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Protein Phosphatase 2A Reduces Cigarette Smoke-Induced Cathepsin S and Loss of Lung Function

Declan F. Doherty[1], Sridesh Nath[2], Justin Poon[2], Robert F. Foronjy[2,3], Michael Ohlmeyer[4,5], Abdoulaye J. Dabo[2,3], Matthias Salathe[6,7], Mark Birrell[8,9], Maria Belvisi[8,9], Nathalie Baumlin[6,7], Michael D. Kim[6,7], Sinéad Weldon[1], Clifford Taggart[1]* and Patrick Geraghty[2,3]*

[1]Airway Innate Immunity Research Group (AiiR), Centre for Experimental Medicine, Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, Northern Ireland, UK; [2]Division of Pulmonary & Critical Care Medicine, Department of Medicine, State University of New York Downstate Medical Centre, Brooklyn, NY, USA; [3]Department of Cell Biology, State University of New York Downstate Medical Centre, Brooklyn, NY, USA; [4]Icahn School of Medicine at Mount Sinai, New York, New York; [5]Atux Iskay LLC, Plainsboro, New Jersey; [6]Department of Internal Medicine, University of Kansas Medical Center, Kansas City, Kansas, USA; [7]Division of Pulmonary, Critical Care, and Sleep Medicine, University of Miami, Miami, Florida, USA; [8]Respiratory Pharmacology Group, Airway Disease Section, National Heart and Lung Institute, Imperial College, London, UK; [9]Respiratory, Inflammation and Autoimmunity, Innovative Medicines and Early Development Biotech Unit, AstraZeneca, London, UK.

*CT and PG share senior authorship

Correspondence to: Patrick Geraghty, PhD; Telephone: (718) 270-3141; Fax: (718) 270-4636; E-mail: Patrick.Geraghty@downstate.edu

Author contributes

Performed experiments: D.F.D., S.N, J.P, M.O., A.J.D., N.B., M.D.K., S.W. and P.G.; Conception and design: R.F.F., M.O., C.T. and P.G.; Analysis and interpretation: D.F.D., S.N, J.P., R.F.F., M.O., M.S., M.B., M.B., N.B., M.D.K., S.W., C.T. and P.G.; Drafting the manuscript for important intellectual content: D.F.D, M.O., M.S., N.B., C.T. and P.G.

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At a Glance Commentary

Cathepsin S (CTSS), a lysosomal cysteine protease with elastase activity across a wide pH range is elevated in COPD clinical samples, but it's role in the disease process is unknown. This study demonstrates that CTSS significantly contributes to cigarette smoke-induced loss of lung function in mice. CTSS expression is negatively regulated by protein phosphatase 2A (PP2A) 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 pharmacological 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.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

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

Objectives: The objective of this study was 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 protein phosphatase 2A (PP2A) activity.

Measurements and Main Results: Here we observed enhanced CTSS expression and activity in mouse lungs following 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 non-smokers and COPD donors and in monocyte-derived macrophages. Modulating PP2A expression or activity, with silencer short interfering RNA 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.

Key words: Cigarette smoke, cathepsin S, phosphatase and chronic obstructive pulmonary disease

Introduction

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 US (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). Though 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 cathepsin (CTS) 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 BALF (10) and plasma of COPD patients (11). Altered CTSS levels are associated with a variety of pathological conditions including cystic fibrosis (CF), 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 protein phosphatase 2A (PP2A), 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 prior to smoke exposure, enhanced CTSS expression and lung inflammation. Equally, normalizing PP2A levels in mice or in human bronchial epithelial (HBE) cells isolated from COPD subjects 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 to 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 four hours daily, five days per week 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 (IP) 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 small molecule activator of PP2A (SMAP; see (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 National Institutes of Health and Institutional Animal

Care and Use Committee (IACUC) 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

Bronchoalveolar lavage fluid (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 ATS/ERS 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 non-smokers and COPD patients were isolated from human organ donor lungs rejected for transplant and fully re-differentiated at the ALI as previously described (29). Consent for research was obtained by the Life Alliance Organ Recovery Agency of the University of Miami. All consents were IRB-approved 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

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 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 ± S.E.M. Data were compared by Student's t test (two-tailed) or by two-way ANOVA and Tukey's post hoc test analysis, using GraphPad Prism Software (Version 6.0h for Mac OS X).

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 and Figure E1). We primarily focused on CTSS as higher levels are observed in the BALF (10) and plasma of COPD patients (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 post exposure 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 ≥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 $FEV_{0.05}$/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 vital capacity that is expired in the first one-twentieth of a second of forced expiration to the full vital capacity. In mice, smoke inhalation typically enhances compliance and reduces $FEV_{0.05}$/FVC levels (Figure 2C). Importantly, *Ctss*$^{-/-}$ mice developed less emphysematous changes following exposure to smoke compare to controls, 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 COPD patients (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 following smoke exposure compared to wild-type mice (Figure 3A). However, *Ctss* expression did not impact eosinophil, T or B cell numbers in the lungs (Figure 3A). Smoke exposure did enhance T 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 (MLI) analysis (Figure 3B). Since 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 to wild type mice (Figure 3C), indicating less elastin degradation. Parenchymal airspace profiling (27) was utilized to demonstrate that *Ctss*$^{-/-}$ mice had a higher alveolar count, reduced loss of alveolar

boundary and reduced ductal destruction compared to 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 COPD patients express more CTSS than cells from non-smokers without COPD partially due to 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 bronchial tissue and is elevated in bronchial tissue from COPD subjects (Figure 4A). To explore further the regulation of CTSS expression, we utilized HBE cells isolated from non-smokers, and COPD subjects. Cells isolated from COPD subjects expressed and secreted more CTSS than cells from non-smokers (Figure 4B-E). The stabilizing RNA-binding protein human antigen R (HuR) and the phosphatase, PP2A, have been linked to the regulation of CTSS expression in atherosclerosis (33) and Alzheimer's disease/Down syndrome (34). Therefore, we examined *CTSS* gene expression and activity in HBE cells following modulation of HuR or PP2A signaling. Loss of HuR expression, with siRNA transfection, did not significantly alter CTSS signaling in HBE cells from non-smokers or COPD subjects (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 a small molecular activator of PP2A (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 (Figure 4D-E).

Other cell types also express CTSS, such as macrophages (10). Human monocytes were isolated from peripheral blood of non-smokers and derived into macrophages. Similar to HBE cells, silencing $PP2A_A$ enhanced CTSS expression and activity in monocyte-derived macrophages (Figure 4F). Alternatively, SMAP treatment enhanced PP2A activity and reduced ERK and CTSS responses (Figure 4G). Therefore, loss of PP2A activity appears 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 short interfering RNA 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 to controls (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

290 $PP2A_A$ in the lungs enhanced inflammation, ERK phosphorylation and CTSS
291 responses in mice (Figure 5B). Alternatively, administration of SMAP to mice
292 reduced smoke-induced immune cell infiltration, ERK phosphorylation, as well as
293 CTSS expression and enzyme activity (Figure 5C).

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

308 To examine whether SMAP treatment prevents the alteration of lung
309 function in response to cigarette smoke, we examined PV loops, compliance and
310 $FEV_{0.05}$/FVC. The PV loop analysis from SMAP treated mice were lower compared
311 to vehicle treated animals, when exposed to smoke (Figure 7A). SMAP treated
312 mice developed less emphysematous changes following exposure to smoke
313 compared to controls, 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 to vehicle treated animals (Figure 7C). SMAP-treated animals had higher alveolar counts and reduced ductal destruction compared to smoke exposed vehicle treated mice (Figure 7D). SMAP administration also prevented the increase in smoke-induced airspace enlargements, determined by MLI analysis (Figure 7E). These SMAP-mediated changes in CTSS levels were observed without changes in inflammation, such as IL1β (36), IFNγ (32) and TNFα (Figure E2A-B). Equally, SMAP administration did not impact smoke- induced *Ctse* or *Ctsg* (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, due to 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 appears 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. Either directly targeting CTSS activity or enhancing PP2A

activity to decrease CTSS expression may represent a plausible means to counter COPD progression. Importantly, pharmacological 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. Single-nucleotide polymorphisms (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 due to toxicity issues (39). In recent years, CTSS has received more attention as a target for multiple diseases (12) and our data here outlines the potential importance of inhibiting CTSS to reduce progression of COPD. Since 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 progression but appear 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 IFNγ (32), TNFα and IL1β (36) all linked to CTSS

expression. However, we did not observe significant changes in these inflammatory mediators following 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 human antigen R, 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 appears 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 (RSV) (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, as 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 utilized in this study inhibit 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 compounds, such as erlotinib, FTY-720, and analogous synthetic sphingolipids, also activate PP2A (44-46) by binding the endogenous PP2A inhibitors, cancerous inhibitor of PP2A (CIP2A) or inhibitor 2 of PP2A (I2PP2A/SET), and de-repressing PP2A activity. These could also be possible therapeutic candidates for the treatment of COPD. Our data with smoke exposure in combination with small molecule activators of PP2A 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 (47). 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 (48). 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 (49). 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 directly. 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 (47). 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, as reduction in CTSS expression prevents loss of lung function, reduces inflammation, 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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Figure Legends

Figure 1. Smoke exposure enhances *Ctss* gene and protein expression in mice lungs.

(A) CTS genes were quantified in C57BL/6J lung tissue, following 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 BALF of C57BL/6J mice following 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 kDa and the active form is 25 kDa. 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 (A.U.) are shown here. Data are represented as mean ± S.E.M, with each measurement performed on 3 separate days from at least 4 animals/group. * denotes p value <0.05, when comparing both treatments connected by a line, determined by student t-tests.

Figure 2. *Ctss* 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-C) Negative pressure-driven forced expiratory and forced oscillation technique maneuvers were performed in all animal groups. (B) Pressure volume loops, (C) compliance and forced expiration (FEV) in the first 0.05 second of forced vital capacity (FVC) was determined in each animal. Data are represented as mean ± S.E.M, where n=10 per group. * denotes a p value <0.05, when comparing both treatments connected by a line, determined by 2-way ANOVA with Tukey's post hoc test.

Figure 3. *Ctss* 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 (MLI) 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 ± S.E.M, where n≥5 per group. * denotes a p value <0.05, when comparing both treatments connected by a line, determined by 2-way ANOVA with Tukey's post hoc test or Student T-Test when comparing only 2 groups.

Figure 4. HBE cells from COPD patients have enhanced CTSS responses due to PP2A inhibition.

(A) Bronchial tissue from non-smokers and COPD subjects were stained for CTSS (red), DAPI (blue) and acetylated tubulin (green) and CTSS staining intensity was quantified. Images are x20 magnification. HBE cells isolated from non-smokers without COPD and COPD individuals were transfected with (B) scrambled or HuR siRNA, (C) scrambled or $PP2A_A$ siRNA and (D) albumin or active PP2A protein or

(E) treated with SMAP. Gene expression of *CTSS* was determined in all cells and CTSS activity quantified in media. Immunoblots were performed to confirm transfection efficiency for (A) HuR and β-Actin and (B-C) ERK phosphorylation as a downstream readout of PP2A activity. (F/G) Peripheral blood monocytes from non-smokers were derived into macrophages and transfected with (F) scrambled or $PP2A_A$ siRNA or (G) treated with SMAP. *CTSS* gene expression, PP2A and CTSS activities and immunoblots were determined. Data are represented as mean ± S.E.M., where each measurement was performed on 3 independent days and with n≥3 subjects per group. * denotes a p value <0.05, when comparing both treatments connected by a line, determined by 2-way ANOVA with Tukey's post hoc test.

Figure 5. Modulating PP2A 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 IP), (B) intranasally administered scrambled or $PP2A_A$ silencer short interfering RNA, or (C) two oral administrations of SMAP daily for 3 days. Mice were euthanized 24 hours post last exposure (n = 5 for each group). BALF cellularity levels were examined in each mouse. Immunoblots were performed for ERK 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 CTSS activity were examined by qPCR and substrate activity assays, respectively. * denotes a p value <0.05, when comparing both treatments connected by a line, determined by 2-way ANOVA with Tukey's post hoc test.

Figure 6. Activating PP2A signaling alters long-term smoke-induced lung *Ctss* expression. A/J mice were exposed to room air and cigarette smoke and two oral administration of SMAP daily for 2 months. Mice were euthanized 24 hours post last exposure (n = 9 for each group). (A) Liver to body weight ratios and whole-body weights were recorded in each group. (B) BALF cellularity levels were examined in each mouse. (C) Lung PP2A activity, (D) lung *Ctss* gene expression and BALF CTSS activity were examined by substrate activity assays, qPCR and substrate activity assays, respectively. * denotes a p value <0.05, when comparing both treatments connected by a line, determined by 2-way ANOVA with Tukey's post hoc test.

Figure 7. Activating PP2A signaling prevents smoke-induced loss of lung function. Mice were exposed to room air and cigarette smoke and two oral administrations of SMAP daily for 2 months. (A/B) Negative pressure-driven forced expiratory and forced oscillation technique maneuvers were performed in all animal groups. (A) Pressure volume loops, (B) compliance and forced expiration extension (FEV) in the first 0.05 second of forced vital capacity (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 (MLI) 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 ± S.E.M,

where n=9 per group. * denotes a p value <0.05, when comparing both treatments connected by a line, determined by 2-way ANOVA with Tukey's post hoc test. (F) Possible signaling mechanism for PP2A regulation of CTSS. Evidence presented in this study indicates that PP2A prevents signaling leading to *CTSS* gene expression but following smoke exposure *CTSS* expression is enhanced and the phosphatase activity of PP2A is diminished. This enhancement of CTSS directly impacts lung function.

Figure 1

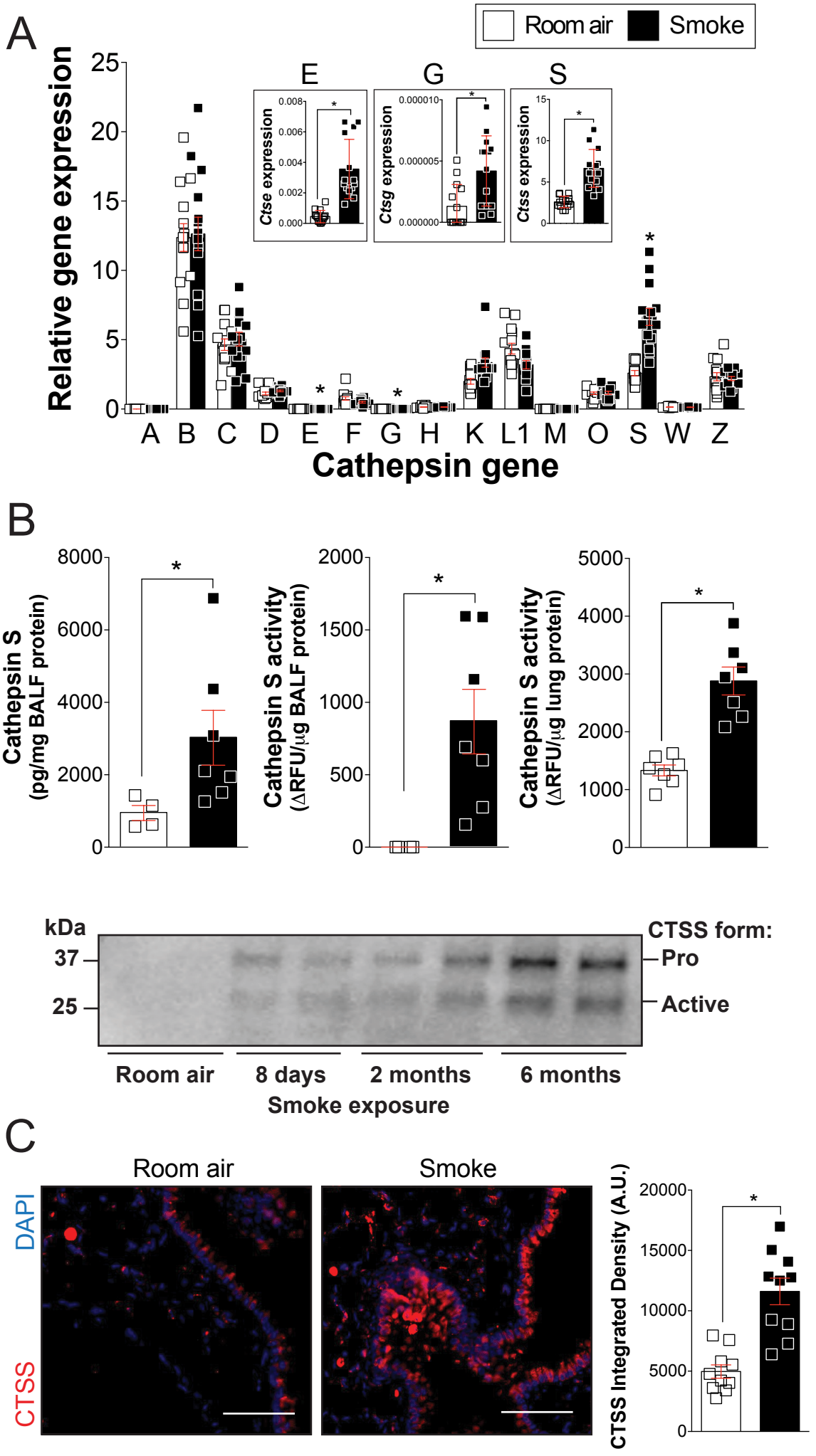

A
Room air
Smoke
Relative gene expression
Cathepsin gene
A B C D E F G H K L1 M O S W Z
E
G
S
Ctse expression
Ctsg expression
Ctss expression
B
Cathepsin S (pg/mg BALF protein)
Cathepsin S activity (ΔRFU/μg BALF protein)
Cathepsin S activity (ΔRFU/μg lung protein)
kDa
37
25
CTSS form:
Pro
Active
Room air
8 days
2 months
6 months
Smoke exposure
C
Room air
Smoke
DAPI
CTSS
CTSS Integrated Density (A.U.)

Figure 2

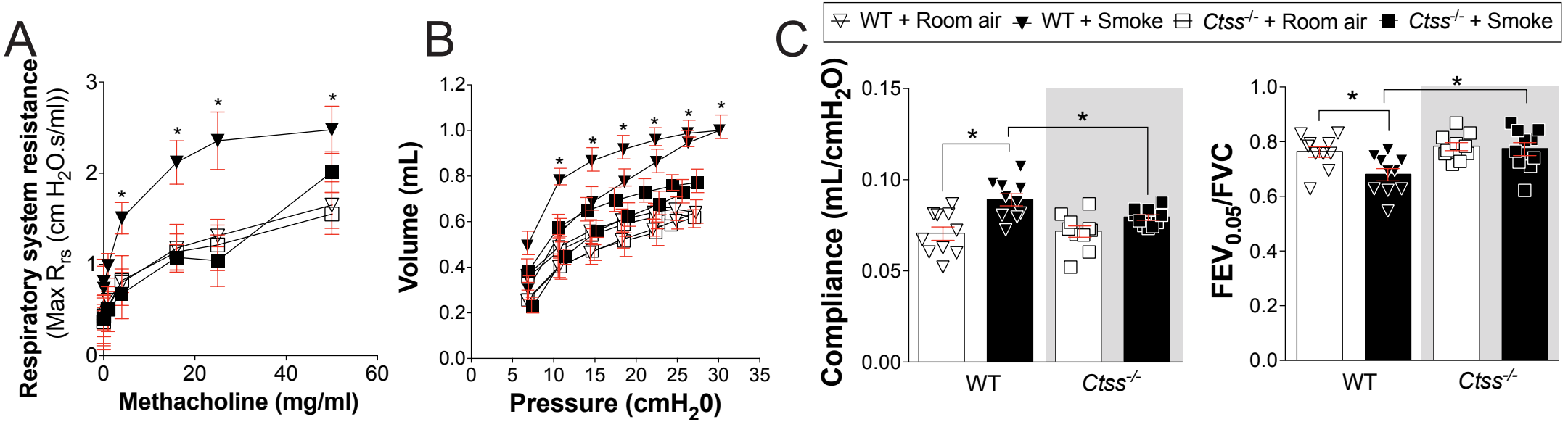

A
Respiratory system resistance
(Max R_{rs} (cm H_2O.s/ml))
Methacholine (mg/ml)
B
Volume (mL)
Pressure (cmH_20)
C
WT + Room air
WT + Smoke
Ctss$^{-/-}$ + Room air
Ctss$^{-/-}$ + Smoke
Compliance (mL/cmH_2O)
$FEV_{0.05}$/FVC
WT
Ctss$^{-/-}$

Figure 3

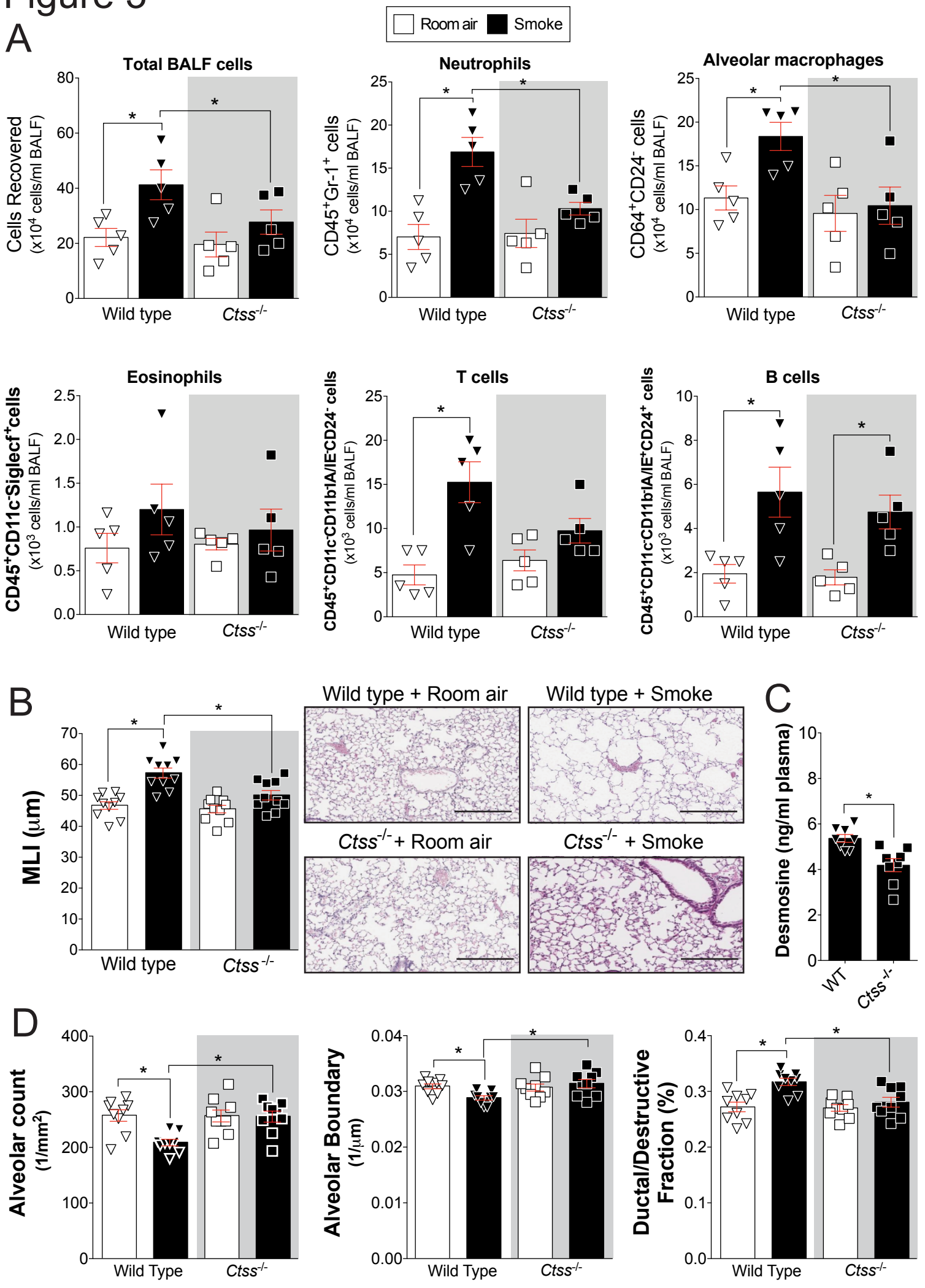

A
Room air
Smoke
Total BALF cells
Cells Recovered (x10^4 cells/ml BALF)
Neutrophils
CD45+Gr-1+ cells (x10^4 cells/ml BALF)
Alveolar macrophages
CD64+CD24- cells (x10^4 cells/ml BALF)
Eosinophils
CD45+CD11c-Siglecf+cells (x10^3 cells/ml BALF)
T cells
CD45+CD11c-CD11b1A/IE-CD24- cells (x10^3 cells/ml BALF)
B cells
CD45+CD11c-CD11b1A/IE+CD24+ cells (x10^3 cells/ml BALF)
Wild type
Ctss-/-
B
MLI (µm)
Wild type + Room air
Wild type + Smoke
Ctss-/- + Room air
Ctss-/- + Smoke
C
Desmosine (ng/ml plasma)
WT
Ctss-/-
D
Alveolar count (1/mm2)
Alveolar Boundary (1/µm)
Ductal/Destructive Fraction (%)
Wild Type

Figure 4

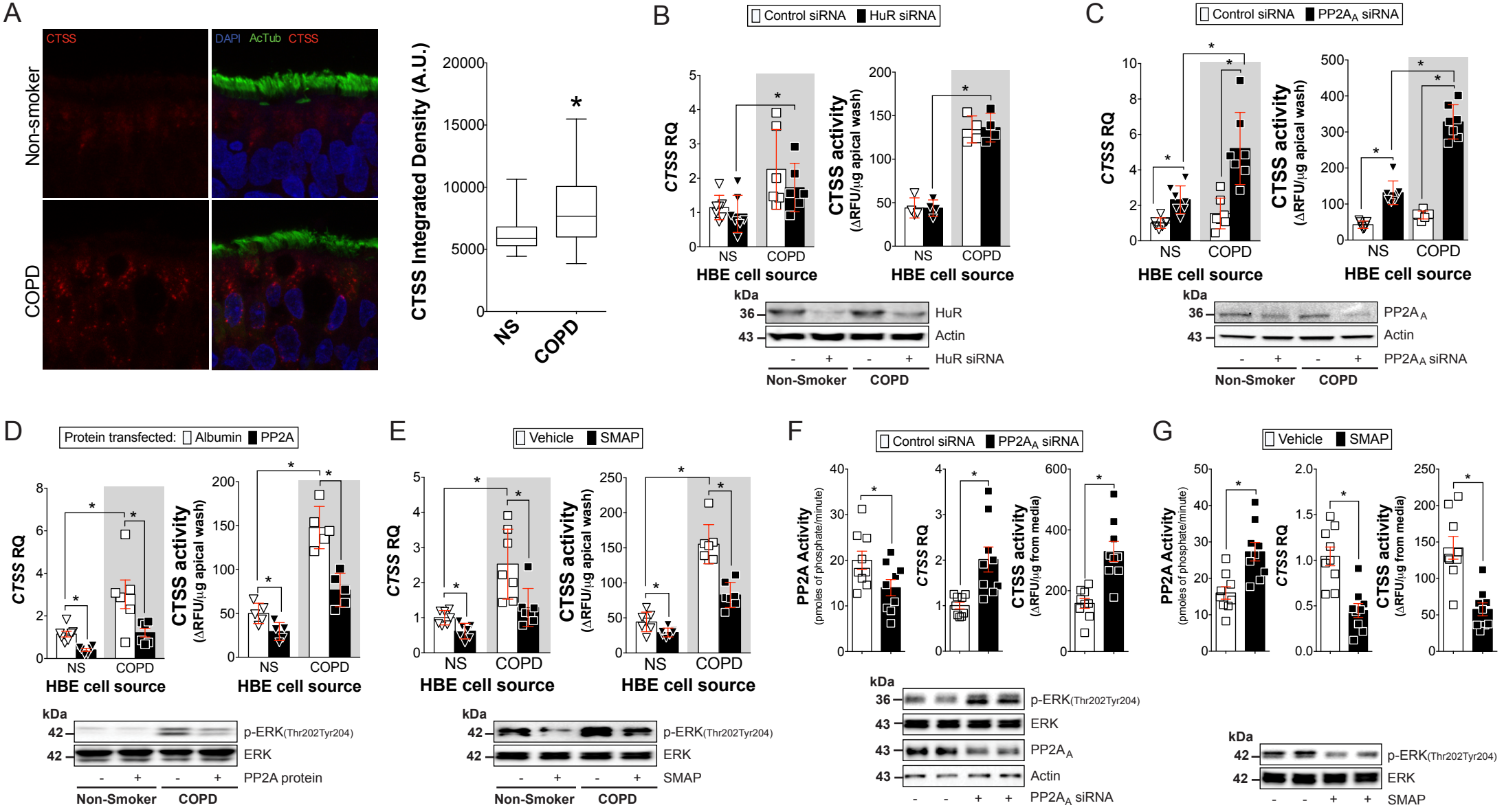

A
CTSS
DAPI AcTub CTSS
Non-smoker
COPD
CTSS Integrated Density (A.U.)
NS
COPD
B
Control siRNA
HuR siRNA
CTSS RQ
CTSS activity (ΔRFU/μg apical wash)
HBE cell source
kDa
HuR
Actin
HuR siRNA
Non-Smoker
COPD
C
Control siRNA
PP2A$_A$ siRNA
PP2A$_A$
PP2A$_A$ siRNA
D
Protein transfected: Albumin PP2A
p-ERK(Thr202Tyr204)
ERK
PP2A protein
E
Vehicle
SMAP
F
PP2A Activity (pmoles of phosphate/minute)
CTSS activity (ΔRFU/μg from media)
G

Figure 5

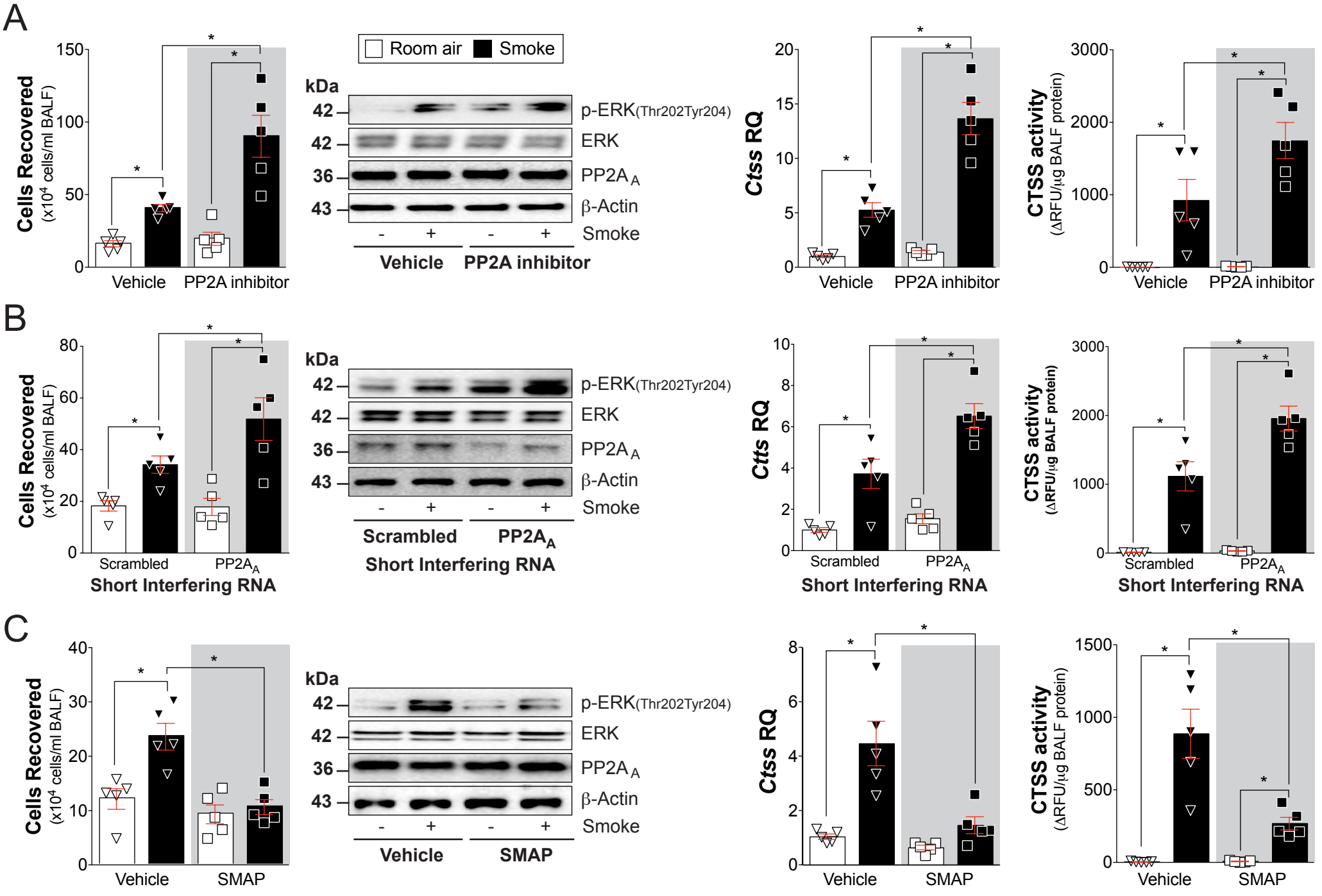

A
Cells Recovered
(x10^4 cells/ml BALF)
150
100
50
0
Vehicle
PP2A inhibitor
Room air
Smoke
kDa
42
42
36
43
p-ERK(Thr202Tyr204)
ERK
PP2A A
β-Actin
Smoke
- + - +
Vehicle
PP2A inhibitor
Ctss RQ
20
15
10
5
0
Vehicle
PP2A inhibitor
CTSS activity
(ΔRFU/μg BALF protein)
3000
2000
1000
0
Vehicle
PP2A inhibitor
B
Cells Recovered
(x10^4 cells/ml BALF)
80
60
40
20
0
Scrambled
PP2A A
Short Interfering RNA
kDa
42
42
36
43
p-ERK(Thr202Tyr204)
ERK
PP2A A
β-Actin
Smoke
- + - +
Scrambled
PP2A A
Short Interfering RNA
Ctts RQ
10
8
6
4
2
0
Scrambled
PP2A A
Short Interfering RNA
CTSS activity
(ΔRFU/μg BALF protein)
3000
2000
1000
0
Scrambled
PP2A A
Short Interfering RNA
C
Cells Recovered
(x10^4 cells/ml BALF)
40
30
20
10
0
Vehicle
SMAP
kDa
42
42
36
43
p-ERK(Thr202Tyr204)
ERK
PP2A A
β-Actin
Smoke
- + - +
Vehicle
SMAP
Ctss RQ
8
6
4
2
0
Vehicle
SMAP
CTSS activity
(ΔRFU/μg BALF protein)
1500
1000
500
0
Vehicle
SMAP

Figure 6

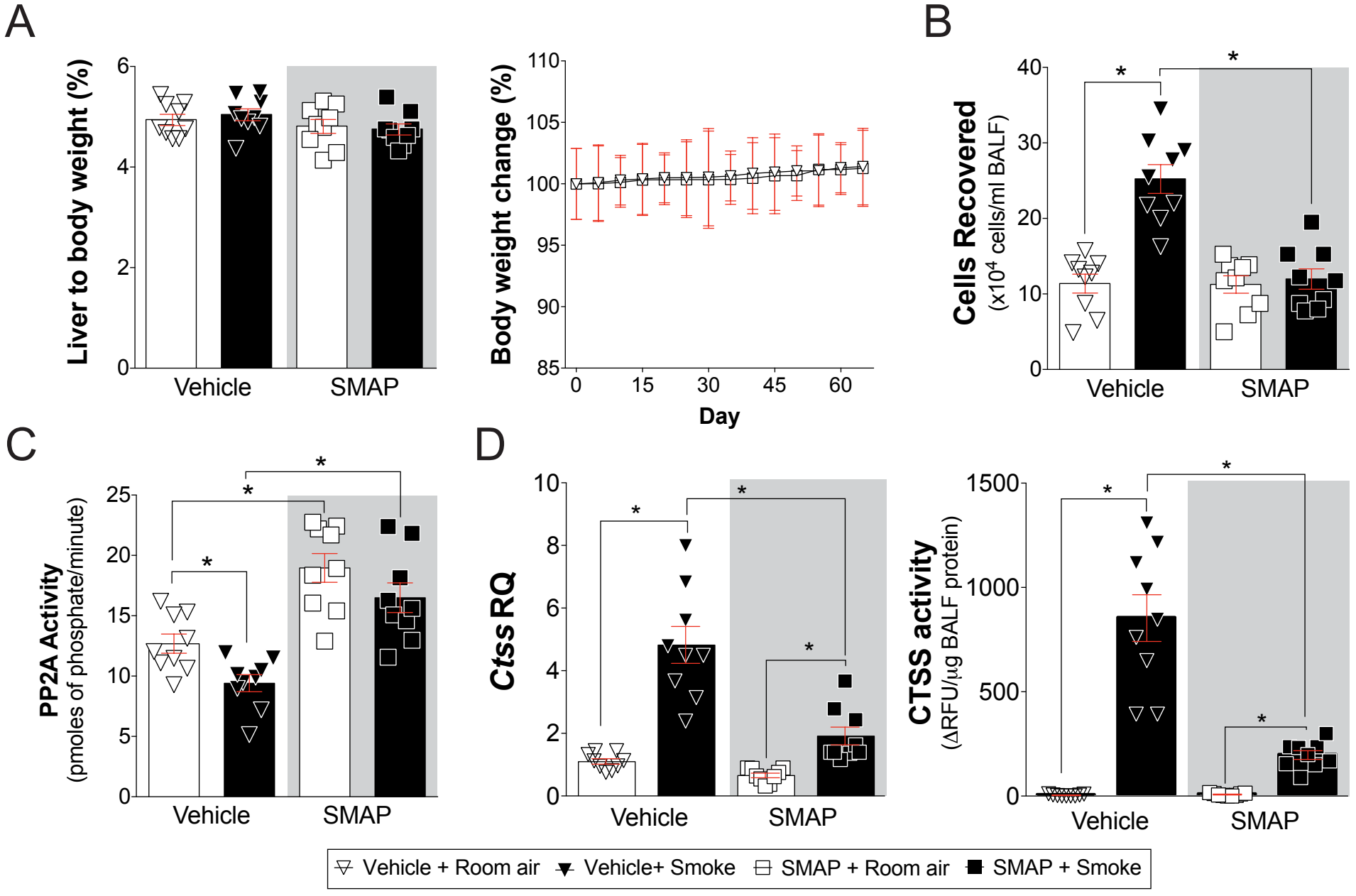

A
Liver to body weight (%)
0
2
4
6
Vehicle
SMAP
Body weight change (%)
85
90
95
100
105
110
0
15
30
45
60
Day
B
Cells Recovered
(x10^4 cells/ml BALF)
0
10
20
30
40
*
*
Vehicle
SMAP
C
PP2A Activity
(pmoles of phosphate/minute)
0
5
10
15
20
25
*
*
*
Vehicle
SMAP
D
Ctss RQ
0
2
4
6
8
10
*
*
*
Vehicle
SMAP
CTSS activity
(ΔRFU/μg BALF protein)
0
500
1000
1500
*
*
*
Vehicle
SMAP
Vehicle + Room air
Vehicle+ Smoke
SMAP + Room air
SMAP + Smoke

Figure 7

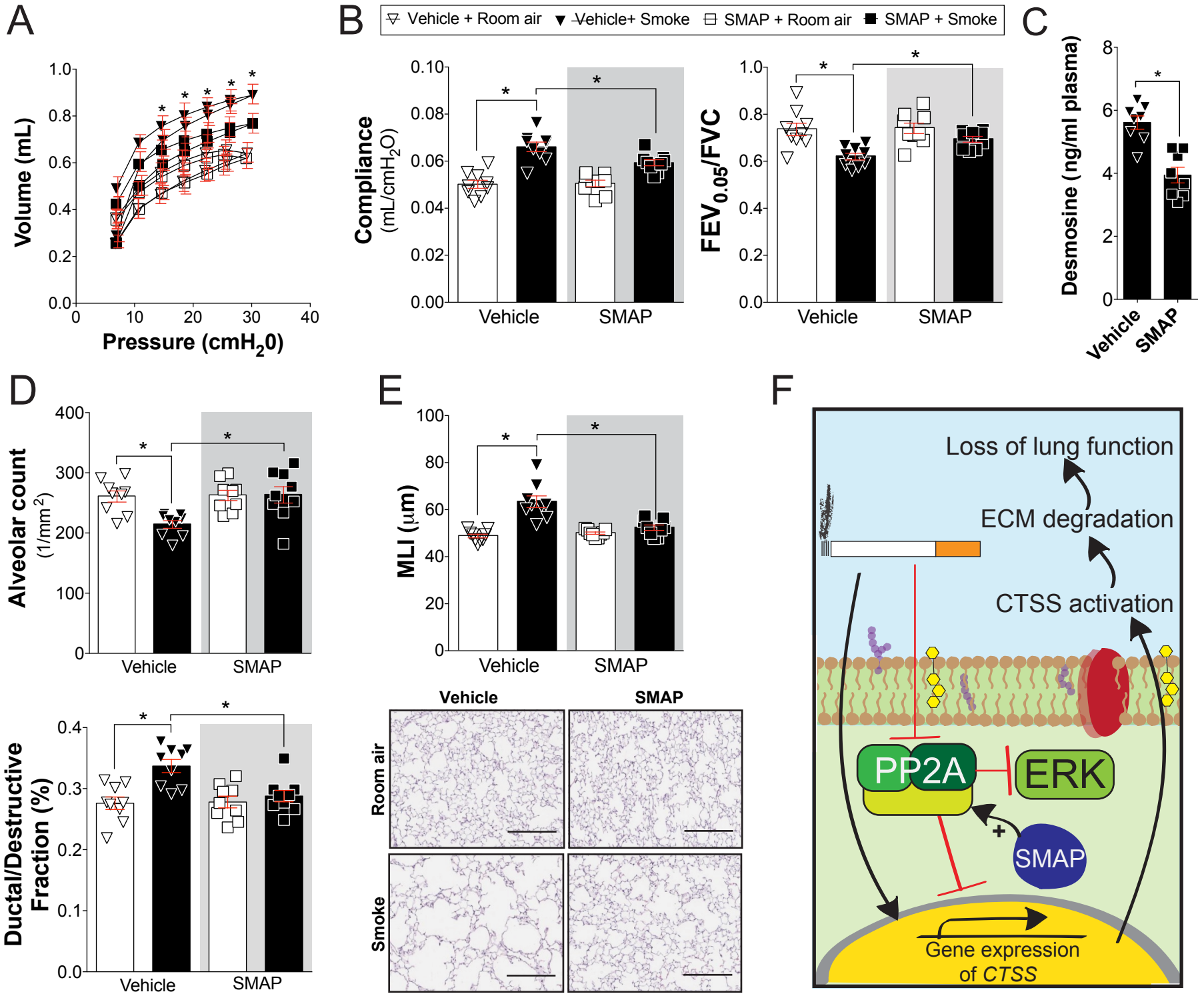

A
Volume (mL)
Pressure (cmH20)
B
Vehicle + Room air
Vehicle+ Smoke
SMAP + Room air
SMAP + Smoke
Compliance (mL/cmH2O)
FEV0.05/FVC
Vehicle
SMAP
C
Desmosine (ng/ml plasma)
Vehicle
SMAP
D
Alveolar count (1/mm2)
Ductal/Destructive Fraction (%)
Vehicle
SMAP
E
MLI (µm)
Vehicle
SMAP
Room air
Smoke
F
Loss of lung function
ECM degradation
CTSS activation
PP2A
ERK
SMAP
Gene expression of CTSS

Figure E1

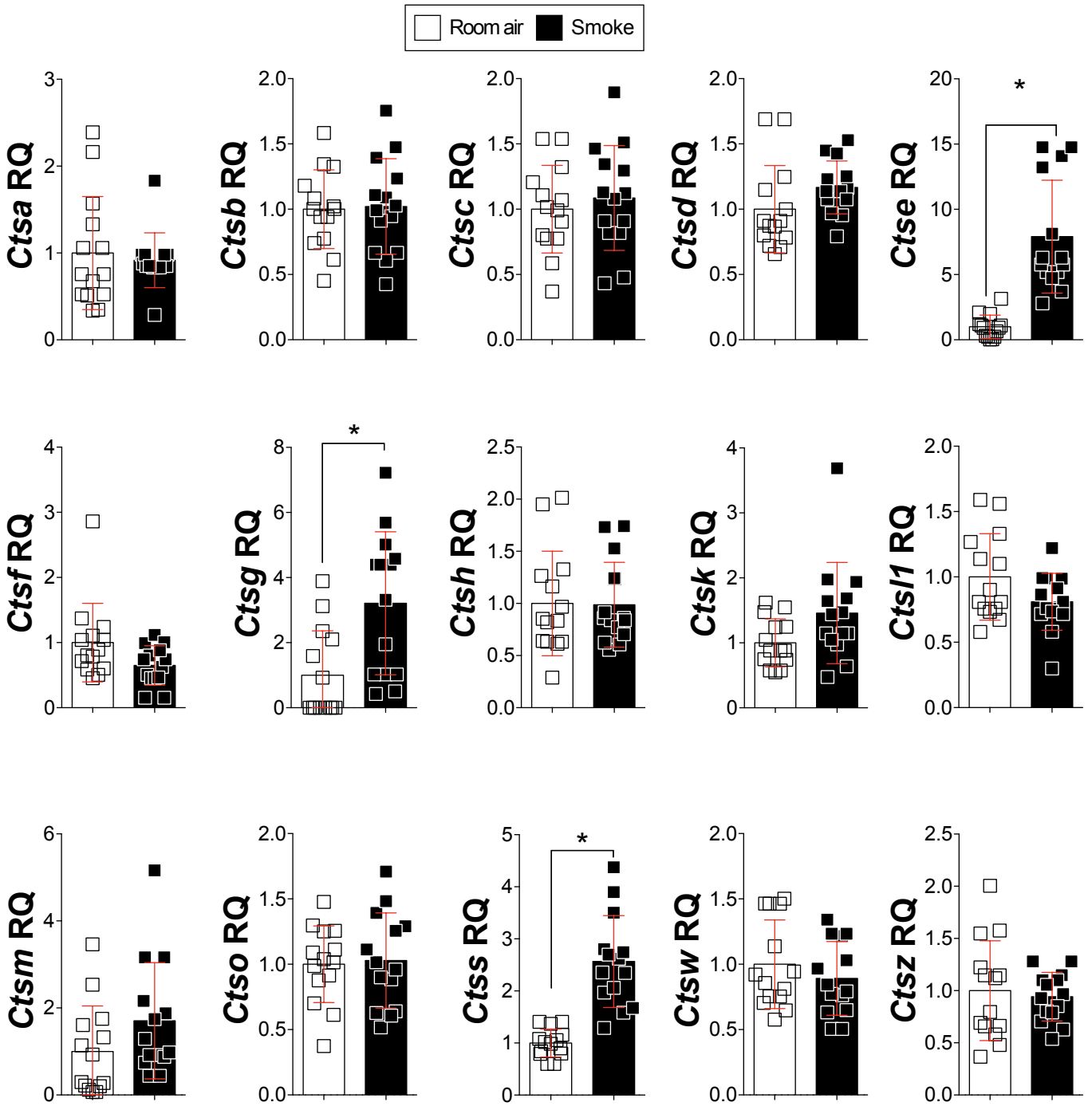
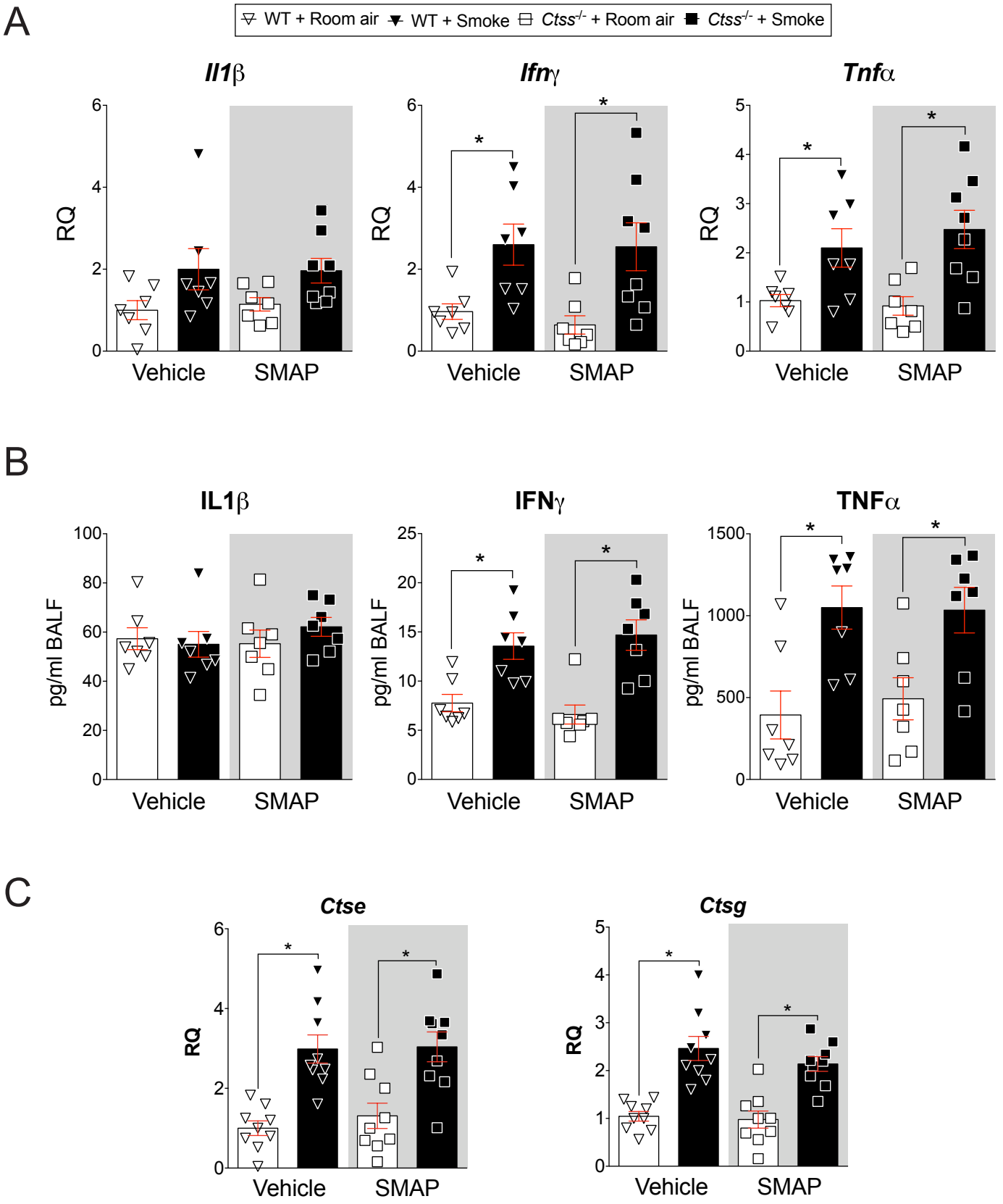

Room air
Smoke
Ctsa RQ
Ctsb RQ
Ctsc RQ
Ctsd RQ
Ctse RQ
Ctsf RQ
Ctsg RQ
Ctsh RQ
Ctsk RQ
Ctsl1 RQ
Ctsm RQ
Ctso RQ
Ctss RQ
Ctsw RQ
Ctsz RQ
*
*
*

Figure E2


A
WT + Room air
WT + Smoke
Ctss-/- + Room air
Ctss-/- + Smoke
Il1β
Ifnγ
Tnfα
RQ
Vehicle
SMAP
B
IL1β
IFNγ
TNFα
pg/ml BALF
Vehicle
SMAP
C
Ctse
Ctsg
RQ
Vehicle
SMAP