

SIRT1 regulates cigarette smoke extract-induced alveolar macrophage polarization and inflammation by inhibiting the TRAF6/NLRP3 signaling pathway

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Abstract. M1 macrophages activated by cigarette smoke extract (CSE) serve a pro-inflammatory role in chronic obstructive pulmonary disease (COPD). The expression of silent information regulator 1 (SIRT1) is decreased in the alveolar macrophages of patients with COPD. However, whether SIRT1 is involved in COPD by regulating macrophage polarization remains unknown. Rat Alveolar Macrophage NR8383 cells were exposed to CSE. Cell Counting Kit-8 assay, western blot assay and ELISA showed that with increasing concentration of CSE, the activity of NR8383 cells and expression of SIRT1 gradually decreased, while the release of inflammatory cytokines TNF α , IL-1 β and IL-6 increased. As shown in western blot or Immunofluorescence assays, exposure to CSE also increased expression levels of the M1 markers inducible nitric oxide synthase and CD86, whereas it downregulated expression of the M2 markers arginase 1 and CD206. In addition, CSE increased expression of TNF receptor associated factor 6 (TRAF6), NOD-like receptor thermal protein domain associated protein 3 (NLRP3) and cleaved caspase-1 protein in NR8383 cells. Overexpression plasmids of SIRT1 and TRAF6 significantly reversed the aforementioned changes induced by CSE. Moreover, immunoprecipitation demonstrated that TRAF6 could bind to NLRP3. The overexpression of TRAF6 notably attenuated the regulatory effects of overexpression of SIRT1 on polarization and inflammation in NR8383 cells. Conversely, overexpression of SIRT1 inhibited

the TRAF6/NLRP3 signaling pathway, thereby suppressing CSE-induced M1 polarization and release of inflammatory factors in NR8383 cells. The present study demonstrates that SIRT1 regulates CSE-induced alveolar macrophage polarization and inflammation by inhibiting the TRAF6/NLRP3 signaling pathway.

Introduction

Chronic obstructive pulmonary disease (COPD) is a prevalent chronic respiratory condition associated with high morbidity, mortality and disability rates, which result in death of approximately 3.2 million individuals annually (1,2). By 2030, COPD is projected to become the third leading cause of mortality worldwide (3). Currently, the clinical treatment for COPD aims to alleviate the clinical symptoms, typically through the use of bronchodilators and oxygen therapy (4). However, COPD faces issues, such as under-diagnosis and misdiagnosis, and there is currently no treatment method to halt the progression of COPD (5). Therefore, it is imperative to study potential mechanisms responsible for the occurrence of COPD and develop novel predictive and therapeutic targets.

Inflammation is the key mechanism in the development of COPD (6). Alveolar macrophages (AMs) are the first line of defense in the lung and play a key role in lung inflammation (7). Studies have shown that in COPD, there is an increase in number of lung macrophages and a decrease in antigen presentation ability (8,9). Smoking is a common risk factor for the development of COPD (10); M1 polarization of lung macrophages is increased in smokers and patients with COPD (11). Therefore, inhibiting cigarette smoke (CS)-induced M1 polarization of AMs may offer a novel approach for anti-inflammatory treatment of COPD.

Silent information regulator 1 (SIRT1) is the mammalian homolog of the yeast silent information regulator 2 protein and is expressed in low levels in the lung of patients with COPD; SIRT1 is associated with a decline in lung function, indicating it is a potential biological marker for the severity of COPD (12). Studies have shown that SIRT1 serves a key role in COPD by regulating oxidative stress, inflammatory responses, autophagy and apoptosis (13-15). The NOD-like receptor thermal protein domain associated protein 3 (NLRP3) signal

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is a classic inflammatory signaling pathway and NLRP3 expression is increased in CS-induced AMs (16). SIRT1 can inhibit the expression of TNF receptor associated factor 6 (TRAF6) (17), which is involved in promoting polarization of M1 macrophages; its expression is also increased in serum of patients with COPD (18). Notably, TRAF6 can bind to NLRP3 and activate its signaling (19,20).

The present study aimed to explore whether SIRT1 can inhibit CS extract (CSE)-induced AM damage via the TRAF6/NLRP3 signaling pathway, thus contributing to its protective role in COPD. The aim of the present study was to elucidate the role and mechanisms of action of SIRT1 in the occurrence and development of COPD and to provide an experimental basis for understanding the pathogenesis COPD. The present results may aid in the development of novel drug and clinical treatments for COPD.

Materials and methods

Preparation of CSE. CSE was prepared by burning three cigarettes [11.0 tar, 1.1 nicotine, 17.0 mg carbon monoxide; Chongqing Hongsheng Industrial (Group) Co., Ltd.]. The smoke was dissolved in serum-free F-12K medium (20 ml; cat. no. 21127022, Gibco; Thermo Fisher Scientific, Inc) and filtered through a 0.22- μ m filter. The resulting 100% CSE was used within 1 h of preparation. F-12K medium was used to adjust the CSE working concentration.

Cell culture and treatment. The rat alveolar macrophage cell line NR8383 (American Type Culture Collection), was cultured in F-12K medium (cat. no. 21127022, Gibco; Thermo Fisher Scientific, Inc) with 15% FBS (Thermo Fisher Scientific, Inc) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO₂. The cells were treated as aforementioned (21) with CSE (5, 10 and 20%) for 24, 48 and 72 h at 37°C, respectively.

Cell transfection. The overexpression plasmids of SIRT1 [Ov-SIRT1; vector, pEX-3(pGCMV/MCS/Neo)], TRAF6 [(Ov-TRAF6; vector, pEX-3(pGCMV/MCS/Neo)] and negative control (NC) plasmid pEX-3(pGCMV/MCS/Neo) were purchased from Genepharma Biotech Corp. The cells were inoculated in a 6-well plate at a density of 1x10⁵ cells/well. Following 24 h culture and upon reaching 75% confluency, the cells were transfected using 2 μ g overexpression plasmid and Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h. After 48 h, western blot analysis was performed to detect transfection efficiency.

Cell Counting Kit (CCK)-8 assay. CCK-8 (BIOSS, cat. no. BA00208) was employed to assess cell viability. NR8383 cells were inoculated into 96-well plates at a density of 3x10⁴/l. Following stimulation with CSE, 10 μ l CCK8 solution was added to each well and incubated at 37°C for 2 h. The optical density at 450 nm was determined by a microplate reader (Bio-Rad Laboratories, Inc.).

ELISA. The concentrations of TNF α (cat. no. JL13202-96T), IL-1 β (cat. no. JL20884-96T) and IL-6 (cat. no. JL20896-96T; all JONLNBIO) in the cell supernatant were measured using ELISA kits according to the manufacturer's instructions.

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Primer	Sequence, 5'→3'
GAPDH	Forward	GGCAAGTTCAACGGCACAGTC
	Reverse	TCGCTCCTGGAAGATGGTGATG
TNF α	Forward	GCCCAGACCCTCACACTCAG
	Reverse	CCGCTTGGTGGTTTGCTACG
IL-1 β	Forward	GACTTCACCATGGAACCCGT
	Reverse	GGAGACTGCCCATTCTCGAC
IL-6	Forward	CACTTCACAAGTCGGAGGCT
	Reverse	TCTGACAGTGCATCATCGCT
TRAF6	Forward	AATCACTTGGCAGGCACTTG
	Reverse	GGAGAGGAGGCATCGCATGG

TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor.

Western blot analysis. NR8383 cells were lysed in RIPA buffer with protease inhibitor cocktail (both Beyotime Institute of Biotechnology). After measuring the protein concentration by BCA assay, 20 μ g/lane protein was electrophoresed on 10% SDS-PAGE and transferred onto PVDF membranes (Amersham Biosciences). The membrane was blocked in 5% BSA (Biofroxx; neoFroxx) at room temperature for 2 h and incubated with anti-SIRT1 (1:1,000; cat. no. ab110304, Abcam), anti-inducible nitric oxide synthase (iNOS; 1:1,500; cat. no. AF0199, Affinity Biosciences), anti-CD86 (1:1,500; cat. no. DF6332, Affinity Biosciences), anti-arginase 1 (Arg-1; 1:1,000; cat. no. DF6657, Affinity Biosciences), anti-CD206 (1:1,500; cat. no. DF4149, Affinity Biosciences), anti-TRAF6 (1:1,000; cat. no. ab40675, Abcam), anti-NLRP3 (1:1,500; cat. no. DF7438, Affinity Biosciences), anti-cleaved caspase-1 (1:1,000; cat. no. AF4022, Affinity Biosciences), anti-pro-caspase-1 (1:1,000; cat. no. DF6148, Affinity Biosciences) and anti-GAPDH (1:2,500; cat. no. ab9485, Abcam) at 4°C overnight. The membrane was incubated with secondary antibody (goat anti-rabbit IgG-HRP; 1:5,000, cat. no. S0001, Affinity Biosciences) at 37°C for 2 h. The protein signals were exposed using ECL reagent (Beyotime Institute of Biotechnology) and analyzed using ImageJ software version 1.50 (National Institutes of Health).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the NR8383 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed using the RevertAid RT kit (cat. no. MR101-02, Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. qPCR was performed at 95°C for 120 sec for initial denaturation, followed by denaturation at 95°C for 15 sec and annealing and extension at 60°C for 30 sec (40 cycles). Gene expression was normalized to GAPDH and relative mRNA expression levels were determined using the 2^{- $\Delta\Delta$ C_q} method (22). The primer sequences were obtained from PrimerBank (pga.mgh.harvard.edu/primerbank) (Table I).

Immunofluorescence assay. Transfected NR8383 cells were inoculated into six-well plates and when the cell fusion reached

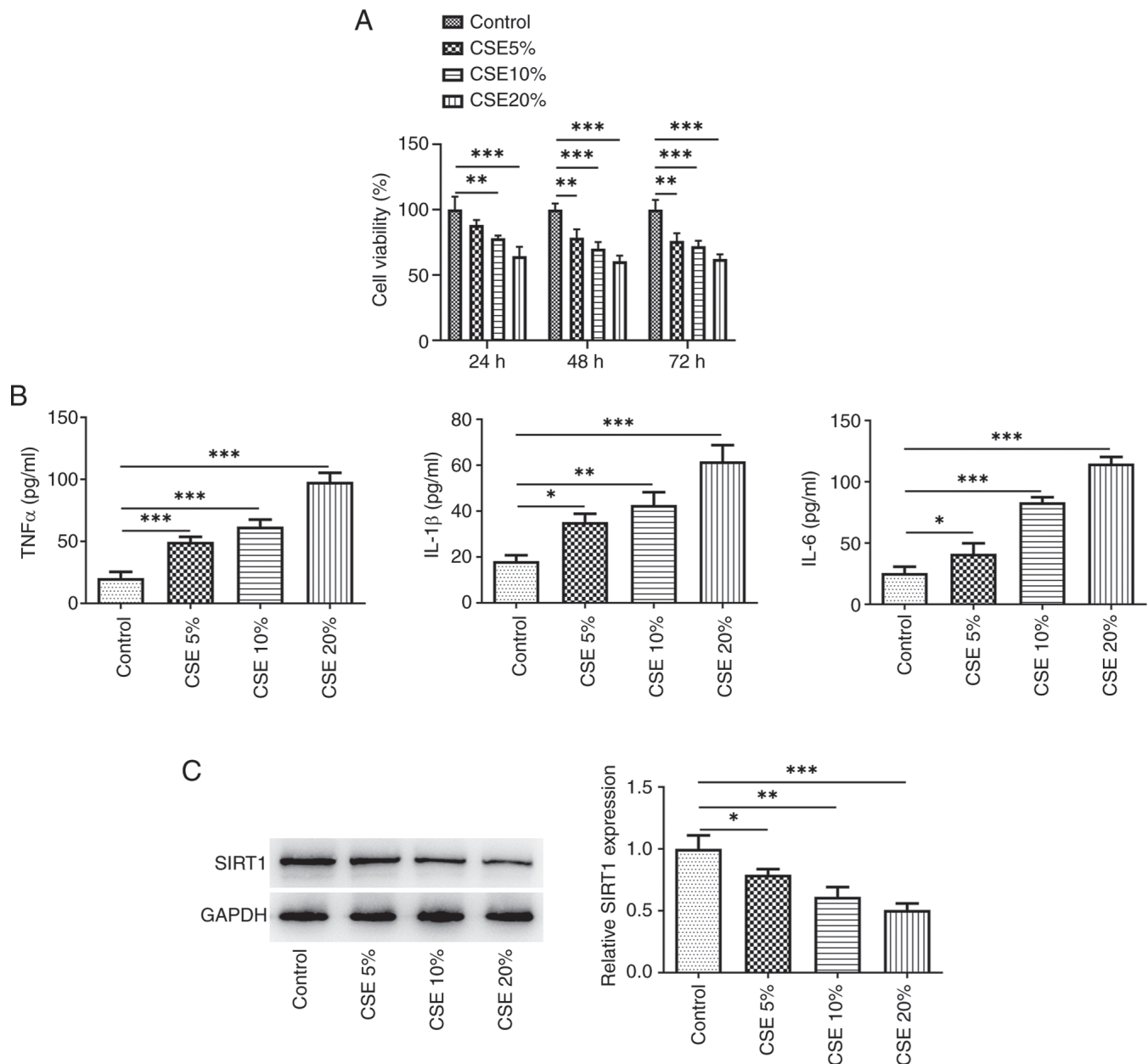


Figure 1. SIRT1 expression is decreased in CSE-induced alveolar macrophages. (A) Cell viability following exposure to various concentrations of CSE. (B) Effect of CSE on the release of TNFα, IL-1β and IL-6 (n=5). (C) Effects of CSE on the expression of SIRT1 (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. Control. SIRT1, silent information regulator 1; CSE, cigarette smoke extract.

80%, the cells were fixed with 4% neutral formaldehyde for 30 min at 4°C. Sealing solution was added to each well followed by incubation at 37°C for 30 min. The primary antibodies [iNOS (1:200; cat. no. AF0199, Affinity Biosciences) and Arg-1 (1:200; cat. no. DF6332, Affinity Biosciences)] were incubated overnight at 4°C. The diluted secondary antibody Goat Anti-Rabbit IgG (H+L) Fluor488-conjugated (1:200; cat. no. S0018, Affinity Biosciences) was incubated at room temperature without light for 1 h. After washing the cells with PBS, nuclei of the cells were re-stained with DAPI solution (Beyotime Institute of Biotechnology) at room temperature for 10 min and observed under a fluorescence microscope (200x) (Nikon Corporation).

Co-immunoprecipitation (Co-IP) assay. NR8383 cell lysates were prepared by IP lysate (Beyotech Institute of

Biotechnology; cat. no. P0013) (150 μl/well) and incubated with anti-TRAF6 (1 μg) (1:100; cat. no. ab137452, Abcam) overnight at 4°C followed by addition of 30 μl protein G Agarose beads (Cytiva) at 4°C for 4 h. After being washed three times with cold wash and once with lysis buffer (both New Cell and Molecular Biotech Co., Ltd.), the complexes were isolated by centrifuging at 1,000 x g at 4°C for 3 min. The immunoprecipitate was resuspended in 30 μl loading buffer. The expression of NLRP3 was detected using western blot analysis as aforementioned.

Statistical analysis. All analyses were performed using GraphPad Prism 9 (Dotmatics) and data are expressed as the mean ± SD of at least three independent experiments. All data were analyzed for normality distribution using the

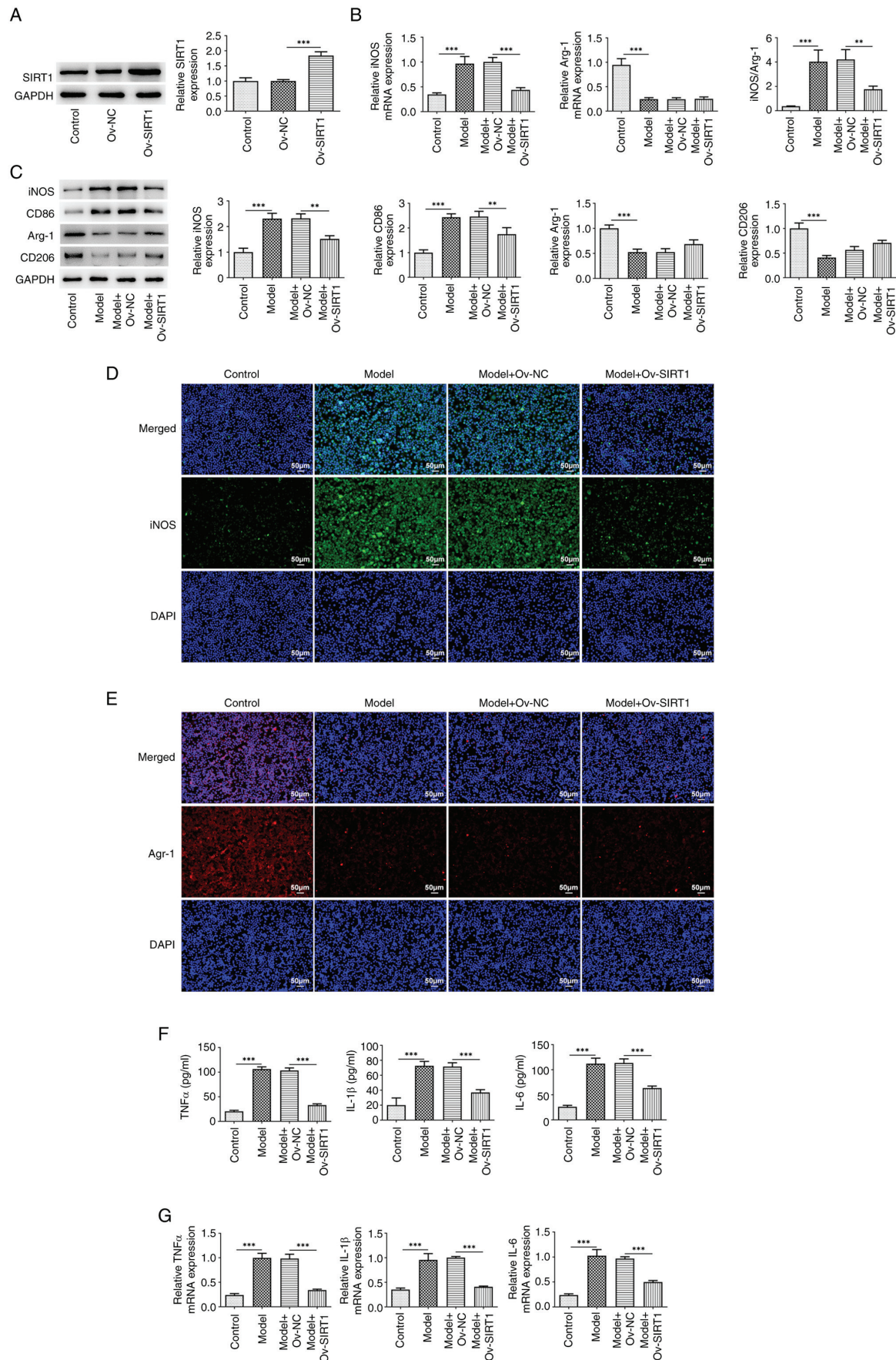


Figure 2. Ov-SIRT1 inhibits CSE-induced alveolar macrophage M1-type polarization and inflammatory release. (A) Transfection efficiency. (B) mRNA levels of macrophage M1-type polarization marker iNOS and the M2-type marker Arg-1 ($n=3$). (C) Protein expression of macrophage M1-type polarization markers iNOS and CD86 and the M2-type markers Arg-1 and CD206. Fluorescence intensities of (D) iNOS and (E) Arg-1. (F) Levels of the cytosolic inflammatory factors TNF α , IL- β and IL-6. (G) mRNA levels of TNF α , IL- β and IL-6 ($n=5$). * $P<0.01$ and *** $P<0.001$. SIRT1, silent information regulator 1; CSE, cigarette smoke extract; iNOS, inducible nitric oxide synthase; Ag-1, arginase 1; Ov, overexpression; NC, negative control.

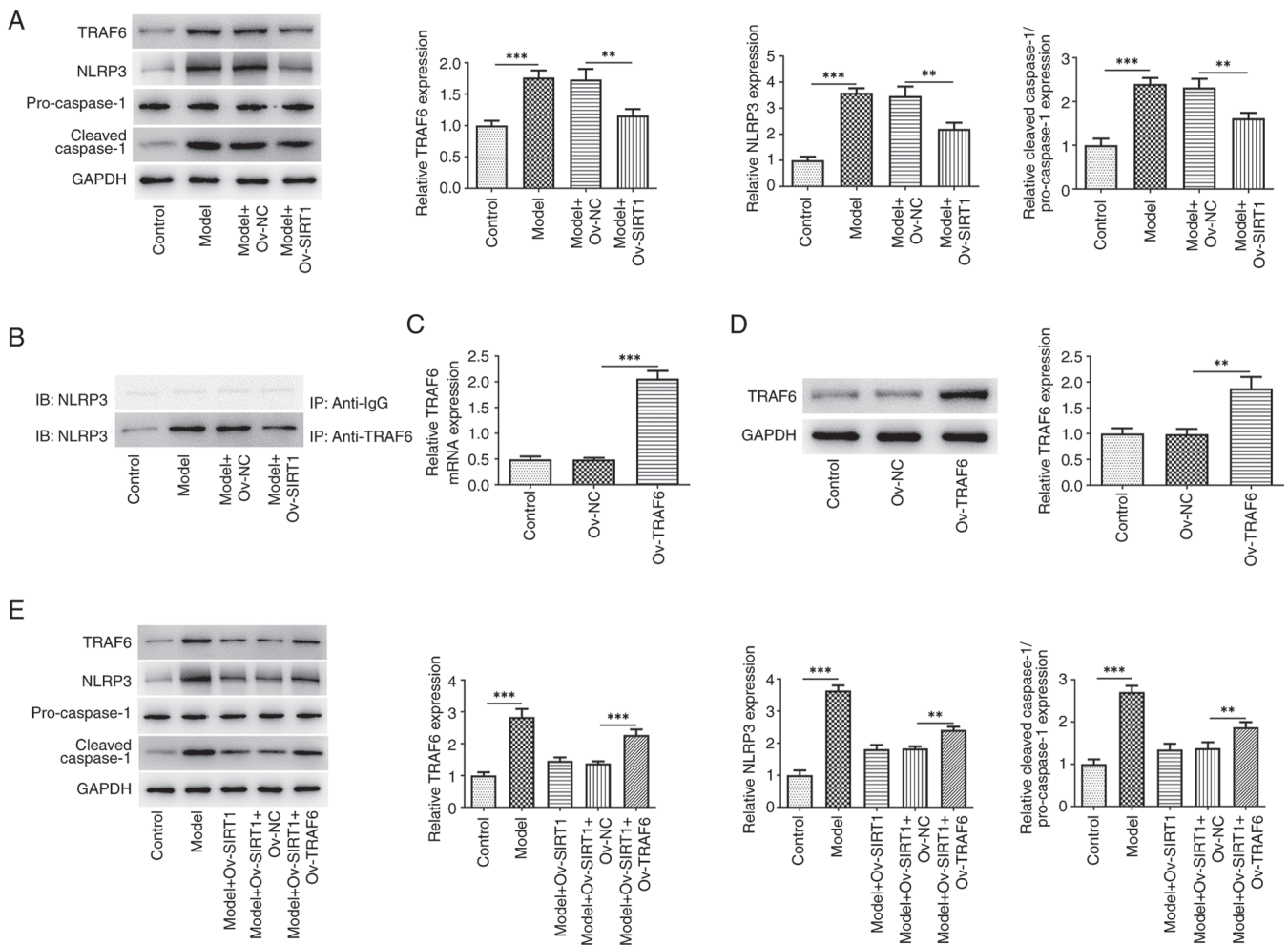


Figure 3. Ov-SIRT1 overexpression inhibits TRAF6/NLRP3 signaling activation in CSE-induced macrophages. (A) TRAF6, NLRP3 and cleaved caspase-1 protein expression in NR8383 cells. (B) Validation of TRAF6 binding ability to NLRP3. TRAF6 (C) protein and (D) mRNA and (E) TRAF6, NLRP3 and cleaved caspase-1 protein expression in NR8383 cells following TRAF6 overexpression. ** $P < 0.01$ and *** $P < 0.001$; $n = 3$. SIRT1, silent information regulator 1; CSE, cigarette smoke extract; TRAF6, TNF receptor-associated factor 6; NLRP3, NOD-like receptor thermal protein domain associated protein 3; Ov, overexpression; NC, negative control; IB, immunoblotting; IP, immunoprecipitation.

Shapiro-Wilk test. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons post hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SIRT1 expression is decreased in CSE-induced AMs. With increasing concentrations of CSE, cell viability gradually decreased at 24 and 48 h, but there was no significant difference between 48 and 72 h (Fig. 1A). Therefore, CSE was used to induce cells for 48 h for subsequent experiments. The secretion of inflammatory cytokines (TNF α , IL- β , and IL-6) was elevated (Fig. 1B), whereas SIRT1 expression exhibited a dose-dependent decrease (Fig. 1C). The greatest effects were observed at 20% CSE, therefore 20% CSE was used in further experiments.

SIRT1 overexpression inhibits CSE-induced AM M1-type polarization and inflammatory factor release. There was a significant increase in SIRT1 expression in cells transfected with Ov-SIRT1 compared with both the control and Ov-NC

groups (Fig. 2A). The detection of macrophage polarization markers indicated a significant elevation in both mRNA (Fig. 2B) and protein expression levels (Fig. 2C) of the M1 marker iNOS following exposure to 20% CSE, along with an enhanced fluorescence intensity of iNOS (Fig. 2D) and augmented expression of CD86 (Fig. 2C). Following exposure to 20% CSE, there was a significant decrease in both mRNA (Fig. 2B) and protein expression levels (Fig. 2C) of the M2 marker Arg-1 accompanied by a reduced fluorescence intensity of Arg-1 (Fig. 2E) and a diminished expression of CD206 (Fig. 2C). The iNOS/Arg-1 ratio exhibited a significant decrease (Fig. 2B). Moreover, exposure to 20% CSE increased expression of TNF α , IL- β and IL-6 in the cells (Fig. 2F and G). SIRT1 overexpression significantly reversed these changes induced by CSE.

SIRT1 overexpression inhibits TRAF6/NLRP3 signaling activation in CSE-induced macrophages. The present study assessed the TRAF6/NLRP3 signaling pathway in macrophages stimulated by CSE. There was a significant increase in the levels of TRAF6, NLRP3 and cleaved caspase-1 proteins in NR8383 cells following exposure to 20% CSE.

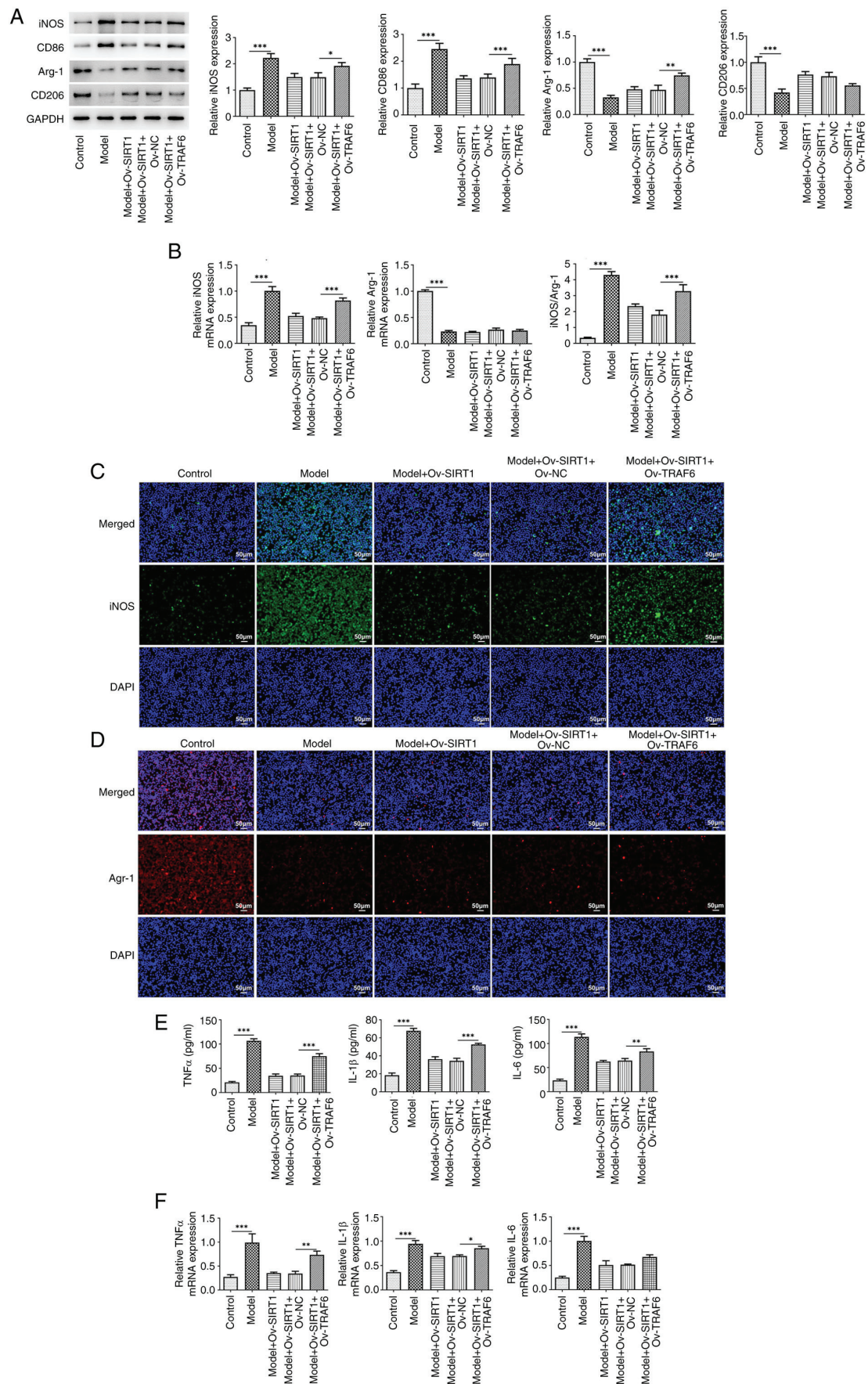


Figure 4. Ov-SIRT1 inhibits CSE-induced alveolar macrophage M1-type polarization and inflammatory release by suppressing TRAF6/NLRP3 signaling. (A) Protein expression of macrophage M1-type polarization markers iNOS and CD86 and the M2-type markers Arg-1 and CD206 (n=3). (B) mRNA levels of iNOS and Arg-1. Fluorescence intensities of (C) iNOS and (D) Arg-1. (E) Levels of cytosolic inflammatory factors TNF α , IL- β and IL-6 (n=5). (F) mRNA levels of TNF α , IL- β and IL-6. *P<0.05, **P<0.01 and ***P<0.001. SIRT1, silent information regulator 1; CSE, cigarette smoke extract; iNOS, inducible nitric oxide synthase; Arg-1, arginase 1; Ov, overexpression; NC, negative control.

Overexpression of SIRT1 significantly reversed this trend (Fig. 3A). IP demonstrated the ability of TRAF6 to interact with NLRP3 (Fig. 3B). Western blot analysis (Fig. 3C) and RT-qPCR (Fig. 3D) revealed a significant increase in both the protein and mRNA levels of TRAF6 in NR8383 cells following transfection with Ov-TRAF6. Moreover, TRAF6 overexpression attenuated the effects of Ov-SIRT1 on expression of TRAF6, NLRP3 and cleaved caspase-1 proteins in CSE-induced macrophages (Fig. 3E).

Overexpression of SIRT1 inhibits CSE-induced AM M1-type polarization and inflammatory factor release by suppressing TRAF6/NLRP3 signaling. Finally, the present study used the TRAF6 overexpression vector to examine whether TRAF6/NLRP3 signaling mediates the role of SIRT1. The results revealed marked suppression of the effects of Ov-SIRT1 on CSE-induced M1 polarization and inflammatory release in NR8383 cells, supported by the upregulated protein expression of the M1 markers iNOS and CD86 and the downregulated protein expression of the M2 markers Arg-1 and CD206 following TRAF6 overexpression (Fig. 4A). Following TRAF6 overexpression, there was an increase in iNOS mRNA levels (Fig. 4B) and fluorescence intensity (Fig. 4C), whereas Arg-1 mRNA levels (Fig. 4B) and fluorescence intensity decreased (Fig. 4D). The release and mRNA levels of inflammatory cytokines (TNF α , IL- β , and IL-6) also exhibited an increase (Fig. 4E and F).

Discussion

The present study investigated the involvement of SIRT1 in AMs induced by CSE. The results revealed a decrease in SIRT1 expression in NR8383 cells induced by CSE. SIRT1 overexpression suppressed M1 polarization, inflammatory factor release and TRAF6/NLRP3 signaling activation in NR8383 cells exposed to CSE. However, enhancing TRAF6 expression markedly diminished the protective effects of SIRT1 overexpression on NR8383 cells.

Inflammation is a key pathological feature of COPD (23). Smoking is a contributing factor for COPD. CS stimulates release of inflammatory mediators and activates pathways associated with inflammation, leading to pulmonary inflammation (24). As the concentration of CSE increased, TNF α , IL- β , and IL-6 release in NR8383 cells increased, accompanied by a gradual decrease in cellular viability.

SIRT1 is involved in oxidative stress and chronic inflammatory responses, known for its anti-aging properties and associated with the development and progression of COPD (25). Previous research has revealed a decrease in SIRT1 levels in pulmonary macrophages of patients with COPD (26), suggesting its potential involvement in pathogenesis of COPD through the modulation of these macrophages. AMs are key components of innate immune responses (27). Depending on environmental stimuli, they are activated classically (also known as macrophage M1 polarization) or alternatively (also known as macrophage M2 polarization). The two polarization directions of AMs suggest two different inflammatory states. M1-type macrophages primarily release inflammatory factors (TNF α , IL- β and IL-6) and promote development of inflammation, and their markers mainly include iNOS and CD86. M2-type macrophages primarily release anti-inflammatory factors (TGF- β

and IL-10) and inhibit progression of inflammation, and their markers mainly include CD206 and Arg-1 (28). Dysregulated macrophage function exacerbates pulmonary inflammation in COPD by affecting initiation of inflammation, disrupting alveolar architecture and remodeling the airways (29). Modulating the balance of M1/M2 macrophage polarization is effective in alleviating COPD (28). Macrophage polarization is one of the pathogenic mechanisms of COPD. Feng and Zheng (10) found that M1 polarization of AMs is inhibited in smokers and patients with COPD and that CS may inhibit LPS-induced M1 polarization of AMs by suppressing NLRP3. Both macrophages from individuals with COPD and (30), and CSE-induced macrophages tend to be M1-type (29). Mu *et al* (31) showed that CSE promotes macrophage polarization towards the M1 type and high-mobility group box-1 is involved in regulation of macrophage polarization. Li *et al* (32) demonstrated that *Fritillaria cirrhosa* D. Don inhibits CSE treatment-induced macrophage M1 polarization, thereby attenuating inflammatory responses. It has been reported that salidroside serves a protective role in COPD by inhibiting JNK/c-Jun to reduce the M1 polarization of AMs induced by CS (7). BML-111 (lipoxin receptor agonist) treatment decreases iNOS levels and increases Arg-1 expression, which has the potential to convert macrophages from a pro-inflammatory M1 to an anti-inflammatory M2 phenotype, thereby preventing COPD (33). Rosiglitazone, an exogenous ligand of PPAR γ , has been shown to inhibit CS-induced M1 macrophage polarization and decrease M1/M2 ratio, thereby attenuating emphysema induced by CS exposure and inflammatory responses (34). The present study demonstrated a significant decrease in inflammatory factor release in NR8383 cells following the overexpression of SIRT1. This reduction was accompanied by a decrease in expression of M1 polarization markers, such as iNOS and CD86, induced by CSE in NR8383 cells, along with an increase in expression of M2 markers, including Arg-1 and CD206. Numerous studies have investigated the role of SIRT1 in COPD: For example, SIRT1 improves COPD by regulating CS-induced autophagy (35), endoplasmic reticulum stress (36), airway remodeling and epithelial-mesenchymal transition (37). To the best of our knowledge, the present study is the first to reveal that SIRT1 has the capability to inhibit the M1 polarization of AMs induced by CSE. This provides novel insight into the role of SIRT1 in COPD.

SIRT1 exerts a protective effect against COPD by influencing downstream signaling molecules. For example, SIRT1 activates the proliferator-activated receptor- γ coactivator-1 α /NF- κ B signaling axis, mitigating oxidative stress induced by CS in mice with COPD (38). SIRT1 has also been shown to inhibit inflammation *in vivo* via the Nrf2/p65 NF- κ B pathway, thereby reversing oxidative stress and inflammation induced by CS (39). Therefore, the present study explored the mechanisms underlying SIRT1-mediated regulation of AM polarization. SIRT1 mediates the toll-like receptor 4 (TLR4)/NF- κ B signaling pathway to regulate the polarization of microglial cells (40). Tetramethylpyrazine improves acute lung injury by inhibiting the TLR4/TRAF6/NF- κ B/NLRP3/caspase-1 signaling pathway (41). This suggests a potential association between SIRT1 and TLR4/TRAF6 and their downstream signaling. Previous research has suggested that TRAF6 can interact with NLRP3, thereby activating the NLRP3 signaling pathway (19). IP experiments demonstrated the ability of

TRAF6 to interact with NLRP3. The present study revealed overexpression of SIRT1 significantly reversed the increased expression of TRAF6, NLRP3 and cleaved caspase-1 induced by exposure to 20% CSE treatment. Conversely, overexpression of TRAF6 attenuated the effects of SIRT1 overexpression on NR8383 cells, indicating that the regulatory role of SIRT1 in CSE-induced polarization and inflammation of NR8383 cells was mediated by the TRAF6/NLRP3 signaling pathway. However, further experimental validation is required to determine whether this process involves other up- and downstream components of the TRAF6/NLRP3 pathway. Additionally, the present study primarily examined the role of SIRT1 in the polarization of AMs induced by CSE at the cellular level. However, *in vitro* experiments may not completely replicate the complexity of the *in vivo* environment. Thus, further studies are warranted to validate the findings of the present study by establishing an animal model of COPD.

In conclusion, the present study conducted a preliminary exploration of the role of SIRT1 in AMs. The overexpression of SIRT1 reduced CSE-induced M1 polarization and inflammatory release in NR8383 cells by inhibiting the TRAF6/NLRP3 signaling pathway.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FY conceptualized the study and wrote the manuscript. HQ was responsible for conceptualization. CQ and BH designed and performed experiments. FG, YL, YT, YM and QY analyzed data and constructed figures. CW designed experiments and edited the manuscript. All authors have read and approved the final manuscript. FY and CW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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