 at 37°C. Sirt3 siRNA and negative control siRNA were from RiboBio (Guangzhou, China; Sirt3 siRNA, sense 5'-GGUGGAAGA AGGUCCAUAUtt-3' and antisense 5'-AUAUGGACCUUC UUCCACCtt-3'). To knockdown Sirt3, RAW 264.7 or Caco-2 with 30–50% confluence was transfected with siRNA as well as Lipofectamine 3000 reagents (Thermo Fisher Scientific, Waltham, MA). Medium was removed at 12 h after the transfection, Knockdown efficiency of Sirt3 were measured by Western blotting. After confirmation of successful knockdown, cells were harvested for the following experiments. RAW 264.7 cells received lipopolysaccharide (LPS, 1 μg/ml, Sigma-Aldrich) to mimic the inflammatory environment in the body when IBD occurs. RAW264.7 macrophages were incubated with HNK (5 μM) or 3-TYP (5 μM) for 24 h.

Sirt3 activity measurement. The lysyl deacetylase activity of Sirt3 was determined using a commercially available kit from Enzo Life Sciences Inc. (Plymouth Meeting, PA) according to the manufacturer's instructions. In briefly, protein samples (30 μg) were incubated with specific substrates for 45 min at 37°C. Next, 25 μl of developer was added to the sample and incubated for an additional 45 min. Sirt3 activity was measured spectrophotometrically using a microtiter plate fluorimeter at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Intestinal barrier function assay. Intestinal barrier function was evaluated by detecting transepithelial resistance (TER) and the flux of FITC-dextran concentration (FD4) in human intestinal Caco-2 cells. In brief, Caco-2 cells (2×10^4) were placed in transwell chambers and cultured until the TER values had plateaued (300 $\Omega \cdot cm^2$). After that, TNF-α (25 ng/ml) was used to simulate cells on the basolateral side for 24 h. Finally, TER was recorded using a Millicell-ERS epithelial voltmeter (Millipore Corp.). Later, FD4 (0.1 mg/ml, Sigma-Aldrich) was added to the upper side and incubated for 2 h. The FD4 concentration was measured using fluorescence spectroscopy at an excitation wavelength of 480 nm and an emission wave length of 520 nm.

Western blotting analysis. Western blot analysis was performed to detect the level of Sirt3. In brief, the colon tissues were homogenized and lysed using lysis buffer (Beyotime Institute of Biotechnology) with addition of protease inhibitor cocktail and phosphatase inhibitors, for 30 min on the ice. The protein was extracted and quantified by BCA assay kit according to the manufacturer's instruction. Protein extract samples (40 μg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidenedifluoride membrane. The membrane was incubated with primary antibody against Sirt3 (Abcam), NF-κB p65, phosphorylated (p)-p65 overnight at 4°C. Finally, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The Western blot bands were reacted with an enhanced chemiluminescence solution (Bio-Rad, Hercules, CA). The intensity of protein bands was quantified using image software (Image-Pro Plus 6.0, Media Cybernetics, Inc., Rockville, MD).

Statistical analysis. SPSS 17.0 was used for statistical analysis and processing all data were presented as means and SD. Statistical significance was identified by using one-way analysis of variance (ANOVA) and Bonferroni's post-hoc analysis. Moreover, Spearman's correlation was used to estimate the association between Sirt3 mRNA and TNF-α mRNA. Analyses were performed using GraphPad Prism 6.0 (GraphPad, San Diego, CA). A p value less than 0.05 was regarded as a statistically significant difference.

Results

Sirt3 was down-regulated in IBD and negatively correlated with intestinal TNF-α. To evaluate expression of Sirt3 in the IBD, colonic biopsies were taken from the pediatric and adult patients. As seen in Fig. 1A, Sirt3 mRNA levels negatively correlated with TNF-α in IBD pediatric patients ($p<0.01$; CD and UC). Next, we examined Sirt3 levels in biopsy specimens of IBD patients by RT-PCR and Western blotting. The results showed Sirt3 transcripts and expression were significantly reduced in pediatric IBD patients compared with those of pediatric control ($p<0.01$; Fig. 1B and C). Moreover, we have observed that Sirt3 transcripts and expression were significantly decreased in adult IBD patients compared with those of adult control as well ($p<0.01$; Fig. 1D and E).

Sirt3 inhibited DSS-induced colitis in mice. To determine the role of Sirt3 in IBD, we developed an DSS-induced colitis in mice, and treated them by Sirt3 activator HHK or Sirt3 inhibitor 3-TYP, respectively. Body weight, DAI values and colon length were used to evaluate the severity of colitis. As shown in Fig. 2A–C, body weight, and colon length were significantly decreased, while DAI was significantly increased in the DSS group compared with those in the NC group ($p<0.01$). However,

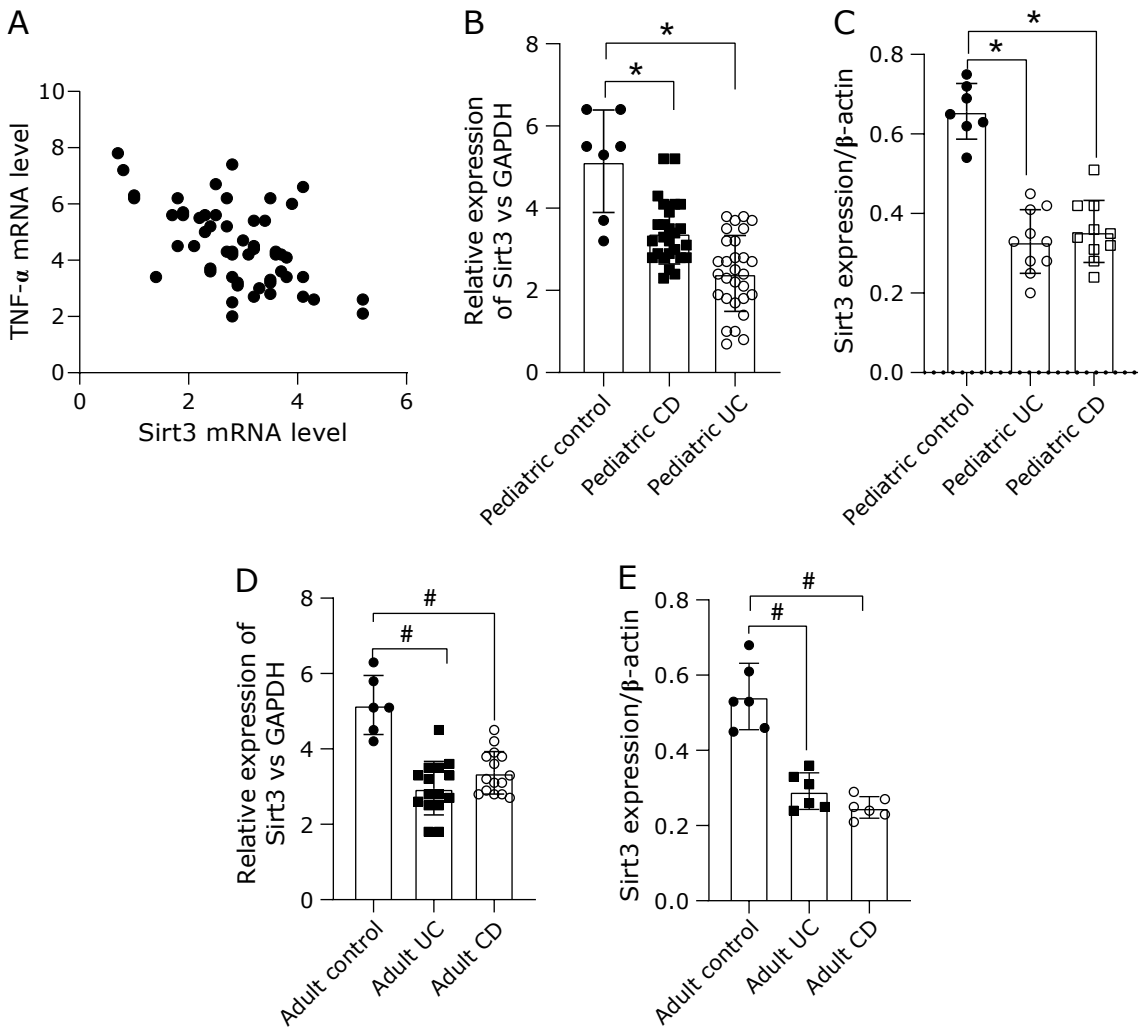

Fig. 1. Sirt3 level was reduced in IBD inflamed intestinal tissues. (A) Sirt3 mRNA levels negatively correlated with intestinal TNF-α mRNA levels. R = –0.5597, the data were from 55 IBD patients. (B) Sirt3 mRNA was evaluated in intestinal biopsies taken from pediatric control (n = 7), pediatric UC patients (n = 29) and pediatric CD patients (n = 26). PCR level was normalized to GAPDH. *p<0.01 vs pediatric control. (C) Quantitative analysis of Sirt3/β-actin ratios in mucosal samples taken from pediatric control (n = 7), pediatric UC patients (n = 10) and pediatric CD patients (n = 10) was performed. *p<0.01 vs pediatric control. (D) Sirt3 mRNA was evaluated in intestinal biopsies taken from adult control (n = 6), adult UC patients (n = 15) and adult CD patients (n = 15). #p<0.01 vs adult control. (E) Quantitative analysis of Sirt3/β-actin ratios in mucosal samples taken from adult control (n = 6), adult UC patients (n = 6) and adult CD patients (n = 6) was performed. #p<0.01 vs adult control. Data indicate mean ± SD.

compared with DSS group, HHK treatment effectively activated Sirt3 and increased its expression, and meanwhile improved colitis symptoms via decreasing DAI scores and increasing body weight and colon length (p<0.01). Moreover, compared with DSS group, 3-TYP significantly inhibited Sirt3 activity and deceased Sirt3 expression, causing an deteriorated colitis symptoms at the same time (p<0.01).

Sirt3 improved histopathological manifestation of DSS-induced colitis in mice. Severity of colonic ulceration and inflammation was further examined by pathological analysis using H&E staining. As shown in Fig. 3, no evidence of pathological damage was observed in the NC group. DSS administrated to mice caused severe tissues injury, including decrease in the number of crypts, surface epithelium lost and infiltration of inflammatory cells in mucosa and submucosa. However, treatment of HNK attenuated pathological changes induced by DSS, and meanwhile reduced its pathological score (p<0.01). Moreover, 3-TYP administration remarkably worsened colonic pathological features, and increased the pathological score compared with that in DSS group (p<0.01).

Sirt3 inhibited oxidative stress and reduced intestinal permeability of DSS-induced colitis mice. To evaluate oxidative stress of colitis mice, colonic MDA, SOD, and GSH concentrations were measured. As shown in Fig. 4, SOD and GSH were significantly decreased, and MDA was significantly increased in the DSS group compared with NC group (p<0.01). Compared with DSS group, HNK significantly increased colonic SOD and GSH, and decreased MDA levels, while 3-TYP remarkably decreased colonic SOD and GSH, and increased MDA levels (p<0.01). Moreover, compared with DSS group, HNK significantly reduced intestinal permeability, while 3-TYP remarkably increased the permeability at same time (p<0.01).

Sirt3 inhibited inflammatory response of DSS-induced colitis mice. To evaluate inflammation in the colitis mice, the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and infiltrative macrophages were measured. As shown in Fig. 5A, TNF-α, IL-1β, and IL-6 were significantly increased in the DSS group compared with those of the NC group (p<0.01). Compared with DSS group, HNK significantly decreased colonic TNF-α, IL-1β, and IL-6, while 3-TYP remarkably increased

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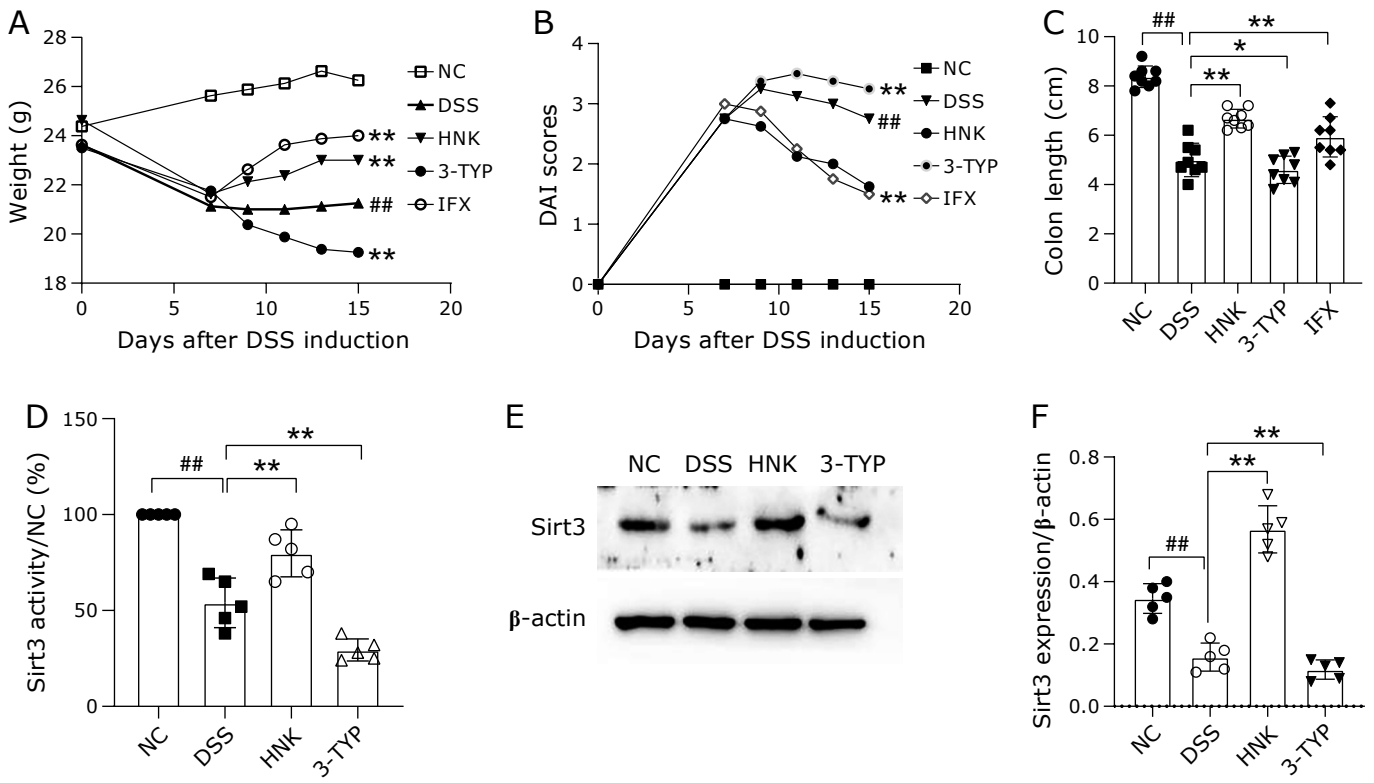

Fig. 2. Sirt3 inhibited DSS-induced colitis in mice. (A) Body weight. (B) DAI scores. (C) Colon length. (D) Sirt3 activity. (E, F) Sirt3 expression. Data are presented as mean ± SD; $n = 8$ per group. $^{\#\#}p<0.01$ vs NC; $^{*}p<0.05$; $^{**}p<0.01$ vs DSS.

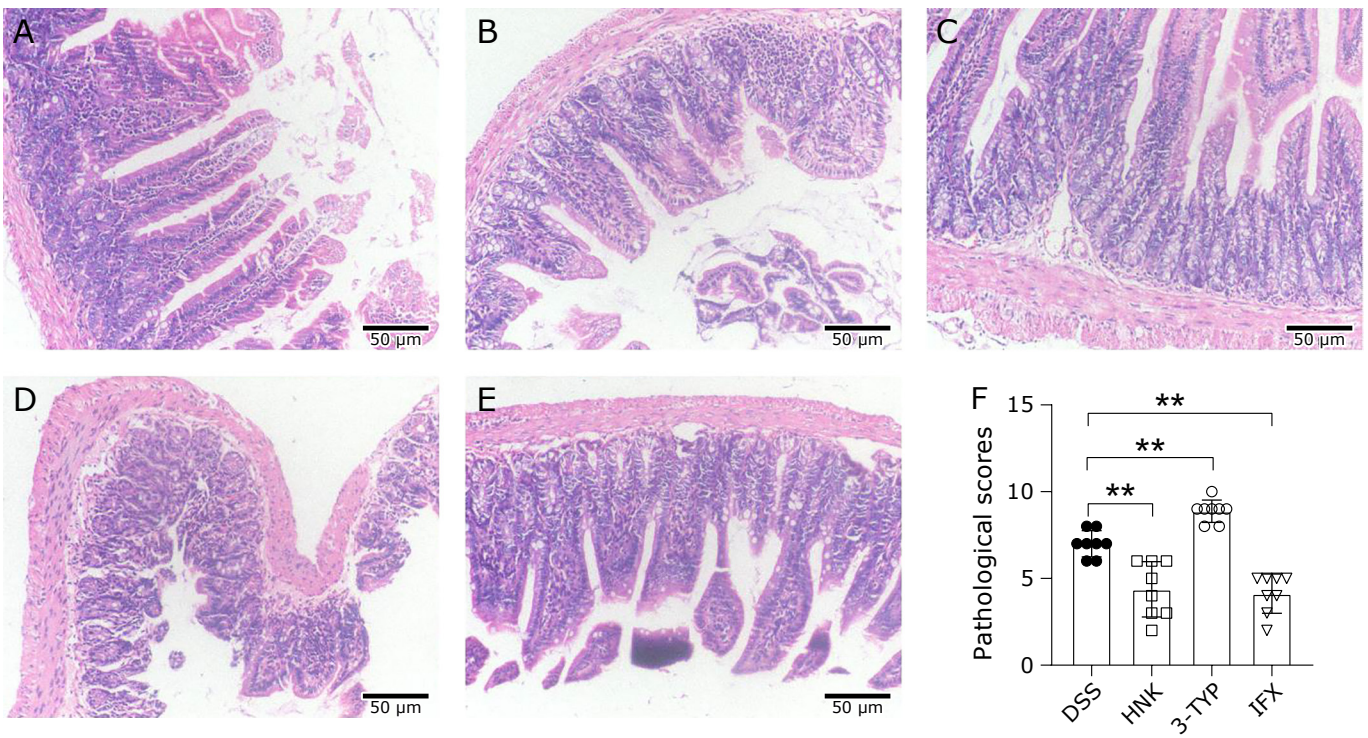

Fig. 3. Sirt3 improved pathological manifestation of DSS-induced colitis in mice. Inhibition of Sirt3 improved pathological damage in the colon. Representative pathological images from different groups of DSS exposed mice were showed (A: NC; B: DSS; C: HNK; D: 3-TYP; E: IFX). Tissue sections were stained with H&E (200×). Pathological scoring was evaluated and presented in (F). Data are presented as mean ± SD; $n = 8$ per group. $^{**}p<0.01$ vs DSS.

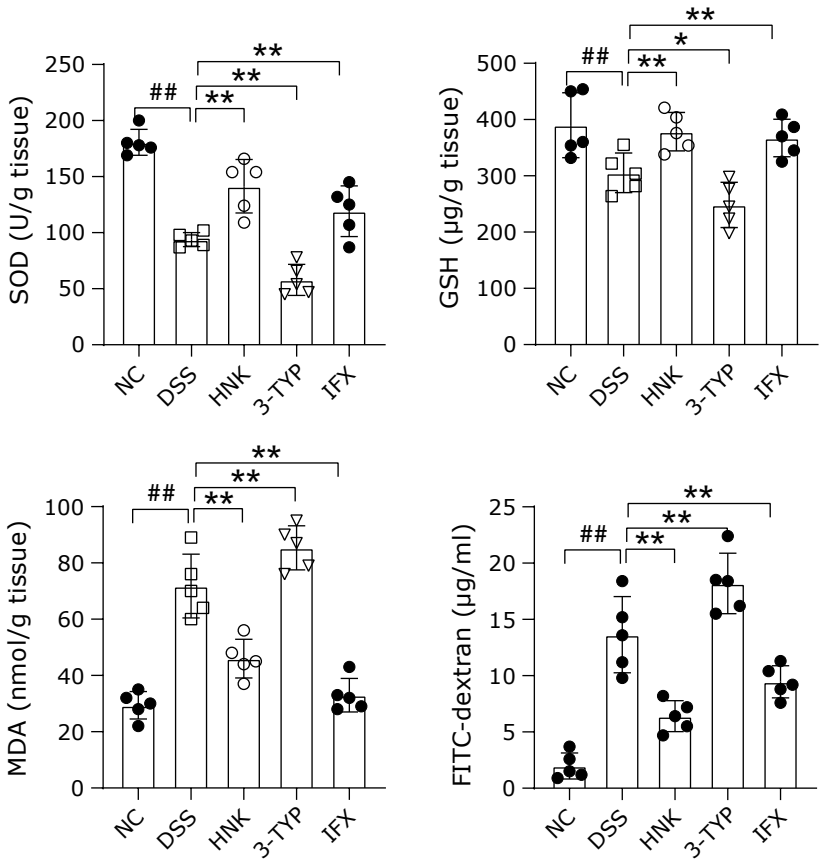

Fig. 4. Sirt3 repressed oxidative stress and reduced intestinal permeability of DSS-induced colitis. Data are presented as mean ± SD; $n = 5$ per group. $^{\#\#}p<0.01$ vs NC; $^{*}p<0.05$; $^{**}p<0.01$ vs DSS.

these cytokines (IL-1β and IL-6, $p<0.01$). Further, we detected macrophages infiltration in colons by immunochemistry and found the numbers of CD11c+ macrophages were sharply increased in colonic samples from DSS mice (Fig. 5B, $p<0.01$). However, compared with DSS group, the numbers of CD11c+ macrophages in those of HNK or 3-TYP group were reduced and increased, respectively ($p<0.01$). Moreover, HNK inhibited NF-κB activation via reducing p-p65 expression, while 3-TYP showed the opposite regulatory effect (Fig. 5C, $p<0.01$). Our data indicated Sirt3 plays an negative role in the inflammatory response of DSS-induced colitis mice.

Sirt3 inhibited pro-inflammatory cytokines secretion of LPS stimulated RAW264.7 cells. Macrophages play an important role in the progression IBD,[6] and drive & amplify colonic inflammatory response via producing TNF-α, IL-1β, and IL-6. To further explore mechanism of anti -inflammatory effect of Sirt3 in IBD, we altered Sirt3 expression or activity via using HNK, 3-TYP or Sirt3 siRNA (Fig. 6A). As shown in Fig. 6B and C, the levels of TNF-α, IL-1β, and IL-6 secreted by RAW264.7 cells were significantly increased after LPS stimulation ($p<0.01$). Incubation of RAW264.7 cells with HNK significantly increased Sirt3 activity and expression, while 3-TYP showed opposite results (Fig. 6B, $p<0.01$). Moreover, siRNA silenced Sirt3 expression and effectively increased pro-inflammatory cytokines production and promoted NF-κB activation via increasing p-p65 expression in the cells received LPS stimulation (Fig. 6C and D, $p<0.01$).

Sirt3 improved barrier function of Caco-2 cells exposed to TNF-α stimulation. Caco-2 cells were used to evaluated intestinal barrier function. As seen in Fig. 7A, after stimulation with TNF-α (50 ng/ml) for 48 h, the monolayer barrier was destroyed, and TER decreased significantly ($p<0.01$). Sirt3 knockdown significantly enhanced TNF-α induced dysfunction in Caco-2 cells ($p<0.01$). We next examined effects of Sirt3 on the paracellular tracer permeability of Caco-2 cell monolayers. As seen in Fig. 7B, consistent with the results of TER analysis, TNF-α stimulation increased the cell bypass of FD4, which was further enhanced by Sirt3 knockdown ($p<0.01$). Our data suggest that Sirt3 mediated intestinal epithelial cell barrier function as well.

Discussion

Accumulating evidences recently document that Sirt3 is associated with many types of human diseases, indicating Sirt3 can be a potential therapeutic target.[14] Sirt3 participates in several types of autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis.[14] In this study, we report protective role of Sirt3 in the IBD. We found significant decrease in Sirt3 mRNA and protein level in inflammatory tissues from adult and pediatric IBD patients compared with corresponding health colon tissues. Moreover, Sirt3 levels negatively correlated with intestinal TNF-α. Our results were consistent with previously reported reduced expression of other Sirt family members.[15] However, the reason why Sirt3 is down-regulated in IBD is unknown and needed to be uncovered in the future. Further, we developed the DSS-induced colitis model in mice, and treated the mice with Sirt3 specific activator and inhibitor, respectively. HNK is a small molecular weight natural compound derived from the bark of magnolia trees, and widely used in a traditional Asian medicinal system.[16]

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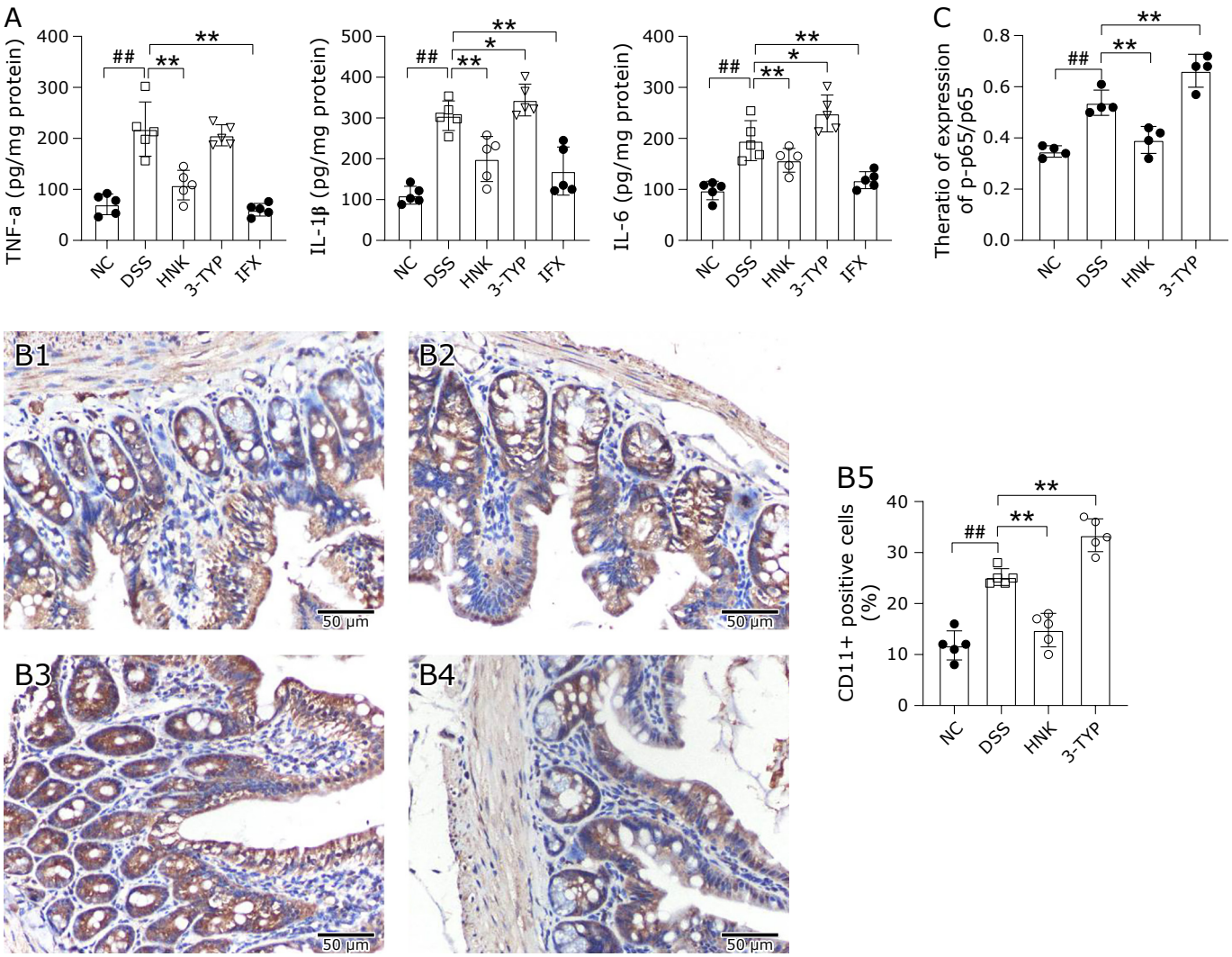

Fig. 5. Sirt3 inhibited inflammatory response of DSS-induced colitis in mice. (A) Pro-inflammatory cytokines production from colonic tissues were assayed by commercial ELISA kits. (B) Sections of colon tissues (B1: NC; B2: DSS; B3: HNK; B4: 3-TYP; B5: semi-quantitative analysis of CD11c expression) were stained with anti-CD11c antibodies, and observed by microscope, 200×. (C) NF-κB p65 and p-p65 expression in colonic tissues were detected by Western blotting. Data are presented as mean ± SD; n = 4–5 per group. $^{\#\#}p<0.01$ vs NC; $^{*}p<0.05$; $^{**}p<0.01$ vs DSS.

Sirt3 is localized to the inner mitochondrial membrane and it is implicated in metabolic regulation of pathological processes, such as inflammation, metabolism and cell cycle regulation.[17] HNK has previously been presented in mitochondria, enhanced Sirt3 expression and bind to Sirt3 to further increase its activity.[17] Moreover, 3-TYP, a Sirt3 -selective inhibitor, has been previously reported to inhibit Sirt3 activity as well as decrease expression.[18] Therefore, HNK and 3-TYP have been widely used to alter Sirt3 function both in *vivo* and *vitro*. In present study, HNK and 3-TYP significantly altered Sirt3 expression or its activity in DSS induced colitis mice, implying that Sirt3 function was effectively changed by using specific medicine. Further study shows HNK exhibited well alleviation of colitis in the mice, evidenced by reduced DAI scores, improved pathological abnormalities in colons. In contrast, 3-TYP showed the opposite results. Several recently published studies showed regulation of Sirt3 is an vital mechanism of protective pharmacological effects. Melatonin attenuates sepsis-induced small-intestine injury by up-regulating Sirt3-mediated oxidative-stress inhibition, mitochondrial protection, and autophagy induction,[18] and moreover, dihydromyricetin protects intestinal barrier integrity through regulating Sirt3.[19] Our results indicate that Sirt3 plays an negative role in the development of colitis, and maybe an promising pharmacological target to facilitate specific medicine development.

In IBD, oxidative stress not only occurs in the inflamed intestinal mucosa but also extends into the deeper layers of the intestinal wall and is mirrored within the systemic circulation.[7] When IBD happens, the generation of oxygen free radicals including MDA increases, and the levels of GSH and SOD decrease, leading to oxidative stress injury on the intestinal mucosa.[20] In IBD patients, a significant decrease of SOD activity and GSH level was seen.[21] Sirt3 has been demonstrated to regulate oxidative stress by modulating key antioxidants. Mechanistically, Sirt3 directly deacetylates the MnSOD 122-lysine residue in the mitochondria, enhancing its ability to neutralize ROS. Moreover, Sirt3 enhances transcription of MnSOD and other oxidative stress responsive genes.[22] In present study, HNK treatment significantly increased levels of SOD and GSH, and deceased MDA in the colonic tissues. In contrast, i.p. administration of 3-TYP to the DSS mice further aggravated oxidative stress, and decreased levels of SOD and GSH. Our data imply Sirt3 inhibits oxidative stress in the IBD.

Intestinal immune imbalance and inflammatory response is

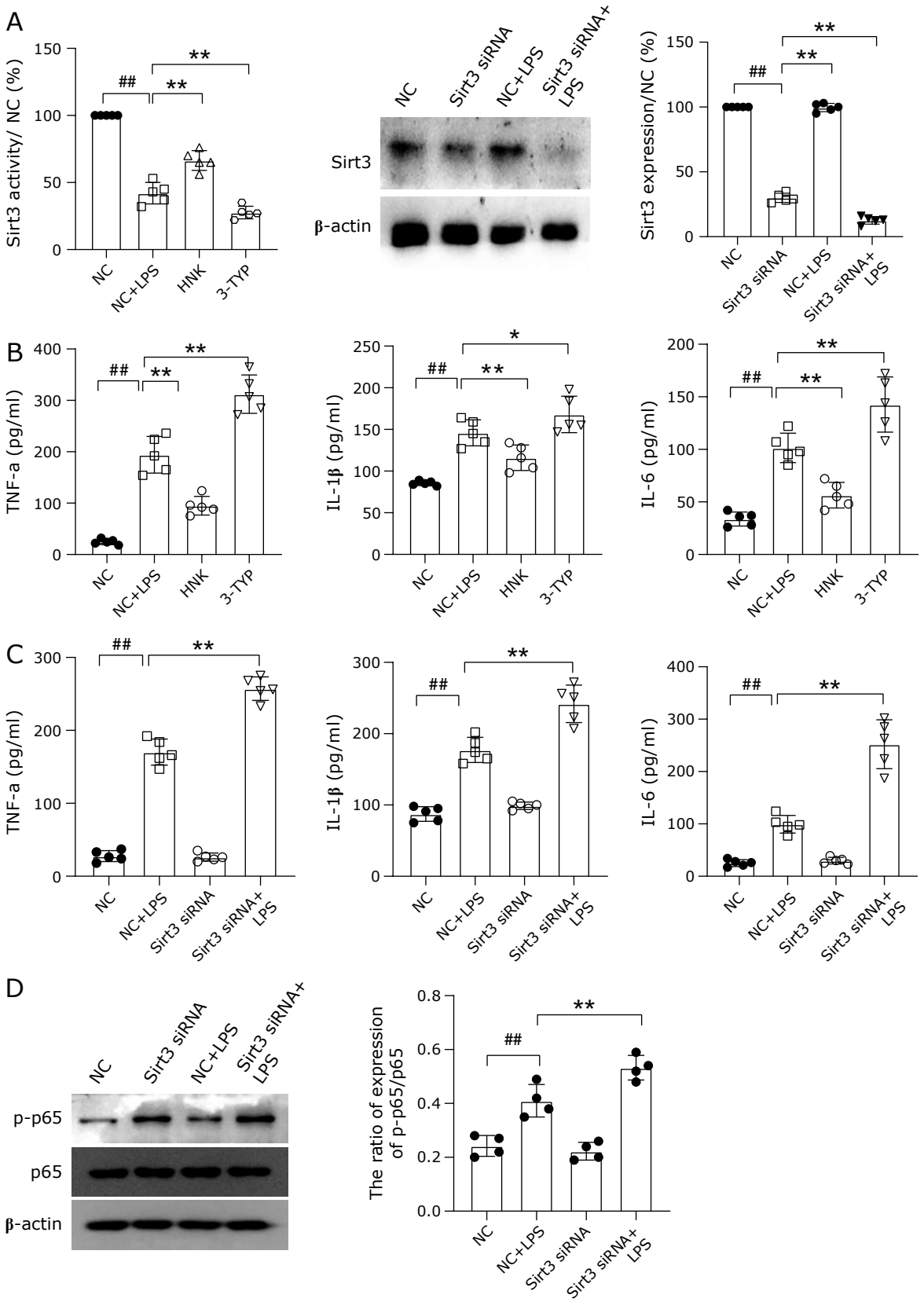

Fig. 6. Sirt3 negatively regulated pro-inflammatory cytokines secretion of LPS stimulated RAW264.7. (A) RAW264.7 cells were transfected with Sirt3 siRNA or treated HNK/3-TYP, and Sirt3 expression/activity was successfully altered. (B, C) Pro-inflammatory cytokines produced by RAW264.7 were assayed by commercial ELISA kits. (D) NF-κB p65 and p-p65 expression of RAW264.7 cells were detected by Western blotting. Data are presented as mean ± SD; $n = 5$ per group. $^{\#\#}p<0.01$ vs NC; $^{**}p<0.01$ vs NC + LPS.

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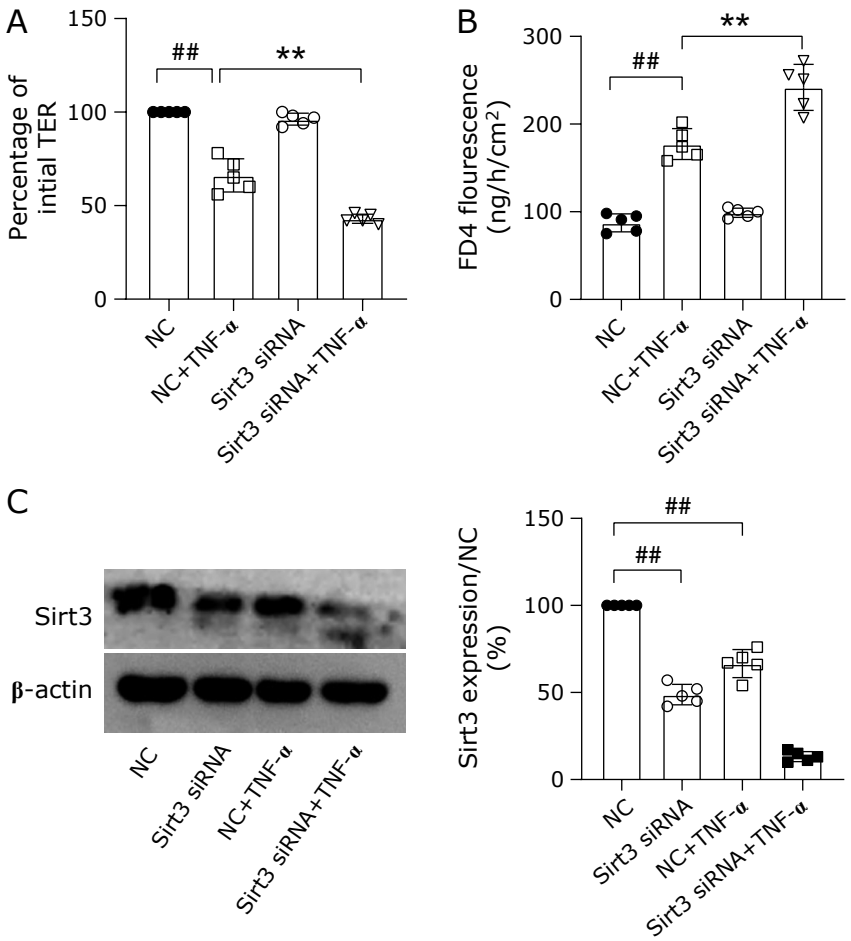

Fig. 7. Sirt3 inhibited TNF-α induced disruption of barrier function *in vitro*. (A) Caco-2 cell monolayers received stimulation by 50 ng/ml TNF-α for 48 h, and then, TER value was measured. (B) Flux of FITC-dextran from Caco-2 were measured. (C) Sirt3 expression was successfully knockdown by siRNA transfection. Data are presented as mean ± SD; $n = 5$ per group. $^{\#\#}p<0.01$ vs NC; $^{**}p<0.01$ vs NC + TNF-α.

another critical characteristic of IBD. Macrophages play an indispensable role in colitis.[6] Macrophages act as a "power forward" in the inflammatory response. In the IBD, macrophages clear the pathogen by enhancing phagocytosis, secreting various pro-inflammatory cytokines to remove pathogens and control the disease process.[6] Pro-inflammatory cytokines, such as TNF-α and IL-6, are the main factors involved in the regulation of immune response in IBD. TNF-α promotes T cell proliferation and differentiation, causing accelerated intestinal inflammation and damaged intestinal barrier.[23] IL-6 is a pro-inflammatory cytokine, and has been shown to play an essential role in the intestinal epithelial barrier. IL-6 modulates intestinal epithelial tight junction via activation of claudin-2 gene.[24,25] Similarly, as one of the most important pro-inflammatory cytokines involved in the pathogenesis of IBD, high plasma level of IL-1β correlates well with severity of intestinal inflammation and disease activity.[26] Pharmacological inhibition of these cytokines ameliorates intestinal inflammation in the IBD.[27] Sirt3 exerts anti-inflammatory effect via acting multi-target in response to tissue stress or disease development. Previously, Sirt3 inhibits NF-κB activity in mammary epithelial cells,[28] and Sirt3 also inhibits NLRP3 to exert anti-inflammatory function.[29] In present study, HNK inhibited macrophage infiltration, reduced TNF-α, IL-1β, and IL-6 levels, and prevented NF-κB activation in DSS induced colitis mice. Interestingly, 3-TYP remarkably deteriorated inflammatory responses via further enhancing macrophage infiltration, NF-κB activation and facilitating these pro-inflammatory cytokines secretion. LPS is an endotoxin derived from gram negative bacteria, and increase the levels of pro-inflammatory cytokines by stimulating Toll-like receptors such as TLR4.[30] In *vitro* study, we used LPS to stimulate Macrophage cell line RAW264.7, and altered Sirt3 activity or expression by HNK, 3-TYP or Sirt3 siRNA. Our results showed that 3-TYP or Sirt3 siRNA significantly promoted TNF-α, IL-1β, and IL-6 secretion in the RAW264.7 stimulated by LPS, while HNK caused the opposite outcome.

Intestinal barrier integrity is a prerequisite for homeostasis of mucosal function, and loss of epithelial integrity was considered to be one pathogenic factor for IBD.[13] In *vivo* study, we found inhibition of Sirt3 activity caused increased intestinal permeability. Further study showed that silencing Sirt3 could enhance TNF-α induced down-regulation of TER and flux of FD4. Therefore, it is suggested that protective of Sirt3 in the developed IBD was independent on its regulatory effects on oxidative stress and inflammation. To the best of our knowledge, this is the first report describing the regulatory effects of Sirt3 in IBD.

In conclusion, the present study demonstrated that Sirt3 exert an protective action in the development of colitis. The underlying mechanism is linked with reducing inflammation, inhibiting oxidative stress, and strengthening intestinal barrier integrity. Sirt3 is an promising therapeutic target in clinical application for IBD therapy.

Author Contributions

Study concept and design: MZ and C-YL; acquisition of data: ZQ, QC, and AD; analysis and interpretation of data: ZQ; drafting of the manuscript: ZQ.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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