

## **Supplementary data**

### **Losartan reduces cigarette smoke-induced airway inflammation and mucus hypersecretion**

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## Supplementary methods

### Lungs

Lung tissue was obtained from organ donors whose lungs were rejected for transplant and recovered for research by the Life Alliance Organ Recovery Agency at the University of Miami (Miami, FL, USA), LifeCenter Northwest (Seattle, WA, USA), and the Midwest Transplant Network (Kansas City, KS, USA). A ring of the trachea or main bronchi was cut and fixed in 10% formalin at 4°C until embedded in paraffin for sectioning and tissue staining. Airways were dissected and the tissue exposed to protease overnight as previously described.<sup>1 2</sup> Cells were harvested the following day and frozen in liquid nitrogen. These cells are considered P0 HBECs.

### Cell culture and drug treatments

Culturing of HBECs at the air-liquid interface (ALI) was performed as described.<sup>1 2</sup> Briefly, P0 HBECs were thawed at 37°C, resuspended in warm bronchial epithelial cell growth medium (BEGM) and spun at 360 x g for 5 min at room temperature. The supernatant was discarded and the cell pellet resuspended in BEGM with Amphotericin B. The density of viable cells plated in a 10 cm dish was approximately 1-2 x 10<sup>6</sup> cells. Once the cells reached confluence, they were trypsinized and plated on Transwell and/or Snapwell inserts at a minimum of 150,000 cells/cm<sup>2</sup> in ALI media.<sup>1</sup> HBECs were maintained submerged for 4-6 days before exposing them to air and allowing them to re-differentiate for a minimum of 28 days before experiments were performed. Fully re-differentiated HBECs are referred to as P1 HBECs. *In vitro* losartan (#61188, MilliporeSigma, St. Louis, MO, USA) treatment at 10 µM was started the day P1 HBECs went to an ALI. Losartan was maintained in the media throughout differentiation. LY2157299 (#S2230, Selleckchem, Houston, TX, USA) at 10 µM or EXP3179 (#18855, Cayman Chemicals, Ann

Arbor, MI, USA) at 5  $\mu$ M was added to the basolateral media of ALI cultures 24 h before CS exposure.

### **Immunofluorescence staining**

Immunofluorescence staining of tissue sections was performed as previously described.<sup>3</sup> Slides were first incubated overnight at 60°C. The following day, a deparaffinization step of 2 x 5 min in xylene was performed, followed by several hydration steps of 2 x 3 min in 100% ethanol, 2 x 1 min in 95% ethanol, and finally, 2 x 1 min in 80% ethanol before rinsing in distilled water. Antigen retrieval was achieved using 10 mM trisodium citrate (#S1804, MilliporeSigma) with 0.05% Tween 20 in distilled water with pH adjusted to 6.0 for 30 min in a water bath at 98°C. The slides were allowed to cool down before a 10 min rinsing step in phosphate buffered saline (PBS) with 0.05% Tween 20. All the following steps were performed on a shaker. 3% bovine serum albumin (BSA; #BP1600-100, ThermoFisher Scientific, Waltham, MA, USA) in PBS was used for 1 h at room temperature (RT) to block for nonspecific bindings. Anti-MUC5AC primary antibody (#MA1-38223, ThermoFisher Scientific) at 0.4  $\mu$ g/mL in 3% BSA was incubated overnight at 4°C. After two washing steps of 10 min with PBS with 0.05% Tween 20, secondary antibody in 3% BSA was applied for 1 h at RT. Two more washing steps of 10 min were performed before adding 2  $\mu$ g/mL Hoechst (#H3569, ThermoFisher Scientific) for 10 min in PBS with 0.05% Tween 20. One final washing step of 10 min was performed before adding Fluoro-Gel with Tris Buffer (#17985-11, Electron Microscopy Sciences, Hatfield, PA, USA). For staining of P1 HBECs, Transwell inserts were fixed with a solution of 50% methanol and 50% acetone for 2 min at -20°C followed by 3 washes of PBS. A solution of 3% BSA was used to block for one hour at RT on a shaker. Immunostaining with primary and secondary antibodies were performed as described

above. P1 HBECs exposed to CS or room air were washed and fixed 48 h after exposure following the same immunostaining protocol. All slides were imaged with a Nikon C2+ confocal microscope (Nikon Instruments, Tokyo, Japan).

### **Quantitative PCR**

P1 HBECs exposed to CS or room air were lysed 24 h after exposure and total RNA was isolated using the E.Z.N.A.® Total RNA Kit (Omega Bio-tek, Norcross, GA, USA). qPCR was performed as described<sup>4,5</sup> using TaqMan Gene Expression Assays (ThermoFisher Scientific) for *MMP9* (Hs00234579\_m1), *MUC5AC* (Hs01365601\_m1) and *TGF-β1* (Hs00998133\_m1), and normalized to reference gene *GAPDH*.

### **MMP-9 activity assay**

MMP-9 activity was measured in 200 μL PBS washes collected 24 h after room air or CS exposure using Human Active MMP-9 Fluorokine E Kit (#F9M00, R&D Systems, Minneapolis, MN, USA) and following manufacturer's instructions for non-activated samples.

### **Ussing chamber**

Cystic fibrosis transmembrane conductance regulator (CFTR) and large conductance, Ca<sup>2+</sup>-activated and voltage-dependent K<sup>+</sup> channel (BK) activities were recorded in Ussing chambers as previously described.<sup>6,7</sup> Briefly, ENaC currents were blocked by amiloride (10 μM; MilliporeSigma) before CFTR currents were stimulated by forskolin (10 μM; MilliporeSigma) and inhibited by CFTR<sub>inh</sub>-172 (10 μM; MilliporeSigma). Prior to measuring BK currents, the basolateral membranes were permeabilized with amphotericin B (20 μM; MilliporeSigma),

Nigericin (10  $\mu$ M; Tocris Bioscience, Minneapolis, MN, USA), and Valinomycin (10  $\mu$ M; Tocris Bioscience). The cells were exposed to a  $K^+$  gradient of 140 mM at the permeabilized basolateral side and 5 mM at the apical side. After ENaC currents were blocked by amiloride (10  $\mu$ M), BK currents were stimulated by ATP (10  $\mu$ M; Tocris Bioscience). The transepithelial membrane potential was clamped at 0 mV (model VCC MC8, Physiologic Instruments, San Diego, CA, USA), using Ag/AgCl electrodes in agar bridges. Signals were digitized and recorded using the Acquire and Analyze revision II module according to the manufacturer's instructions. The experiments were performed at 37°C using heated water jackets and bubbled with a gas mixture of 95%  $O_2$  / 5%  $CO_2$  for CFTR current or air for BK current. The CFTR and BK currents from P1 HBECs on Snapwells were measured at 4 h after the cells were exposed to CS or room air.

#### **Airway surface liquid (ASL) volume measurements**

ASL volume estimation was performed by meniscus scanning as previously published.<sup>5 8</sup> P1 HBECs exposed to CS or room air were scanned 1 h and 4 h after exposure and the delta was plotted.

#### **Ciliary beat frequency (CBF)**

CBF was recorded 4 h after exposure to CS or room air using a high-speed camera and analyzed using the individual Region-of-Interest (ROI) method of SAVA software.<sup>9 10</sup> Ciliary beating was recorded 1-2 mm away from the center of the insert for 2 seconds and four ROIs were plotted.

### **Mucus concentration measurements**

The percent of solids on top of cultures was measured according to published methods of mucus wet and dry weights using an ultra-microbalance (UMX2 Mettler Toledo) capable of detecting accurately to 100 ng.<sup>11 12</sup> Briefly, mucus was lifted off the cultures using a 5-mm diameter Kimwipe paper mesh that was cut with a laser to assure equal size (Epilog Mini 30W laser). All weights were recorded with the mesh in an aluminum boat. The dry weight of the mesh was recorded first, then the mesh was applied onto the cultures and left on the surface for 10 min at 37°C in an incubator before being pulled off and immediately measured again for the wet weight. After an overnight incubation at 60°C (oven), the dry weight with only mucus left was recorded again. All measurements were corrected for elapsed time after pulling off the mesh from the cultures and when taken out of the oven until it was placed onto the scale. Percent mucus solids were calculated as follows:  $[(\text{dry weight-mesh weight}) / (\text{wet weight-mesh weight})] \times 100 - 0.9$ . P1 HBECs exposed to CS or room air were tested 24 h after exposure.

### **Cigarette smoke (CS) exposure**

CS exposure of P1 HBECs was done as previously described.<sup>3 5 13</sup> Briefly, cells were washed with PBS and the media was replaced the day before the exposure. P1 HBECs were exposed to 24 puffs of a Kentucky research cigarette (3R4F) with a volume of 35 mL delivered every 60 seconds using the Vitrocell VC10 smoking robot (Vitrocell, Waldkirch, Germany) following ISO standard 3308. As controls, P1 HBECs were exposed to room air. Nicotine deposition onto the surface of HBECs after CS exposures was validated by mass spectrometry (LS-MS/MS, Florida International University, FL, USA) and showed depositions of approximative 100-120 µM of nicotine onto ALI cultures comparable to *in vivo* deposition of 1-2 cigarettes.<sup>14</sup>

## **Study approval**

The study was approved by the University of Miami Human Subject Research Office and informed consent was obtained from each participant. Clinicaltrials.gov registry under NCT02416102.

## **Human subjects**

The study enrolled a total of 31 participants: 16 healthy never-smokers and 15 current smokers with a smoking history of >10 pack/years and no signs of COPD by PFT with DLCO. FEV1/FVC, FEV1, and FVC values from both groups were in the normal range. Thus, none of the subjects had COPD by spirometric definitions. From those 31 patients, 14 (7 smokers and 7 nonsmokers) completed the study with laboratory test results and only 5 subjects in each group had complete nasal sample data sets. The subjects were aged 35 to 70 years and not taking any angiotensin receptor blockers (ARBs) prior to enrollment. None of the subjects were taking corticosteroids for 6 weeks prior to enrollment, were pregnant, had hypoxemia, untreated arterial hypertension, blood pressure lower than 100 mm Hg systolic or 70 mm Hg diastolic while standing. Furthermore, there was no significant difference in complete blood count (CBC), comprehensive metabolic panel (CMP) or c-reactive protein (CRP) measured in the blood samples, between week 1 and week 8. Participants received 50 mg losartan for 4 weeks and then 100 mg losartan for another 4 weeks.

## **Nasal cells collection**

Nasal cells were collected using sterile cytology brushes (Medical Packaging Corporation, Camarillo, CA, USA). The brushes were introduced into the nasal cavity under direct visual guidance and were placed between the nasal septum and the inferior turbinate. No anesthesia was used. The cells were harvested by a few careful backward-forward and rotary movements before

twirling the brush into 5 mL of sterile PBS in a 15 mL tube to release the cells. The same procedure was repeated 3 times in each nostril. Immediately after the harvest, the tube was centrifuged at 360 x g for 5 min at 4°C. The supernatant was discarded and the remaining pellet was frozen at -80°C until qPCR assay was performed.

### **Statistical analyses**

Data are shown as dot plots / bar graph combinations with means  $\pm$  S.E.M. Differences between two groups were compared by parametric or non-parametric tests as indicated in the figure captions depending on whether the data passed Shapiro-Wilk normality testing. p values for significance were accepted at  $p < 0.05$ . All analyses were performed using Prism (GraphPad Software, San Diego, CA, USA).



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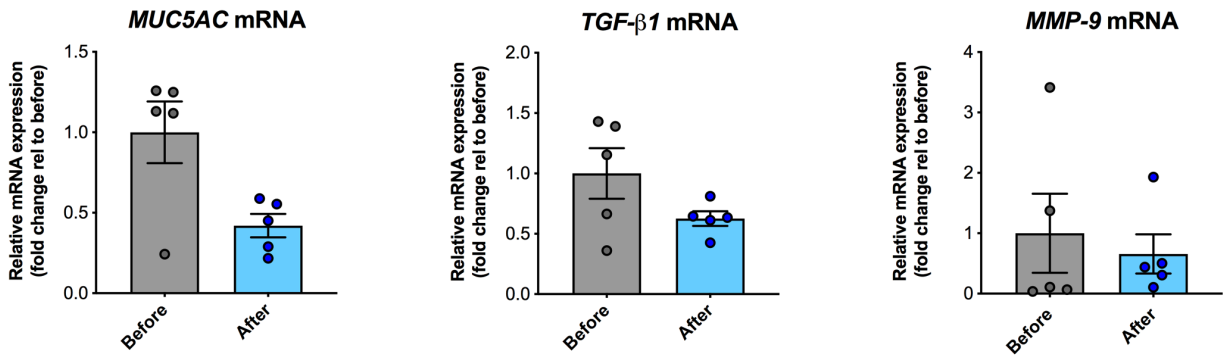
Supplementary table and figures

**Table S1**

	<b>Nonsmokers</b>	<b>Smokers</b>
n	10	7
Age	44.5 ± 2.3	43 ± 2.9
Female	4	2
Male	6	5
Caucasian	8	3
African-American	1	1
Hispanic	1	3
Pack-years	0	18.9 ± 2.1

Supplementary Table S1. Table with demographics of all lung donors used for Figs. 1-5.

**Figure S1**



**Supplementary Figure S1. Human nasal epithelial cells (HNECs) of nonsmokers treated with losartan.** Quantitative mRNA expression of *MUC5AC*, *TGF-β1*, and *MMP-9* of nasal cells from nonsmokers, before losartan treatment and 2 months after (50 mg daily for one month and 100 mg daily for an additional month). Data shown as relative to baseline prior to losartan administration. n=5 subjects for each group. None of the comparisons were significant by Student's t-test after passing Shapiro-Wilk normality test.