

Temporal Evolution of Alveolar Epithelial Cell Dysfunction Induced by Cigarette Smoke in Chronic Obstructive Pulmonary Disease and the Protective Role of SIRT1

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Research Article

Keywords: COPD SIRT1 Alveolar Epithelial Cells Cigarette Exposure Alveolar Homeostasis

Posted Date: February 23rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8753095/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

Chronic obstructive pulmonary disease (COPD) is characterized by pathological alterations including alveolar structure destruction and small airway remodeling. However, the dynamic evolution of alveolar structural damage from the early to advanced stages of COPD remains to be fully elucidated. Meanwhile, SIRT1, a critical regulator of metabolism and cellular stress, warrants further investigation for its potential role in early intervention of COPD.

Methods

This study set out to track how alveolar epithelial cells change as COPD develops from early to advanced stages, using cigarette smoke (CS) as the trigger. We wanted to see what role SIRT1 plays in this entire process.

Results

By establishing a mouse COPD model induced by CS combined with lipopolysaccharide (LPS), and utilizing alveolar epithelial cell-specific SIRT1 gain- and loss-of-function models, we found that during the early stage of CS exposure (2 weeks), aberrant proliferative repair of type II alveolar epithelial cells (AT2) serves as a key driver. SIRT1 activation was able to ameliorate this abnormal proliferative repair in AT2, thereby improving lung function.

Conclusions

Our study shows that at 2 weeks, the number and function of alveolar epithelial cells remained normal. After 4 weeks, however, the number of alveolar epithelial cells decreased, and normal function was gradually lost. Concurrently, the marker for alveolar intermediate cells, KRT8, remained unchanged, suggesting that these intermediate cells may have lost their normal differentiation capacity at this stage. Our work shows that SIRT1 plays a crucial protective role against CS-induced COPD. It seems to work by shielding alveolar epithelial cells from death (apoptosis), dialing down inflammation, helping fix the leaky alveolar barrier, and importantly, by encouraging AT2 cells to properly differentiate into type I cells. So, targeting SIRT1 emerges as a promising strategy. The idea would be to rescue the function and fate of alveolar epithelial cells, which could potentially slow down or improve the course of COPD.

Background

COPD is a common, preventable, and treatable disease characterized by persistent airflow limitation. Its pathological basis primarily includes the destruction of alveolar structures (emphysema) and small

airway alterations". Globally, COPD stands as a predominant cause of morbidity and mortality, representing a major global health challenge which imposes a substantial health and socioeconomic burden. Historically, research has largely centered on the chronic inflammation within the small airways of established COPD. Although current treatments can alleviate symptoms in patients with COPD, they offer no cure. Given the substantial population at risk and those in early disease stages, how to delay or even reverse the progression of COPD has become a critical, unresolved challenge. Our research focuses on the longitudinal observation of structural changes in the lungs throughout the entire disease continuum—from early to advanced stages—rather than solely examining the stable advanced phase. This time-course investigation aims to uncover the dynamics of disease evolution and identify potential therapeutic targets.

CS exposure stands as the predominant cause of COPD. This exposure triggers the irreversible breakdown of alveolar walls and remodels the airways via a web of linked pathological events. Key mechanisms involve persistent oxidative stress, ongoing inflammation, an imbalance between proteases and their inhibitors, and programmed cell death (apoptosis). Within this destructive cascade, alveolar epithelial cells are central players. Special attention falls on AT2 cells, which retain the crucial abilities to proliferate and differentiate. Beyond their essential job of producing pulmonary surfactant, AT2 also serve as the primary cellular source for repair and regeneration following alveolar injury. They uphold alveolar integrity by multiplying and maturing into thin, gas-exchanging type I alveolar epithelial cells (AT1 cells). Substantial evidence indicates that during the pathogenesis of COPD/emphysema, CS exposure can directly induce cellular senescence, apoptosis, autophagy dysregulation, and aberrant epithelial-mesenchymal transition in AT2 cells. These alterations severely impair the regenerative repair capacity and barrier function of AT2 cells, which constitutes the core cellular biological basis for the persistent loss of alveolar structure. Despite these insights, a major gap remains in our understanding. The precise molecular circuitry that commands these fate choices in alveolar epithelial cells after CS—especially how this regulatory network changes over time—is still not clearly mapped.

SIRT1 (Silent information regulator 1), a core member of the highly conserved NAD⁺-dependent class III histone deacetylase family, serves as a central regulator in key biological pathways including cellular energy metabolism, stress response, senescence, apoptosis, and inflammation. In the field of pulmonary diseases, studies have suggested that SIRT1 may exert protective effects against inflammation, apoptosis, and oxidative stress in models of acute lung injury, asthma, and pulmonary fibrosis, likely through the deacetylation of downstream targets such as NF- κ B, p53, and FOXO. Particularly noteworthy is that recent research has unveiled the potential role of SIRT1 in maintaining airway epithelial barrier function in COPD. For instance, studies indicate that traditional Chinese herbal compounds can enhance autophagy by activating the SIRT1/AMPK/FOXO3 signaling axis, thereby protecting the integrity of airway epithelial tight junctions. Furthermore, in sepsis-induced acute lung injury, activation of SIRT1 has been demonstrated to ameliorate dysfunction of the alveolar epithelial barrier. More direct evidence indicates that dysregulation of the SIRT1/p53 and SIRT1/FOXO3a signaling pathways is involved in the senescence process of AT2 in patients with COPD. However, most existing studies have focused on

acute injury models or the airway level. There is still a lack of systematic in vivo experimental evidence regarding how SIRT1 dynamically regulates the survival, senescence, barrier function, and differentiation potential toward AT1 of alveolar epithelial cells (particularly AT2) under the influence of chronic CS exposure, which is a core etiological factor of COPD. Several pressing scientific questions need answers. Over long-term CS exposure, how do alveolar epithelial cell phenotypes change dynamically? This includes tracking markers linked to aging, cell death, and differentiation. Does the protein SIRT1 specifically help maintain the balance and function of these cells, protecting them from smoke damage? If it does, how does it work? Is SIRT1's role directly tied to guiding AT2 cells toward their proper fate? And does this ultimately affect the lung's ability to repair its air sacs?

To find out, we will start by creating a mouse model of long-term smoke exposure. This will let us systematically chart the shifting states of alveolar cells as emphysema develops. Using this model, we will work with genetically modified mice—some that overproduce SIRT1 only in alveolar cells, and others where SIRT1 function is reduced. We'll combine these animal studies with experiments on cells in a dish. This research is expected not only to provide deeper insights into the cellular and molecular mechanisms underlying alveolar destruction in COPD but also to establish a solid theoretical and experimental foundation for future development of novel therapeutic strategies targeting SIRT1 to precisely promote alveolar epithelial repair and restore alveolar homeostasis.

Methods

Experimental Animal Models and Study Design

A total of 110 male C57BL/6N mice (8 weeks old, specific pathogen-free (SPF) grade) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were housed in the animal facility of the Science and Technology Center at the First Affiliated Hospital of Army Medical University and acclimatized for 7 days. The housing conditions were maintained at $25 \pm 2^{\circ}\text{C}$, 40–60% relative humidity, with a 12/12-hour light-dark cycle. Mice were fasted (food and water) for 12 hours prior to modeling. The animal experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Army Medical University (Approval No. AMUWEC20252136). Mice were randomly divided into two groups: a wild-type control group (WT group, $n = 40$) and a cigarette smoke-exposed group (CS group, $n = 70$). Mice in the CS group received intratracheal instillation of lipopolysaccharide (LPS, 2 mg/kg) on day 1 and day 14. LPS was purchased from Sigma-Aldrich (Catalog No. L2630). Prior to intratracheal instillation, mice were anesthetized via intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg). After anesthesia, mice were fixed on an operating platform. Following intratracheal administration, mice were held in an upright position briefly to facilitate even distribution of LPS in both lungs. Cigarette smoke exposure was not performed on the days of LPS administration.

Cigarettes used for exposure were Hongmei brand (produced by Kunming Cigarette Factory, Yunnan Province), with specifications of 15 mg tar, 1.2 mg nicotine, and 13 mg carbon monoxide per cigarette. The CS exposure protocol was as follows: Four cigarettes were combusted per cycle for 12 minutes.

Each exposure session lasted 1 hour (comprising 5 cycles), administered twice daily with an interval of at least 6 hours between sessions. This protocol was continued for 12 consecutive weeks²⁷. CS exposure commenced on the second day of the experiment. Exposure was conducted in a plexiglass chamber measuring 50 cm × 35 cm × 30 cm. Daily CS exposure was performed as scheduled, but was withheld on days when intratracheal instillation of LPS or adeno-associated virus (AAV) was administered.

Body weight was measured for all mice every two weeks. Following measurement, 5 mice were randomly selected from each of the WT and CS-exposed groups for sample collection, according to the following procedure: Mice were anesthetized using 1% pentobarbital sodium. The anesthetized mouse was secured on a surgical platform. The trachea was exposed via dissection and cannulated. A syringe was used to draw 1 mL of ice-cold (4°C) PBS, which was then instilled into the lungs via the tracheal cannula. After three repeated lavages, the Bronchoalveolar Lavage Fluid (BALF) was recovered. The thoracic cavity and heart were exposed. Blood was collected from the right ventricle using an insulin syringe and transferred into an anticoagulant tube. An infusion needle was inserted into the right ventricle, and the right atrial appendage was cut. PBS was perfused until the effluent ran clear. The right lung was then excised, blotted dry, and immediately snap-frozen in liquid nitrogen for subsequent Western Blot (WB) analysis. The perfusate was switched to 4% Paraformaldehyde (PFA). Systemic perfusion fixation was continued until muscle twitching was observed. The left lung was harvested and immersed in 4% PFA for post-fixation, pending further processing. The collected BALF and blood samples were centrifuged in a refrigerated centrifuge (4°C, 3000 rpm, 10 minutes). The supernatant was collected and stored at -80°C for subsequent assays".

At the 4th week of the experiment, mice were subjected to the following grouping and interventions: WT Groups: Based on body weight, mice were divided into three groups using stratified randomization: WT Control (n = 20); WT + SIRT1 Overexpression (n = 5); WT + SIRT1 Inhibition (n = 5)

CS-Exposed Groups: Based on the rate of body weight loss, mice were divided into five groups using stratified randomization: CS Exposure Control (n = 30); CS + SIRT1 Overexpression (n = 10); CS + SIRT1 Inhibition (n = 10); CS + Empty Overexpression Control (n = 5); CS + Empty Inhibition Control (n = 5)

All mice were anesthetized with 1% pentobarbital sodium and secured on an operating platform. Subsequently, the corresponding viral suspension (30 µL per mouse) was administered via intratracheal instillation using a microsyringe: WT+SIRT1 Overexpression and CS+SIRT1 Overexpression groups: Received the SIRT1-overexpressing adeno-associated virus (serotype 6.2FF, titer $\geq 5.00 \times 10^{12}$ vg/mL, rAAV-SP-C-SIRT1-2A-EGFP-WPRE-hGH polyA). WT+SIRT1 Inhibition and CS+SIRT1 Inhibition groups: Received the SIRT1-inhibiting adeno-associated virus (rAAV-U6-shRNA(SIRT1)-CMV-mCherry-SV40 pA, serotype 6.2FF, titer $\geq 5.00 \times 10^{12}$ vg/mL). CS+Empty Overexpression Control group: Received the empty overexpression control virus (rAAV-SP-C-EGFP-WPRE-hGH polyA, serotype 6.2FF, titer 5.00×10^{12} vg/mL). CS+Empty Inhibition Control group: Received the empty inhibition control virus (rAAV-U6-shRNA(scramble)-CMV-mCherry-SV40 pA, serotype 6.2FF, titer 5.00×10^{12} vg/mL)". All remaining groups

that did not receive viral intervention were administered an equal volume (30 µL) of normal saline via intratracheal instillation as a control treatment.

During the subsequent experimental period, body weight was measured for all surviving mice every two weeks. Furthermore, tissue sampling was performed at specified time points (weeks 2, 4, 6, 8, 10, and 12), with 5 mice collected from each of the WT control group and the CS exposure control group at each time point.

HE and MASSON staining

Lung tissues were embedded in paraffin and sectioned at a thickness of 4 µm. The sections were subsequently stained with Hematoxylin and Eosin (H&E) and Masson's trichrome. Stained sections were imaged under an optical microscope at 200× magnification to observe morphological changes and perform quantitative analysis. The specific parameters analyzed were: H&E staining: Used to measure the mean linear intercept (MLI), the number of alveoli per field of view (MAN), and the destruction index (DI). Masson's trichrome staining: Used to assess collagen deposition. For quantification, five random fields of view were selected from each mouse lung section. Measurements were performed using Image-Pro Plus 6.0 software: the total number of alveoli, the number of alveolar walls intersecting a test line, and the area of the field of view were recorded. The following calculations were then applied: $MLI = \text{Total length of the test line} / \text{Number of alveolar walls intersecting the test line}$; $MAN = \text{Total number of alveoli} / \text{Area of the field of view}$; $DI = \text{Number of alveoli with structural destruction} / \text{Total number of alveoli}$. To minimize bias, counting and measurements were conducted in a blinded manner by personnel unaware of the group assignments. Subsequently, all data were compiled and subjected to statistical analysis.

Non-invasive pulmonary function testing

After the 12-week intervention period, pulmonary function in mice was assessed using a whole-body plethysmography (WBP) system, a non-invasive method. Following standardized procedures, the instrument parameters were set and calibrated. Mice were acclimatized to the chamber for 10 minutes, followed by a 20-minute data acquisition session. The measured parameters included minute ventilation (Mvb), Airway stenosis index (Penh), mid-expiratory flow (EF50), and Apnea index (PAU). The data were exported for subsequent organization and analysis.

ELISA

ELISA was performed to measure SIRT1 levels in BALF and serum, using the Mouse NAD-dependent deacetylase sirtuin-1 (Sirt1) ELISA Kit (Cusabio, Cat. No. CSB-E16187m) according to the manufacturer's instructions.

Western Blotting

Mouse lung tissues were lysed using RIPA lysis buffer (Beyotime, Cat. #P0013B). The tissues were further disrupted by sonication on ice. Proteins were separated by SDS-PAGE gels of appropriate concentrations and then transferred onto PVDF membranes. After blocking with 5% skim milk for 2 h, the membranes were washed three times with TBST (≥ 5 min per wash) and incubated overnight at 4 °C with the following primary antibodies: HOPX (Proteintech, 11419-1-AP), CLDN4 (Abcam, ab210796), SIRT1 (SAB, 48501), SIRT1 (Prosci, 5765), HOPX (Santa Cruz, sc-398703), PCNA (Boster, A00125), NLRP3 (Boster, BA3677), E-cadherin (Proteintech, 20874-1-AP), TNF- α (Boster, BA0131), CLDN4 (Proteintech, 16195-1-AP), SP-C (Proteintech, 10774-1-AP), KRT8 (Boster, A01421). The next day, after three washes, the membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit secondary antibody (Boster, BA1054) and HRP-conjugated goat anti-chicken secondary antibody (Abbkine, A21080). Following another three washes, protein bands were visualized and recorded using a chemiluminescence imaging system. Band intensities were analyzed with ImageJ software, normalized, and expressed as fold changes relative to the control group.

Immunofluorescence assay and analysis

After embedding for 30 min, lung tissues were frozen and sectioned at 8–10 μ m thickness. The sections were mounted on slides and air-dried overnight at room temperature. The next day, antigen retrieval was performed using a frozen-section rapid antigen retrieval solution (Beyotime, P0090) for 10 min, followed by treatment with an autofluorescence quenching reagent (Applygen, C1212) for 90 min. Sections were then washed twice with pure water (3 min each), permeabilized with 0.5 Triton X-100 for 30 min, and washed three times (5 min each). After blocking with goat serum for 2 h and three washes (2 min each), the sections were incubated overnight at 4°C with the following primary antibodies: HOPX (Oasis Biofarm, OB-PRT015-02), HOPX (Santa Cruz Biotechnology, sc-398703), SP-C (Proteintech, 10774-1-AP), SIRT1 (Prosci, 5765), Nrf2 (HUABIO, R1312-8-100ul), Cleaved-caspase-3 (Proteintech, 25128-1-AP), CLDN4 (Abcam, ab210796), E-cadherin (Proteintech, 20874-1-AP), TNF- α (Boster, BA0131), CLDN4 (Proteintech, 16195-1-AP), Ki-67 (Boster, PB9026). On the following day, after washing, sections were incubated in the dark for 1 h at room temperature with the corresponding secondary antibodies: Goat anti-chicken IgG (H + L) Alexa Fluor Plus 405 (Invitrogen, A48260), Goat anti-rabbit IgG H&L (Alexa Fluor 488) (Abcam, ab150077), Goat anti-rat IgG H&L (Alexa Fluor 555) (Abcam, ab150158), Alexa Fluor 647-conjugated goat anti-mouse antibody (Beyotime, A0473), Alexa Fluor 555-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific, A-21428), DyLight 594-conjugated goat anti-mouse IgG (Abbkine, A23410), DyLight 680-conjugated AffiniPure goat anti-rabbit IgG (H + L) (Fdbio science, FD0133), AF647-conjugated goat anti-rabbit IgG (H + L) (Beyotime, A0468). After four washes, sections were air-dried in the dark, mounted with DAPI-containing mounting medium, and observed under an Olympus fluorescence microscope. Images were acquired and stored for subsequent analysis. Quantitative assessments, such as counting positive cells and measuring fluorescence intensity, were conducted in a

batch-processing manner using ImageJ software. This was followed by data compilation and statistical evaluation.

Cell culture and treatment

Human A549 cells (Procell; CL-0016) were cultured in DMEM medium supplemented with 10% fetal bovine serum (ZETA LIFE; Z7010FBS-500) and 1% penicillin-streptomycin solution (ZETA LIFE; BM0001-100) at 37 °C in a 5% CO₂ incubator. Based on preliminary experiments, the following working concentrations were used: Cigarette smoke extract (CSE) (PYTHONBIO; AAPR551-A10): 50 µg/mL; LPS (Sigma-Aldrich; L2630): 10 µg/mL; SIRT1 agonist SRT1720 (Beyotime; SC0267-5mg): 5 µM; SIRT1 inhibitor EX527 (Beyotime; SC0281-5mg): 10 µM; Nrf2 agonist NK252 (GLPBIO; GC13058): 10 µM; Nrf2 inhibitor ML385 (GLPBIO; GC19254): 5 µM; The COPD model group was treated with a combination of CSE and LPS, referred to hereafter as the CS group. Experimental Groups: The cells were divided into the following treatment groups: Control group; CS group (CSE + LPS); CS + SRT1720 group; CS + EX527 group; CS + NK252 group; CS + ML385 group; CS + SRT1720 + NK252 group; CS + SRT1720 + ML385 group; CS + EX527 + NK252 group; CS + EX527 + ML385 group; SRT1720 group (agonist alone); EX527 group (inhibitor alone); NK252 group (agonist alone); ML385 group (inhibitor alone); Drug vehicle control group (containing only the four drug vehicles, without cells); Assessment of Cell Proliferation and Apoptosis: Cell proliferation and apoptosis were evaluated using the following assays: Cell Counting Kit-8 (CCK-8) assay; ATPase activity assay; Apoptosis analysis via PI fluorescence staining.

Statistical analysis

Data were processed, analyzed, and visualized using GraphPad Prism software, version 9.5. For normally distributed measurement data, results are reported as the mean ± standard deviation (mean ± SD). An independent samples t-test was used for comparisons between two groups, and one-way analysis of variance (ANOVA) was applied for comparisons across multiple groups. If the data did not conform to a normal distribution, results are presented as the median and interquartile range, and comparisons were made using the Wilcoxon rank-sum test for two groups or the Kruskal-Wallis test for multiple groups. A p-value of less than 0.05 was considered statistically significant. Image analysis was carried out using ImageJ and Image-Pro Plus software (version 6.0).

Results

1. Validation of the COPD mouse model established by CS.

During the 0-4 week period, the body weight of COPD model mice under CS exposure continued to decline, whereas that of WT mice steadily increased (Fig. 1A-B). After 4 weeks, the body weight gain of CS-exposed COPD mice was impeded, showing a persistent downward trend, in sharp contrast to the continued weight increase in WT mice (Fig. 1C-D). Histological examination of lung sections revealed progressively worsening alveolar damage and increased inflammatory cell infiltration in COPD mice over the course of CS exposure, while the lung morphology of WT mice remained largely unchanged over time

(Fig. 1E, H&E staining). Masson's trichrome staining showed that collagen deposition in the lungs of COPD mice intensified with prolonged CS exposure, with no significant changes observed in WT mice (Fig. 1F). Quantitative analysis demonstrated a significant increase in the MLI and the alveolar DI, along with a significant decrease in the MAN per field in the COPD group compared to controls (Fig. 1G-I). We further assessed the dynamic changes in pulmonary inflammation during CS exposure.

Immunofluorescence and Western blot analyses revealed that the expression of the pro-inflammatory cytokine TNF- α was significantly elevated in COPD mice after CS exposure and remained high up to 12 weeks (Fig. 2A-D). Western blot results indicated that the expression levels of TNF- α and the key inflammasome protein NLRP3 were not significantly altered at the early stage (2 weeks) but began to increase markedly from the 4th week onward, sustaining high levels thereafter (Fig. 2C-F). Pulmonary function was evaluated in WT and COPD model mice after 12 weeks. Parameters including MVb, Penh, EF50, and the PAU were measured. The results showed that all pulmonary function indices were impaired in the COPD group, with significant differences compared to the WT group (Fig. 8A-D). These findings collectively confirm the successful establishment of a valid COPD mouse model induced by 12 weeks of CS exposure combined with early intratracheal LPS instillation.

2. CS exposure induces a time-dependent imbalance in the proportions of alveolar epithelial cell subpopulations

We first measured how the different types of alveolar epithelial cells changed over time. Using immunofluorescence and Western blot, we tracked these markers. The AT1 cell marker HOPX stayed fairly steady during the first 2 weeks of smoke exposure. However, it began to drop noticeably starting at week 4. On the other hand, the AT2 cell marker SP-C showed a small uptick at week 2. After that, it fell sharply. This decline in SP-C was much steeper than the drop in HOPX. As a result, the ratio of SP-C to HOPX quickly decreased (Fig. 3A-H). Interestingly, Western blot analysis revealed that protein levels of KRT8, a marker associated with alveolar intermediate cells, did not change significantly at any time point compared to controls (Fig. 3I-J). However, both immunofluorescence and Western blot analyses showed that the expression of CLDN4, another marker associated with intermediate cell differentiation, began to gradually decrease after week 2. What does this tell us? In the development of COPD, AT2 cells show damage and fail to regenerate earlier and more severely than AT1 cells. So, the initial problem isn't just the direct loss of AT1 cells. Instead, the early decline in AT2 cell function is what starts the process of alveolar destruction. The loss of intermediate cells points to a weakened ability for AT2 cells to turn into AT1 cells, which means the repair process is impaired.

The reason for the sustained KRT8 protein expression, however, remains unclear and warrants further study. Prior research has delineated alveolar intermediate cells into five sequential stages: CTGF (connective tissue growth factor)-positive PATs (pre-alveolar type 1 transitional cells), LGALS3 (galectin-3)-positive PATs, KRT8+ ADI (alveolar differentiation intermediate cells), preAT1, and DATP (damage-associated transient progenitors), with the latter two stages expressing CLDN4. In the later phase when both AT1 and AT2 cells decline, the reduced differentiation capacity of AT2 cells would theoretically lead to a decrease in intermediate cell numbers. However, our data show a decrease in CLDN4 while KRT8

expression remains largely unchanged. This discrepancy suggests that the intermediate cell stage marked by KRT8 precedes the stage marked by CLDN4. We propose a hypothesis that cellular differentiation becomes arrested at the KRT8-positive stage, preventing normal progression to subsequent CLDN4-positive stages. This block in differentiation could potentially create a negative feedback loop, further inhibiting the inherent proliferative and repair functions of AT2 cells. Therefore, such differentiation arrest may be the critical mechanism triggering the early regenerative failure observed in SP-C-positive cells, a notion that requires future experimental confirmation.

3. Temporal Changes in Alveolar Epithelial Cell Barrier Function, Proliferation, and Apoptosis Status.

We next looked at proteins important for the barrier function of the alveolar epithelium. With longer CS, their levels progressively declined. Both immunofluorescence and Western blot showed that key tight junction proteins, as well as the adhesion protein E-cadherin, started to drop significantly in both positive cell counts and expression levels after two weeks (Fig. 4A-H). Assessing cellular proliferation revealed a complex pattern. The count of Ki-67-positive cells, a proliferation marker detected by immunofluorescence, was significantly reduced after two weeks of exposure (Fig. 5A, C). In contrast, protein levels of the proliferation marker PCNA, measured by Western blot, showed a significant initial increase at the two-week mark before declining rapidly in subsequent weeks (Fig. 5E-F). Apoptosis assessment indicated that the apoptosis marker cleaved-caspase-3 detected by immunofluorescence showed almost no increase at 2 weeks but began to rise significantly at 4 weeks of CS exposure (Fig. 5B,D).

The temporal changes in barrier function, proliferation, and apoptosis align with the overall alterations in alveolar epithelial cells. However, the peak of inflammatory indicators occurs later than these epithelial changes. Consequently, we propose that inflammation is likely not the primary cause of the alveolar epithelial cell alterations; instead, an earlier initiating stimulus probably triggers this process.

4. Temporal alterations in SIRT1/Nrf2 and validation of SIRT1 gain- and loss-of-function models.

In our previous study, we observed that the expression of SIRT1 decreased early in a COPD mouse model; its protein level increased at week 2 and then gradually declined (Fig. 6 C-D). This suggests that SIRT1 plays a role in the early stages of COPD, and its temporal changes align with the alterations in alveolar epithelial cells. Furthermore, we examined the dynamics of Nrf2 expression and found that its level also rose at week 2 before gradually decreasing (Fig. 6 A-B), a pattern consistent with the changes in SIRT1 and alveolar epithelial cells.

Looking at the data over time, we saw that SIRT1 and Nrf2 levels change together, and this happens early in the disease. The dysfunction of alveolar epithelial cells in smoke-induced COPD clearly gets worse over time. Interestingly, protein levels for both SIRT1 and Nrf2 went up after two weeks of exposure, but then fell later. This timing matches up closely with when the alveolar epithelial cells are lost and their function worsens. These results indicate that problems with the SIRT1/Nrf2 system are an early driver of the disease process, not just a late result. This gives us a reason, based on timing, to look at this system

as a possible central control mechanism. There is also a proposed direct link between the SIRT1 and Nrf2 pathways. Earlier work suggests SIRT1 can activate Nrf2 by modifying both the Nrf2 protein itself and its inhibitor, Keap1. Since Nrf2 controls the cell's main defense against oxidative stress—a key starting point for smoke damage—this creates a logical chain. The pathway connects SIRT1's regulatory role to Nrf2 activation and finally to fighting oxidative damage. This makes it a strong candidate for directly linking smoke exposure (the cause) to oxidative injury in lung cells (the effect). We therefore suggest that in early COPD, the SIRT1/Nrf2 pathway is turned on. It likely helps AT2 cells differentiate and protects AT1 cells from dying. But with continued smoke exposure, this protective system gets worn down and depleted. Eventually, this leads to a failure in the cells' ability to proliferate and repair themselves.

Based on this hypothesis, at the fourth week of cigarette smoke exposure, we intratracheally administered two AAVs: one carrying a lung-specific promoter and green fluorescent protein for SIRT1 overexpression, and the other labeled with red fluorescent protein for SIRT1 inhibition. This gain- and loss-of-function experiment was conducted to test our proposed mechanism.

Body weight monitoring revealed that in both wild-type and COPD model groups, mice injected with the SIRT1-overexpressing virus exhibited higher absolute body weight and weight change rate compared to the saline control group. The control group, in turn, showed higher body weight metrics than mice injected with the SIRT1-inhibiting virus (Fig. 1C-D). Given the known role of SIRT1 in metabolism, this weight difference indirectly indicates successful expression and biological activity of the SIRT1 gain- and loss-of-function viruses.

Since the overexpression virus was tagged with EGFP and the inhibition virus with mCherry, both produce detectable fluorescence. Examination of lung tissue sections under a microscope revealed green fluorescence in the overexpression group and red fluorescence in the inhibition group (Fig. 7A). This visual evidence confirms successful viral transduction in the lung tissue.

We further assessed SIRT1 protein levels in mouse lung tissues using ELISA and Western blot. The measurements showed that SIRT1 expression was significantly elevated in the overexpression group compared to the control group. Conversely, SIRT1 levels in the control group were significantly higher than those in the inhibition group (Fig. 7B-J). Together, these results verify that the viral vectors not only reached the lung tissue but also effectively altered the expression of the target protein as intended.

Additionally, we included control groups receiving blank viruses (carrying fluorescent tags but without SIRT1 intervention). Measurements of body weight, fluorescence, and protein expression showed no significant differences between these blank virus groups and the saline control group, indicating that the observed effects were due to specific SIRT1 modulation rather than the viral vectors themselves.

In summary, we successfully established in vivo gain- and loss-of-function models for SIRT1.

5. Intervening in SIRT1 expression can alter the progression of COPD through alveolar epithelial cells.

Respiratory function in mice was evaluated using non-invasive pulmonary function testing. Measured parameters included minute ventilation (reflecting overall ventilatory capacity), airway narrowing index (where a higher value indicates more severe obstruction), mid-expiratory flow (where a lower value suggests reduced lung elasticity), and the apnea-hypopnea index (where a higher value signifies increased airway resistance or worsened compliance). All pulmonary function parameters were significantly impaired in the COPD model group compared to wild-type mice. In COPD mice, SIRT1 overexpression improved pulmonary function, whereas SIRT1 inhibition exacerbated its decline. Notably, modulating SIRT1 expression in wild-type mice did not significantly affect their lung function, indicating that the role of SIRT1 is specific to the pathological microenvironment induced by cigarette smoke exposure in COPD. Furthermore, no significant difference in pulmonary function was observed between the blank virus control groups and the COPD model group, confirming that the viral vectors themselves did not influence respiratory function (Fig. 8A-D).

HE and MASSON staining of lung tissue sections showed that SIRT1 overexpression reduced cigarette smoke-induced lung damage, including emphysema, inflammatory cell infiltration, and collagen buildup. In contrast, SIRT1 inhibition worsened these pathological changes (Fig. 8E). Further analysis of quantitative measures, such as the mean linear intercept and alveolar destruction index, confirmed these observations. These metrics were significantly better in the SIRT1-overexpression group compared to the cigarette smoke-exposed group, while SIRT1 inhibition led to further decline. No significant differences were found between the blank-virus control groups and the cigarette smoke-exposed group (Fig. 8F-H). These findings indicate that SIRT1 can rescue cigarette smoke-induced emphysematous structural alterations and improve lung function, suggesting a protective role for SIRT1 in cigarette smoke-induced COPD.

Immunofluorescence and Western blot results showed that SIRT1 overexpression increased the numbers of both alveolar type I and type II epithelial cells compared to the COPD model group, while SIRT1 inhibition decreased them. No significant changes were seen between the blank-virus control groups and the COPD model group (Fig. 9). Further analysis indicated that the ratio of the AT2 cell marker SP-C to the AT1 cell marker HOPX was higher in the SIRT1-overexpression group than in the COPD model group, but lower in the inhibition group (Fig. 9D). This suggests that SIRT1 exerts a more pronounced effect on AT2 cells than on AT1 cells, indicating its role in modulating the compositional balance of alveolar epithelial cell subtypes.

6. SIRT1 regulates inflammation, barrier function, proliferation, and apoptosis in alveolar epithelial cells

Western blot analysis of TNF- α showed that SIRT1 overexpression led to higher levels of the TNF- α precursor but lower levels of the active soluble form, compared to the control group. Conversely, SIRT1 inhibition increased both the precursor and the active form. No significant changes were seen in the

blank-virus control groups (Fig. 10A-F). NLRP3 expression was also affected: it decreased with SIRT1 overexpression and increased with SIRT1 inhibition, while blank-virus controls again showed no difference from the control group (Fig. 10D, H, I). Immunofluorescence staining for TNF- α supported these findings, with fewer TNF- α -positive cells after SIRT1 overexpression and more after its inhibition. These findings demonstrate that SIRT1 can ameliorate cigarette smoke-induced inflammatory responses in lung tissue. In a separate analysis of the tight junction protein CLDN4, Western blot showed that CLDN4 expression rose in the SIRT1-overexpression group—even exceeding levels in the wild-type group—but fell in the SIRT1-inhibition group. The blank-virus control groups did not differ from the control group (Fig. 11F-H). Immunofluorescence and Western blot analysis revealed higher E-cadherin expression in the SIRT1-overexpression group and lower expression in the SIRT1-inhibition group. The blank-virus control groups showed no significant change compared to the control group (Fig. 11A-E). This points to a role for SIRT1 in enhancing barrier function. In addition, immunofluorescence staining revealed that the number of Ki-67-positive cells was increased in the SIRT1-overexpression group and decreased in the SIRT1-inhibition group, while no significant differences were observed between the blank-virus control groups and the control group (Fig. 12A-B). Western blot analysis indicated that PCNA expression was elevated in the SIRT1-overexpression group, exceeding that in the wild-type (WT) group, whereas it was reduced in the SIRT1-inhibition group. The blank-virus control groups showed no notable change compared to the control group (Fig. 12C-E). These findings suggest that SIRT1 significantly influences the proliferative and differentiation functions of alveolar epithelial cells in cigarette smoke-induced COPD. Finally, immunofluorescence for cleaved-caspase-3 demonstrated that SIRT1 overexpression reduced alveolar epithelial cell apoptosis, whereas inhibition worsened it. Blank-virus controls again showed no difference from the control group (Fig. 13A, C). This indicates that SIRT1 activation can alleviate apoptosis.

Immunofluorescence analysis indicated that SIRT1 overexpression up-regulated Nrf2 expression, whereas SIRT1 inhibition down-regulated it; no significant differences were observed between the blank-virus control groups and the normal control group (Fig. 13B, D). This suggests a functional link between SIRT1 and Nrf2 in the context of cigarette smoke-induced chronic obstructive pulmonary disease. We subsequently performed cell-based experiments to further investigate the interaction between SIRT1 and Nrf2.

7. In vitro experiments demonstrate the protective role of SIRT1.

Western blot analysis showed that pharmacological treatment effectively activated or inhibited SIRT1 in cells (Fig. 14A-B).

CCK-8 and ATPase activity assays showed that activating either SIRT1 or Nrf2 alone partially restored the drop in cell viability and proliferation caused by CS. Inhibiting either pathway alone made the CS-induced damage worse. When one pathway was activated while the other was inhibited, the restorative effect was weaker, pointing to an interaction between SIRT1 and Nrf2 in fighting CS-induced proliferation problems. A closer look revealed that activating SIRT1 while blocking Nrf2 worked better than activating

Nrf2 while blocking SIRT1. This suggests SIRT1 might have a stronger influence than Nrf2 on regulating cell proliferation in this context. Simultaneous inhibition of both SIRT1 and Nrf2 resulted in a substantial decrease in proliferative capacity, underscoring the critical protective roles both pathways play in normal cells responding to CS injury. Furthermore, treatment with the activators or inhibitors alone caused only a mild reduction in proliferation, indicating low inherent cytotoxicity of the compounds themselves. The results from the vehicle control groups confirmed that the color of the drugs did not significantly interfere with the assay readings.

Regarding apoptosis, CS treatment significantly increased cell apoptosis. Administration of SRT (a SIRT1 activator) or NK (an Nrf2 activator) alone effectively rescued the CS-induced apoptosis. Conversely, treatment with EX (a SIRT1 inhibitor) or ML (an Nrf2 inhibitor) alone exacerbated apoptosis. In the combination treatment experiments, the rescue effect achieved by co-administering SRT and ML (activating SIRT1 while inhibiting Nrf2) was inferior to that achieved by co-administering EX and NK (inhibiting SIRT1 while activating Nrf2). This result suggests that the Nrf2 pathway may play a more critical role than SIRT1 in counteracting CS-induced apoptosis. Control experiments validated the reliability of the assay system: the high apoptosis rate in the positive control group confirmed the effectiveness of the staining method. Meanwhile, the absence of apoptotic signals in the vehicle control groups indicated that the drugs themselves did not react with the apoptosis detection dye, thereby ruling out potential false-positive interference.

Discussion

The main findings of this study are summarized as follows: CS exposure led to progressive decline in lung function and corresponding pathological alterations, accompanied by dynamic changes in alveolar epithelial cells and SIRT1 expression. Alveolar epithelial cells were altered early after CS exposure. Compared to apoptosis, the impairment in the proliferative repair capacity of AT2 cells emerged as an earlier pathological event. The inflammatory response in COPD lung tissues induced by CS exposure occurred later than the changes in alveolar epithelial cells, suggesting that pulmonary inflammation may not be the primary initiator of alveolar epithelial cells. Overexpression of SIRT1 alleviated CS-induced emphysema, improved lung function and alveolar epithelial barrier function, reduced cell apoptosis and inflammation, and significantly enhanced the proliferative repair capacity of lung tissue. It also altered the subtype composition of alveolar epithelial cells in CS-induced COPD. Conversely, inhibition of SIRT1 aggravated the CS-induced damage described above. In vitro studies further confirmed that the SIRT1/Nrf2 pathway exerts protective effects against CS-induced declines in cell proliferation and viability, as well as increased apoptosis. Specifically, SIRT1 played a more prominent role in regulating cell proliferation and viability, whereas Nrf2 had a greater impact on counteracting apoptosis.

Traditionally, COPD has been associated with long-term airway inflammation caused by neutrophils. Our study, using data collected over time, reveals an earlier series of disease events in alveolar epithelial cells after smoke exposure. A key finding is that an imbalance in the ratio of AT2 to AT1 cells, along with a significant drop in SIRT1 protein, was seen as early as 4 weeks into exposure—before any structural

emphysema appeared. This suggests that problems in these lung lining cells are an early driver of COPD, not just a late result. This idea fits with a recent single-cell study that found a unique, inflammatory type of AT2 cell in the lungs of human smokers, indicating these cells change their function right from the start. In more detail, after a brief increase, AT2 cell numbers dropped significantly and earlier than AT1 cells. This caused a quick shift in the balance between their markers, SP-C for AT2 and HOPX for AT1. On the other hand, noticeable rises in the inflammatory signal TNF- α and the key inflammation protein NLRP3 only started after week 4. This clear order of events indicates that in smoke-induced COPD, the alveolar epithelial cells are not just passive targets of inflammation. Instead, the breakdown in their own ability to proliferate and repair themselves likely acts as the initial problem that then triggers the later inflammation and tissue damage.

AT2 cells serve as the resident progenitor cells in the alveoli. Their jobs include producing pulmonary surfactant and, following injury, repairing the alveolar structure by proliferating and differentiating into AT1 cells. Our research identified an early decline in both the number and function of AT2 cells. This significantly weakens the lung's intrinsic repair capacity right from the start of the disease. Looking closer, we found that the expression of CLDN4—a marker for the late stage of intermediate cell differentiation—started to decrease from week 2 of smoke exposure. In contrast, expression of KRT8, a marker for the early differentiation stage, showed no change. This pattern suggests that CS exposure may specifically block a later stage in the AT2-to-AT1 differentiation process. Cells get stuck and cannot complete their transition into fully functional, mature AT1 cells. This blockade has two critical effects. It directly reduces the pool of functional AT1 cells and creates a negative feedback signal that further suppresses the AT2 cells' own ability to proliferate. Together, this establishes a self-perpetuating vicious cycle. This understanding provides a new biological perspective on the "irreversible" structural damage seen in COPD. The disease progresses not only because of ongoing injury from smoke, but equally because of an early and escalating failure of the lung's own regenerative repair system.

Our data provide direct temporal evidence for this "abnormal differentiation" and further suggest that the early loss of SIRT1 may be a key factor triggering this process. Furthermore, the successful alteration of disease progression by intervening with SIRT1 at the early 4-week time point holds significant translational medical importance. It indicates that early interventions targeting alveolar epithelial cell state (such as using SIRT1 agonists) could potentially delay or prevent the onset and progression of COPD before structural lung damage becomes irreversible.

Our findings align with the existing literature, further confirming the protective role of SIRT1 in COPD. Different from previous studies, our longitudinal intervention study provides direct in vivo causal evidence, moving beyond correlative observations. The data support that SIRT1 exerts its protective effects by inhibiting pro-apoptotic and pro-inflammatory pathways, consistent with its known functions. Notably, we found that in the context of CS-induced COPD, SIRT1 has a more pronounced impact on the proliferative and differentiation capacities of alveolar epithelial cells. SIRT1 not only promoted the proliferation of AT2 cells but, more importantly, enhanced their ability to differentiate into AT1 cells. This positive regulation of alveolar epithelial cell stemness and differentiation potential may be the core

mechanism through which SIRT1 achieves long-term protection and promotes structural repair. Our data also suggest an interaction between SIRT1 and Nrf2 under CS-induced COPD conditions, but this relationship is not a simple hierarchical regulation. SIRT1 plays a more dominant role in rescuing cell viability and promoting proliferation, whereas Nrf2 has a greater influence in resisting apoptosis. This indicates that their downstream effectors differ: SIRT1 likely orchestrates a more global regulation of cellular metabolism, stress response, and epigenetic state, providing a foundation for cell survival and proliferation; whereas Nrf2 is more specialized in combating oxidative stress—the direct cause of apoptotic damage.

The clinical significance of this study lies in pointing out a clear therapeutic window for early intervention targeting the proliferative repair capacity of alveolar epithelial cells in at-risk individuals, before the formation of structural emphysema (approximately 4 weeks in this study). For smokers or early-stage COPD patients with preserved lung function, monitoring alveolar epithelial cell function or biomarkers related to SIRT1 activity may aid in the very early identification of disease risk. Secondly, targeting SIRT1 through early intervention, such as with SIRT1 activators (e.g., SIRT1720, resveratrol), may delay or even halt the onset and progression of COPD. This treatment strategy, which addresses the early driver of the disease (impaired epithelial repair), may be more fundamental compared to traditional approaches that primarily target late-stage symptoms (e.g., bronchodilators or anti-inflammatory agents).

However, we also acknowledge several limitations of this study. First, the timing of SIRT1 intervention was relatively late (initiated at 4 weeks, with effects observed at 7–8 weeks), by which time epithelial cell dysfunction had already been initiated. Thus, the effect of genuine early intervention was not observed. Future studies should administer SIRT1 activation at the onset of CS exposure or even earlier, to investigate whether it can completely prevent the development of alveolar damage. Second, the molecular mechanisms underlying the early arrest of proliferation and differentiation remain unclear. Furthermore, the specific signaling pathways through which SIRT1 influences the proliferation and differentiation of alveolar type 2 (AT2) cells require further investigation; subsequent studies need to elucidate the involved pathway molecules. Finally, regarding cell specificity: although we used a virus driven by an alveolar epithelial cell-specific promoter, the role of SIRT1 in other alveolar cell types is equally crucial and should not be overlooked. A comprehensive analysis of SIRT1's cell type-specific roles will facilitate the design of more precise therapeutic strategies.

In summary, this work provides a systematic view of how CS drives COPD. It shows that dysfunction of AT2/AT1 is an early and central event. In this process, the decrease of their ability to proliferate and repair plays a bigger role than cell death (apoptosis) as the main disease mechanism. Here, SIRT1 acts as a central regulator. Overexpressing SIRT1 brings together several protective actions: prevents cell death, reduces inflammation, maintains the alveolar barrier, and, importantly, boosts the regenerative and differentiation potential of AT2 cells. By integrating these effects, SIRT1 overexpression effectively counteracts the damage from CS, ultimately improving both lung function and the structure of lung tissue.

Looking forward, several directions can be further investigated. First, employing high-resolution techniques like single-cell sequencing and spatial transcriptomics to delineate the dynamic transcriptional profiles and epigenetic landscapes of alveolar epithelial cells and their progenitors upon CS exposure. This will help precisely identify the key target cell populations regulated by SIRT1. Second, find the specific downstream effectors of SIRT1, particularly those directly involved in regulating the proliferation and differentiation of AT2. Third, exploring the potential of combining SIRT1 agonists with other therapeutic strategies. Fourth, further validating the role of SIRT1 in more human-relevant models, such as human airway epithelial cells and human-derived lung organoids, and eventually in early-phase clinical trials.

Abbreviations

COPD
Chronic obstructive pulmonary disease
CS
cigarette smoke
LPS
lipopolysaccharide
AT2
type II alveolar epithelial cells
AT1
type I alveolar epithelial cells
SIRT1
Silent information regulator 1
AAV
adeno-associated virus
BALF
Bronchoalveolar Lavage Fluid
WB
Western Blot
MLI
mean linear intercept
MAN
mean number of alveoli per field of view
DI
destruction index
Mvb
minute ventilation
Penh
Airway stenosis index

EF50
mid-expiratory flow
PAU
Apnea index
CSE
Cigarette smoke extract
KRT8
Keratin 8
CLDN4
Claudin 4
Nrf2
Nuclear factor erythroid 2-related factor 2

Declarations

Author's contributions

Wei Xiong and Xiaotian Dai conceived this project. Cheng Liang performed the major animal experiments, cell experiments and molecular experiments. Weijia Zhou and Cheng Liang contributed to the design of animal experiments and animal operations. Wu Li, Qiang Zeng, Lei Xue and Weijia Zhou contributed to the western blot, HE staining and Immunofluorescence analysis of data. Wei Xiong and Xiaotian Dai provided the fund for this study. Cheng Liang, Wei Xiong and Xiaotian Dai wrote and revised the manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by National Key Research and Development Program of China (2024YFA1108900) and National Key Research and Development Program of China (2018YFC2000301).

Availability of data and material

Data and materials can be made available upon written request to the corresponding author.

Ethics approval and consent to participate

All animal studies were performed following guidelines from the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All protocols were approved by the Ethics Committee of the First Affiliated Hospital of Army Medical University (Approval No. AMUWEC20252136).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

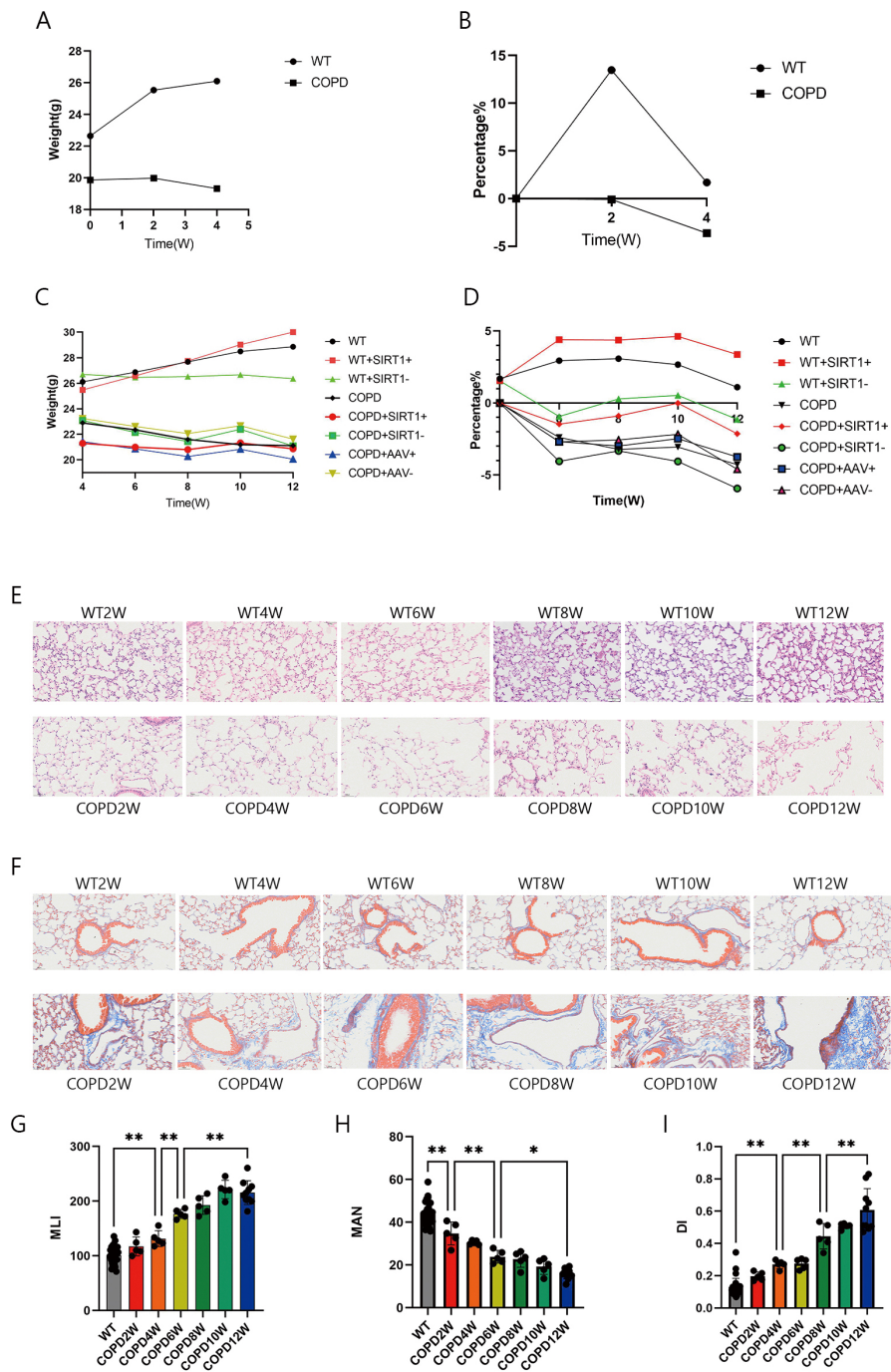


Figure 1

Effects of Cigarette Smoke Exposure on Mouse Body Weight and Lung Histopathological Changes

(A) Absolute change curve of mouse body weight before grouping. (B) Rate of change curve of mouse body weight before grouping. (C) Absolute change curve of mouse body weight after grouping. (D) Rate of change curve of mouse body weight after grouping. (E) H&E staining of mouse lung tissue

(magnification $\times 200$; scale bar = 50 μm). (F) Masson staining of mouse lung tissue (magnification $\times 200$; scale bar = 50 μm). (G) Mean Linear Intercept (MLI) index for each group. (H) Mean Alveolar Number (MAN) per field for each group. (I) Destruction Index (DI) for each group.

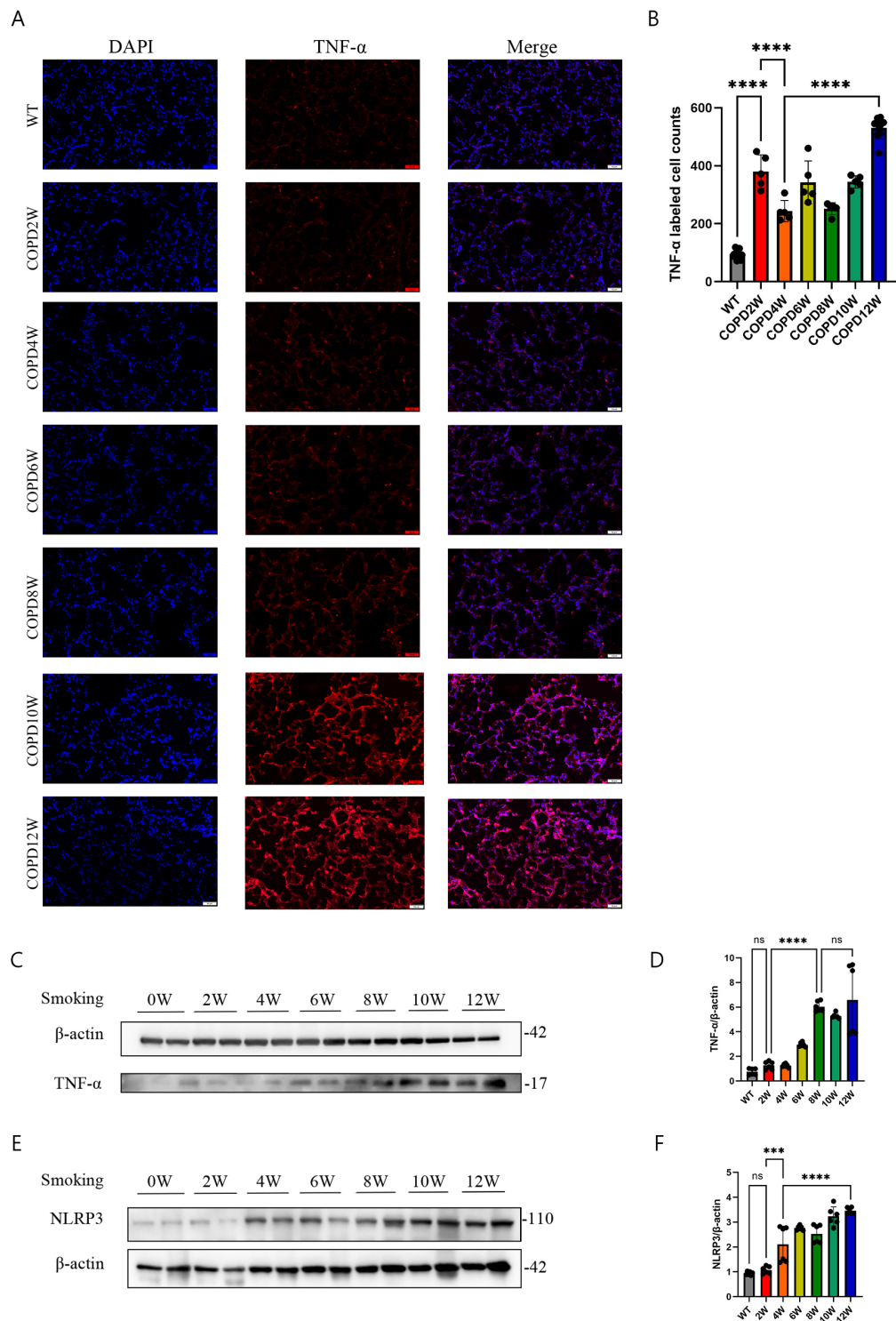


Figure 2

Effects of Cigarette Smoke Exposure on Inflammation in Mice

(A) Immunofluorescence images of TNF- α for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of TNF- α fluorescent positive cells for each group. (C) Expression of TNF- α in lung tissues detected by Western blot for each group. (D) Relative protein expression level of TNF- α / β -actin for each group. (E) Expression of NLRP3 in lung tissues detected by Western blot for each group. (F) Relative protein expression level of NLRP3/ β -actin for each group.

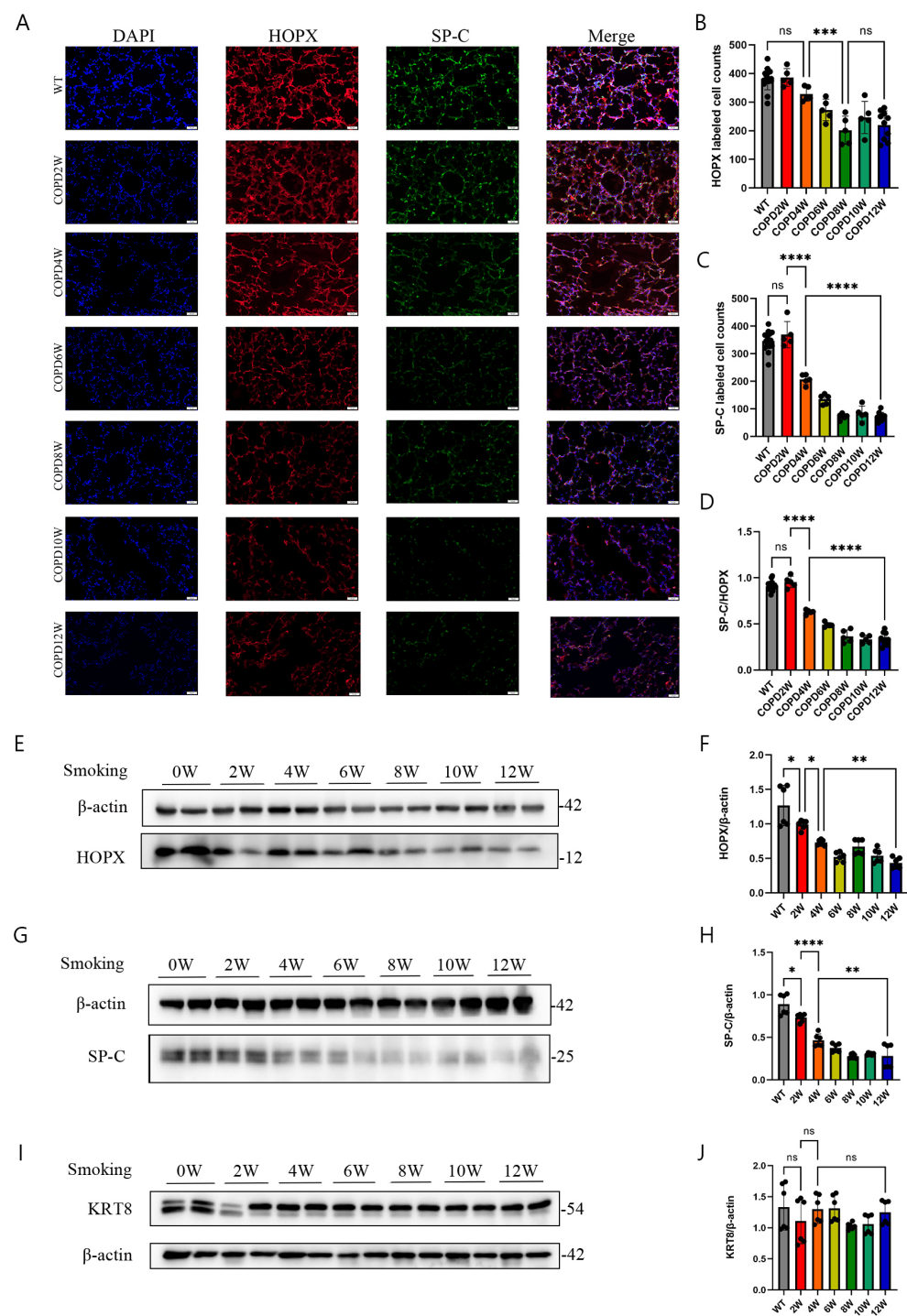


Figure 3

Temporal Changes in Alveolar Epithelial Cells Following CS Exposure

(A) Immunofluorescence images of HOPX and SP-C for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of HOPX fluorescent positive cells for each group. (C) Count of SP-C fluorescent positive cells for each group. (D) Ratio of SP-C/HOPX for each group. (E) Expression of HOPX in lung tissues detected by Western blot for each group. (F) Relative protein expression level of HOPX/ β -actin for each group. (G) Expression of SP-C in lung tissues detected by Western blot for each group. (H) Relative protein expression level of SP-C/ β -actin for each group. (I) Expression of KRT8 in lung tissues detected by Western blot for each group. (J) Relative protein expression level of KRT8/ β -actin for each group.

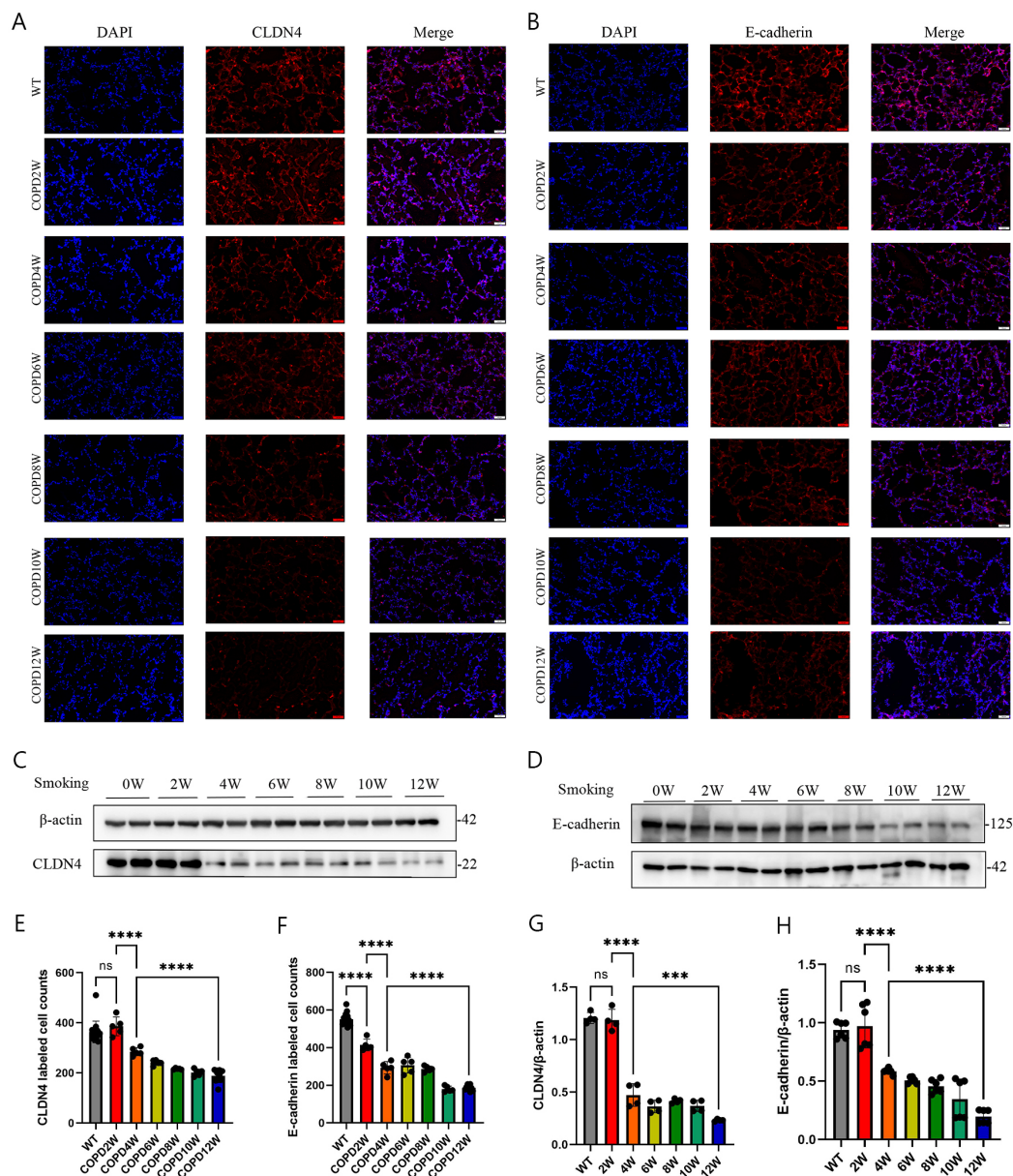


Figure 4

Temporal Changes in Pulmonary Barrier Function Following CS Exposure

(A) Immunofluorescence images of CLDN4 for each group (magnification $\times 200$; scale bar = 50 μm). (B) Immunofluorescence images of E-cadherin for each group (magnification $\times 200$; scale bar = 50 μm). (C) Expression of CLDN4 in lung tissues detected by Western blot for each group. (D) Expression of E-

cadherin in lung tissues detected by Western blot for each group. (E) Count of CLDN4 fluorescent positive cells for each group. (F) Count of E-cadherin fluorescent positive cells for each group. (G) Relative protein expression level of CLDN4/ β -actin for each group. (H) Relative protein expression level of E-cadherin/ β -actin for each group.

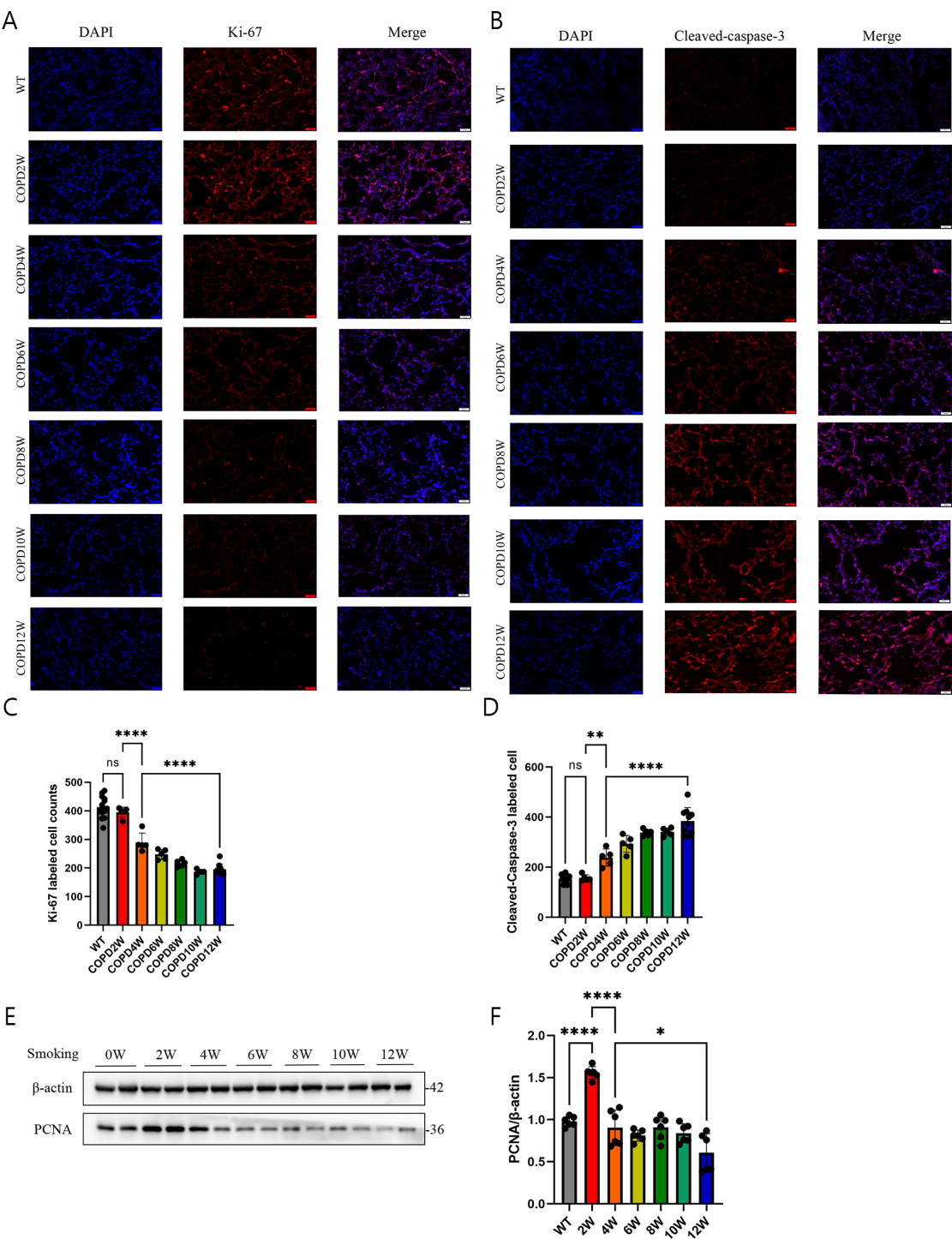


Figure 5

Temporal Changes in Lung Proliferation and Apoptosis Function Following CS Exposure

(A) Immunofluorescence images of Ki-67 for each group (magnification $\times 200$; scale bar = 50 μm). (B) Immunofluorescence images of cleaved-caspase-3 for each group (magnification $\times 200$; scale bar = 50 μm). (C) Count of Ki-67 fluorescent positive cells for each group. (D) Count of cleaved-caspase-3 positive cells for each group. (E) Expression of PCNA in lung tissues detected by Western Blot for each group. (F) Relative protein expression level of PCNA/ β -actin for each group.

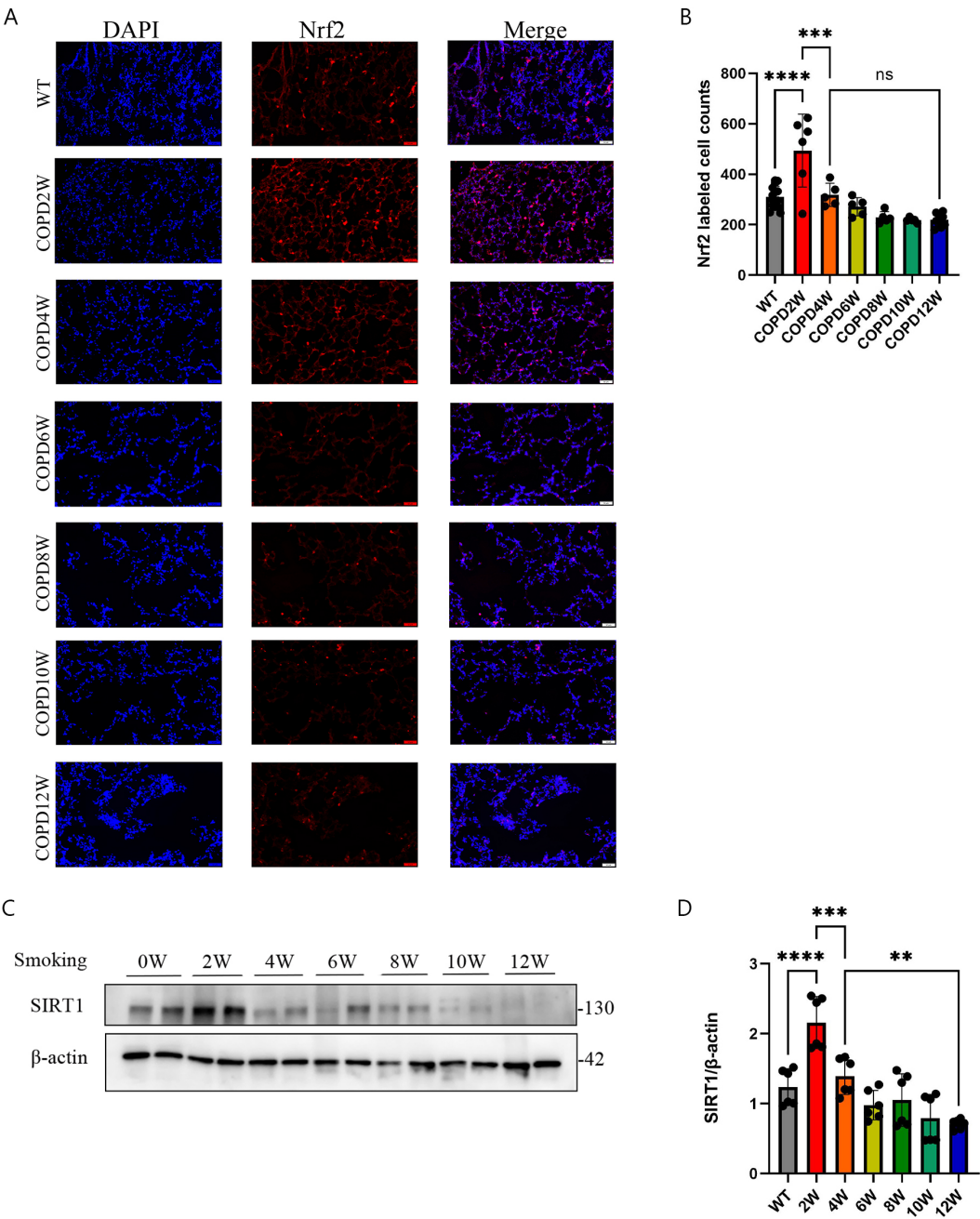


Figure 6

Temporal Changes in SIRT1 and Nrf2 Molecules Following CS Exposure

(A) Immunofluorescence images of Nrf2 for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of Nrf2 fluorescent positive cells for each group. (C) Expression of SIRT1 in lung tissues detected by Western Blot for each group. (D) Relative protein expression level of SIRT1/ β -actin for each group.

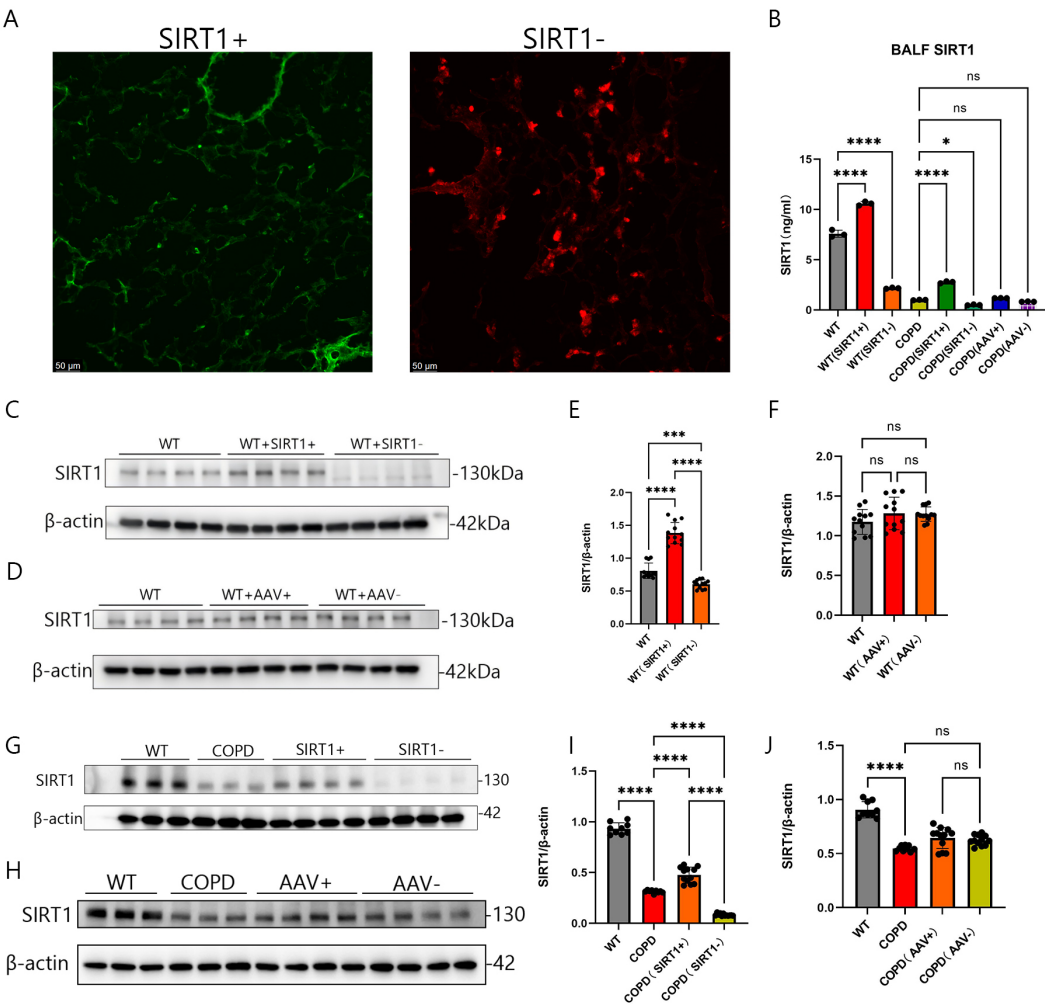


Figure 7

Validation of SIRT1 Overexpression and Inhibition Models

(A) Fluorescent images of lung tissue adenovirus for SIRT1 overexpression and inhibition. (B) Changes in SIRT1 content in bronchoalveolar lavage fluid (BALF) of each group. (C) Expression of SIRT1 in lung tissues of WT, WT + SIRT1 overexpression, and WT + SIRT1 inhibition groups detected by Western Blot. (D) Expression of SIRT1 in lung tissues of WT, WT + blank overexpression (AAV+), and WT + blank inhibition (AAV-) groups detected by Western Blot. (E) Relative protein expression level of SIRT1/ β -actin for each group. (F) Relative protein expression level of SIRT1/ β -actin for each group. (G) Expression of SIRT1 in lung tissues of WT, COPD, COPD + SIRT1 (+), and COPD + SIRT1 (-) groups detected by Western Blot. (H) Expression of SIRT1 in lung tissues of WT, COPD, COPD + AAV (+), and COPD + AAV (-) groups detected by Western Blot. (I) Relative protein expression level of SIRT1/ β -actin for each group. (J) Relative protein expression level of SIRT1/ β -actin for each group.

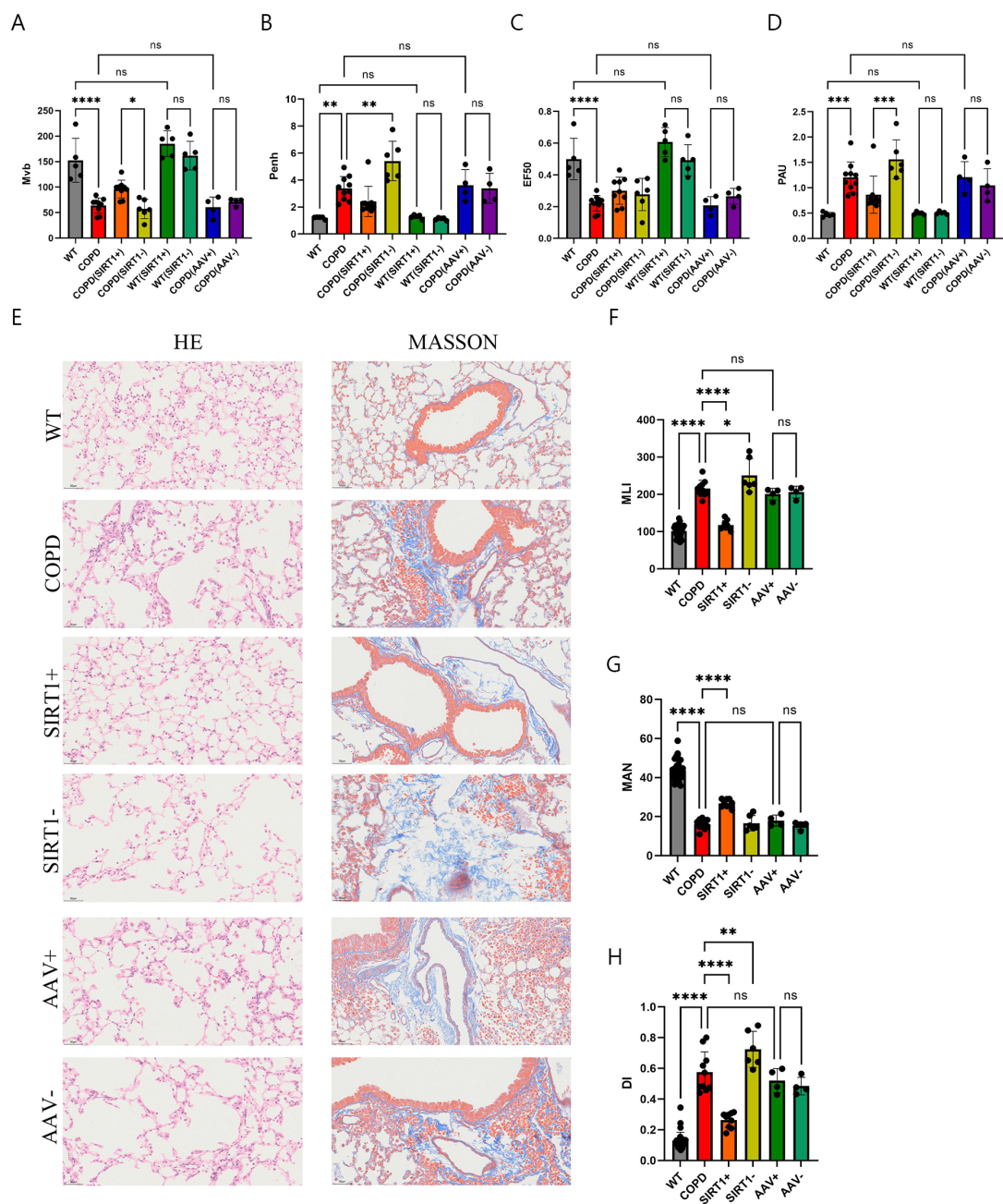


Figure 8

Effects of SIRT1 Overexpression and Inhibition on Lung Function and Lung Histopathology in the COPD Model

(A) Measurement of minute ventilation (Mvb) by non-invasive lung function test for each group. (B) Measurement of airway narrowing index (Penh) by non-invasive lung function test for each group. (C)

Measurement of mid-expiratory flow (EF50) by non-invasive lung function test for each group. (D) Measurement of apnea index (PAU) by non-invasive lung function test for each group. (E) HE and MASSON staining of mouse lung tissues for each group (magnification $\times 200$; scale bar = 50 μm). (F) Mean linear intercept (MLI) index for each group. (G) Number of alveoli per field of view (MAN) index for each group. (H) Alveolar destruction index (DI) for each group.

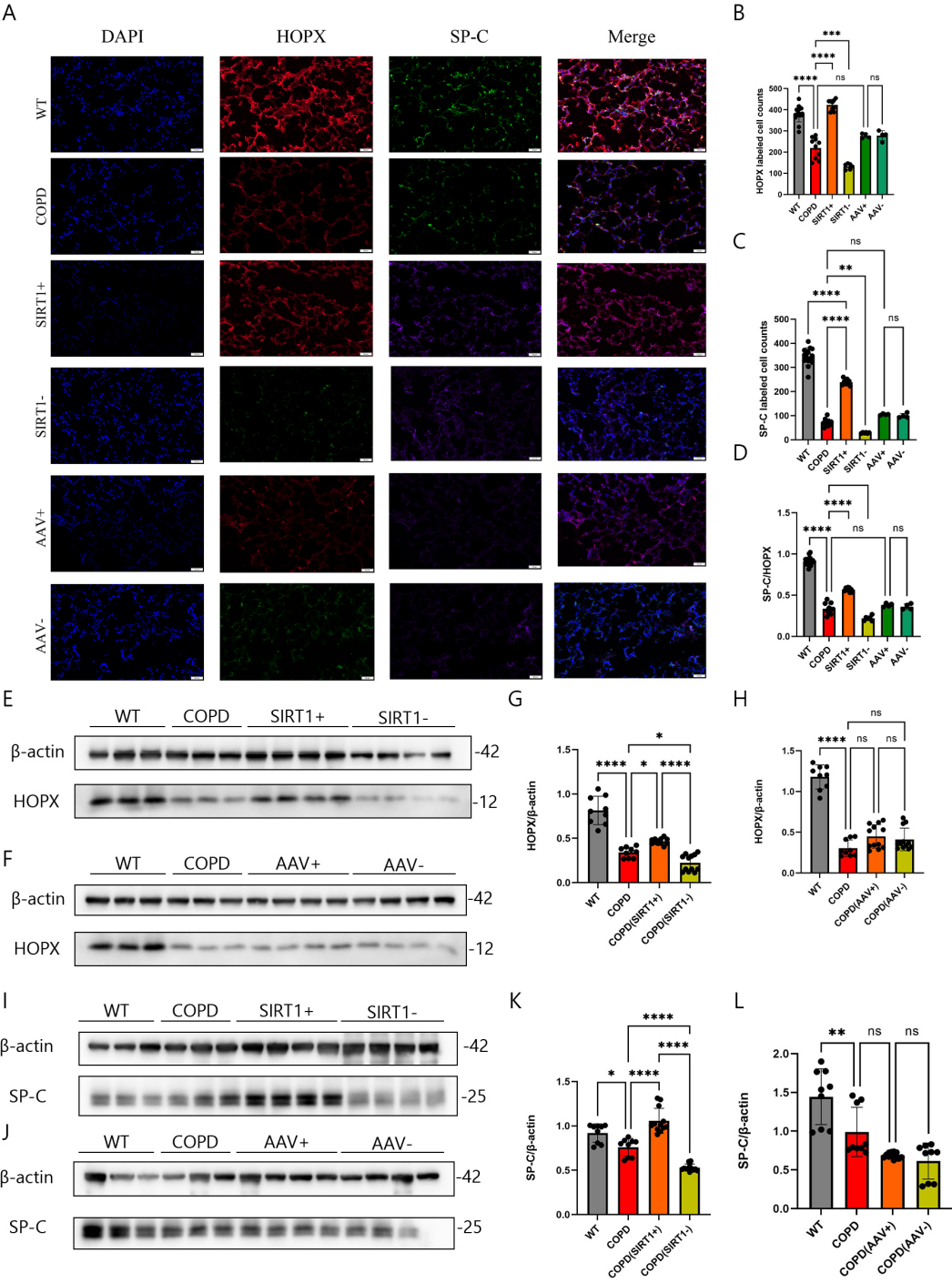


Figure 9

Effects of SIRT1 Overexpression and Inhibition on the Number of Alveolar Epithelial Cells

(A) Immunofluorescence images of HOPX and SP-C for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of HOPX fluorescence-positive cells for each group. (C) Count of SP-C fluorescence-positive cells for each group. (D) SP-C/HOPX ratio for each group. (E) Expression of HOPX in lung tissues of SIRT1 overexpression and inhibition groups detected by Western blot. (F) Expression of HOPX in lung tissues of blank overexpression and inhibition groups detected by Western blot. (G-H) Relative protein expression level of HOPX/ β -actin for each group. (I) Expression of SP-C in lung tissues of SIRT1 overexpression and inhibition groups detected by Western blot. (J) Expression of SP-C in lung tissues of blank overexpression and inhibition groups detected by Western blot. (K-L) Relative protein expression level of SP-C/ β -actin for each group.

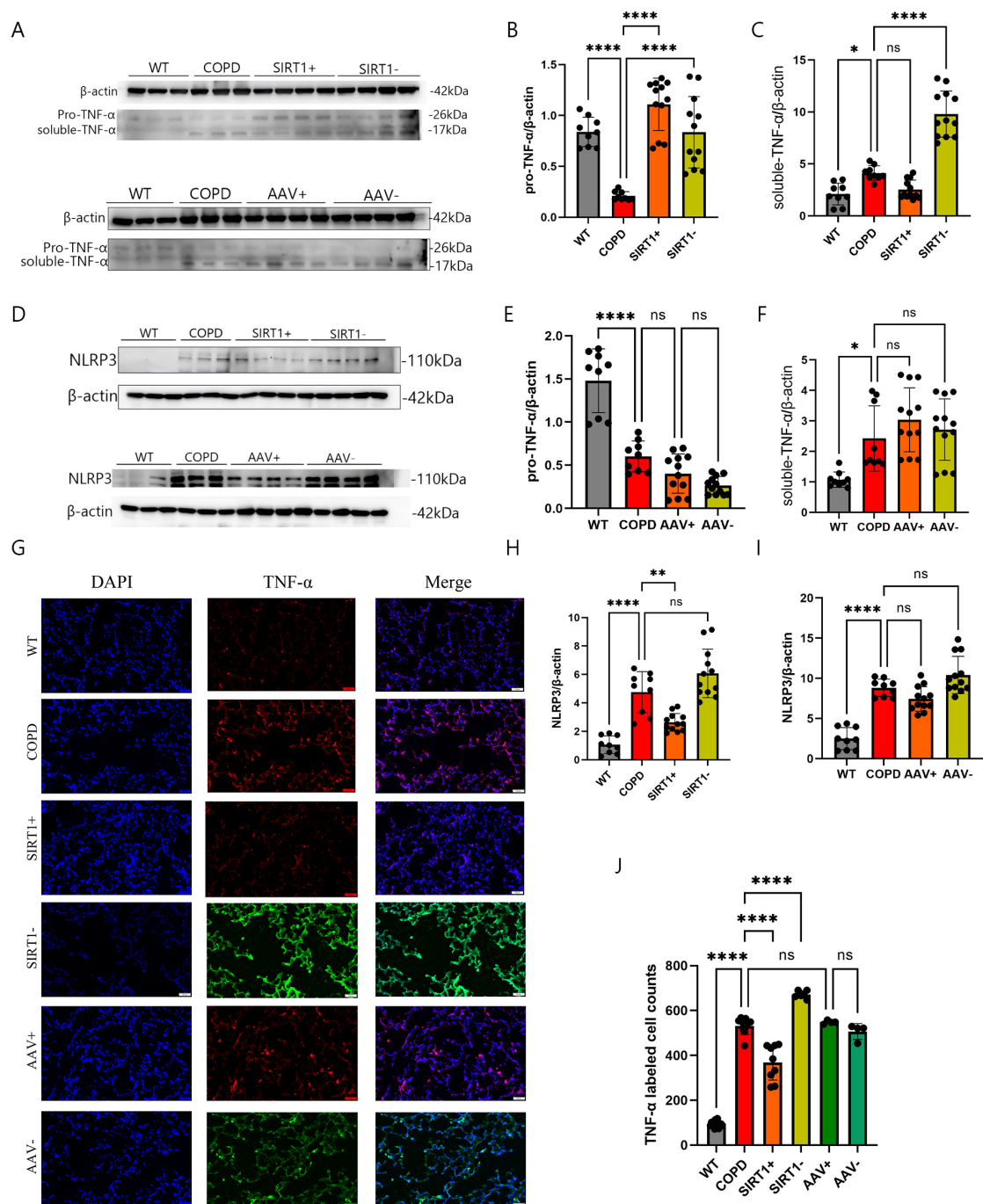


Figure 10

Effects of SIRT1 Overexpression and Inhibition on Inflammation in the Cigarette Smoke (CS)-Exposed COPD Model

(A) Expression of TNF- α in lung tissues of each group detected by Western blot. (B) Relative protein expression level of pro-TNF- α / β -actin for each group. (C) Relative protein expression level of soluble-

TNF- α / β -actin for each group. (D) Expression of NLRP3 in lung tissues of each group detected by Western blot. (E) Relative protein expression level of pro-NLRP3/ β -actin for each group. (F) Relative protein expression level of soluble-NLRP3/ β -actin for each group. (G) Immunofluorescence images of TNF- α for each group (magnification $\times 200$; scale bar = 50 μ m). (H-I) Relative protein expression level of NLRP3/ β -actin for each group. (J) Count of TNF- α fluorescence-positive cells for each group.

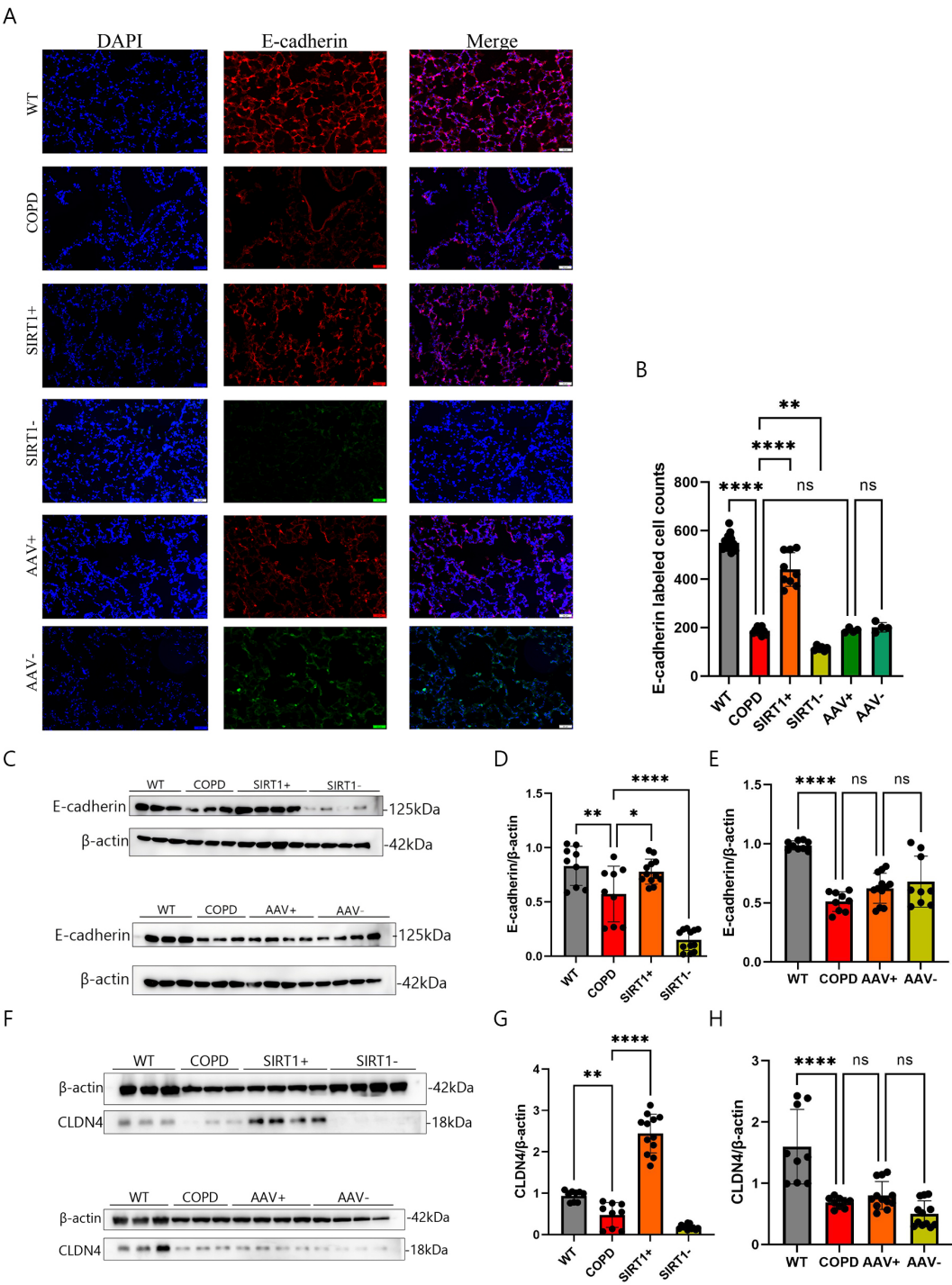


Figure 11

Effects of SIRT1 Overexpression and Inhibition on Barrier Function in the Cigarette Smoke (CS)-Exposed COPD Model

(A) Immunofluorescence images of E-cadherin for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of E-cadherin fluorescence-positive cells for each group. (C) Expression of E-cadherin in lung tissues of each group detected by Western blot. (D-E) Relative protein expression level of E-cadherin/ β -actin for each group. (F) Expression of CLDN4 in lung tissues of each group detected by Western blot. (G-H) Relative protein expression level of CLDN4/ β -actin for each group.

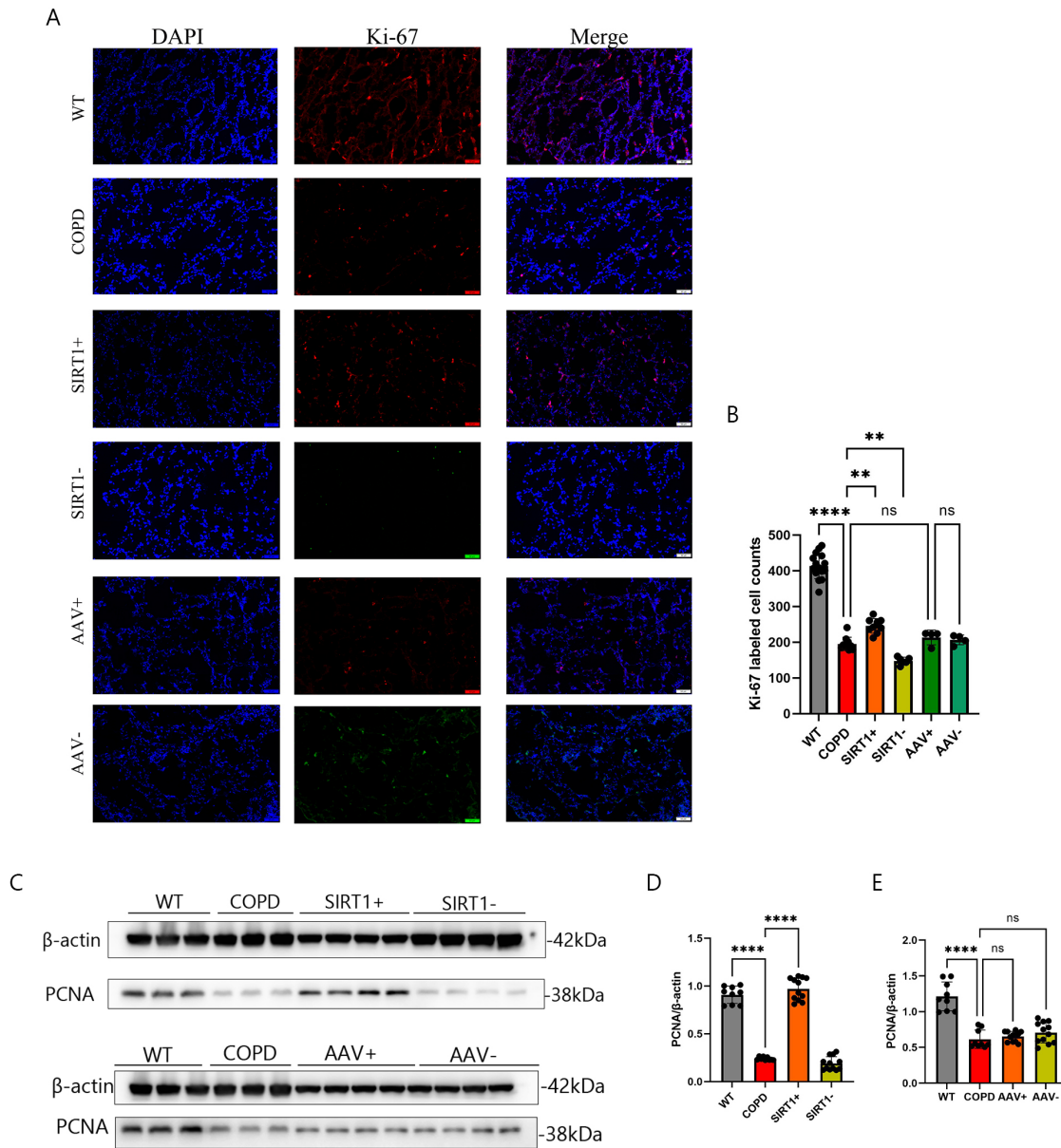


Figure 12

Effects of SIRT1 Overexpression and Inhibition on Proliferative Function in the Cigarette Smoke (CS)-Exposed COPD Model

(A) Immunofluorescence images of Ki-67 for each group (magnification $\times 200$; scale bar = 50 μm). (B) Count of Ki-67 fluorescence-positive cells for each group. (C) Expression of PCNA in lung tissues of each

group detected by Western blot. (D-E) Relative protein expression level of PCNA/ β -actin for each group.

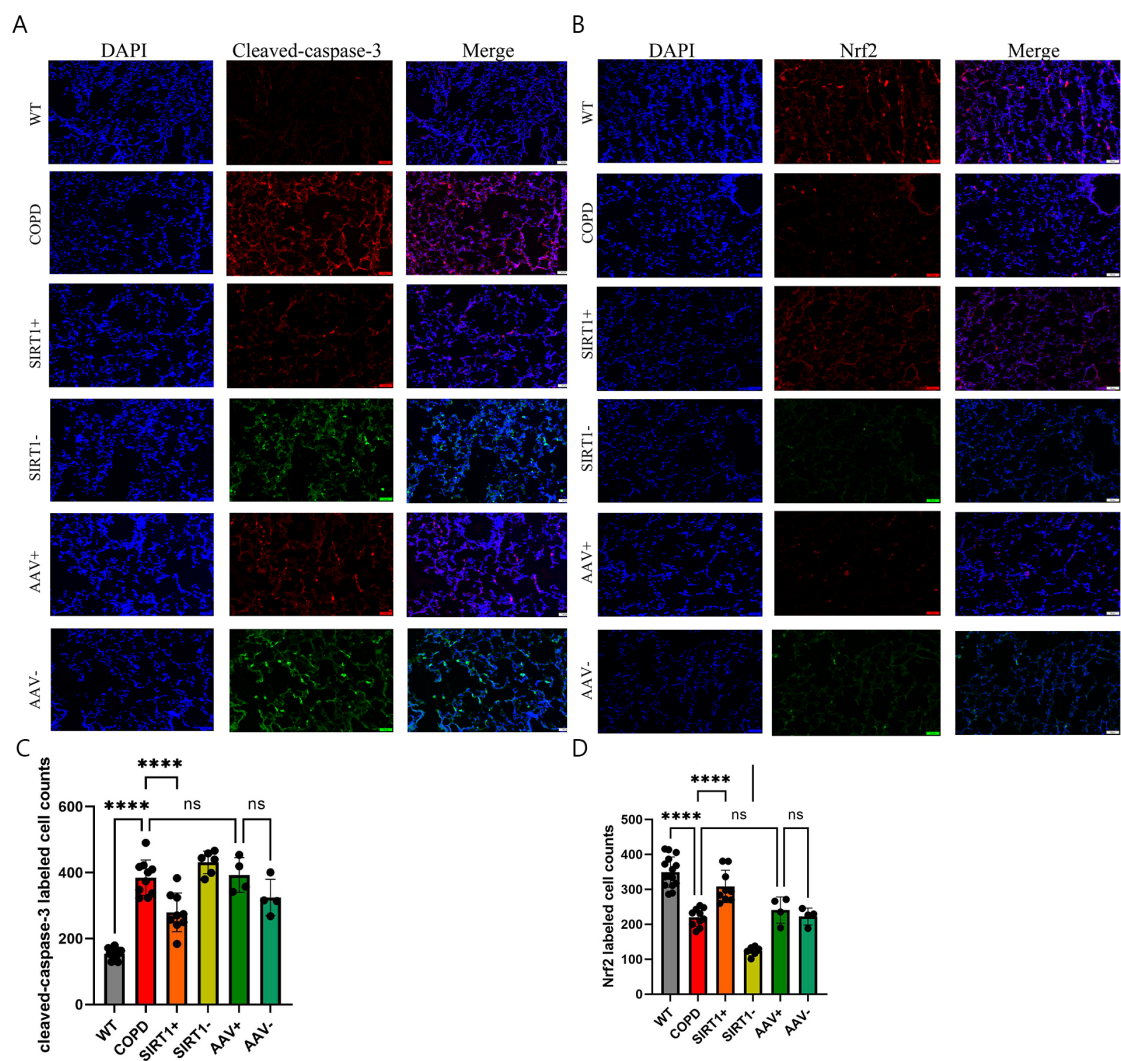


Figure 13

Effects of SIRT1 Overexpression and Inhibition on Apoptotic Function and Nrf2 in the Cigarette Smoke (CS)-Exposed COPD Model

(A) Immunofluorescence images of Cleaved-caspase-3 for each group (magnification $\times 200$; scale bar = 50 μm). (B) Immunofluorescence images of Nrf2 for each group (magnification $\times 200$; scale bar = 50 μm). (C) Count of Cleaved-caspase-3 fluorescence-positive cells for each group. (D-E) Count of Nrf2 fluorescence-positive cells for each group.

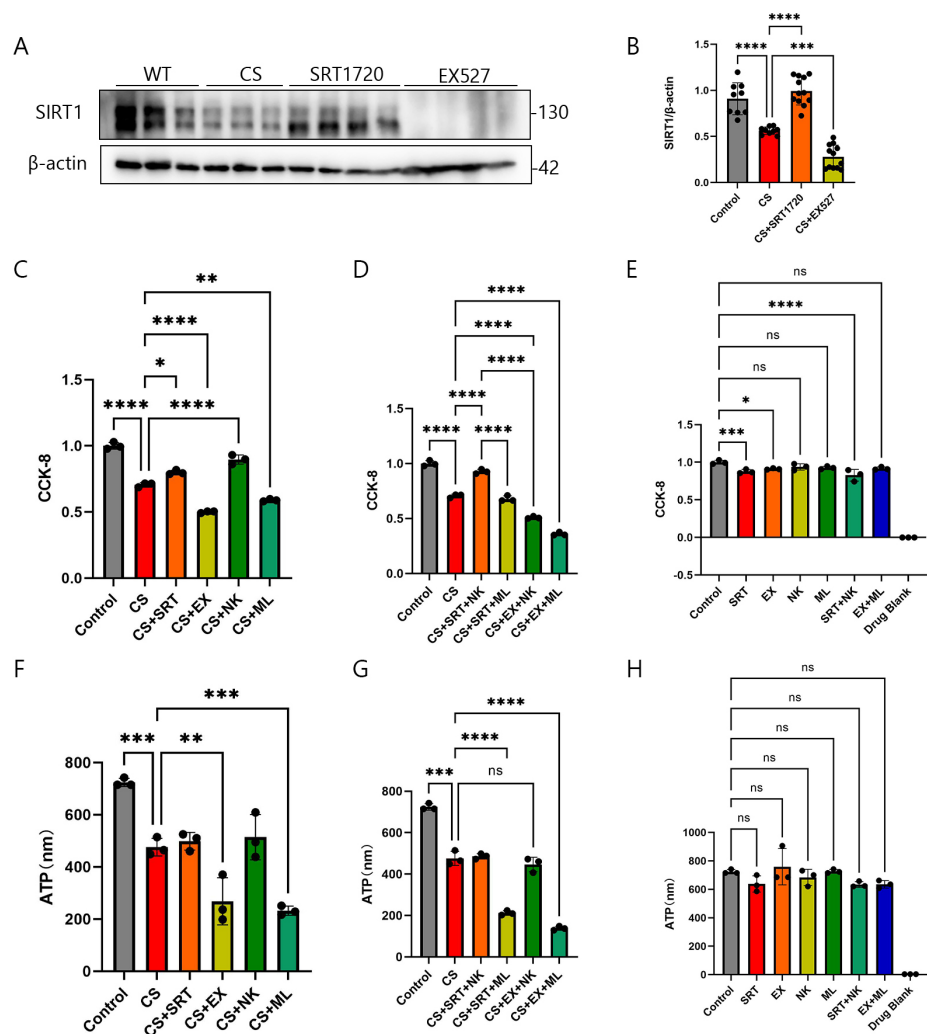


Figure 14

Validation of the SIRT1 Agonist and Inhibitor Models in A549 Cells and CCK-8 and Cell Viability Assays

(A) Expression of SIRT1 in A549 cells after treatment with WT, CS, SIRT1 agonist (SRT1720), and SIRT1 inhibitor (EX527), detected by Western Blot. (B) Relative protein expression level of SIRT1/ β -actin for each group. (C-E) Cell survival rate measured by CCK-8 assay for each group. (F-H) ATP content measured by cell viability assay for each group.

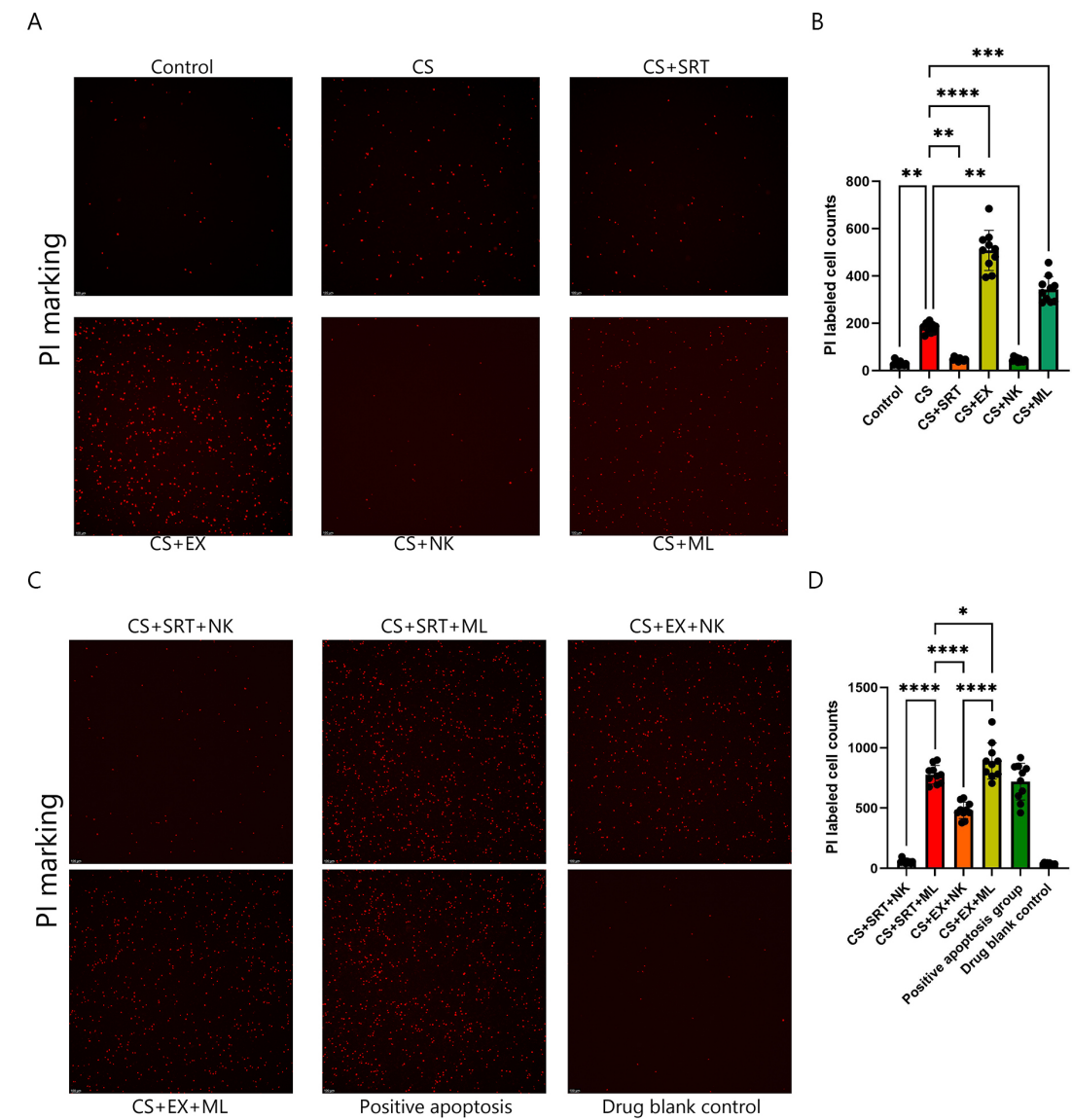


Figure 15

PI Fluorescence Cell Apoptosis Results in A549 Cells for Each Group

(A, C) PI fluorescence images of cell apoptosis for each group (magnification $\times 100$; scale bar = 100 μm).

(B, D) Count of PI fluorescence-positive cells for each group.