

Novel phosphodiesterase-4 inhibitor mufemilast protects against smoke inhalation-induced lung injury

Bin Liu

iamicehe@163.com

Characteristic Medical Center of Chinese People's Armed Police Force

Long Yang

Tianjin University

Ningning Shao

Tianjin University

Wei Fu

Tianjin Hemay Pharmaceutical Co., Ltd.

Shunjun Tang

Tianjin University

Aiyi Hao

Tianjin University

Hesheng Zhang

Tianjin Hemay Pharmaceutical Co., Ltd.

Richard Jones

Tianjin Hemay Pharmaceutical Co., Ltd.

Shaobo Chen

Characteristic Medical Center of Chinese People's Armed Police Force

Bin Su

Characteristic Medical Center of Chinese People's Armed Police Force

Peter Barnes

Imperial College London

Jinrui Dong

Tianjin University

Research Article

Keywords: Smoke inhalation injury, PDE4, Multi-omics, Inflammation, Fibrosis

Posted Date: August 27th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10644113/v1>

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Additional Declarations: Competing interest reported. Dr. R. Jones, Hesheng Zhang and Wei Fu are employees of Hemay Pharmaceutical Co., Ltd., which may be considered a potential conflict of interest.

Abstract

Introduction:

Smoke inhalation injury (SII) is a leading cause of acute lung injury and subsequent fibrotic remodeling, yet effective targeted therapies remain limited. Phosphodiesterase 4 (PDE4) signaling has been implicated in inflammatory and fibrotic lung diseases; however, its contribution to SII pathogenesis remains poorly understood.

Objectives

This study aimed to elucidate the role of PDE4 signaling, particularly PDE4B, in the development of smoke-induced acute and chronic lung injury and to evaluate the therapeutic potential of PDE4 inhibition.

Methods

Acute and chronic mouse models of wood smoke exposure were established to investigate the pathological role of PDE4 signaling in SII. Pharmacological inhibition using mufemilast and adeno-associated virus-mediated Pde4b knockdown were employed to assess therapeutic efficacy. Histological, molecular, transcriptomic, and metabolomic analyses were performed in murine tissues and human samples. Mechanistic studies were conducted in human airway epithelial cells exposed to smoke extract.

Results

Wood smoke exposure induced severe pulmonary inflammation, alveolar damage, mucus hypersecretion, and activation of NF- κ B, STAT3, and PDE4B signaling. Mufemilast markedly attenuated acute lung inflammation and exhibited superior anti-inflammatory efficacy compared with dexamethasone. Transcriptomic analyses demonstrated broad suppression of inflammatory pathways following PDE4 inhibition. In vitro, mufemilast alleviated smoke extract-induced oxidative stress, ferroptosis, and epithelial cell injury. In chronic exposure models, mufemilast significantly reduced pulmonary fibrosis and airway remodeling. Genetic silencing of Pde4b recapitulated the protective effects of pharmacological inhibition. Metabolomic profiling identified conserved metabolic alterations in murine and human SII, including decreased levels of D-pinitol.

Conclusion

PDE4B is a critical regulator of smoke-induced pulmonary inflammation, epithelial injury, and fibrotic remodeling. Pharmacological or genetic inhibition of PDE4B confers robust protection against both

acute and chronic manifestations of SII, supporting PDE4B-targeted therapy as a promising treatment strategy for smoke-induced lung injury and related fibrotic lung diseases.

Introduction

Inhalation injury is an acute injury to the respiratory tract caused by exposure to toxic combustion byproducts in smoke, and contributes to approximately one-third of extensive burns and 8% of burn-related hospital admissions [1]. Independently of cutaneous burns, smoke inhalation injury (SII) significantly increases mortality independently of cutaneous burns and accounts for 80–90% of burn-related deaths[2, 3]. As a result of global warming, there is an increasing frequency and duration of wildfires that has been linked to higher incidence and severity of respiratory and cardiovascular diseases[4]. SII is characterized by its sudden onset complex pathophysiology and prolonged effects. This involves a heterogeneous mixture of toxic, carcinogenic, and irritant volatile chemicals as well as inhalable particulate matter that can penetrate deep into the airways and reach the systemic circulation[5, 6]. The ensuing inflammatory response involves both pulmonary and systemic inflammation, leading to genotoxicity, immune dysregulation, and activation of innate immune cell populations [7, 8]. With persistent inflammation, the lung injury progresses to tissue remodeling that may progress to pulmonary fibrosis [9, 10]. The increased risk of pulmonary fibrosis among rescue workers in the World Trade Center 9/11 incident, who were exposed to high levels of particulates, is well documented [11]. Despite advances in SII management, morbidity and mortality remain high, and therapeutic options for managing SII are limited. The glucocorticoid dexamethasone, a commonly used anti-inflammatory agent, has limited efficacy in preventing long-term fibrosis and is frequently associated with adverse side effects, especially with prolonged use [12]. This underscores the need for alternative treatments that target both the acute inflammatory response and the SII-induced chronic fibrotic remodeling.

Cyclic nucleotide phosphodiesterase (PDE) plays a crucial role in intracellular signaling by metabolizing the second messenger cyclic-3',5'-adenosine monophosphate (cAMP) and cyclic guanosine-3',5'-monophosphate (cGMP). The PDE superfamily consists of 11 gene families that comprise more than 100 isoforms with diverse functions[13]. Among these, the PDE4 enzyme family, is of particularly relevant in pulmonary disease, as they selectively hydrolyze cAMP into 5'-AMP and thereby regulate inflammation, immune responses, and fibrotic pathways [14]. Roflumilast, a selective PDE4 inhibitor (PDE4i), is the only PDE4i that has been approved for the treatment of severe chronic obstructive pulmonary disease (COPD) [15], a chronic inflammatory condition due to chronic exposure to cigarette smoke and inhaled PM that shares many pathological features with SII[16]. Two other PDE4i, apremilast and crisaborole have been approved for psoriatic arthritis and mild-to-moderate atopic dermatitis, respectively; but their use in pulmonary diseases remains under investigation[17]. A PDE4B selective inhibitor, nerandomilast, has recently been approved for the treatment of idiopathic pulmonary fibrosis (IPF) and progressive pulmonary fibrosis[18]. The clinical utility of oral PDE4iis frequently limited by their on-target adverse effects, including gastrointestinal symptoms (nausea, vomiting, diarrhea) and headaches, which are dose-limiting and remain a key challenge to their broader therapeutic

application[19]. Mufemilast is a novel PDE4i that has recently been approved for the treatment of psoriasis and currently in phase II clinical trial for inflammatory bowel disease (NCT05486104, August 3, 2022). Mufemilast has been well tolerated in long-term clinical trials [20].

Despite the known benefits of PDE4i in lung diseases, their therapeutic potential for managing acute lung injury and subsequent fibrosis has not been explored. Additionally, while prior studies have primarily focused on the effects PDE4i in cigarette smoke induced lung responses, this study is the first to explore the role of PDE4 in response to wood smoke exposure. This work is particularly relevant in the context of wildfires, as exposure to wildfire wood smoke has been linked to heightened lung inflammation, exacerbations of COPD, and other respiratory conditions[4]. Building on previous studies [21] and our preliminary results, we developed two models to investigate SII: an acute inflammation model at 3 days post-exposure of wood smoke exposure and a pulmonary fibrosis model at 21 days post-exposure. To determine the therapeutic value of PDE4i in these models, we assessed its effects on inflammation, fibrosis, and tissue regeneration using a combination of animal and cellular studies. Additionally, we integrated serum metabolomics from both mice and patients exposed to wood smoke, along with lung transcriptomics, to uncover the molecular mechanisms and systemic responses that underlie PDE4i-mediated protection to identify novel targets, biomarkers and strategies for clinical intervention in SII.

Material and methods

Mice. Animal care and experimental protocols conformed to the recommendations of the 8th Edition of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH, revised 2011). All procedures were performed in accordance with the Animal Management Rules of China (Documentation 55, 20 01, Ministry of Health, the People's Republic of China).

C57BL/6J mice (males, 8 weeks) were used to generate the SII model. In our preliminary studies, a mouse model was established using a custom-designed smoke-exposure system. Apple wood was used as the smoke-generating fuel and placed in a smoke generator chamber. The system was maintained at 37°C, with the carbon monoxide (CO) concentration controlled at 3,000 ppm[22]. Mice were placed in an exposure chamber connected to a smoke generator. After initiating combustion, animals were exposed to smoke for 15 min. Following exposure, the mice were immediately transferred to a fresh-air environment for recovery. The animals were kept at the Tianjin University Laboratory Animal Center and housed at 21–25°C, with 40–50% humidity, a 12-h light: dark cycle, and free access to drinking water and standard feed. The animals were monitored daily.

Antibodies. The following antibodies were used in this study: ACTA2 (Abcam, ab124964); ERK1/2 (Cell Signaling, 4695); phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴; Cell Signaling, 4370); fibronectin (FN; Abcam, ab268020); NF-κB (Cell Signaling, 3033); phospho-NF-κB (Ser⁵³⁶; Cell Signaling, 3031); STAT3 (Cell Signaling, 9039); phospho-STAT3 (Tyr⁷⁰⁵; Cell Signaling, 9045); GAPDH (Cell Signaling, 2118); PDE4B (Abcam, ab170939; Invitrogen, MA5-25677); HRP-conjugated secondary antibodies (rabbit, Cell Signaling, 7074; mouse, Abcam, ab205719); Alexa Fluor 488-conjugated rabbit secondary antibody

(Abcam, ab150077); PE rat anti-mouse Siglec-F (BD Biosciences, 552126); PE-Cy7 rat anti-mouse CD11b (BD Biosciences, 557657); FITC rat anti-mouse CD19 (BD Biosciences, 557398); PerCP rat anti-mouse GR-1 (BioLegend, 108426); Brilliant Violet 605 anti-mouse CD3 (BioLegend, 100237); APC anti-mouse CD45 (BioLegend, 103112); and Pacific Blue anti-mouse CD11c (BioLegend, 117322).

Mufemilast. Mufemilast was provided by Hemay Pharmaceutical Technology Co., Ltd. (CAS: 1255909-03-5).

Chemicals. AWE was purchased from Shanxi Shuo Hang Biotechnology Co., Ltd. 4',6-diamidino-2-phenylindole (DAPI, D1306, Thermo Fisher), Dexamethasone (Dex, D137736, Aladdin), Paraformaldehyde (PFA, 28908; Thermo Fisher), Triton X-100 (T8787, Sigma).

Cell Culture. Human non-small cell lung cancer cells (A549) and normal human lung epithelial cells (BEAS-2B) were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For stimulation, cells were treated with 10% AWE, Dex, or mufemilast for 24 h, as described in the text and/or Fig. legends.

Blood routine examination and biochemical parameters. Fresh blood was collected by cardiac puncture at the designated postoperative time points. Samples were divided into two portions and stored in either anticoagulant (EDTA-K2) or non-anticoagulant tubes. Blood collected in anticoagulant tubes was analyzed for complete blood count. Samples in non-anticoagulant tubes were allowed to clot at room temperature for 1 h, after which serum was isolated. Biochemical parameters related to SII-associated organ damage, including liver function (ALT, AST, ALP) and kidney function ((UA, CREA, urea, blood urea nitrogen (BUN)), were measured using an automated biochemical analyzer (cobas C311, Roche, Basel, Switzerland).

Analysis of the bronchoalveolar lavage fluid (BALF) total and differential cell counts. Following euthanasia, the trachea was cannulated and the chest cavity opened to collect BALF from the right lung. The lung was lavaged three times with 1.5 mL normal saline, and the recovered fluid was pooled. The BALF was centrifuged, and the supernatant was stored at -80°C for subsequent analyses. Red blood cells in the cell pellet were lysed, and the total cell count was determined. For differential cell counts, cells were incubated with an antibody master mix at room temperature in the dark for 30 min and analyzed by flow cytometry. Immune cells were gated as CD45⁺ cells; macrophages were gated as CD11c⁺ and Siglec-F⁺ cells; eosinophils were gated as CD11c⁻ and Siglec-F⁺ cells; neutrophils were gated as GR-1⁺ and CD11b⁺ cells; lymphocytes were gated as CD3⁺ and CD19⁻ cells.

mRNA expression analysis by RT-qPCR. Total RNA was extracted from snap-frozen tissues using the RNeasy™ Animal RNA Isolation Kit with Spin Column (R0027, Beyotime). cDNA was synthesized using the cDNA Synthesis Master Mix (D7185, Beyotime). Quantitative PCR was performed in duplicate with SYBR Green (D7260, Beyotime) on a CFX96 system (Bio-Rad) for 40 cycles. Gene expression levels were

normalized to Gapdh mRNA, and relative fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are provided in **Table S1**.

Western blotting. Mouse tissues were surgically excised, washed in cold PBS, and homogenized on ice in lysis buffer to prepare tissue lysates. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% skimmed milk powder, incubated overnight at 4°C with primary antibodies, and washed with TBS containing 0.1% Tween-20. After incubation with HRP-conjugated anti-rabbit secondary antibodies for 2 h at room temperature, protein bands were visualized using an ECL detection system. Band intensities were quantified by densitometry with ImageJ software.

Flow cytometry. Cell death was assessed using the Dead Cell Apoptosis Kit with Annexin V-FITC and PI (CA1020; Beyotime). Lipid peroxidation was evaluated by staining with Liperfluo dye (L248; Dojindo), and intracellular Fe^{2+} levels were measured using FerroOrange dye (F374; Dojindo). Stained cells were analyzed by flow cytometry (Beckman) and processed with CytExpert software. For each sample, 10,000 events were initially gated based on FSC/SSC. Debris (SSC-A vs FSC-A) and doublets (FSC-H vs FSC-A) were excluded. Thresholds for positive and negative staining were set at 10^4 .

ROS Detection. Cells were seeded in a 24-well plate at 5×10^3 cells per well. Twenty-four hours after treatment with AWE, Dex, or mufemilast, cells were washed and incubated with 10 μ M DCFDA (S0033, Beyotime) for 30 min at 37°C in the dark, then rinsed with the provided dilution buffer according to the manufacturer's instructions. Fluorescent signals from live cells with positive DCF staining were captured using a fluorescence microscope (Nikon) with a filter set suitable for FITC.

Mitochondrial membrane potential detection. Cells were seeded in a 24-well plate at 5×10^3 cells per well. Twenty-four hours after treatment with AWE, Dex, or iPDE4, cells were washed and incubated with 200 nM MitoTracker Red CMXRos (C-1049B; Beyotime) for 30 min at 37°C in the dark, then rinsed with the supplied dilution buffer according to the manufacturers instructions. Mitochondrial membrane potential was visualized in live cells with positive MitoTracker staining using a fluorescence microscope (Nikon) equipped with a filter set suitable for PE.

Histology and Immunohistochemistry. Mouse tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned at 7 μ m thickness. Sections were deparaffinized with xylene and rehydrated through graded ethanol. Hematoxylin and eosin (H&E), Masson's trichrome, and Prussian Blue staining were performed following the manufacturers' protocols. After staining, sections were washed with distilled water, dehydrated in ethanol, cleared in xylene, and mounted with neutral resin. For immunohistochemistry, antigen retrieval was performed using citrate buffer, and sections were incubated overnight at 4°C with an anti-mouse PDE4B antibody. Fibrosis areas were quantified using ImageJ software, and fibrotic severity was graded using the modified Ashcroft score [23]. Two independent analysts, blinded to treatment groups, performed the scoring, and the results were averaged for statistical analysis.

ELISA. The levels of TNF- α , IL-1 β , IL-6, IL-8, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (g-GT), uric acid (UA), creatinine (CRE) and blood urea nitrogen (BUN), in serum were determined by Quantikine sandwich ELISA kits (mlbio, Shanghai, China). The cytokine concentration was measured at 450 nm wavelength of absorbance, and calculated by GraphPad Prism linear regression analysis.

Micro-computed tomography (CT). Mice were anesthetized via intraperitoneal injection of 1% pentobarbital sodium and positioned supine on a dedicated imaging bed for lung CT scanning using a SkyScan 1276 system (Bruker micro-CT, Germany). Preliminary images were acquired, with the lungs specifically targeted for non-gated imaging. Spiral CT acquisition and 3D reconstruction were performed using a high-resolution protocol with the following parameters: 80 kV, 200 μ A tube current, 0.25 mm aluminum X-ray filter, 8 μ m pixel size, 0.40 degree rotation step, and event synchronization at 650 ms.

Gene Knockdown with AAV-shRNA construction and preparation. AAV-LungM3 carrying the PDE4B shRNA (GGAGAAGGCCACAGCTATTTC) or the non-targeting control shRNA (GCACTACCAGAGCTAACTCA) were generated by Obio Technology (Shanghai, China). 5×10^{10} viral genome copies diluted in 50 μ L sterile saline were injected into each mouse lung via intratracheal. After three weeks, mice were exposed to smoke. Body weight and survival were monitored every day. 3 or 21 days post-exposure, subsequent experiments were conducted.

Metabolic profiling. The serum samples (100 μ L) from human and mice were placed in the EP tubes and resuspended with prechilled 80% methanol by well vortex. Then the samples were incubated on ice for 5 min and centrifuged at 15,000 g, 4°C for 20 min. Some of supernatant was diluted to final concentration containing 53% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then were centrifuged at 15000 g, 4°C for 20 min. The Q Exactive HF-X mass spectrometer was operated in positive/negative polarity mode to detect metabolites eluted from the column. The Compound Discoverer 3.1 (CD3.1, Thermo Fisher) software was used for the acquired MS data pretreatments. The KEGG and HMDB databases were used to annotate the metabolites with the exact molecular mass data (m/z) of samples. Student's t test was used to detect the differences in metabolite concentrations between 2 groups. Volcano plots were used to filter metabolites of interest, which were based on $\log_2$ (FoldChange) and $-\log_{10}(P \text{ value})$ of metabolites by ggplot2 in R language. For clustering heatmaps, the data were normalized using Z scores of the intensity areas of differential metabolites and were plotted by Pheatmap package in R language. The correlation between differential metabolites was analyzed by cor() in R language (method = Pearson). Statistically significant of correlation between differential metabolites was calculated by cor.mtest() in R language. The experiments were supported by Novogene Co., Ltd. (Beijing, China).

RNA-Seq and bioinformatics analysis. The RNA extraction, reverse transcription, cDNA library construction and sequencing were performed by Novogene Company. Reads were mapped to mouse genome mm10 using Hisat2 software (v2.0.4). Mapped reads were assigned to mouse RefSeq genes using feature Counts and NCBI mouse annotation build 37.2. DESeq2 was used for differential gene

expression analysis among treatment groups. GSEA was performed on genes ranked by their differential expression T-statistic using the cluster Profiler package in R with GO, KEGG.

Human serum sample. In total, serum samples from 20 patients admitted to the emergency department with SII caused by indoor fire smoke exposure (male: n = 13, average age of 49.9 years old; Female: n = 7, average age of 48.6 years old) and 20 healthy individuals (male: n = 9, average age of 43.2 years old; Female: n = 11, average age of 50.8 years old) were analyzed. Detailed information is provided in **Table S2**.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 10.0). For comparisons between two groups, a two-tailed Student's t-test was used. For comparisons involving more than two groups, one-way ANOVA followed by Tukey's post hoc test was applied. *P* value < 0.05 was considered statistically significant. For all bar graphs, the mean $\pm$ SEM was plotted unless otherwise indicated.

Results

Serum metabolomic profiles after SII in mouse and human

First, based on previous studies and our preliminary experimental findings, we established both an acute inflammatory model (3 days post-smoke exposure) and a chronic pulmonary fibrosis model (21 days post-exposure) via a smoke exposure system (**Fig. S1**), in order to investigate the progression of disease following smoke inhalation. To validate the reliability of these models, we collected serum samples from patients with burn-associated inhalation injury at 3 days post-injury, as well as from mice at the corresponding 3-day time point, and performed metabolomic analyses. Principal component analysis (PCA) of serum metabolite profiles in mice with SII compared to SA controls, showed distinct metabolic alterations in response to smoke exposure. Metabolite profiles from patients with smoke injury compared to healthy controls show a similar pattern, indicating cross-species similarities (**Fig. 1A, B**). Volcano plots depicting differentially regulated metabolites in human and mouse serum, identifying key metabolites upregulated in SII, show association with inflammation, oxidative stress, and metabolic disturbances (**Fig. 1C, D**). KEGG pathway enrichment analysis revealed significant perturbations in amino acid and lipid metabolism, as well as xenobiotic biodegradation, which reflects immune activation and oxidative stress that are characteristic features of SII. Notably, metabolic reprogramming in SII was associated with the upregulation of pathways that are related to lipid and carbohydrate metabolism, along with cell growth (**Fig. 1E, F**). A conserved set of metabolites was identified across species, and this highlighted the presence of common metabolic pathways in SII in mouse and human (**Fig. 1G, H**). The chord diagram (**Fig. S1C, D**) depicts the metabolite correlations. Among these, D-Pinitol (AUC > 0.7) —an anti-inflammatory inositol derivative[24, 25]—was the only metabolite that was consistently down-regulated in both humans and mice, and thus may be a promising potential diagnostic biomarker for early metabolic disruption following smoke exposure (**Fig. S1E-G**). Pathway enrichment analyses revealed the significant involvement of tryptophan and oxocarboxylic acid metabolism (**Fig. 1I, J**), this

suggests metabolic reprogramming in response to smoke inhalation. Tryptophan metabolism—which is closely linked to immune modulation and oxidative stress—was upregulated, which suggests its role in inflammation and cellular repair.

Building on these metabolomic findings, we next investigated the expression of PDE4 family genes in lung tissue to identify potential molecular regulators linking metabolic reprogramming and inflammatory signaling. Among the PDE4 isoforms, *Pde4b* emerged as the predominant subtype significantly upregulated in lung tissue following smoke exposure (**Fig. 1K, L**), aligning with previous studies implicating PDE4 signaling in inflammatory lung diseases and immune regulation. These findings position PDE4B as a key downstream effector and potential therapeutic target in SII-associated metabolic and inflammatory dysregulation.

Figure 1 Metabolomic profiling of SII reveals key metabolic alterations and potential biomarkers. (**A, B**) PCA of serum metabolite profiles (**A**, human, n = 20/group; **B**, mice, n = 7/group). (**C, D**) Volcano plots show the same differential metabolites in different species (**C**, human; **D**, mice). (**E, F**) Functional annotation analysis of differential metabolites (**E**, human; **F**, mice). (**G, H**) Heatmap of differential metabolites (**G**, human; **H**, mice). (**K, L**) RT-qPCR analysis of *Pde4* isoforms (*Pde4a*, *Pde4b*, *Pde4c*, and *Pde4d*) in lung tissues (**K**, Day3, **L**, Day21).

Adeno-associated virus (AAV)-mediated PDE4B knockdown confirms isoform-specific role in modulating acute lung inflammation

To directly validate the isoform-specific contribution of PDE4B to smoke-induced lung injury, we utilized adeno-associated virus (AAV)-mediated knockdown to target epithelial PDE4B expression in mouse lungs with AAV null vector as a control. Mice were administered AAV-PDE4B-shRNA via oropharyngeal instillation 3 weeks prior to smoke exposure, and this ensured sustained silencing across airway epithelial cells at the time of SII exposure (**Fig. 2A**). RT-qPCR results confirmed that AAV vector was able to suppress SII-induced PDE4B overexpression in mouse lungs (**Fig. 2B**). Histological assessment on day 3 post-smoke exposure revealed markedly reduced alveolar destruction, inflammatory cell infiltration, and mucus overproduction in *Pde4b*-knockdown mice compared to SII controls (**Fig. 2C**). These findings reinforced the protective effect of PDE4B suppression on the pulmonary structure. Flow cytometry-based analysis revealed decreased neutrophil and macrophage recruitment in BALF samples, which indicated systemic anti-inflammatory effects (**Fig. 2D**). RT-qPCR and ELISA of lung tissues and BALF confirmed significant reductions in proinflammatory cytokine levels in the PDE4B deficient mice (**Fig. 2E, F**), which mimicked the findings of pharmacological PDE4 inhibition with mufemilast. Western blot analysis further demonstrated the attenuation of the activation of STAT3 and NF- κ B pathways in PDE4B-deficient mice, and this supported the hypothesis that PDE4B is a central node role in inflammation-amplifying during acute SII (**Fig. 2G**).

Figure 2 Epithelial-specific knockdown of Pde4b by AAV delivery attenuates SII-induced acute lung injury. (A) Schematic showing AAV administration via intratracheal and SII model establishment in mice. (B) RT-qPCR analysis of PDE4B in lung tissues. (C) Representative H&E (scale bar: 200 μ m) and PAS (scale bar: 100 μ m) images of the lungs. (D) Differential cells count in the BALF. Means $\pm$ SEM, n = 5 shown. (E) ELISA-based analysis of cytokines in the BALF. Means $\pm$ SEM, n = 5 shown (F) Heatmap of the expression of key inflammatory genes in the lung. (G) Western blot images of p-STAT3, p-NF- κ B, and PDE4B in lung tissues. (H) ELISA analysis of cytokines in the serum. Means $\pm$ SEM, n = 5 shown. ****P < 0.0001. Statistics obtained from 1-way ANOVA. (macrophages: Mac, eosinophils: Eso, neutrophils: Neu, lymphocytes: Lym).

Epithelial PDE4B silencing ameliorates SII-induced fibrotic remodeling and oxidative injury

To determine whether PDE4B contributes to long-term fibrotic response to wood smoke, we extended the knockdown model to a 21-day SII exposure (Fig. 3A). RT-qPCR results confirmed that the AAV-PDE4B-shRNA vector suppressed SII-induced PDE4B overexpression in mouse lungs, whereas the control null vector was ineffective (Fig. 3B). Masson's trichrome staining revealed extensive collagen deposition, interstitial thickening, and alveolar distortion in SII mice, all of which were markedly reduced in the *Pde4b*-depleted animals (Fig. 3C-E). RT-qPCR demonstrated significant downregulation of fibrotic markers, including collagen *1a1* (*Col1a1*), *Col1a2*, *Acta2*, and *Fn*, in the *Pde4b*-knockdown group (Fig. 3F). These findings paralleled those observed with pharmacological inhibition with mufemilast. Western blotting revealed suppressed ERK activation in PDE4B-depleted lungs, which indicated reduced fibroblast activation and enhanced ECM production (Fig. 3G). Prussian blue staining of lung tissues showed decreased iron deposition in the *Pde4b*-depleted group, which was consistent with a reduced oxidative burden (Fig. 3H). Complementary assays revealed lower lipid peroxidation and Fe²⁺ levels, alongside partially reduced GSH levels (Fig. 3H-J). These results emphasize the persistent pathological role of PDE4B in both the acute and chronic phases of lung injury in response to wood smoke, which reinforces that targeted PDE4B inhibition-whether pharmacological or genetic-may simultaneously alleviate inflammation and fibrosis in SII.

Early Intervention with mufemilast attenuated SII-induced inflammation and immunity

We next investigated whether inhibiting the excessive activation of PDE4 could alleviate lung injury induced by smoke exposure. Here we used mufemilast, a novel PDE 4 inhibitor, which exhibits a high selectivity in inhibiting the PDE4B subtype. In the dose-dependent protection experiments, we found that the 20 mg/kg dose exhibited a more pronounced protective effect and was therefore selected for subsequent experiments (Fig. S2). We compared the effects of mufemilast (20 mg/kg) and dexamethasone (Dex; 0.25 mg/kg[26], intraperitoneally 30 min post-exposure) on pulmonary

inflammation (3 days post–smoke exposure) and associated secondary hepatic and renal responses. Histological sections stained with hematoxylin and eosin (H&E) revealed marked inflammation and tissue injury in the lungs, to a lesser extent, in the liver and kidneys. Exposure to smoke compared to room air caused extensive infiltration with inflammatory cells (particularly neutrophils and macrophages) in the lungs and airways, with alveolar damage, and increased mucus production in the airways, which were markedly reduced by mufemilast and Dex (Fig. 4B, D). Milder but similar damage was observed in the liver and kidneys, which was also alleviated by mufemilast (**Fig. S3**).

Smoke exposure compared to sham air (SA) exposure significantly increases lung leukocytes, especially neutrophils and macrophages, and elevated lymphocyte counts may reflect ongoing immune regulation or tissue repair. PDE4 inhibition with mufemilast effectively suppressed the SII-induced increased mRNA expression of proinflammatory cytokines, including tumor necrosis factor (*Tnf*) α , interleukin (*Il*)1 β , and *Il*6 in the lungs and showed anti-inflammatory effects comparable to Dex (Fig. 4C). This anti-inflammatory response was further supported by reduced levels of TNF- α , IL-1 β , IL-6, and IL-8 in bronchoalveolar lavage fluid (BALF), indicating a marked anti-inflammatory effect in the lungs (Fig. 4D). Mufemilast also mitigated the accumulation of neutrophils and macrophages in the BALF and circulation, indicating modulation of innate immune cell recruitment and systemic inflammation (Fig. 4E). Consistent with the previous research results, wood smoke selectively increased the mRNA expression of the isoform *Pde4b*, but had no effect on *Pde4a*, *Pde4c* or *Pde4d* (Fig. 4F–H). PDE4 inhibition attenuated the activation of STAT3 (p-STAT3) and NF- κ B (p-NF- κ B) signaling, which are key transcriptional regulators of inflammation, thereby disrupting downstream inflammatory cascades in lungs (Fig. 4G). Micro-CT images revealed mild thickening of the pulmonary interstitial markings in the SII group, which remained largely normal without noticeable inflammatory infiltration following mufemilast treatment (Fig. 4I and **Supplemental video 1–4**).

As with pulmonary inflammation, systemic cytokine profiling revealed significantly elevated mRNA serum levels of *Tnfa*, *Il1b*, *Il6*, and *Il8* in SII mice (**Fig. S3J, K**), which was markedly reduced by mufemilast. H&E staining also showed reduction of inflammatory cells in liver and kidney tissues after mufemilast (**Fig. S3A, B**). Markers of liver and kidney injury were also elevated after SII and were attenuated by mufemilast treatment (**Fig. S3, C, D**). *Pde4b* mRNA was also the predominant upregulated isoform in the liver and kidney tissues (**Fig. S3, G–I**), and mirrored the pulmonary expression profile of PDE isoforms. Similarly, serum from patients with SII also showed biochemical evidence of liver and kidney damage, indicating multiorgan involvement (**Fig. S3E, F**).

These findings highlight that PDE4B is a critical mediator that links the pulmonary and extrapulmonary inflammatory responses in SII in mice. Therapeutic inhibition of PDE4B not only ameliorated lung injury and immune cell infiltration but also attenuated systemic cytokine release. In contrast, Dex was less effective in reducing inflammation induced by acute wood smoke exposure. Collectively, these data suggest that PDE4B is a promising therapeutic target for controlling both local and systemic inflammation in SII.

Intervention with mufemilast attenuated SII-induced pulmonary fibrosis and dysfunction

The acute-phase findings demonstrated that PDE4 inhibition after smoke exposure alleviated smoke-induced inflammation by suppressing STAT3 and NF- κ B signaling, reducing cytokine release, and limiting leukocyte infiltration, thereby preserving lung structure and function. However, unresolved inflammation may progress to chronic fibrotic remodeling, which is characterized by fibroblast activation, ECM deposition, and irreversible tissue scarring. To investigate whether the acute protective effects persisted during the chronic phase, we examined pulmonary fibrosis and oxidative injury in a 21-day smoke inhalation-induced fibrosis model (Fig. 5A). Masson's trichrome staining revealed extensive collagen deposition and interstitial thickening in the SII group, which confirmed progressive fibrosis. Mufemilast effectively reduced collagen accumulation comparable to Dex (Fig. 5B-D). Micro-CT imaging further revealed markedly thickened and increased interstitial markings with patchy peripheral infiltrates and blurred margins in SII lungs. In contrast the mufemilast group showed only minor peripheral infiltrates, indicating a substantially preserved lung parenchymal structure, whereas the Dex group showed thickened and increased peripheral infiltrates (Fig. 5E and **Supplemental video 5–8**). Consistent with the histological findings, RT-qPCR results revealed a marked upregulation of fibrotic markers, e.g., *Col1a1*, *Col1a2*, *Acta2*, fibronectin (*Fn*), which were strongly suppressed by mufemilast and, to a lesser extent, by Dex (Fig. 5F). Thus, PDE4 inhibition not only dampened early inflammation, but also disrupted the chronic profibrotic signaling axis. Isoform analysis confirmed that *Pde4b* remained the dominant isoform upregulated in fibrotic lungs, whereas *Pde4a* and *Pde4d* showed modest elevations and *Pde4c* remained unchanged (Fig. 5G). Immunohistochemistry further validated this persistent *Pde4b* upregulation, with minimal expression in controls but robust airway epithelial and peribronchial staining on day 3 post-SII, persisting in fibrotic lesions on day 21 (Fig. 5H). Fibrosis was associated with the persistent activation of the ERK pathway (p-ERK), a downstream effector of inflammatory signaling that promotes fibroblast activation and ECM production (Fig. 5I). Chronic fibrosis was accompanied by oxidative stress and iron accumulation. Lipid peroxidation (LPO) and free Fe^{2+} levels were markedly elevated, while the antioxidant glutathione (GSH) was depleted, indicating disrupted redox homeostasis. PDE4 inhibition alleviated LPO and iron accumulation and partially restored GSH levels (**Fig. S4B-D**). Prussian blue staining (**Fig. S4A**) confirmed prominent iron deposition and change of ferroptosis markers in the SII lungs, which was mitigated by mufemilast.

Transcriptomic evidence for anti-inflammatory effects of PDE4 inhibition

To elucidate the mechanism underlying the beneficial of PDE4 inhibition in SII, transcriptome RNA-seq was performed on the lungs of control (SA), SII, and SII+mufemilast mice. The Venn diagram revealed that 12,925 genes were common across all three groups (Fig. 6A). Volcano plot analysis identified 540 upregulated and 697 downregulated genes in the SII group compared to the SA group, and 611

upregulated and 310 downregulated genes in the SII+mufemilast group compared to the SII group (Fig. 6B, C). The heatmap displayed the genetic similarities between the SA and SII+mufemilast groups, which differed from those in the SII group, and suggested that PDE4 inhibition influences a broad spectrum of genes involved in inflammation and cell signaling; the restoration of gene expression closer to the baseline levels seen in the control group (Fig. 6D). GO analysis indicated that SII induced robust activation of immune and inflammatory pathways, including leukocyte migration, chemokine signaling, and extracellular matrix (ECM) remodeling. Notably, ECM and basement membrane-associated genes were upregulated, which suggests the initiation of early lung tissue remodeling and a molecular basis for subsequent fibrotic progression (Fig. 6E, F). KEGG pathway analysis showed the downregulation of pathways related to cytochrome P450 and glutathione metabolism in SII, highlighting an impaired oxidative stress and detoxification response (Fig. 6G, H). Further GSEA enrichment analysis revealed the key pathways influenced by PDE4 inhibition. The Hippo signaling pathway, which regulates cell proliferation and fibrosis[27], was activated in the SII group and partially normalized in the SII+mufemilast group (Fig. 6I). Additionally, the NOD-like receptor signaling pathway, which is central to immune activation, was significantly modulated by PDE4 inhibition, and this underscores the role of innate immunity in SII pathogenesis (Fig. 6I). Together, these results highlight that PDE4 inhibition modulates immune, oxidative, and fibrotic pathways, mitigating inflammation and fibrosis in an acute murine model of SII.

Transcriptomic evidence for anti-fibrotic and reparative effects of PDE4 Inhibition

To investigate the molecular mechanisms underlying chronic lung remodeling after SII, we performed transcriptomic profiling of lung tissues 21 days post-exposure. Differential expression analysis identified a large set of dysregulated genes in the SII group compared to the SA control group, and reflecting the persistent activation of inflammatory and fibrotic pathways. Transcriptomic profiling revealed distinct alterations in gene expression that were induced by SII and modulated by mufemilast. A Venn diagram demonstrated the overlapping and unique gene expression patterns among the SA, SII, and SII+mufemilast groups, which highlighted both conserved and treatment-specific transcriptional changes (Fig. 7A). Volcano plots identified 1,237 differentially expressed genes (DEGs) between SA and SII, including key genes that are involved in inflammation, immune activation, and epithelial-barrier disruption (Fig. 7B). PDE4 inhibition reversed a subset of these genes, with 611 upregulated and 310 downregulated in SII+mufemilast compared to SII (Fig. 7C). Heatmap clustering showed that the transcriptional profile of SII+ mufemilast mice closely resembled that of SA mice, suggesting the broad restorative effect of PDE4 inhibition on smoke-induced gene dysregulation (Fig. 7D). GO enrichment analysis indicated that SII prominently activated immune and inflammatory pathways, including leukocyte migration, chemokine signaling, and extracellular matrix remodeling, consistent with acute lung injury and epithelial disruption (Fig. 7E). PDE4 inhibition attenuated the enrichment of these pathways, reflecting the restoration of immune homeostasis and epithelial integrity (Fig. 7F). KEGG pathway analysis revealed that smoke exposure impaired oxidative stress and detoxification pathways,

notably cytochrome P450 and glutathione metabolism, indicating ongoing inflammation and epithelial barrier disruption[28], and normalized to PDE4 inhibition (Fig. 7G, H). Furthermore, Reactome enrichment suggested that PDE4 inhibition modulates lipid metabolism and immune response pathways and integrates the regulation of proliferation, fibrosis, and innate immune responses in SII pathogenesis (Fig. 7I). Taken together, these results indicate that PDE4 inhibition broadly counteracts SII-induced transcriptional dysregulation by targeting inflammation, immune signaling, and early fibrotic remodeling pathways, and thereby contributes to lung protection.

In vitro studies of the protective effects of the mufemilast

To investigate the cytoprotective potential of PDE4 inhibition in smoke-exposed lung epithelial cells, we employed the human bronchial epithelial BEAS-2B and alveolar epithelial A549 cell lines (**Supplemental Fig. 5**), which were treated with apple wood extract (AWE). A dose-response study was conducted to identify the optimal concentration of mufemilast capable of for mitigating smoke-induced cytotoxicity. We observed a significant concentration-dependent protective effect of the mufemilast against 10% AWE-induced damage (Fig. 8A). Notably, treatment with 2 μ M mufemilast induced maximal improvement in viability and was thus chosen for subsequent cellular assays (Fig. 8A).

All cells were treated with 2 μ M mufemilast or 5 μ M Dex[29] following exposure to 10% AWE. Mufemilast increased the survival of epithelial and alveolar cells (Fig. 8B) and reduced the release of lactate dehydrogenase (LDH), a marker of cell damage (Fig. 8C). PDE4A and PDE4B are the main subtypes of PDE4 expressed after cell damage, and were inhibited by mufemilast and Dex. Flow cytometry analysis revealed a significant reduction in apoptosis in the mufemilast-treated group compared to that in the AWE-only group (Fig. 8E), indicating that PDE4 inhibition protects against smoke-induced cell injury and apoptosis. PDE4 inhibition reduced oxidative stress, as evidenced by the reduced lipid peroxidation and ferrous ion (Fe²⁺) accumulation in mufemilast-treated cells (Fig. 8E-G). ROS production, which was significantly increased by AWE exposure, was reduced by approximately 50% following PDE4 inhibition (Fig. 8H). AWE exposure significantly decreased mitochondrial membrane potential ($\Delta\Psi$ m). Treatment with mufemilast and Dex restored $\Delta\Psi$ m to baseline levels (Fig. 8I). This suggests that PDE4 inhibition alleviates both the oxidative stress and mitochondrial dysfunction induced by smoke exposure.

Discussion

Inhalation injury, specifically SII, produces a distinct pathology due to [1, 2]exposure to toxic combustion byproducts in smoke. Despite substantial research on the role of PDE family members, particularly PDE4, in acute or chronic inflammatory conditions [30], this study is the first to investigate the involvement of PDE4 in the context of SII, a crucial distinction that underscores the importance of understanding how PDE4 modulates both acute and chronic respiratory pathologies.

Acute exposure to wood smoke induces a rapid and robust inflammatory response in the lungs [6], characterized by increased infiltration of neutrophils and macrophages, elevated pro-inflammatory

cytokine expression, and epithelial barrier damage[31]. In our in vivo murine model of SII, these pathological features were consistently observed within 72 hours of exposure. Therapeutic administration of the PDE4 inhibitor mufemilast 30 minutes after exposure markedly suppressed lung inflammation, as evidenced by the attenuation of proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, and the down-regulation of key inflammatory signaling pathways including NF- κ B and STAT3. These effects were comparable to, and in several aspects exceeded, those achieved by Dex, an anti-inflammatory glucocorticoid routinely employed in clinical practice. Transcriptomic profiling of lung tissues further substantiated this connection by revealing that enhanced immune system activation, ECM remodeling, and oxidative stress are key pathogenic mechanisms, all of which are consistent with prior reports of acute lung inflammatory responses after smoke exposure[32]. Lung transcriptomic profiling confirmed a significant restoration of immune homeostasis by mufemilast, with reversal of gene signatures related to leukocyte recruitment, chemokine signaling, and extracellular matrix remodeling. Collectively, these findings highlight the potent anti-inflammatory capacity of PDE4 inhibition in the acute phase of SII.

Persistent inflammation is a well-established precursor to fibrotic remodeling in the lungs [33]. In our chronic SII model, mice displayed extensive interstitial fibrosis, collagen deposition, and progressive architectural distortion of the lung parenchyma by day 21 post-exposure. Mufemilast significantly mitigated these fibrotic features, as shown by reduced expression of genes linked to fibrosis. These antifibrotic effects were further corroborated by histological assessments and micro-CT imaging, which showed preserved alveolar integrity and reduced interstitial thickening in the mufemilast group. Transcriptomic analyses from fibrotic lungs revealed that PDE4 inhibition dampened chronic immune activation and ECM remodeling, while reactivating antioxidant and mitochondrial repair pathways. However, glucocorticoids remain the mainstay of clinical intervention, but their immunosuppressive effects and limited efficacy in fibrotic remodeling underscore the need for more targeted therapies [29, 32], reinforcing the therapeutic value of targeting PDE4 in chronic smoke-induced lung injury. Integrative transcriptomic analyses of lung tissue further elucidated the transition from acute inflammation to chronic fibrosis, which highlights the dynamic interplay between immune system activation, ECM remodeling, and oxidative damage. Early in the course of injury, we observed a significant upregulation of immune signaling pathways, including NOD-like receptors, chemokines, and cytokine-receptor interactions, which are consistent with the recruitment and activation of neutrophils, macrophages, and lymphocytes that were identified in BALF and peripheral blood. This might be related to the activation of the Hippo pathway [27], highlighting the need for us to frequently monitor the continuous pathophysiological process of SII.

Smoke inhalation impairs the integrity of the airway epithelial cell monolayer, due to the disruption, primarily, of ion transport mechanisms [34]. This, in turn, impairs mucociliary clearance and epithelial cell regeneration, thereby perpetuating inflammation and fibrotic progression [34, 35]. Upregulation of PDE4B and dysregulation of cAMP signaling are mechanistically connected to the dysfunction of ion channels, such as transmembrane conductance regulator and epithelial Na channel (ENaC), which are critical for airway surface liquid homeostasis [36]. Our transcriptomic data revealed the enrichment of pathways

involved in epithelial cell repair, polarization, and ECM remodeling, which underscores the ongoing epithelial cell regeneration in response to injury. Our data showed that mufemilast not only mitigated inflammatory and oxidative stress responses, but also likely restored epithelial barrier integrity and ion-transport function, promoted mucosal defense, and facilitated tissue repair. If replicated in humans, this multifaceted evidence would position PDE4 as a pivotal node linking ion transport disruption, immune system activation, and epithelial cell remodeling in the pathogenesis of SII.

Beyond pulmonary effects, SII also induces systemic organ dysfunction, particularly involving the liver and kidneys. Several studies have highlighted the extensive damage to these organs in the context of SII, which have shown that smoke inhalation leads to significant liver and kidney injury, with inflammation and elevated systemic cytokine levels being prominent markers of organ dysfunction [37, 38]. Our histological and biochemical assessments showed that mufemilast effectively reduced hepatic and renal damage markers and suppressed systemic cytokine elevations. Remarkably, PDE4B expression was significantly upregulated in both liver and kidney tissues, mirroring its induction in the lungs. Genetic silencing of *Pde4b* in airway epithelial cells not only protected against pulmonary injury but also attenuated systemic inflammation, highlighting PDE4B as a molecular conduit linking local and systemic pathophysiology in SII.

A critical aspect of our study was the integration of across species serum metabolomic profiling to delineate systemic biochemical alterations. Although we do not have the serum data after PDE4 inhibitor treatment, we have found that differentially regulated metabolites revealed significant associations with signaling pathways regulated by the PDE family. D-Pinitol, the sole consistently downregulated metabolite, maintains intracellular cAMP and regulates redox balance [39], suggesting that the disruption of cAMP homeostasis may exacerbates oxidative stress and inflammation. In contrast, the upregulated metabolite LysoPI(18:2(9Z,12Z)/0:0) (a class of lysophospholipids derived from phosphatidylinositol) activates protein kinase C [40], which intersects with PDE-regulated pathways to promote epithelial-barrier dysfunction and inflammation [41]. Given the established role of PDE4 in cAMP hydrolysis, pulmonary inflammation, and fibrotic responses [42], our findings suggest that metabolic dysregulation modulates PDE4 activity and downstream signaling. Thus, PDE4 constitutes a key link between systemic metabolic reprogramming and lung-specific fibrotic and inflammatory responses, and represents a promising therapeutic target in SII. The concurrent upregulation of metabolites that are associated with lipid peroxidation, amino acid metabolism, and oxidative stress was also evident indicating a coordinated disruption of redox homeostasis and immune-metabolic regulation. In this respect, perturbations in oxocarboxylic acid metabolism, particularly TCA cycle intermediates, are closely linked to oxidative stress and inflammatory responses in SII, driving the pathology towards pulmonary fibrosis [43]. These metabolic changes may exacerbate the inflammatory and fibrotic changes by impairing energy homeostasis and contributing to tissue remodeling and dysfunction [44]. A previous investigation established that, tryptophan metabolism is activated by *Mycobacterium tuberculosis*, particularly via the indoleamine 2, 3-dioxygenase (IDO) pathway, to generate indole derivatives that modulate immune responses and oxidative stress; this highlights the metabolic link between injury repair and regulation of inflammation [45].

This metabolic reprogramming intersects with PDE4-regulated pathways, which modulate intracellular cAMP levels that are crucial for controlling inflammatory and fibrotic signaling cascades. Transcriptomic profiling of lung tissues further substantiated this connection by revealing that enhanced immune system activation, ECM remodeling, and oxidative stress are key pathogenic mechanisms, all of which are consistent with prior reports of acute lung inflammatory responses after smoke exposure. Building on this mechanistic insight, we demonstrated using an acute and chronic model of SII in mice, that inhibition of PDE4 with mufemilast and genetic silencing of the *Pde4b* gene (the predominant *Pde4* isoform upregulated by SII), effectively reversed these pathological alterations by attenuating inflammatory signaling, restoring mitochondrial function, and reducing fibrotic remodeling. SII triggers an acute and frequently severe inflammatory response in the lung [46], and this is likely accompanied by systemic organ dysfunction and, after chronic exposure, progressive fibrosis. Mufemilast appears to be more effective than a clinically used glucocorticoid, particularly in reducing fibrosis, suggesting that PDE4 inhibition is a promising therapeutic strategy to modulate both acute inflammation and chronic fibrotic remodeling.

Integrative transcriptomic analyses of lung tissue further elucidated the transition from acute inflammation to chronic fibrosis, which highlights the dynamic interplay between immune system activation, ECM remodeling, and oxidative damage. Early in the course of injury, we observed a significant upregulation of immune signaling pathways, including NOD-like receptors, chemokines, and cytokine-receptor interactions, which are consistent with the recruitment and activation of neutrophils, macrophages, and lymphocytes that were identified in peripheral lung, BALF and peripheral blood. These inflammatory responses are tightly regulated by intracellular signaling cascades, particularly those controlled by cAMP levels.

PDE4 inhibitors, due to their subunit catalytic conformation and broad-spectrum non-selective inhibitory effects, often cause side effects such as nausea and vomiting, in clinical use [17]. Currently, the main approach is to reduce the side effects of treatment for respiratory systems through the development of PDE4 subtype inhibitors or inhaled delivery to reduce systemic exposure. Ensifentrine, an inhaled PDE3 and PDE4 inhibitor, has been approved for treatment of COPD, but it has not been shown to have anti-inflammatory effects in COPD as PDE4 is only weakly inhibited [47]. Nerandomilast, a PDE4B selective inhibitor, has recently been approved for the treatment of idiopathic pulmonary fibrosis. PDE4B has emerged as the predominant phosphodiesterase isoform driving SII pathophysiology, exhibits marked upregulation in lung epithelial cells, and infiltrates immune populations during both acute and chronic phases.

Given the growing incidence of wildfires and urban air pollution worldwide, SII has emerged as a significant public health concern. The ability of PDE4B inhibition to modulate both acute and chronic pathological events in SII provides a compelling rationale for its clinical translation. Moreover, the mechanistic parallels between SII and other chronic lung diseases, particularly COPD and IPF, suggest that insights from this model may extend beyond the context of smoke injury [16]. Indeed, existing

clinical experience with PDE4 inhibitors in COPD, coupled with the more favorable safety profile of mufemilast, supports the feasibility of repurposing PDE4B-targeted therapies for broader indications.

Despite the strengths of this study, several limitations merit acknowledgment. The diagnostic and prognostic utility of D-pinitol and other metabolite biomarkers must be validated in larger, diverse patient cohorts. Furthermore, long-term safety and efficacy studies of PDE4B inhibition in chronic pulmonary injury models are essential prior to clinical application. Emerging technologies such as single-cell transcriptomics and spatial multi-omics should be employed to resolve cellular heterogeneity and local microenvironmental dynamics in SII. Additionally, the potential therapeutic synergy between PDE4B inhibitors and ferroptosis modulators warrants future exploration, given the observed link between PDE4B activity and redox imbalance.

In summary, this study delineated the comprehensive molecular landscape of the SII in mice after acute and chronic exposure and establishes PDE4B as a central integrator of inflammatory, fibrotic, and metabolic dysregulation. These insights provide a strong rationale to evaluate PDE4-targeted therapies to treat SII in humans and their potential to prevent progression to irreversible pulmonary fibrosis. Future work should explore the long-term benefits and safety of PDE4B inhibition in larger preclinical models and clinical trials, as well as investigate the interplay between PDE4B signaling and ferroptosis pathways to fully elucidate and harness its therapeutic potential.

Abbreviations

SII
Smoke inhalation injury
PDE
Phosphodiesterase
cAMP
Cyclic-3',5'-adenosine monophosphate
cGMP
Cyclic guanosine-3',5'-monophosphate
PDE4i
Pde4 inhibitor
COPD
Chronic obstructive pulmonary disease
IPF
Idiopathic pulmonary fibrosis
CO
Carbon monoxide
BALF
Bronchoalveolar lavage fluid
Dex

Dexamethasone
TNF
Tumor necrosis factor
IL
Interleukin
ECM
Extracellular matrix
ALT
Alanine aminotransferase
AST
Aspartate aminotransferase
ALP
Alkaline phosphatase
g-GT
Gamma-glutamyl transpeptidase
UA
Uric acid
CRE
Creatinine
BUN
Blood urea nitrogen
WBC
Wbcwhite blood cell
NEU
Neutrophil
LYM
Lymphocyte
MONO
Monocytes
EOS
Eosinophil

Declarations

Ethics approval and consent to participate

Animal experiments were approved by the Animal Ethics Committee of the Tianjin University Laboratory Animal Center (Tianjin, the People's Republic of China; Approval No. TJUE-2025-013). Experiments involving human serum samples were approved by the Ethics Committee of Characteristic Medical Center of Chinese People's Armed Police Force (Approval NO. 2025-0023.1). In total, serum samples from 20 patients with SII caused by indoor fire and 20 healthy individuals were analyzed. The

requirement for informed consent was waived by the Ethics Committee of Characteristic Medical Center of Chinese People's Armed Police Force due to the retrospective nature of the study.

Author contributions

Conceptualization: Liu B., Dong J., Yang L., Methodology: Dong J., Yang L. Investigation: Liu B., Dong J., Yang L., Jones R. Visualization: Yang L., Shao N. Dong J., Tang S., Hao A. Funding acquisition: Liu B., Dong J., Zhang H. Supervision: Liu B., Dong J. Writing-original draft: Yang L., Writing-review & editing: Dong J., Liu B., Jones R., Barnes PJ..

Funding

This work was funded by National Natural Science Foundation of China (grant number 82200655); Key Laboratory of Medical Rescue Key Technology and Equipment, Ministry of Emergency Management (grant number YJBZZJTJU202403); Tianjin Key Medical Discipline Construction Project (grant number TJYXZDXK-3-001D); Natural Science Foundation of Tianjin, China (grant numbers 22JCYBJC00730, 23JCYBJC01560, and 23JCQNJC01590).

Acknowledgments

We would like to thank Wei Fu and Hesheng Zhang (Tianjin Hemay Pharmaceutical Technology) for providing mufemilast. Image items used in this work were created in Figdraw:
<https://www.Fig.draw.com/static/index.html#/>

Consent for publication

Not applicable.

Competing interests

Dr. R. Jones, Hesheng Zhang and Wei Fu are employees of Hemay Pharmaceutical Co., Ltd., which may be considered a potential conflict of interest.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Figures

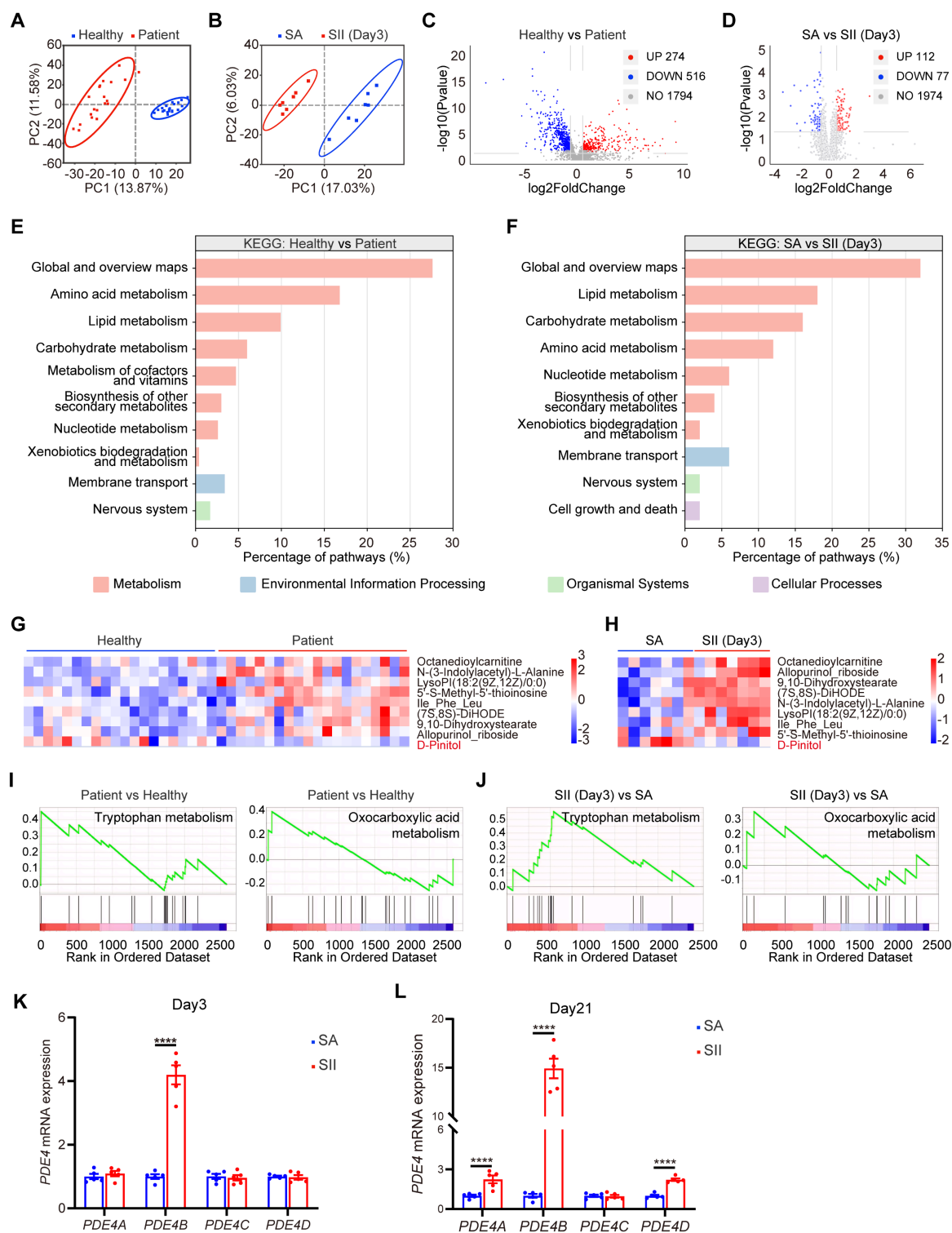


Figure 1

Metabolomic profiling of SII reveals key metabolic alterations and potential biomarkers. (A, B) PCA of serum metabolite profiles (A, human, n=20/group; B, mice, n=7/group). (C, D) Volcano plots show the same differential metabolites in different species (C, human; D, mice). (E, F) Functional annotation analysis of differential metabolites (E, human; F, mice). (G, H) Heatmap of differential metabolites (G,

human; H, mice). (K, L) RT-qPCR analysis of *Pde4* isoforms (*Pde4a*, *Pde4b*, *Pde4c*, and *Pde4d*) in lung tissues (K, Day3, L, Day21).

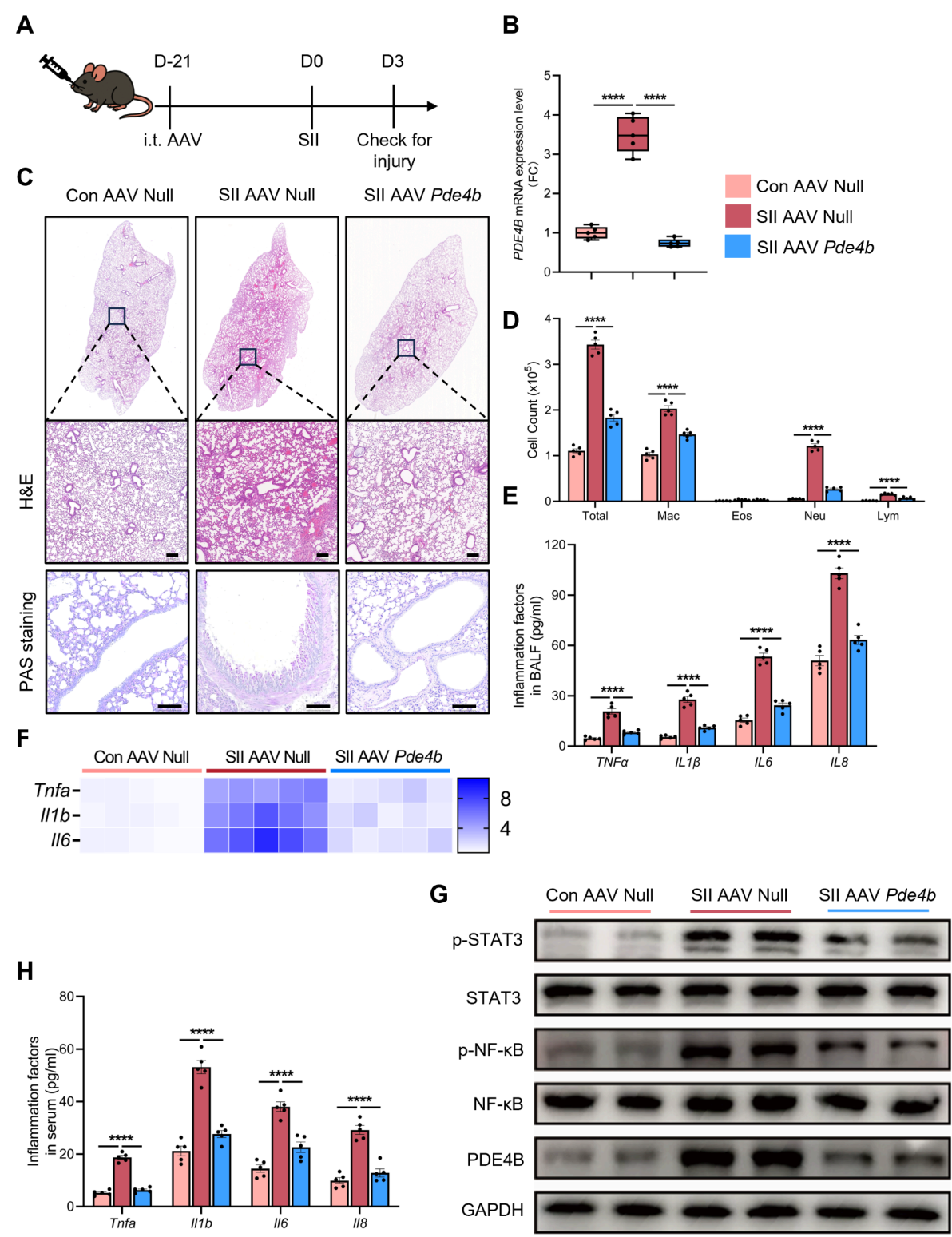


Figure 2

Epithelial-specific knockdown of *Pde4b* by AAV delivery attenuates SII-induced acute lung injury. (A) Schematic showing AAV administration via intratracheal and SII model establishment in mice. (B) RT-

qPCR analysis of PDE4B in lung tissues. **(C)** Representative H&E (scale bar: 200 μ m) and PAS (scale bar: 100 μ m) images of the lungs. **(D)** Differential cells count in the BALF. Means $\pm$ SEM, n=5 shown. **(E)** ELISA-based analysis of cytokines in the BALF. Means $\pm$ SEM, n=5 shown **(F)** Heatmap of the expression of key inflammatory genes in the lung. **(G)** Western blot images of p-STAT3, p-NF- κ B, and PDE4B in lung tissues. **(H)** ELISA analysis of cytokines in the serum. Means $\pm$ SEM, n=5 shown. ****P < 0.0001. Statistics obtained from 1-way ANOVA. (macrophages: Mac, eosinophils: Eso, neutrophils: Neu, lymphocytes: Lym).

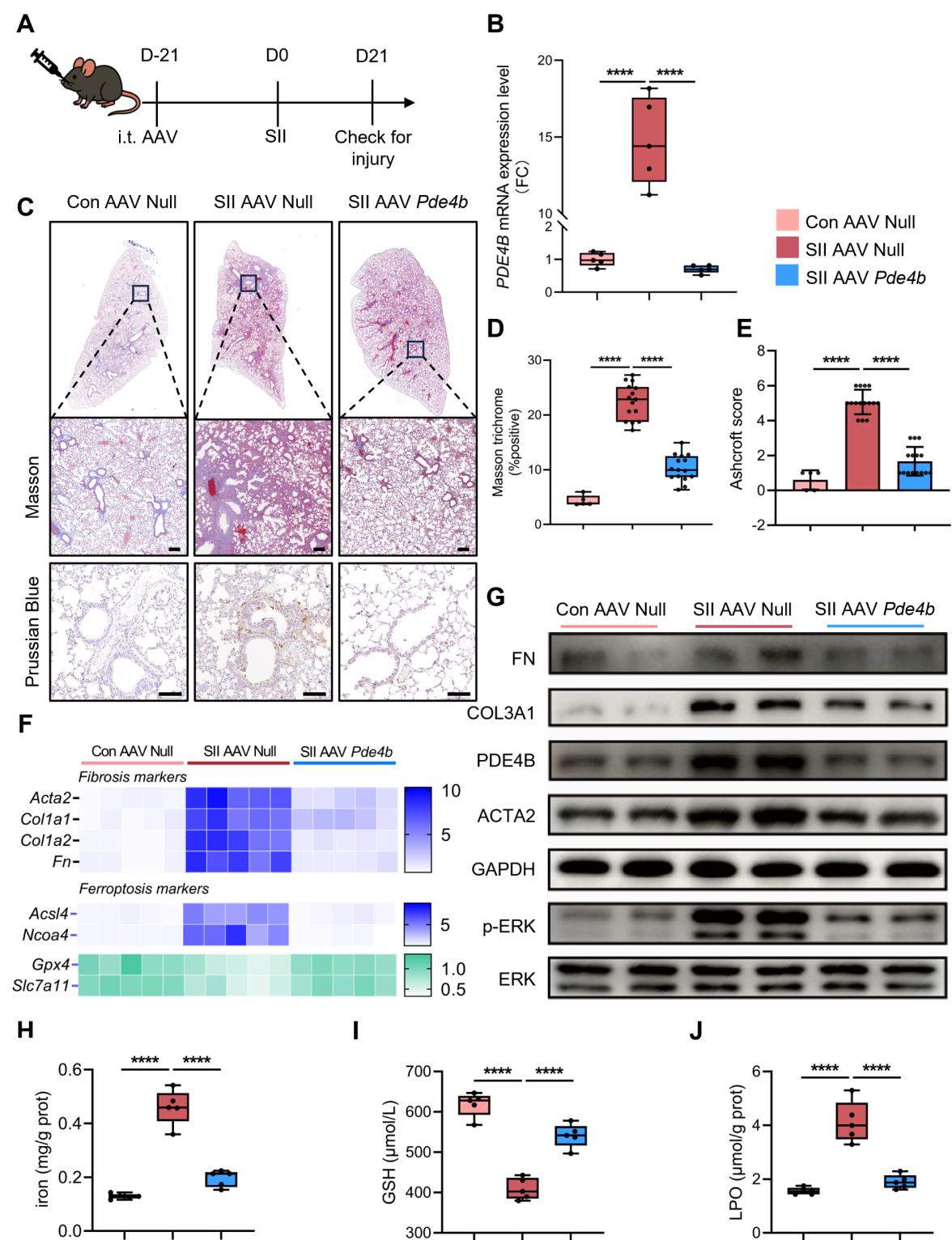


Figure 3

Knockdown of *Pde4b* in airway epithelial cells by AAV delivery attenuates lung pathology in a chronic model of SII. (A) Schematic showing AAV delivery via intratracheal and SII model establishment in mice. (B) RT-qPCR analysis of *Pde4b* in lung tissues. (C) Representative Masson (scale bar: 200 μ m) and Prussian blue staining (scale bar: 100 μ m) images of the lungs connective tissue in blue and iron deposition in brown. (D, E) Quantified changes of fibrotic areas (D) and Ashcroft scale (E) (n = 5, 15, 15, 15). (F) Heatmap of the expression of key fibrosis and ferroptosis genes in the lung. (G) Western blot image of p-ERK, ACTA2, PDE4B, COL3A1, and FN in lung tissues. (H-J) Lung iron levels (H), lung GSH content (I), and lung LPO content (J). Panels H-J: means $\pm$ SEM, n=5 shown. **** $P < 0.0001$. Statistics obtained from 1-way ANOVA

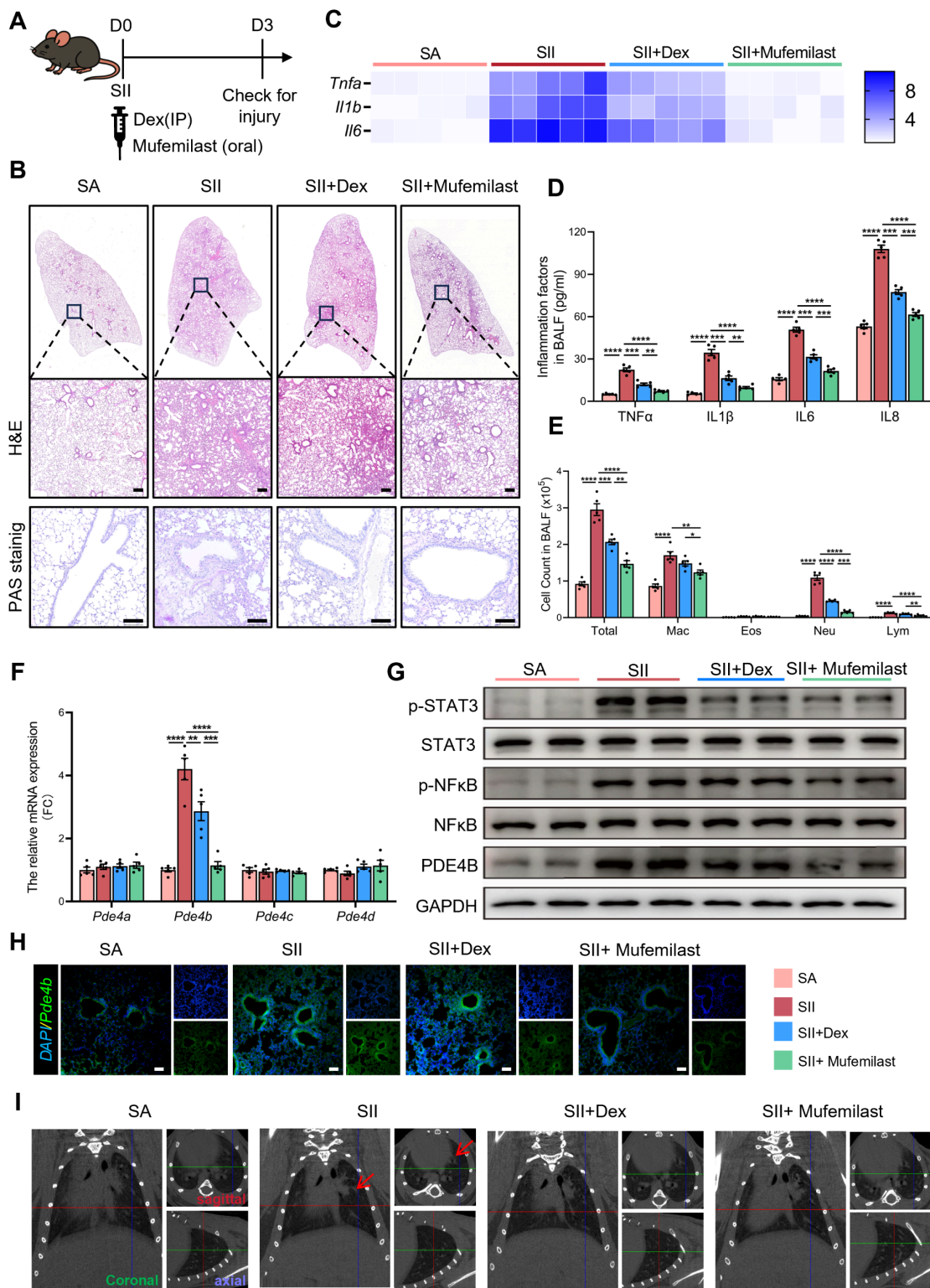


Figure 4

Mufemilast prevents inflammation and promotes lung repair in SII-induced acute injury. Exposure to smoke (SII) and sham air (SA) and after treatment with dexamethasone (Dex) or mufemilast. **(A)** Schematic diagram of SII model establishment and the treatment strategy in mice. **(B)** Representative H&E (scale bar: 200µm) and periodic acid Schiff (PAS) to show mucin (scale bar: 100µm) images of lungs showing tissue injury after SII. **(C)** Heatmap of the expression of key inflammatory genes in the

lung parenchyma. **(D)** ELISA-based cytokine analysis of bronchoalveolar lavage fluid (BALF). Means $\pm$ SEM, n=5 shown **(E)** Differential cells counts in BALF: macrophages: Mac, eosinophils: Eos, neutrophils: Neu, lymphocytes: Lym. Means $\pm$ SEM, n=5 shown **(F)** RT-qPCR analysis of *Pde4* isoforms (*Pde4a*, *Pde4b*, *Pde4c*, and *Pde4d*) in lung tissues. Means $\pm$ SEM, n=5 shown **(G)** Representative Western blot images of p-STAT3, p-NF- κ B, and PDE4B in lung tissues. **(H)** Representative immunofluorescence images of lungs (Green: *Pde4b*; Blue: DAPI nuclear stain) (scale bar: 200 μ m). **(I)** Representative micro-CT images of live mice lungs showing changes in lung tissue density (red arrows: thickened pulmonary interstitium). * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 (1-way ANOVA).

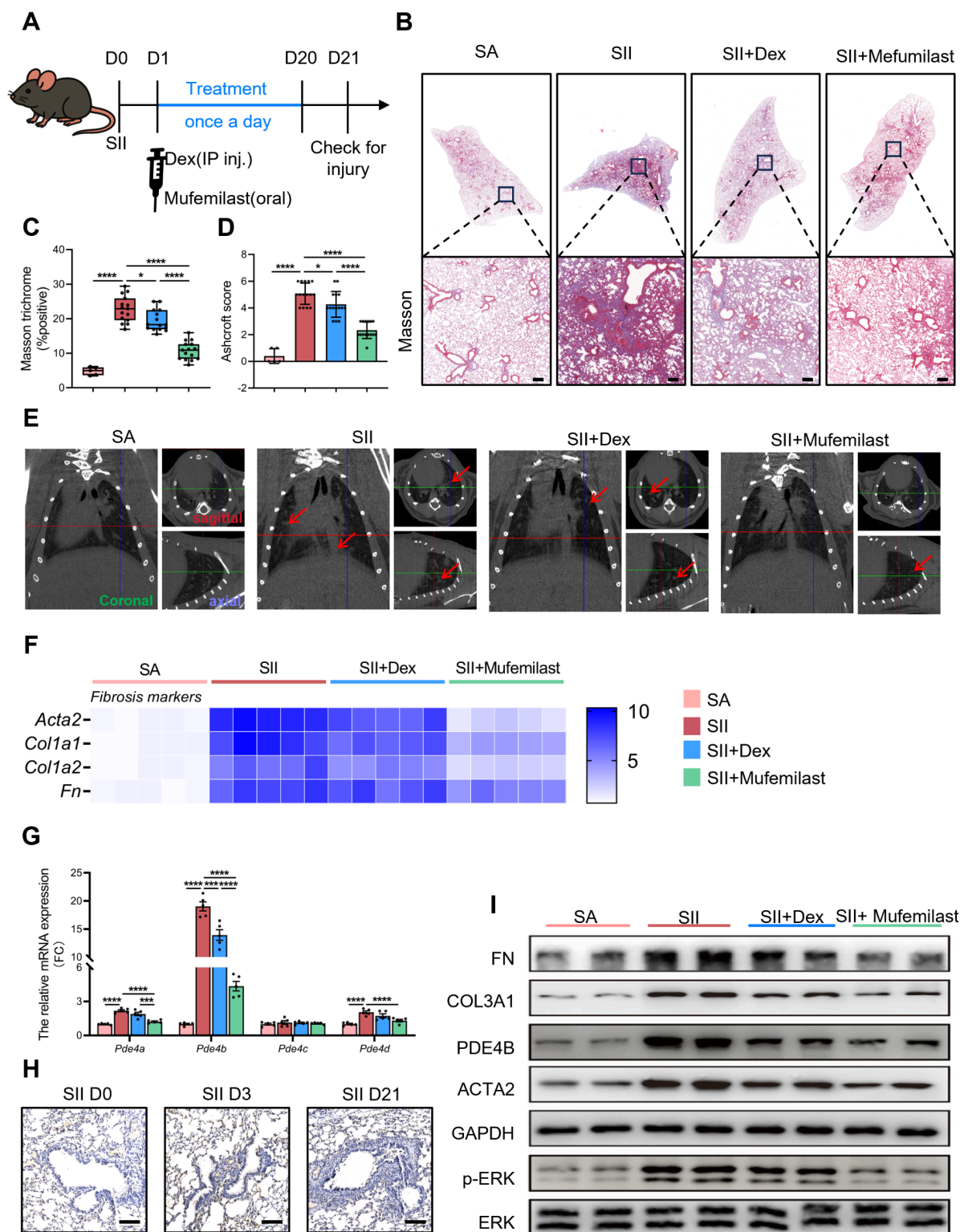


Figure 5

Mufemilast prevented fibrosis and promoted lung repair in a chronic murine model of SII. (A) Schematic showing SII model protocol and the treatment strategy in mice. (B) Representative Masson trichrome stain showing connective tissue in blue images of the lungs (scale bar: 200 μ m). (C, D) Quantified changes of fibrotic areas (C) and Ashcroft scale (D) (n = 5, 15, 15, 15). (E) Representative micro-CT images of live mice lungs show that mufemilast reduced patchy peripheral infiltrates and

blurred margins ((red arrows: thickened pulmonary interstitium and ground-glass opacity). (F) Heatmap of the expression of key fibrosis genes in the lung. (G) RT-qPCR analysis of *Pde4* isoforms (*Pde4a*, *Pde4b*, *Pde4c*, and *Pde4d*) in lung tissue Means $\pm$ SEM, n=5 shown. (H) Representative images of immunohistochemical analysis of the lungs (scale bar: 200 μ m). (I) Western blot image of p-ERK, ACTA2, PDE4B, COL3A1, and FN in lung tissues. * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$. Statistics obtained from 1-way ANOVA.

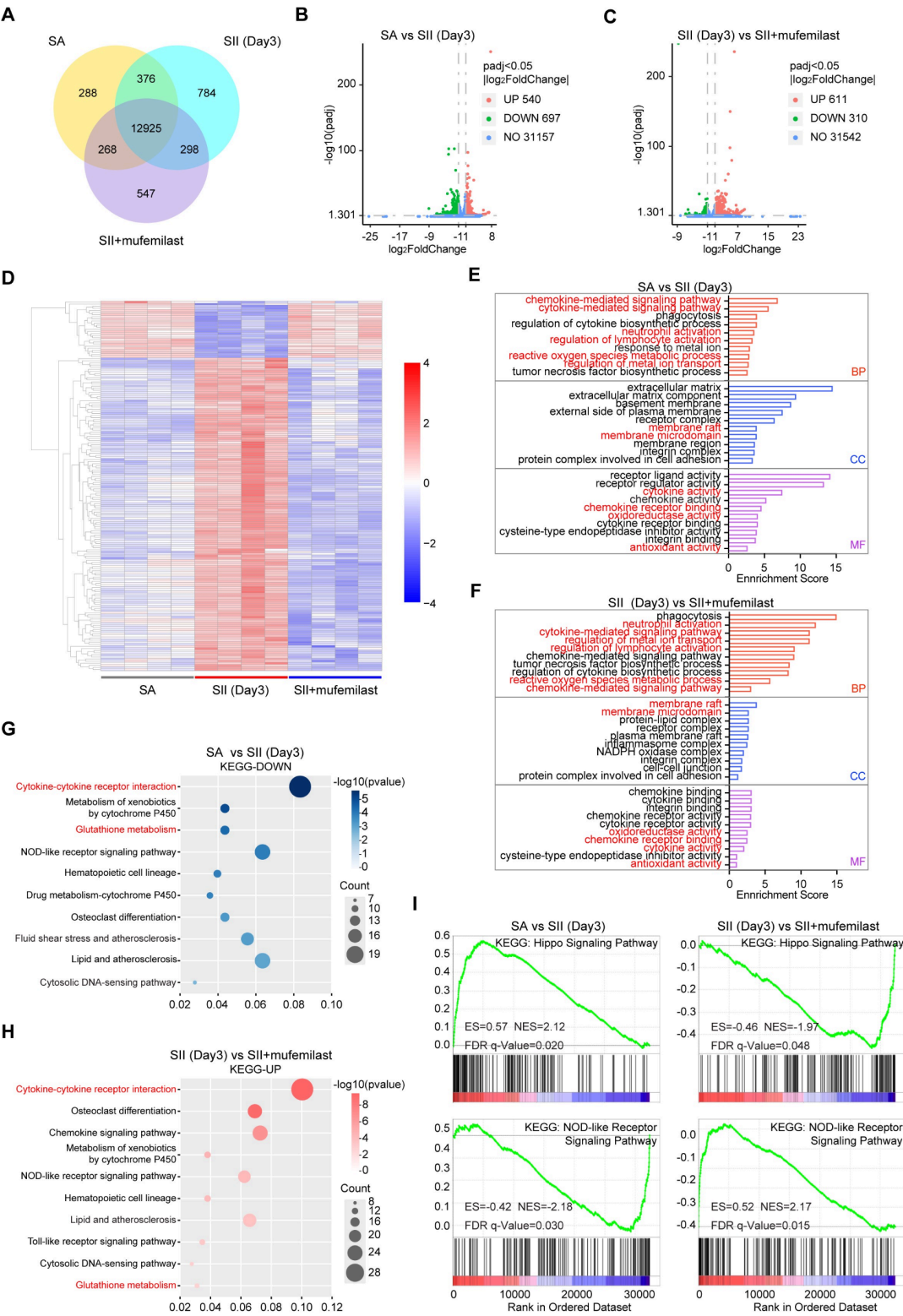


Figure 6

Effect of mufemilast on transcriptomic changes in the lungs of mice subjected acutely to SII. (A) Venn diagram illustrating the number of co-expressed genes. (B, C) Volcano map displaying the DEGs between the SA and SII groups (B), or the SII and SII+mufemilast (C) groups. (D) Heatmap showing the DEGs among the SA, SII, and SII+mufemilast groups. (E) GO enrichment analysis of the DEGs between the SA and SII groups. (F) GO enrichment analysis of the DEGs between the SII and SII+mufemilast groups. (G) KEGG enrichment analysis of the upregulated pathways in the SA compared to the SII group for each DEG. (H) KEGG enrichment analysis of downregulated pathways in the SII compared to the SII+mufemilast group, stratified by the DEGs. (I) GSEA enrichment analysis identified the potential GO or KEEG pathway.

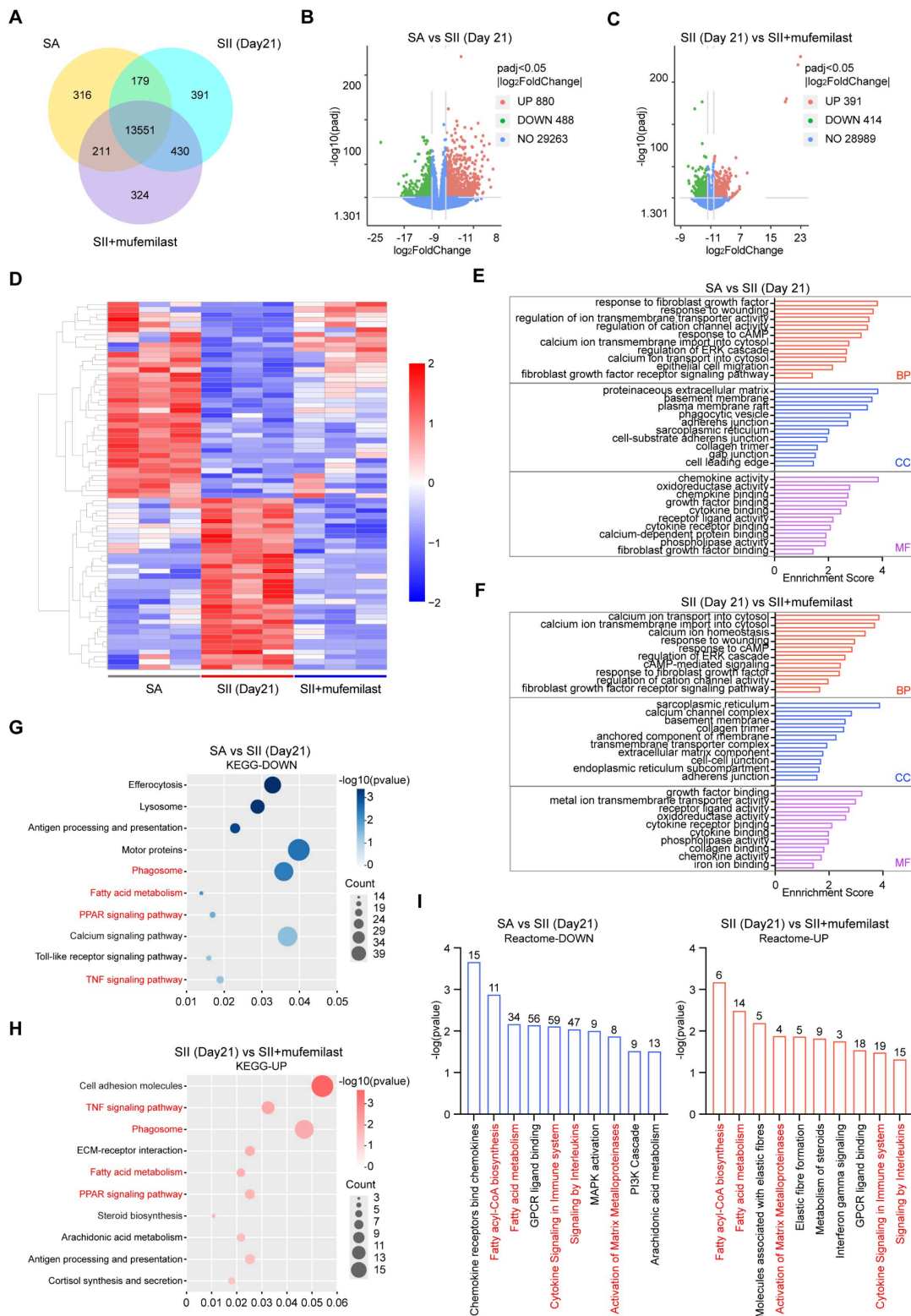


Figure 7

Effect of mufemilast on transcriptomic changes in the lungs of mice subjected chronically to SII. (A) Venn diagram illustrating the number of co-expressed genes. **(B, C)** A volcano map of the DEGs between the SA and SII groups **(B)**, or the SII and SII+mufemilast **(C)** groups. **(D)** Heatmap showing the DEGs among the SA, SII, and SII+mufemilast groups. **(E)** GO enrichment analysis of the DEGs between the SA and SII groups. **(F)** GO enrichment analysis of the DEGs between the SII and SII+mufemilast groups. **(G)**

KEGG enrichment analysis of upregulated pathways in the SA compared to the SII group for each DEG. (H) KEGG enrichment analysis of downregulated pathways in the SII compared to SII+mufemilast group stratified by the DEGs. (I) Reactome enrichment analysis identified the potential GO or KEEG pathways.

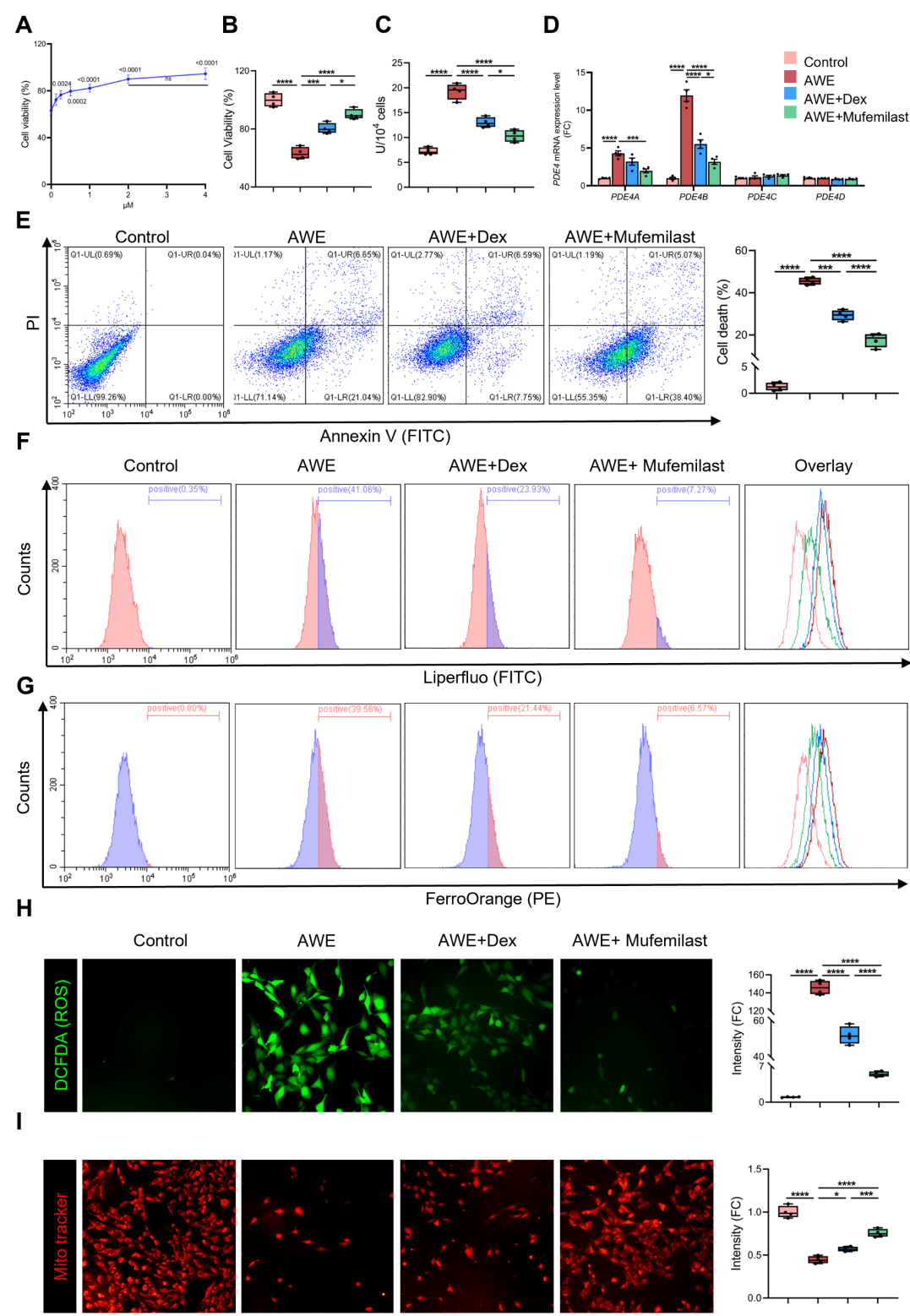


Figure 8

Mufemilast ameliorates oxidative stress and cell death. (A) Effects of AWE stimulation and mufemilast dose-dependent treatment of BEAS-2B cell viability was examined using the Cell Counting Kit-8 assay. (B, C) Effects of AWE stimulation and mufemilast and Dex treatment of BEAS-2B cell viability was examined using the CCK8 assay (B, n = 4) and LDH assay (C, n = 4). (D) RT-qPCR analysis of *PDE4* isoforms (*PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D*). (E) Cell-death assay was performed using Annexin V/PI staining (n = 4). (F, G) Flow cytometry-based analysis of lipid peroxidation by Liperfluo staining (F, n = 4) and cellular Fe²⁺ levels by FerroOrange staining on cells (G, n = 4). (H, I) Representative fluorescent images and quantification of DCFDA (2',7'-dichlorofluorescein diacetate) (H, n = 4) and Mito-Tracker Red CMXRos (I, n = 4) (scale bar: 200 μm). Means ±SEM, n=4 shown. **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001. Statistics obtained from 1-way ANOVA.

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