Online Data Supplement for

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

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Supplementary Methods:

Animal models

Male *Ctss*$^{-/-}$ mice (obtained from Professor Chris Scott, School of Pharmacy, Queen's University Belfast, Northern Ireland), on a C57BL/6J background, were maintained in a specific pathogen-free facility at SUNY Downstate Medical Centre. Animals were bred with heterozygous mating pairs and wild type littermates were used as controls. Male mice, 8-week-old, were used at the initiation point for all experiments and each experimental group had at least 10 animals per group. Mice were exposed to cigarette smoke in a chamber (Teague Enterprises, Davis, CA, USA) for four hours a day, five days per week at a total particulate matter concentration of 80-120 mg/m^3. Smoke exposure was continued for up to 6 months. The University of Kentucky reference research cigarettes 3R4F (Lexington, KY, USA) were used to generate cigarette smoke. An additional group of wild type (Jackson Laboratories, Ellsworth, MA, USA) mice, on a C57BL/6J background, were intraperitoneally (ip) injected with 100 µl of 0.2% ethanol

containing 2 μg/kg of okadaic acid (O-2220, LC Labs, Woburn, MA) or 0.2% ethanol alone. Alternatively, PP2A activity was enhanced in mice by oral administration of 50 mg/kg of a bioavailable small molecule activator of PP2A (SMAP; see (1)) twice daily, one-hour prior to smoke exposure and one-hour after smoke exposure. Animals intranasally received 7.4 nmol negative control (scrambled) or $PP2A_A$ (mouse *Ppp2r1a*) silencer short, interfering RNA (siRNA; Life Technologies, Grand Island, NY) in PBS, and 48 hours later were exposed to smoke. Mice administered okadaic acid, *Ppp2r1a* interfering RNA or SMAP were exposed to cigarette smoke for 3 days, as outlined above. Long-term smoke exposures (2 months) in combination with SMAP administration was also performed in both male and female A/J mice, 8-weeks old at initiation of experiments. All animal experiments were performed with approval from SUNY Downstate's Institutional Animal Care and Use Committee. This study was performed 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 exposed to long term smoke inhalation were anesthetized with an ip injection of ketamine/xylazine hydrochloride solution (100/10 mg/kg; MilliporeSigma Burlington, MA, USA). Animals were tracheostomized and connected via an endotracheal cannula to the SCIREQ flexiVent system (SCIREQ Inc., Montreal, Canada). After initiating mechanical ventilation, animals were paralyzed with a 1 mg/kg pancuronium bromide (MilliporeSigma) via ip injection and several pulmonary function measurements (pressure

volume loops, lung compliance and forced expiration extension (FEV) in the first 0.05 seconds of forced vital capacity (FVC)) were determined as previously described (2). Airway responses to increasing doses of methacholine (MilliporeSigma) were assessed with the Flexivent system following pulmonary function measurements.

Lung immune cell measurements

Bronchoalveolar lavage fluid (BALF) and BALF cells were obtained from animals of each group. Flow analysis was performed on BALF cells to identify neutrophils, alveolar macrophages, eosinophils, T and B cells using the method and same antibodies described by Yu et al (3). Neutrophils were distinguished from all other leukocytes based on their expression of Gr-1. Alveolar macrophages were $CD64^{+}CD24^{-}$, eosinophils were $CD45^{+}CD11c^{-}Siglecf^{+}$, T cells were $CD45^{+}CD11c^{-}CD11b^{-}IA/IE^{-}CD24^{-}$ and B cells were $CD45^{+}CD11c^{-}CD11b^{-}IA/IE^{+}CD24^{+}$. Absolute cell numbers were recorded and corrected to BALF volume collected.

Histology

Following euthanasia by cervical dislocation, the lungs underwent pressure-fixation and morphometric analysis in accordance with our previously published protocol (4) and in accordance with the ATS/ERS issue statement on quantitative assessment of lung structure (5). Fixed sections (4-μm) of paraffin-embedded lungs were H&E stained. Mean linear intercept analysis was performed as previously described (6). Alveolar counts and boundary size and ductal destructive measurements were performed on images of the H&E stained tissues, as previously described (7).

Cell culture

HBE cells were isolated from appropriately consented donors whose lungs were found unsuitable for transplantation. The 9 non-smokers donors had no known airway diseases, 7 females and 2 males at 25-58 years of age. From a racial aspect, there were 7 Caucasians and 2 African Americans. The 6 COPD donors had macro-histology evidence of disease, 3 females and 3 males at 41-60 years of age. From a racial aspect, there were 4 Caucasians and 2 African Americans. 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. For lungs from patients with disease, the diagnosis of COPD was made by clinical criteria before the death of the patient. The diagnosis of COPD was provided by the clinician taking care of the patient. Since no confirmatory lung function was available, sample classified and cells grouping was determined by signs of emphysema. All COPD patients had a significant smoking history and upon dissection their lungs had macro-pathological evidence of emphysema. Following cell expansion, cells were transfected with purified PP2A protein (MilliporeSigma) using Pro-Ject transfection reagent (Pierce, Thermo Scientific, Springfield Township, NJ) or Pro-Ject with albumin as per the manufacturers' instructions and as outlined previously (8). Cells were also transfected with scrambled control siRNA or HuR or $PP2A_A$ specific siRNA (All siRNA was purchased from Qiagen, Gaithersburg, MD). Alternatively, cells were treated with 1 μM SMAP. Twenty-four hours later, cells were collected for protein and RNA analysis and apical surface of cells were washed and collection with the media for CTSS activity.

Mononuclear cells were isolated from heparinized venous peripheral blood obtained from healthy non-smoker volunteers, 4 females and 5 males between 41-62 years of age.

Briefly, density gradient centrifugation was conducted in Lymoprep (AXIS-SHIELD PoC AS, Oslo, Norway) to separate the red cell pellet containing the neutrophil population from the monolayer. The mononuclear cell band was aspirated and washed three times in RPMI 1640 medium. Monocytes were purified using the EasySep human CD14 selection mixture, as recommended by manufacturers (StemCell Technologies, Cambridge, MA). Monocytes were then cultured in RPMI 1640 containing 40% autologous serum, penicillin G (final concentration 100 U/ml), and streptomycin sulfate (final concentration 100 µg/ml) at 37°C in a 5% CO_2 atmosphere for 9 days. Transfection with siRNA for the A subunit of PP2A ($PP2A_A$) or negative control scrambled was performed with JetPei transfection reagent (Polyplus-transfection, Illkirch-Graffenstaden, France) and $PP2A_A$, CTSS and ERK levels were determined 48 hours later. Media was collected for CTSS activity analysis.

Immunofluorescence staining

Fresh human bronchial tissue was fixed in 10% formalin, embedded in paraffin block and cut at 5 µm thickness. Sections were deparaffinized in Xylene for 10 min, 100% ethanol for 6 minutes, 95% ethanol for 2 minutes, 80% ethanol for 2 minutes and rinsed in distilled water. Antigen retrieval was performed using tri-sodium citrate at pH 6 for 30 minutes in a 98 ^oC water bath. After allowing the sections to cool down to room temperature, they were rinsed in PBS containing 0.05% Tween 20 (PBST) and blocked in 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Anti-Human CTSS (R&D Systems, Minneapolis, MN, USA) and acetylated tubulin (MilliporeSigma) antibodies were added following manufacturer's recommendation in 3% BSA and incubated overnight at 4 ^oC on a rocking shaker. Sections were then washed in PBS with tween 3 times for 10 minutes

and incubated with secondary antibody (ThermoFisher Scientific) in 3% BSA for 1 hour at room temperature on a rocking shaker. Finally, sections were washed 2 times for 10 minutes in PBST and Hoechst dye was added in PBST for an additional 10 minutes. After a final wash in PBST for 10 minutes, mounting media was added, and the sections were allowed to dry for 24 hours before visualization and image acquisition using a Nikon C2+ confocal microscope equipped with a 20X objective. Quantification was performed using ImageJ software (NIH, Bethesda, MD, USA). For each section, three different images were acquired. Integrated density values (IDV) were measured within three different 100 x 50 μm region of interest for each image and normalized to the maximum IDV for each processed batch to account for batch-to-batch variations. To correct for different cell numbers per field, the data were also normalized to IDV/cell. Data was represented an IDV using arbitrary units. Mouse lung tissue was also examined for CTSS immunofluorescence in the same manner as outlined here, with anti-rat CTSS goat polyclonal IgG antibody (Santa Cruz Biotechnology, Dallas, TX, USA).

Intracellular signaling

Protein was collected from lung tissue by bead beater disruption (Minibeadbeater-16, BioSpec Products, Bartlesville, OK, USA). Tissue was placed in lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT, 0.2% Igepal CA-630) with 50mg of 1 mm diameter Zirconia beads (BioSpec Products, Bartlesville, OK, USA) and disrupted for 30 seconds in the bead beater. HBE cells were also lysed with the same buffer. Soluble proteins were collected following a 10-minute centrifugation at 13,000 x g at 4^{o}C. Immunoblots for ERK phosphorylation (Thr202/Tyr204 and total ERK), the A subunit of PP2A, HuR and β-Actin (all antibodies from Cell Signaling Technologies,

Beverly, MA, USA) were performed to confirm equal levels of protein were loaded per sample. ERK phosphorylation was examined to demonstrate altered downstream changes in PP2A signaling. Chemiluminescence detection was performed using the Bio-Rad Laboratories Molecular Imager ChemiDoc XRS+ imaging system. PP2A activity was determined using the Millipore PP2A activity assay (Cat # 17-313, MilliporeSigma).

CTSS measurements

Gene expression was performed by qPCR using Taqman probes (Life technologies/Applied Biosystems, Carlsbad, CA, USA). Data were analyzed using the ΔΔCT method, with normalization to β-actin, and are presented in arbitrary units. Most qPCR results are represented as relative quantification (RQ) compared to room air or non-smoker controls and corrected to actin levels. For Figure 1A, gene expression was relative to each cathepsin gene corrected for actin levels. To achieve this the values for *Ctsd* were adjusted to a mean of 1 and all other genes were adjusted relative to this group. CTSS ELISAs were performed on BALF using the CTSS ELISA kit (R&D Systems) and were corrected to BALF protein concentrations. CTSS immunoblots were also performed on BALF to visualize the pro and active forms of CTSS. Total protein was determined using Pierce BCA Protein Assay kits (ThermoFisher). CTSS activity was determined in BALF, lung protein homogenates, cell media or cell surface apical washes and corrected to total protein content of sample, as previously described (9). Plasma desmosine levels were determined by ELISA using the Biorbyt mouse desmosine ELISA kit, as recommended by manufacturers (Cat # orb409382; Biorbyt Ltd, San Francisco, CA, USA).

Cytokine measurement

IL1β, IFNγ and TNFα levels were measured in BALF using Luminex analysis with beads assay (EMD Millipore, Billerica, MA) on the BioRad Bio-Plex 200 system (BioRad, Hercules, CA). Data was expressed as pg/ml BALF. Each target was also analyzed by qPCR as outline above.

Statistical analyses

Data are expressed as dot plots with the means ± S.E.M highlighted. Differences between two groups were compared by Student's t test (two-tailed). Experiments with more than 2 groups were analyzed by 2-way ANOVA with Tukey's post hoc test analysis. p values for significance were set at 0.05 and all significant changes were noted with *. All analysis was performed using GraphPad Prism Software (Version 6.0h for Mac OS X).

Supplementary Figure legends:

Supplementary Figure 1. Smoke exposure enhances *Ctse*, *Ctsg* and *Ctss* gene expressions in mice lungs. 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. Data are represented as mean ± S.E.M, with each measurement performed on 3 separate days from at least 13 animals/group. Expression is relative to room air group per gene. * denotes p value <0.05, when comparing both treatments connected by a line, determined by student t-tests.

Supplementary Figure 2. Signaling unaltered following the activation of PP2A during long-term smoke. Mice were exposed to 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) *Il1β*, *Ifnγ* and *Tnfα* genes were quantified in C57BL/6J

lung tissue, following 2 months exposure to room air and cigarette smoke, by qPCR and are shown as relative gene expression. (B) IL1β, IFNγ and TNFα levels were quantified in BALF by Luminex bead assays. (C) *Ctse* and *Ctsg* gene expression was determined in lung samples. Data are represented as mean ± S.E.M, with each measurement performed on 3 separate days from at least 7 animals/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.

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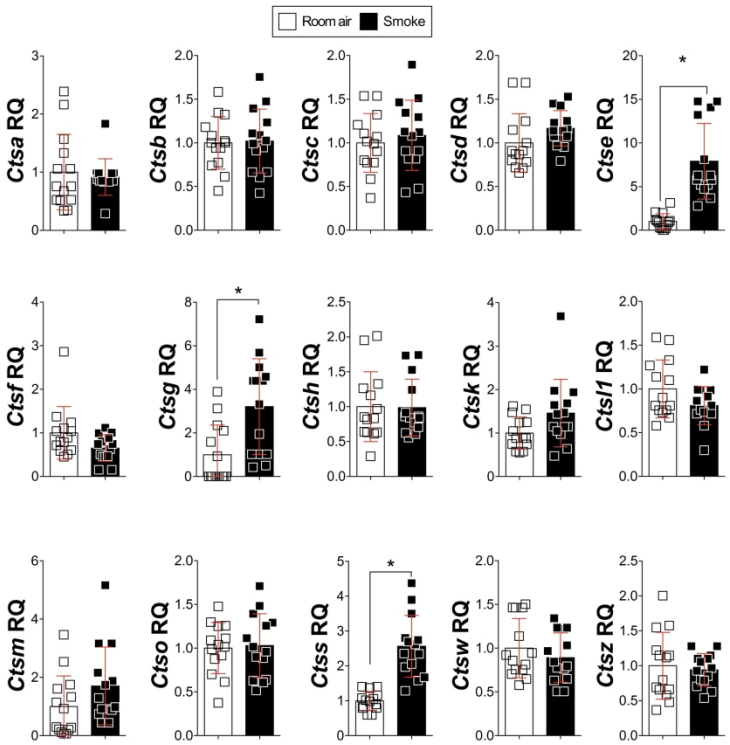
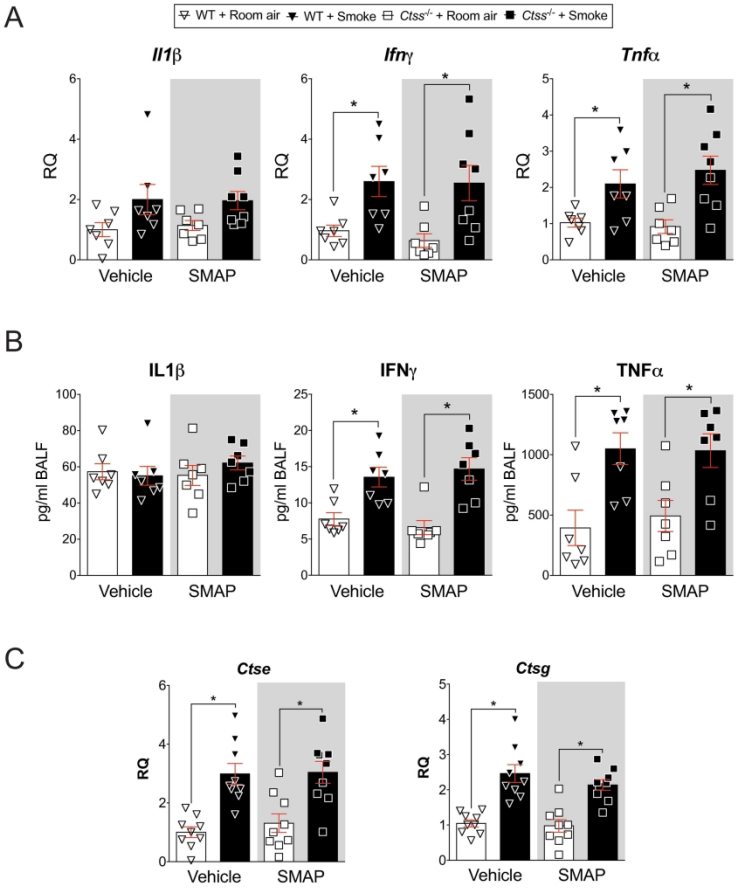
Figure E1



Smoke exposure enhances *Ctse*, *Ctsg* and *Ctss* gene expressions in mice lungs

210x297mm (300 x 300 DPI)

Figure E2



Signaling unaltered following the activation of PP2A during long-term smoke

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