Journal of Inflammation Research

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Metformin Ameliorates Ulcerative Colitis Through Inhibiting NLRP3 Inflammasome Activation

Run Cao[1,*], Jing Jing[1,*], Yuting Ma[1,2,*], Wenqing Qi[1], Xinyu Huang[1], Chaofang Zhang[1], Zhizhuo Lu[1], Jiayi He[1], Guiling Wang[1], Yuanfang Ma[1], Hailong Zhang[1]

[1]Joint National Laboratory for Antibody Drug Engineering, the First Affiliated Hospital, Henan University, Kaifeng, Henan, People's Republic of China; [2]Xuzhou Central Hospital, Xuzhou, Jiangsu, People's Republic of China

*These authors contributed equally to this work

Correspondence: Hailong Zhang; Yuanfang Ma, Joint National Laboratory for Antibody Drug Engineering, the First Affiliated Hospital, Henan University, Kaifeng, Henan, People's Republic of China, Email hailong6891@163.com; mayf@henu.edu.cn

Purpose: Metformin (Met) is widely used to treat a variety of diseases, but its role in ulcerative colitis (UC) has not been fully elucidated. This study aimed to clarify the effect of Met on UC, exploring its relationship with NLRP3 inflammasome and elucidating the potential mechanisms.

Methods: C57BL/6J mice were administrated with DSS solution to establish UC model. Disease Activity Index (DAI) and hematoxylin and eosin staining (HE) were performed to evaluate the impact of Met on UC model. Enzyme-linked immunosorbent assay (ELISA), Reverse transcription - quantitative polymerase chain reaction (RT-qPCR), Western blotting (WB), immunohistochemistry, and immunofluorescence were used to detect NLRP3 inflammasome activation in vivo. Furthermore, in vitro, bone marrow-derived macrophages (BMDMs) selected to clarify the role of Met on NLRP3 inflammasome activation and the underlying mechanisms.

Results: In vivo, Met could significantly inhibit the development of UC, characterized by decreased DAI, increased body weight and colorectal length, and the repair of damaged tissue. Met could also block macrophage infiltration and subsequently reduced the level of IL-1β, NLRP3, and Caspase-1 in the colorectal tissue, which were mainly expressed by macrophages. In addition, the level of IL-1β in serum was remarkedly down-regulated by Met. In vitro, Met could inhibit NLRP3 inflammasome activation and subsequently dampen the maturation of pro-caspase-1 and pro-IL-1β. Moreover, Met could simultaneously suppress the activation of NF-κB/p65 signaling pathway and disrupt the formation of ASC speck. At last, Met exhibited an anti-oxidant effect, along with upregulating the level of UCP2 and NCF1.

Conclusion: Met significantly ameliorated UC by inhibiting NLRP3 inflammasome activation in macrophages. The underlying mechanisms not only involved the inhibition of NF-κB signaling pathway activation (first signal), but was also associated with up-regulation of UCP2 and NCF1 levels and thus the repression of ROS generation (second signal).

Keywords: metformin, NF-κB, NLRP3 inflammasome, ROS, ulcerative colitis

Introduction

As an inflammatory bowel disease (IBD), Ulcerative colitis (UC) primarily affects the colorectum and is characterised by continuous inflammation of the colonal mucosal and submucosal. It poses a global healthcare challenge, showing a consistent rise in cases.[1] At present, UC treatment included non- targeted therapies (such as aminosalicylic acid, glucocorticoids, and immunomodulators) and targeted therapies (such as anti TNF, antiIL-12/IL-23, and antialpha 4 β 7 integrin).[2] In addition, recent studies have also shown that the melanocortin plays a potential therapeutic role in the treatment of UC.[3] The mechanisms might be that the melanocortin could regulate the balance of inflammation-related cytokines. It could reduce the inflammatory response by inhibiting the production of pro - inflammatory cytokines such

Received: 26 October 2024
Accepted: 24 January 2025
Published: 5 February 2025

as tumor necrosis factor-α (TNF-α) and interleukin - 1β (IL-1β).[4–6] Although there are many methods, there is no effective method for this disease, urgent search for new potential treatments.[7]

NLRP3 inflammasome is a multiprotein complex, which comprises NLRP3 sensors, the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), the effector protein caspase-1, and NEK7 (NIMA-related kinase 7), a key regulator of NLRP3 inflammasome activation.[8] The activation of the NLRP3 inflammasome requires two signals: a priming signal and an activation signal. TLR4 activation induces phosphorylation-mediated nuclear translocation of nuclear transcription factor kappa-B (NF-κB) p65, stimulating innate immune responses, which serves as the priming signal for NLRP3 inflammasome activation.[9] Consequently, the upregulation of NLRP3 gene expression and Interleukin-1 beta (IL-1β) and Interleukin-18 (IL-18) occurs. Subsequently, various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) such as adenosine triphosphate (ATP), Monosodium urate (MSU), Nigericin (Nig) mitochondrial reactive oxygen species (mtROS), and mitochondrial DNA (mtDNA) induce NLRP3 oligomerization, and subsequently recruit ASC and pro-caspase-1 to form the NLRP3 inflammasome.[10,11] After being activated, it mediates the generation of inflammation in the body and plays a role in fighting against infections. However, overactivated NLRP3 inflammasome is involved in a variety of autoimmune diseases, such as atherosclerosis, UC and other inflammatory diseases.[12]

Current research has found that NLRP3 inflammasome activation and/or elevation of pro-inflammatory cytokines are the main pathological mechanisms of UC.[13] The essential role of NLRP3 inflammasome in the pathogenesis of UC has been explored in mouse model induced by dextran sulfate sodium (DSS). It has been demonstrated that the depletion of NLRP3 downregulates the levels of IL-1β and IL-18 in the colorectum and ameliorates DSS-induced inflammation.[14] However, one study had also found that depletion of NLRP3 made mice more sensitive to DSS-induced colitis, which may be related to the gut microbiota.[15] These results suggest that the role of NLRP3 inflammasome in UC models is debatable. In addition, our previous research found that in DSS - induced UC, NLRP3 inflammasome activation was enhanced, pro-inflammatory cytokines expression was increased and immune cell infiltration in colorectum tissue was significantly increased.[15,16] This suggests that blocking the activation of NLRP3 inflammasome or downregulating the release of inflammatory factors may improve the occurrence and development of UC.

Metformin (Met), a synthetic derivative of guanidine, has over 50 years of clinical experience in treating type 2 diabetes.[17] But recent preclinical and clinical studies have shown that Met also ameliorates chronic inflammation by improving metabolic parameters such as hyperglycemia, insulin resistance, and atherogenic dyslipidemia, and Met also has a direct anti-inflammatory effect.[18] Extensive research has been focused on demonstrating the anti-inflammatory effects of Met in cellular studies, animal models, patient records, and randomized clinical trials. Recent studies have found that co-administration of empagliflozin and Met demonstrates a protective anti-inflammatory effect on the colorectum in DSS-induced rats and improves the macroscopic and microscopic characteristics of the ulcerative colorectum.[19] Met in combination with other small molecules also plays an important role in the treatment of UC through different mechanisms, such as MCC950,[20] HSP90.[21] In addition, previous studies have also shown that Met inhibits mitochondrial fission, reduces oxidative stress, and prevents NLRP3 inflammasome activation, thereby alleviating adipose cell dysfunction.[22] Met is believed to have significant anti-inflammatory effects by inhibiting NF-κB activation and nuclear translocation.[23] Met downregulates inflammatory cytokines in inflamed human intestinal epithelial HT-29 cells in a dose-dependent manner and suppresses azoxymethane-induced colorectal aberrant crypt foci in mice.[24] Over the years, multiple studies have identified Met as a potential candidate for the treatment of UC.[25] However, the underlying mechanisms of Met intervention in UC remain unclear.

In this study, we established a mouse UC model by DSS and employed the effect of Met on this model. We verified the protective effect of Met by evaluating changes in body weight, DAI scores, and the levels of inflammatory factors IL-1β and IL-18 in serum and colorectum tissues. Additionally, we investigated the effect of Met on the NLRP3 inflammasome in LPS-induced macrophages. These findings reveal novel regulatory mechanisms for the treatment of UC and lay the foundation for the potential application of Met in the prevention and treatment of UC patient populations.

Materials and Methods

Mice

Male C57BL/6J mice aged 6–8 weeks were procured from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). They were housed in a sterile environment with ad libitum access to food and water. Following one week of acclimatization, the mice were considered suitable for the formal experiments. Furthermore, all animal procedures were approved by the Animal Experimental Ethics Committee of Henan University (HUSOM-2018-303), and complied with the relevant requirements of the People's Republic of China "Laboratory animals - General code of animal welfare" (GB/T 42011–2022).

DSS-Induced UC

The methodology for inducing ulcerative colitis with DSS has been outlined in previous literature.[16] In brief, UC was induced by administering a 3% (w/v) solution of DSS (molecular weight 36000–50000, MP Biomedical, USA) for a duration of 7 days. The mice were randomly divided into three groups based on their body weight: the normal group (Normal, n=6), the DSS group (DSS, n=6), and the Met group (administered intragastrically at 500 mg/kg/day, n=6).

The Disease Activity Index (DAI) scores, incorporating parameters such as weight loss, stool consistency, and presence of occult blood, were evaluated as previously described.[16] On the seventh day, all mice were anaesthetised with isoflurance and blood was obtained, after which they were euthanised. Additionally, the colorectum of all mice was photographed and measured for length. Subsequently, the colorectum was divided into two sections. One section was fixed in 4% paraformaldehyde for histological analysis, and the other section was rapidly frozen in liquid nitrogen for biochemical detection.

Cell Preparation and Stimulation

Bone marrow-derived macrophages (BMDMs) were obtained from 6-8-week-old C57BL/6 mice according to a previous study.[26] The cells were cultured in RIPI-1640 medium supplemented with 10% FBS and 30% supernatant from L929 cells. Do not change the medium during cell culture. Replace half of the bone marrow macrophage induction medium after the 3rd day of culture, replace all the medium on the 5th day, and use it for subsequent experiments on the 7th day.

To activate the NLRP3 inflammasome, 1×10^6 BMDMs were seeded in 6-well plates and incubated for 16 hours. The medium was then replaced with opti-MEM, and the cells were stimulated with LPS (200 ng/mL) for 2 hours. Subsequently, the cells were treated with Met for 1 hour. And with various NLRP3 activators (2.5 mm ATP for 30 min, 10 μM nigericin for 30 min, 400μg/mL Alum for 5 h, or 300 μg/mL MSU for 5 h). For NLRC4 inflammasomes activation, 10 ng of flagellin (Invivogen, tlrl-epstla-5) was transfected into cells using DOTAP, followed by incubation for 6 h. For AIM2 inflammasome activation, the cells were transfected with 0.5 μg/mL poly(dA:dT) via Lipofectamine 2000 for 4 h.

Western Blotting

Cells and colorectum samples were lysed using Radio Immunoprecipitation Assay (RIPA) buffer containing protease inhibitors. Proteins from the supernatants were extracted, separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes. Western blotting were performed using primary antibodies against IL-1β (Abcam, ab9722), P20 (CST, 89332S), Pro-caspase-1 (Abcam, ab179515), F4/80 (Abcam, ab6640), NLRP3 (Abcam, ab263899), IKKα (CST, 61295S), ASC (CST, 67824S), P17 (CST, 63124S), UCP2 (CST, 89326S), AIM2 (CST, 12948S), p65 (CST, 8242S), β-actin (Abclonal, AC004), P-P65 (CST, 3033S), P-IKKαβ (CST, 2697S), GAPDH (Abclonal, AC002), Brg-1 (CST, 49360S), NCF-1 (Abclonal, A1148). The membranes were incubated with primary antibodies at 4°C overnight, and then with the corresponding secondary antibodies for 2 hours at room temperature. Protein bands were visualized using PierceTM ECL Western Blotting Substrate (ThermoFisher Scientific, USA) and imaged using an automatic chemiluminescence imaging system (Tanon 5200, Tanon, Shanghai, China).

ELISA for Cytokines Examination

Supernatants from cell culture or serum from mice were collected to detect the level of IL-1β (#88-7013-77) and TNF-α (#88-7324-76) using the corresponding ELISA kits (Invitrogen, ThermoFisher Scientific, USA). The procedure was performed according to the manufacturer's instructions.

Fluorescence Staining

For p65 assay, BMDMs (5×10^5 per dish) were plated in glass-bottom cell culture dishes and incubated overnight. The following day, the cells were stimulated with LPS (200 ng/mL) for 2 hours and treated with Met. And then the cells were washed twice with precooled PBS. Subsequently, stained with p65 at 4°C overnight, then washed three times, stained cell nuclei with DAPI (Solarbio, 10 μg/mL), and analyzed by confocal microscopy after washing.

For the ASC speck assay, BMDMs (5×10^5 per dish) were plated in glass-bottom cell culture dishes and incubated overnight. The following day, the cells were stimulated with LPS (200 ng/mL) for 2 hours and then treated with Met. Then the cells were fixed with paraformaldehyde, permeabilized with 0.25% Triton X-100 and incubated with ASC antibodies overnight. The following day, the cells were incubated with a fluorescent secondary antibody for 1 h. After being counterstained with DAPI, the cells were observed using a fluorescence microscope.

For UCP2 and NCF1: After treatment with ATP, the cells washed twice with precooled PBS, stained with UCP2 or NCF1 at 4°C overnight, and then the cells washed three times, stained cell nuclei with DAPI (Solarbio, 10 μg/mL), and analyzed by confocal microscopy after washing.

ASC Oligomerization

BMDMs were stimulated with LPS (200 ng/mL) for 2 hours and then treated with Met for 1h, followed by stimulation with ATP for 30 minutes. Cells were washed twice with precooled PBS, and 300 μL of lysis buffer was added, with incubation on ice for 30 minutes. The lysate was centrifuged at 340 g for 10 minutes at 4°C, the supernatant was discarded, and the pellets were washed twice with precooled PBS. Disuccinimidyl suberate (2 mm, Adamas-beta) was added to the pellets, and samples were rotated at 15 rpm at room temperature for 30 minutes. The samples were then centrifuged at 340 g for 10 minutes at 4°C, and the supernatant was discarded. The pellets were resuspended in SDS sample buffer and incubated in a water bath at 100°C for 10 minutes. Finally, the samples were analyzed by immunoblotting.

ROS Production

BMDMs were plated in 6-well plates and treated as described above. Cells were incubated with DCFH-DA (Beyotime, 10 μM) at 37°C for 30 minutes, protected from light. Cells were then harvested, washed twice with PBS, and digested with trypsin. After centrifugation at 500 g for 5 minutes at 4°C, the supernatant was discarded, and cell pellets were washed twice with precooled PBS. Fixed samples were analyzed by flow cytometry.

Histological Examination

After fixation with 4% paraformaldehyde for 24 hours, colorectal tissues were embedded in paraffin to obtain paraffin sections. These sections were then stained with hematoxylin and eosin (H&E) observed under an optical microscope. As for Immunohistochemical experiment on colorectal tissue, IL-1β, NLRP3 and F4/80 were diluted at a ratio of 1:100 and incubated overnight at 4 °C. The next day, washed three times and adding biotinylated secondary antibody and incubate at room temperature for 1 hour, washed three times. Subsequently, adding SABC (Strept Avidin-Biotin Complex) onto the tissue and incubated for 30 minutes at room temperature. After color development with DAB (3,3′-Diaminobenzidine), stain the nuclei with hematoxylin. Histopathological scores were analyzed according to established criteria.

Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM) from three independent experiments. Statistical significance was assessed using one-way ANOVA in GraphPad Prism 6.0 software, followed by post hoc testing (Tukey's test). A *p-value* of ≤ 0.05 (*), ≤ 0.01 (**), or ≤ 0.001 (***) was considered significant.

Results

Met Treatment Attenuates DSS-Induced UC in Mice

First, we explored the effects of Met in vivo, we administered 3% DSS in the drinking water of mice for 7 days, resulting in severe illness characterized by notable body weight loss, rectal bleeding, and diarrhea (Figure 1A). Met significantly mitigated the loss of body weight and reduced the DAI (Figure 1B). Additionally, histological examination revealed that Met attenuated the reduction in colorectum length induced by DSS (Figure 1C and D). Histopathological analysis revealed serious pathological changes in colorectal tissues of mice in the DSS group, such as crypt deformation, goblet cell deletion, monocyte infiltration, mucosal injury and necrosis (Figure 1E and F). However, Met could significantly ameliorate the above pathological changes.

Met Reduces Macrophage Infiltration in Colorectum Tissue and Inhibits NLRP3 Inflammasome Activation

Abnormal activation of NLRP3 inflammasome and their mediated pro-inflammatory cytokine production play an important role in UC. We found that Met significantly reduced serum levels of IL-1β (Figure 2A) Similarly, Met decreased mRNA and protein levels of IL-1β, NLRP3, and caspase-1 compared to DSS treatment alone (Figure 2B–E, Supplementary Figure 1). NLRP3 inflammasome activation is one of the major forms of macrophage activation, to further explore the role of NLRP3inflammasome in colorectal tissue of Met, by IHC, we found that Met reduced macrophage infiltration (Figure 2F and G). Additionally, co-localization studies of F4/80 with NLRP3 demonstrated that Met significantly decreased NLRP3 production and activation in macrophages (Figure 2H and I). These results collectively suggest that Met suppresses macrophage infiltration and inhibits NLRP3 inflammasome activation in colorectum tissue.

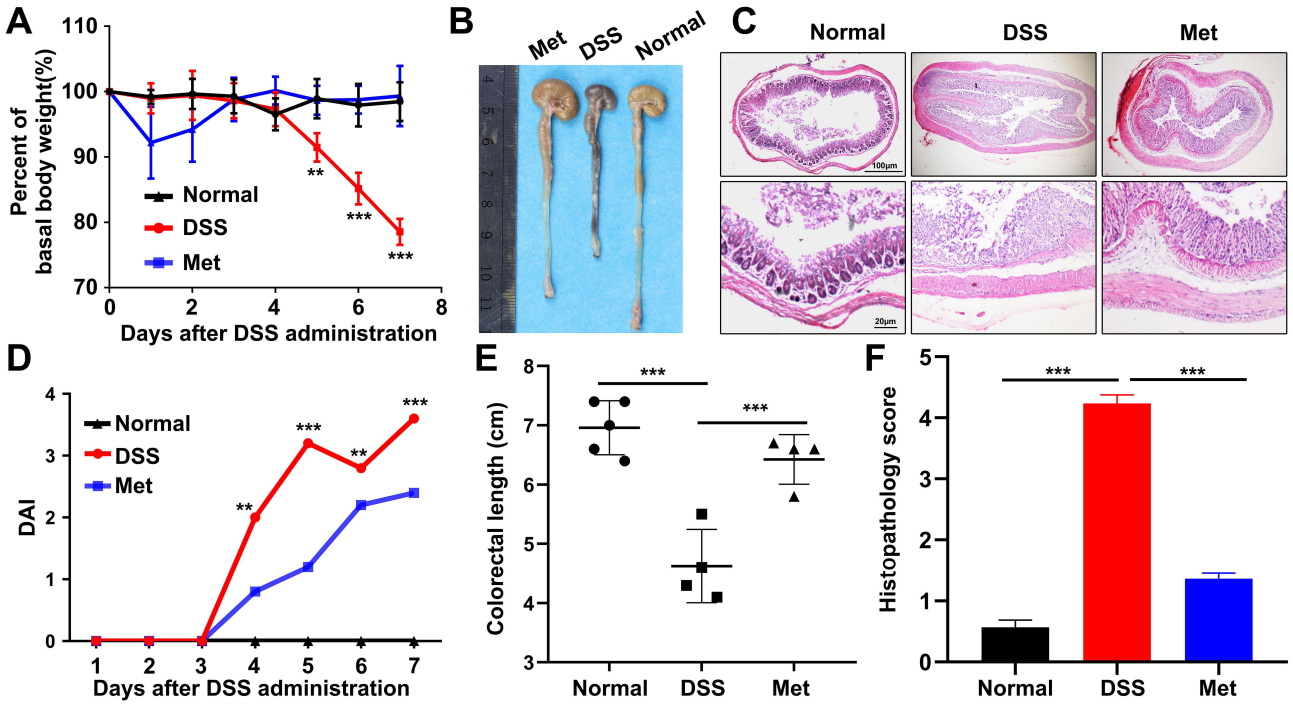

Figure 1 Met treatment attenuates DSS-induced UC in mice. Mice were given 3% DSS in drinking water for 7 d to induce acute colitis. Body weight loss (**A**). DAI (**B**) Macroscopic appearances and colorectum lengths of the mice were measured (**C** and **D**). Representative H&E-stained colorectum sections (**E** and **F**). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Values are mean ± SEM. All of the experiment repeated three times and shown the representative result.

https://doi.org/10.2147/JIR.S503033

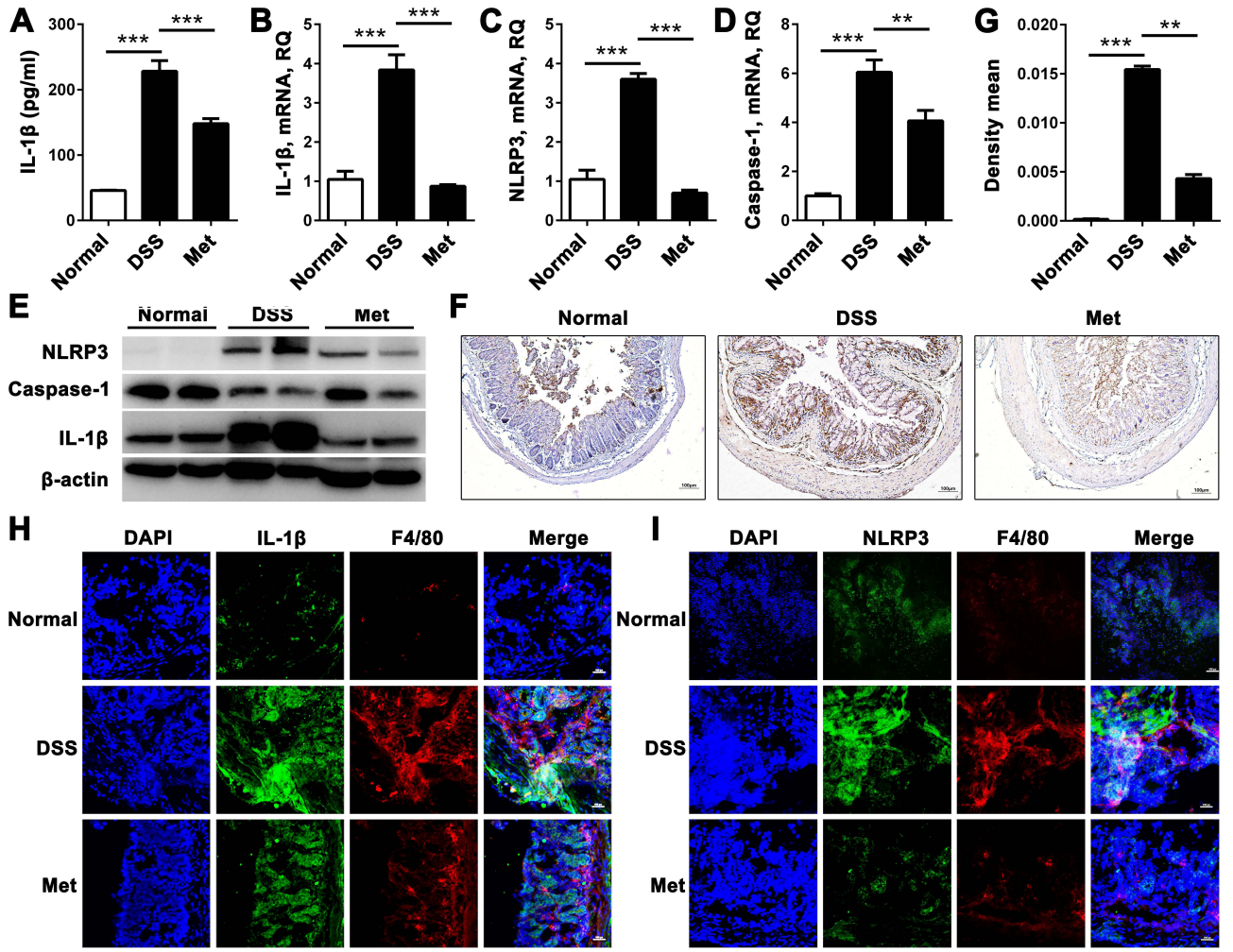

Figure 2 Met reduces macrophage infiltration in colorectum tissue and inhibits NLRP3 inflammasome activation. Serum levels of IL-1β were analyzed by ELISA (**A**). The gene levels of IL-1β, NLRP3 and caspase-1 in colorectum tissues were analyzed by RT-qPCR (**B**–**D**). The protein levels of IL-1β, NLRP3 and caspase-1 in colorectum tissues was detected by WB (**E**). Representative IHC stained colorectum sections (**F** and **G**). Scale bar corresponds to 500 μm and 100 μm. Results of F4/80/NLRP3/IL-1β colocalization labeled macrophage infiltration in colorectum tissues by IF (**H** and **I**). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Values are mean ± SEM. All of the experiment repeated three times and shown the representative result.

Met Suppresses NLRP3 Inflammasome-Mediated IL-1β Secretion and Caspase-1 Activation

To further explore whether Met amelioration of UC is associated with inhibition of NLRP3 inflammasome activation, we carried out in vitro experiments. We treated lipopolysaccharide (LPS)-primed BMDMs with Met prior to stimulation with ATP. Met inhibited caspase-1 activation (P20) and IL-1β maturation (P17) induced by ATP in a dose-dependent manner (Figure 3A and B). However, the production of TNF-α, an inflammasome-independent cytokine, remained unaffected by Met (Figure 3C). To determine if Met selectively affected ATP-induced NLRP3 inflammasome activation, we also tested other irritants (such as Nig, MSU, and alum). And we found Met effectively inhibited P20 and P17 induced by all examined agonists similar to ATP (Figure 3D and E, Supplementary Figure 2). These results indicate that Met is a potent and broad inhibitor of NLRP3 inflammasome activation, suggesting its anti-inflammasome in suppressing NLRP3 inflammasome activation and subsequent IL-1β production.

Met Inhibits NLRP3 Inflammasome Activation via the NF-κB Signaling Pathway

NLRP3 inflammasome mediated cytokine release requires two key stages: priming step: Ligands of Toll-like receptors (TLR), such as lipopolysaccharide LPS, bind to their homologous receptors resulting in the translocation of the

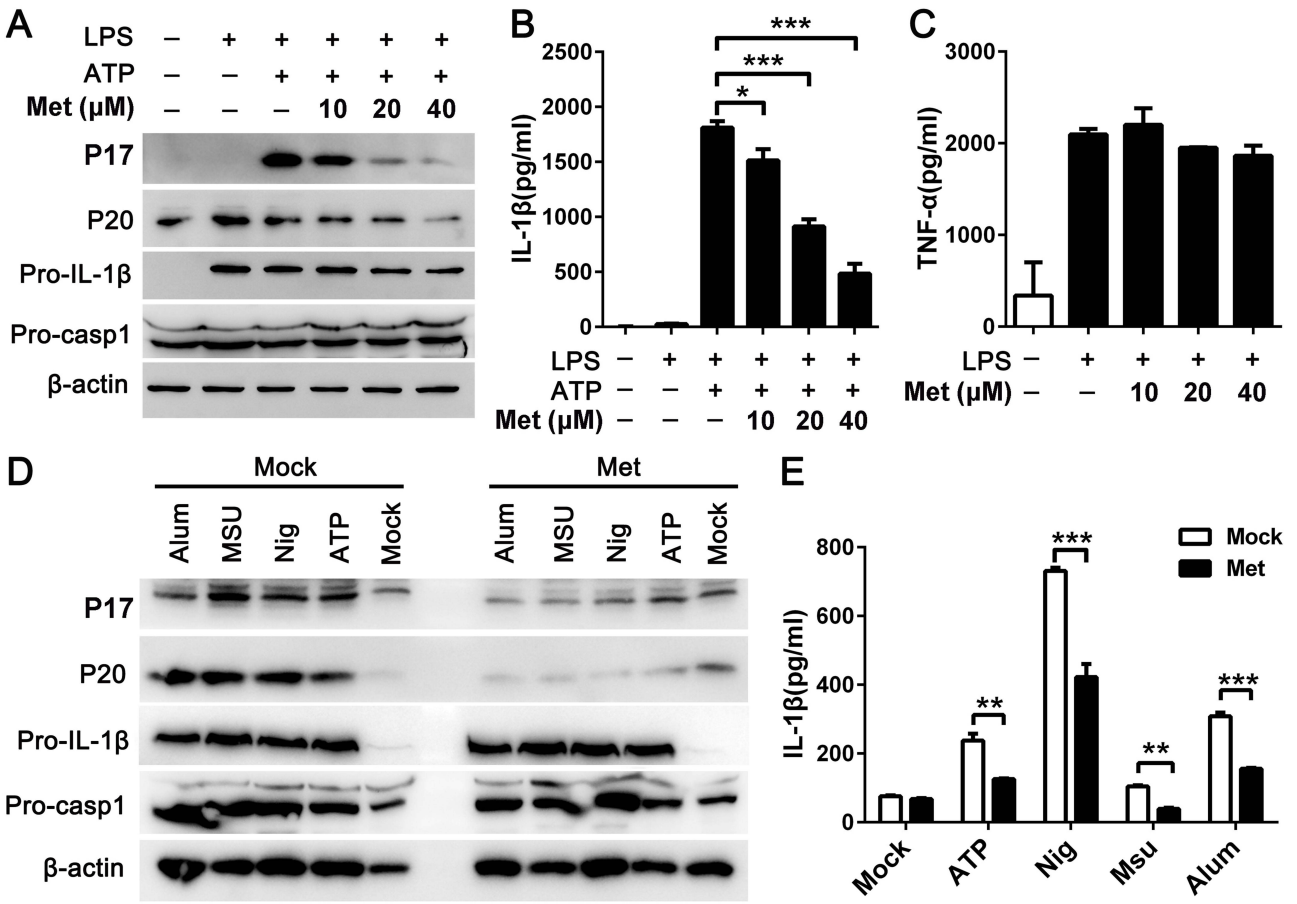

Figure 3 Met suppresses NLRP3 inflammasome-mediated IL-1β secretion and caspase-1 activation. LPS-primed BMDMs were incubated with Met (10 μM, 20 μM, 40 μM unless otherwise indicated) for 1h, followed by treatment with various NLRP3inflammasome agonists. WB analysis of cleaved IL-1β (P17) and cleaved caspase-1 (P20) in culture supernatants (Sup) and analysis of pro-IL-1β and pro-caspase-1 in lysates of BMDMs (Input) (**A**). IL-1β and TNF-α secretion in the culture supernatants was determined by ELISA (**B** and **C**). Using different NLRP3 inflammasome agonists, WB analysis of P17, P20, pro-IL-1β and pro-caspase-1 (**D**). IL-1β secretion in the culture supernatants was determined by ELISA(**E**). Data were analyzed by 1-way ANOVA with Tukey's post hoc test and were expressed as mean ± SEM of three independent experiments (n = 3 in each group). **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

transcription factor NF-κB to the nucleus, Then the expression of NLRP3, IL-1β and IL-18 precursors (pro-IL-1β and pro-IL-18) was induced. To investigate whether the NF-κB pathway mediated the inhibitory effect of Met on the inflammatory response, we assessed the protein levels of NF-κB and Iκκα by WB, and we found the levels of phosphorylated P65 and Iκκα/β (p-p65 and p-Iκκα/β) were significantly elevated after LPS stimulation but were dose-dependently reduced by Met (Figure 4A). NF-κB activation typically involves the nuclear translocation of NF-κB factors. Analysis of NF-κB factor distribution in the cytoplasm and nucleus by WB revealed that nuclear p65 levels were markedly decreased in the presence of Met. Conversely, p65 expression in cytoplasmic extracts remained largely unchanged following Met treatment, suggesting that Met inhibits NF-κB activation by preventing p65 nuclear translocation (Figure 4B and C, Supplementary Figure 3).

Then we examined the levels of genes related to the NLRP3 inflammasome under a signal stimulus. Our found that Met inhibited the expression of NLRP3, IL-1β, and caspase-1 (Figure 5A–D). Additionally, we examined other inflammasomes and found that Met specifically inhibited NLRP3 protein expression (Figure 5E, Supplementary Figure 4), this implies that Met may play a role in suppressing inflammation through the NF-κB signaling pathway.

Met Inhibits NLRP3-Mediated ASC Oligomerization and ASC Formation

We further investigated ASC oligomerization, a common mechanism of NLRP3 inflammasome activation. ASC molecules within the cell aggregate in the cytoplasm to form supramolecular polymers. This process is tightly regulated

https://doi.org/10.2147/JIR.S503033

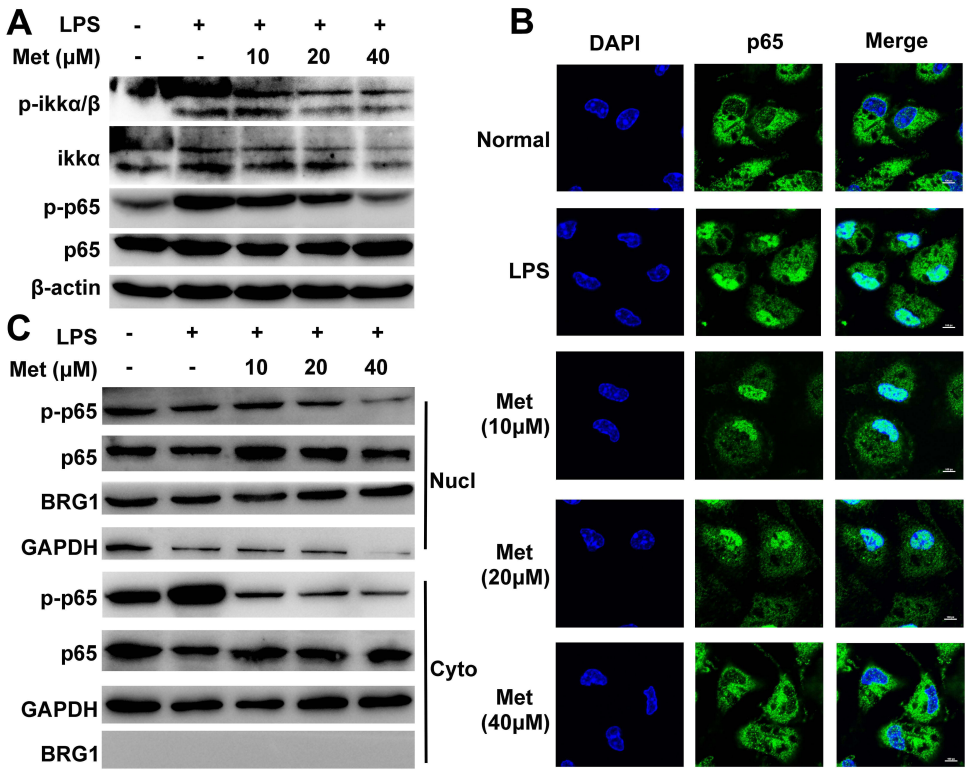

Figure 4 Met suppressed the LPS-induced NF-κB Signaling Pathway. LPS-primed BMDMs were incubated with Met (10 μM, 20 μM, 40μM unless otherwise indicated) for 1 h, cell lysates were immunoblotted for cleaved p65, p-p65, ikkα, p ikkα/β and β-actin served as loading control (**A**), p65 was detected by immunofluorescence(**B**), The cytoplasm and nucleus were separated by the kit and p65, p-p65, GAPDH and BRG1 served as loading control (**C**).

and occurs rapidly. Typically, only one ASC speck is formed per cell. We found that Met significantly attenuated the formation of this ASC complex (Figure 6A). Furthermore, upon NLRP3 inflammasome activation by ATP, ASC condensed into large cytosolic specks in each cell, a process inhibited by Met in BMDMs (Figure 6B and C). To confirm these findings, we isolated BMDMs from ASC knockout mice and observed that the effect of Met disappeared (Figure 6D and E, Supplementary Figure 5). These results indicated that Met inhibits NLRP3-mediated ASC oligomerization and ASC speck straining.

Met Inhibited ROS Production Mainly by Up-Regulating UCP2 and NCF1 Expression

ROS play a critical role in the activation of the NLRP3 inflammasome. Thus, we evaluated ROS levels in BMDMs following treatment with Met. Remarkably, we observed a significant reduction in ROS levels (Figure 7A and B). UCP2 and NCF1 are inner mitochondrial membrane proteins known to directly mitigate ROS generation. We found that Met promotes the expressions of UCP2 and NCF1 by IF (Figure 7C). Moreover, the same results were also observed in WB (Figure 7D, Supplementary Figure 6).

Discussion

UC is a chronic inflammatory condition affecting the rectum and colorectum, with varying severity among individuals.[26] As of 2023, the global prevalence of UC was estimated at 5 million cases, and its incidence continues to rise worldwide.[27] Current therapeutic approaches primarily involve anti-inflammatory medications such as aminosalicylic acid preparations and glucocorticoids, along with immune-modulating drugs like immunosuppressants, aimed at controlling inflammation and reducing symptoms.[28] Despite expanding treatment options, 10–20% of patients still require proctocolectomy due to medically refractory disease. Hence, there is an imperative to discover safer and more effective drugs for UC treatment.[29]

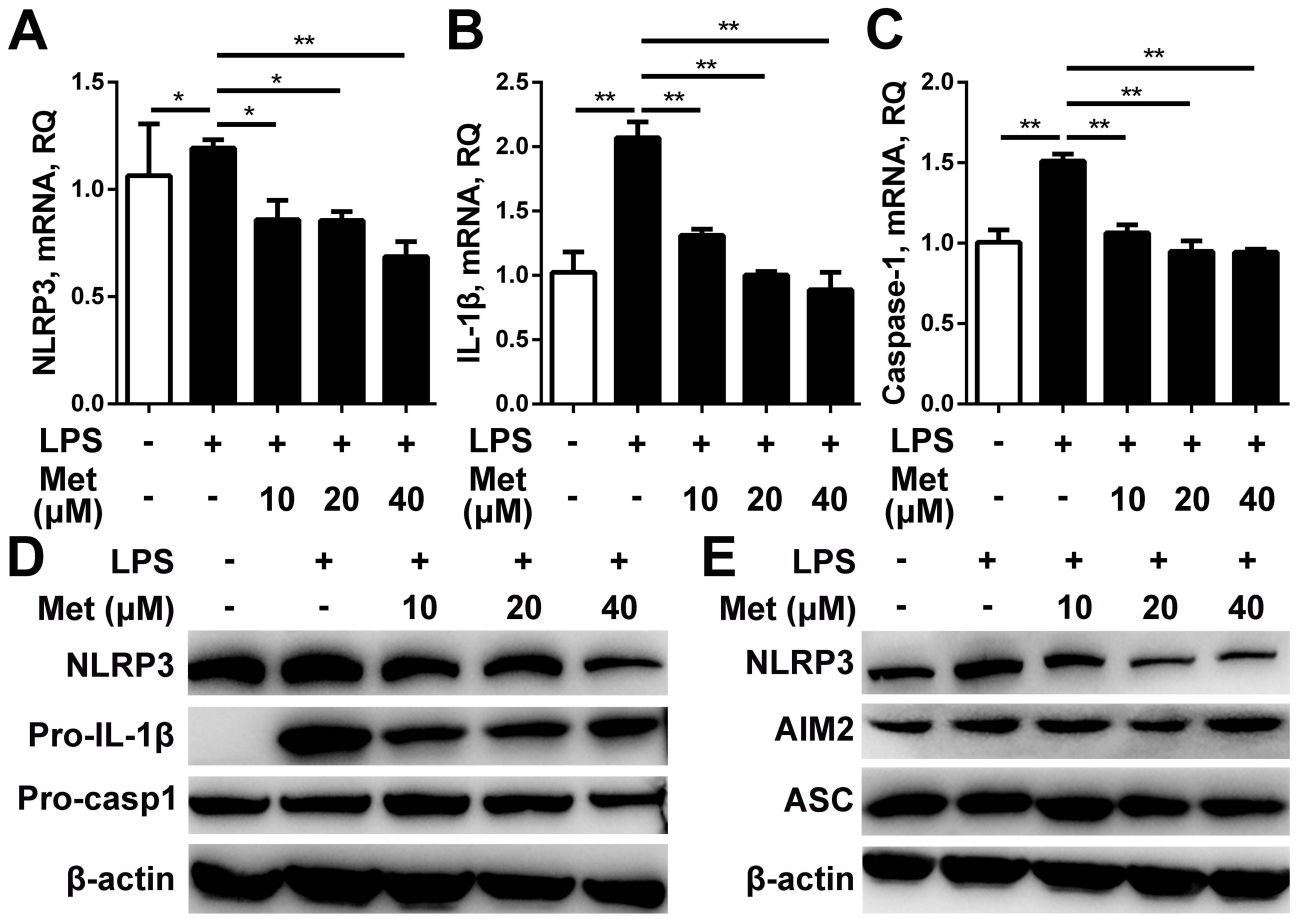

Figure 5 Met inhibited NLRP3inflammasome in a NF-κB-dependent manner. LPS-primed BMDMs were incubated with Met (10 μM, 20 μM, 40μM unless otherwise indicated) for1h, cell RNA was PCR for NLRP3, IL-1β, caspase-1 (**A-C**), NLRP3, Pro-casp1, Pro-IL-1β was detected by WB (**D**), NLRP3, AMI2, ASC was detected by WB (**E**). * $P \leq 0.05$, *** $P \leq 0.001$. Values are mean ± SEM. All of the experiment repeated three times and shown the representative result.

The pathogenesis of UC is intricate, with the uncontrolled inflammatory response of the intestine being a central feature. Elevated levels of NLRP3 and IL-1β have been observed in UC mice, while NLRP3 deficiency in mice leads to decreased levels of NLRP3 and IL-1β, accompanied by alleviation of UC symptoms.[30–32] Consequently, targeting NLRP3 inflammasome activation emerges as a promising therapeutic strategy for UC. In our study, we also observed up-regulation of gene and protein levels of NLRP3 and IL-1β in the colorectum tissue of UC mice. In Met group, we observed a significant reduction in serum inflammatory factor levels, improved inflammatory infiltration of colorectal mucosa, and restoration of colorectal epithelial structure. Furthermore, immunofluorescence analysis revealed co-localization of IL-1β and F4/80, as well as NLRP3 and F4/80 in colorectum tissue, indicating the involvement of macrophages in the inflammatory response. Met treatment resulted in reduced co-localization signals, suggesting alleviation of inflammatory factor production. These findings collectively demonstrate the beneficial effects of Met in ameliorating UC in mice. However, there are also limitations in our study. We did not detect the changes in macrophages in peripheral blood, lymph nodes and the spleen after Met treatment.

As widely acknowledged, the NLRP3 inflammasome-mediated cytokine release involves two crucial stages: priming and activation. During the priming step, ligands of Toll-like receptors (TLR), such as LPS, bind to their respective receptors, leading to the translocation of the transcription factor NF-κB into the nucleus. Previous studies have suggested that Met was found to block NF-κB signal transduction in colitis mice, thereby reducing the expression of IL-1β, IL-6, gamma interferon, and TNF-α, inhibiting the activity of cyclooxygenase-2 and inducible nitric oxide synthase, and subsequently alleviating inflammation.[33] In our study, we also observed that Met affected the expression of pro-IL-1β and NLRP3 at both gene and protein levels. Additionally, Met influenced the expression of p65 and its nuclear translocation.

https://doi.org/10.2147/JIR.S503033

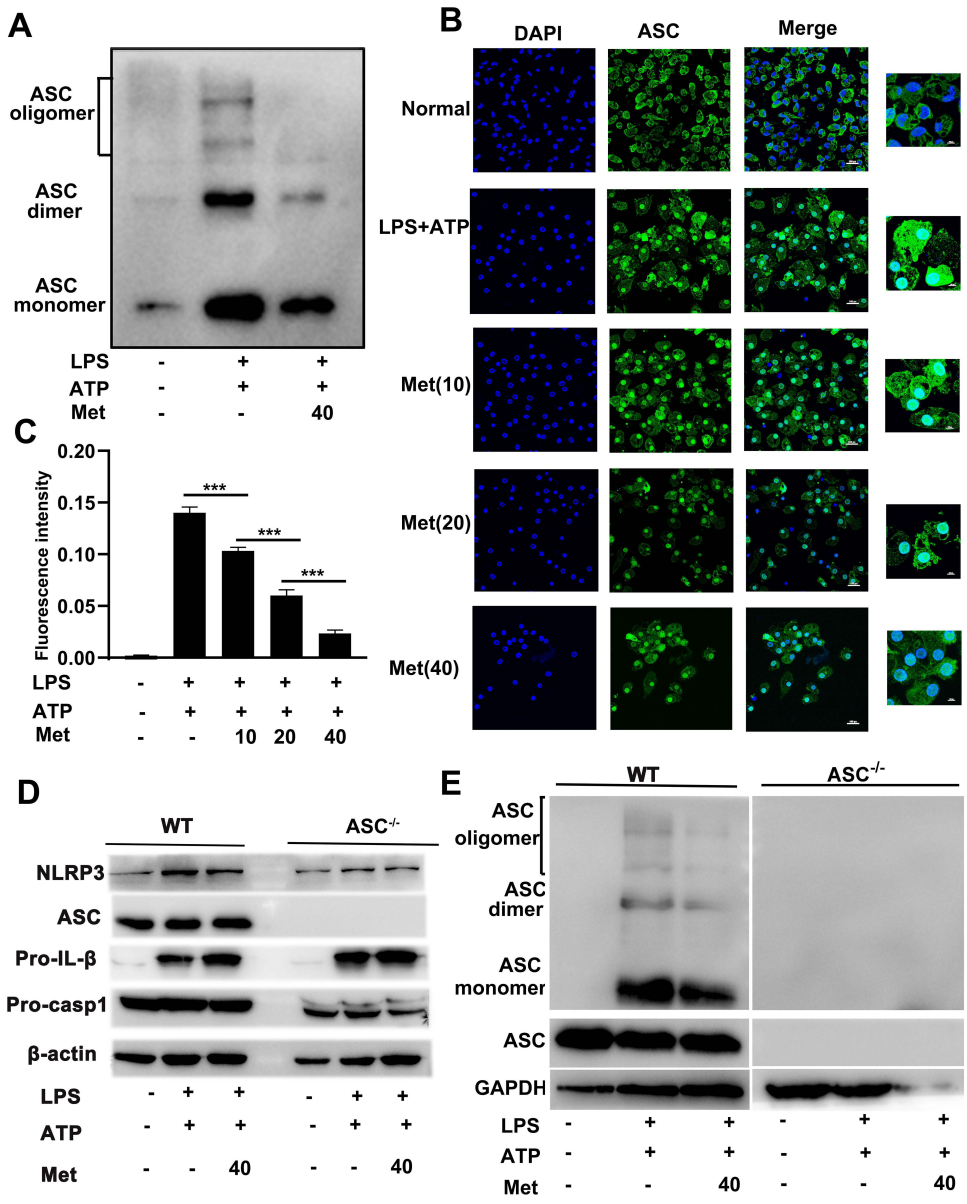

Figure 6 Met inhibits ASC oligomerization and ASC speck formation. LPS-primed BMDMs were treated with 10 μM, 20 μM and 40μM Met and then stimulated with ATP. ASC oligomerization by WB (**A**). Representative immunofluorescence images of ASC speck formation by confocal microscopy (**B** and **C**). ASC knockout mice, cell lysates were immunoblotted for NLRP3, Pro-casp1, Pro-IL-1β, ASC and β-actin served as loading control (**D**), ASC oligomerization by immunoblotting (**E**). Scale bars represent 200mm(panel). *** $P \leq 0.001$. Values are mean ± SEM. All of the experiment repeated three times and shown the representative result.

This reveals that Met may play a protective role through the NF-κB signaling pathway. In the activation step, proteins responsible for recognizing PAMPs or DAMPs activate the NLRP3 inflammasome, leading to the aggregation and cleavage of pro-caspase-1 into mature caspase-1. Subsequently, pro-IL-1β and pro-IL-18 are cleaved to produce the inflammatory cytokines IL-1β and IL-18.[34,35] In this study, we discovered that Met inhibited the activation of NLRP3 inflammasomes in primary macrophages, thereby blocking caspase-1 cleavage and the release of cytokines IL-1β in a dose-dependent manner. Furthermore, Met significantly suppressed the release of IL-1β induced by other DAMPs.

ASC, as one of the crucial components of the NLRP3 inflammasome, interacts with homologous proteins via its N-terminal PYD domain and C-terminal CARD domain to recruit NLRP3 and the precursor of caspase-1, thereby completing the assembly.[36] The formation of ASC specks promotes the autocleavage of the caspase-1 precursor into active caspase-1. During the activation of the inflammasome, all ASC molecules within the cell aggregate in the cytoplasm to form supramolecular polymers. This process is tightly regulated and occurs rapidly. Typically, only one

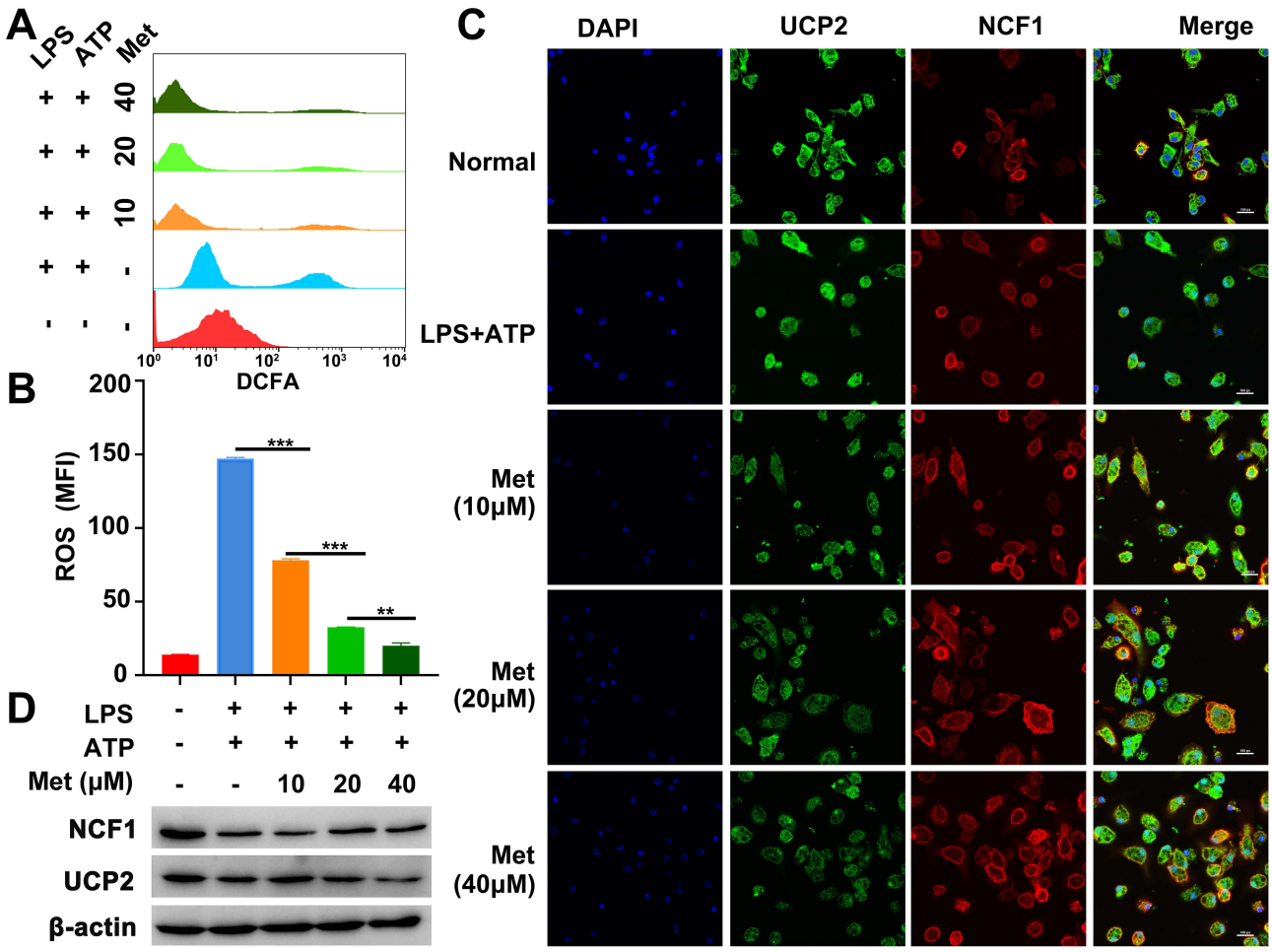

Figure 7 Met inhibited ROS production mainly by up-regulating UCP2 and NCF1 expression. FACS analysis of ROS generation from LPS-primed BMDMs treated for 1 h with various doses of Met (**A**). The fluorescence intensity was determined by FlowJo. (**B**). Immunofluorescence analysis of UCP2 and NCF1 expression (**C**). WB analysis of UCP2 and NCF1 expression (**D**). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Values are mean ± SEM. Data are representative of three independent experiments.

ASC speck is formed per cell.[37] Our research results indicated that Met could inhibit the formation of ASC oligomers and ASC specks. However, how Met regulates ASC oligomers and which domain of ASC it functions through requires further investigation. Additionally, in our study, we also conducted verification in ASC knockout mice and further confirmed that Met can inhibit NLRP3 inflammasome activation by suppressing ASC oligomerization.

The generation of ROS are key factors triggering the activation of the NLRP3 inflammasome.[38] ROS is primarily produced through processes such as NADPH oxidation, mitochondrial respiratory chain activity, and lipid peroxidation of mitochondrial membranes. Studies have demonstrated that suppressing ROS production can hinder NLRP3 inflammasome activation.[39] In our study, we discovered that Met could significantly inhibit the release of ROS in BMDMs cells in a dose-dependent manner. Uncoupling protein 2 (UCP2), which is located in the inner mitochondrial membrane, mainly transports protons from the intermembrane space of mitochondria into the matrix, thereby reducing the mitochondrial membrane potential.[40] UCP2 plays an important role in regulating ATP production in mitochondria, maintaining calcium homeostasis, and eliminating ROS.[41] In this study, we found that Met significantly promotes the expression of UCP2 protein, indicating that Met may be involved in the UCP2-ROS signaling pathway in mitochondria. Prior research has indicated that elevating UCP2 expression in cardiomyocytes decreases mtROS levels and suppresses NLRP3 inflammasome activation.[42] Furthermore, NCF1 can also impede NLRP3 inflammasome activation by negatively modulating ROS expression.[43] We also found that Met can promote the expression of NCF1. Further elucidation suggests that Met may inhibit the activation of the NLRP3 inflammasome by suppressing the release of ROS, thereby alleviating

https://doi.org/10.2147/JIR.S503033

the occurrence and development of UC. Nevertheless, we merely detected the level of ROS. Other parameters such as mitochondrial membrane potential, mitochondrial DNA, and mitochondrial respiratory chain were not evaluated.

Conclusion

In conclusion, Met significantly ameliorated UC by inhibiting NLRP3 inflammasome activation in macrophages. The underlying mechanisms not only involved the inhibition of NF-κB signaling pathway activation (first signal), but was also associated with up-regulation of UCP2 and NCF1 levels and thus the repression of ROS generation (second signal).

Data Sharing Statement

Data will be made available on request.

Ethics Approval

All animal procedures were approved by the Animal Experimental Ethics Committee of Henan University (HUSOM-2018-303), and complied with the relevant requirements of the People's Republic of China "Laboratory animals - General code of animal welfare" (GB/T 42011-2022).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This research was funded by the Key project of National Natural Science Foundation (No. U22A20382), the National Natural Science Foundation of China (82100897), the Natural Science Foundation of Henan Province (No. 222300420118), the China Postdoctoral Science Foundation (No. 2021M701061), the medical science and technology research in Henan Province (SBGJ202102196 and SBGJ202103095), the Key Scientific Research Project Plan of Colleges and Universities in Henan Province (21A310002).

Disclosure

The authors declare that they have no competing interests in this work.

References

1. Kaplan GG. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol*. 2015;12(12):720–727. doi:10.1038/nrgastro.2015.150
2. Eisenstein M. Ulcerative colitis: towards remission. *Nature*. 2018;563(7730):S33. doi:10.1038/d41586-018-07276-2
3. Gravina AG, Pellegrino R, Durante T, et al. The melanocortin system in inflammatory bowel diseases: insights into its mechanisms and therapeutic potentials. *Cells*. 2023;12(14):1889. doi:10.3390/cells12141889
4. Gravina AG, Panarese I, Trotta MC, et al. Melanocortin 3,5 receptors immunohistochemical expression in colorectal mucosa of inflammatory bowel disease patients: a matter of disease activity? *World J Gastroenterol*. 2024;30(9):1132–1142. doi:10.3748/wjg.v30.i9.1132
5. Miler M, Živanović J, Kovačević S, et al. Citrus flavanone effects on the Nrf2-Keap1/GSK3/NF-κB/nlrp3 regulation and corticotroph-stress hormone loop in the old pituitary. *Int J mol Sci*. 2024;25(16):8918. doi:10.3390/ijms25168918
6. Hiramoto K, Yamate Y, Yokoyama S. Ultraviolet B eye irradiation aggravates atopic dermatitis via adrenocorticotropic hormone and NLRP3 inflammasome in NC/Nga mice. *Photodermatol Photoimmunol Photomed*. 2018;34(3):200–210. doi:10.1111/phpp.12372
7. Hirten RP, Sands BE. New Therapeutics for Ulcerative Colitis. *Annu Rev Med*. 2021;72:199–213. doi:10.1146/annurev-med-052919-120048
8. Hooftman A, Angiari S, Hester S, et al. The immunomodulatory metabolite itaconate modifies NLRP3 and inhibits inflammasome activation. *Cell Metab*. 2020;32(3):468–478.e7. doi:10.1016/j.cmet.2020.07.016
9. Fu J, Wu H. Structural mechanisms of NLRP3 inflammasome assembly and activation. *Annu Rev Immunol*. 2023;41:301–316. doi:10.1146/annurev-immunol-081022-021207
10. Mangan MSJ, Olhava EJ, Roush WR, et al. Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov*. 2018;17(8):588–606. doi:10.1038/nrd.2018.97
11. Xian H, Watari K, Sanchez-Lopez E, et al. Oxidized DNA fragments exit mitochondria via mPTP- and VDAC-dependent channels to activate NLRP3 inflammasome and interferon signaling. *Immunity*. 2022;55(8):1370–1385.e8. doi:10.1016/j.immuni.2022.06.007

12. Lv Q, Xing Y, Liu J, et al. Lonicerin targets EZH2 to alleviate ulcerative colitis by autophagy-mediated NLRP3 inflammasome inactivation. *Acta Pharm Sin B*. 2021;11(9):2880–2899. doi:10.1016/j.apsb.2021.03.011
13. Paradies G, Paradies V, Ruggiero FM, et al. Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease. *World J Gastroenterol*. 2014;20(39):14205–14218. doi:10.3748/wjg.v20.i39.14205
14. Ali FEM, Ibrahim IM, Ghogar OM, et al. Therapeutic interventions target the NLRP3 inflammasome in ulcerative colitis: comprehensive study. *World J Gastroenterol*. 2023;29(6):1026–1053. doi:10.3748/wjg.v29.i6.1026
15. Huang J, Dai M, He, M et al. Treatment of Ulcerative Colitis by Cationic Liposome Delivered NLRP3 siRNA. *Int J Nanomedicine*. 2023, Aug 16;18:4647–4662. doi:10.2147/IJN.S413149
16. Cao R, Ma Y, Li S, et al. 1,25$(OH)_2D_3$alleviates DSS-induced ulcerative colitis via inhibiting NLRP3 inflammasome activation. *J Leukoc Biol*. 2020;108(1):283–295. doi:10.1002/JLB.3MA0320-406RR
17. LaMoia TE, Shulman GI. Cellular and molecular mechanisms of metformin action. *Endocr Rev*. 2021;42(1):77–96. doi:10.1210/endrev/bnaa023
18. Xian H, Liu Y, Rundberg Nilsson A, et al. Metformin inhibition of mitochondrial ATP and DNA synthesis abrogates NLRP3 inflammasome activation and pulmonary inflammation. *Immunity*. 2021;54(7):1463–1477.e11. doi:10.1016/j.immuni.2021.05.004
19. Youssef ME, Abd El-Fattah EE, Abdelhamid AM, et al. Interference with the AMPKα/mTOR/NLRP3 Signaling and the IL-23/IL-17 axis effectively protects against the dextran sulfate sodium intoxication in rats: a new paradigm in empagliflozin and metformin reprofiling for the management of ulcerative colitis. *Front Pharmacol*. 2021;12:719984. doi:10.3389/fphar.2021.719984
20. Saber S, El-Kader EMA. Novel complementary coloprotective effects of metformin and MCC950 by modulating HSP90/NLRP3 interaction and inducing autophagy in rats. *Inflammopharmacology*. 2021;29(1):237–251. doi:10.1007/s10787-020-00730-6
21. Shaaban AA, Abdelhamid AM, Shaker ME, et al. Combining the HSP90 inhibitor Tas-116 with metformin effectively degrades the NLRP3 and attenuates inflammasome activation in rats: a new management paradigm for ulcerative colitis. *Biomed Pharmacother*. 2022;153:113247. doi:10.1016/j.biopha.2022.113247
22. Yang F, Qin Y, Wang Y, et al. Metformin inhibits the NLRP3 inflammasome via AMPK/mTOR-dependent effects in diabetic cardiomyopathy. *Int J Biol Sci*. 2019;15(5):1010–1019. doi:10.7150/ijbs.29680
23. Abdi M, Pasbakhsh P, Shabani M, et al. Metformin therapy attenuates pro-inflammatory microglia by inhibiting NF-κB in cuprizone demyelinating mouse model of multiple sclerosis. *Neurotox Res*. 2021;39(6):1732–1746. doi:10.1007/s12640-021-00417-y
24. Zakikhani M, Dowling RJ, Sonenberg N, et al. The effects of adiponectin and metformin on prostate and colorectum neoplasia involve activation of AMP-activated protein kinase. *Cancer Prev Res*. 2008;1(5):369–375. doi:10.1158/1940-6207
25. Villumsen M, Schelde AB, Jimenez-Solem E, et al. GLP-1 based therapies and disease course of inflammatory bowel disease. *EClinicalMedicine*. 2021;37:100979. doi:10.1016/j.eclinm.2021.100979
26. Gros B, Kaplan GG. Ulcerative colitis in adults: a review. *JAMA*. 2023;330(10):951–965. doi:10.1001/jama.2023.15389
27. Ochoa B, McMahon L. Surgery for ulcerative colitis. *Semin Pediatr Surg*. 2024;33(2):151404. doi:10.1016/j.sempedsurg.2024.151404
28. Wangchuk P, Yeshi K, Loukas A. Ulcerative colitis: clinical biomarkers, therapeutic targets, and emerging treatments. *Trends Pharmacol Sci*. 2024;45(10):892–903. doi:10.1016/j.tips.2024.08.003
29. Marafini I, Roseira J, Duijvestein M. Treatment options for refractory ulcerative colitis: small molecules, big effects. *United European Gastroenterol J*. 2024;12(5):539–540. doi:10.1002/ueg2.12585
30. Li X, Wu X, Wang Q, et al. Sanguinarine ameliorates DSS induced ulcerative colitis by inhibiting NLRP3 inflammasome activation and modulating intestinal microbiota in C57BL/6 mice. *Phytomedicine*. 2022;104:154321. doi:10.1016/j.phymed.2022.154321
31. Mai CT, Wu MM, Wang CL, et al. Palmatine attenuated dextran sulfate sodium (DSS)-induced colitis via promoting mitophagy-mediated NLRP3 inflammasome inactivation. *Mol Immunol*. 2019;105:76–85. doi:10.1016/j.molimm.2018.10.015
32. Zeng B, Huang Y, Chen S, et al. Dextran sodium sulfate potentiates NLRP3 inflammasome activation by modulating the KCa3.1 potassium channel in a mouse model of colitis. *Cell mol Immunol*. 2022;19(8):925–943. doi:10.1038/s41423-022-00891-0
33. Zhan X, Li Q, Xu G, et al. The mechanism of NLRP3 inflammasome activation and its pharmacological inhibitors. *Front Immunol*. 2023;13:1109938. doi:10.3389/fimmu.2022.1109938
34. Bai D, Du J, Bu X, et al. ALDOA maintains NLRP3 inflammasome activation by controlling AMPK activation. *Autophagy*. 2022;18(7):1673–1693. doi:10.1080/15548627.2021.1997051
35. Wang L, Cui J. Palmitoylation promotes chaperone-mediated autophagic degradation of NLRP3 to modulate inflammation. *Autophagy*. 2023;19 (10):2821–2823. doi:10.1080/15548627.2023.2187957
36. Dick MS, Sborgi L, Rühl S, et al. ASC filament formation serves as a signal amplification mechanism for inflammasomes. *Nat Commun*. 2016;7:11929. doi:10.1038/ncomms11929
37. Vanaja SK, Russo AJ, Behl B, et al. Bacterial outer membrane vesicles mediate cytosolic localization of LPS and Caspase-11 activation. *Cell*. 2016;165(5):1106–1119. doi:10.1016/j.cell.2016.04.015
38. Liu Y, Huang Y, Xu C, et al. Mitochondrial dysfunction and therapeutic perspectives in cardiovascular diseases. *Int J mol Sci*. 2022;23(24):16053. doi:10.3390/ijms232416053
39. Liu X, Li M, Chen Z, et al. Mitochondrial calpain-1 activates NLRP3 inflammasome by cleaving ATP5A1 and inducing mitochondrial ROS in CVB3-induced myocarditis. *Basic Res Cardiol*. 2022;117(1):40. doi:10.1007/s00395-022-00948-1
40. Moon JS, Lee S, Park MA, et al. UCP2-induced fatty acid synthase promotes NLRP3 inflammasome activation during sepsis. *J Clin Invest*. 2015;125(2):665–680. doi:10.1172/JCI78253
41. Forte M, Bianchi F, Cotugno M, et al. An interplay between UCP2 and ROS protects cells from high-salt-induced injury through autophagy stimulation. *Cell Death Dis*. 2021;12(10):919. doi:10.1038/s41419-021-04188-4
42. Wang D, Yu X, Gao K, et al. Sweroside alleviates pressure overload-induced heart failure through targeting CaMKIIδ to inhibit ROS-mediated NF-κB/NLRP3 in cardiomyocytes. *Redox Biol*. 2024;74:103223. doi:10.1016/j.redox.2024.103223
43. Luo H, Urbonaviciute V, Saei AA, et al. NCF1-dependent production of ROS protects against lupus by regulating plasmacytoid dendritic cell development and functions. *JCI Insight*. 2023;8(7):e164875. doi:10.1172/jci.insight.164875