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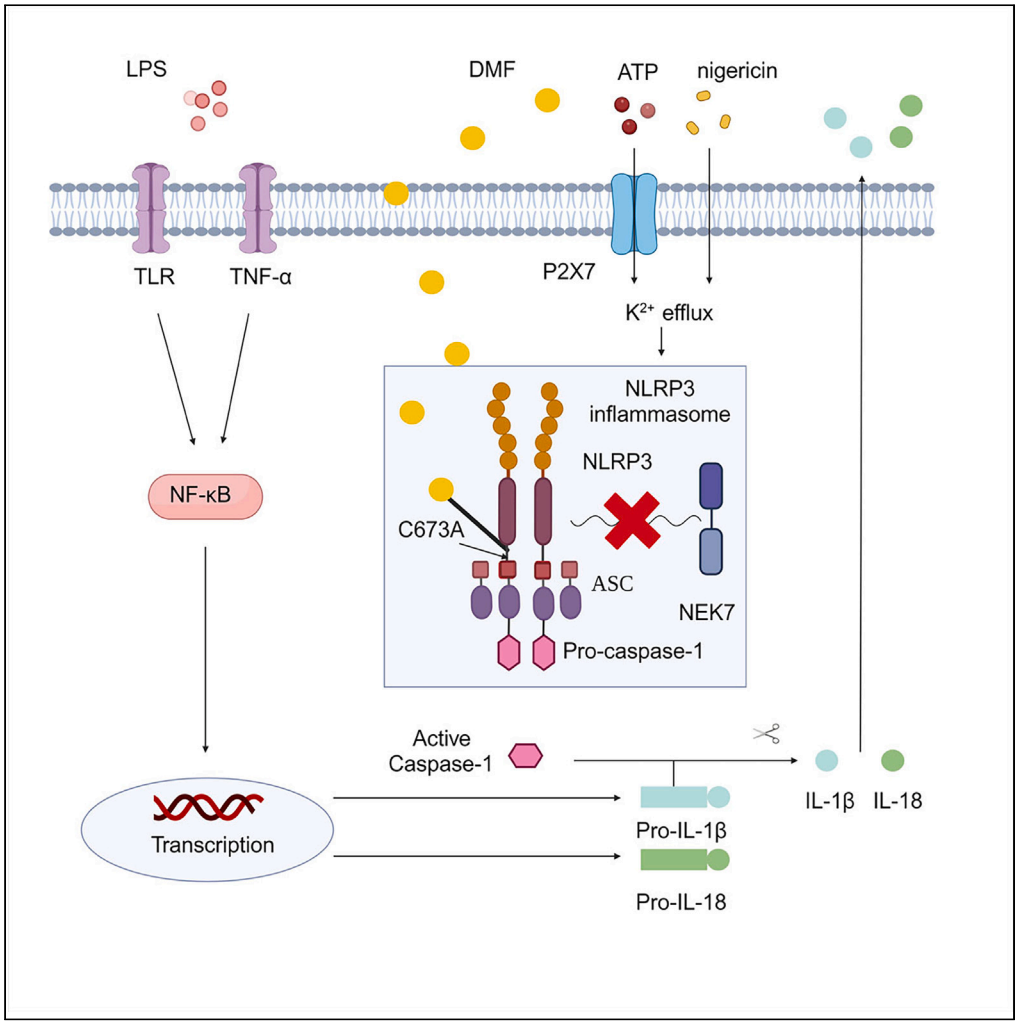

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Highlights

Dimethyl fumarate inhibits NLRP3 inflammasome activation in macrophages

Dimethyl fumarate targets Cys673 of NLRP3 to block NLRP3 inflammasome assembly

Dimethyl fumarate relieves ulcerative colitis by targeting NLRP3

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Dimethyl fumarate covalently modifies Cys673 of NLRP3 to exert anti-inflammatory effects

Huiting Hu,[1,3] Yuqian Cai,[2,3] Yuanfang Shi,[1] Shengyu Zhang,[1] Xiaoxuan Yu,[1] Tonghui Ma,[1,*] and Shanting Liao[1,4,*]

SUMMARY

The NLRP3 inflammasome plays a pivotal role in various chronic inflammation-driven human diseases. However, no drugs specifically targeting NLRP3 inflammasome have been approved by the Food and Drug Administration (FDA) of the United States. In our current study, we showed that dimethyl fumarate (DMF) efficiently suppressed the activation of the NLRP3 inflammasome induced by multiple agonists and covalently modified Cys673 of NLRP3, thereby impeding the interaction between NLRP3 and NEK7. The inhibitory effect of DMF was nullified by anaplerosis of the Cys673 mutant (but not the wild-type) NLRP3 in *Nlrp3*$^{-/-}$ THP-1 cells. *In vivo* experiments, DMF demonstrated protective effects in the dextran sodium sulfate (DSS)-induced ulcerative colitis of WT mice, but not in *Nlrp3*$^{-/-}$ mice. In summary, our study identified DMF as a direct covalent inhibitor of NLRP3 and a potential candidate for the treatment of NLRP3 inflammasome-mediated diseases.

INTRODUCTION

The NLRP3 inflammasome, a pivotal component of innate immunity, is a multi-protein complex that assembles in the cytoplasm or on the membranes of cellular organelles, such as endolysosomes or the *trans*-Golgi.[1–4] This inflammasome responds to invading microbes and endogenous host products associated with cellular stress and injury.[5,6] The canonical NLRP3 inflammasome comprises the innate immune sensor NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and pro-Caspase-1.[7]

In macrophages, activation of the NLRP3 inflammasome involves both a priming step and an activation step.[8,9] The priming step primarily entails NF-κB activation through Toll-like receptor (TLR) pathways, promoting the transcription and expression of NLRP3, pro-IL-1β, and pro-IL-18. This step is often investigated *in vitro* using lipopolysaccharide (LPS). The activation step involves the assembly and activation of the NLRP3 inflammasome triggered by a cascade of signals in response to stimuli from pathogenic microorganisms, such as viruses, bacteria, or various danger signals, including cholesterol crystals, urate crystals, β-amyloid peptides, ceramide, and palmitate. The assembled NLRP3 inflammasome activates the protease Caspase-1, leading to the cleavage of pro-IL-1β and pro-IL-18, facilitating the release of IL-1β and IL-18. K^+ efflux, Ca^{2+} mobilization, and mitochondrial damage have all been implicated in NLRP3 inflammasome assembly and activation.[5,10,11] Furthermore, imiquimod and CL097 can activate the canonical NLRP3 inflammasome independently of K^+ efflux.[12,13] NEK7 (NIMA-related kinase 7) is a key regulator of NLRP3 activation, interacting with NLRP3 to facilitate its interaction with ASC, promoting NLRP3 inflammasome assembly and activation.[14–16] NEK7 participates in all NLRP3 inflammasome stimulators, including adenosine triphosphate (ATP), nigericin, monosodium urate crystals (MSU), and alum, playing a vital role in NLRP3 inflammasome activation.[17]

Recognizing a broad range of risk signals from various sources, the NLRP3 inflammasome is the most extensively studied inflammasome implicated in the development of numerous chronic inflammatory diseases, including ulcerative colitis, diabetes, cardiovascular disease, neurodegenerative diseases, autoimmune diseases, and metabolic disorders.[18–21] Consequently, the NLRP3 inflammasome is considered a potential drug target for treating inflammatory diseases.

The NLRP3 inflammasome is implicated in numerous human diseases, prompting significant research into NLRP3 inflammasome inhibitors. Several small-molecule compounds have demonstrated inhibitory effects on the NLRP3 inflammasome, falling into three categories: inhibitors targeting the upstream signaling of NLRP3 inflammasome (e.g., Glyburide, 16673-34-0, JC124, FC11A-2), inhibitors acting on the constituents of the NLRP3 inflammasome (e.g., Parthenolide, VX-740, VX-765, Bay 11–7082, β-Hydroxybutyrate, Licochalcone B), and direct inhibitors of the NLRP3 protein (e.g., MCC950, CY-09, OLT1177, tranilast, oridonin, itaconate, RRx-001).[22–27] While some of these compounds have shown promising therapeutic effects on NLRP3-related diseases in both *in vitro* and *in vivo* studies, none have been approved by Food and Drug Administration (FDA) of United States. Consequently, there is an urgent need to develop highly safe NLRP3 inflammasome inhibitors for clinical testing.

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Dimethyl fumarate (DMF) has garnered approval as a first-line treatment for relapsing-remitting multiple sclerosis (RRMS) in various countries, including the United States, Germany, and China.[28] Over recent years, oral administration of DMF has demonstrated a significant reduction in the relapse rate of RRMS, establishing itself as a prominent oral immunomodulatory drug for treating RRMS.[29] While the precise mechanism of DMF in RRMS remains incompletely understood, it is widely acknowledged that DMF exerts its beneficial effects through the regulation of Nrf2-dependent and independent pathways.[30,31] Initially approved in Europe as a psoriasis drug, DMF has been reported to prevent the processing and oligomerization of Gasdermin D, thereby limiting pore formation, cytokine release, and cell death.[32] Additionally, DMF has been shown to inhibit both the priming and activation steps of NLRP3 activation.[33–35]

In our current study, we discovered that DMF specifically inhibited the activation of the NLRP3 inflammasome. DMF achieved this by covalently binding to cysteine 673 (Cys673) of NLRP3, disrupting the interaction between NLRP3 and NEK7. Consequently, DMF impedes the oligomerization of ASC, leading to the inhibition of NLRP3 inflammasome assembly and the subsequent activation of Caspase-1, IL-1β and IL-18 release. Furthermore, in a mouse model of dextran sodium sulfate (DSS)-induced ulcerative colitis, DMF demonstrated notable therapeutic effects in WT mice, but not in *Nlrp3*$^{-/-}$ mice. Collectively, as an established clinical drug with a high safety profile, DMF had the potential to be developed as a promising candidate for the treatment of NLRP3-mediated inflammatory diseases.

RESULTS

DMF inhibits the activation of NLRP3 inflammasome

In our quest to identify potential candidates for the therapy of NLRP3-related diseases, we sought NLRP3 inhibitors and discovered that DMF could effectively block the activation of the NLRP3 inflammasome. To assess the impact of DMF on the NLRP3 inflammasome, we evaluated the cytotoxicity of DMF in THP-1 cells or bone marrow-derived macrophages (BMDMs). Notably, cell viability remained largely unchanged at doses below 80 μM (Figure S1).

For the activation of the NLRP3 inflammasome, THP-1 cells or BMDMs were first primed with LPS and then pre-treated with various concentrations of DMF before stimulation with ATP or nigericin. Our findings demonstrated that DMF significantly inhibited Caspase-1 activation and IL-1β secretion induced by ATP or nigericin in LPS-primed THP-1 cells or BMDMs (Figures 1A, 1B, and S2). Additionally, enzyme-linked immunosorbent assay (ELISA) revealed a substantial reduction in the levels of IL-1β and IL-18 in the cell supernatants, further confirming that DMF effectively suppressed the release of these inflammatory cytokines (Figures 1C–1F). In summary, these results clearly established that DMF prevented ATP or nigericin induced activation of the NLRP3 inflammasome in both human and mouse macrophages.

To explore whether DMF exhibited a broad-spectrum anti-NLRP3 inflammasome effect, we investigated its impact on the activation induced by multiple agonists. Our results demonstrated that DMF reduced Caspase-1 activation and IL-1β release triggered by canonical NLRP3 inflammasome agonists such as ATP, nigericin, and MSU (Figure 1G). Collectively, these findings suggested that DMF possessed the ability to inhibit NLRP3 inflammasome activation induced by various agonists.

DMF blocks NLRP3 inflammasome assembly

We then investigated whether DMF inhibited NLRP3 inflammasome activation by regulating NF-κB-dependent NLRP3 or pro-IL-1β expression. In our experimental conditions, BMDMs were treated with DMF for 45 min after 3 h of LPS priming. DMF significantly suppressed ATP-induced IL-1β release and Caspase-1 activation. However, it had no effect on NLRP3 and pro-IL-1β expression, as well as TNF-α release (Figures 2A, S3A, and S3B). This suggested that the expression of NLRP3 or pro-IL-1β was not downregulated when DMF inhibited NLRP3 inflammasome activation. When BMDMs were treated with DMF for 45 min before LPS stimulation, DMF also significantly suppressed ATP-induced IL-1β release and Caspase-1 activation, mildly inhibited pro-IL-1β expression, and TNF-α production but had no effects on NLRP3 expression (Figures 2A, S3A, and S3B). It is reported that DMF can activate Nrf2,[36,37] we further tested the effect of DMF on Nrf2. The results indicated that DMF treatment of 45 min failed to promote Nrf2 protein and mRNA expression (Figures S4A and S4B). While when the administration time was extended to 24 h, DMF significantly increased Nrf2 protein expression but not mRNA level (Figures S4A and S4B). In addition, the expressions of Nrf2 target genes, including *Nqo1, Gclm, Hmox1, Gsr, Pgd, Taldo1*, were not significantly changed by treatment with DMF for 45 min, but were significantly increased by treatment with DMF for 24 h (Figures S4C–S4H). These results showed that DMF exerted an anti-inflammatory effect mainly by inhibiting NLRP3 inflammasome activation at the early stage of administration, while long-term treatment was by activating Nrf2. Therefore, inhibition of NLRP3 inflammasome activation is an important mechanism for DMF to exert its anti-inflammatory effects, and it is necessary to further study the target and regulatory mechanism of DMF in inhibiting NLRP3 inflammasome activation. These results indicated that, under our experimental conditions, DMF inhibited NLRP3 inflammasome activation rather than the priming step.

Next, we explored the mechanism by which DMF inhibited NLRP3 inflammasome activation. ASC recruitment and subsequent oligomerization are crucial steps for NLRP3 inflammasome activation, leading to the cleavage and activation of pro-Caspase-1.[38] Co-immunoprecipitation experiments revealed that DMF significantly inhibited the interaction between ASC and NLRP3 (Figure 2B). Cross-linking experiments also demonstrated a marked decrease in the expressions of ASC dimers and polymers after DMF treatment (Figure 2C). Additionally, immunofluorescence results indicated that DMF significantly inhibited the formation of ASC specks (Figure 2D).

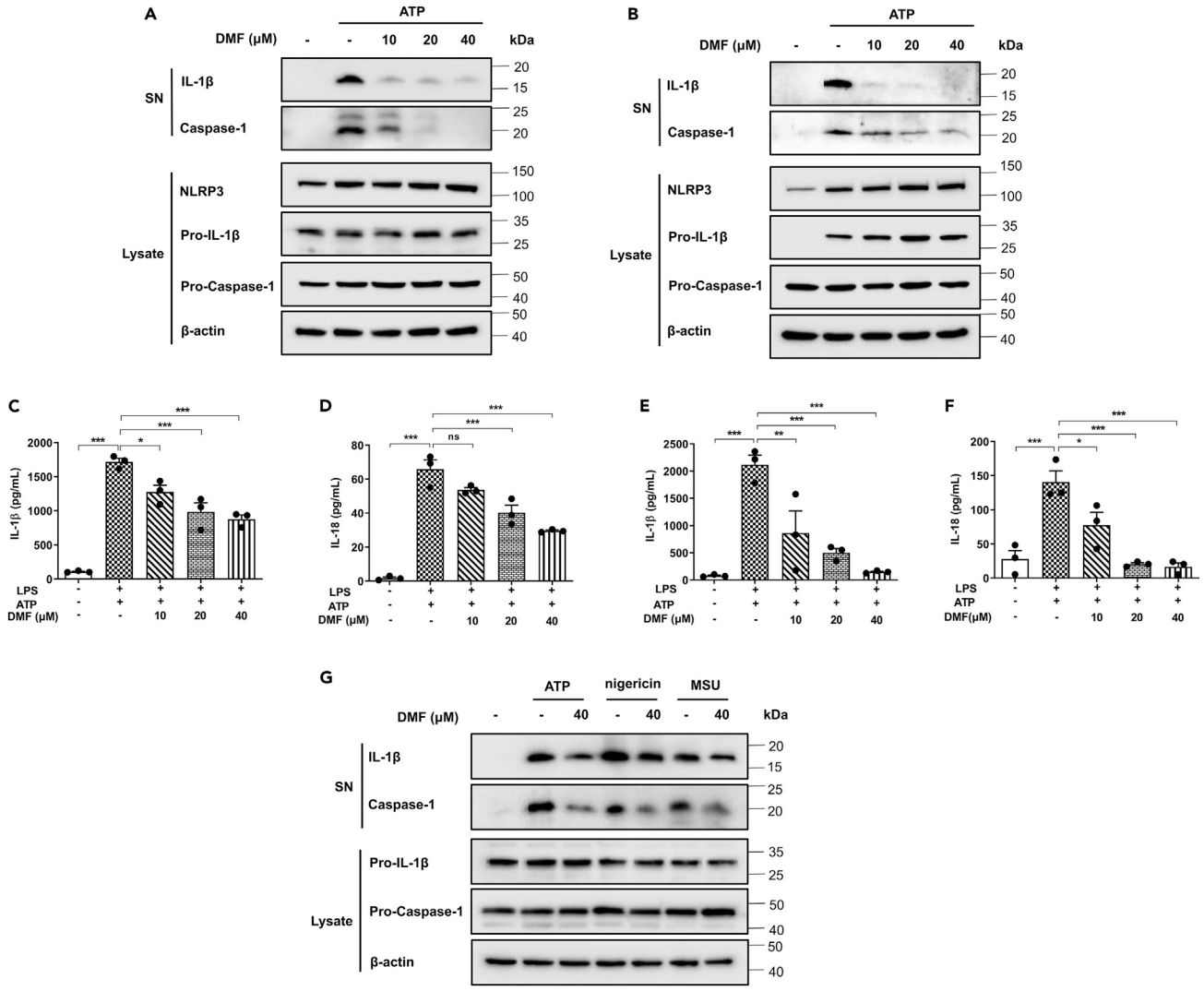

Figure 1. DMF inhibits the activation of NLRP3 inflammasome

(A and B) Immunoblot analysis of IL-1β and caspase-1 (p20) in culture supernatants (SN) of LPS-primed THP-1 cells (A) and BMDMs (B) treated with different doses (above lanes) of DMF and then stimulated with ATP.

(C–F) ELISA of IL-1β and IL-18 in supernatants of LPS-primed THP-1 cells (C and D) and BMDMs (E and F) treated with different doses of DMF and then stimulated with ATP.

(G) Immunoblot analysis of the indicated proteins in lysates from BMDMs treated with LPS for 3 h and stimulated with different doses of DMF and then stimulated with ATP, nigericin, and MSU. Data are representative of three independent experiments (A, B, G) or are expressed as mean ± SEM from three biological duplicates (C–F). Statistical analysis was performed using one-way ANOVA: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. ns, not significant.

These findings suggested that DMF might directly act on ASC oligomerization or upstream of inflammasome assembly to inhibit NLRP3 inflammasome activation.

DMF does not directly target the ASC oligomerization

Given that ASC oligomerization is crucial for the activation of NLRP3 and AIM2 inflammasomes but unnecessary for the NLRC4 inflammasome activation,[39,40] we delved deeper into understanding whether DMF's suppressive effect on NLRP3 inflammasome activation was directly linked to targeting ASC oligomerization. To explore this, we investigated the effect of DMF on the activation of NLRC4 and AIM2 inflammasomes induced by *Salmonella typhimurium* (*Salmonella*) infection and poly (dA:dT) transfection, respectively. The results revealed that DMF could indeed inhibit Caspase-1 activation and IL-1β release triggered by *Salmonella* infection at a dose of 40 μM (Figures 3A and 3B). However, DMF did not inhibit the poly (dA:dT) transfection-induced activation of the AIM2 inflammasome (Figures 3C and 3D). These findings strongly suggested that DMF's anti-NLRP3 inflammasome effect did not involve targeting ASC oligomerization.

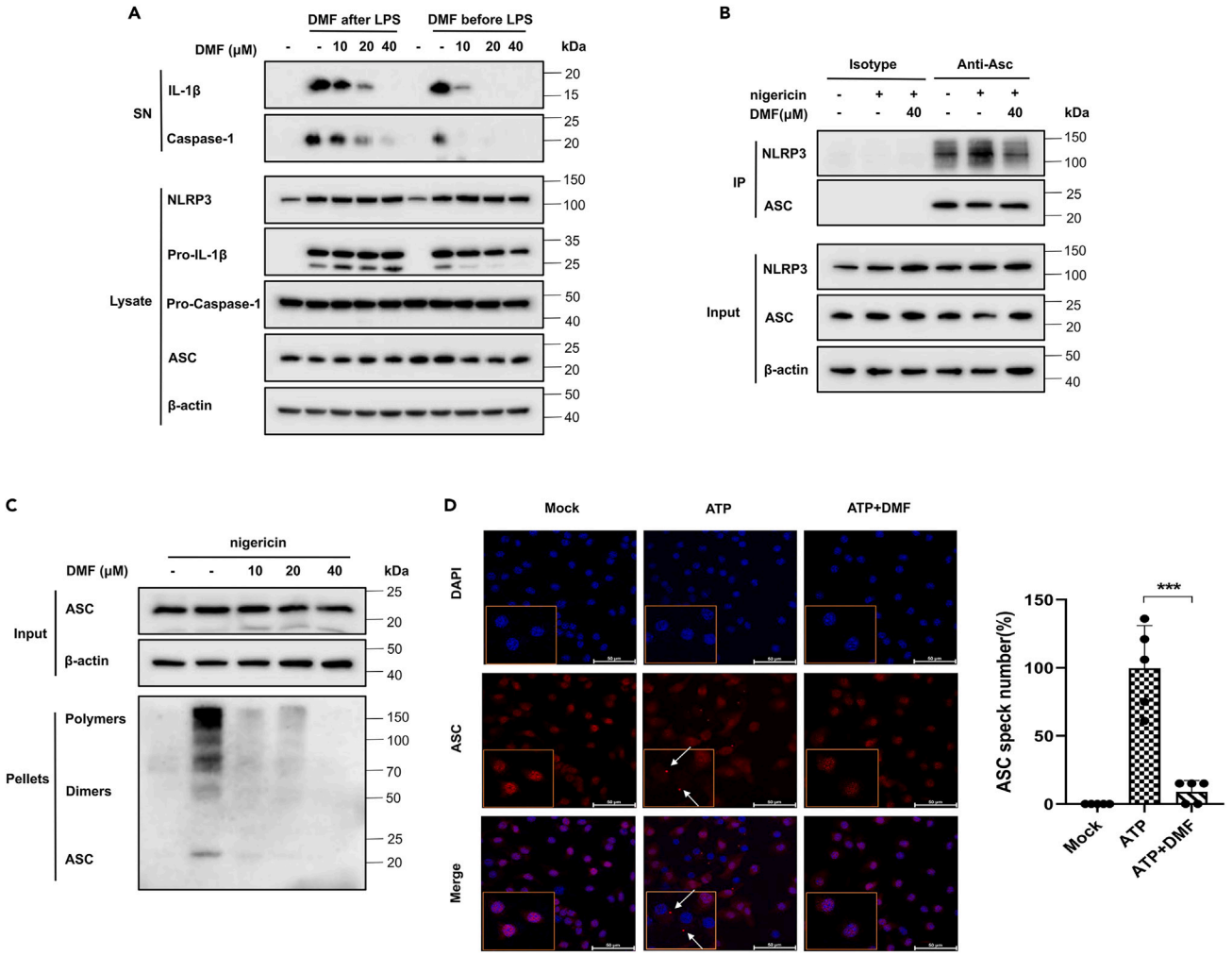

Figure 2. DMF blocks NLRP3 inflammasome assembly

(A) Immunoblot analysis of the indicated proteins in lysates from BMDMs treated with LPS for 3 h and treated with various doses of DMF for 45 min (DMF after LPS) or BMDMs treated with various doses of DMF for 45 min and then stimulated with LPS for 3 h (DMF before LPS), and then stimulated with ATP.

(B) An endogenous co-immunoprecipitation with ASC antibody or isotype antibody was performed in LPS-primed THP-1 cells stimulated with nigericin in the absence or presence of DMF.

(C) Western blotting analysis of cross-linked ASC in the NP-40-insoluble pellet of THP-1 cells stimulated with nigericin in the absence or presence of DMF.

(D) Confocal microscopy analysis in LPS-primed BMDMs treated with DMF (40 μM) and then stimulated with ATP, followed by incubation with fluorescent secondary antibody. Nuclei were stained with DAPI. Data are representative of three independent experiments (A-C) or are expressed as mean ± SEM from five biological duplicates (D). Statistical analysis was performed using one-way ANOVA: ***$p < 0.001$.

DMF inhibits NLRP3 inflammasome assembly by blocking NLRP3-NEK7 interaction

Several molecular and cellular events have been proposed as necessary upstream pathways for NLRP3 inflammasome activation, including K^+ efflux, Ca^{2+} signaling, and mitochondrial dysfunction.[41] We investigated whether DMF regulated these upstream events of NLRP3 inflammasome activation. Our results demonstrated that DMF treatment did not affect the upstream signals for NLRP3 inflammasome activation, such as K^+ efflux and Ca^{2+} mobilization (Figures 4A and 4B). Additionally, DMF inhibited IL-1β release elicited by imiquimod (a K^+ efflux-independent activator of NLRP3) in LPS-primed BMDMs, indicating that DMF inhibited NLRP3 inflammasome activation independently of K^+ efflux (Figure S5). While mitochondrial damage-induced reactive oxygen species (ROS) are known to activate the NLRP3 inflammasome, DMF had no amelioratory effect on mitochondrial damage under our experimental conditions (Figure S6). In summary, these findings suggested that DMF inhibited NLRP3 inflammasome activation by preventing inflammasome assembly rather than targeting these upstream signaling events.

To delve into the mechanism by which DMF inhibited NLRP3 inflammasome activation, we examined whether DMF inhibited inflammasome assembly. An essential step in the assembly of the NLRP3 inflammasome is the interaction between NEK7 and NLRP3.[14] This interaction is crucial for subsequent NLRP3 oligomerization and recruitment of ASC to NLRP3. We investigated whether DMF could inhibit the formation

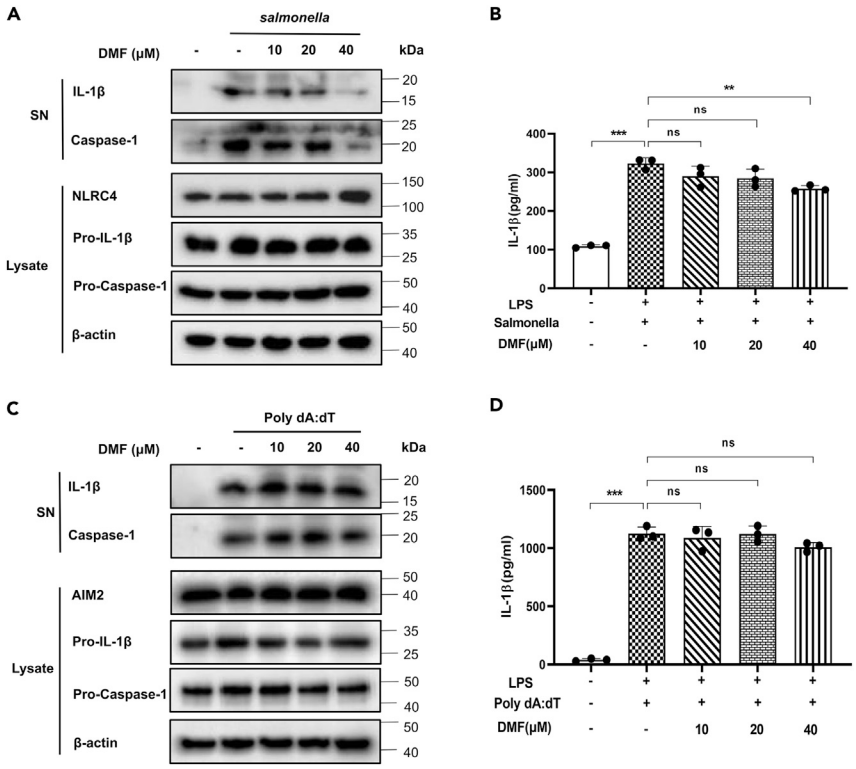

Figure 3. DMF does not directly target the ASC oligomerization

(A and B) Immunoblot analysis (A) of IL-1β and Caspase-1 (p20) or ELISA (B) of IL-1β in SN of LPS-primed THP-1 cells treated with different doses of DMF and then stimulated with *salmonella*.

(C and D) Immunoblot analysis (C) of IL-1β and Caspase-1 (p20) or ELISA (D) of IL-1β in SN of LPS-primed THP-1 cells treated with different doses of DMF and then stimulated with poly (dA:dT) in the absence or presence of DMF. Data are representative of three independent experiments (A, C) or are expressed as mean ± SEM from three biological duplicates (B, D). Statistical analysis was performed using one-way ANOVA: $^{**}p < 0.01$ and $^{***}p < 0.001$. ns, not significant.

of the endogenous NLRP3-inflammasome complex and found that DMF blocked the endogenous NEK7-NLRP3 interaction (Figure 4C). Furthermore, we validated the interaction between NEK7 and NLRP3 in 293T cells and observed that DMF hindered the NEK7-NLRP3 interaction (Figure 4D). Therefore, it was postulated that DMF prevented NLRP3 inflammasome assembly by directly inhibiting the NLRP3-NEK7 interaction.

DMF directly binds to Cys673 of NLRP3

DMF exhibited no direct targeting of ASC oligomerization and had no significant impact on the upstream signals associated with NLRP3 inflammasome activation, including NF-κB signaling, K^+ efflux, Ca^{2+} signaling, and mitochondrial dysfunction. Despite this, DMF notably inhibited NLRP3 inflammasome activation by blocking the interaction between NLRP3 and NEK7. Thus, we hypothesized that the specific effect of DMF on NLRP3 inflammasome activation was exerted by directly targeting NLRP3 or NEK7 rather than ASC or Caspase-1, which are shared components with the NLRC4 and AIM2 inflammasomes.

NLRP3 has previously been identified as a protein directly regulated by various compounds, including MCC950, oridonin, tranilast, CY-09, and itaconate.[7,23–25,42] Notably, NLRP3 has been reported to covalently bind with numerous α, β-unsaturated ketone structures.[4,22] Building on this, we speculated that NLRP3 might undergo covalent modification by DMF. To verify this, LC-MS/MS analysis was conducted, revealing that DMF indeed directly bound to Cys673 of NLRP3 (Figures 5A and S7A). Modification of NLRP3 Cys673 was also identified in the synthetic peptide (MDHMVSSFCIENCHR) incubated with DMF (Figure 5B). Molecular docking results further supported this finding, demonstrating that DMF could covalently bind to Cys673, consistent with the LC-MS/MS results (Figure S7B).

Cys673 is situated in the helical domain 2 (HD2) of NLRP3, representing one of the surfaces where NLRP3 interacts with NEK7. This discovery suggested that the modification of NLRP3 at Cys673 by DMF is a possible mechanism for the inhibition of NLRP3 inflammasome activation. The direct modification likely disrupted the interaction between NLRP3 and NEK7, consequently inhibiting the activation of the NLRP3 inflammasome.

To verify whether DMF covalently bound to Cys673 of NLRP3 and subsequently inhibited the interaction between NEK7 and NLRP3, we co-transfected HA-NEK7 with wild-type Flag-NLRP3 (WT NLRP3) or Cys673 mutant Flag-NLRP3 (C673A NLRP3) plasmids into 293T cells. The results revealed that NEK7 could still bind to NLRP3 after cells were transfected with C673A NLRP3, while DMF could not hinder the interaction

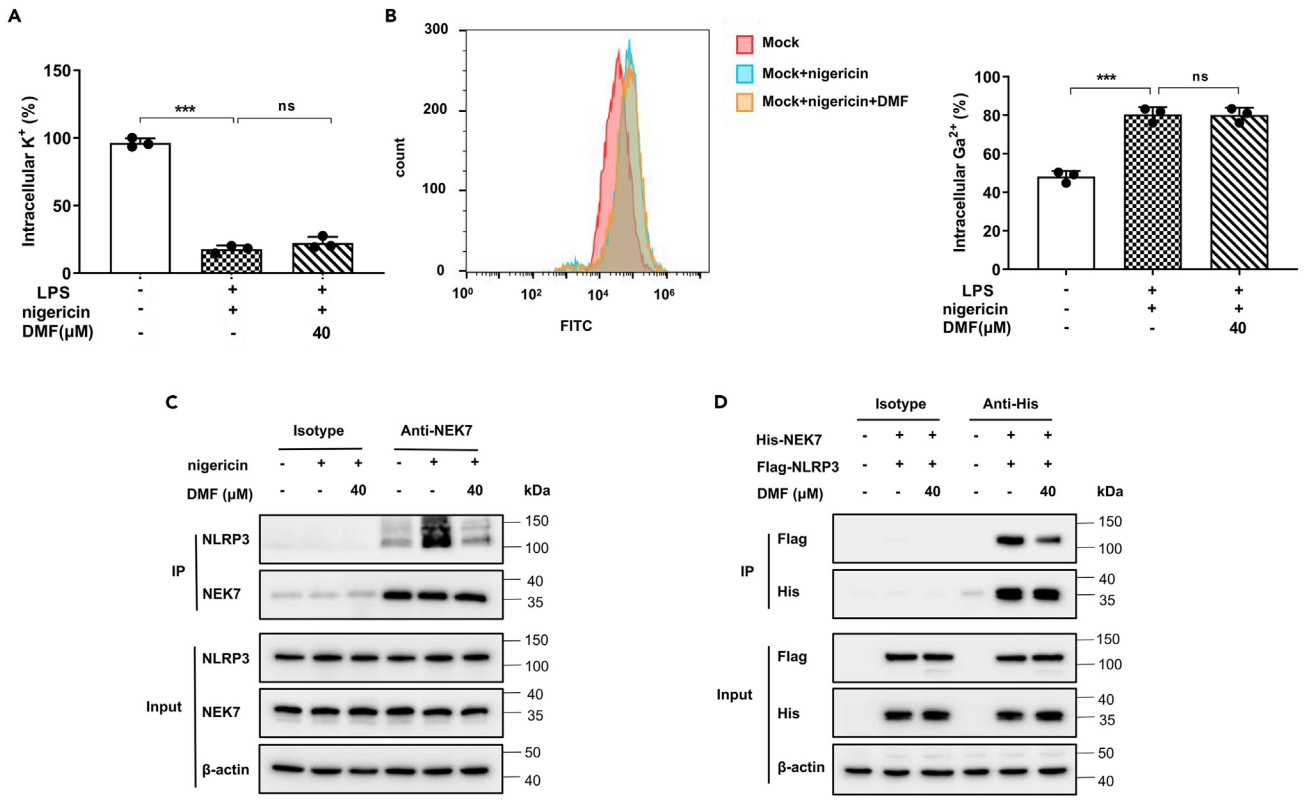

Figure 4. DMF inhibits NLRP3 inflammasome assembly by blocking NLRP3-NEK7 interaction

(A) Qualification of K^+ efflux in LPS-primed THP-1 cells treated or untreated with DMF and then stimulated with nigericin.

(B) Flow cytometric analysis of intracellular Ca^{2+} level detected by Ca^{2+}-sensitive fluorescence indicator Fluo-4 AM in LPS-primed THP-1 cells treated or untreated with DMF and then stimulated with nigericin.

(C) An endogenous IP with NEK7 antibody or isotype antibody was performed in LPS-primed THP-1 cells stimulated with nigericin in the absence or presence of DMF.

(D) IP and western blot analysis of NEK7-NLRP3 interaction in 293T cells. Data are expressed as mean ± SEM from three biological duplicates (A, B) or are representative of three independent experiments (C, D). Statistical analysis was performed using one-way ANOVA: ***$p < 0.001$. ns, not significant.

between NEK7 and C673A NLRP3 (Figure 5C). This implied that the mutation of Cys673 abolished the binding of NLRP3 to DMF, resulting in the inability of DMF to inhibit the interaction between NEK7 and NLRP3.

To further confirm the effect of the covalent binding of DMF to Cys673 of NLRP3 on inflammasome activation, we reconstituted the NLRP3 inflammasome system in 293T cells. As expected, the Cys673 mutant NLRP3 did not affect Caspase-1 cleavage and IL-1β release (Figures 5D and 5E). However, we found that DMF could inhibit the NLRP3 inflammasome activation reconstituted with WT NLRP3 but not with C673A mutant NLRP3 in 293T cells (Figures 5D and 5E). We further reconstituted the NLRP3 inflammasome with lentivirus encoding WT NLRP3 or C673A NLRP3 in *Nlrp3*$^{-/-}$ THP-1 cells. Consistently, the results indicated that the ability of C673A NLRP3 to induce Caspase-1 activation and IL-1β release was not decreased by DMF treatment (Figures 5F and 5G). Taken together, these results suggested that Cys673 served as a binding site for DMF to inhibit NLRP3 inflammasome activation.

DMF relieves DSS-induced ulcerative colitis

The NLRP3 inflammasome is widely distributed in colonic macrophages, and blocking NLRP3 inflammasome activation has been shown to effectively ameliorate ulcerative colitis. To further validate whether NLRP3 was a direct target of DMF *in vivo*, we established a mouse ulcerative colitis model induced by DSS in both WT and *Nlrp3*$^{-/-}$ mice.

DMF and MCC950 treatment significantly ameliorated weight loss and shortened colon lengths in WT mice, but these effects were not observed in *Nlrp3*$^{-/-}$ mice (Figures 6A–6E). Histopathological analysis of mouse colons revealed that DSS induced mucosal barrier disruption and inflammatory cell infiltration, which was improved after treatment with DMF or MCC950 in WT mice (Figure 6F). However, these improvements were not significant in *Nlrp3*$^{-/-}$ mice (Figure 6G). Additionally, ELISA results demonstrated that DMF or MCC950 significantly inhibited IL-1β release in the colon tissue of DSS-induced WT mice, but had no significant effect in *Nlrp3*$^{-/-}$ mice (Figure 6H).

In conclusion, these results indicated that DMF could target NLRP3 to reverse the pathological process of DSS-induced ulcerative colitis.

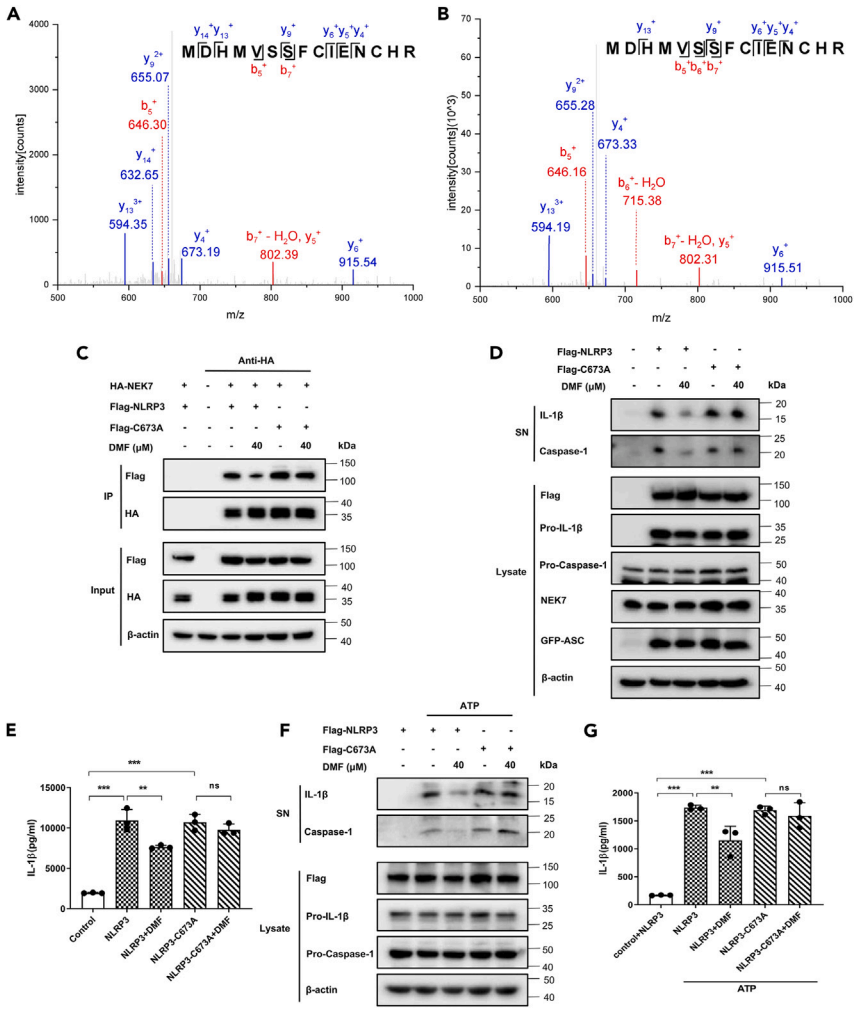

Figure 5. DMF directly binds to Cys673 of NLRP3

(A) Representative LC-MS/MS spectra showing a covalent modification of the cysteine 673-containing NLRP3 peptide by DMF (+144.0419 Da).

(B) Representative LC-MS/MS spectra showing a covalent modification of the second cysteine (equivalent to Cys673 of NLRP3) of synthetic peptide (MDHMVSSFCIENCHR) by DMF (+144.0419 Da).

(C) IP and Western blotting analysis of the interaction between NEK7 and WT or mutant NLRP3 in 293T cells.

(D and E) Western blotting analysis (D) of IL-1β and activated caspase-1 (p20) or ELISA of IL-1β (E) in SN from 293T cells 24 h after reconstituted with Pro-IL-1β, Pro-Caspase1, GFP-ASC and Flag-NLRP3 or Flag-NLRP3 C673A.

(F and G) Western blotting analysis (F) of IL-1β and activated caspase-1 (p20) or ELISA of IL-1β (G) in SN from LPS-primed *Nlrp3*$^{-/-}$ THP-1 cells reconstituted with WT or C673A NLRP3 that were treated or untreated with DMF (40 μM) for 45 min and then stimulated with ATP. Data are representative of three independent experiments (C, D, F) or are expressed as mean ± SEM from three biological duplicates (E, G). Statistical analysis was performed using one-way ANOVA: **$p < 0.01$ and ***$p < 0.001$. ns, not significant.

DISCUSSION

The NLRP3 inflammasome, a crucial component of innate immunity, plays a critical role in various inflammation-associated diseases, including ulcerative colitis, Type 2 diabetes, gout, Alzheimer's disease, and non-alcoholic fatty liver disease.[43,44] This signaling platform responds to both pathogens and sterile triggers, leading to the expression and release of proinflammatory cytokines and triggering a cascade of inflammatory responses.[45] Consequently, inhibiting NLRP3 inflammasome activation is considered a promising strategy for intervening in relevant inflammatory diseases.[46]

DMF, known as an immunomodulatory drug, is widely used for the treatment of multiple sclerosis with a proven high safety profile in clinical practice.[29] In the present study, it was discovered that DMF exhibited selective and potent inhibitory activity against NLRP3 inflammasome activation both *in vitro* and *in vivo*. This finding suggested that DMF might serve as a small, multifunctional molecule for studying the biological functions of NLRP3 and exploring its therapeutic effects in inflammatory diseases.

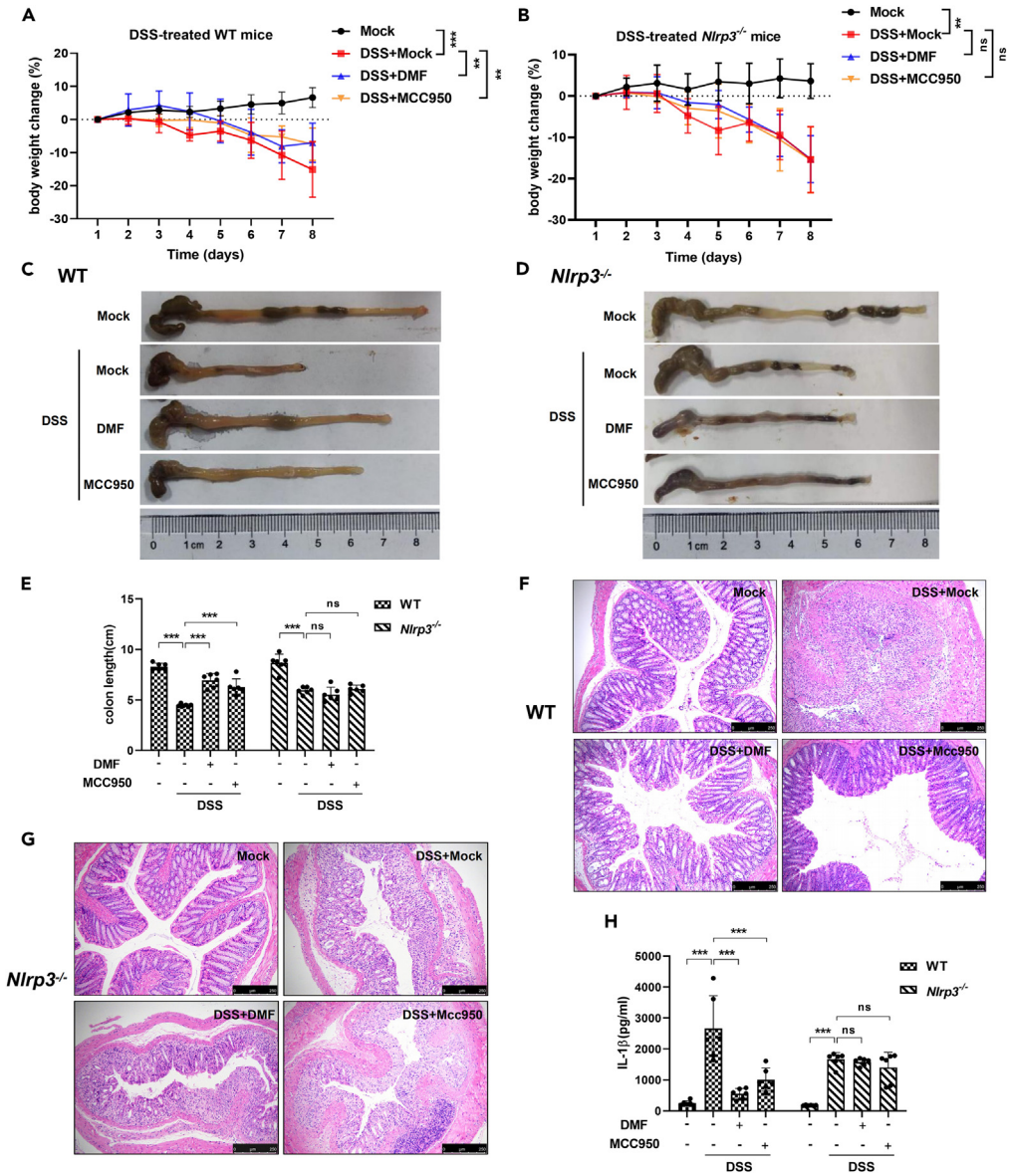

Figure 6. DMF relieves DSS-induced ulcerative colitis

(A and B) WT C57BL/6 (A) and *Nlrp3*$^{-/-}$ (B) mice were given 2.5% DSS in the drinking water in the presence or absence of DMF (50 mg/kg) or MCC950 (50 mg/kg) for 8 days, and body weights of the mice were measured (n = 6 for each group).

(C–G) Representative WT C57BL/6 (C) and *Nlrp3*$^{-/-}$ (D) mice colon images, the colon lengths (E, n = 6 for each group), and H&E-stained colon sections (F and G, n = 6 for each group) were measured 8 days after treatment with DSS plus vehicle, DMF (50 mg/kg) or MCC950 (50 mg/kg).

(H) ELISA assay of IL-1β in colon tissues (n = 6 for each group). Data are expressed as mean ± SEM (n = 6) (A, B, E, H). Statistical analysis was performed using one-way ANOVA: **p < 0.01 and ***p < 0.001. ns, not significant.

NLRP3 inflammasome activation involves two distinct steps: the priming step and the activation step. Previous studies have reported that treatment with DMF inhibits the nuclear translocation of NF-κB in various immune cells.[33,35,47–50] Additionally, DMF has been shown to activate Nrf2, limiting the production of IL-1β and IL-6 with less impact on TNF-α and NLRP3.[35] In the current study, it was observed that DMF treatment prior to LPS priming slightly inhibited the levels of pro-IL-1β and TNF-α. However, treatment after LPS priming had no effect on the expressions of NLRP3, pro-IL-1β, and TNF-α. Furthermore, DMF significantly suppressed ATP-, nigericin-, and MSU-induced cleavage of Caspase-1 and IL-1β release, suggesting that DMF directly inhibited the activation step of the NLRP3 inflammasome.

In addition to NLRP3 inflammasome, other inflammasomes, such as NLRC4 and AIM2, can also assemble in response to *Salmonella* and poly (dA:dT), respectively, contributing to Caspase-1 cleavage and IL-1β release.[51,52] DMF was found to inhibit the activation of NLRP3 inflammasome but not AIM2 inflammasome, indicating that DMF did not act on ASC or its downstream components but rather directly on NLRP3 or

its upstream components. Considering that DMF has reported antibacterial effects,[53,54] it was speculated that high concentrations of DMF might inhibit NLRC4 inflammasome activation by inhibiting *Salmonella* growth.

Subsequent investigation into the effect of DMF on upstream signals of NLRP3 inflammasome activation revealed that DMF had no effect on K^+ efflux, Ca^{2+} mobilization or mitochondrial damage. These results suggested that DMF inhibited NLRP3 inflammasome activation by hindering its assembly. Supporting this notion, DMF was found to inhibit the NLRP3-ASC interaction and ASC oligomerization, both of which are markers for inflammasome assembly. As a crucial step for NLRP3 inflammasome assembly, endogenous interaction between NLRP3 and NEK7 is inhibited by DMF treatment in macrophages.[6,14,55,56] Furthermore, DMF also suppressed exogenous NLRP3-NEK7 interaction, demonstrating that DMF targeted NLRP3 or NEK7 to prevent NLRP3 inflammasome assembly.

NLRP3 is a core component of the inflammasome and an important target for small-molecule drugs. Previous studies have reported that oridonin and itaconate, both containing α, β-unsaturated carbonyl units, can covalently bind to C279 and C548 of NLRP3, respectively, inhibiting its activation.[25,42] Therefore, it is possible that DMF's ability to inhibit inflammasome activation also stems from its α, β-unsaturated carbonyl unit covalently binding to NLRP3. Mass spectrometry results showed that DMF directly targeted C673 of NLRP3. The NLRP3-NEK7 interaction was prevented by DMF administration in WT NLRP3, while the efficacy of DMF was abrogated in C673A NLRP3. Moreover, the decrease in Caspase-1 cleavage and IL-1β release induced by DMF treatment was also successfully abolished in C673A NLRP3 cells, indicating that DMF directly bound to NLRP3 to prevent NLRP3-NEK7 interaction. Therefore, this research provided evidence that DMF covalently bound to C673 of NLRP3 to suppress NLRP3 inflammasome assembly and held promise for treating inflammasome-related diseases.

Ulcerative colitis, characterized by continuous mucosal congestion, swelling, erosion, and small shallow ulcers in the intestine, is a non-specific inflammatory disease.[57,58] Excessive activation of the NLRP3 inflammasome has been closely associated with the occurrence and development of ulcerative colitis.[59–61] Therefore, inhibiting NLRP3 inflammasome activation is considered a crucial strategy for treating ulcerative colitis. The present research explored the therapeutic role of DMF in a mouse model of DSS-induced colitis.

The results demonstrated that oral administration of DMF significantly ameliorated DSS-induced colitis n WT mice, as evidenced by improvements in body weight, colon length, and histopathological examination, but not in *Nlrp3*$^{-/-}$ mice. IL-1β, a critical proinflammatory factor dependent on NLRP3 inflammasome activation, was also decreased by treatment with DMF in the DSS-induced colon of WT mice, but not in *Nlrp3*$^{-/-}$ mice. These findings suggested that the anti-colitis effect of DMF was exerted by targeting NLRP3.

In conclusion, this study identified DMF as a promising and potent NLRP3 inhibitor. DMF effectively inhibited NLRP3 inflammasome activation both *in vitro* and *in vivo*. The research revealed that DMF directly targeted NLRP3 and disrupted the NLRP3-NEK7 interaction. Consequently, DMF inhibited NLRP3 inflammasome assembly and activation, leading to notable anti-inflammatory effects. Moreover, DMF demonstrated efficacy similar to MCC950 in inhibiting NLRP3 inflammasome activation in DSS-induced colitis WT mice, but these effects were not observed in *Nlrp3*$^{-/-}$ mice. Importantly, DMF is already approved for use in multiple sclerosis in the United States, Germany, and China, indicating its high clinical safety. This research expanded the potential role of DMF as a pharmacological approach for the treatment of NLRP3-related diseases.

Limitations of the study

The primary limitation of our study lies in the extrapolation of DMF's potential in alleviating NLRP3 inflammasome-mediated diseases to clinical applications. While our investigations demonstrated a robust anti-inflammatory effect of DMF in human and mouse macrophages, as well as mouse models, its specific clinical efficacy in treating NLRP3 inflammasome-related inflammatory diseases warrants further exploration. Given the pivotal role of the NLRP3 inflammasome in innate immunity and its implication in various chronic inflammation-driven human diseases beyond ulcerative colitis, it is imperative to investigate whether DMF, functioning as a direct covalent inhibitor of NLRP3, can ameliorate other inflammatory conditions. This broader exploration will enhance our understanding of the potential therapeutic applications of DMF across a spectrum of inflammatory diseases.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Biasizzo, M., and Kopitar-Jerala, N. (2020). Interplay Between NLRP3 Inflammasome and Autophagy. Front. Immunol. *11*, 591803. https://doi.org/10.3389/fimmu.2020.591803.
2. Lee, B., Hoyle, C., Wellens, R., Green, J.P., Martin-Sanchez, F., Williams, D.M., Matchett, B.J., Seoane, P.I., Bennett, H., Adamson, A., et al. (2023). Disruptions in endocytic traffic contribute to the activation of the NLRP3 inflammasome. Sci. Signal. *16*, eabm7134. https://doi.org/10.1126/scisignal.abm7134.
3. Zhang, Z., Venditti, R., Ran, L., Liu, Z., Vivot, K., Schürmann, A., Bonifacino, J.S., De-Matteis, M.A., and Ricci, R. (2023). Distinct changes in endosomal composition promote NLRP3 inflammasome activation. Nat. Immunol. *24*, 30–41. https://doi.org/10.1038/s41590-022-01355-3.
4. Schmacke, N.A., O'Duill, F., Gaidt, M.M., Szymanska, I., Kamper, J.M., Schmid-Burgk, J.L., Mädler, S.C., Mackens-Kiani, T., Kozaki, T., Chauhan, D., et al. (2022). IKKβ primes inflammasome formation by recruiting NLRP3 to the trans-Golgi network. Immunity *55*, 2271–2284.e7. https://doi.org/10.1016/j.immuni.2022.10.021.
5. Broz, P., and Dixit, V.M. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. Nat. Rev. Immunol. *16*, 407–420. https://doi.org/10.1038/nri.2016.58.
6. Sharif, H., Wang, L., Wang, W.L., Magupalli, V.G., Andreeva, L., Qiao, Q., Hauenstein, A.V., Wu, Z., Núñez, G., Mao, Y., and Wu, H. (2019). Structural mechanism for NEK7-licensed activation of NLRP3 inflammasome. Nature *570*, 338–343. https://doi.org/10.1038/s41586-019-1295-z.
7. Hooftman, A., Angiari, S., Hester, S., Corcoran, S.E., Runtsch, M.C., Ling, C., Ruzek, M.C., Slivka, P.F., McGettrick, A.F., Banahan, K., et al. (2020). The Immunomodulatory Metabolite Itaconate Modifies NLRP3 and Inhibits Inflammasome Activation. Cell Metabol. *32*, 468–478.e7. https://doi.org/10.1016/j.cmet.2020.07.016.
8. Jo, E.K., Kim, J.K., Shin, D.M., and Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. Cell. Mol. Immunol. *13*, 148–159. https://doi.org/10.1038/cmi.2015.95.
9. Huang, Y., Xu, W., and Zhou, R. (2021). NLRP3 inflammasome activation and cell death. Cell. Mol. Immunol. *18*, 2114–2127. https://doi.org/10.1038/s41423-021-00740-6.
10. Ren, G., Zhang, X., Xiao, Y., Zhang, W., Wang, Y., Ma, W., Wang, X., Song, P., Lai, L., Chen, H., et al. (2019). ABRO1 promotes NLRP3 inflammasome activation through regulation of NLRP3 deubiquitination. EMBO J. *38*, e100376. https://doi.org/10.15252/embj.2018100376.
11. Martín-Sánchez, F., Diamond, C., Zeitler, M., Gomez, A.I., Baroja-Mazo, A., Bagnall, J., Spiller, D., Mortellaro, A., White, M., Daniels, M.J.D., et al. (2016). Inflammasome-dependent IL-1β release depends upon membrane permeabilisation. Cell Death Differ. *23*, 1219–1231. https://doi.org/10.1038/cdd.2015.176.
12. Groß, C.J., Mishra, R., Schneider, K.S., Médard, G., Wettmarshausen, J., Dittlein, D.C., Shi, H., Gorka, O., Koenig, P.A., Fromm, S., et al. (2016). K^+ Efflux-Independent NLRP3 Inflammasome Activation by Small Molecules Targeting Mitochondria. Immunity *45*, 761–773. https://doi.org/10.1016/j.immuni.2016.08.010.
13. Wang, Y., Wang, C., He, Q., Chen, G., Yu, J., Cang, J., and Zhong, M. (2023). Inhibition of sphingosine-1-phosphate receptor 3 suppresses ATP-induced NLRP3 inflammasome activation in macrophages via TWIK2-mediated potassium efflux. Front. Immunol. *14*, 1090202. https://doi.org/10.3389/fimmu.2023.1090202.
14. He, Y., Zeng, M.Y., Yang, D., Motro, B., and Núñez, G. (2016). NEK7 is an essential mediator of NLRP3 activation downstream of

potassium efflux. Nature *530*, 354–357. https://doi.org/10.1038/nature16959.
15. Shi, H., Wang, Y., Li, X., Zhan, X., Tang, M., Fina, M., Su, L., Pratt, D., Bu, C.H., Hildebrand, S., et al. (2016). NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. Nat. Immunol. *17*, 250–258. https://doi.org/10.1038/ni.3333.
16. Schmid-Burgk, J.L., Chauhan, D., Schmidt, T., Ebert, T.S., Reinhardt, J., Endl, E., and Hornung, V. (2016). A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an essential component of NLRP3 inflammasome activation. J. Biol. Chem. *291*, 103–109. https://doi.org/10.1074/jbc.C115.700492.
17. Pavillard, L.E., Marín-Aguilar, F., Bullon, P., and Cordero, M.D. (2018). Cardiovascular diseases, NLRP3 inflammasome, and western dietary patterns. Pharmacol. Res. *131*, 44–50. https://doi.org/10.1016/j.phrs.2018.03.018.
18. Takahashi, M. (2022). NLRP3 inflammasome as a key driver of vascular disease. Cardiovasc. Res. *118*, 372–385. https://doi.org/10.1093/cvr/cvab010.
19. Holbrook, J.A., Jarosz-Griffiths, H.H., Caseley, E., Lara-Reyna, S., Poulter, J.A., Williams-Gray, C.H., Peckham, D., and McDermott, M.F. (2021). Neurodegenerative Disease and the NLRP3 Inflammasome. Front. Pharmacol. *12*, 643254. https://doi.org/10.3389/fphar.2021.643254.
20. Zhen, Y., and Zhang, H. (2019). NLRP3 Inflammasome and Inflammatory Bowel Disease. Front. Immunol. *10*, 276. https://doi.org/10.3389/fimmu.2019.00276.
21. Chen, L., Cao, S.Q., Lin, Z.M., He, S.J., and Zuo, J.P. (2021). NOD-like receptors in autoimmune diseases. Acta Pharmacol. Sin. *42*, 1742–1756. https://doi.org/10.1038/s41401-020-00603-2.
22. Zahid, A., Li, B., Kombe, A.J.K., Jin, T., and Tao, J. (2019). Pharmacological Inhibitors of the NLRP3 Inflammasome. Front. Immunol. *10*, 2538. https://doi.org/10.3389/fimmu.2019.02538.
23. Jiang, H., He, H., Chen, Y., Huang, W., Cheng, J., Ye, J., Wang, A., Tao, J., Wang, C., Liu, Q., et al. (2017). Identification of a selective and direct NLRP3 inhibitor to treat inflammatory disorders. J. Exp. Med. *214*, 3219–3238. https://doi.org/10.1084/jem.20171419.
24. Huang, Y., Jiang, H., Chen, Y., Wang, X., Yang, Y., Tao, J., Deng, X., Liang, G., Zhang, H., Jiang, W., and Zhou, R. (2018). Tranilast directly targets NLRP3 to treat inflammasome-driven diseases. EMBO Mol. Med. *10*, e8689. https://doi.org/10.15252/emmm.201708689.
25. He, H., Jiang, H., Chen, Y., Ye, J., Wang, A., Wang, C., Liu, Q., Liang, G., Deng, X., Jiang, W., and Zhou, R. (2018). Oridonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity. Nat. Commun. *9*, 2550. https://doi.org/10.1038/s41467-018-04947-6.
26. Li, Q., Feng, H., Wang, H., Wang, Y., Mou, W., Xu, G., Zhang, P., Li, R., Shi, W., Wang, Z., et al. (2022). Licochalcone B specifically inhibits the NLRP3 inflammasome by disrupting NEK7-NLRP3 interaction. EMBO Rep. *23*, e53499. https://doi.org/10.15252/embr.202153499.
27. Chen, Y., He, H., Lin, B., Chen, Y., Deng, X., Jiang, W., and Zhou, R. (2021). RRx-001 ameliorates inflammatory diseases by acting as a potent covalent NLRP3 inhibitor. Cell. Mol. Immunol. *18*, 1425–1436. https://doi.org/10.1038/s41423-021-00683-y.
28. Saidu, N.E.B., Kavian, N., Leroy, K., Jacob, C., Nicco, C., Batteux, F., and Alexandre, J. (2019). Dimethyl fumarate, a two-edged drug: Current status and future directions. Med. Res. Rev. *39*, 1923–1952. https://doi.org/10.1002/med.21567.
29. Kourakis, S., Timpani, C.A., de Haan, J.B., Gueven, N., Fischer, D., and Rybalka, E. (2020). Dimethyl Fumarate and Its Esters: A Drug with Broad Clinical Utility. Pharmaceuticals *13*, 306. https://doi.org/10.3390/ph13100306.
30. Oh, C.J., Park, S., Kim, J.Y., Kim, H.J., Jeoung, N.H., Choi, Y.K., Go, Y., Park, K.G., and Lee, I.K. (2014). Dimethylfumarate attenuates restenosis after acute vascular injury by cell-specific and Nrf2-dependent mechanisms. Redox Biol. *2*, 855–864. https://doi.org/10.1016/j.redox.
31. Grzegorzewska, A.P., Seta, F., Han, R., Czajka, C.A., Makino, K., Stawski, L., Isenberg, J.S., Browning, J.L., and Trojanowska, M. (2017). Dimethyl Fumarate ameliorates pulmonary arterial hypertension and lung fibrosis by targeting multiple pathways. Sci. Rep. *7*, 41605. https://doi.org/10.1038/srep41605.
32. Humphries, F., Shmuel-Galia, L., Ketelut-Carneiro, N., Li, S., Wang, B., Nemmara, V.V., Wilson, R., Jiang, Z., Khalighinejad, F., Muneeruddin, K., et al. (2020). Succination inactivates gasdermin D and blocks pyroptosis. Science *369*, 1633–1637. https://doi.org/10.1126/science.abb9818.
33. Tastan, B., Arioz, B.I., Tufekci, K.U., Tarakcioglu, E., Gonul, C.P., Genc, K., and Genc, S. (2021). Dimethyl Fumarate Alleviates NLRP3 Inflammasome Activation in Microglia and Sickness Behavior in LPS-Challenged Mice. Front. Immunol. *12*, 737065. https://doi.org/10.3389/fimmu.2021.737065.
34. Li, H., Li, M., Dong, C., and Liu, B. (2022). Dimethyl fumarate ameliorates lipopolysaccharide-induced acute lung injury by inhibiting NLRP3 inflammasome-mediated pyroptosis through enhancing Nrf2 signaling. Toxicol. Res. *11*, 437–450. https://doi.org/10.1093/toxres/tfac020.
35. Hoyle, C., Green, J.P., Allan, S.M., Brough, D., and Lemarchand, E. (2022). Itaconate and fumarate derivatives inhibit priming and activation of the canonical NLRP3 inflammasome in macrophages. Immunology *165*, 460–480. https://doi.org/10.1111/imm.13454.
36. Yan, N., Xu, Z., Qu, C., and Zhang, J. (2021). Dimethyl fumarate improves cognitive deficits in chronic cerebral hypoperfusion rats by alleviating inflammation, oxidative stress, and ferroptosis via NRF2/ARE/NF-κB signal pathway. Int. Immunopharm. *98*, 107844. https://doi.org/10.1016/j.intimp.2021.107844.
37. Shimizu, H., Takayama, K., Yamada, K., Suzumura, A., Sato, T., Nishio, Y., Ito, M., Ushida, H., Nishiguchi, K.M., Takeuchi, M., and Kaneko, H. (2022). Dimethyl Fumarate Protects Retinal Pigment Epithelium from Blue Light-Induced Oxidative Damage via the Nrf2 Pathway. Antioxidants *12*, 45. https://doi.org/10.3390/antiox12010045.
38. Soriano-Teruel, P.M., García-Laínez, G., Marco-Salvador, M., Pardo, J., Arias, M., DeFord, C., Merfort, I., Vicent, M.J., Pelegrín, P., Sancho, M., and Orzáez, M. (2021). Identification of an ASC oligomerization inhibitor for the treatment of inflammatory diseases. Cell Death Dis. *12*, 1155. https://doi.org/10.1038/s41419-021-04420-1.
39. Mariathasan, S., Newton, K., Monack, D.M., Vucic, D., French, D.M., Lee, W.P., Roose-Girma, M., Erickson, S., and Dixit, V.M. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature *430*, 213–218. https://doi.org/10.1038/nature02664.
40. Ozaki, E., Campbell, M., and Doyle, S.L. (2015). Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. J. Inflamm. Res. *8*, 15–27. https://doi.org/10.2147/JIR.S51250.
41. He, Y., Hara, H., and Núñez, G. (2016). Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends Biochem. Sci. *41*, 1012–1021. https://doi.org/10.1016/j.tibs.2016.09.002.
42. Bambouskova, M., Potuckova, L., Paulenda, T., Kerndl, M., Mogilenko, D.A., Lizotte, K., Swain, A., Hayes, S., Sheldon, R.D., Kim, H., et al. (2021). Itaconate confers tolerance to late NLRP3 inflammasome activation. Cell Rep. *34*, 108756. https://doi.org/10.1016/j.celrep.2021.108756.
43. Liu, H., Zhan, X., Xu, G., Wang, Z., Li, R., Wang, Y., Qin, Q., Shi, W., Hou, X., Yang, R., et al. (2021). Cryptotanshinone specifically suppresses NLRP3 inflammasome activation and protects against inflammasome-mediated diseases. Pharmacol. Res. *164*, 105384. https://doi.org/10.1016/j.phrs.2020.105384.
44. Zhao, Q., Bi, Y., Guo, J., Liu, Y.X., Zhong, J., Pan, L.R., Tan, Y., and Yu, X.J. (2021). Pristimerin protects against inflammation and metabolic disorder in mice through inhibition of NLRP3 inflammasome activation. Acta Pharmacol. Sin. *42*, 975–986. https://doi.org/10.1038/s41401-020-00527-x.
45. Guo, C., Xie, S., Chi, Z., Zhang, J., Liu, Y., Zhang, L., Zheng, M., Zhang, X., Xia, D., Ke, Y., et al. (2016). Bile Acids Control Inflammation and Metabolic Disorder through Inhibition of NLRP3 Inflammasome. Immunity *45*, 802–816. https://doi.org/10.1016/j.immuni.2016.09.008.
46. Vong, C.T., Tseng, H.H.L., Yao, P., Yu, H., Wang, S., Zhong, Z., and Wang, Y. (2021). Specific NLRP3 inflammasome inhibitors: promising therapeutic agents for inflammatory diseases. Drug Discov. Today *26*, 1394–1408. https://doi.org/10.1016/j.drudis.2021.02.01833.
47. Tang, Y., Wang, C., Chen, S., Li, L., Zhong, X., Zhang, J., Feng, Y., Wang, L., Chen, J., Yu, M., et al. (2022). Dimethyl fumarate attenuates LPS induced septic acute kidney injury by suppression of NFκB p65 phosphorylation and macrophage activation. Int. Immunopharm. *102*, 108395. https://doi.org/10.1016/j.intimp.2021.108395.
48. Cross, S.A., Cook, D.R., Chi, A.W.S., Vance, P.J., Kolson, L.L., Wong, B.J., Jordan-Sciutto, K.L., and Kolson, D.L. (2011). Dimethyl fumarate, an immune modulator and inducer of the antioxidant response, suppresses HIV replication and macrophage-mediated neurotoxicity: a novel candidate for HIV neuroprotection. J. Immunol. *187*, 5015–5025. https://doi.org/10.4049/jimmunol.1101868.
49. Wilms, H., Sievers, J., Rickert, U., Rostami-Yazdi, M., Mrowietz, U., and Lucius, R. (2010). Dimethylfumarate inhibits microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1beta, TNF-alpha and IL-6 in an in-vitro model of brain

inflammation. J. Neuroinflammation *7*, 30. https://doi.org/10.1186/1742-2094-7-30.

50. Liu, X., Zhou, W., Zhang, X., Lu, P., Du, Q., Tao, L., Ding, Y., Wang, Y., and Hu, R. (2016). Dimethyl fumarate ameliorates dextran sulfate sodium-induced murine experimental colitis by activating Nrf2 and suppressing NLRP3 inflammasome activation. Biochem. Pharmacol. *112*, 37–49. https://doi.org/10.1016/j.bcp.2016.05.002.
51. Liu, W., Liu, X., Li, Y., Zhao, J., Liu, Z., Hu, Z., Wang, Y., Yao, Y., Miller, A.W., Su, B., et al. (2017). LRRK2 promotes the activation of NLRC4 inflammasome during Salmonella Typhimurium infection. J. Exp. Med. *214*, 3051–3066. https://doi.org/10.1084/jem.20170014.
52. Tang, T., Lang, X., Xu, C., Wang, X., Gong, T., Yang, Y., Cui, J., Bai, L., Wang, J., Jiang, W., and Zhou, R. (2017). CLICs-dependent chloride efflux is an essential and proximal upstream event for NLRP3 inflammasome activation. Nat. Commun. *8*, 202. https://doi.org/10.1038/s41467-017-00227-x.
53. Ali, M., Bonay, M., Vanhee, V., Vinit, S., and Deramaudt, T.B. (2020). Comparative effectiveness of 4 natural and chemical activators of Nrf2 on inflammation, oxidative stress, macrophage polarization, and bactericidal activity in an *in vitro* macrophage infection model. PLoS One *15*, e0234484. https://doi.org/10.1371/journal.pone.0234484.
54. Kwon, H.K., Yu, K.E., Cahill, S.V., Alder, K.D., Dussik, C.M., Kim, S.H., Sharma, L., Back, J., Oh, I., and Lee, F.Y. (2022). Concurrent targeting of glycolysis in bacteria and host cell inflammation in septic arthritis. EMBO Mol. Med. *14*, e15284. https://doi.org/10.15252/emmm.202115284.
55. Place, D.E., and Kanneganti, T.D. (2018). Recent advances in inflammasome biology. Curr. Opin. Immunol. *50*, 32–38. https://doi.org/10.1016/j.coi.2017.10.011.
56. Chen, X., Liu, G., Yuan, Y., Wu, G., Wang, S., and Yuan, L. (2019). NEK7 interacts with NLRP3 to modulate the pyroptosis in inflammatory bowel disease via NF-κB signaling. Cell Death Dis. *10*, 906. https://doi.org/10.1038/s41419-019-2157-1.
57. Niu, T., De Rosny, C., Chautard, S., Rey, A., Patoli, D., Groslambert, M., Cosson, C., Lagrange, B., Zhang, Z., Visvikis, O., et al. (2021). NLRP3 phosphorylation in its LRR domain critically regulates inflammasome assembly. Nat. Commun. *12*, 5862. https://doi.org/10.1038/s41467-021-26142-w.
58. Dong, S., Zhu, M., Wang, K., Zhao, X., Hu, L., Jing, W., Lu, H., and Wang, S. (2021). Dihydromyricetin improves DSS-induced colitis in mice via modulation of fecal-bacteria-related bile acid metabolism. Pharmacol. Res. *171*, 105767. https://doi.org/10.1016/j.phrs.2021.105767.
59. Liu, Q., Zuo, R., Wang, K., Nong, F.F., Fu, Y.J., Huang, S.W., Pan, Z.F., Zhang, Y., Luo, X., Deng, X.L., et al. (2020). Oroxindin inhibits macrophage NLRP3 inflammasome activation in DSS-induced ulcerative colitis in mice via suppressing TXNIP-dependent NF-κB pathway. Acta Pharmacol. Sin. *41*, 771–781. https://doi.org/10.1038/s41401-019-0335-4.
60. Shen, H.H., Yang, Y.X., Meng, X., Luo, X.Y., Li, X.M., Shuai, Z.W., Ye, D.Q., and Pan, H.F. (2018). NLRP3: A promising therapeutic target for autoimmune diseases. Autoimmun. Rev. *17*, 694–702. https://doi.org/10.1016/j.autrev.2018.01.020.
61. Ruiz, P.A., Morón, B., Becker, H.M., Lang, S., Atrott, K., Spalinger, M.R., Scharl, M., Wojtal, K.A., Fischbeck-Terhalle, A., Frey-Wagner, I., et al. (2017). Titanium dioxide nanoparticles exacerbate DSS-induced colitis: role of the NLRP3 inflammasome. Gut *66*, 1216–1224. https://doi.org/10.1136/gutjnl-2015-310297.
62. Liao, S.T., Han, C., Xu, D.Q., Fu, X.W., Wang, J.S., and Kong, L.Y. (2019). 4-Octyl itaconate inhibits aerobic glycolysis by targeting GAPDH to exert anti-inflammatory effects. Nat. Commun. *10*, 5091. https://doi.org/10.1038/s41467-019-13078-5.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-NLRP3/NALP3	AdipoGen	Cat#AG-20B-0014; RRID: AB_2490202
anti-pro-Caspase-1	abcam	Cat#ab179515; RRID: AB_2884954
anti-mouse-pro-IL-1β/cleaved-IL-1β	abcam	Cat#ab234437; RRID: AB_2936228
anti-NEK7	abcam	Cat#ab133514; RRID: AB_2877625
anti-Nrf2	abcam	Cat#ab137550; RRID: AB_2687540
anti-human-AIM2	Cell Signaling Technology	Cat#12948; RRID: AB_2798067
anti-NLRC4	Cell Signaling Technology	Cat#12421; RRID: AB_2797906
anti-human-pro-IL-1β	Cell Signaling Technology	Cat#12703; RRID: AB_2737350
anti-human-cleaved-IL-1β	Cell Signaling Technology	Cat#83186; RRID: AB_2800010
anti-human-cleaved-Caspase-1	Cell Signaling Technology	Cat#4199; RRID: AB_1903916
anti-mouse-cleaved-Caspase-1	abmart	Cat#M025280; RRID: AB_3095728
anti-Flag	Yeasen	Cat#30503ES61; RRID: AB_3095723
Mouse IgG	Yeasen	Cat#36111ES10; RRID: AB_3095724
anit-ASC	Proteintech	Cat#10500-1-AP; RRID: AB_2174862
anti-β-actin	Proteintech	Cat#66009-1-Ig; RRID: AB_2687938
anti-HIS	Proteintech	Cat#66005-1-Ig; RRID: AB_11232599
anti-HA	Proteintech	Cat#51064-2-AP; RRID: AB_11042321
anti-GFP	Proteintech	Cat#66002-1-Ig; RRID: AB_11182611
Rabbit IgG	Proteintech	Cat#30000-0-AP; RRID: AB_2819035
Bacterial and virus strains		
salmonella typhimurium	BNCC	Cat#108207
Chemicals, peptides, and recombinant proteins		
DMF (Dimethyl fumarate)	MCE	Cat#HY-17363
PMA (phorbol-12-myristate-13-acetate)	Yeasen	Cat#50601ES03
LPS (lipopolysaccharide)	Sigma	Cat#L2880
ATP	Sigma	Cat#A1852
MSU	Sigma	Cat#U0881
Poly (dA:dT)	InvivoGen	Cat#tlr-patn
Nigericin	InvivoGen	Cat#tlrl-nig
DSS (dextran sulphate sodium salt)	MP Blomedicals	Cat#160110
Protein A/G Magnetic Beads	MCE	Cat#HY-K0202
Lipo8000™ Transfection Reagent	Beyotime	Cat#C0533
M-CSF (macrophage colony stimulating factor)	PEPROTECH	Cat#315-02
Fluo-4 AM	Beyotime	Cat#S1060
MCC950	MCE	Cat#HY-12815A
DAPI	Solarbio	Cat#S2110
RIPA	Solarbio	Cat#R0010
Nonidet P-40 Substitute	Beyotime	Cat#ST2045
protease inhibitor Cocktail	Yeasen	Cat#20124ES03
JC-1	Beyotime	Cat#C2005

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNA isolater Total RNA Extraction Reagent	Vazyme	Cat#R401-01
Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR	Yeasen	Cat#11141ES60
Hieff® qPCR SYBR Green Master Mix	Yeasen	Cat#11202ES08
Critical commercial assays		
Mouse TNF-α ELISA Kit	Absin	Cat#abs520010
Human IL-1β ELISA Kit	FCMACS	Cat#fms-elh002
Mouse IL-1β ELISA Kit	Novus	Cat#VAL601
Human IL-18 ELISA Kit	Novus	Cat#7620
Mouse IL-18 ELISA Kit	Novus	Cat#7625
CCK8 Kit	Yeasen	Cat#40203ES60
BCA Protein Quantification Kit	Yeasen	Cat#20201ES76
Experimental models: Cell lines		
THP-1	ATCC	TIB-202
293T	ATCC	CRL-3216
Experimental models: Organisms/strains		
Mouse: C57BL/6J	GemPharmatech Co., Ltd. (Nanjing, China)	NO.N000013
Mouse: *Nlrp3*$^{-/-}$	GemPharmatech Co., Ltd. (Nanjing, China)	NO.T010873
Oligonucleotides		
Primers for *Nqo1*, *Hmox1*, *Gsr*, *Pgd*, *Gclm*, *Taldo1*, *Nfe2l2*, *β-actin*, see Table S1	This paper	N/A
Software and algorithms		
GraphPad Prism 8.0	GraphPad Software	Prism - GraphPad
FlowJo software (v10.0.7) Article	FlowJo software	Download FlowJo | FlowJo, LLC

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shanting Liao (liaoshanting@njucm.edu.cn.).

Materials availability

This study did not generate new materials or reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon reasonable request.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All experiments were conducted utilizing 8-week-old male C57BL/6J and *Nlrp3*$^{-/-}$ mice (18-25 g), procured from GemPharmatech Co., Ltd. (Nanjing, China). The mice were maintained under specific pathogen-free conditions and housed in a controlled environment with a 12-hour photoperiod (lights on at 09:00 a.m. and off at 09:00 p.m.), at a temperature range of 21-25°C, with *ad libitum* access to food and water. Ethical considerations were strictly observed, and all animal experiments were conducted following the approval of the University Committee on Use and Care of Animals at Nanjing University of Chinese Medicine (Approval No. 202109A039).

METHOD DETAILS

Cell preparation and stimulation

BMDMs were isolated from the bone marrow of 6-8-week-old male C57BL/6J mice and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 25 ng/mL murine M-CSF for 6 days. 293T and THP-1 cells were cultured in DMEM and RPMI-1640 medium, respectively, both supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator (Thermo Scientific) containing 5% CO_2. 293T and THP-1 cell lines were authenticated by short tandem repeat (STR) profiling by Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. THP-1 cell line was isolated from the peripheral blood of a 1-year-old boy with acute monocytic leukemia. 293T cell line was isolated from human embryonic kidney, so cell sex could not be identified. All cell lines were routinely tested for mycoplasma contamination. Prior to inflammatory activation, THP-1 cells were treated with 50 ng/mL PMA for 3 hours and incubated overnight. To induce activation of NLRP3, NLRC4, and AIM2 inflammasomes, THP-1 cells were primed with 1 μg/mL LPS (100 ng/mL LPS for BMDMs) for 3 hours. For NLRP3 inflammasome activation, DMF was added to the culture medium, followed by a 45-minutes incubation, after which the cells were stimulated as follows: 5 mM ATP for 45 minutes (2.5 mM for BMDMs), 5 μM nigericin for 45 minutes, 0.25 mg/mL MSU for 6 hours, and 20 μg/mL imiquimod for 45 minutes. For NLRC4 inflammasome, cells were treated with DMF for 45 minutes and then stimulated with *Salmonella* for 1 hour. For AIM2 inflammasome, cells were treated with DMF for 45 minutes, followed by transfection of 0.5 μg/mL poly (dA:dT) into the cells using Lipo8000 for 6 hours.

Plasmid generation and lentiviral infection

sgRNAs targeting the human NLRP3 gene (sequences: ATTGAAGTCGATCATTAGCG and AATGATCGACTTCAATGGGG) were designed, cloned into the LentiCRISPRv2 vector, and subsequently packaged into lentivirus.

To deplete NLRP3, THP-1 cells were infected with lentivirus (using 4 μg/mL polybrene) and selected with puromycin for 5 days. For the overexpression of NLRP3 in CRISPR-mediated NLRP3-depleted (*Nlrp3*$^{-/-}$) THP-1 cells, cDNAs encoding wild-type or C673A mutant human NLRP3 were synonymous mutated at PAM sequences of sgRNA target regions and cloned into a lentiviral vector expressing EGFP. The resulting lentivirus was used for transduction, and cells overexpressing WT or C673A mutant NLRP3 were sorted using FACS based on green fluorescence emission. The expression of NLRP3 was assessed through Western blotting analysis.

ELISA

The expressions of IL-1β, IL-18, and TNF-α in supernatants from cell culture and colon tissue homogenates were assessed using corresponding ELISA kits according to the manufacturer's instructions.

Western blotting analysis

Western blotting analysis was performed following established protocols.[62] Cells were lysed in RIPA buffer containing complete protease and phosphatase inhibitors. The lysates were transferred to a 1.5-mL tube and heat-denatured in 2× Laemmli sample buffer. To concentrate supernatants for Western blotting, an equal volume of methanol and a quarter volume of chloroform were added, followed by centrifugation at 12,000 rpm for 5 minutes at room temperature. After discarding the supernatants, pellets were resuspended in an equal volume of methanol, centrifuged again, and dried at 55°C for 5 minutes. The remaining pellets were resuspended in 1× loading buffer and heated at 95°C for 10 minutes. Samples were stored at -80°C until use. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies at room temperature. Immunoreactive bands were visualized using ECL Substrates on an Azure Biosystems c500. β-actin served as a loading control.

Reconstitution of NLRP3 inflammasome in 293T cells

293T cells were seeded at 2×10^5 /mL into 48-well plates or 6-cm culture dishes in DMEM complete medium. After 24 hours, GFP-ASC, Myc-pro-Caspase-1, pro-IL-1β, and Flag-NLRP3 or Flag-NLRP3 mutant (C673A) plasmids were transfected into cells by Lipo8000 for 24 hours. Cells were treated with or without DMF, and supernatants or lysates were detected by Western blotting analysis or ELISA.

Reconstitution of NLRP3 inflammasome in *Nlrp3*$^{-/-}$ THP-1 cells

Nlrp3$^{-/-}$ THP-1 cells were transfected with lentivirus encoding NLRP3 and NLRP3 mutants (C673A). After 6 hours, the supernatants were replaced by a complete medium and continually incubated for 66 hours. Subsequently, cells were stimulated with LPS/ATP in the absence or presence of DMF, and the supernatants were analyzed by ELISA.

Immunofluorescence staining

BMDMs were seeded at 1.8×10^6 cells/mL in 12-well cell slides. After NLRP3 inflammasome activation and treatment, the medium was removed, and the slides were fixed with paraformaldehyde for 10 minutes. Following fixation, slides were washed three times with PBS supplemented with 0.05% Triton. BMDMs were then blocked with 1% BSA and 0.05% Triton in PBS for 1 hour. Subsequently, BMDMs were incubated with anti-ASC primary antibody overnight at 4°C. The following day, after three washes with PBS supplemented with 0.05% Triton,

BMDMs were incubated with a fluorescent secondary antibody for 2 hours. After rinsing with PBS supplemented with 0.05% Triton, cells were incubated with DAPI. Confocal microscopy was performed using a Laser TCS SP8.

Co-Immunoprecipitation

For the endogenous interaction assay, THP-1 cells were stimulated, lysed with NP-40 lysis buffer (containing complete protease and phosphatase inhibitors), and incubated with primary antibodies at 4°C for 2 hours. Protein A/G magnetic beads were washed with PBS supplemented with 0.5% Triton three times and then incubated with cell lysates at 4°C for 2 hours. Immunoprecipitate were detected by Western blotting analysis. For the exogenous interaction assay, 293T cells were transfected with plasmids in 6-cm culture dishes using Lipo8000. After 24 hours, cells were treated with or without DMF, lysed with NP-40 lysis buffer (containing complete protease and phosphatase inhibitors), and subjected to immunoprecipitation with anti-His or anti-HA antibodies. The resulting proteins were then analyzed by Western blotting.

ASC oligomerization assay

THP-1 cells were seeded in 6-cm culture dishes at a density of 1×10^6 cells/mL. The next day, the medium was replaced, and cells were primed with 1 μg/mL LPS for 3 hours. Subsequently, cells were treated with or without DMF for 45 minutes and then stimulated with nigericin for an additional 45 minutes. After removing the supernatants, cells were rinsed in ice-cold PBS and lysed with NP-40 for 30 minutes. The lysates were centrifuged at 330×g for 10 minutes at 4°C. Pellets were washed twice with 2 mL ice-cold PBS, resuspended in 500 μL PBS, and added with 2 mM disuccinimidyl suberate. The samples were incubated with rotation at room temperature for 30 minutes and then centrifuged at 330×g for 10 minutes at 4°C. The resulting cross-linked pellets were resuspended in 30 μL 1× sample buffer, boiled, and analyzed by Western blotting.

Intracellular K^+ determination

To determine intracellular K^+ levels, THP-1 cells were seeded in 6-well dishes and stimulated as usual. Following stimulation, the culture medium was discarded, and cells were washed twice with potassium-free buffer (139 mM NaCl, 1.7 mM NaH_2PO_4, and 10 mM Na_2HPO_4, pH 7.2). Subsequently, 5 mL of ultrapure HNO_3 was added for cell lysis, and cell lysates were collected in glass bottles. Samples were boiled at 100°C for 30 minutes, diluted to 10 mL with ddH_2O, and analyzed for intracellular K^+ levels using Agilent 7800 inductively coupled plasma mass spectrometry (ICP-MS).

Intracellular Ca^{2+} and mitochondrial membrane potential determination

To determine intracellular Ca^{2+} levels and mitochondrial membrane potential, THP-1 cells were seeded in 12-well dishes and stimulated as usual. After stimulation, the culture medium was discarded, and cells were washed twice with PBS. For Ca^{2+} detection, the Fluo-4 AM calcium fluorescence probe was diluted to a final concentration of 2 μM in PBS. Subsequently, 500 μL of the Fluo-4 AM solution was added to each well, and cells were incubated at 37°C for 30 minutes in the dark. After incubation, cells were washed twice with PBS, resuspended in 300 μL PBS buffer, collected in a 1.5-mL centrifuge tube, and filtered through a nylon membrane. For mitochondrial membrane potential determination, the JC-1 fluorescence probe was diluted to a final concentration of 1 μg/mL in PBS and the JC-1 solution was used in a similar manner. After incubation, cells were washed, resuspended, collected, and filtered. The fluorescence of Fluo-4 AM and JC-1 was analyzed by flow cytometry to determine changes in intracellular Ca^{2+} concentration and mitochondrial membrane potential.

Analysis of NLRP3 modification by DMF

293T cells were transfected with a plasmid encoding human Flag-NLRP3 using Lipo8000 for 24 hours. Following transfection, cells were treated with 40 μM DMF for 1.5 hours. Subsequently, cells were washed with PBS and lysed using NP-40 buffer. Immunoprecipitation of Flag-NLRP3 in cell lysates was performed using anti-Flag antibody and Protein A/G Magnetic Beads. The immune complexes were eluted, boiled for 5 minutes, and separated by SDS-PAGE gel. The gel was stained with Coomassie blue, and bands corresponding to Flag-NLRP3 were excised. The excised bands were subjected to in-gel digestion by trypsin.

Samples were analyzed in an Q ExactiveTM Plus coupled online to an EASY-nLC 1000 UPLC system (Thermo). The MS/MS data were analyzed using Proteome Discoverer 1.3. The UniProt database was used to search tandem mass spectra. Precursor mass error was set to 10 ppm, while fragment ions was detected with a tolerance of 0.6 Da. The peptide confidence was set high, and the peptide ion score was set at >20.

Quantitative real-time PCR

Total RNA was isolated from THP-1 cells by extraction with RNA isolater Total RNA Extraction Reagent. RNA (1 μg) of each sample was used for reverse transcription with Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR according to the manufacturer's instructions. qPCR was performed using Hieff® qPCR SYBR Green Master Mix in a QuantStudio™ 3 Real-Time PCR Instrument. The relative gene expression levels were determined by the ΔΔCt method using *β-actin* as a reference gene. All primer sequences were listed in Table S1.

DSS-induced colitis

Briefly, 8-week-old male C57BL/6 and *Nlrp3*$^{-/-}$ mice (18-25 g) were randomly assigned into four groups as follows: the control + vehicle (Con), untreated colitis + vehicle (model), colitis + DMF (DMF, 50 mg/kg), and colitis + MCC950 (MCC950, 50 mg/kg) groups. Colitis was induced by 2.5% (w/v) DSS in drinking water continuously for 7 days. DMF or MCC950 was dissolved in sodium carboxymethyl cellulose (CMC-Na) and gavaged to the appropriate groups for seven consecutive days. The control and model groups received the same volume of CMC-Na. The mice were monitored, and their body weight was measured daily. After 7 days, the mice were weighed and subsequently euthanized after anesthetized with isoflurane, and the colons were excised and measured. The colons were snap-frozen in liquid nitrogen for pathological examination and IL-1β level detection.

QUANTIFICATION AND STATISTICAL ANALYSIS

Briefly, Graphpad software 8 (Prism) was used to perform all statistical analyses. Unpaired, two-tailed Student's t-test was applied for two-group comparisons. Furthermore, one-way ANOVA was used for multiple-group comparisons. Data were presented as the mean ± standard deviation. $p < 0.05$ was considered to be statistically significant.