ORIGINAL ARTICLE

Protein Phosphatase 2A Reduces Cigarette Smoke–induced Cathepsin S and Loss of Lung Function

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Abstract

Rationale: CTSS (cathepsin S) is a cysteine protease that is observed at higher concentrations in BAL fluid and plasma of subjects with chronic obstructive pulmonary disease (COPD).

Objectives: To investigate whether CTSS is involved in the pathogenesis of cigarette smoke–induced COPD and determine whether targeting upstream signaling could prevent the disease.

Methods: CTSS expression was investigated in animal and human tissue and cell models of COPD. $Ctss^{-/-}$ mice were exposed to long-term cigarette smoke and forced oscillation and expiratory measurements were recorded. Animals were administered chemical modulators of PP2A (protein phosphatase 2A) activity.

Measurements and Main Results: Here we observed enhanced CTSS expression and activity in mouse lungs after exposure to cigarette smoke. $Ctss^{-/-}$ mice were resistant to cigarette smoke–induced inflammation, airway hyperresponsiveness, airspace enlargements, and loss of lung function. CTSS expression was negatively regulated by PP2A in human bronchial epithelial cells isolated from healthy nonsmokers and COPD donors and in monocyte-derived macrophages. Modulating PP2A expression or activity, with silencer siRNA or a chemical inhibitor or activator, during acute smoke exposure in mice altered inflammatory responses and CTSS expression and activity in the lung. Enhancement of PP2A activity prevented chronic smoke-induced COPD in mice.

Conclusions: Our study indicates that the decrease in PP2A activity that occurs in COPD contributes to elevated CTSS expression in the lungs and results in impaired lung function. Enhancing PP2A activity represents a feasible therapeutic approach to reduce CTSS activity and counter smoke-induced lung disease.

Keywords: cigarette smoke; cathepsin S; phosphatase; chronic obstructive pulmonary disease

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At a Glance Commentary

Scientific Knowledge on the Subject: CTSS (cathepsin S), a lysosomal cysteine protease with elastase activity across a wide pH range, is elevated in chronic obstructive pulmonary disease (COPD) clinical samples, but its role in the disease process is unknown.

What This Study Adds to the Field: This study demonstrates that CTSS significantly contributes to cigarette smoke–induced loss of lung function in mice. CTSS expression is negatively regulated by PP2A (protein phosphatase 2A), but PP2A activity is inhibited by prolonged exposure to cigarette smoke. Chemical activation of PP2A reduces induction of CTSS expression in the lung and loss of lung function. Thus, these findings demonstrate a major role of CTSS and PP2A in smoke-induced COPD and identify a new potential therapeutic target to treat COPD. Finally, our results and approaches suggest that pharmacologic activation of important upstream signaling enzymes, such as phosphatases (PP2A), that negatively regulate key effectors associated with COPD progression, such as CTSS, may represent an alternative and possibly complementary approach to direct effector enzyme inhibition.

Lifelong cigarette smoke exposure decreases pulmonary function in susceptible smokers leading to the onset and progression of chronic obstructive pulmonary disease (COPD) (1). COPD is currently the third leading cause of death in the United States (2) and is a major global health problem. Exposure to cigarette smoke is the primary environmental factor associated with COPD formation in the developed world. Cellular responses triggered by cigarette smoke cause the release of inflammatory and proteolytic mediators that contribute to the pathogenesis of COPD (3). Although the role of proteases in COPD is well established, much of the research has focused on serine elastase and matrix metalloproteinases (MMP) (4, 5). In particular, the role of the CTS (cathepsin) family of enzymes, which are highly expressed in COPD, remains to be determined.

Several CTS are induced by smoke inhalation and are linked to emphysema development, including CTS E (6), G (7), K (8), and S (9). CTSS is a lysosomal cysteine protease that exerts elastase activity across a wide range of pH in alveolar macrophages, fibroblasts, and epithelial cells. CTSS activity is significantly elevated in the BAL fluid (BALF) (10) and plasma of patients with COPD (11). Altered CTSS levels are associated with a variety of pathologic conditions including cystic fibrosis, arthritis, cancer, and cardiovascular disease (12). CTSS has multiple functional roles, including major histocompatibility complex class II antigen presentation (13), and it can also cleave and inactivate key innate immunity proteins, such as β-defensins 2 and 3 (14), secretory leukocyte protease inhibitor (15), and lactoferrin (16). Unlike other CTS, CTSS has activity at a neutral pH (17) and increased levels of CTSS would have proteolytic activity in a healthy lung. Therefore, determining the stimuli that increase CTSS activity may provide key insights into the pathogenesis of lung diseases.

In view of the potential link between CTSS and COPD progression, we explored whether cigarette smoke alters CTSS signaling and determined whether CTSS impairs lung function and structure. Here we demonstrate that smoke exposure triggers robust *Ctss* expression and enhanced proteolytic activity in the lungs of mice. Using *Ctss*$^{-/-}$ mice, we determined that *Ctss* expression directly impacts cigarette smoke–induced changes in pulmonary physiology. One plausible mechanism for smoke induction of CTSS expression is inactivation of PP2A (protein phosphatase 2A), a phosphatase that regulates inflammatory and proteolytic responses (18–20). Chronic smoke exposure diminishes lung PP2A responses and coincides with airspace enlargement in response to smoke (19, 21). Inhibition of PP2A in mice before smoke exposure enhanced CTSS expression and lung inflammation. Equally, normalizing PP2A levels in mice or in human bronchial epithelial (HBE) cells isolated from subjects with COPD reduced CTSS expression and secretion. Chemical activation of PP2A prevents cigarette smoke–induced loss of lung function in mice and this study presents data showing PP2A regulation of CTSS that alters lung immune and proteolytic responses responsible for airway injury and function.

Methods

Detailed and expanded methodology is included in the online supplement.

Animal Models

Ctss$^{-/-}$ mice, on a C57BL/6J background, were exposed to cigarette smoke in a chamber (Teague Enterprises) for 4 hours daily, 5 d/wk at a total particulate matter concentration of 80–120 mg/m^3 with the University of Kentucky reference research cigarettes 3R4F (Lexington). An additional group of wild-type mice were intraperitoneally injected with 2 μg/kg of okadaic acid (LC Labs) or intranasal delivery of 7.4 nmol PP2A$_A$ (mouse *Ppp2r1a*) silencer short, interfering RNA (Life Technologies). PP2A activity was enhanced in mice by oral administration of 50 mg/kg of a bioavailable SMAP (small molecule activator of PP2A) (*see* Reference 22) twice daily. All animal experiments were performed with approval from SUNY Downstate's Institutional Animal Care and Use Committee and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH and Institutional Animal Care and Use Committee guidelines and according to the Declaration of Helsinki conventions for the use and care of animals.

Forced Oscillation and Expiratory Measurements

Mice were anesthetized, tracheostomized, and connected via an endotracheal cannula to the SCIREQ flexiVent system (SCIREQ Inc.). Animals were paralyzed and pulmonary function measured (23). Airway responses to increasing doses of methacholine were assessed.

Histology and Lung Immune Cell Measurements

BALF and BALF cells were obtained from animals of each group and assessed by flow cytometry (24). Lungs underwent pressure fixation and morphometric analysis in accordance with the American Thoracic Society/European Respiratory Society issue statement (25). Mean linear intercept analysis was performed (26). Alveolar counts, boundary size, and ductal

destructive measurements were performed (27). Sections from human bronchial tissue (28) and mouse lung tissue were stained for CTSS.

Cell Culture

HBE cells from nonsmokers and patients with COPD were isolated from human organ donor lungs rejected for transplant and fully redifferentiated at the air–liquid interface as previously described (29). Consent for research was obtained by the Life Alliance Organ Recovery Agency of the University of Miami. All consents were approved by the institutional review board and conformed to the Declaration of Helsinki. Cells were transfected with purified PP2A protein (Millipore) using Pro-Ject transfection reagent (Pierce) as per the manufacturers' instructions (18). Cells were also transfected with PP2A or human antigen R (HuR)-specific siRNA. Alternatively, cell media were supplemented with 1 μM SMAP. Monocyte-derived macrophages were also examined for PP2A regulation of CTSS.

PP2A and CTSS Measurements

Immunoblots for ERK (extracellular signal–regulated kinase) phosphorylation (Thr202/Tyr204 and total ERK), the A subunit of PP2A and β-Actin (Cell Signaling Technologies), were performed. PP2A activity was determined using the Millipore PP2A activity assay (17–313; Millipore). Gene expression was performed by qPCR using Taqman probes (Applied Biosystems). CTSS concentrations were determined in BALF using a CTSS ELISA kit (R&D Systems) and immunoblots. CTSS activity was determined, as previously described (30).

Statistical Analyses

Data are expressed as mean ± SEM. Data were compared by Student's *t* test (two-tailed) or by two-way ANOVA and Tukey *post hoc* test analysis, using Prism Software version 6.0h for Mac OS X (GraphPad).

Results

Cigarette Smoke Enhances CTS Expressions and CTSS Activity in Mouse Lungs

To investigate the impact of cigarette smoke on CTS expressions, C57BL/6J animals were exposed to cigarette smoke daily for several time points. Lung CTS expressions were determined by qPCR and CTSS was further analyzed by ELISA, substrate activity assays, and immunoblots. First, the gene expression of all CTS family members was examined in the lungs of mice exposed to smoke for 6 months, with gene expression relative to each other CTS gene. *Ctse*, *Ctsg*, and *Ctss* were significantly altered by smoke exposure in the lungs (Figure 1A; *see* Figure E1 in the online supplement). We primarily focused on CTSS because higher levels are observed in the BALF (10) and plasma of patients with COPD (11). Smoke exposure resulted in a significant increase in CTSS levels and activity in BALF (Figure 1B). Lung tissue analysis also confirmed that there is elevated CTSS activity within the tissue of smoke-exposed animals (Figure 1B). Western blot analysis confirmed elevated CTSS proteins levels in BALF from mice exposed to cigarette smoke, as early as 8 days postexposure and remained high throughout exposure (Figure 1B). Immunofluorescence evaluation demonstrated that CTSS is elevated in smoke-exposed mice and CTSS is located in immune and epithelial cells (Figure 1C). Therefore, smoke exposure elevates several CTS genes in the lungs.

Ctss Deficiency Prevents Smoke-induced Loss of Lung Function in Mice

To determine whether *Ctss* expression impacted on airway resistance and lung function in mice, $Ctss^{-/-}$ mice and their wild-type littermates were exposed to cigarette smoke daily for 6 months. Airway resistance was assessed by methacholine challenge test. At every methacholine dose greater than or equal to 4 mg/ml, $Ctss^{-/-}$ mice exposed to cigarette smoke showed significantly lower respiratory resistance than wild-type mice exposed to cigarette smoke (Figure 2A). To examine how *Ctss* deficiency altered lung function in response to cigarette smoke, pressure–volume (PV) loops, compliance, and forced expiratory volume in 0.05 seconds ($FEV_{0.05}$)/FVC were determined as previously described (23). A PV loop that shifts up and to the left, suggests an emphysematous lung as observed in wild-type mice exposed to smoke (Figure 2B). However, the PV loop from $Ctss^{-/-}$ mice exposed to smoke did not shift up. Lung compliance is a measure of the lung's ability to stretch and expand, and $FEV_{0.05}$/FVC is the proportion of the animal's VC that is expired in the first one-twentieth of a second of forced expiration to the full VC. In mice, smoke inhalation typically enhances compliance and reduces $FEV_{0.05}$/FVC levels (Figure 2C). Importantly, $Ctss^{-/-}$ mice developed less emphysematous changes after exposure to smoke compare with control animals, with reduced smoke-induced changes in lung function in all three parameters observed in these mice.

Immune cell infiltration is frequently observed in the lungs of patients with COPD (31). Total BALF immune cell counts were significantly increased in smoke-exposed wild-type mice but not in $Ctss^{-/-}$ mice (Figure 3A). Lung macrophages and neutrophils were reduced in $Ctss^{-/-}$ mice after smoke exposure compared with wild-type mice (Figure 3A). However, *Ctss* expression did not impact eosinophil, T-cell, or B-cell numbers in the lungs (Figure 3A). Smoke exposure did enhance T-cell and B-cell frequency in the airways, in a CTSS-independent manner (Figure 3A). Morphometric quantification demonstrated that the loss of *Ctss* expression prevented the increase in smoke-induced airspace enlargements, determined by mean linear intercept analysis (Figure 3B). Because CTSS is a potent elastase, elastin degradation was investigated by quantifying plasma levels of desmosine, an amino acid found in elastin. Smoke-exposed $Ctss^{-/-}$ mice had reduced desmosine in their plasma compared with wild-type mice (Figure 3C), indicating less elastin degradation. Parenchymal airspace profiling (27) was used to demonstrate that $Ctss^{-/-}$ mice had a higher alveolar count, reduced loss of alveolar boundary, and reduced ductal destruction compared with smoke-exposed wild-type mice (Figure 3D). Therefore, *Ctss* expression impacts on lung function, inflammation, elastin degradation, and lung tissue remodeling during chronic cigarette smoke exposure.

HBE Cells Isolated from Patients with COPD Express More CTSS Than Cells from Nonsmokers without COPD Partially Because of Altered PP2A Signaling

Previous work has identified airway epithelial cells as a source of pulmonary CTSS (32). Here, we further investigated CTSS levels in human bronchial tissue to confirm the presence of CTSS and elevated levels in COPD samples. Immunofluorescence analysis demonstrated that CTSS is expressed by

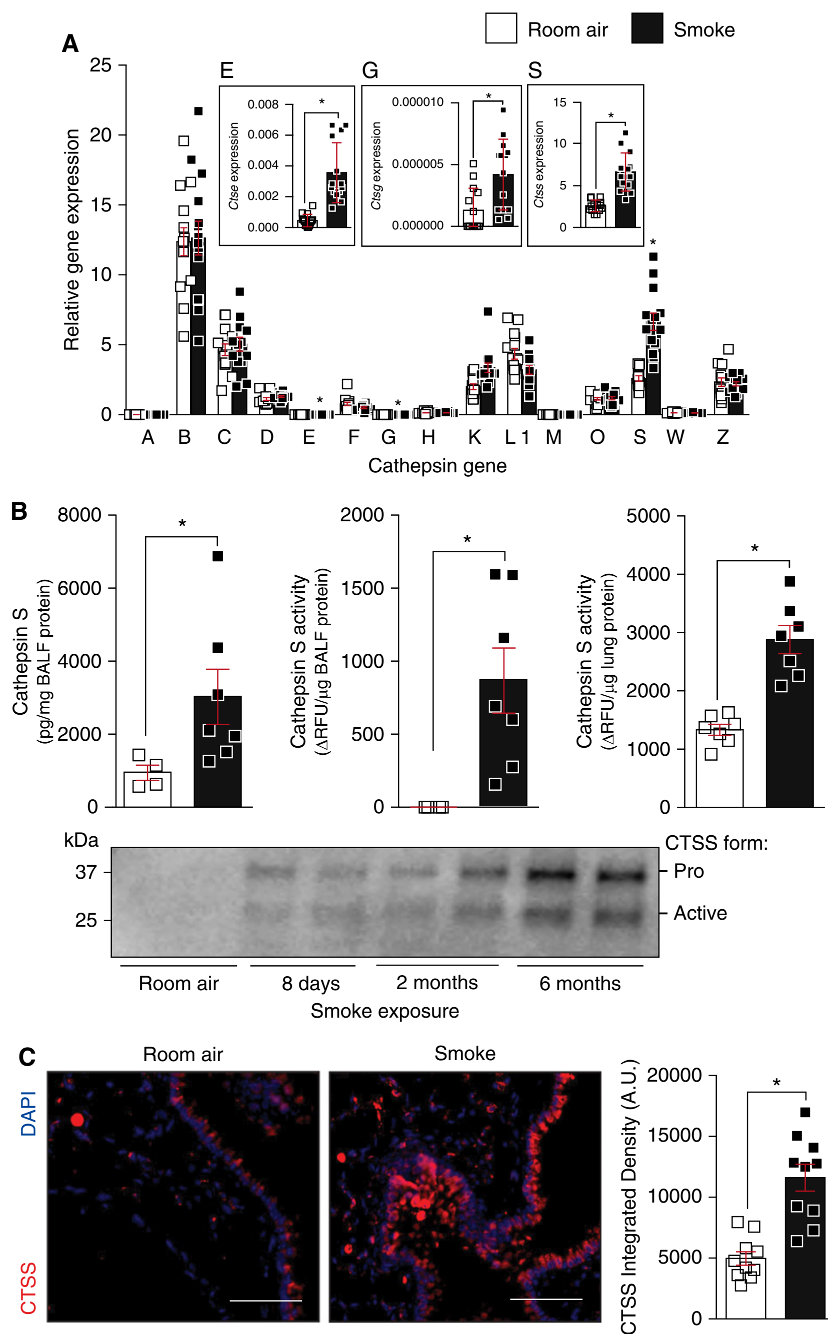

Figure 1. Smoke exposure enhances *Ctss* (cathepsin S) gene and protein expression in mice lungs. (*A*) CTS genes were quantified in C57BL/6J lung tissue, after 6 months exposure to room air and cigarette smoke, by qPCR and are shown as relative gene expression to each CTS gene. (*B*) CTSS protein and activity were quantified in the BAL fluid (BALF) of C57BL/6J mice, after 6 months exposure to room air and cigarette smoke, by ELISA and substrate activity assays, respectively. CTSS activity was also determined in total lung tissue protein. Immunoblots were also performed on BALF from C57BL/6J mice exposed to cigarette smoke for 0, 8 days, 2 months, or 6 months. The CTSS pro form is 37 kD, and the active form is 25 kD. Every lane represents an individual mouse. (*C*) Immunofluorescence was performed on lung tissue from room air– and smoke-exposed mice for CTSS and DAPI. Comparative images of the two mouse groups are presented here (scale bars, 150 μm). CTSS fluorescence intensity was determined and arbitrary units are shown here. Data are represented as mean ± SEM, with each measurement performed on 3 separate days from at least four animals/group. *$P < 0.05$, when comparing both treatments connected by a line, determined by Student's *t* tests. AU = arbitrary units; RFU = relative fluorescence units.

bronchial tissue and is elevated in bronchial tissue from subjects with COPD (Figure 4A). To explore further the regulation of CTSS expression, we used HBE cells isolated from nonsmokers, and subjects with COPD. Cells isolated from subjects with COPD expressed and secreted more CTSS than cells from nonsmokers (Figures 4B–4E). The stabilizing RNA-binding protein HuR and the phosphatase, PP2A, have been linked to the regulation of CTSS expression in atherosclerosis (33) and Alzheimer disease/Down syndrome (34). Therefore, we examined *CTSS* gene expression and activity in HBE cells after modulation of HuR or PP2A signaling. Loss of HuR expression, with siRNA transfection, did not significantly alter CTSS signaling in HBE cells from nonsmokers or subjects with COPD (Figure 4B). However, transfecting siRNA specific for the A subunit of PP2A ($PP2A_A$) (Figure 4C) or PP2A protein into HBE cells (Figure 4D) or the treatment of HBE cells with SMAP (Figure 4E) significantly altered CTSS expression and activity in both cell groups. Modulated PP2A signaling was confirmed by the regulation of ERK phosphorylation in these cells, with reduced ERK phosphorylation observed when PP2A is active (Figures 4D and 4E).

Other cell types also express CTSS, such as macrophages (10). Human macrophages were derived from monocytes isolated from peripheral blood of nonsmokers. Similar to HBE cells, silencing PP2AA enhanced CTSS expression and activity in these macrophages (Figure 4F). Alternatively, SMAP treatment enhanced PP2A activity and reduced ERK and CTSS responses (Figure 4G). Therefore, loss of PP2A activity seems to result in enhanced CTSS expression and enzyme activity, possibly contributing to disease development.

Triggering PP2A Responses Prevents Smoke-induced CTSS Expression in Mice

To examine PP2A modulation and acute smoke effects on *Ctss* expression, wild-type mice were exposed to cigarette smoke daily for 3 days while they were administered daily injections of the phosphatase inhibitor okadaic acid, intranasal delivery of $PP2A_A$ silencer siRNA, or twice daily oral administration of SMAP (18, 19, 22). Mice treated with okadaic acid had significantly higher infiltrating immune cells into the lung after smoke exposure compared with

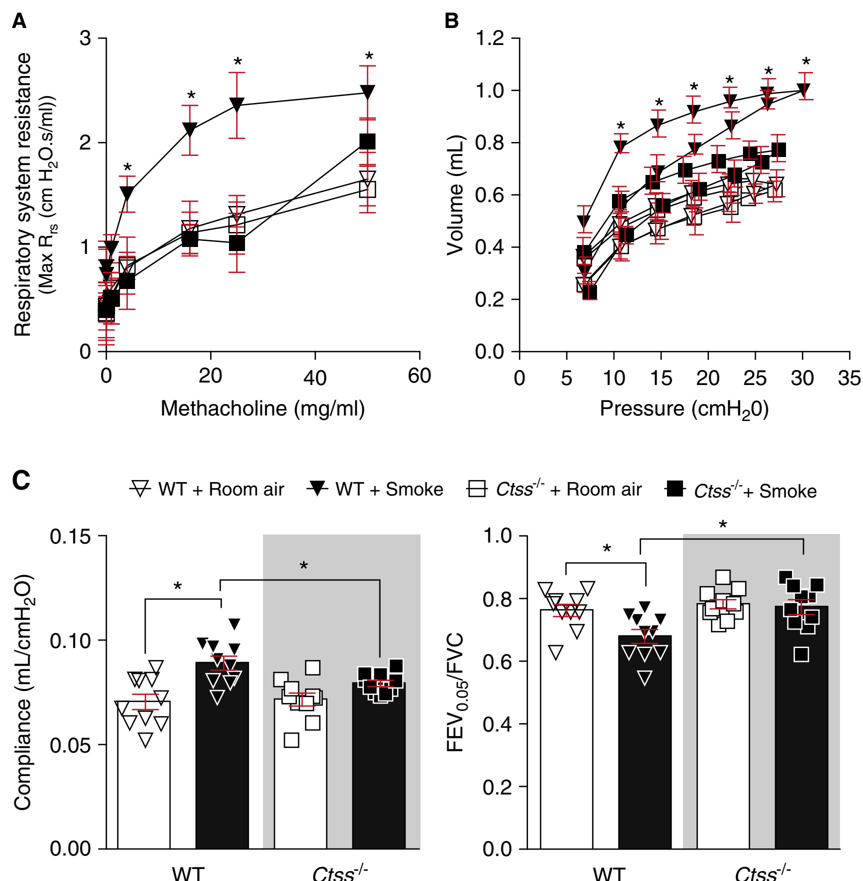

Figure 2. *Ctss* (cathepsin S) deficiency prevents smoke-induced loss of lung function in mice. Wild-type and *Ctss*$^{-/-}$ mice were exposed to room air and cigarette smoke for 6 months. (*A*) Animals were challenged for airway resistance by a dose response of methacholine. (*B* and *C*) Negative pressure-driven forced expiratory and forced oscillation technique maneuvers were performed in all animal groups. (*B*) Pressure–volume loops and (*C*) compliance and forced expiratory volume in 0.05 seconds ($FEV_{0.05}$)/FVC were determined in each animal. Data are represented as mean ± SEM, where $n = 10$ per group. *$P < 0.05$, when comparing both treatments connected by a line, determined by two-way ANOVA with Tukey *post hoc* test. WT = wild type.

control animals (Figure 5A). Okadaic acid treatment also enhanced lung ERK phosphorylation. In response to cigarette smoke, lung *Ctss* gene expression and BALF CTSS activity were significantly increased in okadaic acid–treated mice. Similarly, silencing $PP2A_A$ in the lungs enhanced inflammation, ERK phosphorylation, and CTSS responses in mice (Figure 5B). Alternatively, administration of SMAP to mice reduced smoke-induced immune cell infiltration, ERK phosphorylation, and CTSS expression and enzyme activity (Figure 5C).

To determine the long-term effects of SMAP treatment on lung function, wild-type mice were administered SMAP twice daily during 2-month exposures to cigarette smoke. A/J mice were chosen because they are more sensitive to cigarette smoke–induced emphysema-like symptoms than other mouse backgrounds (35). Animal weight was recorded throughout the study and liver-to-body-weight ratio was measured at the end, as indicators of chemically induced changes to organs. No significant changes in weight were observed between groups (Figure 6A). Treatment with SMAP reduced smoke-induced immune cell infiltration into the airways (Figure 6B) and prevented smoke-induced inhibition of PP2A activity within the lungs (Figure 6C), which coincided with reduced lung *Ctss* gene and protein release into the airways during smoke exposure (Figure 6D). As expected, SMAP treatment was not able to completely block smoke-induced CTSS responses (Figure 6D). Nevertheless, it showed the importance of PP2A in regulating CTSS.

To examine whether SMAP treatment prevents the alteration of lung function in response to cigarette smoke, we examined PV loops, compliance, and $FEV_{0.05}$/FVC. The PV loop analysis from SMAP-treated mice were lower compared with vehicle treated animals, when exposed to smoke (Figure 7A). SMAP-treated mice developed less emphysematous changes after exposure to smoke compared with control animals, with reduced smoke-induced changes in lung function in compliance and $FEV_{0.05}$/FVC (Figure 7B). SMAP administration reduced desmosine levels in their plasma compared with vehicle-treated animals (Figure 7C). SMAP-treated animals had higher alveolar counts and reduced ductal destruction compared with smoke-exposed vehicle treated mice (Figure 7D). SMAP administration also prevented the increase in smoke-induced airspace enlargements, determined by mean linear intercept analysis (Figure 7E). These SMAP-mediated changes in CTSS levels were observed without changes in inflammation, such as IL-1β (36), IFN-γ (32), and tumor necrosis factor-α (*see* Figures E2A and E2B). Equally, SMAP administration did not impact smoke-induced *Ctse* or *Ctsg* (*see* Figure E2C). Therefore, SMAP treatment impacts on lung function, inflammation, elastin degradation, and lung tissue remodeling during chronic cigarette smoke exposure.

Discussion

Here, we establish that cigarette smoke enhances CTSS levels and activity, at least partly, because of a reduction in PP2A activity. Furthermore, CTSS contributes to cigarette smoke–induced COPD (Figure 7F). *Ctss*$^{-/-}$ mice were resistant to cigarette smoke–induced loss of lung function. Elevated levels of CTSS are observed in the lungs of mice from 8 days after the initiation of smoke inhalation and persisted throughout exposure. Expression of CTSS in the airway epithelium seems to be regulated by PP2A and not HuR. However, it is possible that HuR stabilizes CTSS mRNA in other cell types, as previously reported (33). Therefore, we propose that CTSS promotes the loss of lung function in COPD and also modulates pulmonary inflammatory responses.

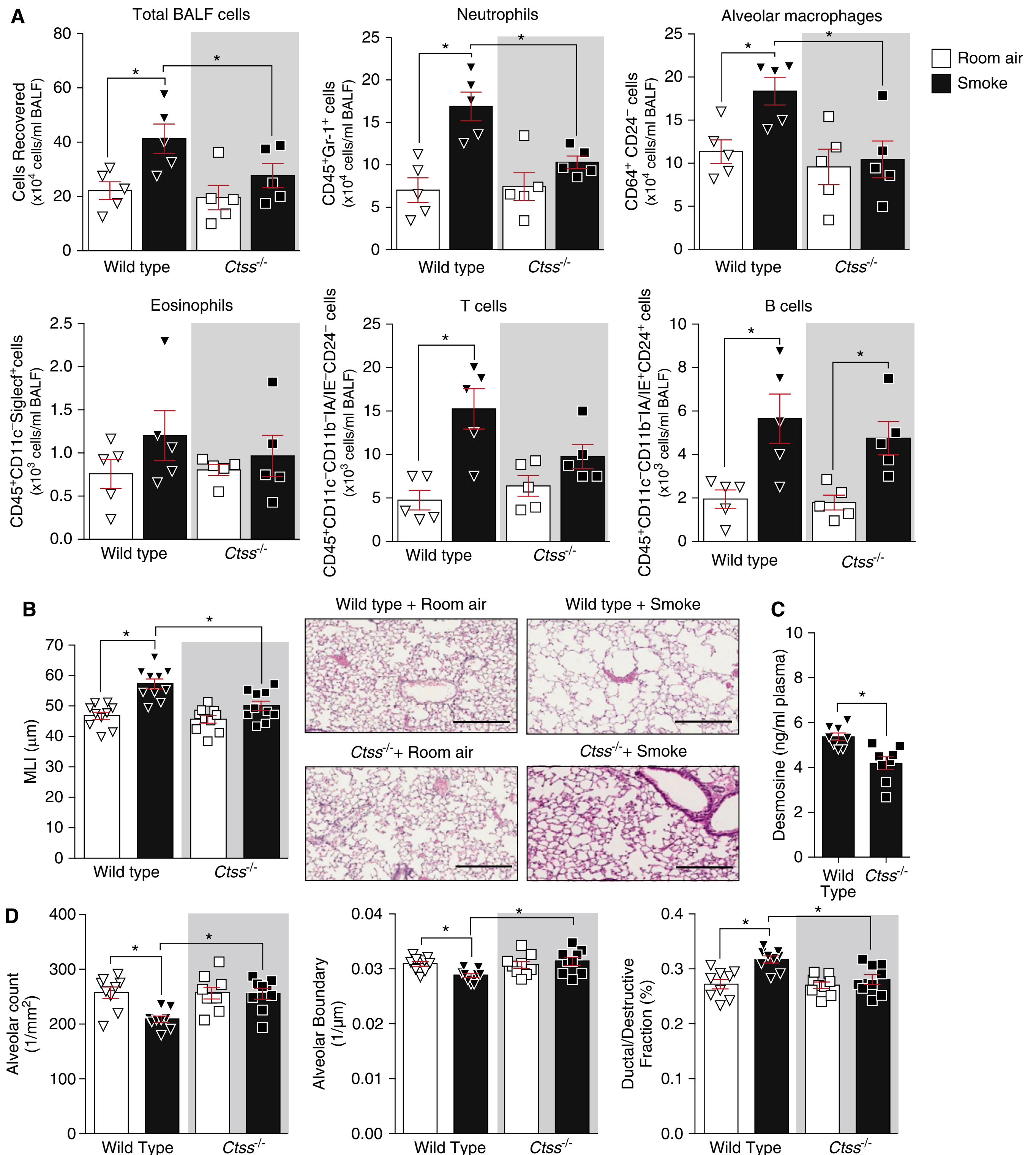

Figure 3. *Ctss* (cathepsin S) deficiency prevents smoke-induced lung immune cell infiltration and airspace enlargements in mice. Wild-type and *Ctss*$^{-/-}$ mice were exposed to room air and cigarette smoke for 6 months. (*A*) BALF total immune cells, neutrophils, alveolar macrophages, eosinophils, T cells, and B cells were quantified in each group by flow cytometry. (*B*) Mean linear intercepts were measured in the lungs of the mice to assess air space size, and comparative histology images of the four mouse groups are presented here (scale bars, 40 μm). (*C*) Plasma desmosine levels were determined in smoke-exposed animals by ELISA. (*D*) Alveolar count, alveolar boundary, and ductal/destructive fractions were quantified in each animal by parenchymal airspace profiling. Data are represented as mean ± SEM, where $n \geq 5$ per group. $^*P < 0.05$, when comparing both treatments connected by a line, determined by two-way ANOVA with Tukey *post hoc* test or Student's *t* test when comparing only two groups. BALF = BAL fluid; MLI = mean linear intercept.

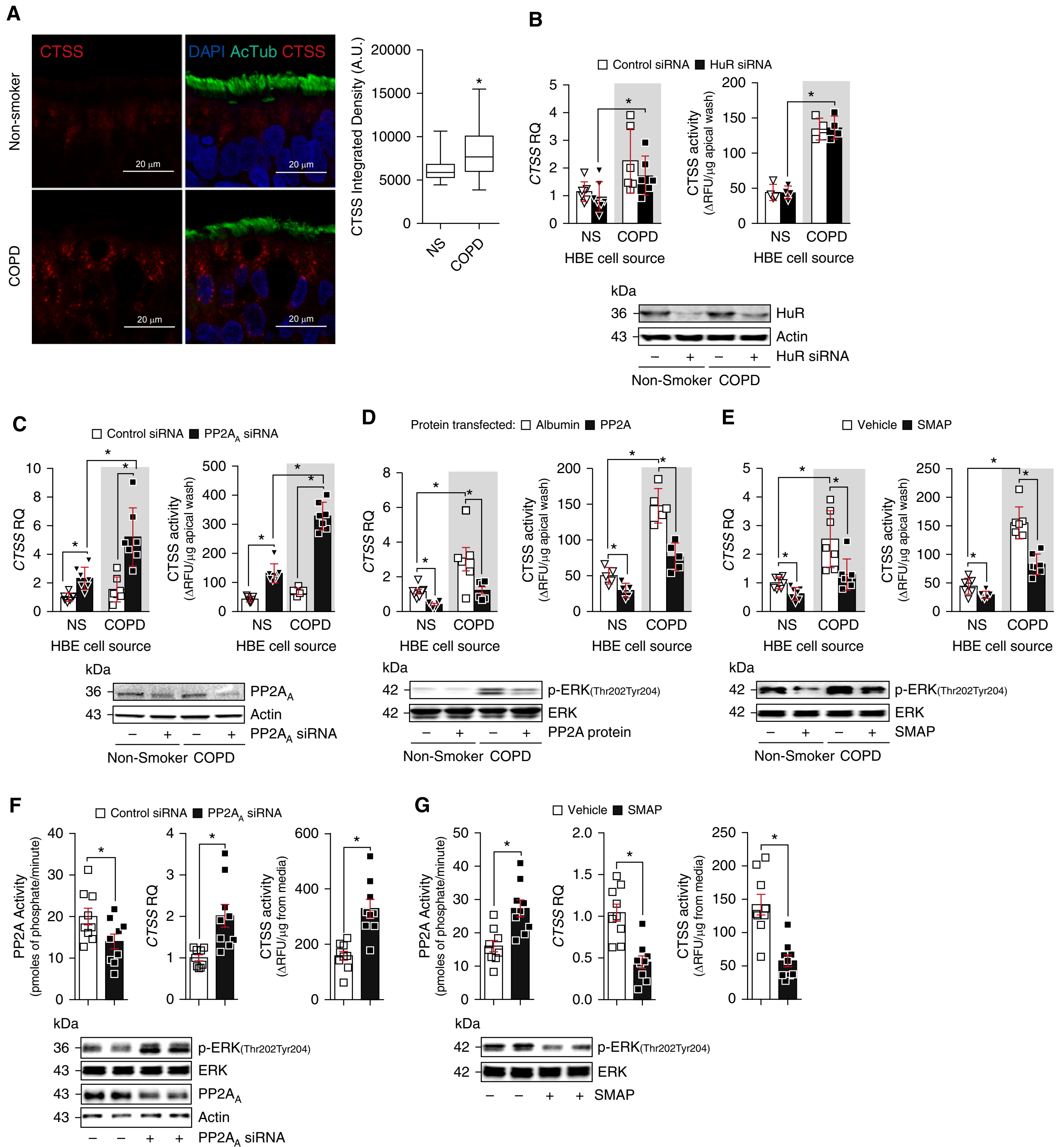

Figure 4. Human bronchial epithelial (HBE) cells from patients with chronic obstructive pulmonary disease (COPD) have enhanced CTSS (cathepsin S) responses because of PP2A (protein phosphatase 2A) inhibition. (*A*) Bronchial tissue from nonsmokers and subjects with COPD were stained for CTSS (red), DAPI (blue), and acetylated tubulin (AcTub; green) and CTSS staining intensity was quantified. (*B–E*) HBE cells isolated from nonsmokers without COPD and individuals with COPD were transfected with scrambled or human antigen R (HuR) siRNA (*B*), scrambled or $PP2A_A$ siRNA (*C*), and albumin or active PP2A protein (*D*) or treated with SMAP (small molecule activator of PP2A) (*E*). Gene expression of *CTSS* was determined in all cells and CTSS activity quantified in media. Immunoblots were performed to confirm transfection efficiency for (*B*) HuR, (*C* and *F*) $PP2A_A$, and (*D–G*) ERK (extracellular signal–regulated kinase) phosphorylation as a downstream readout of PP2A activity. (*F* and *G*) Macrophages derived from peripheral blood

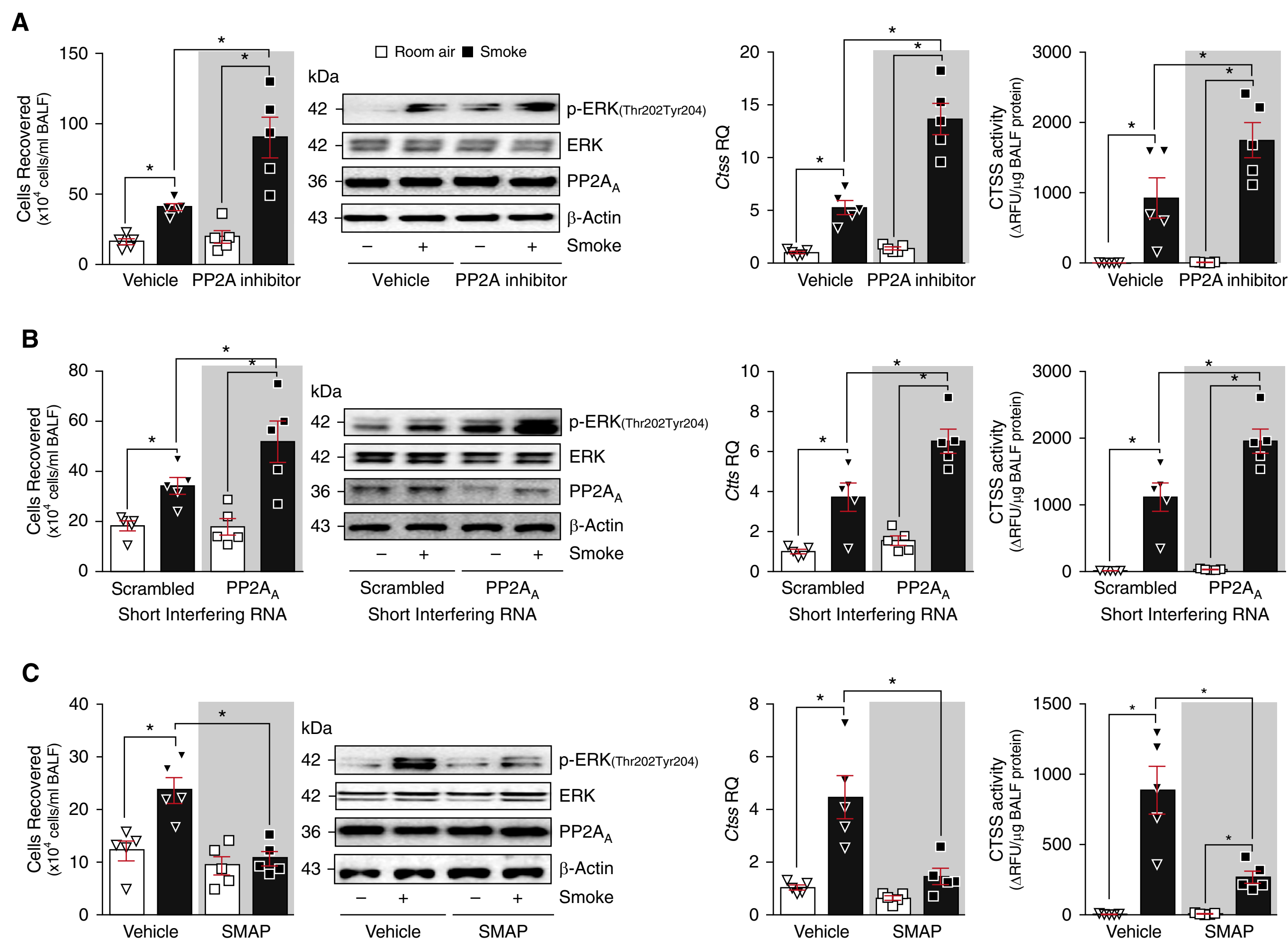

Figure 5. Modulating PP2A (protein phosphatase 2A) signaling alters acute smoke-induced lung *Ctss* expression. Mice were exposed to room air and cigarette smoke and either (*A*) daily injections of okadaic acid (2 μg/kg, i.p.), (*B*) intranasally administered scrambled or PP2A$_A$ silencer siRNA, or (*C*) two oral administrations of small molecule activator of PP2A daily for 3 days. Mice were killed 24 hours after the last exposure ($n = 5$ for each group). BAL fluid (BALF) cellularity levels were examined in each mouse. Immunoblots were performed for ERK (extracellular signal–regulated kinase) phosphorylation as a downstream readout of PP2A and total levels of PP2A$_A$ and β-actin were included as controls. Lung *Ctss* gene expression and BALF cathepsin S activity were examined by qPCR and substrate activity assays, respectively. *$P < 0.05$, when comparing both treatments connected by a line, determined by two-way ANOVA with Tukey *post hoc* test. CTSS = cathepsin S; RQ = relative quantification; SMAP = small molecule activator of PP2A.

Either directly targeting CTSS activity or enhancing PP2A activity to decrease CTSS expression may represent a plausible means to counter COPD progression. Importantly, pharmacologic reactivation of the endogenous enzyme, PP2A, negatively regulates CTSS expression and prevented smoke-induced loss of lung function.

Neutrophil elastase and MMPs are the most frequent proteases implicated in the pathogenesis of COPD. SNPs in MMP1, MMP9 (37), and MMP12 (38) are associated with COPD. However, of the numerous protease inhibitory molecules tested, only one elastase inhibitor, Sivelestat (ONO-5046), is currently approved for the treatment of acute lung injury but not COPD because of toxicity issues (39). In recent years, CTSS has received more attention as a target for multiple diseases (12) and our data here outline the potential importance of inhibiting CTSS to reduce progression of COPD. Because CTSS activity is elevated in COPD patient samples (10, 11) and CTSS is activated at a neutral pH (17), increased levels of CTSS would have proteolytic activity in a healthy lung and may be a critical step in establishing early stage COPD. We and others have demonstrated that CTSE and CTSG are also enhanced by smoke exposure (6, 9). Both CTSE (6) and CTSG (7) play important roles in disease

Figure 4. (Continued). monocytes from nonsmokers were transfected with scrambled or PP2A$_A$ siRNA (*F*) or treated with SMAP (*G*). *CTSS* gene expression and PP2A and CTSS activities were determined, and immunoblots were performed. Data are represented as mean ± SEM, where each measurement was performed on 3 independent days and with $n \geq 3$ subjects per group. *$P < 0.05$, when comparing both treatments connected by a line, determined by two-way ANOVA with Tukey *post hoc* test. AU = arbitrary units; NS = nonsmoker; RFU = relative fluorescence units; RQ = relative quantification.

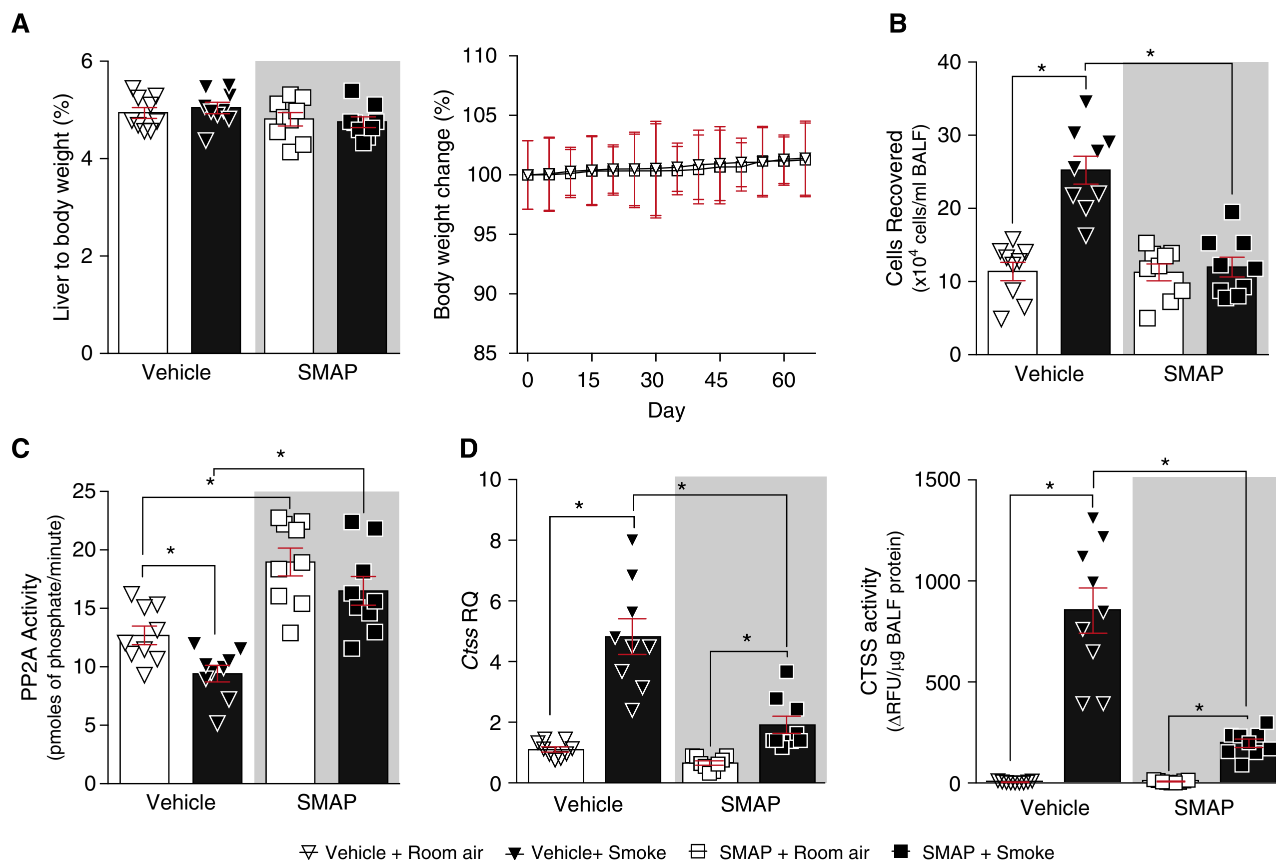

Figure 6. Activating PP2A (protein phosphatase 2A) signaling alters long-term smoke-induced lung *Ctss* expression. A/J mice were exposed to room air and cigarette smoke and two oral administrations of small molecule activator of PP2A daily for 2 months. Mice were killed 24 hours after the last exposure ($n = 9$ for each group). (*A*) Liver-to-body-weight ratios and whole-body weights were recorded in each group. (*B*) BAL fluid (BALF) cellularity levels were examined in each mouse. (*C*) Lung PP2A activity, (*D*, left) lung *Ctss* gene expression, and (*D*, right) BALF CTSS activity were examined by substrate activity assays, qPCR, and substrate activity assays, respectively. $^*P < 0.05$, when comparing both treatments connected by a line, determined by two-way ANOVA with Tukey *post hoc* test. CTSS = cathepsin S; RQ = relative quantification; SMAP = small molecule activator of PP2A.

progression but seem not to be regulated at the transcriptional level by SMAP treatment. Our results establish the role of CTSS in early disease development and suggest that targeting this protease could be an effective therapeutic strategy in COPD.

We explored several mechanisms to determine how smoke exposure enhanced CTSS expression in the lungs. Inflammatory mediators can influence CTSS expression, with IFN-γ (32), tumor necrosis factor-α, and IL-1β (36) all linked to CTSS expression. However, we did not observe significant changes in these inflammatory mediators after SMAP administration but cannot rule out these or other unidentified factors regulating CTSS levels in COPD. We also explored HuR and PP2A as potential regulators of CTSS. Editing of RNA integrity is associated with the progression of multiple diseases, including cardiovascular disease (33). Recruitment of the stabilizing RNA-binding protein HuR, to the 3′ UTR of the CTSS transcript, enhances *CTSS* mRNA stability and expression (33). HuR expression did not impact CTSS expression in HBE cells in this study. However, we cannot completely rule out the possibility of HuR or other RNA stabilizing proteins playing a role on *Ctss* expression in smoke-exposed lungs. Cigarette smoke extract alters HuR expression to modulate SNAIL signaling in small airway epithelial cells (40). It is conceivable that HuR could exert similar effects to stabilize and enhance *CTSS* expression in the COPD lung.

Investigating how the mRNA stability of key COPD-associated genes alters the initiation and progression of this disease is an important future area of study. In our findings, however, PP2A seems to be the primary factor responsible for changes in *CTSS* expression. We previously observed increased *Ctss* expression and reduced PP2A activity in mice exposed to smoke while infected with respiratory syncytial virus (9). In this current study, we directly show that the loss of PP2A signaling is responsible for elevated CTSS expression in mice and HBE cells. This is important, because inhibition of PP2A coincides with multiple changes in the lungs, including immune responses (19), mucus production (41), protease expression (18), and corticosteroid sensitivity (42). The SMAP compound used in this study inhibits tumor formation via activation of PP2A (22, 43). The SMAP compound activates PP2A by binding to the A subunit of PP2A, promoting conformational changes, which increase cellular phosphatase activity (22) and promoting PP2A holoenzyme (ABC subunit) assembly and perturbs interactions with endogenous PP2A inhibitors. Other

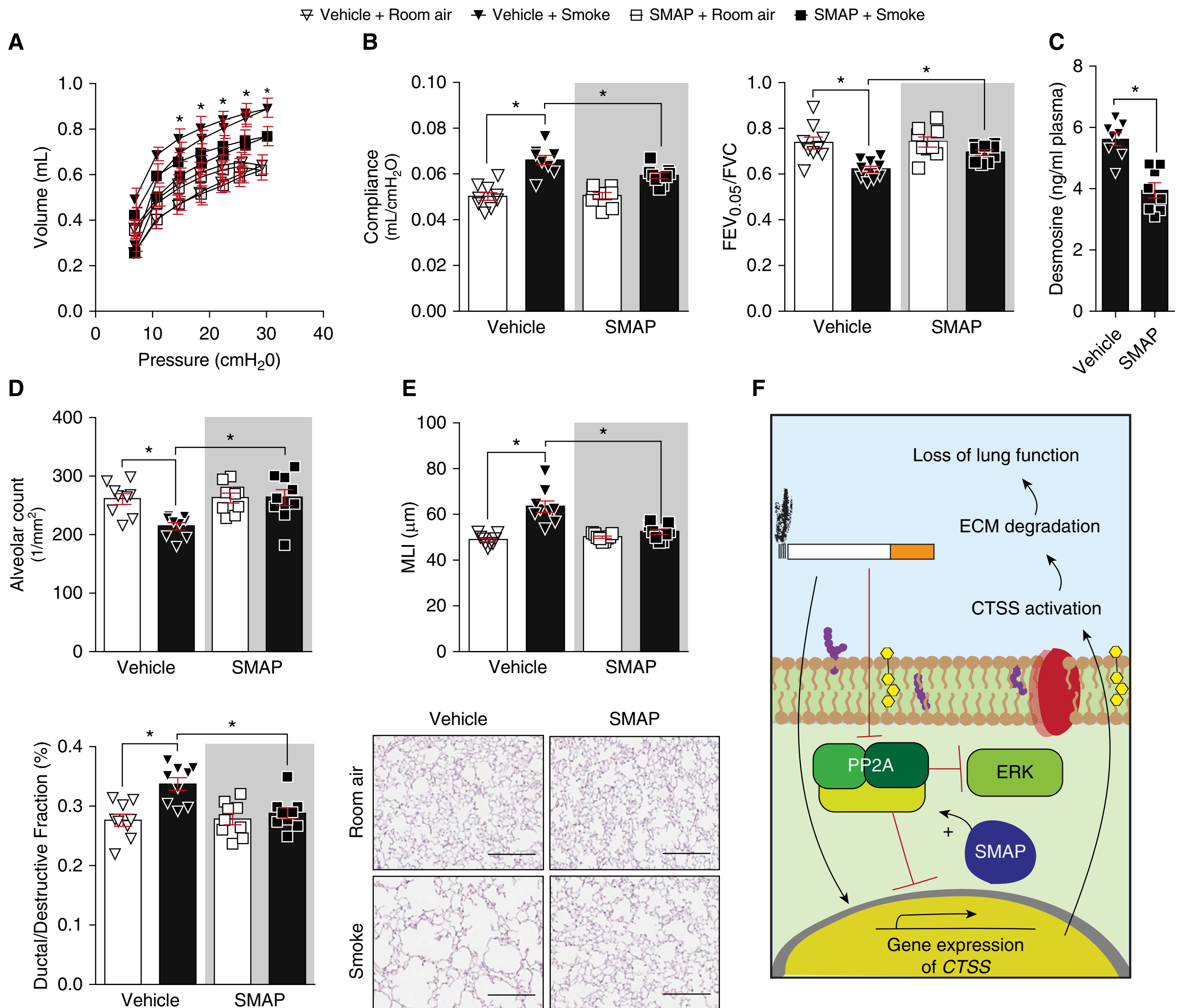

Figure 7. Activating PP2A (protein phosphatase 2A) signaling prevents smoke-induced loss of lung function. Mice were exposed to room air and cigarette smoke and two oral administrations of small molecule activator of PP2A daily for 2 months. (*A* and *B*) Negative pressure-driven forced expiratory and forced oscillation technique maneuvers were performed in all animal groups. (*A*) Pressure–volume loops and (*B*) compliance and forced expiratory volume in 0.05 seconds ($FEV_{0.05}$)/FVC were determined in each animal. (*C*) Plasma desmosine levels were assessed in smoke-exposed animals by ELISA. (*D*) Alveolar count and ductal/destructive fractions were quantified in each animal by parenchymal airspace profiling. (*E*) Mean linear intercepts were measured in the lungs of the mice to assess air space size, and comparative histology images of the four mouse groups are presented here (scale bars, 40 μm). Data are represented as mean ± SEM, where $n = 9$ per group. $^*P < 0.05$, when comparing both treatments connected by a line, determined by two-way ANOVA with Tukey *post hoc* test. (*F*) Illustration of the possible signaling mechanism for PP2A regulation of CTSS (cathepsin S). Evidence presented in this study indicates that PP2A prevents signaling leading to *CTSS* gene expression, but after smoke exposure, *CTSS* expression is enhanced and the phosphatase activity of PP2A is diminished. This enhancement of CTSS directly impacts lung function. ECM = extracellular matrix; ERK = extracellular signal–regulated kinase; MLI = mean linear intercept; SMAP = small molecule activator of PP2A.

compounds, such as FTY-720, erlotinib, and analogous synthetic sphingolipids, also activate PP2A (21, 44, 45) by binding the endogenous PP2A inhibitors, inhibitor 2 of PP2A (I2PP2A/SET) or cancerous inhibitor of PP2A (CIP2A), and derepressing PP2A activity. These could also be possible therapeutic candidates for the treatment of COPD. Our data with smoke exposure in combination with SMAP suggests that this class of compounds could be considered for the treatment of smoke-associated diseases and warrant further preclinical investigations.

In addition, direct CTSS enzyme inhibitors are currently being investigated in multiple disease models. For example, RO5459072, a CTSS inhibitor, suppresses

systemic and peripheral disease-associated mechanisms of autoimmune tissue injury in mice (46). RO5459072 also reduced CD4 T-cell and dendritic cell activation, and autoantibody production in a preclinical model of spontaneous systemic lupus erythematosus and lupus nephritis (47). CTSS inhibition also reduces the inflammatory responses of macrophages by causing these cells to secrete less proinflammatory cytokines and express less MHC class II and CD80 (48). Thus, the therapeutic benefits of reducing CTSS activity may be achieved in two ways: upstream by exploiting the negative regulation of CTSS transcription via PP2A activation, as shown in the present study; and directly by inhibiting CTSS enzyme activity. Combination therapy potential of SMAPs and CTSS inhibitors may be beneficial in several ways (i.e., allow reduced dosing of CTSS inhibitors to minimize its potential toxicity and targeting the neutrophil pool of CTSS) (46). Advancing our current studies, we will focus on combinational therapy potential, including the use of CTSS inhibitors and other therapeutic agents.

Together, our data identify PP2A's negative regulation of CTSS as an important factor in smoke-induced COPD, because reduction in CTSS expression prevents loss of lung function, reduces inflammation, and slows the degradation of elastin and lung tissue remodeling. Indeed, our work highlights that targeting the PP2A/CTSS pathway may limit smoke-induced COPD. ■

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