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Research Paper

SOD1 suppresses pro-inflammatory immune responses by protecting against oxidative stress in colitis

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ABSTRACT

Superoxide dismutase 1 (SOD1) binds copper and zinc ions and is one of three superoxide dismutases responsible for destroying free superoxide radicals in the body. Reactive oxygen species (ROS), including free superoxide radicals, play important roles in colitis. However, the role of SOD1 in oxidative stress under colitis remains unclear. Here, we examined the role of SOD1 in the DSS-induced mouse model of colitis. SOD1 deficiency resulted in severe oxidative stress with body weight loss, epithelial barrier disruption and decreased antioxidant enzyme activities. The levels of neutrophils, monocytes, pro-inflammatory $CD11c^+$ macrophages and $CD11b^+CD103^-$ dendritic cells (DCs) were increased, while anti-inflammatory $CD206^+$ macrophages and $CD11b^-CD103^+$ DCs were decreased, in DSS-treated SOD1-knockout (KO) mice compared to DSS-treated wild-type mice. Furthermore, rescue of SOD activity in SOD1-KO mice by oral gavage of *B. amyloliquefaciens* SOD (BA SOD) significantly ameliorated enhanced DSS-induced colitis in these mice by suppressing p38-MAPK/NF-κB signaling, which can induce inflammation and apoptosis. Taken together, our results suggest that SOD1-mediated inhibitory responses play a crucial role in limiting the development of DSS-induced colitis, and that BA SOD is a promising candidate for treating colitis.

1. Introduction

Superoxide dismutase 1 (SOD1) is an enzyme that is important in defending tissues against oxidative stress; it acts by catalyzing the conversion of superoxide radicals (O_{2^-}) to hydrogen peroxide, which can then be further reduced to water [1]. Among the known antioxidant enzymes, SOD is thought to play a central role through its ability to scavenge superoxide anions, which represent the primary reactive oxygen species (ROS) generated from molecular oxygen in cells. ROS can adversely affect various important classes of biological molecules, such as nucleic acids, lipids and proteins, thereby altering the normal redox status and increasing oxidative stress [2]. Accumulating evidence shows that oxidative stress plays an essential role in the pathogenesis and progression of inflammatory bowel disease (IBD). In recent years, increasing attention has been paid to the role of ROS in the pathogenesis of IBD [3–6]. Thus, the removal of ROS by SODs has been suggested as an effective and preventive strategy against IBD [7]. SOD1 deficiency is thought to form a link between ROS and inflammation in various pathogeneses, such as amyotrophic lateral sclerosis (ALS) [8]. In addition, genetic polymorphisms of SOD1 have been associated with the susceptibility to ulcerative colitis (UC). For example, the G allele of the SOD1 A251G polymorphism decreases the risk of UC, suggesting that this allele has a protective role [9]. However, further work is needed to explore how these polymorphisms are linked to oxidative stress and/or specific phenotypes. IBD is generally thought to result from a complex interplay of susceptibility and immune system dysregulation [10]. However, the role of SOD1 in IBD has not yet been fully explored in an *in vivo* colitis model.

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The innate immune system, which is the first line of defense against invading pathogens, combines the actions of various mechanisms ranging from physical barriers to cellular components [11]. In IBD, the recruitment of inflammatory cells into the inflamed intestine is a key mechanism that contributes to perpetuating and amplifying the inflammatory damage [12]. Macrophages and dendritic cells (DCs) are largely responsible for the innate immune response in the intestinal mucosa [13]. The mouse large intestine contains four distinct DC subsets that can be identified based on their expression levels of CD11b and CD103 [14]. $CD103^{+}CD11b$- DCs modulate the intestinal lymphocyte-mediated secretion of IFN-γ to trigger the expression of IFN-γ-inducible epithelial genes, including those encoding various well-characterized anti-inflammatory molecules that contribute to containing intestinal inflammation [15]. Macrophages express CD206, which is a marker associated with anti-inflammatory macrophage function [16], and $CD11c^{+}$ macrophages express pro-inflammatory cytokines [17]. In DSS-induced colitis, the inflammatory lesions exhibit massive production of pro-inflammatory cytokines. $CD11b^{+}$ DCs, macrophages and neutrophils produce various pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-12, IL-17 and IFN-γ [18]. IL-10 is a predominant anti-inflammatory cytokine that plays an essential role in maintaining gastrointestinal homeostasis [19]. During the development of colitis, inflammation dysregulates the coordinated balance between mucosal immune cells and various soluble factors. Notably, however, this may be significantly reduced by treatment with SOD1 [20].

In the present study, we examined whether SOD1 can protect against colitis by modulating innate immune responses, tested whether SOD1 deficiency could be rescued by administration of BA SOD and sought to elucidate the underlying anti-inflammatory immune responses. We report that, under DSS-induced colitis, SOD1 protects the histological mucosal barrier, balances the immune system and inhibits inflammation by blocking immune cell entry via regulation of the intestinal immune response. We also demonstrate that administration of BA SOD to DSS-treated SOD1-knockout (KO) mice protects against colitis by inhibiting p38-MAPK/NF-κB signaling. In summary, we herein elucidate the exact role of SOD1 using an *in vivo* genetic model and *in vitro* culture, and revealed the anti-inflammatory mechanisms of BA SOD in DSS-induced acute colitis.

2. Materials and methods

2.1. Mice

$SOD1^{+/-}$ mice (B6.129S7-Sod1tm1Leb/DnJ) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and crossbred to generate $SOD1^{+/+}$ and $SOD1^{-/-}$ mice. Six-week-old male littermate mice were used for this study. All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) of Ewha Womans University and followed National Research Council Guidelines.

2.2. Cell culture and small interfering RNA (siRNA)

The HCT-116 human colorectal carcinoma cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Hyclone) at 37 °C in 5% CO_2 saturated humidity. The predesigned SOD1 siRNA (Bioneer) and control siRNA (Bioneer) were transfected using the NEON microporation system (Invitrogen). Each electroporation was performed in a 10-μl tip containing 5×10^6 cells. Optimal electroporation results were achieved using a single 20-ms pulse at 1530 V. The gene silencing efficiency was examined by Western blot analysis after 48 h of transfection. Where indicated, cells were stimulated with LPS (1 μg/ml; Sigma Aldrich) for 3 h or 24 h.

2.3. Bacillus amyloliquefaciens (BA) SOD

BA SOD and the endospores used in this study were provided by GenoFocus, Inc. (Daejeon, South Korea). BA SOD is an enzyme produced by *B. amyloliquefaciens* GF423 (patent accession number KCTC 13222BP), which was obtained as a pure isolate from Binex Co., Ltd. BA SOD and endospores were prepared as described by Kang et al. [21]. Briefly, *B. amyloliquefaciens* GF424 was grown in tryptic soy broth at 37 °C for 24 h. The culture supernatant was collected by centrifugation and filtered through a 0.25-μM pore-size membrane (Sartorius) and then concentrated 10-fold by ultrafiltration using a polyethersulfone membrane (Sartorius) with a molecular weight cut-off of 10 kDa. Enteric coating was conducted as follows: concentrate was mixed with the same volume of 0.1% [w/v] shellac solution dissolved in distilled water and 5% ethanol, and the mixture was lyophilized. For preparation of endospores, *B. amyloliquefaciens* was grown in tryptic soy broth at 37 °C for about 48 h. The precipitate obtained by centrifugation at 7500 rpm for 10 min at 4 °C was washed with phosphate-buffered saline (PBS) solution (Sigma Aldrich), and the endospores were purified from the precipitate. Mice were administrated with vehicle (PBS), BA SOD (20 U) or BA SOD (20 U) + spores (1×10^7) daily by oral gavage for a total of 16 days beginning 1 week prior to colitis induction. To assess the therapeutic effect, a subset of mice were administrated with vehicle (PBS) or BA SOD (20 U) + spores (1×10^7) for 7 days after DSS induction.

2.4. Induction and assessment of DSS-induced colitis

Colitis was induced by exposing mice to 2% (w/v) DSS (molecular weight 36–50 kDa; MP Biomedicals)-containing drinking water for 9 days. The colon length was measured at day 16. The severity of each DSS case was assessed daily using a disease activity index (DAI) [22]. Body weight loss, stool consistency and fecal bleeding were scored on a scale of 0–4, with 4 indicating the highest severity, and the DAI was calculated as the combined score of: (weight loss, stool consistency and fecal bleeding)/3. Histological severity was scored based on a scoring system in which crypt abscesses and epithelial erosion are scored on a scale of 0–3 [23]. The histological score was calculated as: the combined scores from four sections/4.

2.5. Hematoxylin and eosin (H & E) staining

Formalin-fixed colon samples were dehydrated in an ethyl alcohol series (70–100% ethyl alcohol), dealcoholized in xylene, embedded in paraffin and cut into 5-μm sections. Colon tissues were deparaffinized in xylene, rehydrated in an ethyl alcohol series (100-70%) and stained with H&E. The slides were dehydrated, dealcoholized, mounted using mounting medium (Leica Biosystems), and assessed for inflammation and tissue damage using an Olympus microscope (Olympus).

2.6. Immunohistochemistry

We performed immunofluorescence staining on 5-μm-thick cryosections that were prepared from formalin-fixed tissues and mounted on slides. For staining, we used anti-E-cadherin (R&D Systems) and goat IgG isotype control (Santa Cruz) as the primary antibodies and anti-goat Alexa 594 (Invitrogen) as the secondary antibody. Nuclei were counterstained with DAPI. Digital immunostaining images were captured and analyzed using an LSM780 confocal microscope (Carl Zeiss).

2.7. Isolation of colonic lamina propria (LP) cells

The large intestines of mice were removed and washed in ice-cold PBS (Invitrogen), and the fat was removed. The intestines were opened longitudinally, washed in PBS, and cut into 1-cm sections. Whole colonic segments were prepared by incubation with 5 ml of an enzyme mixture containing 0.5 M EDTA (Duchefa) and 1 M DTT (Goldbio) in

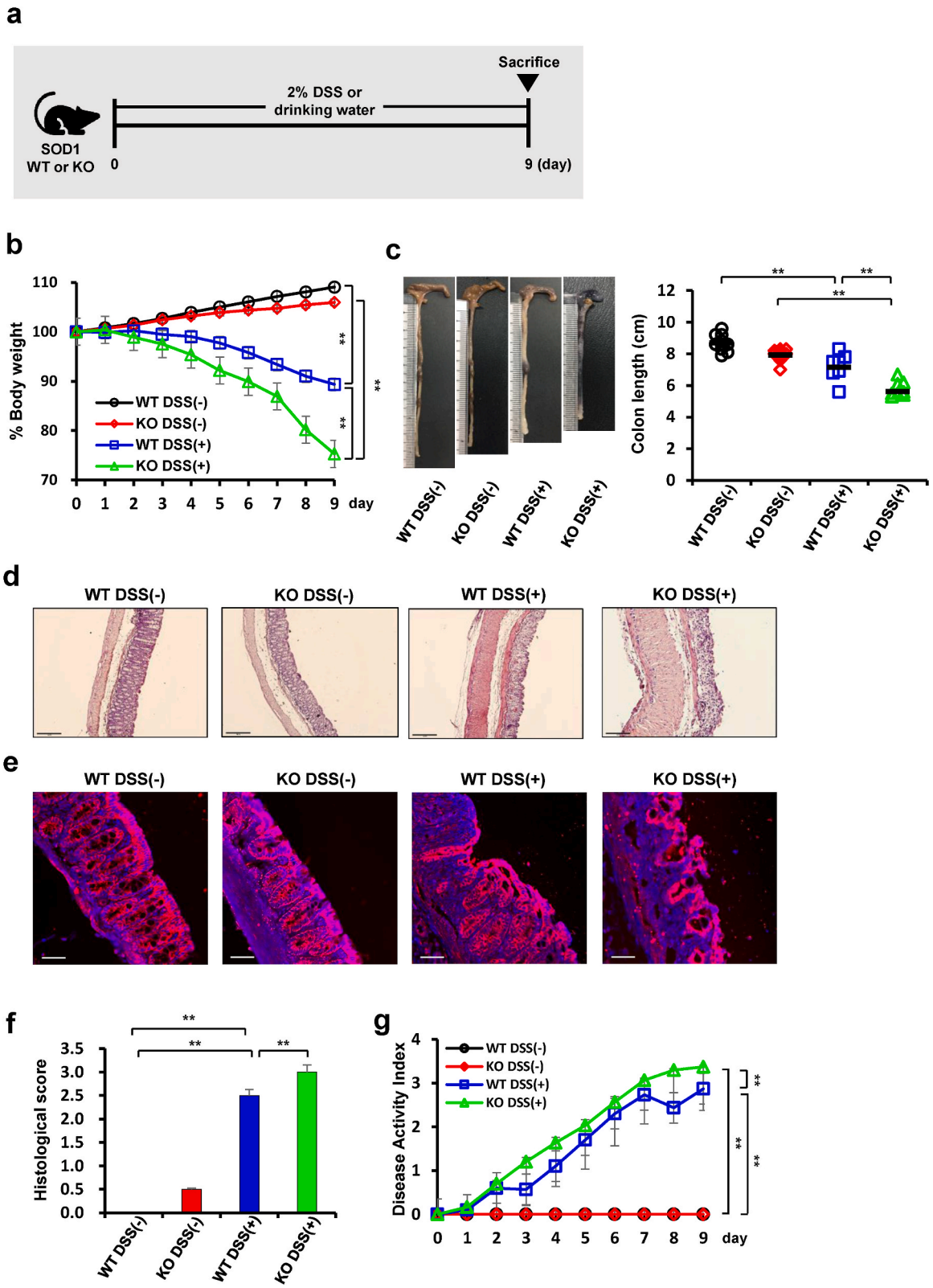

Fig. 1. SOD1 deficiency promotes DSS-induced colitis in mice. (a) Experimental scheme. SOD1 WT and KO mice were exposed to 2% DSS dissolved in their drinking water for 9 days, while normal control mice received distilled water. (b) Change in weight over time is expressed as the percentage of the initial body weight. (c) The colon length was measured at day 16. (d) Representative microscopic images of H&E-stained colon sections. Scale bars, 100 μm. (e) Representative microscopic images of immunofluorescence-stained colon sections. E-cadherin (red staining) and DAPI-stained cell nuclei (blue staining) are shown. Scale bars, 100 μm. (f) For histological scoring, a measure of crypt abscesses and epithelial erosion was calculated. (g) DAI, which is a measure of weight loss, stool consistency and blood in the stool, was calculated. n = 8–10 mice per group. Results were significantly different at the $^{**}P < 0.01$ level, as assessed by one-way ANOVA followed by Bonferroni correction. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Hank's balanced salt solution without calcium or magnesium (Welgene) for 30 min at 37 °C with gentle mixing. Cell suspensions were prepared by filtration using a 100-μm cell strainer (SPL Life Sciences), 5 ml of PBS was added, the filtration step was repeated, and the remaining tissue was cut into 0.5-mm sections using scissors. The pieces were digested with 5 ml of enzyme mixture containing collagenase D (Roche), DNase I (Sigma Aldrich), and Dispase II (Sigma Aldrich) in Hank's balanced salt solution with calcium and magnesium, using a gentleMACS C tube

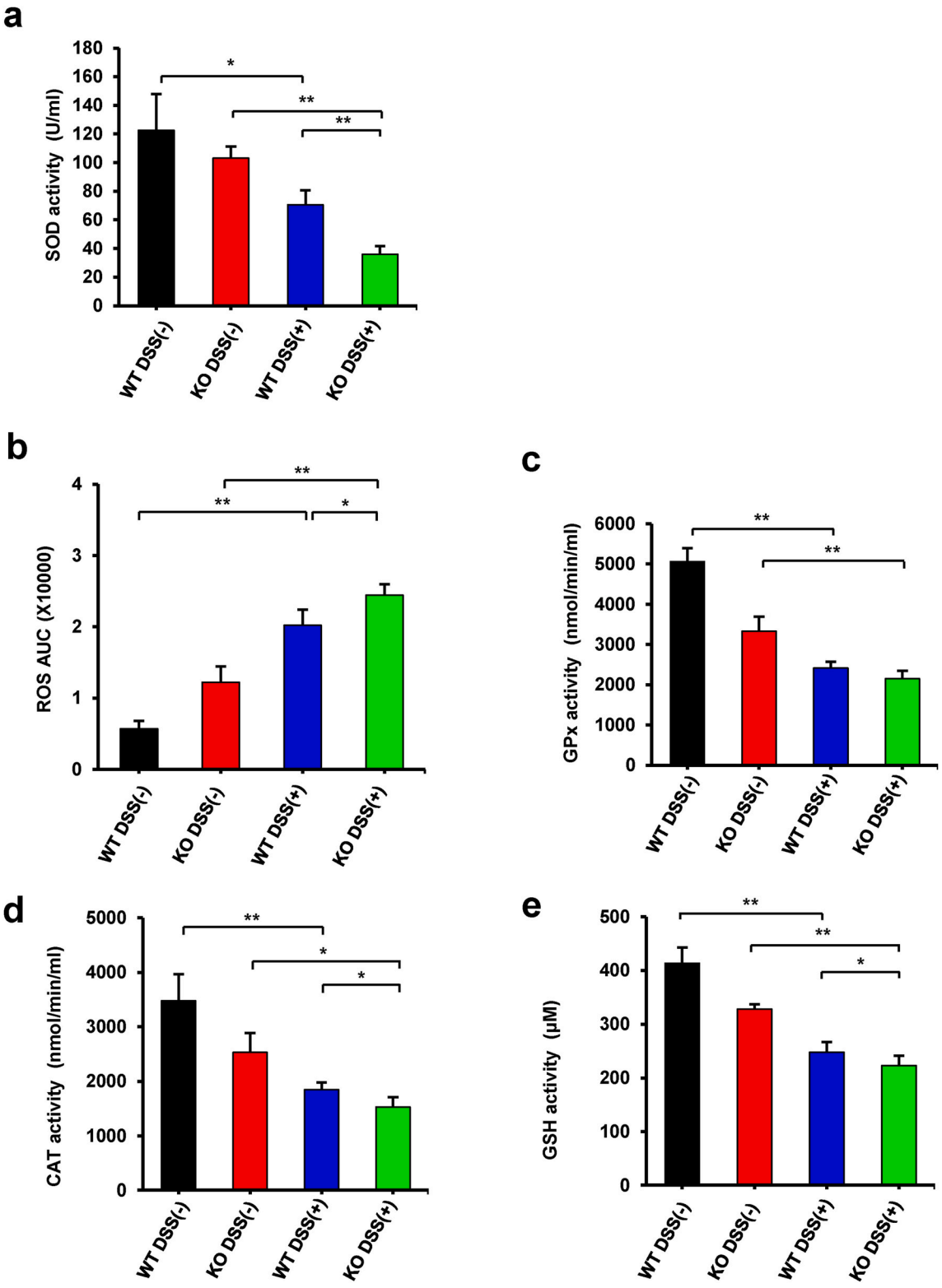

Fig. 2. SOD1 deficiency reduces antioxidant enzyme activities. SOD1 WT or KO mice were exposed to 2% DSS dissolved in their drinking water for 9 days, while normal control mice received only distilled water. (a) SOD activity in erythrocytes. (b) ROS level in plasma. (c–e) Catalytic enzyme activities of (c) GPx, (d) CAT and (e) GSH in erythrocytes. n = 5–10 mice per group. Results were significantly different at the $^{*}P < 0.05$ and $^{**}P < 0.01$ levels, as assessed by one-way ANOVA followed by Bonferroni correction.

attached to a gentleMACS Dissociator (Miltenyi Biotec). The mixture was incubated for 30 min at 37 °C with automated rotation and filtered using a 70-μm cell strainer (SPL Life Sciences), and the digestion step (5 ml enzyme mixture) was repeated. The sample was centrifuged at 13,000 rpm for 10 min at 4 °C, and the cell pellets were collected.

2.8. Flow cytometric analysis

Cell-surface markers were assessed by staining cells. Blocking was performed with purified anti-mouse CD16/32 (Fc block, BD Biosciences), and the samples were incubated for 30 min at 4 °C with fluorochrome-conjugated antibodies against CD45 (30-F11), CD3 (17A2), CD11b (M1/70), Ly6C (HK1.4), CD11c (N418), MHCII (M5/114.15.2), Ly6G (1A8), CD103 (2.00E+07) and CD206 (C068C2) at a 1:400 dilution and F4/80 (BM8) at a 1:100 dilution. These antibodies were purchased from BioLegend. Monitoring of apoptosis was performed by staining with anti-Annexin V antibody and PI (BD Biosciences). Cells negative for PI staining but positive for anti-Annexin V staining were defined as early apoptotic cells, and those double positive for PI and anti-Annexin V staining were defined as late apoptotic cells. Stained cells were analyzed using a BD LSRFortessa flow cytometer (BD Biosciences) and the data were processed using the FlowJo software (Tree Star).

2.9. ROS level and antioxidant enzyme activity analysis

To measure the ROS level in plasma, we performed a luminol-dependent chemiluminescence (LmCL) assay. Plasma was stored at −80 °C and used as soon as possible (within 10 min) after thawing. The ROS level was measured by chemiluminescence amplified by luminol (5-amino-2,3-dihydro-1,4-Phtha-zinedione; Sigma Aldrich) using a chemiluminescence Fluoroskan Ascent FL (Thermo-Labsystems) at 37 °C. Intracellular ROS was measured with the ROS-specific fluorescent dye, CM-H2DCFDA (5 μM; Invitrogen). Colonic cells (pre-stained with the indicated antibodies) and HCT 116 cells were incubated for 15 min with 5 μM CM-H2DCFDA and washed twice with PBS. The activities of SOD, GPx, CAT and GSH were measured in erythrocytes using the appropriate assay kits (Cayman).

2.10. Milliplex map panel enzymatic activity assays

The plasma levels of IFN-γ, TNF-α, IL-6, IL-1β and IL-10 were assessed using a mouse cytokine magnetic bead panel 96-well plate assay (Cat #MHSTCMAG-70K-09; EMD Millipore) per the manufacturer's instructions. Briefly, the plate was washed and the wells were loaded with (as appropriate): 25 μL of each standard or control, 25 μL of assay buffer, 25 μL of whole lysate and 25 μL of premixed cytokine panel beads. The plate was sealed, covered with aluminum foil and incubated overnight at 4 °C. The plate was then washed twice, 25 μL of detection antibodies were added to each well, the plate was covered with foil and shaking-incubation was performed at room temperature (RT). After 1 h, 25 μL of streptavidin-phycoerythrin was added to each well and the plate was covered with foil and incubated with shaking for 30 min. The plate was washed twice, 150 μL of the provided drive fluid was added to each well, and the plate was subjected to 5 min of shaking. The plate was run on a MAGPIX® machine, and the xPonenent® Software (EMD Millipore Corporation) was used to read and analyze the data. Briefly, the software analyzed median fluorescent intensity data using a five-parameter logistic or spline curve-fitting method to calculate the concentration of each cytokine in each sample.

2.11. Western blot analysis

Protein lysates were subjected to 4%–15% gradient gel electrophoresis and the resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). The membranes were blocked for 1 h at room temperature in 5% skim milk or BSA (bovine serum albumin) in 1X Tris-buffered saline with 0.1% Tween 20 (TBST), and then incubated with specific antibodies. For Western blot analysis, we used antibodies against the following: p65, p-p65, Caspase 3, cleaved Caspase 3, phosphorylated p38MAPK (p-p38MAPK), p38MAPK (all from Cell Signaling), Bcl-2 (Bioss), SOD1 (Bioss), GAPDH (GeneTex) and β-actin (Santa Cruz). The membrane was washed with 1X TBST, and then incubated with secondary antibodies for 1 h at RT. The bands were visualized with enhanced chemiluminescence reagents (Ab Frontier).

2.12. Statistical analysis

Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) with Bonferroni correction were performed using the InStat (version 4.0) for Windows software package (GraphPad). Statistical significance was indicated by $P < 0.05$.

3. Results

3.1. SOD1 deficiency exacerbates DSS-induced colitis

To elucidate whether SOD1 plays a protective role in DSS-induced colitis, we treated SOD1 wild-type (WT) and KO mice with 2% DSS-containing drinking water for 9 days (Fig. 1a). As expected, DSS-treated SOD1 KO mice showed progressive weight loss from day 5, and this was correlated with shortened colon length (a marker of intestinal inflammation) (Fig. 1b and c). Histological analysis of colon sections showed more severe inflammation with loss of crypts in DSS-induced SOD1 KO (+DSS) mice compared to SOD1 WT (+DSS) mice (Fig. 1d). The expression of E-cadherin, which is the core component of epithelial adherent junctions and is essential for tissue development, differentiation and maintenance [24], was also decreased in SOD1 KO (+DSS) mice (Fig. 1e). The severity of DSS-induced colitis was determined according to a previously reported scoring system [23], and our results revealed that SOD1 KO (+DSS) mice showed more severe scores than WT (+DSS) mice (Fig. 1f). The disease activity index (DAI) [22] was also significantly higher in SOD1 KO (+DSS) mice than in WT (+DSS) mice (Fig. 1g). These results demonstrate that SOD1 deficiency is associated with increased severity of DSS-induced colitis in mice, with enhanced disruption of the epithelial barrier.

3.2. SOD1 deficiency decreases antioxidant enzyme activities

Intestinal inflammation is accompanied by the massive production of ROS by activated immune cells, which contributes to inflammation-associated tissue destruction [25]. Among the three forms of SOD (SOD1, SOD2, SOD3), approximately 70% of all SOD is expressed as SOD1; this enzyme acts as a superoxide dismutase and can also convert superoxide to hydrogen peroxide, which is then converted to water via a reaction catalyzed by CAT or GPx [26]. We thus evaluated whether SOD1 deficiency affected the activities of total SOD and various catalytic enzymes *in vivo*. We found that SOD activity was decreased by DSS treatment of both SOD1 WT and SOD1 KO mice, with SOD1 KO mice showing a greater decrease in SOD activity compared to WT mice (Fig. 2a). We next used luminol-dependent chemiluminescence to analyze total ROS levels in plasma. As expected, the ROS level was significantly higher in SOD1 KO mice compared to WT mice (Fig. 2b). The activities of the tested catalytic enzymes, including GPx (glutathione peroxidase), CAT (catalase) and GSH (glutathione), were markedly lower in SOD1 KO (+DSS) mice compared to WT (+DSS) mice (Fig. 2c–e). These results suggest that SOD1 deficiency decreases antioxidant enzyme activities, and that SOD1 is the important antioxidant enzyme responsible for inhibiting oxidative stress in DSS-induced colitis.

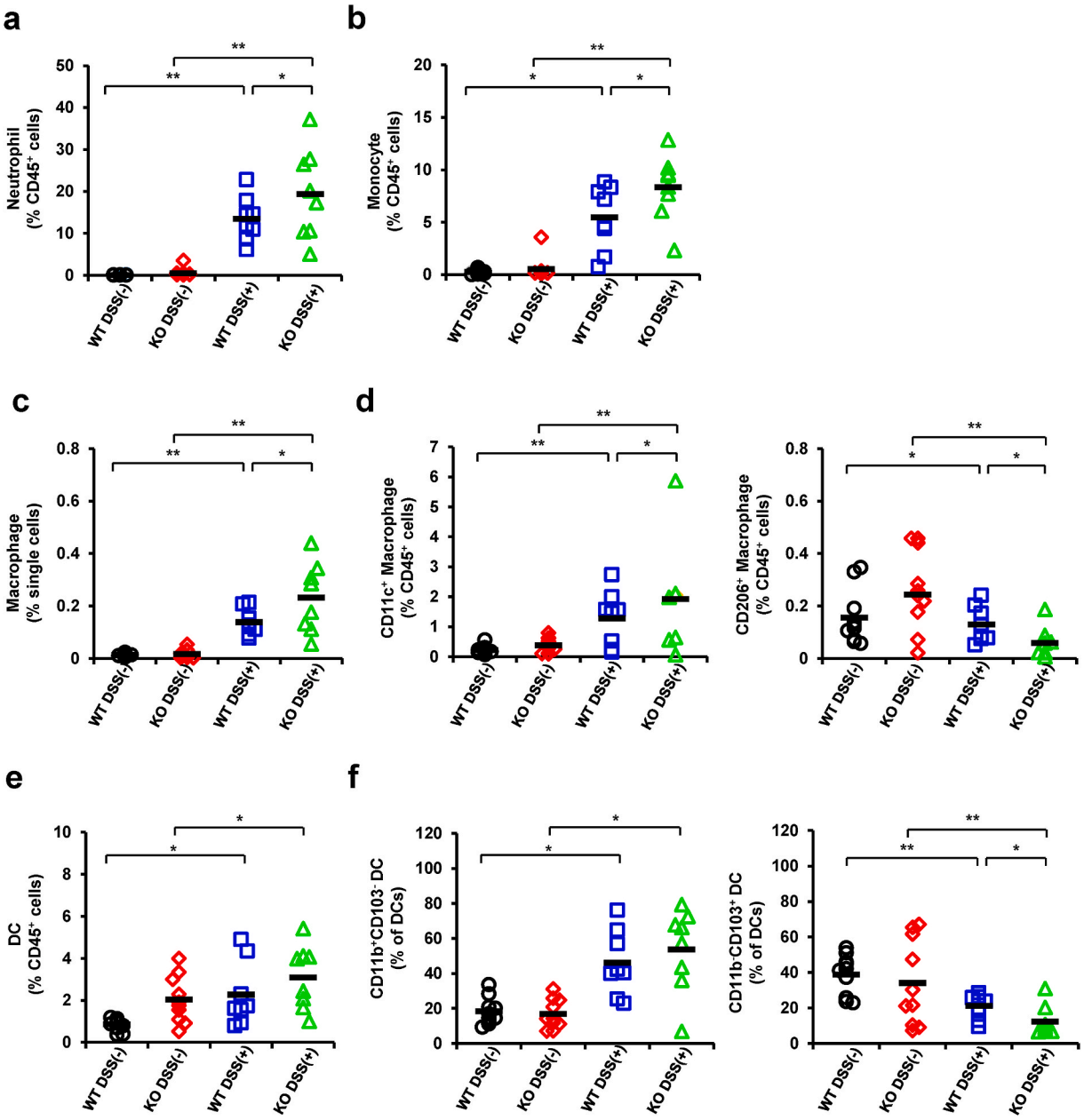

Fig. 3. SOD1 deficiency increases innate immune cell infiltration. Representative flow cytometric analysis of cell populations in the colons of DSS-induced SOD1 WT and SOD1 KO mice. Colonic lamina propria (LP) cells were isolated after 9 days of 2% DSS colitis induction. (a) Neutrophils, (b) monocytes, (c) macrophages, (d) $CD11c^+$ and $CD206^+$ macrophages, (e) DCs, (f) $CD11b^+CD103^-$ DCs and CD11b-$CD103^+$ DCs. n = 8–10 mice per group. Student's *t*-test was used for the comparison; each symbol represents a single mouse, and the average is indicated by horizontal lines. $^*P < 0.05$, $^{**}P < 0.01$.

3.3. SOD1 deficiency increases the infiltration of pro-inflammatory immune cells

As in IBD, the well-controlled balance of the intestinal immune system is disturbed at all levels in DSS-induced colitis [27]. Therefore, we analyzed whether the symptom severity observed in SOD1 KO (+DSS) mice reflected an increase in intestinal immune cell infiltration. We analyzed the immune cell compositions in the lamina propria (LP) of colons after 9 days of DSS treatment in SOD1 WT and SOD1 KO mice. A flow cytometric gating strategy was used to identify LP cells (Supplementary Fig. 1). We found that the proportions and absolute number of neutrophils, monocytes and macrophages were dramatically increased in SOD1 KO (+DSS) mice compared to WT (+DSS) mice (Fig. 3a–c, and Supplementary Fig. 2a–c). Moreover, the proportion and number of $CD11c^+$ macrophages were increased, whereas those of $CD206^+$ macrophages were decreased in SOD1 KO (+DSS) mice compared to WT (+DSS) mice (Fig. 3d and Supplementary Fig. 3d). The number of DCs was significantly increased in SOD1 KO (+DSS) mice compared to WT (+DSS) mice (Fig. 3e and Supplementary Fig. 2e). More importantly, the number of $CD11b^+$ $CD103^-$ DCs was also significantly increased in SOD1 KO (+DSS) mice (Supplementary Fig. 2f). In contrast, the proportion of the anti-inflammatory CD11b-$CD103^+$ DC subset was significantly decreased in SOD1 KO (+DSS) mice compared to WT (+DSS) mice (Fig. 3f and Supplementary Fig. 2f). These results suggest that SOD1 deficiency induces inflammation by increasing the infiltration of pro-inflammatory immune cells in DSS-induced colitis.

3.4. BA SOD administration ameliorates the severity of colitis

Since SOD1 deficiency increases DSS-induced colitis, we investigated

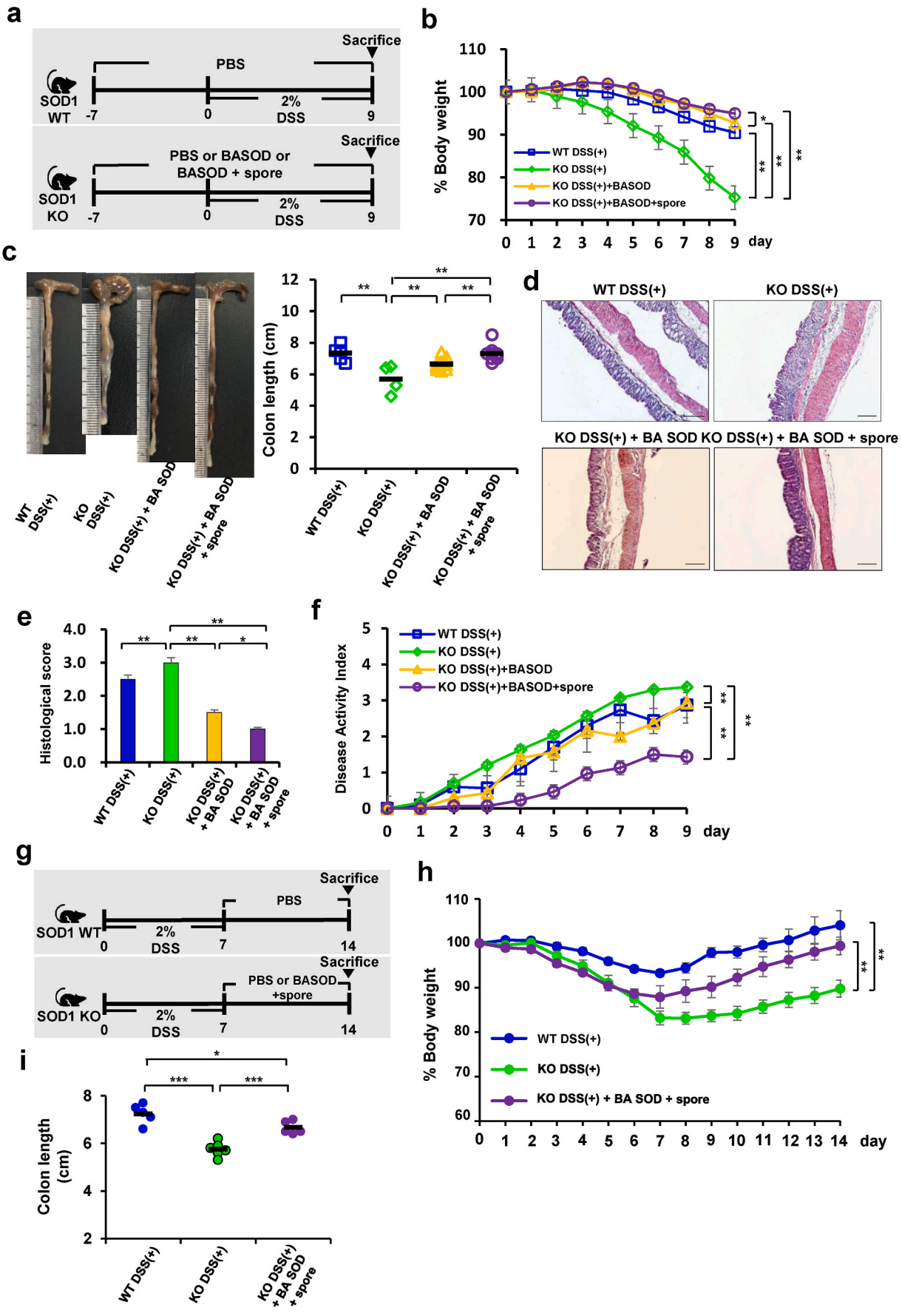

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Fig. 4. BA SOD administration ameliorates DSS-induced colitis. (a) Experimental scheme. Mice were orally administrated with either phosphate-buffered saline (PBS), BA SOD (20 U) or BA SOD + spores (BA SOD 20 U + 1×10^7 spores) daily for a total of 16 days. For induction of acute colitis, the mice were exposed to 2% DSS dissolved in their drinking water for the last 9 days of the treatment period. (b) Change in weight over time is expressed as the percentage of the initial body weight. (c) The colon length was measured at day 16. Each symbol represents a single mouse. (d) Representative H&E-stained colon sections are shown. Scale bars, 100 μm. (e) For histological scoring, a measure of crypt abscesses and epithelial erosion was calculated. (f) DAI, which is a measure of weight loss, stool consistency and blood in the stool, was calculated. (g) Experimental scheme. Mice were exposed to 2% DSS dissolved in drinking water for 7 days and then orally administrated with either PBS or BA SOD + spores (BA SOD 20 U + 1×10^7 spores) daily for 7 days. (h) Change in weight over time is expressed as the percentage of the initial body weight. (i) The colon length was measured at day 14. n = 5–10 mice per group. Each symbol represents a single mouse. Results were significantly different at the $^{**}P < 0.01$ level, as assessed by one-way ANOVA followed by Bonferroni correction.

whether rescuing SOD1 by administrating exogenous BA SOD daily for 1 week prior to DSS induction and continuing daily treatments during DSS induction (Fig. 4a) could alleviate the severity of DSS-induced colitis in our SOD1 KO mice. BA SOD, an antioxidant enzyme produced by *B. amyloliquefaciens* GF423 produces spores to resist adverse conditions. It has been reported that oral gavage of BA SOD and spores ameliorated oxidative stress and inflammatory responses on γ-radiation-induced oxidative stress and DSS-induced ulcerative colitis [21]. A preliminary experiment designed to determine the effective concentration of BA SOD revealed that administration of 20 U or 60 U BA SOD significantly restored the body weight loss, colon length shortening and DAI compared to PBS-treated WT (+DSS) mice, and further showed that there was no significant difference in these parameters or epithelial disruption between the BA SOD 20 U- and 60 U-treated groups (Supplementary Fig. 3). Based on these preliminary findings, we treated SOD1 KO (+DSS) mice with PBS, BA SOD (20 U/day) or BA SOD + spores (1×10^7) by oral gavage for 16 days, with DSS administered as described above during the last 9 days (Fig. 4a). SOD1 KO (+DSS) mice showed progressive weight loss from day 5 to day 9 and exhibited shortened colon lengths (Fig. 4b and c). However, the administration of BA SOD or BA SOD + spores to SOD1 KO (+DSS) mice significantly ameliorated their body weight loss (Fig. 4b) and colon length shortening (Fig. 4c). Histological analysis of colon sections from WT (+DSS) and SOD1 KO (+DSS) mice showed severe inflammation with loss of crypts and infiltration of immune cells, whereas the corresponding mice treated with BA SOD or BA SOD + spores exhibited decreases in the epithelial damage associated with DSS treatment (Fig. 4d). The histological scores of BA SOD- and BA SOD + spore-treated SOD1 KO (+DSS) mice were significantly lower than those of the SOD1 KO (+DSS) mice (Fig. 4e). The results of the DAI score analysis (Fig. 4f) were similar to those obtained from our histological analysis. To further demonstrate the therapeutic effects of BA SOD, SOD1 KO mice were treated with BA SOD + spores daily for 7 days after 2% DSS induction (Fig. 4g). The administration of BA SOD + spores to SOD1 KO (+DSS) mice significantly ameliorated their body weight loss (Fig. 4h) and colon length shortening (Fig. 4i) compared to PBS-treated SOD1 KO (+DSS) mice. Taken together, these results indicate that BA SOD and BA SOD + spores could inhibit DSS-induced colitis by protecting against epithelial barrier disruption, and that BA SOD may have potential as a therapeutic candidate for the treatment of colitis.

3.5. BA SOD administration increases antioxidant enzyme activity

Next, we examined whether the administration of BA SOD to SOD1 KO (+DSS) mice could reduce ROS production and increase endogenous antioxidant enzyme activities. Interestingly, the administration of BA SOD or BA SOD + spores to SOD1 KO (+DSS) mice restored endogenous SOD activity (Fig. 5a) and dramatically reduced the plasma ROS levels (Fig. 5b). Intracellular ROS was measured using the ROS-specific fluorescence dye, CM-H2DCFDA. Our results showed that following administration of BA SOD or BA SOD + spore to colitis-induced SOD1 KO mice, the intracellular ROS levels of immune cells (neutrophils, macrophages, monocytes) and non-immune cells (epithelial cells) were reduced (Fig. 5c and d). These results suggest that both immune and non-immune cells are the source of ROS production, and that BA SOD administration could reduce ROS production in these cells. In addition, administration of BA SOD or BA SOD + spores markedly increased the activities of GPx, CAT and GSH (Fig. 5e–g). Our data therefore demonstrate that rescue of SOD1 with BA SOD or BA SOD + spores can inhibit ROS production and enhance endogenous antioxidant enzyme activities in DSS-induced colitis.

3.6. BA SOD administration inhibits the infiltration of pro-inflammatory immune cells

We next analyzed whether the administration of BA SOD to SOD1 KO (+DSS) mice could inhibit the infiltration of pro-inflammatory immune cells. As expected, the proportions and numbers of neutrophils, monocytes and macrophages were significantly decreased by the administration of BA SOD or BA SOD + spores to SOD1 KO (+DSS) mice (Fig. 6a–c and Supplementary Fig. 4a–c). Compared to SOD1 KO (+DSS) mice, BA SOD- or BA SOD + spore-treated SOD1 KO (+DSS) mice also showed decreases in the percentage and number of $CD11c^+$ macrophages, but increases in the percentage and number of $CD206^+$ macrophages (Fig. 6d and Supplementary Fig. 4d). As expected, the proportion and number of DCs were significantly increased in KO (+DSS) mice compared to WT (+DSS) mice; however, this change was significantly decreased by the administration of BA SOD or BA SOD + spores (Fig. 6e). More importantly, the proportion and number of $CD11b^+CD103^-$ DCs were significantly decreased in BA SOD- or BA SOD + spore-treated SOD1 KO (+DSS) mice compared to their untreated counterparts (Fig. 6f). Although the number of $CD11b^-CD103^+$ DCs was not increased by BA SOD + spore administration (Supplementary Fig. 4f), the proportion of $CD11b^-CD103^+$ DCs was significantly increased in BA SOD- and BA SOD + spore-treated SOD1 KO (+DSS) mice compared to SOD1 KO (+DSS) mice (Fig. 6f). Together, these results demonstrate that administration of BA SOD to SOD1 KO mice ameliorates DSS-induce colitis by inhibiting intestinal pro-inflammatory immune cell infiltration.

3.7. BA SOD administration inhibits the production of pro-inflammatory cytokines

Cytokines play a crucial role in the pathogenesis of IBDs, such as Crohn's disease and UC, where they control multiple aspects of the inflammatory response [28]. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in IBD impedes the resolution of inflammation, perpetuating the disease and tissue destruction [29]. Since our results indicate that SOD1 plays a crucial role in protecting against DSS-induced colitis, we next analyzed the plasma levels of various pro-inflammatory and anti-inflammatory cytokines in DSS-induced SOD1 WT and KO mice. As expected, the pro-inflammatory cytokines, IFN-γ, TNF-α, IL-6 and IL-1β were significantly elevated in SOD1 KO (+DSS) mice compared to WT (+DSS) mice (Fig. 7a). Interestingly, the administration of either BA SOD or BA SOD + spores to SOD1 KO (+DSS) mice led to profound decreases in the levels of these cytokines compared, to those in the corresponding untreated mice (Fig. 7a). In contrast, the anti-inflammatory cytokine, IL-10, was markedly decreased in SOD1 KO (+DSS) mice compared to WT (+DSS) mice, and the administration of BA SOD or BA SOD + spores to SOD1 KO (+DSS) mice markedly increased the IL-10 level (Fig. 7b). Collectively, our findings suggest that SOD1 plays a major role in

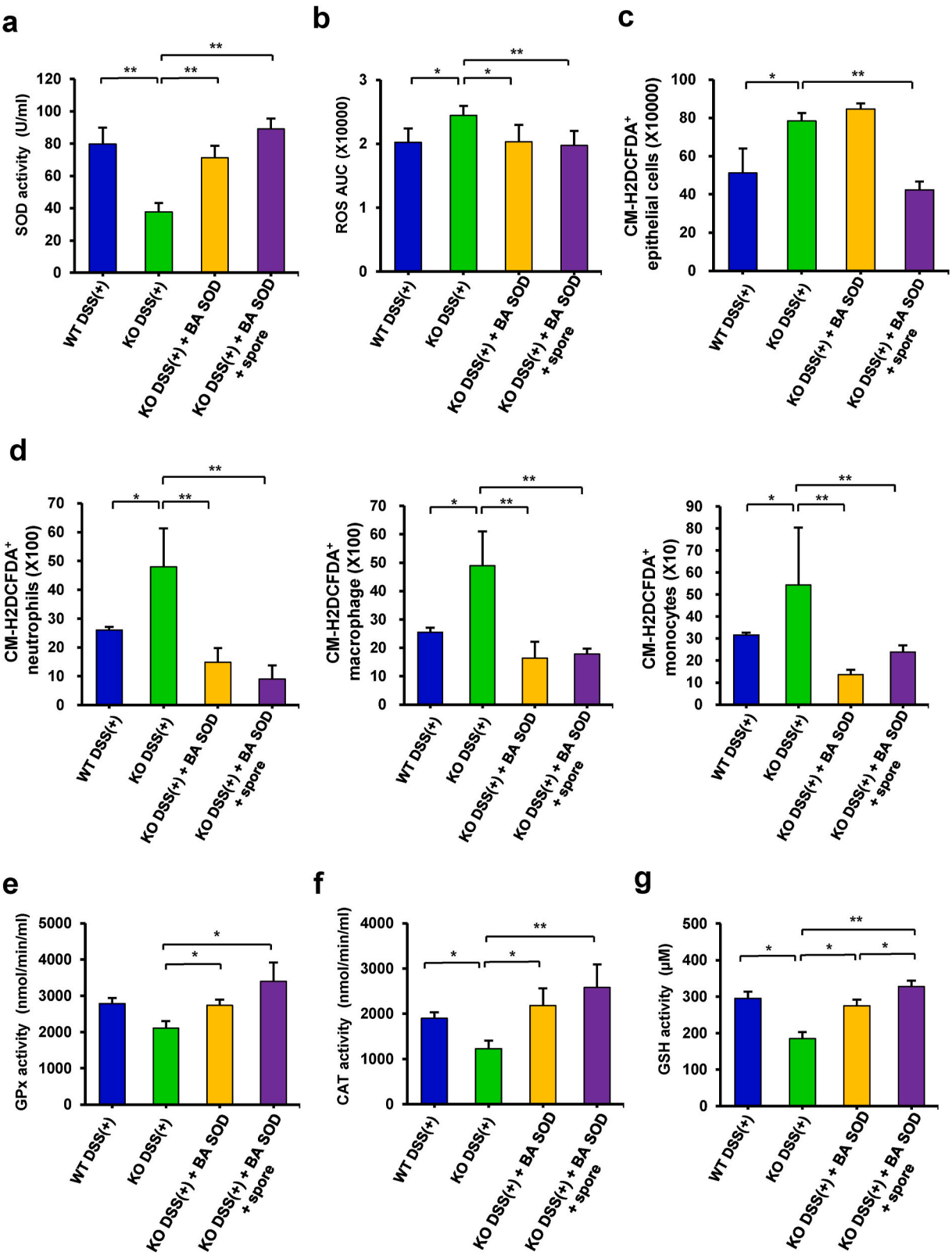

Fig. 5. BA SOD administration increases antioxidant enzyme activities. Mice were orally administrated with BA SOD (20 U) or BA SOD + spores (BA SOD 20 U + 1×10^7 spores) daily for a total of 16 days. For induction of acute colitis, mice were exposed to 2% DSS dissolved in their drinking water for the last 9 days of the treatment period. Colon lamina propria (LP) cells were isolated on day 16 after the end of colitis induction. (a) SOD activity in erythrocytes. (b) ROS levels in plasma. (c) The number of CM-H2DCFDA$^+$ epithelial cells. (d) The number of CM-H2DCFDA$^+$ cells [neutrophils [53], macrophages (*middle*) and monocytes (*right*)]. (e–g) Catalytic enzyme activities of (e) GPx, (f) CAT and (g) GSH in erythrocytes. n = 8 mice per group. Results were significantly different at the $^*P < 0.05$ and $^{**}P < 0.01$ levels, as assessed by one-way ANOVA followed by Bonferroni correction.

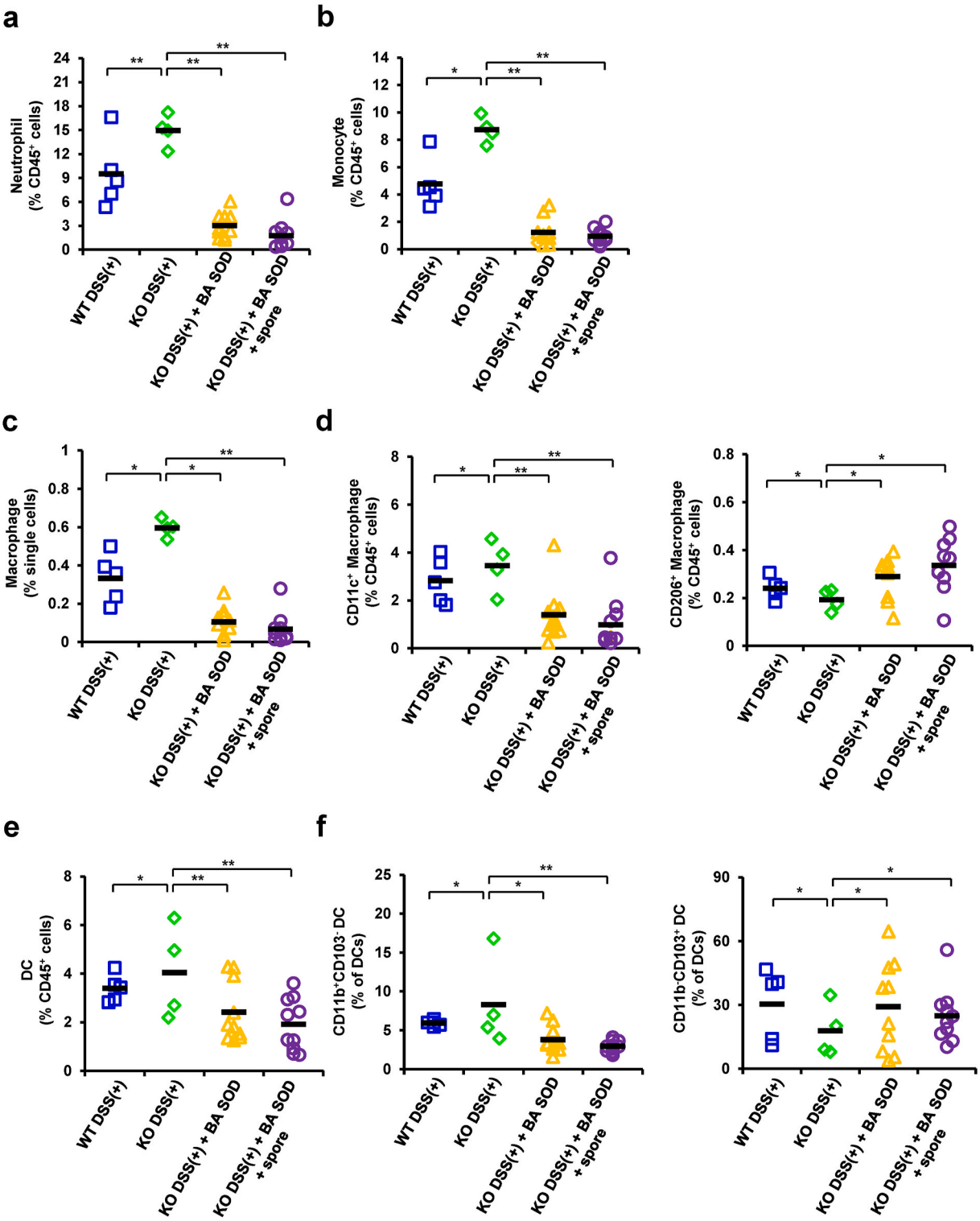

Fig. 6. BA SOD administration inhibits intestinal innate immune cell infiltration. SOD1 KO mice were orally administrated with BA SOD (20 U) or BA SOD + spores (BA SOD 20 U + 1×10^7 spores) daily for a total of 16 days. For induction of acute colitis, mice were exposed to 2% DSS dissolved in their drinking water for the last 9 days of the treatment period. LP cells were isolated at day 16 after the end of colitis induction. (a) Neutrophils, (b) monocytes, (c) macrophages, (d) $CD11c^+$ macrophages and $CD206^+$ macrophages, (e) DCs, (f) $CD11b^+CD103^-$ DCs and CD11b-$CD103^+$ DCs. n = 4–10 mice per group. Student's *t*-test was used for the comparison; each symbol represents a single mouse, and the average is indicated by horizontal lines. $^*P < 0.05$, $^{**}P < 0.01$.

inhibiting pro-inflammatory cytokine production, and that BA SOD may have potential as a therapeutic candidate for the treatment of colitis.

3.8. BA SOD administration suppresses p38-MAPK/NF-κB signaling

To further explore the molecular mechanism underlying the ability of BA SOD to modulate DSS-induced inflammatory responses, we performed *in vitro* experiments with HCT 116 cell lines. To mimic the colitis

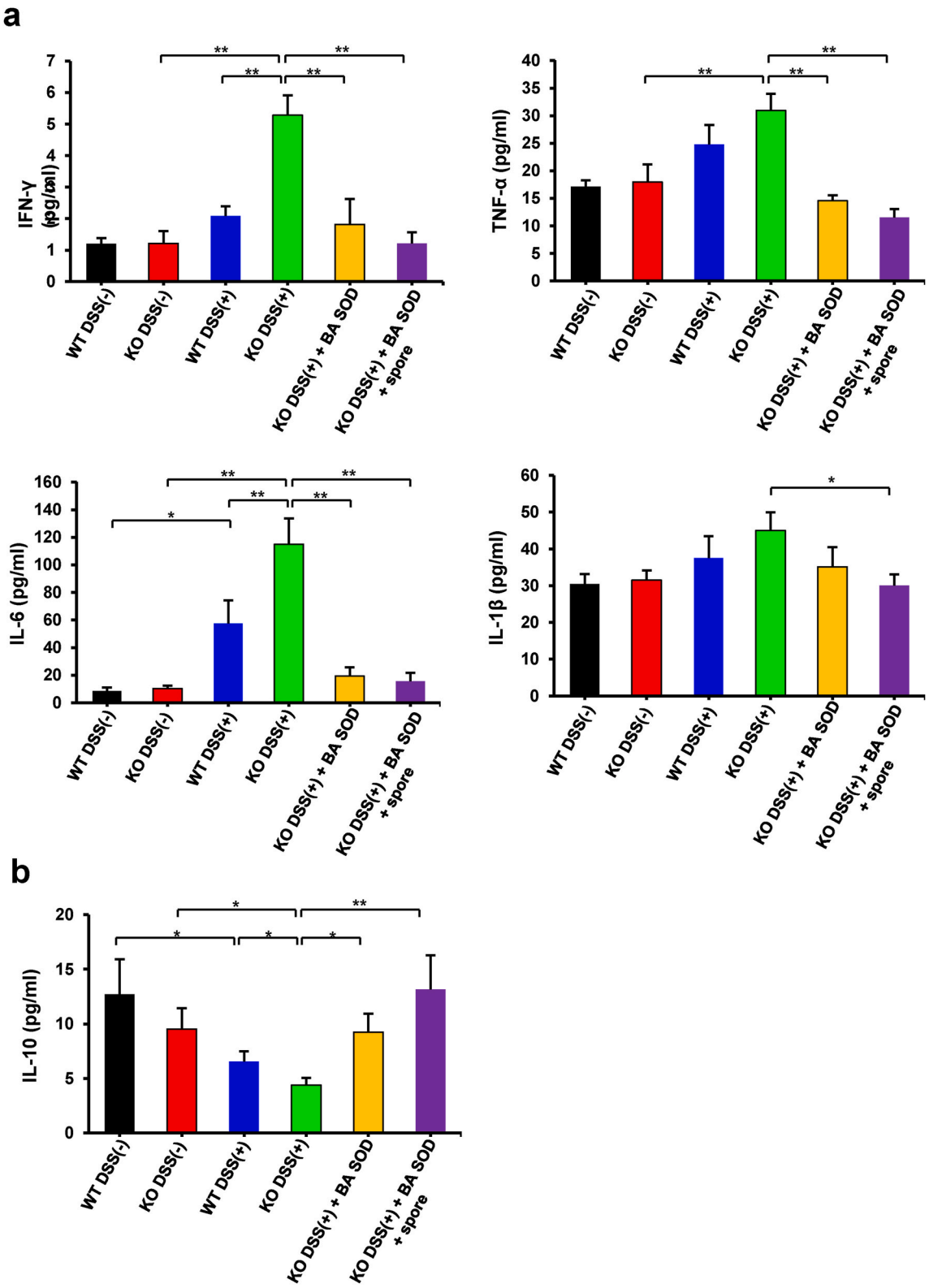

Fig. 7. BA SOD administration attenuates pro-inflammatory cytokines and augments anti-inflammatory cytokines. Mice were orally administrated with either BA SOD (20 U) or BA SOD + spores (BA SOD 20 U + 1×10^7 spores) daily for a total of 16 days. For induction of acute colitis, mice were exposed to 2% DSS dissolved in their drinking water for the last 9 days of the treatment period. LP cells were isolated at day 16 after the end of colitis induction. (a) The levels of IFN-γ, TNF-α, IL-6 and IL-1β. (b) The level of IL-10. n = 10 mice per group. Results were significantly different at the $^*P < 0.05$ and $^{**}P < 0.01$ levels, as assessed by one-way ANOVA followed by Bonferroni correction.

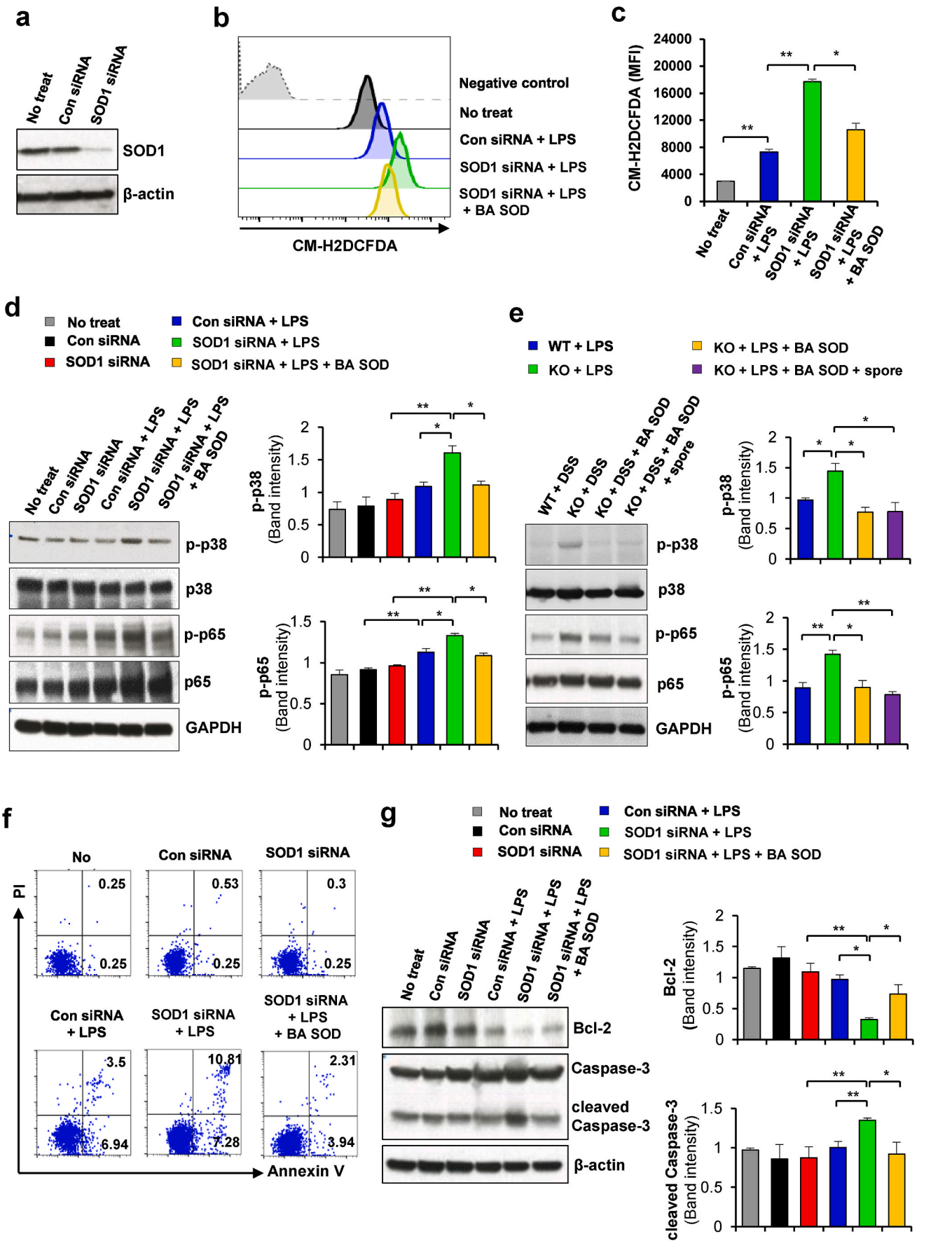

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Fig. 8. BA SOD administration suppresses the p38-MAPK/NF-κB signaling pathways. HCT 116 cells were transfected with SOD1 siRNA for 48 h, pretreated with BA SOD (4 U) for 1 h and stimulated with LPS for 3 or 24 h. (a) Western blot analysis of SOD1 in HCT 116 cells transfected with SOD1 siRNA for 48 h. (b, c) Representative FACS histograms (b) and mean fluorescence intensity (MFI) (c) for ROS production after incubation with CM-H2DCFDA. (d) Western blot analysis of phosphorylated p38 (p-p38), p38, phosphorylated p65 (p-p65), p65 and GAPDH in SOD1 siRNA-transfected HCT 116 cells in response to LPS and BA SOD. (e) Western blot analysis of p-p38, p38, p-p65, p65 and β-actin. Protein lysates were extracted from the colons of SOD1 WT (+DSS) or BA SOD (20 U) - or BA SOD + spores (BA SOD 20 U + 1×10^7 spores)-administrated SOD1 KO (+DSS) mice. (f) Representative FACS dot plots for propidium iodide (PI) and Annexin V staining after treatment with LPS and BA SOD. (g) Western blot analysis of Bcl-2, Caspase-3, cleaved Caspase-3 and β-actin in SOD1 siRNA-transfected HCT 116 cells in response to LPS and BA SOD. (d, e, g) Data were normalized to GAPDH or β-actin band intensities and are presented as mean ± SEM of three independent experiments. Student's *t*-test was used for the comparison; *$p < 0.05$; **$p < 0.01$.

condition, LPS (lipopolysaccharide) was used as stimulator. HCT 116 cells were transfected with SOD1 siRNA, pretreated with BA SOD and stimulated with LPS. The expression level of SOD1 was dramatically downregulated in cells transfected with SOD1 siRNA compared to the control siRNA (Fig. 8a). Analysis of LPS-induced ROS production revealed that the ROS level was increased in the SOD1 siRNA group compared to the control siRNA group. However, BA SOD treatment reduced the ROS level in the SOD1 siRNA group (Fig. 8b and c). Previous studies demonstrated that the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways are involved in the pathogenesis of IBD [26,30–32]. In our *in vitro* experimental system, we found that the protein levels of phosphorylated p38 (p-p38) and phosphorylated p65 (p-p65) were increased in the SOD1 siRNA group compared to the control siRNA group, and that BA SOD recovered these changes (Fig. 8d). We obtained similar results *in vivo* using DSS-induced colitis mice. The increased levels of p-p38 and p-p65 seen in SOD1 KO (+DSS) mice were reduced by BA SOD administration (Fig. 8e). p38-MAPK pathway is a key regulator of cell apoptosis and it has been shown that p38-MAPK signaling promotes cell death [33], and activated NF-κB signaling pathway activates caspase 3 and leading to cell apoptosis in intestinal epithelial cells [26]. We found that both early apoptotic (PI^- Annexin V^+) and late apoptotic (PI^+ Annexin V^+) cells were increased by SOD1 siRNA transfection compared to control siRNA, and this effect was reduced by BA SOD treatment (Fig. 8f). The protein level of Bcl-2 were decreased and those of cleaved Caspase-3 were increased in the SOD1 siRNA group compared to the control siRNA group, and these changes were rescued by BA SOD treatment (Fig. 8g). Taken together, our results suggest that BA SOD administration suppresses oxidative stress, inflammatory responses and apoptosis through inhibiting p38-MAPK/NF-κB signaling in epithelial cells.

4. Discussion

In this study, we provide evidence that deficiency of SOD1 exacerbates DSS-induced colitis in mice through increases in ROS-induced oxidative stress and pro-inflammatory immune responses, and that administration of BA SOD may protect against DSS-induced colitis. Our results suggest that SOD1-mediated inhibitory responses are crucial in limiting the development of colitis.

Among the immune-regulatory factors, oxidative stress has been proposed as a major mechanism in the pathophysiology of IBD [34]. It has been established that chronic intestinal inflammation is associated with overproduction of reactive oxygen, and that this leads to oxidative stress, which has been implicated in several human diseases, including IBD [35]. An increased sensitivity to H2O2, increased lipid peroxidation and decreased superoxide anion radical levels have also been observed in patients with gastric reflux disease [36,37]. Decreased SOD activities are associated with increased inflammatory processes, suggesting that inflammation may be caused by insufficient antioxidant activity in patients with IBD [4,38]. Although SOD1 is known to have tolerogenic activity in IBD patients, the specific role of SOD1 in limiting oxidative stress during the development of colitis was previously unknown. Here, we report that SOD1 deficiency increases ROS levels, thereby providing a direct connection to oxidative stress. The activities of key catalytic enzymes, such as GPx, CAT and GSH, were also significantly decreased in SOD1 KO (+DSS) mice compared to WT (+DSS) mice. Our results suggest that SOD1-mediated inhibitory responses play a crucial role in limiting oxidative stress during the development of colitis.

Phenotypes common to both UC and CD include chronic inflammation and a dysregulated immune response; therefore, much of the research on IBD pathogenesis has focused on the immune system. The pathogenesis of both UC and CD involve genetic factors, changes in the gut microbiome, and components of the immune response, including cytokines and immune cells [28]. The gut immune system has the challenge of responding to pathogens while remaining relatively unresponsive to food antigens and the commensal microflora [39]. Different subsets of macrophages have distinct effects on the severity of colitis in animal models. For example, $F4/80^+CD206^+$-polarized macrophages protect mice from DSS-induced colitis, whereas $CD11b^+CD11c^+$-polarized macrophages contribute to disease pathogenesis [40]. Intestinal $CD11b^+$ DCs increase the production of IL-6 and IL-23, which promote Th17 development. CD11b-$CD103^+$ DCs help balance the development of Treg cells versus Th1 cells from naïve T cells in intestinal inflammation [15]. Here, we show that SOD1 deficiency increases the DSS exposure-induced entry of pro-inflammatory immune cells, such as neutrophils, monocytes, $CD11c^+$ macrophages and $CD11b^+CD103^-$ DCs, but decreases the levels of anti-inflammatory $CD206^+$ macrophages and $CD11b^-$-$CD103^+$ DCs. Intestinal immune cells also influence mucosal barriers through the production of cytokines or direct cell–cell contact [41]. Anti-cytokine therapies involving TNF-specific agents form an important cornerstone of clinical therapy for both Crohn's disease and UC. TNF-specific antibodies suppress chronic intestinal inflammation and may induce mucosal healing in IBD [42], and IL-6 can exert pro-inflammatory functions by activating multiple target cells, including APCs and T cells [43]. IL-10 is an anti-inflammatory cytokine that inhibits both antigen presentation and the subsequent release of pro-inflammatory cytokines [44]. Recent work showed that loss-of-function mutations in the genes encoding IL-10 and IL-10R are associated with a very early-onset form of IBD that is characterized by severe intractable enterocolitis in infants [45]. Here, we report that the pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β, were increased and the anti-inflammatory cytokine, IL-10, was decreased in SOD1 KO (+DSS) mice compared to WT (+DSS) mice. These results suggest that increased infiltration of pro-inflammatory immune cells induces pro-inflammatory cytokine secretion, leading to the promotion of intestinal inflammation.

A diet containing a strain of *B. subtilis* was shown to significantly increase the serum or tissue levels of SOD in several fish strains [46,47], and recent studies examined the properties of *B. amyloliquefaciens*, including its antioxidant effects and probiotic potential [48–50]. *B. amyloliquefaciens* has also been reported to be useful in the management of IBD [51]. BA SOD in an antioxidant enzyme produced by *B. amyloliquefaciens* GF423. Kang et al. reported the effect of BA SOD on γ-radiation-induced oxidative stress and DSS-induced ulcerative colitis in WT mice, and they showed that SOD enzyme and spores contribute to reducing oxidative stress and inflammatory responses [21]. However, the molecular mechanism through which BA SOD modulates DSS-induced colitis has not been elucidated. We herein demonstrate that administration of BA SOD and spores to SOD1 KO mice promotes antioxidant defenses, protects against disruption of the mucosal barrier and suppresses pro-inflammatory immune responses in the DSS-induced mouse model of colitis. Furthermore, co-administration of BA SOD

and spores to SOD1 KO colitic mice ameliorates the severity of the induced colitis. Several therapeutics commonly used in IBD treatment, including immunosuppressants, corticosteroids and anti-TNF-α antibodies, could also affect IBD progression by interfering with cellular oxidative stress and cytokine production. Experimental data shows that these drugs may effectively scavenge free radicals, increase the anti-oxidative capacity of cells, influence multiple signaling pathways (e.g., MAPK and NF-κB) and downregulate pro-oxidative enzyme and cytokines [26,30,31,52]. However, further work is needed to fully examine the anti-oxidative and anti-inflammatory effectiveness of these agents. We herein demonstrate that exogenous supplementation of BA SOD and spores to SOD1 KO mice protects against disruption of the mucosal barrier in DSS-induced acute colitis by suppressing p38-MAPK/NF-κB signaling.

In conclusion, our findings indicate that BA SOD might be useful as a dietary supplement for the treatment of acute colitis. These results may inform the future development of BA SOD as an alternative antioxidant enzyme in efforts to treat IBD.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101760.

Author contributions

J.H., J.J., O.K. and G.T.O. proposed the concept, contributed to the study design and wrote the manuscript with help from S.J., S.H.M., M.Y. P., D.Y.Y., J.H.K., J.E.K., M.H. P., E.J.K. and J.G.P. All authors actively discussed and reviewed the manuscript.

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