Article

Sinensetin Attenuated Macrophagic NLRP3 Inflammasomes Formation *via* SIRT1-NRF2 Signaling

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ABSTRACT: Macrophage-mediated inflammation plays essential roles in multiple-organ injury. Sinensetin (SNS) at least exhibits anti-inflammation, antioxidant, and antitumor properties. However, the underlying mechanism of SNS-targeted macrophage-mediated inflammation remains elusive. In the present study, our results showed that SNS suppressed lipopolysaccharide (LPS)-induced inflammation to ameliorate lung and liver injuries. Mechanistically, SNS significantly inhibited M1-type macrophage polarization and its NLRP3 inflammasome formation to significantly decrease tumor necrosis factor α (TNFα) and IL-6 expression, while increasing IL-10 expression. Moreover, SNS interacted and activated SIRT1 to promote NRF2 and its target gene SOD2 transcription, which subsequently decreased LPS-induced inflammation. SIRT1 knockdown impaired the effects of SNS on the inhibition of macrophage polarization, NLRP3 inflammasome formation, and NRF2/SOD2 signaling. Taken together, our results showed that SNS is a potential and promising natural active ingredient to ameliorate inflammatory injury *via* activating SIRT1/NRF2/SOD2 signaling.
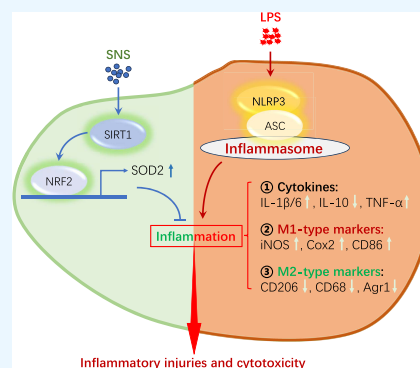
1. INTRODUCTION

Accumulating evidence has highlighted that the active ingredients derived from natural products or traditional Chinese medicine have been evaluated in treating inflammatory diseases and cancer.[1] Flavonoid derives from various vegetables and fruits, at least including green tea, citrus fruits, and even onion. Accumulating evidence indicated that flavonoid plays important roles in antivirus and antitumor response and in improving cardiovascular diseases.[2] Recently, emerging studies highlighted that sinensetin (SNS), one of the flavone aglycones, exhibits various bioactivities on anti-inflammation, antioxidant, antitumor antimicrobial, and antiobesity.[3,4] SNS served as an inhibitor to target GLUT1 to facilitate sorafenib-induced cytotoxicity of cancer cells, which might contribute to overcoming chemoresistance in cancer treatment.[4] Previous studies showed that SNS-activated transcription factor signals transducers and activators of transcription 1α (STAT1α) to decrease inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNFα), and cyclooxygenase-2 (COX2) expression, which might possess a promising and potential role in anti-inflammatory treatments.[5] Recently, SNS decreased IL-1β-induced inflammatory cytokines in human osteoarthritis chondrocytes, such as COX2, iNOS, TNFα, and IL-6, but it also decreased MMP9 and MMP13 to attenuate extracellular matrix degradation, which together ameliorates osteoarthritis.[6] SNS also integrates NF-κB and MAPK signals to ameliorate influenza-induced inflammation.[7] Inflammatory injuries, such as acute lung

injury, possessed a total mortality of about 40%;[8] acute liver failure would be fatal with a mortality of 80−85%.[9] For instance, a high risk of acute lung injury includes genetic factors (ACE2, associated with the COVID-19 pandemic), virulence factors, race differences, and environmental factors, which would be potentiated by the ventilator-induced lung injury.[8] However, the effect of SNS on attenuating inflammation to ameliorate acute lung/liver injuries remains elusive.

Macrophages exhibit multiple and essential roles in inflammatory injury of the lungs, liver, and cardiovascular system and metabolic disease.[10,11] Macrophage-induced auto-inflammation and injury mainly depended on regulating NLRP3-related inflammatory signalings.[12,13] Notch1-YAP loop positively contributes to macrophage M1-type polarization to exacerbate liver injury.[14] Bacterial infection activated macrophage infiltration and AKT/NF-κB signaling to induce fatal lung injury.[15] Bone marrow-derived macrophage (BMDM)-related NLRP3 inflammasome would be suppressed by the natural compound Bergapten *via* promoting mitophagy

and mitochondrial homeostasis.[16] Loss of AMPKα1/FOXO3 signaling in macrophage would aggravate post-injury muscle regeneration and acute lung injury.[17] Promoter-specific interactions of NF-κB and suppressor of cytokine signaling-1 (SOCS1) promoted the transcription of NF-κB downstream pro-inflammatory genes, which promoted macrophage induced inflammatory injuries.[18] Indeed, previous studies indicated that SNS inactivated NF-κB signaling to exhibit anti-inflammation and protect against inflammatory cytotoxicity.[6,7,19] However, the effect and underlying mechanism of SNS on macrophage-induced inflammatory injury remain largely unknown.

In the present study, we evaluated the effect and explore the underlying mechanism of SNS on regulating macrophage polarization and its subsequent inflammation in LPS-induced inflammatory injury *in vitro* and *in vivo* models.

2. METHODS AND MATERIALS

2.1. Cell Culture and Treatments. The mouse mononuclear macrophage RAW264.7 cells were purchased from Beyotime Company (Cat. C7505, Shanghai, China). RAW264.7 cells were maintained in high-glucose DMEM (GIBCO), supplemented with 10% fetal bovine serum (Cat. C0226, Beyotime, Shanghai, China) and 1% penicillin-streptomycin (Cat. C0222, Beyotime, Shanghai, China). RAW264.7 cells were cultured in a 37 °C, 5% CO_2 cellular incubator. The 100 ng/mL LPS (Sigma, lot. L4516) with or without different dosages of SNS (Sigma, lot. SML1787) was used to treat RAW264.7 cells for 6 h (mRNA level detection) or 24 h (protein level detection), and then following subsequent detections.[20] The SIRT1 siRNA sequence was 5′-TGATTGGCACCGATCCTCG-3′, and then SIRT1 knockdown was carried out as previously described.[21] All adenovirus plasmids construction and adenovirus packages were constructed and purchased from Shanghai ObioTechonology Company (Shanghai, China). After infecting RAW264.7 cells for 48 h, the cell growth medium was refreshed, and subsequent experiments were performed.

2.2. Real-Time Polymerase Chain Reaction (PCR). Total RNA of cells or lung and liver tissues were isolated by RNAeasy Plus Animal RNA Isolation Kit with Spin Column (Cat. R0032, Beyotime, Shanghai, China) as the manufacturer's guideline. In brief, samples were lysed in lysis solution within the kit and then stewed for 1 min at room temperature. Binding buffer was then added and gently mixed well. The mixture was then carefully transferred into spin columns for centrifugation at 12,000g, 30 s. Filtrate was collected and transferred into a new Eppendorf (EP) tube. 0.7 mL of binding buffer was added and gently mixed well. The mixture was then carefully transferred into spin columns for centrifugation at 12,000g for 30 s. To the spin columns was added 0.6 mL of washing buffer I for centrifugation at 12,000g for 30 s, followed by washing buffer II with the same procedure twice. After discarding the filtrate, centrifugation was performed at 14,000g for 120 s. Total RNA was washed down from spin columns by RNA eluent and collected in a new EP tube for subsequent experiments. Total RNA was reverse transcribed by the BeyoRT II First Strand cDNA Synthesis Kit with gDNA Eraser (Cat. D7170L, Beyotime, Shanghai, China) according to the manufacturer's guideline. A special gene was amplified by the BeyoFast SYBR Green qPCR Mix (Low ROX) kit (Cat. D7262-25 mL, Beyotime, Shanghai, China) according to the manufacturer's guideline.

2.3. Immunofluorescence. Immunofluorescence was performed as previously described.[22] In brief, tissue slices were permeabilized with Triton X-100 solution (Cat. P0096-100 mL, Beyotime, Shanghai, China); then, they were blocked with QuickBlock Blocking Buffer for Immunol Staining (Cat. P0260, Beyotime, Shanghai, China). After incubating the primary antibodies overnight at 4 °C, Alexa Fluor 488 and Alexa Fluor 647 secondary antibodies were incubated for 1 h at room temperature. Images were captured by a fluorescence microscope (Nikon, Japan). Fluorescence intensity was quantified by ImageJ as previously described.[23] The primary antibodies were used as follows: anti-NLRP3 (Cat. AF2155, Beyotime, Shanghai, China), anti-ASC (Cat. AF6234, Beyotime, Shanghai, China), anti-NRF2 (Cat. AF7623, Beyotime, Shanghai, China), anti-SOD2 (Cat. PK08370, Abmart, Shanghai, China), anti-SIRT1 (Cat. AF5300, Beyotime, Shanghai, China), anti-Cd11b (Cat. AF6396, Beyotime, Shanghai, China), anti-F4/80 (Cat. AG4753, Beyotime, Shanghai, China), anti-SOD2 (Cat. PK08370, Abmart, Shanghai, China).

2.4. Molecular Docking. Molecular docking of SNS and SIRT1 was carried out as previously described.[24] Briefly, we acquired the SNS structure from the PubChem database and SIRT1 three-dimensional (3D) protein structure from the PDB database, and then docking was performed by using AutoDock4 software.

2.5. ELISA. Whole blood was stewed overnight at 4 °C and then centrifuged for 10 min at 3000 rpm and room temperature. Serum was carefully transferred into a new EP tube for subsequent ELISA experiments which were carried out according to the manufacturer's guideline. The ELISA kit of mouse TNFα was purchased from NEOBIOSCIENCE Company (Cat. EMC102a(H).96, Shenzhen, China). The ELISA kit of mouse IL6 and IL1β was purchased from Beyotime Company (Cat. PI326 and Cat. PI301, Shanghai, China). Aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Abcam Company (Cat. ab285263 and Cat. ab263882, Cambridge, United Kingdom).

2.6. *In Vivo* Experiment. C57BL/6 male mice (8 weeks old) were purchased from the Guangdong Medical Laboratory Animal Center. All of the mice were housed at the Animal Research Center of Guangzhou Medical University. All animal experiments were reviewed and approved by The Animal Ethics Committee of Guangzhou Medical University. All of the mice received human care as being housed and bred in the standard specific pathogen-free environment at 22 ± 2° with 50 ± 10% humidity and every 12 h light/dark switch. A 0.25 mg/kg/day LPS plus 80 mg/mL D-Gal mixture was intraperitoneally injected into the mice for 4 days (totally equal to 1 mg/kg) to construct the inflammatory injuries model, SNS (low dose at 12.5 mg/kg/day, medium dose at 25 mg/kg/day, high dose at 50 mg/kg/day) for 4 days (totally equal to 50 mg/kg at a low dose, 100 mg/kg at a medium dose, and 200 mg/kg at a high dose). All of the mice were anesthetized after 24 h injections, and then tissues and blood samples were collected for subsequent examinations.

2.7. H&E Staining. Paraffin slices were bathed within xylene for 10 min twice and gradient alcohol (100, 90, 80, 70%) for 5 min each. After rinsing in ddH$_2$O for 3 min, paraffin slices were stained in hematoxylin (Cat. C0105M, Beyotime, Shanghai, China) for 10 min and then rinsed in ddH$_2$O for 10 min twice. After fast differentiation by acid alcohol for 30 s, eosin (Cat. C0105M, Beyotime, Shanghai, China) staining was
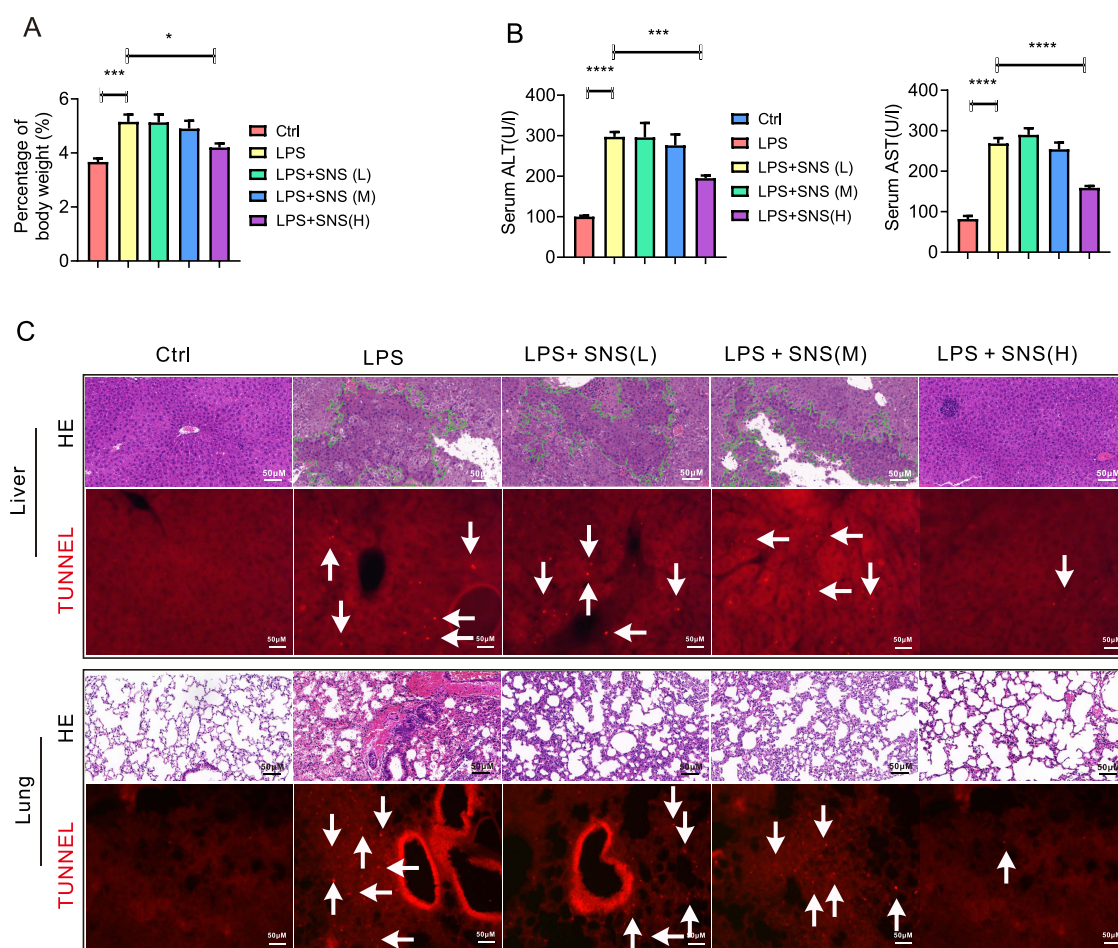
Figure 1. SNS-ameliorated LPS-induced inflammatory injuries and cytotoxicity. Mice were injected with 0.25 mg/kg/day LPS for 4 days, or 0.25 mg/kg/day LPS and SNS (low dose at 12.5 mg/kg/day, medium dose at 25 mg/kg/day, high dose at 50 mg/kg/day) for 4 days. (A) Changing of the ratio of liver/body weight (%) after LPS with/without SNS treatments. (B) Serum ALT and AST levels after indicated treatment. (C) H&E staining and TUNEL staining in the liver and lungs after indicated treatment. $n = 6$, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

carried out for 2 min and then rinsed in 70% alcohol for 2 min. After dehydration, transparently, all slices were sealed by the poly(vinylpyrrolidone) mounting medium kit (Cat. C0185, Beyotime, Shanghai, China).

2.8. TUNEL Assay. Paraffin slices were bathed within xylene for 10 min twice and gradient alcohol (100, 90, 80, 70%) for 5 min each. After rinsing in ddH$_2$O for 3 min, paraffin slices underwent TUNEL assay by using one-step TUNEL Apoptosis Assay Kit (Cat. C1090, Beyotime, Shanghai, China) according to the manufacturer's guideline.

2.9. Statistical Analyses. Data included in the present study were presented as mean ± S.D. *T* test and one-way ANOVA were used to analyze the statistical difference. *p* value less than 0.05 was set as statistical difference.

3. RESULTS

3.1. SNS-Ameliorated LPS-Induced Inflammatory Injuries and Cytotoxicity. We first investigated the effect of SNS on LPS-induced inflammatory injury in mice. Our results showed that LPS significantly elevated the ratio of liver/body weight (%), SNS dose-dependently decreased the ratio of liver/body weight (%), and there was a significant difference in the high dose of SNS treatment (Figure 1A). LPS also significantly elevated the ALT and AST serum levels, SNS dose-dependently decreased the ALT and AST serum levels,

and there was a significant difference in the high dose of SNS treatment (Figure 1B). LPS administration dramatically increased the infiltration of inflammatory cells in the lungs and liver, which is accompanied by apoptosis in the lungs and liver (Figure 1C). SNS dose-dependently reversed the LPS-induced inflammatory infiltration and injuries in the lungs and liver (Figure 1C).

3.2. SNS-Suppressed LPS-Induced Macrophage Infiltration and Subsequent Inflammation. We then wonder about the type of inflammatory cells that infiltrated in lungs and liver. Our results showed that LPS induced almost 3-fold upregulation of TNFα, IL6, and IL1β serum levels, respectively, compared to the control group (Figure 2A). SNS dose-dependently decreased the TNFα, IL6, and IL1β serum levels, respectively, and there was a significant difference in the high dose of SNS treatment (Figure 2A). There was a slight upregulation of IL1β serum level at low-dose SNS treatment compared to the LPS model group, but it had no statistical difference (Figure 2A). LPS positively increased the monocyte marker Cd11b expression in liver, which correlated with the upregulation of macrophage marker F4/80 expression in liver (Figure 2B). Low-dose and medium-dose SNS had no effect on regulating the Cd11b and F4/80 expression in liver, while the high-dose SNS dramatically decreased the Cd11b and F4/80 expression in liver (Figure 2B).
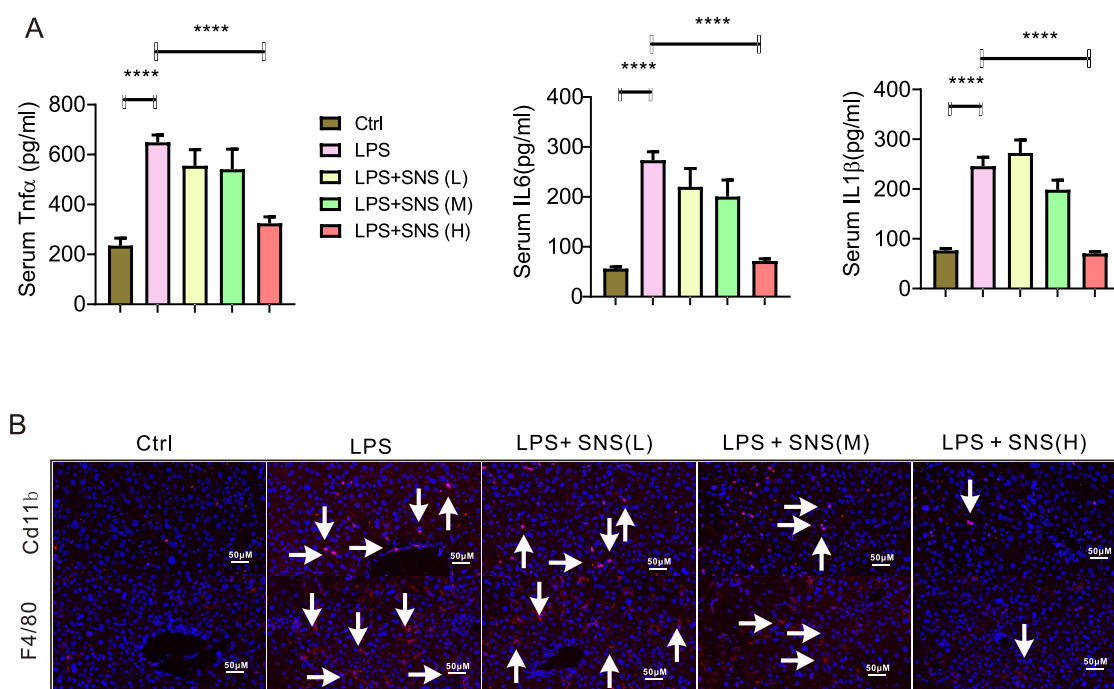
Figure 2. SNS-suppressed LPS-induced macrophage infiltration and subsequent inflammation. Mice were injected with 0.25 mg/kg/day LPS for 4 days, or 0.25 mg/kg/day LPS and SNS (low dose at 12.5 mg/kg/day, medium dose at 25 mg/kg/day, high dose at 50 mg/kg/day) for 4 days. (A) Serum TNF-α, IL-1β, and IL-6 after indicated treatment. (B) Macrophage infiltration in liver after indicated treatment. Cd11b and F4/80 were red, and DAPI was blue. $n = 6$, ****$p < 0.0001$.

3.3. SNS-Regulated Macrophage Polarization and Its Activities.

We then evaluated the effect of SNS on regulating macrophage polarization and its activities. Our results further showed that LPS significantly increased the expression of M1-type macrophage markers, including iNOS, COX2, and CD86 (Figure 3A). Low-dose SNS combined with LPS treatment slightly increased the iNOS mRNA expression compared to the LPS model group, but it had no statistical difference (Figure 3A). SNS dose-dependently reversed LPS-induced upregulation of iNOS, COX2, and CD86 mRNA expression respectively, and there was a significant difference in the high-dose SNS treatment (Figure 3A). LPS significantly decreased the expression of M2-type macrophage markers, including Cd206, Cd68, and Agr1 (Figure 3B). SNS dose-dependently reversed LPS-induced inhibition of Cd206, Cd68, and Agr1 mRNA expression, respectively, and there was a significant difference in the high-dose SNS treatment (Figure 3B). Our results further showed that LPS significantly and subsequently increased the pro-inflammatory cytokines TNFα and IL6 mRNA expression but decreased the anti-inflammatory cytokine IL-10 mRNA expression (Figure 3C). Low-dose and medium-dose SNS had no effect on regulating the TNFα, IL6, and IL-10 mRNA expression, but the high-dose SNS significantly decreased the TNFα and IL6 mRNA expression but increased the IL-10 mRNA expression (Figure 3C).

3.4. SNS-Inhibited Macrophagic NLRP3 Inflammasome Formation.

Our results next showed that in LPS-induced NLRP3 and ASC protein levels in the RAW264.7 cells, low-dose and medium-dose SNS had no effect on decreasing the expression of NLRP3, while high-dose SNS obviously decreased cytoplasmic and nucleus protein levels of NLRP3 (Figure 4A−C). Low-dose SNS also had no effect on decreasing the expression of ASC, while medium-dose and high-dose SNS obviously decreased cytoplasmic and nucleus

protein levels of ASC (Figure 4A−C). LPS significantly elevated the NLRP3 and ASC mRNA expression in RAW264.7 cells, low-dose SNS seemed to be further enhancing this effect by LPS, yet it had no statistical difference (Figure 4D). Otherwise, low-dose SNS had no effect on regulating LPS-induced IL-1β mRNA expression (Figure 4D). Medium-dose SNS had no effect on regulating LPS-induced NLRP3, ASC, or IL-1β mRNA expression, respectively, while high-dose SNS significantly and dramatically decreased LPS-induced NLRP3, ASC, or IL-1β mRNA expression, respectively (Figure 4D).

3.5. SNS-Activated SIRT1 to Amplified NRF2 Anti-Inflammation Signaling.

We then explored the underlying mechanism of SNS in regulating macrophage-induced inflammation. Our results showed that SNS interacted with the conserved activator binding site of SIRT1 (Figure 5A and Supporting Table 1) at THR154, THR166, and GLY183. Besides, high-dose SNS significantly reversed LPS-induced inhibition of SIRT1 mRNA expression, but not low-dose or medium-dose SNS (Figure 5B). Interestingly, high-dose SNS significantly reversed LPS-induced inhibition of NRF2 protein expression and nucleus sublocation, but not low-dose or medium-dose SNS (Figure 5C), which was in line with the effect of SNS on regulating NRF2 target molecular SOD2 protein expression (Figure 5D−F).

3.6. SIRT1 Knockdown-Attenuated SNS-Induced Anti-Inflammation Signaling.

We next conducted SIRT1 knockdown to confirm the SNS bio-functions. Our results solidly confirm that SIRT1 mRNA and protein level were successfully and significantly silent in RAW264.7 cells (Figure 6A,B). Our results further showed that SIRT1 knockdown partly and significantly abolished the effect of SNS on NRF2 upregulation and nucleus translocation rather than its change in the cytoplasm (Figure 6C). Moreover, SIRT1 knockdown almost dramatically abolished the effect of SNS on NRF2 target
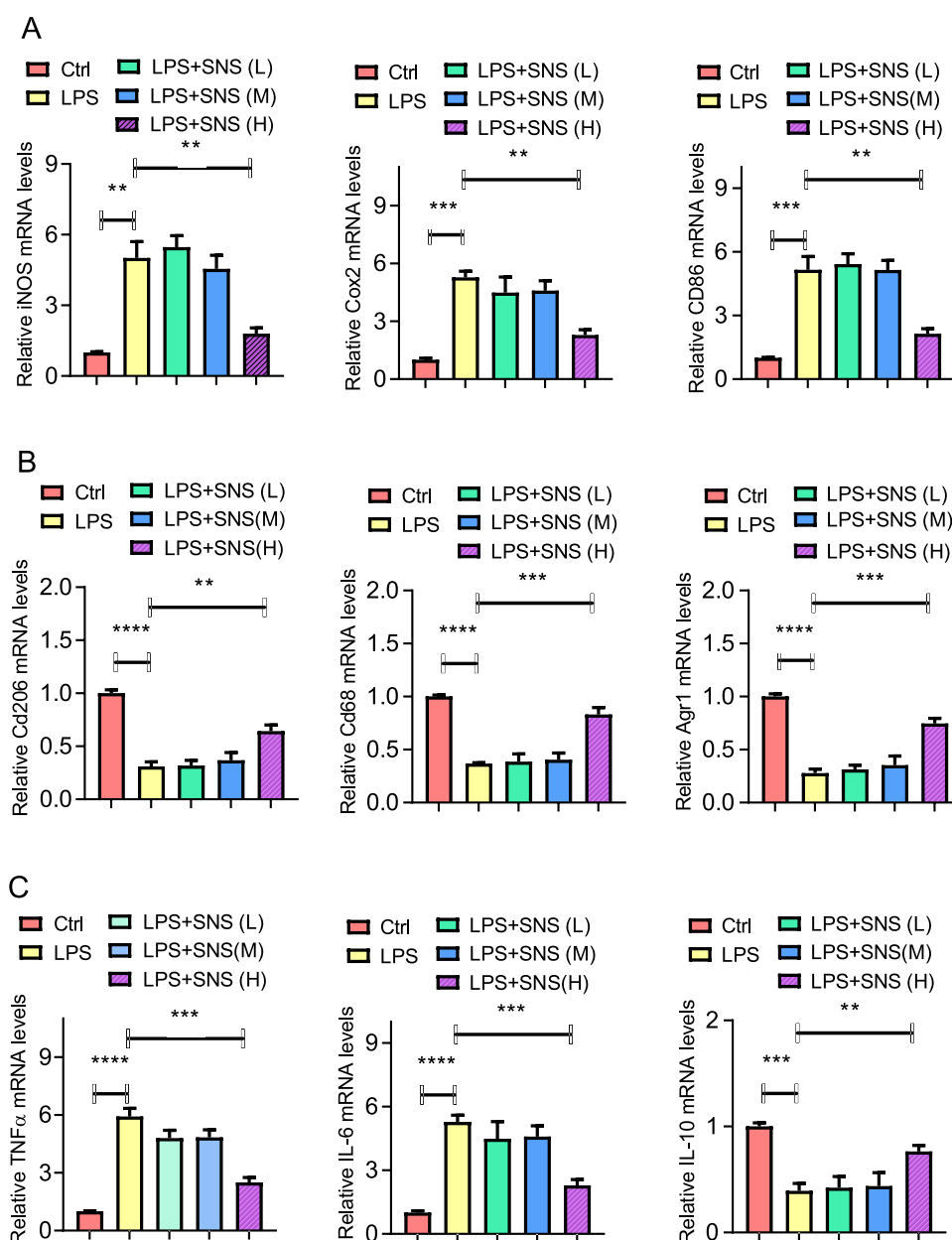
Figure 3. SNS-regulated macrophage RAW264.7 cell polarization and its activities. RAW264.7 cells were treated with 100 ng/mL LPS for 6 h, or 100 ng/mL LPS and SNS (low dose at 12.5 μM, medium dose at 25 μM, high dose at 50 μM) for 6 h. (A) Quantification of M1 macrophage markers mRNA expression after indicated treatments. (B) Quantification of M2 macrophage markers mRNA expression after indicated treatments. (C) Quantification of inflammatory cytokines mRNA expression after indicated treatments. $n = 4$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

molecular SOD2 upregulation in RAW264.7 cells (Figure 6D). SIRT1 knockdown significantly abolished the effect of SNS on decreasing M1-type macrophagic markers iNOS, Cox2, and CD86 mRNA expression (Figure 7A), while restoring M2-type macrophagic markers Cd206, Cd68, and Agr1 mRNA expression (Figure 7B), which was also accompanied by abolishing SNS-induced downregulation of TNFα and IL6 mRNA expression, upregulation of IL-10 mRNA expression (Figure 7C). Moreover, SIRT1 knockdown significantly abolished the effect of SNS on decreasing NLRP3 inflammasome markers NLRP3 and ASC expression (Figure 8A,B), which was also accompanied by abolishing SNS-induced downregulation of IL-1β mRNA expression (Figure 8B). Moreover, Sirt1 overexpression significantly upregulated NRF2 and Cd206, while having no effect on NLRP3, IL-1β, IL-10,

and iNOS. LPS significantly decreased the expression of SIRT1, NRF2, IL-10, and CD206, while significantly elevating NLRP3, IL-1β, and iNOS, yet this effect of LPS would be reversed and attenuated by overexpression of SIRT1 (Figure 8C).

4. DISCUSSION

Inflammatory injury positively correlated with mortality risk in cardiovascular and digestive diseases, even primary infection.[25] The natural active ingredients, including berberine and saikosaponin A/D, exhibit potential and promising prospects in the prevention and treatment of chronic liver disease and liver cancer.[1] SNS served as an effective anti-inflammatory compound that reasoned us to investigate its effects on protecting against inflammatory injury. In the present study,
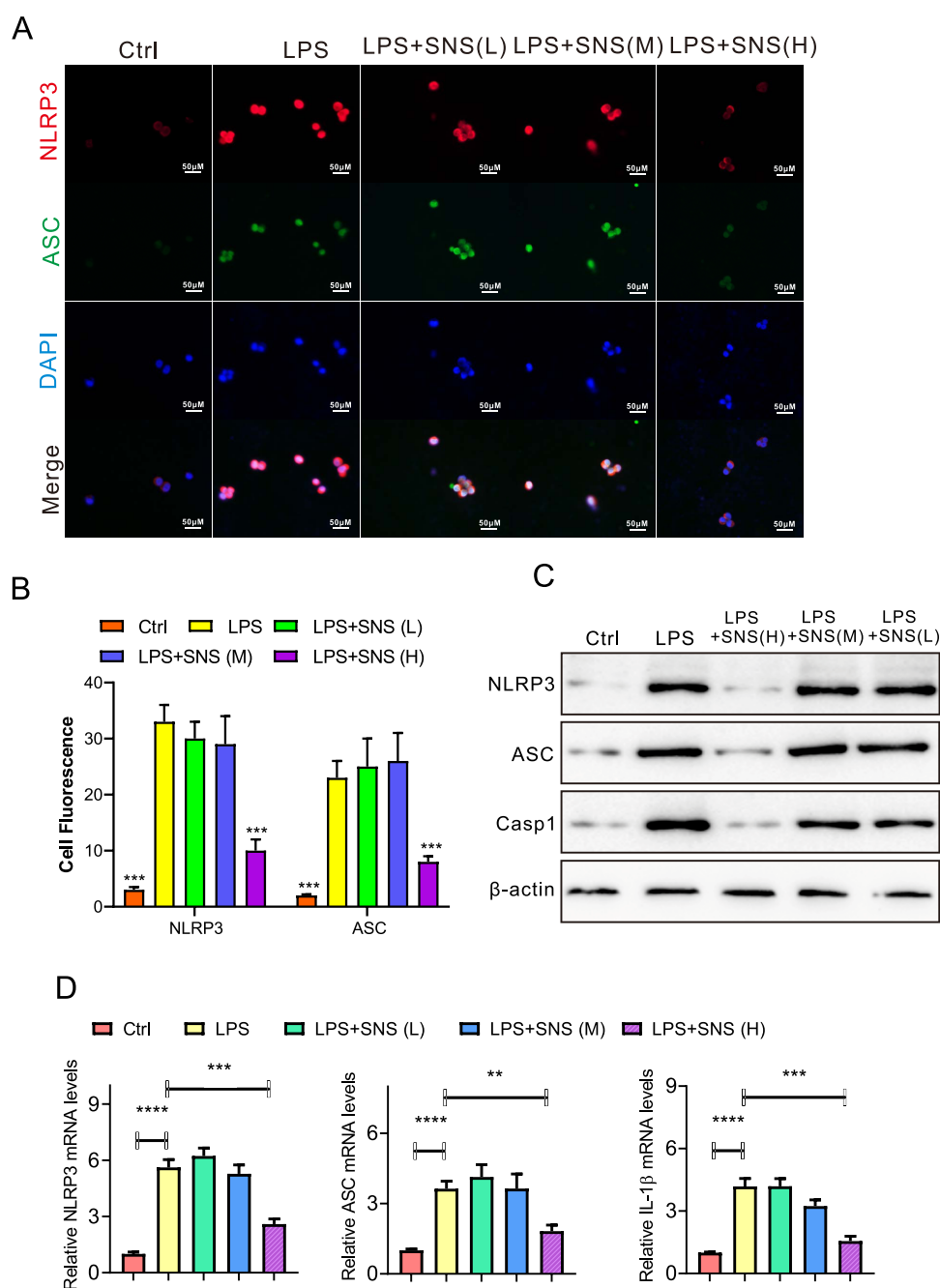
Figure 4. SNS-inhibited NLRP3 inflammasomes formation in RAW264.7 cells. RAW264.7 cells were treated with 100 ng/mL LPS for 6 h (mRNA level detection) or 24 h (protein level detection), or 100 ng/mL LPS and SNS (low dose at 12.5 μM, medium dose at 25 μM, high dose at 50 μM) for 6 h (mRNA level detection) or 24 h (protein level detection). (A) Immunofluorescence analysis of the NLRP3 inflammasomes markers after indicated treatment. NLRP3 was red, ASC was green, and DAPI was blue. (B) Fluorescence intensity of (A) quantified by ImageJ. (C) Western blot analysis of NLRP3, ASC, and caspase 1 after indicated treatments. (D) Quantification of NLRP3 inflammasome-related genes mRNA expression after indicated treatments. $N = 6$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

our data first confirmed that SNS significantly decreased the infiltration of inflammatory cells and subsequent cytotoxicity in LPS-induced lung and liver inflammatory injuries. In line with our findings, previous studies had indicated that SNS might inhibit Wnt/β-Catenin signaling or PI3K-AKT signaling to intervene in the initiation and progression of pulmonary fibrosis.[26] Otherwise, SNS would target p53-mediated AMPK/mTOR signaling to trigger autophagy and apoptosis of Hepatocellular Carcinoma Cells.[27] SNS also inhibited VEGF/VEGFR2/AKT signaling to impaired angiogenesis in

liver cancer.[28] Thus, we and others showed that SNS possesses a protective role in lung and liver diseases.

Accumulating evidence highlighted that macrophage was involved in and initiated the inflammatory injury.[10,13] To uncover the underlying mechanism of SNS on ameliorating inflammatory injuries, we then wonder about the special immunocyte type that first infiltrated into the lungs and liver, which in turn can be targeted to SNS. To date, the effect of SNS on mediating immunocytes activities remains largely elusive. It is reported that flavonoids, but not SNS, significantly decreased LPS-induced inflammatory cytokines in macro-
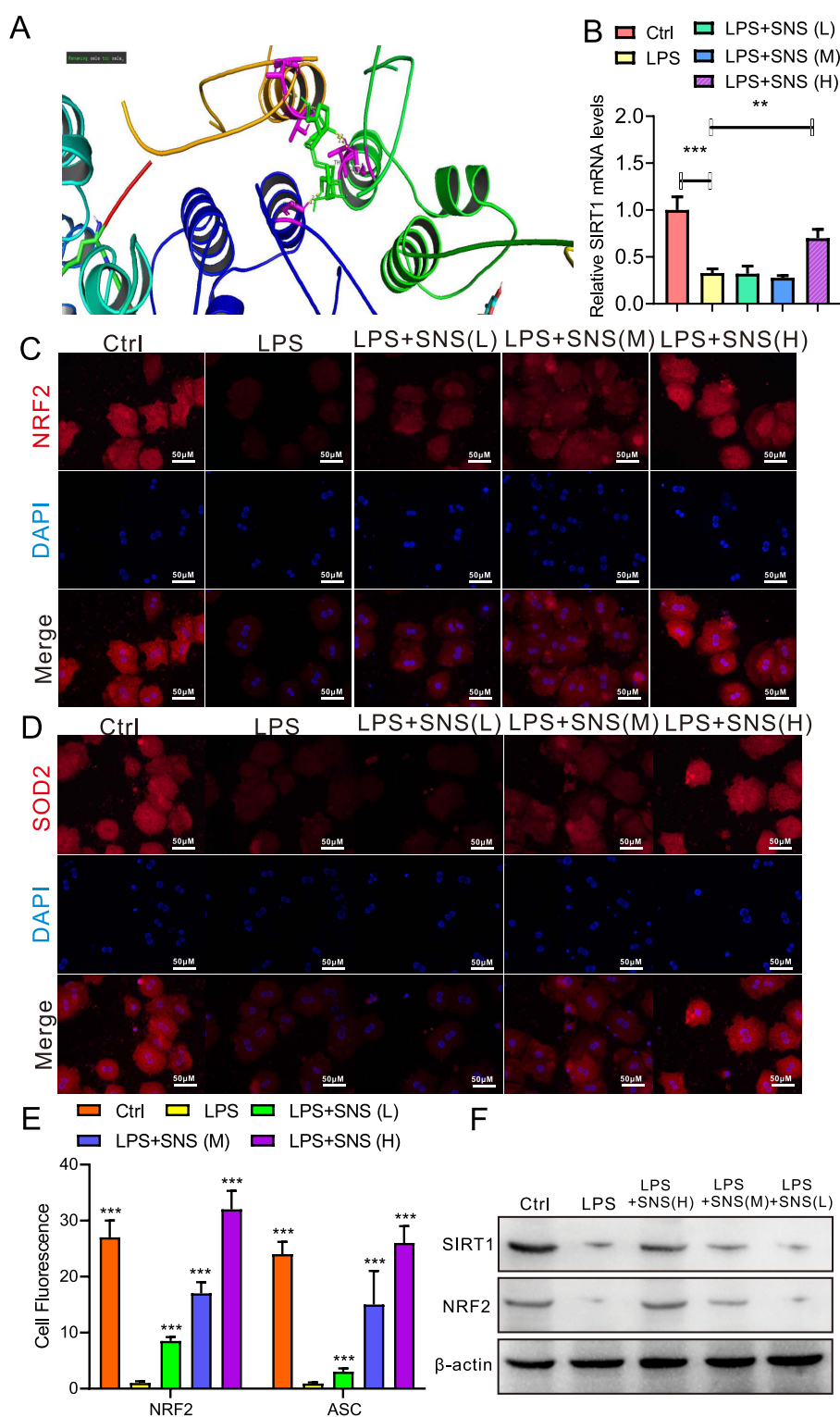
Figure 5. SNS-inhibited NLRP3 inflammasomes formation in RAW264.7 cells. RAW264.7 cells were treated with 100 ng/mL LPS for 6 h (mRNA level detection) or 24 h (protein level detection), or 100 ng/mL LPS and SNS (low dose at 12.5 μM, medium dose at 25 μM, high dose at 50 μM) for 6 h (mRNA level detection) or 24 h (protein level detection). (A) In silico docking analysis for the interaction of SNS and SIRT1. (B) Quantification of SIRT1 mRNA expression after indicated treatments. (C, D) Immunofluorescence analysis of the NRF2 and SOD2 after indicated treatment. NRF2 and SOD2 were red, and DAPI was blue. (E) Fluorescence intensity of (C) and (D) quantified by ImageJ. (F) Western blot analysis of SIRT1 and NRF2 after indicated treatments. $n = 7$, ** $p < 0.01$, *** $p < 0.001$.

phages *via* bioinformatics and *in vitro* approach, such as TNF-α and IL-6 production.[29] However, others reported that SNS performed as an immunity enhancer counters immunosuppression via elevating transcription and secretion of IFN-γ, IL-

2, and IL-6.[30] To further confirm the role of SNS in regulating macrophagic activities in inflammatory injury, we performed *in vitro* and *in vivo* studies and then found that macrophage indeed largely infiltrated in LPS-induced injury sites, which
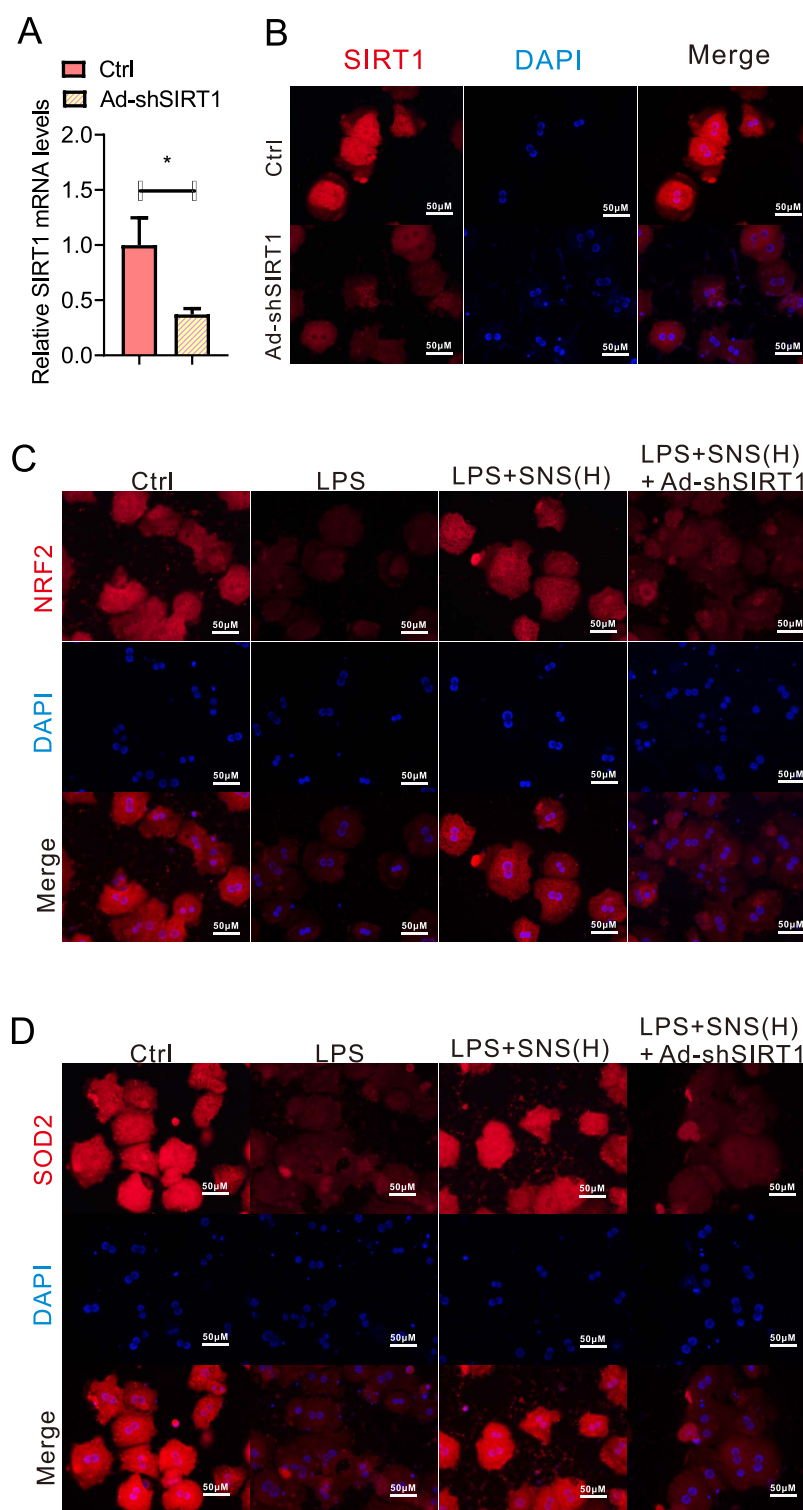
Figure 6. SIRT1 silence would attenuate SNS-induced NRF2 signaling in RAW264.7 cells. RAW264.7 cells were treated with 100 ng/mL LPS for 24 h, or 100 ng/mL LPS and SNS (low dose at 12.5 μM, medium dose at 25 μM, high dose at 50 μM) for 24 h. (A) Quantification of SIRT1 mRNA expression to examine the knockdown efficiency. (B) After SIRT1 silence, immunofluorescence SIRT1 protein level to examine the knockdown efficiency. SIRT1 was red, and DAPI was blue. (C, D) After SIRT1 silence, immunofluorescence analysis of the NRF2 and SOD2 after indicated treatment, respectively. NRF2 and SOD2 were red, and DAPI was blue. $n = 7$, *$p < 0.05$.

accompanied with releasing massive pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Our results then showed that SNS played an anti-inflammation role to significantly inhibit macrophage infiltration, M1-type polarization, and releasing pro-inflammatory cytokines. We did not confirm the effect of SNS on regulating immune response under normal condition. Otherwise, we and others might suggest that the immunomodulatory effects of SNS are under a complex context that should be further explored in future studies.
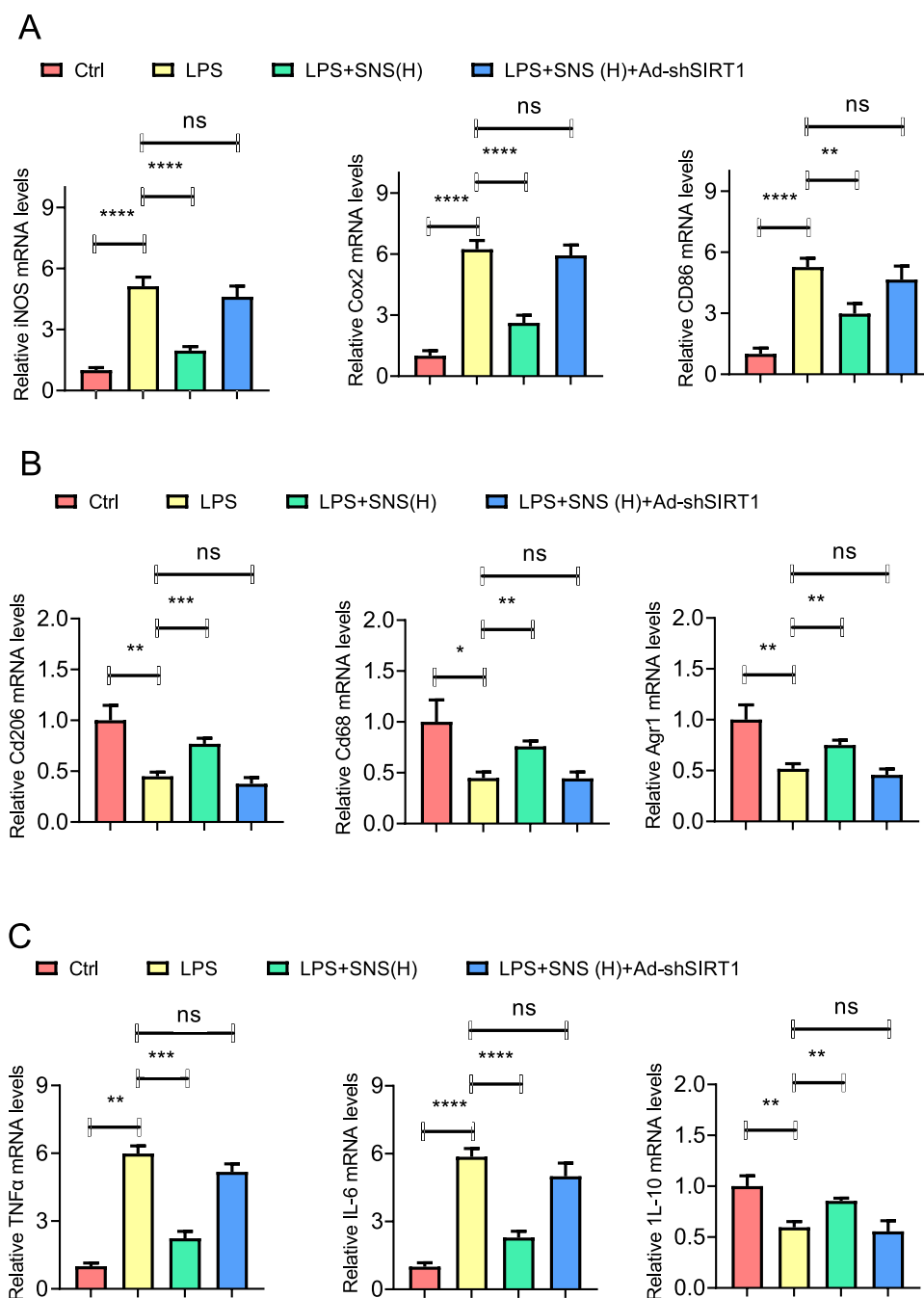
Figure 7. SIRT1 silence would attenuate SNS-induced inhibition of macrophage polarization and its inflammation in RAW264.7 cells. RAW264.7 cells were treated with 100 ng/mL LPS for 6 h, or 100 ng/mL LPS and SNS (low dose at 12.5 μM, medium dose at 25 μM, high dose at 50 μM) for 6 h. (A) After SIRT1 silence, quantification of M1 macrophage markers mRNA expression after indicated treatments. (B) After SIRT1 silence, quantification of M2 macrophage markers mRNA expression after indicated treatments. (C) After SIRT1 silence, quantification of inflammatory cytokines mRNA expression after indicated treatments. $n = 7$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

In the present study, when we explore the underlying mechanism of SNS in regulating inflammation, we unexpectedly found that SNS actually interacted with the conserved activator binding site of SIRT1. The Sirtuin family acts as the NAD$^+$ cofactor which belongs to class III Histone deacetylase (HDAC).[31] As the first cataloged member of the Sirtuin family, SIRT1 exhibits various bioactivities in anti-inflammation, antioxidant, and antitumor.[32,33] SIRT1 deletion caused excessive inflammation and oxidative stress in multiple organs, which subsequently contribute to chronic inflammatory diseases.[34] These previous studies reasoned us to investigate

the effect of SNS on regulating SIRT1 expression and its activities. In line with the previous study, our results further showed that SNS rescued SIRT1 expression under an LPS-induced inflammatory injury model. Accumulating studies had indicated that NRF2 served as a key downstream target of SIRT1 that played important roles in antioxidative stress and immunomodulation. For example, SIRT1 presented as a predominant regulator to promote Nrf2 expression in diabetic complications treatment.[33] Astragaloside IV activated not only SIRT1/NRF2 signaling to inhibit oxidative stress but also SIRT1 to suppress NLRP3-mediated inflammatory cytokines
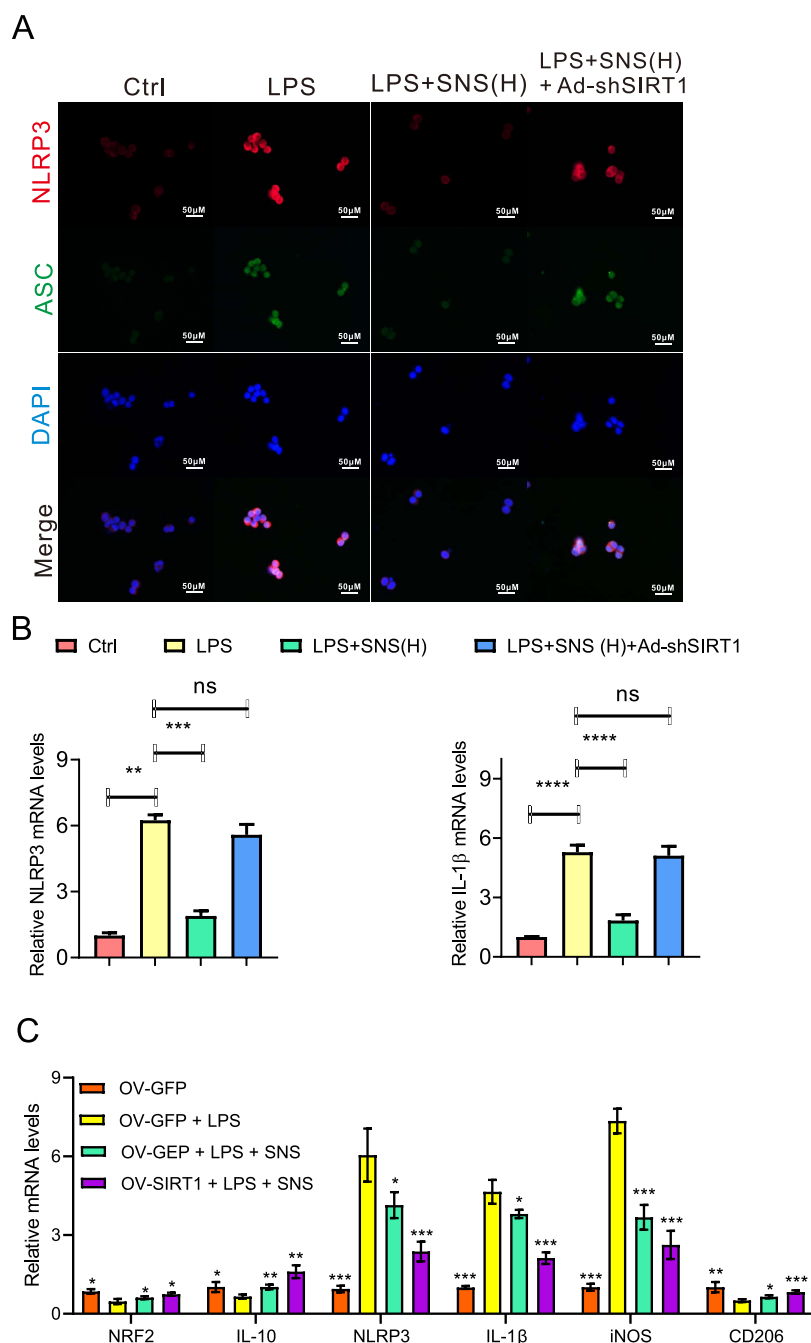
Figure 8. SIRT1 silence would impair SNS-induced inhibition of macrophagic NLRP3 inflammasomes formation in RAW264.7 cells. RAW264.7 cells were treated with 100 ng/mL LPS for 6 h (mRNA level detection) or 24 h (protein level detection), or 100 ng/mL LPS and SNS (low dose at 12.5 μM, medium dose at 25 μM, high dose at 50 μM) for 6 h (mRNA level detection). (A) After SIRT1 silence, immunofluorescence analysis of the NLRP3 and ASC after indicated treatment. NLRP3 was red, ASC was green, and DAPI was blue. (B) After SIRT1 silence, quantification of NLRP3 and IL-1β mRNA expression after indicated treatments. (C) After SIRT1 overexpression, quantification of NRF2, IL-10, NLRP3, IL-1β, iNOS, and CD206 mRNA expression after indicated treatments. n = 6, ****p < 0.0001.

releasing, including IL-1, TNF-α, and IL-6.[35] In line with these previous findings, our results also suggested that SNS might activate SIRT1/NRF2 signaling to promote SOD2 transcription and then ameliorate LPS-induced inflammatory injuries.

NLRP3 inflammasome activation is the key molecular event in M1 macrophage polarization releasing pro-inflammatory cytokines. For instance, LPS promoted M1-type macrophage polarization to promote NLRP3 inflammasome formation to release pro-inflammatory cytokines IL-1β and IL-18.[36] SIRT1

overexpression would decrease endogenous LPS production and inhibit macrophagic NLRP3 inflammasome formation to suppress NF-κB-induced inflammation, which together to alleviate hepatic inflammatory injury and fibrosis.[37] Naringenin activated transcription factor EB to suppress NLRP3 inflammasome formation, which in turn facilitates M2 macrophage polarization.[38] In line with these previous findings, our result also showed that SNS significantly activated SIRT1 to facilitate M2 macrophage polarization and inhibit NLRP3 inflammasome formation.

5. CONCLUSIONS

This study reports an active natural compound SNS that protected against LPS-induced inflammatory injuries in the lungs and liver. In the molecular context, SNS targeted SIRT1 to activate NRF2/SOD2 signaling to induce M0 macrophage facilitating M2 macrophage polarization and inhibiting NLRP3 inflammasome formation, subsequently decreasing the pro-inflammatory cytokines TNFα and IL6 expression, while increased anti-inflammatory cytokine IL-10 expression. Taken together, our results provided a potential and promising strategy for inflammatory injury treatment.

ASSOCIATED CONTENT

Data Availability Statement

The original data in this manuscript are available upon contacting the corresponding author.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03319.

SNS interacted with the conserved activator binding site of SIRT1 (PDF)

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Author Contributions

G.L. conceived the present study. L.L., K.D., Z.G., and H.F. performed experiments. L.L. and D.Z. analyzed data. L.L. prepared the manuscript draft. G.L. reviewed the manuscripts and modified the language. L.L. submitted the manuscript.

Notes

The authors declare no competing financial interest.
The studies involving animal experiments were reviewed and approved by The Animal Ethics Committee of Guangzhou Medical University.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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