SUPPLEMENTARY MATERIAL

Nicotine promotes e-cigarette vapour-induced lung inflammation and structural alterations

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SUPPLEMENTARY METHODS

Cell culture

All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C in a cell incubator and used 24 h after seeding. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), lactate dehydrogenase activity (LDH, cytotoxic assay) and apoptosis assay, cells were seeded in 96-well plates in the following concentrations: 10,000 cells/well for A549, human pulmonary arterial smooth muscle cells (hPASMC), and primary mouse pulmonary arterial smooth muscle cells (mPASMC), 20,000 cells/well for human bronchial epithelial cells (HBEpC), and 140,000 cells/well for mouse alveolar epithelial type II cells (mATII) cells. For analyses of cell confluence and the number of dead cells (BOBO™-3 iodide staining), mPASMC and hPASMC cells were seeded in 96-well plates (both cell types 10,000 cells/well), for the BrdU

proliferation assay in 24-well plates (both cell types 20,000 cells/well), for the migration assay in 2-well Ibidi culture-inserts (Ibidi GmbH, Gräfelfing, Germany) (2,500 cells/well of hPASMC and 5,000 cells/well of mPASMC), and for the real-time PCR, Western blot and microarray in 6-well plates (both cell types 60,000 cells/well). 140,000 cells/well and 3,000,000 cells/well of mATII were seeded in 96-well plates and 6-well plates for the glutathione assay and real-time PCR/microarray, respectively. The following media were used in the study: DMEM (Thermo Fisher Scientific, Waltham, USA) with 10% FCS (Sigma-Aldrich, Burlington, USA) for A549 and mATII cells, airway epithelial cell growth medium (PromoCell, Heidelberg, Germany) with 10% FCS for HBEpC, smooth muscle cell growth medium (PromoCell, Heidelberg, Germany) with supplement and 10% FCS for mPASMC and without FCS for hPASMC.

Isolation of primary mouse pulmonary arterial smooth muscle cells (mPASMC)

mPASMC were isolated from precapillary pulmonary arterial vessels as described previously [1]. Briefly, 3ml of M199 (Medium 199, Invitrogen, Carlsbad, USA) containing 5mg/ml low melting point agarose, 5mg/ml Fe_3O_4 (Sigma-Aldrich, Burlington, USA), and 1% penicillin/streptomycin was injected into the pulmonary artery and the lung was fixated via agarose injection into the trachea. Subsequently, the lung was cut with scissors for 5 min into tiny pieces, which were maintained in 10ml PBS. The pieces from the pulmonary arterial vessels containing Fe_3O_4 (Sigma-Aldrich, Burlington, USA) were collected using a magnet in a magnetic holder and digested in 10ml of M199, containing 80U/ml collagenase (Sigma-Aldrich, Burlington, USA) at 37°C for 60 min. The tissue mixture was disrupted by passing it through 15 and 18-gauge needles 5–6 times each. The resulting suspension, containing the medial and intimal layer of the pulmonary arterial vessels attached to Fe_3O_4 (Sigma-Aldrich, Burlington, USA) particles, was rinsed three times with M199 (Thermo Fisher Scientific,

Waltham, USA), containing 10% FCS, in the magnetic holder. These pieces were re-suspended in smooth muscle cell growth medium (PromoCell, Heidelberg, Germany) with a supplement and 10% FCS, transferred to culture flasks and incubated at 37°C in a cell incubator to induce outgrowth of smooth muscle cells. Upon reaching 80% confluence, the grown mPASMC were trypsinised and seeded into new culture flasks (=first passage). The experiments were performed with mPASMC in the second passage.

Isolation of primary human pulmonary artery smooth muscle cells (hPASMC)

The hPASMC were isolated from pulmonary arteries of donor lung transplants. The studies were approved by the ethics committee of the Justus-Liebig University (AZ 58/15, AZ 10/06). Briefly, pulmonary arteries were dissected from the lung transplant. Then, the medial layer was separated from the adventitial and endothelial layers under a microscope. The medial layer of the arteries was minced using scalpels, transferred to Petri dishes, and cultured with 10ml of smooth muscle cell growth medium (PromoCell, Heidelberg, Germany) containing a supplement (PromoCell, Heidelberg, Germany) to induce outgrowth of hPASMC.

Isolation of primary mouse alveolar epithelial type II cells (mATII)

The mATII cells were isolated from mouse lungs using a modification of the method described previously [2]. After perfusion of the lung through the pulmonary artery with 3ml of saline, 2ml of dispase (Corning, Corning, USA) was instilled intratracheally. The lungs were removed from the chest cavity, placed in a 50ml falcon tube containing 2ml of dispase and incubated for 30 min at 37°C. Then, the lungs were transferred to a 50ml falcon tube containing 2ml DMEM (Thermo Fisher Scientific, Waltham, USA), 0.04mg/ml DNase (Sigma-Aldrich, Burlington, USA) and 1ml FCS (Sigma-Aldrich,

Burlington, USA) to stop dispase activity. After a brief stirring, the lungs were transferred to a 100 mm culture dish. The heart, oesophagus and trachea were carefully removed, and the lung tissue was minced using two scissors. The cell suspension was filtered through a 70µm, 40µm and 10µm nylon mesh cell strainer and centrifuged at 200g for 10 min (15°C). After centrifugation, the supernatant was discarded, and the cell pellet was re-suspended with 1ml DMEM (Thermo Fisher Scientific, Waltham, USA). Crude cell suspensions were purified by the negative selection of immune cells using 100 mm tissue culture plates, coated with a mixture of 0.75µg/ml CD16/32 (#553142, BD Biosciences, Franklin Lakes, USA) and 0.75µg/ml CD45 (#553076, BD Biosciences, Franklin Lakes, USA). After incubation for 45 min at 37°C, the cell suspension was placed in uncoated 100 mm tissue culture plates and incubated for 45 min at 37°C. The cell suspension was collected in a 50ml falcon tube and centrifuged at 200g for 10min (15°C). Afterwards, the cell suspension was treated with 2ml of erythrocyte red blood cell lysing buffer (BD Biosciences, Franklin Lakes, USA) for 5 min. The reaction was stopped by adding 4ml of DMEM. The suspension was centrifuged at 200g for 10 min (15°C). The mATII were re-suspended in DMEM medium (Thermo Fisher Scientific, Waltham, USA) plus 2% L-glutamate (PAN-Biotech GmbH, Aidenbach, Germany), 1% penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and 10% FCS (Thermo Fisher Scientific, Waltham, USA) and cultured on 3µg/cm² fibronectin-coated plates (Sigma-Aldrich, Burlington, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT metabolic assay was used to evaluate the metabolic activity after 24 h incubation with ECVE or CSE according the manufacturer's protocol (Sigma-Aldrich, Burlington, USA).

LDH cytotoxicity assay

24 h after seeding, cells were treated with ECVE, NF ECVE or CSE for 24 h. Twenty-four hours after treatment with different doses of ECVE, NF ECVE or CSE, the CyQUANT™ LDH Cytotoxicity Assay (Thermo Fisher Scientific, Waltham, USA) was performed using 50µl cell medium collected from the samples according to the manufacturer's instructions. Control cells were treated with a medium without ECVE, NF ECVE or CSE.

Proliferation assay

Cells were cultured in 24-well plates. Twenty-four hours after seeding, cells were treated with different doses of ECVE, NF ECVE or CSE and simultaneously incubated with bromodeoxyuridine [BrdU (colorimetric) Cell Proliferation ELISA] assay (Roche, Basel, Switzerland) for 24 h according to the manufacturer's instructions. To quantify proliferation, absorbance was measured with a microplate reader (Tecan Group Ltd, Männedorf, Switzerland) at 370 nm (absorbance at 450 nm was measured as a reference). Control cells were treated with a medium without ECVE, NF ECVE or CSE.

Wound healing assay

Culture-inserts (Ibidi culture-inserts, 2-well) were inserted into the bottom of 24-well plates to provide two cell culture reservoirs and 2,500 hPASMC and 5,000 mPASMC were seeded per reservoir. The wound healing assay was initiated by removing the culture-insert 24 h after seeding. Subsequently, the cells were treated with different doses of ECVE, NF ECVE or CSE for 16 h. Control cells were treated with a medium without ECVE, NF ECVE or CSE. Simultaneously with the application of ECVE, NF ECVE, CSE or the control medium, the wound closure (cell migration) was recorded

using an IncuCyte ZOOM™ live cell imaging system (Essen BioScience, Inc., Ann Arbor, USA) and pictures were acquired every hour for 16 h. This system measured scratch closure in real-time and automatically calculated the relative wound density within the initially vacant area at each time point.

Apoptosis assay

For the assessment of apoptosis, an IncuCyte ZOOM (Essen BioScience Inc.) system and an annexin XII-based polarity-sensitive probe from Kinetic Apoptosis Kit (#ab129817, Abcam, Cambridge, UK) were used according to the manufacturer's instructions. Twenty-four hours after seeding, cells were treated with different doses of ECVE, NF ECVE or CSE for 24 h. Control cells were treated with a medium without ECVE, NF ECVE or CSE. Twenty-four hours after exposure of the cells to ECVE, NF ECVE, CSE or the control medium, the fluorescent signal from the annexin XII-based polarity-sensitive probe was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Dead cell count

To determine dead cells, 10,000 cells/well of mPASMC and hPASMC were seeded in a 96-well plate. Twenty-four hours after seeding, cells were treated with different doses of ECVE, NF ECVE or CSE for 24 h. Control cells were treated with a medium without ECVE, NF ECVE or CSE. Twenty-four hours after exposure to ECVE, NF ECVE, CSE or the control medium, the fluorescent signal from BOBO™-3 Iodide (Thermo Fisher Scientific, Waltham, USA) was collected by an IncuCyte ZOOM and analysed using the Incucyte® integrated analysis software (Essen BioScience Inc., Ann Arbor, USA). BOBO™-3 Iodide penetrates cells with diminished plasma membrane integrity, resulting in a 100–1000-fold increase in fluorescence upon binding to DNA.

Quantitative real-time PCR

Quantitative real-time PCR of mPASMC and mATII cells was performed 24 h after exposure of the cells to different doses of ECVE, NF ECVE or the control medium (medium without ECVE and NF ECVE). Cells were treated with ECVE, NF ECVE, CSE or the control medium 24 h after seeding. The mRNA was isolated from the mPASMC and mATII by using the RNeasy Mini Kit (Qiagen, Hilden, German) according to the manufacturer's instructions. The 1,000ng mRNA was used to synthesise cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Feldkirchen, Germany). Quantitative real-time PCR was performed with the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) according to the manufacturer's instructions.

All primers used in the current study are listed in Supplemental Table 1. Expression levels of target genes were normalised by concurrent measurement of the reference gene *beta-2-microglobulin* (*B2M*).

Supplemental Table 1.

Primer	Sequences
B2M (Beta2-microglobulin)	AGCCCAAGACCGTCTACTGG
	TTCTTTCTGCGTGCATAAATTG
Bcl2 (B cell leukemia/lymphoma 2)	TGGGATGCCTTTGTGGAACT
	TTGGCAATTCCTGGTTCGGT
Ccna1 (Cyclin A1)	AAGAACCTGAGAAGCAGGGC
	CAGGGTCTCTGTGCGAAGTT
Nos2 (Inducible nitric oxide synthase)	TGATGTGCTGCCTCTGGCT
	AATCTCGGTGCCCATGTACC
Mmp9 (Matrix metallopeptidase 9)	CAGCCGACTTTTGTGGTCTTC
	GTCGAAATGGGCATCTCCCT
Timp3 (Tissue inhibitor of metalloproteinase 3)	TCCAAACACTACGCCTGCAT
	CTGCTTGCTGCCTTTGACTG
Traf1 (TNF receptor-associated factor 1)	GCGCACAGTGTGAGAAGAGA
	AGAGAACTCTGGGCTCCGAT

Microarray experiments

Twenty-four hours after exposure of the cells to ECVE, NF ECVE or control medium (medium without ECVE and NF ECVE), microarray analysis (GEO accession number GSE202215) of mPASMC and mATII cells was performed (project number GEn-1543) by using oligonucleotide spotted microarray slides (Agilent Technologies, Santa Clara, USA). Purified total RNA was amplified and Cy3-labelled using the LIRAK kit (Agilent Technologies, Santa Clara, USA) following the kit instructions. 200ng of total RNA was used per reaction. The Cy-labelled aRNA was hybridised overnight to 8x60K 60 mer oligonucleotide spotted microarray slides (design ID 028005). Hybridisation and subsequent washing and drying of the slides were performed following the Agilent hybridisation protocol. The dried slides were scanned at 2µm/pixel resolution using the InnoScan 900 (Innopsys, Carbonne, France). Image analysis was performed with the Mapix 6.5.0 software, and calculated values for all spots were saved as GenePix results files. The data were evaluated using the R-software (R 3.5.1) and the limma package (limma 2.14) from BioConductor. Log mean spot signals were taken for further analysis. Data were background corrected using the NormExp procedure on the negative control spots and quantile-normalised before averaging [3]. Genes were ranked for differential expression using a moderated t-statistic. Pathway analyses were done using gene set tests on the ranks of the t-values.

Protein isolation and Western blot analysis

Western blot analysis was performed using mPASMC lysates after exposure of the cells to ECVE, NF ECVE or control medium (medium without ECVE and NF ECVE) for 24 h. Cells were treated with ECVE, NF ECVE, CSE or control medium 24 h after seeding. The blots were blotted for ubiquitin-binding protein p62 (p62, SPC-219,

StressMarq Biosciences, Victoria, Canada), microtubule-associated proteins 1A/1B light chain 3B (LC3II, # SPC-217, StressMarq Biosciences, Victoria, Canada), PCNA (#13110, RRID: AB_2636979, Cell Signaling, Danvers, CA, USA). The protein expression level of β-actin (#A5316; RRID: AB_476743, Sigma-Aldrich, Burlington, USA) was used as the loading control. The dilution of primary antibodies was 1:1,000 for p62, LC3II and PCNA and 1:50,000 for β-actin. The dilution of the corresponding secondary antibodies was 1:5,000. The incubation time for the primary antibodies was overnight at 4°C and 1h at room temperature for the corresponding secondary antibodies.

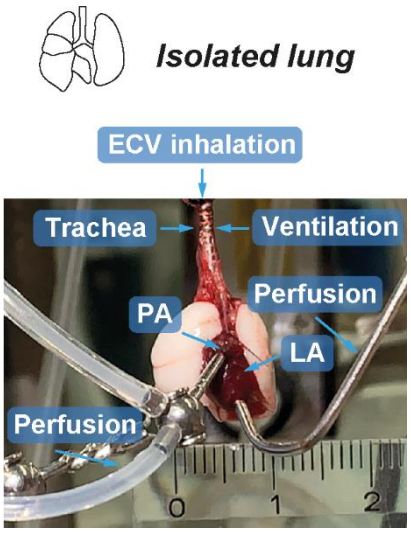
Glutathione assay

Total glutathione (GSH and GSH/GSSG ratios) was measured in mATII cell lysates after exposure to ECVE, or NF ECVE, CSE or control medium (medium without ECVE and NF ECVE) for 24 h by a GSH/GSSG-Glo™ assay (Promega, Madison, United States) following the manufacturer's instructions. Luminescence was measured by a microplate reader (Tecan Group Ltd, Männedorf, Switzerland). Cells were treated with ECVE, NF ECVE, CSE or control medium 24 h after seeding.

E-cigarette vapor application in isolated ventilated and perfused mouse lungs

To isolate the lungs, mice were intubated through a tracheostomy under deep anaesthesia with ketamine (100mg/kg body weight, i.p.) and xylazine (20mg/kg body weight, i.p.) after anticoagulation with heparin (2,500U/kg body weight). Mice were ventilated with a normoxic gas mixture (21.0% O_2, 5.3%CO_2, balanced with N_2, 10µl/g body weight tidal volume, 90 breaths/min and 3 cm H_2O positive end expiratory pressure) using a piston pump (Minivent Type 845, Hugo Sachs Elektronik, March-Hugstetten, Germany) while the chest was opened and the lung and heart were

prepared. Lung perfusion with Krebs-Henseleit buffer [120 mM NaCl, 4.3 mM KCl, 1.1 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 13.3 mM glucose, 5% (w/v) hydroxyethyl amylopectin (molecular weight 200,000Da)] was initiated through a catheter inserted into the pulmonary artery using a peristaltic pump (ISM834A V2.10, Ismatec, Wertheim, Germany). After the lung and heart removal from the chest cavity, the lung/heart preparation was freely suspended on a scale to measure its weight changes, and a catheter was inserted in the left atrium for collection of the outflow perfusate (Supplementary methods figure 1). Outflow pressure was fixed at 2 cm H_2O. Pulmonary arterial pressure, pulmonary venous pressure, ventilation pressure and weight were monitored continuously during the experiment.

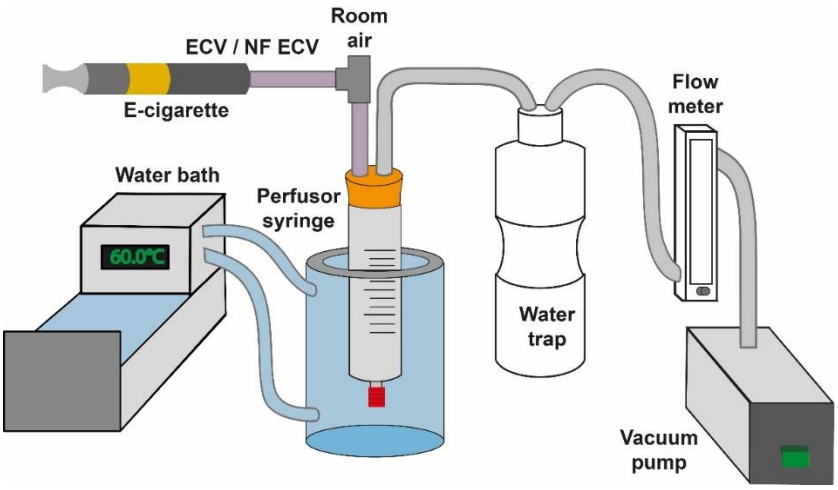


Supplementary methods figure 1. Experimental setup. Nicotine-containing e-cigarette vapour (ECV) was applied in a repetitive manner 3 times for 5 min each, via the trachea in the isolated ventilated and perfused mouse lung system. The lung was ventilated via the trachea and perfused via the pulmonary artery (PA) with recirculating the perfusate by a catheter in the left atrium (LA).

After preparation of the lung and an initial steady-state perfusion period, the lungs were ventilated five times for 10 min with a hypoxic gas mixture (1% O_2, 5.3% CO_2, balanced

with N_2) to determine HPV, each time followed by a ventilation period with a normoxic gas mixture (21% O_2, 5.3% CO_2, balanced with N_2). The normoxic interval between the first and second hypoxic manoeuvre was 25 min; subsequent interval durations lasted 50 min. The K_{fc} was determined by increasing the venous pressure from 2 to 12cm H_2O for 8 min ("pressure challenge"). The first pressure challenge was performed during normoxic ventilation 7 min after the first hypoxic manoeuvre and was used as a reference for all subsequent pressure challenges. After the second hypoxic manoeuvre (which was used as a reference for all ensuing hypoxic manoeuvres), the following protocol was applied to the lung 3 times: 1) normoxic ventilation stage: 10 min of ventilation, intratracheal application of ECV or NF ECV for 5 min, followed by 17 min of ventilation, 8 min of pressure challenge and 10 min of ventilation; 2) subsequent hypoxic ventilation stage: 10 min of ventilation. ECV or NF ECV was produced by Joyetech eVic Primo Mini e-cigarette (0.56Ohm, 4.1V, 30W, Joyetech, Riccardo Retail GmbH, Neubrandenburg, Germany) and drawn with normoxic air into a perfusor syringe using a vacuum pump (see Supplementary methods figure 2). The puffing condition was 4 puffs of 5 sec duration (20 sec in total). For the application of ECV or NF ECV in the isolated lung, the vapour was delivered via the inspiratory tube by a syringe driver, mimicking the ventilation mode of vapour-free ventilation. During the application, the expiratory tube of the ventilation was closed until the "normal" inspiratory volume was reached. After reaching the inspiratory volume, the expiratory tube was opened to release the ECV or NF ECV-air mixture. This procedure lasted 2 sec and was repeated 150 times for 5 min. Thus, ventilation with normoxic air was guaranteed during the intratracheal application of vapour. The PEEP (positive end-expiratory pressure) of 3cm H_2O was constant throughout the procedure. The same protocol was performed for control experiments, but only with normoxic air delivery without ECV or NF ECV. The strength of HPV was determined as the maximum

increase of PAP during hypoxia [1]. The K_{fc} was calculated from the slope of the lungs' weight gain induced by the increased lungs' fluid content during the pressure challenge [4].



Supplementary methods figure 2. Preparation of e-cigarette vapour with or without nicotine (ECV or NF ECV) for inhalative application to the isolated lung via a syringe driver. Joyetech eVic-Primo Mini e-cigarettes were used to produce ECV or NF ECV. ECV or NF ECV was drawn into the syringe by a vacuum pump at a flow rate of 1000ml/min to a final volume of 50ml ECV or NF ECV. The puffing condition was four puffs of 5 sec each, 20 sec in total. The syringe was kept in a cylinder heated to 60°C to prevent condensation of the vapour until delivery to the lung. During the inhalation, the syringe containing ECV, NF ECV or normoxic air was kept under an ultraviolet light lamp to maintain a temperature of 37°C.

Animals for *in vivo* e-cigarette vapour exposure

Twelve mice per group were exposed to ECV, NF ECV or room air. All animals were used for the determination of *in vivo* hemodynamic and lung function measurements and for micro-computed tomography (µCT) imaging. Mice from each group were randomly assigned to echocardiography (n=7), histological (n=6), immunohistochemical (n=5-6), multiplex (n=10) and FACS (n=5) analyses. The number of experiments (n) for *in vivo* lung function and hemodynamics, echocardiography and µCT may differ from the initial n due to technical issues (e.g., dislocation of the measurement catheter).

Flow cytometry

Bronchoalveolar lavage (BAL) was collected, and the cell pellets were incubated with Fc block (Miltenyi Bitech, Bergisch Gladbach, Germany) and stained with CD45APC-Cy7 (clone 30-F11; BioLegend, Fell, Germany), CD11b Pacific Blue (clone M1/70; BioLegend, Fell, Germany), GR-1 PerCy7 (clone RB6-8C5; BioLegend, Fell, Germany), Ly6G FITC (clone 1A8, BioLegend, Fell, Germany) or SiglecF PE (clone E50/2440; BD Biosciences, Franklin Lakes, USA) anti-mouse antibodies in the dark for 15 min at 4°C; they were then washed with a fluorescence-activated cell sorting (FACS) buffer. Flow cytometry was performed with an LSRII flow cytometer (BD Biosciences, Franklin Lakes, USA) using the DIVA software (BD Biosciences, Franklin Lakes, USA) as previously described [5, 6]. The gating strategy was as follows: the total BAL cells were first gated with forward scatter (FSC) and side scatter (SSC). The lymphocytes were gated from the total BAL cells based on the low FSC and low SSC within the CD45+ cluster. The mononuclear phagocytes (MonPh) were gated based on the higher FSC and/or SSC within the CD45+ cluster. From the MonPh cluster, the $CD11c^{+}Gr\text{-}1^{-}$ population was sub-gated to differentiate resident macrophages (rAMs: $CD11b^{hi}Siglec\text{-}F^{hi}$) and exudate macrophages (ExMAs: $CD11b^{hi}SiglecF^{low}$). From the

MonPh cluster, the $CD11c^{-}Gr\text{-}1^{+}$ population was sub-gated to identify neutrophils ($GR\text{-}1^{+}Ly6G^{hi}$) and monocytes ($GR\text{-}1^{+}Ly6G^{-}$).

Multiplex assay

A custom-made mouse magnetic bead-based multiplex assay was used to analyse the levels of selected inflammatory mediators in BAL fluid (BALF) according to the manufacturer's protocol (R&D Systems, Minneapolis, USA). For the experiments, BALF was used after sedimentation of the cells by centrifugation at 300g for 10 min at 4°C). The assay was conducted using the Bio-Plex 200 instrument (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) and analysed with the Bio-Plex Manager software (Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

***In vivo* hemodynamics, echocardiography, histology and micro-computed tomography (µCT)**

Pulmonary vascular function was determined by *in vivo* haemodynamics using a Millar catheter (Model SPR-671 pressure catheter; Millar Instruments, Inc.; Houston, USA) and the PowerLab system with the LabChart 7.0 software (ADInstruments GmbH, Oxford, UK) [7]. Transthoracic echocardiography was performed using a VEVO770 or VEVO2100 system (Visualsonics, Toronto, Canada) [1].

For histological analysis, paraffin blocks were cut in 3µm sections, de-paraffinised, rehydrated, and stained with haematoxylin and eosin (H&E), following routine protocols. Septal wall thickness and mean linear intercept were assessed on H&E-stained slides using uniform random sampling and the Qwin alveolar morphometry software (Leica Microsystems, Wetzlar, Germany) as previously described[8].

In vivo µCT images were acquired using a Quantum GX micro-CT scanner (PerkinElmer, Waltham, USA) in mice under isoflurane anaesthesia. Reconstructed volumes were loaded into the Analyze Pro software (Analyze Direct, Overland Park, USA) and processed by a single observer. Lung segmentation and quantitative analysis of the CT density and functional residual capacity were performed as described previously [7].

In vivo lung function measurements

Measurements of *in vivo* lung function parameters were performed as previously described [9, 10] using the FlexiVent system equipped with an FX2 module (SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada) at a positive end-expiratory pressure (PEEP) of 3 cm H_2O, following the manufacturer's recommendations. Before the lung function measurement, deep inflation was performed by inflating the lung with air up to a pressure of 40 cm H_2O (from initially 3 cm H_2O) over 3 sec and then holding at 40 cm H_2O for an additional 3 sec [11]. This manoeuvre recruited closed lung areas, and the lung volume history was standardised [11-13]. The deep inflation manoeuvre was also used to determine the total inspiratory capacity. The overall respiratory system resistance, a single compartment model perimeter, was measured using a single frequency forced oscillation (SnapShot-150) perturbation as previously described [12]. The static compliance measurement was calculated from a respiratory pressure-volume (P-V) loop as previously described[12, 14]. The P-V manoeuvre included stepwise lung inflation through eight steps of increasing pressure from 3 to 40 cm H_2O with a 1-sec hold at each step. It was similarly followed by stepwise deflation back to the PEEP pressure of 3 cm H_2O. The whole manoeuvre lasted 16 sec. The P-V loop was constructed by recording volume changes and plotting it with pressure values at each holding step [12, 14]. The FlexiVent software automatically

calculated the lung function parameters associated with a perturbation. The software also provided a coefficient of determination (COD), which reflects the fit of the mathematical model to the dataset. Each dataset with an insufficient COD was labelled and excluded by the software as previously described [12]. The results were presented as an average of at least three repeated measurements with the COD above 0.90.

Exposure of mice to conventional cigarette smoke (CS)

Adult mice were exposed to mainstream smoke from 3R4F cigarettes (Kentucky University, Lexington, USA) produced by a smoke generator (Burkhart, Wedel, Germany) at a concentration of 140mg particulate matter/m^3 for 6 h as in [8, 9]. The blood samples to measure nicotine and cotinine concentration were taken immediately after removing the animals from the smoking chamber.

Plasma nicotine and cotinine concentration

Plasma nicotine and cotinine concentrations were measured after exposure of mice to ECV or CS for 6 h by liquid chromatography with tandem mass spectrometry by ABS Laboratories (Welwyn Garden City, UK). The samples to measure nicotine and cotinine concentration were taken immediately after removing the animals from the exposure chamber. Under deep anaesthesia with ketamine (100mg/kg body weight, i.p.) and xylazine (20mg/kg body weight, i.p.) after anticoagulation with heparin (2,500U/kg body weight), abdominal cavity of the mouse was opened and a blood sample was taken from abdominal aorta. Then the blood samples were centrifuged at 2000 g for 10 min (4°C).

Immunohistochemistry

Paraffin-embedded mouse lungs were cut into 3µm thick sections, deparaffinised and rehydrated following standard protocols. Antigen retrieval was performed using the Rodent Decloaker solution (Biocare Medical LLC, Pacheco, USA), and unspecific binding was blocked with a 10% BSA solution. The slides were incubated with primary antibodies (CD45: ab10559, Abcam Cambridge, UK; dilution 1:200 and CD3: #RBK 024-05, Zytomed Systems GmbH, Berlin, Germany; dilution 1:200) diluted in antibody diluent (Zytomed Systems GmbH, Berlin, Germany) overnight at 4°C. A ZytoChem Plus phosphatase polymer kit (Zytomed Systems GmbH, Berlin, Germany) and Warp Red Chromogen substrate kit (Zytomed Systems GmbH, Berlin, Germany) were used to visualise the staining according to the manufacturer's protocols. Counterstaining was performed with CAT Haematoxylin solution (Biocare Medical LLC, Pacheco, USA). The number of cells was determined in randomly selected fields using the Qwin software (Leica Microsystems, Wetzlar, Germany).

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