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ORIGINAL RESEARCH

NLRP3 Mediates NOXs-Induced Iron Overload and Inflammation but Not Oxidative Damage in Colons of DSS-Treated Mice

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Background: Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) with chronic and recurrent characteristics caused by multiple reasons, including iron overload, intestinal inflammation, and barrier dysfunction. Here, we investigated the effects of chemical inhibition of NOXs and NLRP3 activity on colonic iron metabolism and inflammatory reactions in a murine model of dextran sodium sulfate (DSS)-induced ulcerative colitis.

Methods: The mice were randomly divided into five groups: normal control group, DSS-induced ulcerative colitis model group (DSS), DSS + Dapansutrile group, DSS + Diphenyleneiodonium chloride group, and DSS + Dapansutrile + Diphenyleneiodonium chloride group. On day 14, the mice were euthanized. Tissues were collected and analyzed to determine the effects of chemical inhibition of NOXs and NLRP3 activity on colonic iron metabolism and inflammatory reactions of dextran sodium sulfate-induced ulcerative colitis. Measurements such as weight, disease activity index, HE staining, Prussian blue staining, immunohistochemical and immunofluorescence, ELISA, flow cytometry detection, Western blot, and Quantitative Real-Time PCR were conducted.

Results: Chemical inhibition of NOXs and NLRP3 in vivo could significantly reduce colonic iron overload and macrophage infiltration, thus alleviating colonic inflammatory response and tissue damage. Notably, the inhibition of NOXs significantly inhibited the expression of NLRP3 and oxidative damage, but the inhibition of NLRP3 had no significant effect on the expression of NOXs and oxidative damage, suggesting NOXs may exert their effects other than oxidative damage through NLRP3.

Conclusion: To our knowledge, this work is the first to reveal that NLRP3 mediates NOXs-induced colonic iron overload and inflammation rather than oxidative damage in ulcerative colitis murine model, suggesting that the NOXs might promote ulcerative colitis by inducing colonic iron overload and macrophage infiltration dependent or partially dependent on NLRP3, as well as oxidative damage independent of NLRP3, which imply that both NOXs and NLRP3 are attractive targets for anti-colitis therapy.

Keywords: ulcerative colitis, NADPH oxidases, NLRP3 inflammasome, iron overload, ROS, inflammation

Introduction

Ulcerative colitis (UC) is a chronically relapsing inflammatory disease of the colon and rectum, which typically presents clinically with recurrent abdominal pain, diarrhea, rectal bleeding, and weight loss.[1] In recent years, the prevalence of UC has continued to increase worldwide, which seriously threatens people's health.[2] Although the pathogenesis of UC is not fully understood, its occurrence is closely linked to genetics, gut microbiota, immune response, diet, and environmental factors.[3] Many treatments have been developed to treat this disease. In clinical practice, the short-term goals of the therapy at UC are easy to achieve. However, there are still many difficulties and challenges in achieving its long-term and

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ultimate treatment goals.[4] Therefore, the development of safer and more effective methods for UC prevention and treatment warrants further study.

Iron is one of the essential trace elements in organisms and participates in many physiological processes. Iron homeostasis is maintained by the precision regulation of iron metabolism at the cellular and systemic levels, and its disorders such as iron overload can lead to oxidative stress and inflammatory responses, which in turn lead to the occurrence of various diseases.[5] The role of iron overload in ulcerative colitis has attracted much attention in recent years, and an increasing amount of evidence indicates that iron overload may play an important role in the pathogenesis of UC, while the exact mechanism of triggering iron overload remains unclear.

NADPH oxidases (NOXs) are complexes containing several regulatory protein subunits. The NOX family proteins have 7 subtypes, including NOX1-5 and DUOX1/2. NOXs exist in phagocytes and non-phagocytes, and the generation of reactive oxygen species (ROS) is described as their only function.[6] It is well known that the basic level of ROS plays an important role in maintaining normal physiological functions, while excessive ROS production can result in cell and tissue oxidative damage.[7] In addition, redundant ROS is also considered one of the key factors driving iron metabolism disorders and inflammation reactions.[8] However, the role of NOXs in ulcerative colitis remains incompletely understood, and whether it participates in regulating iron overload and inflammatory response in UC requires further study.

The definition of inflammasome was first proposed in 2002,[9] and the NLR family pyrin domain containing 3 (NLRP3) inflammasome is one of the most thoroughly studied.[10] There is a variety of danger signals that activate the NLRP3 inflammasome, especially ROS, which acts as an intermediate trigger for NLRP3 inflammasome activation, exacerbating the subsequent inflammatory cascade and cellular and tissue damage.[11] Although the contribution of NLRP3 to ulcerative colitis has been well documented in vitro and in vivo studies,[12] the mechanisms of NLRP3 inflammasome activation and its mechanisms of action in ulcerative colitis have not been fully elucidated. For instance, whether the ROS-producing enzymes NOXs regulate iron metabolism and inflammation through NLRP3 in ulcerative colitis remains largely unexplored.

Among various chemically induced colitis models, the dextran sulfate sodium (DSS)-induced colitis model is widely used because of its simplicity and many similarities with human ulcerative colitis.[13] In this study, for the first time, the dextran sulfate sodium (DSS)-induced colitis mice model was used to investigate the roles of NOXs and NLRP3 in iron metabolism, inflammation response, and oxidative damage in colitis, as well as the interaction between them. We found that NLRP3 mediates NOXs-induced colonic iron overload and macrophage infiltration but not oxidative damage in colons of DSS-treated mice, which ultimately leads to the development of colitis, suggesting that except for the pharmacological inhibition of NOXs and NLRP3, the antioxidants such as vitamin E and iron removal or a low-iron diet are potential adjuvant therapeutic strategies for the treatment of UC.

Materials and Methods

Main Chemicals and Reagents

Dextran sodium sulfate (DSS) was purchased from Meilunbio (Dalian, China). Dapansutrile, Diphenyleneiodonium chloride (DPI) and Dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from MedChemExpress (New Jersey, USA). Eosin (H&E) solutions were obtained from Sigma Aldrich (St Louis, MO, USA). ELISA kits for mouse 4-HNE, Hepcidin, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β, IL-8, IL-18, C-reaction protein (CRP) and Iron Assay kits were purchased from mlBio (Shanghai, China). RIPA lysis buffer and phosphatase were obtained from Millipore (Darmstadt, Germany) and protease inhibitor cocktails were obtained from Roche (Basel, Swiss). The BCA protein quantification kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies (anti-NOX1, anti-NOX2, anti-NOX4, anti-NLRP3, anti- anti-Fpn1, anti-FTH, anti-SLC7A11, anti-GPX4, anti-F4/80, anti-CD163, anti-CD68) were purchased from Bioss (Beijing, China) and secondary antibodies were obtained from Servicebio (Wuhan, China). Prussian Blue Staining Kits and Oxidized glutathione (GSSG) assay kits were purchased from Solarbio (Beijing, China). Malondialdehyde (MDA) assay kits and glutathione (GSH) assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China).

Animals and Experimental Design

Animal studies were conducted according to protocols approved by the Yunnan Luoyu Biotechnology Co., Ltd of Experimental Animal Ethics on 10 October 2023. The project follows the ARRIVE Guidelines for Animal Research: In vivo Experimental Reports, and adheres to the ethical principles and requirements stipulated in relevant laws and regulations such as the International Association of Veterinary Editors' Consensus on Author Guidelines for Animal Ethics and Welfare, the Yunnan Province Experimental Animal Management Regulations, and the Yunnan Province Experimental Animal License Management Measures. The ethical committee's identification number was PZ20231001. Thirty specific pathogen-free (SPF) male C57BL/6J mice (20 ± 2 g) were acquired from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China), given free access to food and water at a temperature of 23 ± 2 °C and kept for 12 h dark/light cycle. After one week of conventional adaptive feeding, the mice were randomly divided into 5 groups (n = 6), namely, normal control group (CTRL), DSS-induced ulcerative colitis model group (DSS), DSS + Dapansutrile group (DPS), DSS + Diphenyleneiodonium chloride group (DPI), and DSS + Dapansutrile + Diphenyleneiodonium chloride group (DPS+DPI). Mice in the CTRL group were given free drinking distilled water for 14 days, and mice in DSS, DPS, DPI and DPS+DPI groups were given drinking water with 3% (w/v) DSS for 7 days, which was replaced with distilled water for the following 7 days, meanwhile, mice in DPS, DPI and DPS+DPI groups were intraperitoneally injected with DPI (20mg/kg/d) and/or Dapansutrile (0.00001mg/kg/d) for 14 days, respectively. The dose of DSS in water and intraperitoneal injection doses of Dapansutrile and Diphenyleneiodonium chloride were chosen according to previous reports.[14,15]

All mice were weighed, observed, and recorded daily (eg the stool characteristics, bloody stools, and body weight changes) for 14 days. The mice were euthanized on the Fourteenth day and samples, including serum and colon, were collected. Distal colonic tissues were washed with ice physiological saline. After photographing and measuring the colon length, part of the colon was used to prepare cell suspension for flow cytometry, part of the colon was fixed in 4% paraformaldehyde, and the serum and remaining colon were immediately frozen at −80 °C for future studies.

Disease Activity Index (DAI) Assessment

DAI was evaluated based on changes in body weight, fecal characteristics, and fecal occult blood of mice, according to the study of Wu et al.[16] The scoring criteria were shown as follows: percentage weight loss (0 points, weight unchanged; 1 point, weight loss for 1–5%; 2 points, weight loss for 5–10%; 3 points, weight loss for 10–15%; 4 points, weight loss for>15%), fecal consistency (0 points, normal fecal; 2 points, loose fecal; 4 points, diarrhea), and hematochezia (0 points, fecal occult blood negative; 2 points, fecal occult blood positive; 4 points, gross bloody stool). According to the three indicators for comprehensive scoring, the DAI score was obtained by averaging the total score of the three indicators.

HE Staining and Histological Assessment

The colon fixed with paraformaldehyde was dehydrated, embedded in paraffin, and sliced at a thickness of 5 μm. After being dewaxed with xylene and ethanol, these slices were subjected to hematoxylin and eosin (H&E) according to the kit manufacturer's instructions. Images were obtained using Holographic Scanning Imaging Equipment (3DHISTECH, Pannoramic MIDI, Hungarian), which were used for for observing and analyzing the pathological changes of the colon tissue.

Prussian Blue Staining

After deparaffinized, the 5 μm-thick slices of colonic tissue were stained with Prussian blue, and the specific detection method was performed according to the kit manufacturer's instructions. The images were obtained using Holographic Scanning Imaging Equipment (3DHISTECH, Pannoramic MIDI, Hungarian) and used for iron deposition analysis.

Immunohistochemical and Immunofluorescence

After deparaffinized, the 5 μm-thick slices of colonic tissue were stained with anti-NLRP3 antibody (1:80), anti-NOX1 antibody (1:80), anti-NOX2 antibody (1:80) and anti-NOX4 antibody (1:1000). For immunofluorescence, slices were

incubated in anti-NLRP3 antibody (1:80) and antibodies against NOX1, NOX2 and NOX4 (1:80). Next, slices were incubated with 488-conjugated Goat anti-Rabbit IgG (1:800) at room temperature in the dark for 60 min. Finally, the fluorescent mounting medium containing DAPI was applied to seal up the slides, images were obtained through an upright fluorescence microscope (BX53, Olympus, Japan), and positive cells were analyzed by Image J software (Bethesda, MD, USA).

Serum and Colonic Parameter Measurements

The serum levels of TNF-α, IL-1β, IL-8, IL-18 and IL-6, as well as colon 4-HNE, were measured using ELISA kits. The contents of colon MDA, GSH and GSSG were carried out using biochemical kits. All parameters were determined according to the manufacturers' instructions.

Flow Cytometry Detection of Colon ROS

Fresh colon tissue of the mouse was collected and cut into pieces, which were digested with a 0.2% collagenase I and hyaluronidase-containing digestive solution for 30 min at 37°C. The cells were incubated in 0.1% DCFH-DA at 37°C in darkness for 30 min after centrifugation at 1200r for 3 min. After being washed 3 times in PBS and resuspended in 500 μL PBS, the cells were tested on an ACEA Novocyte 2060R flow cytometer to measure the content of ROS.

Western Blot

Total protein was extracted from colon tissues with RIPA lysis buffer (P0013B, Beyotime, China) and quantified by a BCA protein kit (PC0020, Solarbio, China). Equal amounts of protein samples from each well were separated using 8–10% gradient SDS–PAGE gel and 4% concentrated gel and then transferred to PVDF membranes. After being blocked with 5% non-fat milk, the membranes were incubated with primary antibodies against NLRP3 (1:1000), NOX1 (1:1000), NOX2 (1:1000), NOX4 (1:1000), FTH (1:500), SLC7A11 (1:1000), GPX4 (1:1000), or Fpn1 (1:1000) at 4°C overnight. Next, they were incubated with HRP-conjugated secondary antibodies for 2 h. After washing with TBST 3 times, the enhanced chemiluminescence (ECL) method was utilized to detect the membranes, and images were obtained using the Imaging System (Tanon, Shanghai, China). ImageJ software was used to analyze the grayscale values of each group, and GAPDH was used as an internal control protein.

Quantitative Real-Time PCR for Colon Tissue

Total RNA was extracted from colon tissue according to the manufacturer's instructions of the Trizol Reagent kit. cDNA was then produced using a FastKing RT Kit based on the manufacturer's explanatory notes, which was used for Quantitative real-time PCR (qRT-PCR) with Taq Pro Universal SYBR qPCR Master Mix. The $2^{-\Delta\Delta CT}$ method was used to normalize the expression level of each target gene was normalized to the housekeeping gene GAPDH, and the sequences of primers are shown in Supplementary Table 1.

Statistical Analysis

All data analyses were performed on GraphPad Prism version 8 software, and the data were expressed as mean ±standard deviation. The significant differences ($P < 0.05$) between means of data sets were determined by one-way ANOVA or *t*-test.

Results

NOXs and NLRP3 are Upregulated in Colons of DSS-Treated Mice

DSS-induced UC in mice is the most widely used animal model, as the resulting pathological features correspond well to UC in humans.[13] We thus utilized a DSS-induced colitis mouse model for molecular biology research to identify important proteins that are dysregulated in the inflamed colon, which could be used as novel potential therapeutic targets. DSS-treated mice were given 3% w/v DSS for 7 days, whereas control mice were given distilled water for 7 days (Supplementary Figure 1A). During the experimental progress, compared with the control group, the mice in the DSS

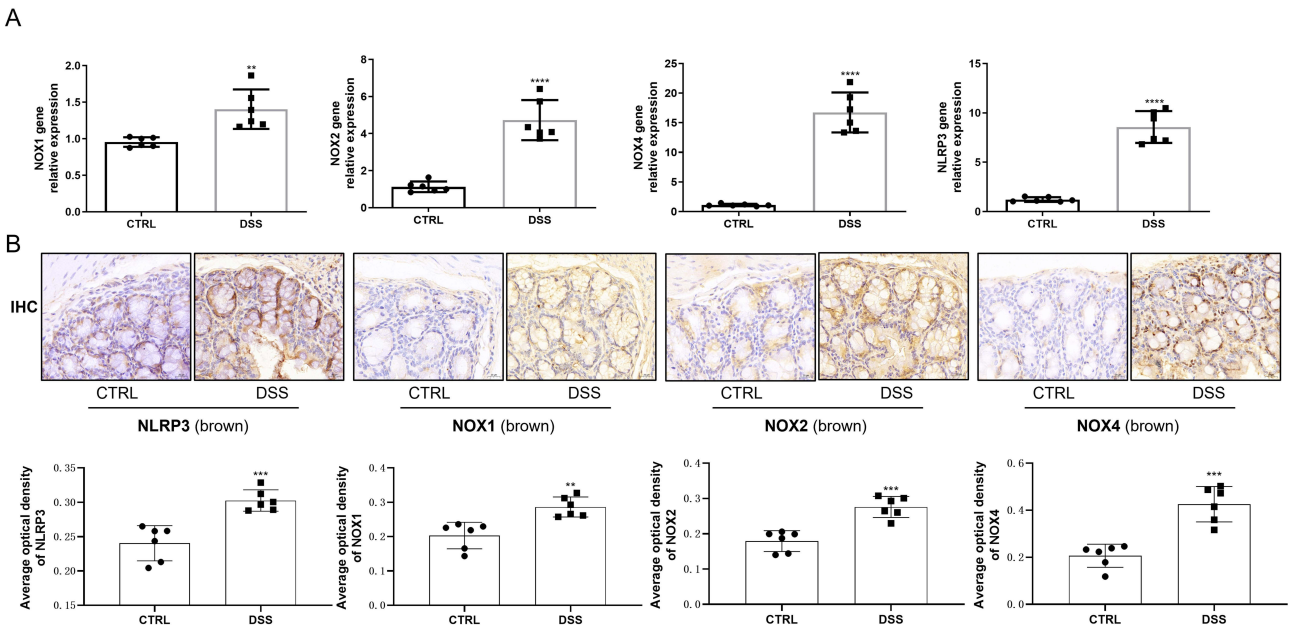

Figure 1 NOXs and NLRP3 are upregulated in colons of DSS-treated mice. (**A**) The expression of mRNA for NOX1, NOX2, NOX4, and NLRP3 in the colon was detected by qRT-PCR. (**B**) IHC staining and the quantification of NLRP3, NOX1, NOX2 and NOX4 in the colon. Scale bars, 20 μm. The results are shown as mean ± SD (n = 6 per group). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus the control group.

group continued to lose body weight and developed diarrhea and hematochezia. These symptoms were most severe on the 7th day, for instance, body weight change and DAI score of the DSS group were significantly lower and higher than that in the control group (Supplementary Figure 1B and C), indicating that the murine model of ulcerative colitis was successfully established, and the mice were killed at the end of the 7th day of modeling to collect colon tissues.

HE staining showed increased tissue damage and infiltration of inflammatory cells in colons of DSS-treated mice with increased pathology scores (Supplementary Figure 1D). In addition, the upregulation of NOXs and NLRP3 in colons of DSS-treated mice was confirmed by RT-qPCR and immunohistochemical (IHC) staining. These data show that the gene and protein levels of NOXs and NLRP3 were significantly increased as colitis progressed (Figure 1A and B). However, further studies are needed to determine whether the upregulation of NOXs and NLRP3 is the cause of colitis development or the negative feedback protective mechanism initiated by the colon tissue.

Pharmacological Inhibition of NOXs and NLRP3 Ameliorates DSS-Induced Colitis

To determine the role of NOXs and NLRP3 in DSS-induced colitis, Diphenyleneiodonium chloride (DPI) and Dapansutrile were used to inhibit NOXs and NLRP3 in vivo, respectively. Control mice were given distilled water for 14 days, while in the first 7 days, DSS-treated mice were given 3% w/v DSS that was replaced with distilled water for the following 7 days. Meanwhile, the DSS-treated mice were administered with DPI (20 mg/kg) and/or Dapansutrile (0.00001mg/kg) daily by intraperitoneal injection for 14 days (Figure 2A).

Compared to the control group, mice in the DSS group were presented with significant weight loss, DAI score increase, and shortening of colon length (Figure 2B–D). However, DPI and Dapansutrile significantly ameliorated weight loss caused by DSS (Figure 2B), and the DAI score of mice in the DPI and Dapansutrile group was lower than that in the DSS group (Figure 2C), implying that DSS-induced symptoms such as diarrhea, hematochezia, and weight loss were relieved by the inhibition of NOXs and NLRP3. Furthermore, DPI and Dapansutrile dramatically reduced DSS-induced shortening of colon length (Figure 2D), indicating that the inhibition of NOXs and NLRP3 can be against the shortening of colon length caused by DSS. Taken together, these results suggest that the inhibition of NOXs and NLRP3 prevent DSS-induced colitis in mice, hinting that the upregulation of NOXs and NLRP3 are causes of colitis development rather than protective mechanisms utilized by the host in the context of colitis.

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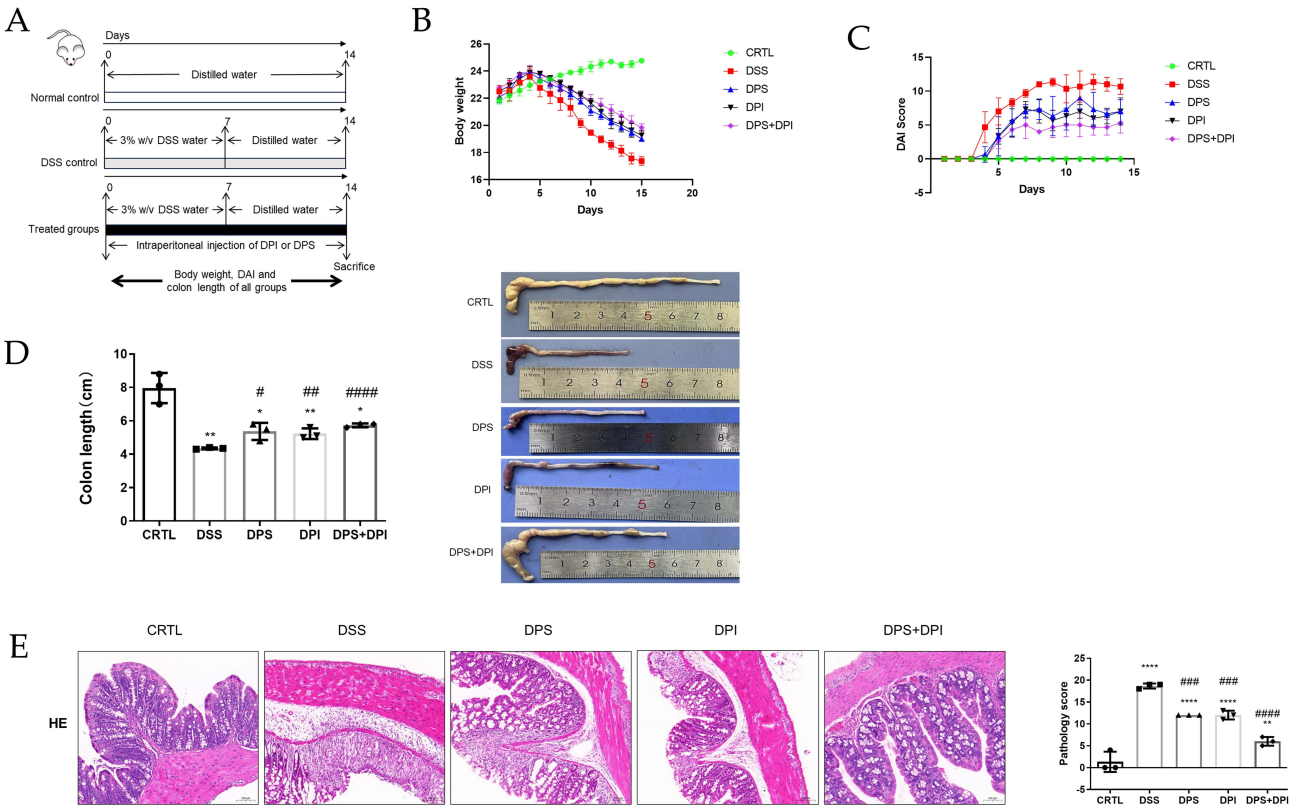

Figure 2 DPS and DPI suppress the symptoms of DSS-induced colitis mice model. (**A**) Schematic diagram of DPS and DPI supplementation; (**B**) Daily body weight of mice; (**C**) DAI of colitis; (**D**)Colon length; (**E**) Colon tissues from mice on day 14 were evaluated by H&E staining and histologic score analysis. Scale bar, 100 μm. The results are shown as mean ± SD (n = 3–6 per group). **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 versus the control group; #*P* < 0.05, ##*P* < 0.01, ####*P* < 0.001, #####*P* < 0.0001 versus DSS-induced colitis group.

DPI and Dapansutrile Decreased Inflammation in the Colon

Next, we examined whether DPI and Dapansutrile decreased inflammation in colons of DSS-treated mice. HE staining showed that compared to the control group, mice in the DSS group presented increased colonic tissue damage and inflammatory cell infiltration (Figure 2E). However, DPI and Dapansutrile significantly mitigated colonic tissue damage and infiltration of inflammatory cells (Figure 2E). The colonic tissue damage was accompanied by augmented expression of colonic pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-18, and TNF-α, which were both significantly restrained by DPI and Dapansutrile (Figure 3A). The same phenomenon was observed with the level change of serum IL-1β, IL-6, IL-8, IL-18, and TNF-α, as well as serum CRP and WBC, indicators of bacterial infections (Figure 3B and C). Moreover, large numbers of macrophages, including CD68 positive cells, F4/80 positive cells, and CD163 positive cells, infiltrated into the mucosa and epithelial layer of the damaged colon, the infiltration of which was dramatically suppressed by DPI and Dapansutrile (Figure 4A–C). Taken together, these results suggest that NOXs and NLRP3 play an important regulatory role in ulcerative colitis, and even hint that they may play a role in the barrier function of intestinal or gut microbiota homeostasis.

Inhibition of NOXs and NLRP3 Alleviated Colonic Iron Overload in the Colon

It is well known that iron metabolism is crucial for intestinal homeostasis, while intestinal inflammation is possibly due to disorders of iron metabolism such as iron overload.[17] We further explored the harmful effect of DSS on the iron homeostasis of mice and the underlying protective effect of DPI and Dapansutrile on that. As shown in the figure (Figure 5A), iron deposition in colons of mice was induced by DSS, which was alleviated by DPI and Dapansutrile treatment, and the increased iron was also found in the serum of DSS-treated mice, but was significantly reduced by DPI and Dapansutrile treatment (Figure 5B), proving that inhibition of NOXs and NLRP3 can alleviate DSS-induced iron

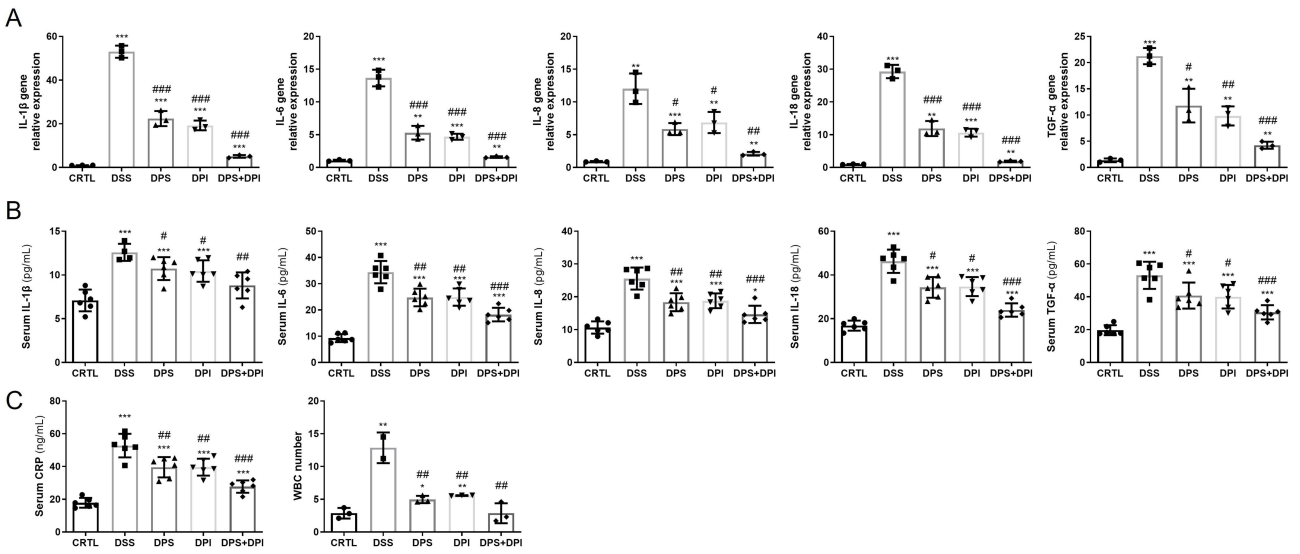

Figure 3 DPS and DPI decreased inflammation in colons of DSS-treated mice. (**A**) The expression of mRNA for IL-1β, IL-6, IL-8, IL-18, and TGF-α in the colon was detected by qRT-PCR; (**B**) The level of serum IL-1β, IL-6, IL-8, IL-18, and TGF-α was measured by ELISA. (**C**) The level of serum CRP and the number of WBC were determined by ELISA and hematology analyzer, respectively. The results are shown as mean ± SD (n = 3–6 per group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus the control group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 versus DSS-induced colitis group.

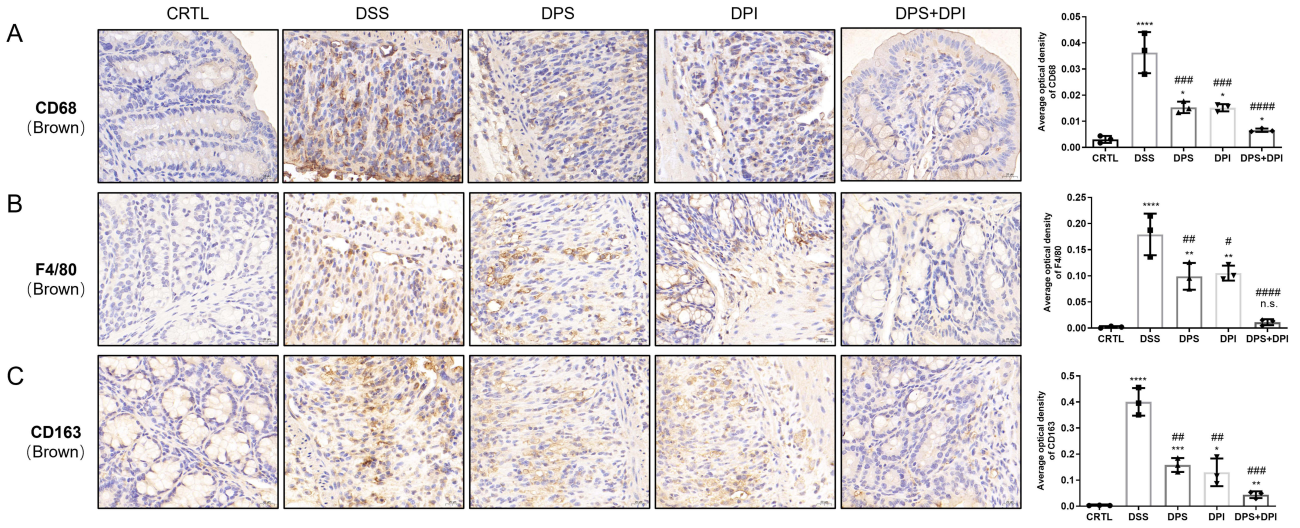

Figure 4 DPS and DPI decreased the infiltration of CD68, F4/80, and CD163 positive cells in colons of DSS-treated mice. IHC staining and the quantification of CD68 (**A**), F4/80 (**B**) and CD163 (**C**) in the colon. Scale bar, 20 μm. The results are shown as mean ± SD (n = 3–6 per group). n.s. no significance, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 versus the control group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, #####*P* < 0.0001 versus DSS-induced colitis group.

overload. The results suggest that NOXs and NLRP3 mediate DSS-induced iron overload. It is worth noting that DSS significantly inhibited FTH expression in colons of mice, which was attenuated by DPI and Dapansutrile (Figure 5C), indicating that NOXs and NLRP3 mediate DSS-induced downregulation of FTH. Previous studies have shown that FTH also has ferroxidase activity, which can convert ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}) to reduce iron toxicity.[18] These results suggest that NOXs and NLRP3 mediate DSS-induced iron overload, which may contribute to increased iron toxicity.

Hepcidin, also known as an inflammatory marker, is a switch that regulates systemic iron metabolism by targeting Fpn and inducing its degradation, resulting in decreased iron absorption from the intestine and increased intracellular iron retention,[19] while the upregulation of Fpn can promote iron excretion, ultimately alleviating iron toxicity.[20] We thus further analyzed the role of DSS in the modulation of hepcidin and Fpn, as well as the effects of DPI and Dapansutrile on

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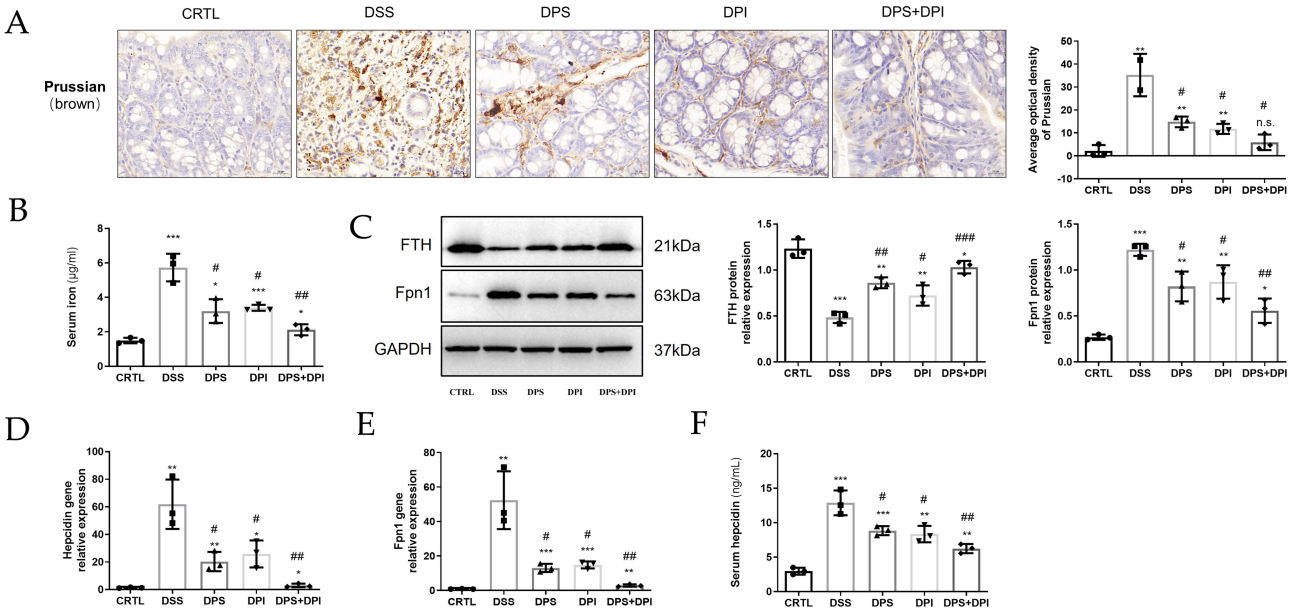

Figure 5 DPS and DPI decreased colonic iron overload and iron toxicity in the colon. (**A**) Prussian blue staining and the qualification data in the colon. Scale bar, 20 μm. (**B**) The level of serum iron was measured by ELISA. (**C**) Western blot analysis of FTH and FPN1 levels in the colon and the quantification data. GAPDH was used as a loading control. (**D** and **E**) The expression of mRNA for hepcidin and Fpn1 in the colon was detected by qRT-PCR. (**F**) The level of serum hepcidin was measured by ELISA. The results are shown as mean ± SD (n = 3–6 per group). n.s. no significance, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus DSS-induced colitis group.

that. We found that DSS significantly upregulated the gene expression of the hepcidin and Fpn1 in the colons of mice (Figure 5D and E), as well as the upregulation of Fpn1 protein expression and the serum hepcidin levels (Figure 5C and F), which were significantly attenuated by DPI and Dapansutrile treatment (Figure 5C–F). Our findings suggest that NOXs and NLRP3 mediate DSS-induced iron overload not through the hepcidin/Fpn1 signaling pathway, and the upregulation of hepcidin and Fpn1 seems to be protective mechanisms rather than causes of iron overload.

Inhibition of NOXs Attenuates DSS-Induced NLRP3 Expression in the Colon

To identify the interaction of NOXs and NLRP3, we examined the effect of DPI on the expression of NLRP3 and the impact of Dapansutrile on NOXs expression. RT-qPCR results showed that compared with the DSS group, mice treated with DPI showed a decrease in NOXs mRNA as well as NLRP3 mRNA, while Dapansutrile decreased NLRP3 mRNA expression but had no effect on the expression of NOXs mRNA in the colons of DSS-treated mice (Figure 6A). Moreover, immunofluorescence and immunoblotting results showed that the protein levels of NOXs in DSS-treated mice decreased upon DPI inhibiting against NOXs, and the expression of NLRP3 protein induced by DSS was also inhibited by DPI treatment (Figure 6B and C). However, Dapansutrile significantly inhibited the induction of NLRP3 protein expression by DSS but had no impact on the expression of NOXs protein (Figure 6B and C). These data suggest that NOXs are involved in regulating NLRP3 in colons of DSS-treated mice, and imply that NOXs may contribute to DSS-induced colitis by regulating NLRP3.

NOXs Promote Oxidative Damage in Colons of DSS-Treated Mice Independent of NLRP3

Oxidative damage is a fundamental mechanism of many diseases, including ulcerative colitis.[21] DSS significantly impaired the antioxidant capacity of the mouse colon by inhibiting SLC7A11 and GPX4 (Figure 7A–E). As shown in the figure (Figure 7F), compared with the normal control group, ROS levels were significantly increased in colons of DSS-treated mice, and DPI treatment inhibited the DSS-induced production of ROS. At the same time, DPI also significantly restored the DSS-mediated decrease in the antioxidant GSH, the increase in the oxidant GSSG, and the reduction in the ratio of GSH/GSSG in the colons of mice (Figure 7G–I), as well as reduced the content of oxidative

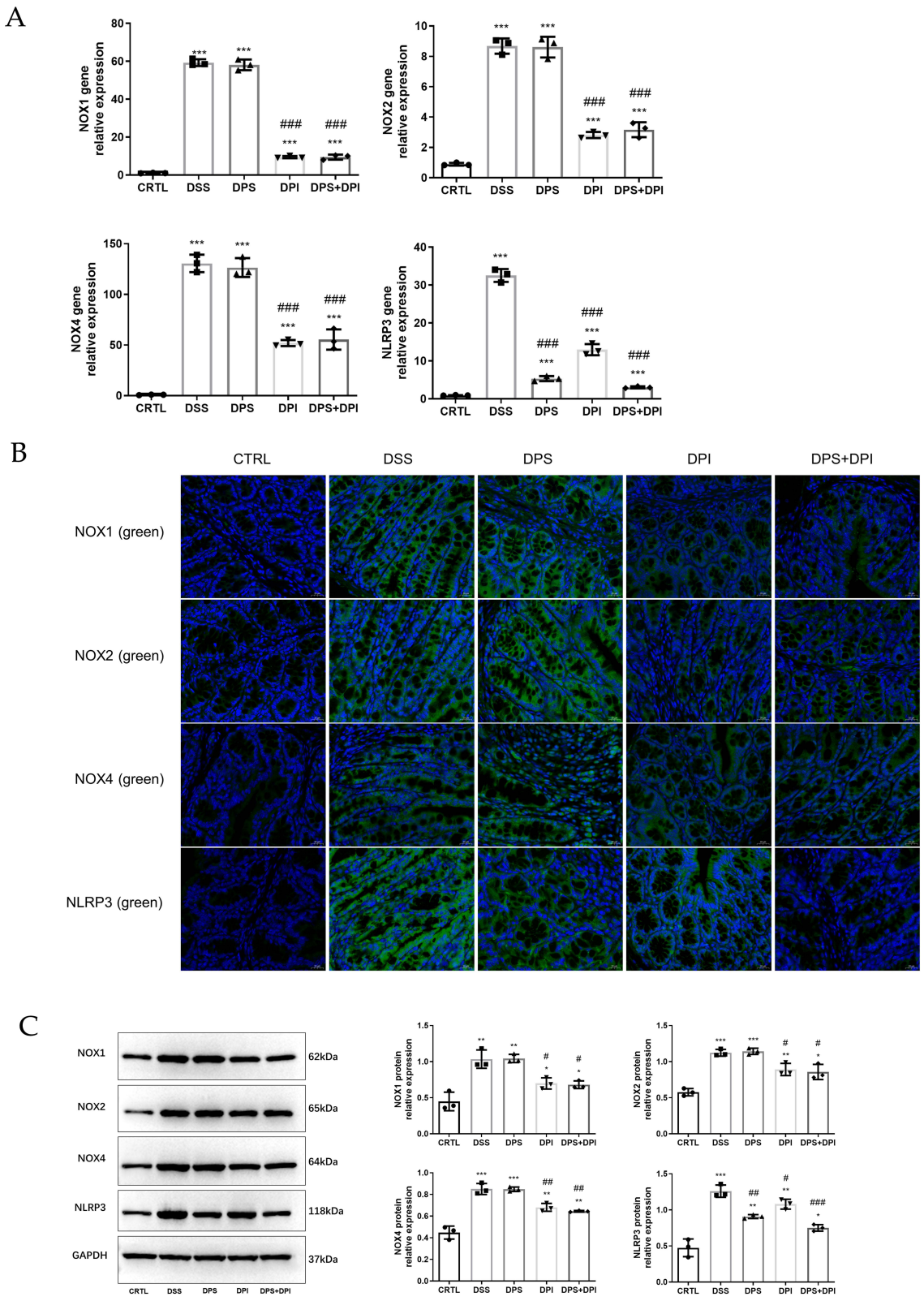

Figure 6 Inhibition of NOXs attenuates DSS-induced NLRP3 expression in colon. (**A**) The expression of mRNA for NOX1, NOX2, NOX4 and NLRP3 in the colon was detected by qRT-PCR. (**B**) Representative immunofluorescence images of colon sections stained with NOX1, NOX2, NOX4 and NLRP3 (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar, 20 μm. (**C**) Western blot analysis of NOX1, NOX2, NOX4 and NLRP3 levels in the colon and the quantification data. GAPDH was used as a loading control. The results are shown as mean ± SD (n = 3–6 per group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus the control group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 versus DSS-induced colitis group.

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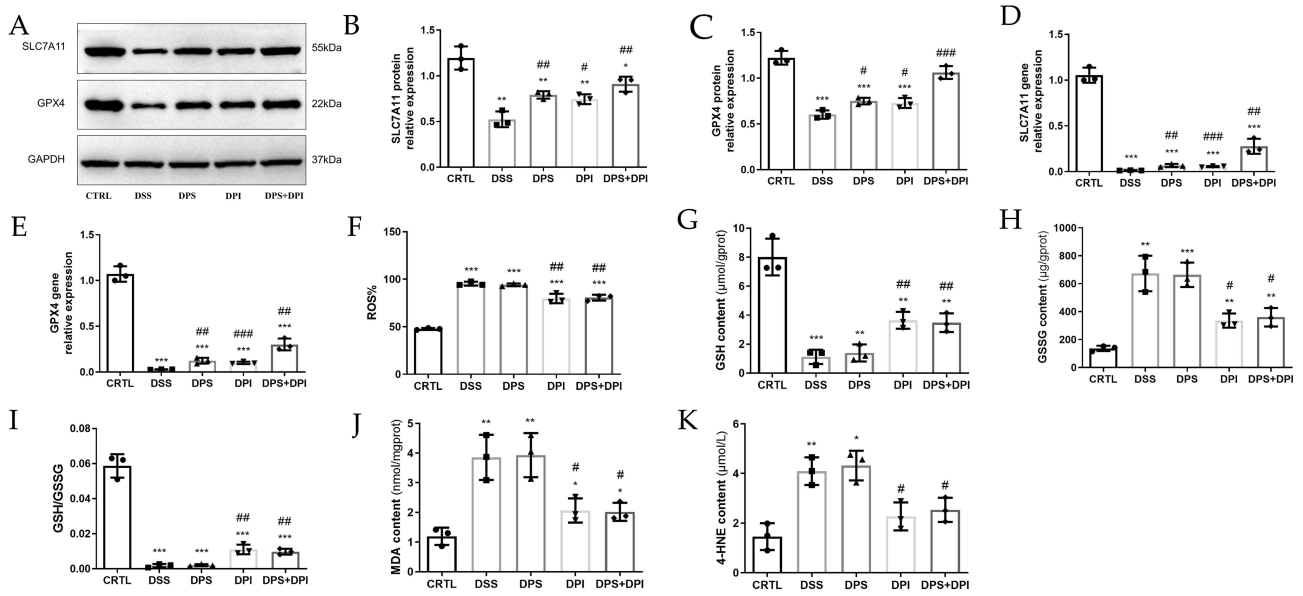

Figure 7 NOXs promote oxidative damage in colons of DSS-treated mice independent of NLRP3. (**A–C**) Western blot analysis of SLC7A11 and GPX4 levels in the colon and the quantification data. GAPDH was used as a loading control. (**D–E**) The expression of mRNA for SLC7A11 and GPX4 in the colon was detected by qRT-PCR. (**F**) Determination of relative ROS levels in the colon by flow cytometry. (**G–I**) GSH, GSSG content in the colon and the ratio of GSH to GSSG. (**J**) MDA content in the colon. (**K**) The level of 4-HNE in the colon was measured by ELISA. The results are shown as mean ± SD (n = 3–6 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.001$ versus DSS-induced colitis group.

damage markers malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the colon (Figure 7J and K). However, Dapansutrile has no significant effect on the above indicators despite restoring antioxidant capacity by upregulating SLC7A11 and GPX4 (Figure 7A–E). These data suggest that NOXs aggravate DSS-induced colitis by promoting oxidative damage independent of NLRP3.

Discussion

Iron overload and inflammation have attracted widespread attention over the past decade due to their important regulatory roles during the development and progression of various diseases.[22,23] This work is the first to investigate the roles of NOXs and NLRP3 in the regulation of colonic iron overload and macrophage infiltration in DSS-induced colitis. We showed here that DSS induced colonic iron overload and macrophage infiltration in mice, which is consistent with the findings of Gao et al and Zhang et al[24,25] Besides that, we also found that NOXs and NLRP3 were significantly upregulated in colons of DSS-treated mice. However, whether NOXs and NLRP3 participate in the regulation of colonic iron overload and macrophage infiltration in UC remains unclear.

Although the role of NOXs participating in the regulation of iron metabolism has not attracted widespread attention, it has been recognized for many years that NOXs are major reactive oxygen species (ROS)-producing enzymes, while ROS is regarded as one of the key factors driving iron metabolism disorders.[8] It is then reasonable rather than exaggeration to consider NOXs as an upstream factor of iron metabolism in UC. The regulation of iron homeostasis plays a crucial role in maintaining normal intestinal function. Disruption of iron homeostasis, particularly iron overload, can contribute to the development of colitis through its significant involvement in modulating oxidative stress and inflammatory pathways.[26,27] Indeed, as shown here, the chemical inhibition of NOXs significantly improved iron overload and macrophage infiltration in colons of DSS-treated mice and effectively ameliorated DSS-induced colitis. Moreover, it is noteworthy that the data of the NOXs inhibition in vivo indicates that NOXs mediate DSS-induced bacterial infection, suggesting that NOXs overexpression may affect the gut microbiome and damage intestinal barrier function, which leads to the translocation of bacteria into the circulation.

On the other hand, the NOD-like receptor protein 3 (NLRP3) inflammasome is one of the most intensively studied inflammasomes to date. Although NLRP3-mediated inflammation involved in ulcerative colitis has been well reported

both in vivo and in vitro studies.[10,28] Its relationship with iron metabolism is poorly understood, and its role and underlying mechanisms in colonic iron overload in ulcerative colitis are even less reported. However, our data on the chemical inhibition of NLRP3 in vivo suggest that NLRP3 mediates iron overload and macrophage infiltration in colons of DSS-treated mice, promoting colitis. It is worth noting that iron overload is one of the key factors driving ferroptosis, and NLRP3 has been widely reported to play a vital role in regulating pyroptosis,[29] which may trigger a discussion on the underlying mechanisms of crosstalk between ferroptosis and pyroptosis in ulcerative colitis.

Systemic iron balance is regulated by hepcidin, a cysteine-rich polypeptide, which can bind to the iron export protein ferroportin (Fpn) and then stimulate its degradation, thereby reducing iron absorption from the intestine into circulation and the intracellular iron release, ultimately resulting in increased cellular iron absorption and iron retention in cells.[5] That is why either overexpression or deficiency of hepcidin can cause iron overload in cells and tissues. Previous reports showed that hepcidin is upregulated in response to high iron levels and inflammatory stimuli, while it is suppressed during periods of increased erythropoietic activity.[30] On the other hand, intracellular iron metabolism is controlled by the action of iron response proteins (IRPs)-iron reactive element (IRE) system. When the level of intracellular iron is deficient, IRPs can interact with IRE to promote iron uptake by upregulating the iron uptake protein TfR1 and inhibit the storage and output of iron by downregulating the iron storage protein FTH and the iron export protein Fpn. However, when intracellular iron is excessive, the effects are the opposite.[20] Therefore, besides being regulated by hepcidin, FPN also responds to intracellular iron levels.

Although this study did not provide definite answers to the underlying mechanism of NOXs and NLRP3-mediated colonic iron overload, their contributions to the upregulation of colonic hepcidin and Fpn1, as well as serum iron and hepcidin, suggest to us that NOXs and NLRP3 may act directly through iron uptake receptors or related molecules (such as TfR1 or DMT1). Meanwhile, this explains why hepcidin and Fpn1 were both upregulated at the same time. As mentioned above, hepcidin overexpression in response to iron signals, hence decreasing/which reduces iron absorption from the intestine by regulating Fpn, while iron overload can also activate the intracellular iron metabolism regulation mechanisms to upregulate Fpn, promoting iron excretion to alleviate iron toxicity.[20,30] These studies imply that the upregulation of hepcidin and Fpn1 seems not to be the cause of colonic iron deposition and colitis, but rather a negative feedback protection mechanism initiated by colon tissue.

In addition, it is worth noting that the data of our ulcerative colitis murine model clearly showed that both NOXs and NLRP3 contribute to colonic iron deposition, while they do not promote FTH expression but instead contribute to FTH inhibition, indicating that the downregulation of FTH may be mainly attributed to its response to NOXs and NLRP3 signals, rather than a response to cellular iron content. Previous reports showed that FTH has the activity of ferroxidase, catalyzing the conversion of ferrous iron to ferric iron and promoting the safe incorporation of iron into the ferritin shell, thereby reducing free iron levels and ROS levels, ultimately reducing iron toxicity.[18] Therefore, the NOXs and NLRP3-mediated downregulation of FTH here seems not to be a negative feedback protection mechanism initiated by colon tissue, but rather a cause of colitis.

Various danger signals can activate the NLRP3 inflammasome, especially ROS, which acts as an intermediate trigger for NLRP3 inflammasome activation that can exacerbate the subsequent inflammatory cascade, resulting in cellular and tissue damage.[31] However, whether NOXs, known as ROS-generating enzymes, regulate the development and progression of UC through NLRP3 remains largely unexplored. As shown in this article, chemical inhibition of NOXs significantly inhibited the upregulation of colonic NLRP3 in our ulcerative colitis murine model, while the inhibition of NLRP3 had no apparent effect on colonic NOXs, indicating that NOXs may exert their effects in UC through NLRP3. However, whether the contribution of NOXs to the development of UC is completely or partially dependent on NLRP3 signaling remains unresolved, and further studies are needed.

Finally, we were surprised not to see that NLRP3 mediates NOXs-induced oxidative damage. Oxidative damage is a fundamental mechanism of many diseases, including ulcerative colitis.[21] It is generally accepted that oxidative damage is caused by an imbalance in the generation and degradation of ROS.[32] A recent study has shown that the lipid peroxidation product 4-hydroxynonenal inhibits NLRP3 inflammasome activation and macrophage pyroptosis,[33] while the role of NLRP3 on lipid peroxidation remains poorly understood. Our data clearly showed a unique inhibitory effect of chemical inhibition of NLRP3 on FTH, SLC7A11 and GPX4 in vivo in colons of DSS-treated mice. As mentioned

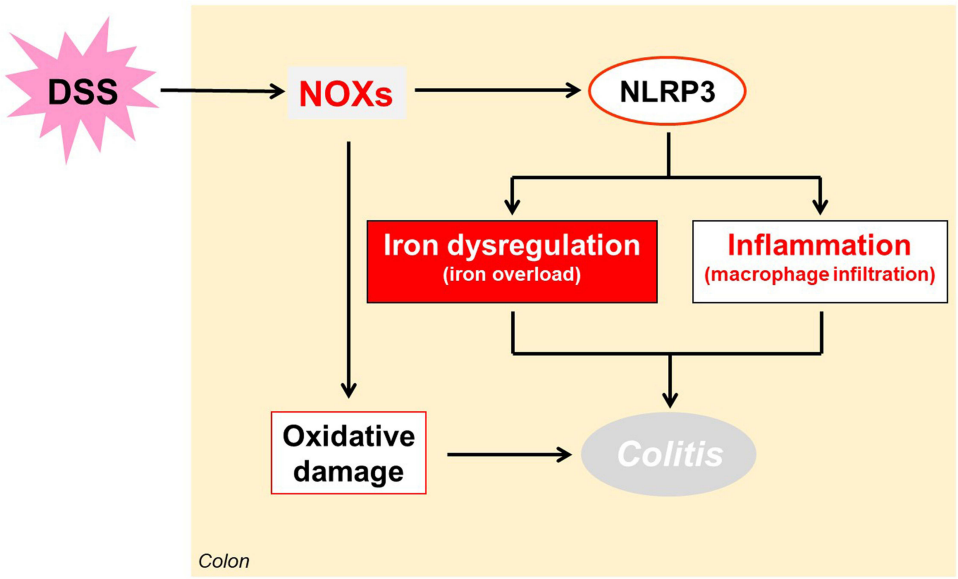

Figure 8 Scheme of the role of NOXs and NLRP3 in DSS-induced colitis.
Abbreviations: DSS, dextran sodium sulfate; NOXs, NADPH oxidases; NLRP3, NLR family pyrin domain containing 3.

above, FTH has ferroxidase activity, converting iron from the divalent form (Fe^{2+}) to the trivalent form (Fe^{3+}) and reducing the production of free radicals within cells, thereby preventing the accumulation of ROS.[18] In addition, SLC7A11 maintains the activity of GPX4 by promoting the synthesis of GSH, and GPX4 removes intracellular peroxides such as lipid peroxide by catalyzing the reaction of reduced glutathione (GSH).[34] It is worth surprising that the effects of NLRP3 on reactive oxidant species ROS, antioxidant GSH, and MDA and 4-HNE, makers of oxidative damage, were not to be observed. Therefore, whether there are compensatory mechanisms to counteract the downregulation of peroxide levels, such as by upregulating lipoxygenase, requires further investigation.

Conclusion

In conclusion, to our knowledge, this work is the first to reveal that NLRP3 mediates NOXs-induced colonic iron dysregulation (eg iron overload) and inflammation (eg macrophage infiltration) rather than oxidative damage in ulcerative colitis murine model, suggesting that the NOXs might promote ulcerative colitis by inducing colonic iron overload and macrophage infiltration dependent or partially dependent on NLRP3, as well as oxidative damage independent of NLRP3, which imply that both NOXs and NLRP3 are attractive targets for anti-colitis therapy. The summary scheme is shown in Figure 8.

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Disclosure

Linna Yu and Meng Wang contributed equally to this work and should be considered co-first authors. The authors report no conflicts of interest in this work.

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