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Inhibition of LSD1 phosphorylation alleviates colitis symptoms induced by dextran sulfate sodium

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Inflammatory Bowel Disease is caused by an acute or chronic dysfunction of the mucosal inflammatory system in the intestinal tract. In line with the results of our previous study, wherein we found that the PKCα-LSD1-NF-κB signaling plays a critical role in the prolonged activation of the inflammatory response, we aimed to investigate the effect of signaling on colitis in the present study. *Lsd1* S112A knock-in (*Lsd1*$^{SA/SA}$) mice, harboring a deficiency in phosphorylation by PKCα, exhibited less severe colitis symptoms and a relatively intact colonic epithelial lining in dextran sulfate sodium (DSS)-induced colitis models. Additionally, a reduction in pro-inflammatory gene expression and immune cell recruitment into damaged colon tissues in *Lsd1*$^{SA/SA}$ mice was observed upon DSS administration. Furthermore, LSD1 inhibition alleviated colitis symptoms and reduced colonic inflammatory responses. Both LSD1 phosphorylation and its activity jointly play a role in the progression of DSS-induced colitis. Therefore, the inhibition of LSD1 activity could potentially protect against the colonic inflammatory response. [BMB Reports 2020; 53(7): 385-390]

INTRODUCTION

Inflammatory Bowel Disease (IBD) is an idiopathic and relapsing inflammatory disorder occurring in the gastrointestinal (GI) tract, and has two major subtypes, namely Crohn's disease and ulcerative colitis (1, 2). Despite symptom similarity, Crohn's disease and ulcerative colitis show distinctive characteristics in damaged regions, pro-inflammatory cytokine profiles and histological phenotypes (1, 2). Crohn's disease shows a non-continuous damaged region in the GI tract (3-5). Conversely, ulcerative colitis involves an extensive area, from the proximal to distal colon, in a continuous fashion (3-5). Various animal models have become useful tools to investigate the mediator of the mucosal immune response and immunological processes underlying acute or chronic intestinal inflammation, improving the understanding of IBD (6, 7). The dextran sulfate sodium (DSS)-induced colitis model is the most widely used in IBD research, even though most IBD experimental models are not appropriate due to disease complexity (8, 9).

DSS is a negatively charged sulfated polysaccharide that can be easily and rapidly administered as a water-soluble agent (10, 11). Although the exact mechanism underlying colitis induction by DSS is unclear, the administration of DSS solution damages the epithelial monolayer cells and mucosal layer in the large intestine. Consequently, gut microbiota enter the colonic lumen and induce a radical immunity imbalance (9). Massive pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, are also released in mouse colon tissues with DSS-induced acute or chronic colitis (6, 12). Therefore, pro-inflammatory cytokines have become a useful maker for colitis in animal models.

Nuclear factor-κB (NF-κB) plays a key role in the regulation of intestinal inflammation and colitis-associated cancer (13-15). The aberrant regulations of canonical and non-canonical NF-κB signaling have been well studied in IBD patients (13-15). This condition induces the release of vast amounts of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which induces tissue damage and immune cell infiltration (16, 17). Post-translational modification of NF-κB by LSD1, NSD1/FBXL11, and PRMT5 is associated with its diverse functions (18-20).

Lysine-specific demethylase 1 (LSD1, also called KDM1A) catalyzes the demethylation of histone H3 (21). H3K4me2 is generally associated with gene activation, while H3K9me2 is associated with gene suppression. Thus, LSD1 acts as a transcriptional activator or inhibitor, depending on the residues of the target histone H3 (22-24). In addition to its well-known H3 demethylase function, LSD1 also demethylates non-histone proteins, including p53, HIF-1α, and the p65 subunit of NF-κB

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(25-27). LSD1 cellular function is controlled by phosphorylation, mediated by several kinases including PLK1, CK2/WIP1, and PKCα (18, 28, 29). Particularly, LSD1 phosphorylation on the 112th serine residue in mice (analogous to 111th serine in human LSD1) by PKCα, was identified as a critical regulatory point for LSD1 functioning in circadian rhythm and inflammatory signaling (18, 30). In inflammatory signaling, lipopolysaccharide (LPS) stimulation of immune cells like macrophages activates PKCα, which then phosphorylates LSD1 on serine-112 (18, 28, 29). Although LSD1 phosphorylation does not alter demethylase activity, it modulates interaction of LSD1 with substrate (18). Phosphorylated LSD1 interacts with monomethylated p65 and removes the methyl group, leading to the stabilization of NF-κB, consequently mediating prolonged inflammatory gene expression (18). Subsequently, *Lsd1*$^{SA/SA}$ mice harboring substitution of 112th serine to alanine, exhibited alleviated symptoms than wild-type mice in the sepsis model (18).

We investigate whether the suppression of the LSD1-NF-κB pathway could reduce colitis and whether an LSD1 inhibitor could alleviate ulcerative colitis symptoms in mice. Consequently, we found that both lack of LSD1 phosphorylation and inhibition of LSD1 activity reduced colitis symptoms, infiltration of immune cells, and pro-inflammatory gene expression in mice.

RESULTS

Lack of LSD1 phosphorylation alleviates the symptoms of DSS-induced acute colitis

Our previous study revealed the involvement of LSD1 phosphorylation on 112th serine residue in the prolonged activation of the NF-κB (18). Here, we investigated whether the phosphorylation plays a role in colitis (where uncontrolled inflammation is the main cause of the disease) using a DSS-induced colitis mouse model. *Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ mice were administered a 2% DSS solution for 5 days (injury phase), followed by normal drinking water for 5 days (recovery phase) (Fig. 1A), which is a modified protocol from Wirtz *et al*. (31). *Lsd1*$^{+/+}$ mice showed progressive weight loss up to 10 days after DSS administration, reaching about 80% of their original body weight. Whereas *Lsd1*$^{SA/SA}$ mice recovered earlier from body weight loss, while exhibiting much milder body weight loss than *Lsd1*$^{+/+}$ mice (Fig. 1B). Disease activity index (DAI) scores increased during DSS treatment in both genotypes, but started to decrease earlier in *Lsd1*$^{SA/SA}$ than in *Lsd1*$^{+/+}$ mice (Fig. 1C), indicating that *Lsd1*$^{SA/SA}$ mice are less susceptible to DSS-induced colitis compared to their wild-type control.

We then obtained the colon tissues from four groups of mice (*Lsd1*$^{+/+}$ versus *Lsd1*$^{SA/SA}$ mice, with and without DSS administration) at 10 days from the start of DSS administration and examined the tissue appearance. Colon length was similar in the absence of DSS administration, in both *Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ mice. DSS administration induced colon length shortening in both genotypes, however, the phenotype was less prominent in *Lsd1*$^{SA/SA}$ mice compared to *Lsd1*$^{+/+}$ mice (Fig. 1D and 1E). The expression of Ki-67 and proliferating cell nuclear antigen (PCNA), marker proteins of cell proliferation, were increased in *Lsd1*$^{+/+}$ colon tissues during DSS administration, whereas the increase was not significant in *Lsd1*$^{SA/SA}$ colon tissues (Fig. 1F and 1G). These results indicate that the increased cell proliferation in *Lsd1*$^{+/+}$ colonic epithelium restores epithelium from DSS-induced damage, and that the reduced increase in Ki-67 and PCNA expression represents less damage in *Lsd1*$^{SA/SA}$ mice. Considered collectively, these data suggest that a lack of LSD1 phosphorylation on 112th serine residue alleviates the symptoms of DSS-induced colitis in mice.

Lsd1$^{SA/SA}$ mice exhibit a reduced colonic inflammatory response upon DSS administration

We then subjected the colon tissues, which had undergone the injury and recovery phases, to hematoxylin & eosin (H&E) staining and examined the detailed histological features. Most

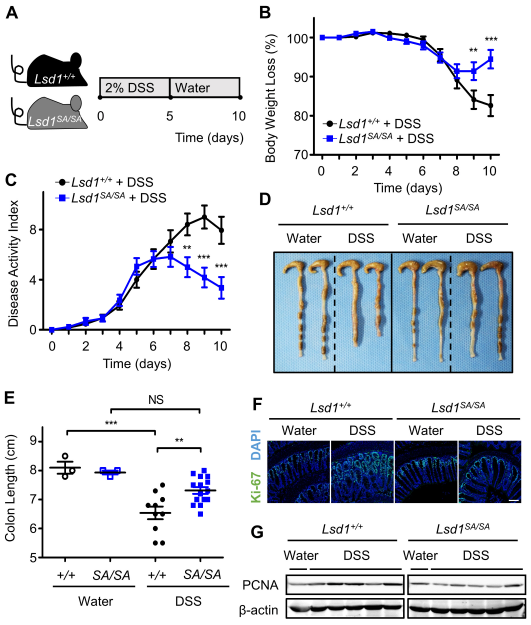

Fig. 1. Susceptibility analyses for DSS-induced colitis by comparing *Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ mice. (A) Schematic diagram of the DSS-induced colitis model. Male mice received 2% DSS in drinking water for 5 days, followed by normal drinking water for 5 days. (B) Body weight loss was measured relative to the initial weight (n = 17 per group). (C) The DAI of colitis was monitored daily (n = 17 per group). (D) Representative pictures of the colon. Colon tissue from each group was obtained 10 days after the first DSS treatment. (E) Graph of colon length. Each symbol represents an individual mouse from each group. (F) Immunofluorescence staining of Ki-67 in colon tissues. Nuclei were stained with DAPI. Scale bar = 100 μm. (G) Immunoblot analysis of PCNA in colons from *Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ mice. β-actin is an equal loading control. Each lane represents a mouse. **P < 0.01, ***P < 0.001 for whole figures.

of the colonic epithelium of *Lsd1*$^{+/+}$ mice were severely damaged and disrupted from DSS administration. Whereas the colon of *Lsd1*$^{SA/SA}$ mice showed less damaged and relatively intact epithelial structure compared to that of *Lsd1*$^{+/+}$ mice (Fig. 2A). Furthermore, histopathological score, measured by macroscopic images of H&E stained whole colonic sections, was also lower in *Lsd1*$^{SA/SA}$ mice (Fig. 2B). Therefore, these data imply that *Lsd1*$^{SA/SA}$ mice exert a protective effect against the intestinal epithelial damage induced by DSS administration in colon tissue. To evaluate the extent of the inflammatory response in the colon, we measured the mRNA levels of proinflammatory cytokines. The mRNA levels of the *Il-1β*, *Il-6*, and *Il-8* genes in colon tissues were similar between *Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ mice from the control group (without DSS administration) and increased two- to five-fold in *Lsd1*$^{+/+}$ mice with DSS administration (Fig. 2C). However, the increase in mRNA levels of all three genes, during DSS administration in *Lsd1*$^{SA/SA}$ mice, was relatively low compared to the control group (Fig. 2C). Additionally, the level of iNOS proteins induced by DSS administration was significantly reduced in *Lsd1*$^{SA/SA}$ mice, compared to that of *Lsd1*$^{+/+}$ mice (Fig. 2D). As additional criteria of the inflammatory response, we examined the infiltration of immune cells in the colon tissues by staining the sections with antibodies against CD4 (T cell), Gr-1 (neutrophil), and F4/80 (macrophage). Fluorescence signals from all three antibodies were clearly detected in the immunofluorescence data of *Lsd1*$^{+/+}$ colon tissues, indicating infiltration of these immune cells to the colon tissue (Fig. 2E). Colon tissues of *Lsd1*$^{SA/SA}$ mice showed much less fluorescence signals from all three antibodies. Collectively, these data suggest that *Lsd1*$^{SA/SA}$ mice are less susceptible to epithelial damage of the colon tissues, induced by DSS administration, with a reduced inflammatory response.

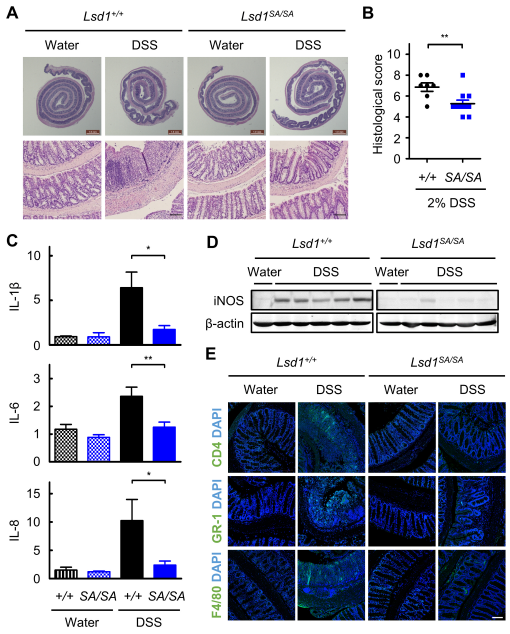

Fig. 2. Analysis of histology and inflammatory response of *Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ mice in DSS-induced colitis. (A) Microscopic images of H&E stained colonic sections. Scale bars, 1 mm (upper panel) and 200 μm (lower panel). (B) Histological scores were determined based on the mucosal hyperemia, surface epithelium loss, and crypt damage. **P < 0.01. Each symbol represents an individual mouse from each group. (C) RT-qPCR data for the expression of pro-inflammatory *Il-1β*, *Il-6*, and *Il-8* genes. (*Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ with water, n = 3; *Lsd1*$^{+/+}$ with DSS, n = 5; and *Lsd1*$^{SA/SA}$ with DSS, n = 7), *P < 0.05, **P < 0.01. (D) Immunoblot analysis of iNOS in colons from *Lsd1*$^{+/+}$ and *Lsd1*$^{SA/SA}$ mice. β-actin is an equal loading control. Each lane represents a mouse. (E) Immunofluorescence staining for CD4, GR-1, and F4/80 in colon tissues. Nuclei were stained with DAPI. Scale bar = 100 μm.

Inhibition of LSD1 activity alleviates colitis symptom by reducing inflammatory responses in mouse colon tissue

We then used GSK-LSD1, a demethylase inhibitor of LSD1, to investigate whether the inhibition of LSD1 activity could alleviate colitis symptoms. GSK-LSD1 (5 mg/kg body weight) was injected intraperitoneally on days 0, 1, and 3 of DSS administration (Fig. 3A). The control group was injected with the same volume of PBS instead of GSK-LSD1. The GSK-LSD1 injection group showed milder symptoms than the PBS group 8 days after acute colitis induction, based on body weight recovery and DAI scores (Fig. 3B and 3C). Colon length shortening was also less severe in the GSK-LSD1 injection group compared to the PBS control (Fig. 3D and 3E). Consistent with the less severe colitis symptoms, Ki-67 expression (representing cell proliferation in colonic epithelium) decreased in the GSK-LSD1 injection group (Fig. 3F). Thus, these results demonstrate the protective effect of LSD1 inhibition in mice, against DSS-induced acute colitis.

Considering that GSK-LSD1 injections produced similar results to *Lsd1*$^{SA/SA}$ mice, colon tissues were subjected to a histological analysis. DSS-administration almost completely destroyed the crypt structure of the colonic epithelium, whereas GSK-LSD1 injection protected colon tissues from damage, resulting in significantly intact crypt structures (Fig. 4A). Histological score also revealed less severe mucosal hyperemia and tissue damage in the GSK-LSD1 injection group, compared to the control group (Fig. 4B). Additionally, we identified a decrease in mRNA levels of proinflammatory cytokines and in immune cell infiltration levels in the GSK-LSD1 injection group, as a measure of the degree of inflammatory response (Fig. 4C and 4D). In summary, DSS destroys the protective barrier of the colonic epithelium, causing an inflammatory response that induces colitis. The absence of LSD1 phosphorylation in *Lsd1*$^{SA/SA}$ mice, or the injection of an LSD1 inhibitor in wild-type mice, reduced the degree of inflammatory responses, which attenuates the progression of colitis due to DSS (Fig. 4E).

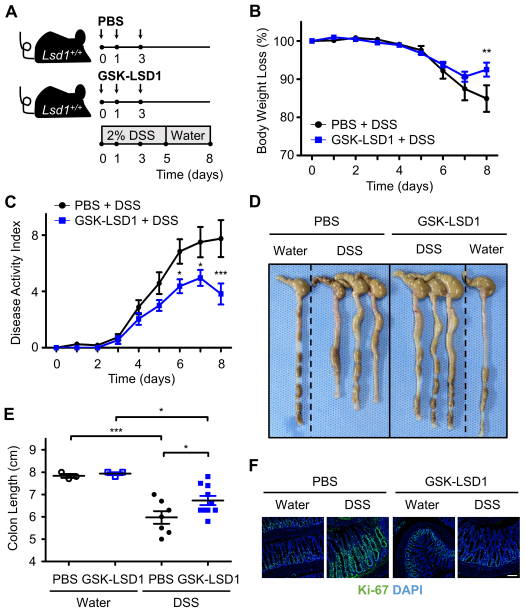

Fig. 3. Effect of GSK-LSD1 on alleviating DSS-induced colitis symptoms. (A) Schematic diagram of a DSS-induced colitis model. All groups were intraperitoneally injected with 5 mg/kg GSK-LSD1 or PBS, in equal volumes, on days 0, 1, and 3. (B) Body weight loss of mice was measured relative to initial weight. (C) The DAI of colitis was monitored daily. Statistical analysis used two-way ANOVA (PBS + DSS, n = 12; GSK-LSD1 + DSS, n = 11), *P < 0.05, ***P < 0.001. (D) Colon tissues of each group were obtained 8 days after the first DSS treatment. (E) Graph of colon length. *P < 0.05, ***P < 0.001. Each symbol represents an individual mouse from each group. (F) Immunofluorescence staining of Ki-67 in colon tissues. Nuclei were stained with DAPI. Scale bar = 100 μm.

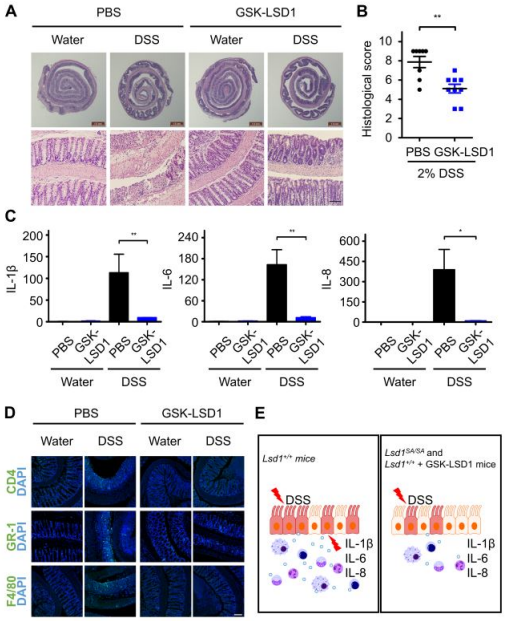

Fig. 4. Effect of GSK-LSD1 on the inflammatory response in DSS-induced colitis. (A) Microscopic images of H&E stained colonic sections. Scale bars, 1 mm (upper panel) and 200 μm (lower panel). (B) Histological scores were measured based on the mucosal hyperemia, surface epithelium loss, and crypt damage. **P < 0.01. Each symbol represents an individual mouse from each group. (C) The amount of pro-inflammatory cytokines *Il-1β*, *Il-6*, and *Il-8* was analyzed with RT-qPCR, 8 days after the start of the experiment. *P < 0.05 and **P < 0.01, (n = 3 per group). (D) Immunofluorescence staining of CD4, GR-1, and F4/80 in colon tissues. Nuclei were stained with DAPI. Scale bar = 100 μm. (E) Graphical overview of this study.

DISCUSSION

Uncontrolled inflammation is a well-known trigger of human IBD (7, 8). In this study, we observed the relief of colitis symptoms along with the downregulation of the inflammatory response in *Lsd1*$^{SA/SA}$ mice and *Lsd1*$^{+/+}$ mice injected with GSK-LSD1. These results indicate that LSD1 phosphorylation and the demethylase activity of LSD1 participate in the progression of inflammatory responses in the experimental colitis model. Thus, the inhibition of LSD1 phosphorylation or LSD1 activity may be utilized to control human IBD.

Recent studies indicated that LSD1 phosphorylation on 112th serine (111th in human) is crucial for a variety of functions, including epithelial-mesenchymal transition (EMT), cancer metastasis, circadian rhythmicity and inflammation (18, 30, 32, 33). Ectopic expression of the LSD1 S111D mutant, mimicking the phosphorylated form of LSD1, promoted the metastatic capacity of MDA-MB-231 breast cancer cells in nude mice (32). Additionally, PKCθ-mediated phosphorylation of LSD1 on the residue was critical for its demethylase and EMT promoting activity in breast cancer (33). PKCα is the first enzyme identified as a kinase, responsible for LSD1 phosphorylation on the residue, in the regulation of the circadian cycle (30). LSD1 phosphorylation by PKCα is also critical for the prolonged activation of NF-κB signaling (18). Intestinal epithelial cells express at least 10 PKC isoforms; not only "classic" PKC isoforms like PKCα, but also "novel" PKC isoenzymes like PKCθ (34). Although PKCα phosphorylated LSD1 upon LPS stimulation (18), DSS-induced colitis is a complex process involving a number of proinflammatory agents. We therefore cannot rule out the possibility that LSD1 is phosphorylated by PKCα as well as other kinases, including PKCθ, in colitis models.

In the previous study we successfully used GSK-LSD1, an inhibitor of LSD1 demethylase activity, to reduce systemic inflammation induced by LPS or CLP (cecal ligation and puncture) (18). The demethylase activity of LSD1 is required for demethylation of methyl-p65, a subunit of the NF-κB transcription factor, resulting in the stabilization of p65 and

prolonged activation of NF-κB. Inhibition of LSD1 by GSK-LSD1 resulted in the accumulation of mono-methylated p65, which was eventually degraded by the ubiquitin-proteasome system resulting in premature termination of the NF-κB-mediated inflammatory responses. In this study, we identified the beneficial effect of GSK-LSD1 on the DSS-induced colitis mouse model. Relief of colitis symptoms was similar to what we saw in *Lsd1*$^{SA/SA}$ mice. Since *Lsd1*$^{SA/SA}$ is a whole-body knock-in mouse, it is not possible to pinpoint which cell type (i.e., colonic epithelial cells or immune cells) is involved in alleviating colitis symptoms through the lack of LSD1 phosphorylation or LSD1 inhibition. Since cytokine crosstalk between immune and epithelial cells affects colitis, the suppression of inflammatory responses in both cell types, through the inhibition of LSD1 phosphorylation or its activity, most likely contributes to the relief of colitis symptoms.

Currently, LSD1 is considered a validated epigenetic target for anticancer drug development, with many LSD1 inhibitors in clinical trials as anticancer agents for AML (acute myeloid leukemia), SCLC (small-cell-lung cancer) and other solid tumors (35, 36). In this study, we showed the potential of LSD1 inhibitors as anti-inflammatory agents that can alleviate colitis symptoms. Since high levels of LSD1 expression have been identified in inflammation-associated cancers, including colorectal cancer (36), the LSD1 inhibitor, GSK-LSD1, has therapeutic potential in colitis and may be good candidates for limiting colitis-associated cancer (CAC) progression, by targeting the microenvironment as well as the cancer itself.

MATERIALS AND METHODS

Mice

Generation of *Lsd1*$^{SA/SA}$ mice has been described previously (30). Mice were handled in accordance with the animal handling guidelines of Sookmyung Women's University. Procedures were approved by the Institutional Animal Care and Use Committee of the University (SMWU-IACUC-1711-029).

DSS-induced colitis

Male mice with corresponding age (7-9 weeks) and body weight (20-25 g) received 2% DSS (MP Biomedicals, M.W. 36-50 kDa) in drinking water for 5 days, followed by normal drinking water for 5 days. In the LSD1 inhibition groups, wild-type mice were intraperitoneally injected with the LSD1 inhibitors, GSK-LSD1 (5 mg/kg body weight, Sigma) or PBS, on days 0, 1, and 3 of 2% DSS administration. Control mice received DSS-free drinking water. The DAI of colitis was monitored daily, based on Supplementary Table 1.

Hematoxylin & Eosin (H&E) and immunofluorescence staining

Mouse colon tissues were fixed in a 4% paraformaldehyde solution at 4°C for 2 days and embedded in paraffin. All samples were cut into 4 μm sections for H&E staining. Microscopic images were obtained through light microscopy (Olympus IX71 and Leica M80). Histological scoring was based on three categories, namely, mucosal hyperemia, surface epithelium loss, and crypt damage (Supplementary Table 2). Immune cell infiltration into colon epithelium was analyzed with immunofluorescence staining. The primary antibodies used were anti-Ki67 (Abcam, ab15580), anti-CD4 (BD Biosciences, #553047), anti-Gr-1 (Serotech, #MCA2387GA), and anti-F4/80 (Serotech, #MCA497GA).

Quantitative RT-PCR of mRNAs from colon tissues

Mouse colon tissues were homogenized in a Trizol reagent (Life Technologies) and total RNA was isolated using the GeneJET RNA Purification Kit (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the cDNA synthesis kit (Thermo Fisher Scientific). Primers used for mouse genes are in Supplementary Table 3.

Western blotting

Colon tissues were washed with ice-cold PBS and solution B (2.7 mM KCl, 150 mM NaCl, 1.2 mM KH_2PO_4, 680 mM Na_2HPO_4, 1.5 mM EDTA, and 1 mM DTT). Fragments of the distal portion were then homogenized in RIPA buffer (150 mM NaCl, 20 mM Tris pH 7.5, 0.1% SDS, 1% NP-40, and 2 mM EDTA). Proteins were resolved on 8-15% gradient gels and transferred onto a nitrocellulose membrane (GE Healthcare). The primary antibodies used were anti-β-actin (AB Frontier), anti-iNOS (Merck Millipore), and anti-PCNA (Abcam).

Statistical analysis

Statistical analyses were performed with GraphPad Prism v5.01 software. The statistical significance of the two groups was analyzed by two-way ANOVA (Body weight and DAI) or Mann-Whitney test (Histological score). One-way ANOVA (Newman keule's test) was used for the statistical analysis among four groups. Data were expressed as the mean ± SEM.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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