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ORGAN TOXICITY AND MECHANISMS

Combined biological effects and lung proteomics analysis in mice reveal different toxic impacts of electronic cigarette aerosol and combustible cigarette smoke on the respiratory system

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Abstract

Combustible cigarettes produce many toxic substances that have been linked to diseases, such as lung cancer and chronic obstructive pulmonary disease. For those smokers unable or unwilling to quit, electronic cigarettes (e-cigarettes) could be used as an alternative to cigarettes. However, the effects and mechanisms of e-cigarette aerosol (ECA) on respiratory function have not been fully elucidated, and in vivo studies of its safety are limited compared to cigarette smoke (CS). In this article, we chose nicotine levels as dosing references and C57BL/6 mice for a 10-week subchronic inhalation toxicity study. A comprehensive set of toxicological endpoints was used to study the effect of exposure. Both CS (6 mg/kg) and ECA (6 or 12 mg/kg) inhalation had decreased the animal's lung function and increased levels of inflammation markers, along with pathological changes in the airways and lungs, with ECA displaying a relatively small effect at the same dose. Proteomic analysis of lung tissue showed greater overall protein changes by CS than that of ECA, with more severe inflammatory network perturbations. Compared with ECA, KEGG analysis of CS revealed upregulation of more inflammatory and virus-related pathways. Protein–protein interactions (PPI) showed that both ECA and CS significantly changed ribosome and complement system-related proteins in mouse lung tissue. The results support that e-cigarette aerosol is less harmful to the respiratory system than cigarette smoke at the same dose using this animal model, thus providing additional evidence for the relative safety of e-cigarettes.

Keywords Electronic cigarettes · Combustible cigarettes · Proteomics · Respiratory system · Inhalation · In vivo

Introduction

Even though combustible cigarette smoking rates have decreased in many countries, it is still a substantial contributor to a variety of health problems (Creamer et al. 2019). Worldwide smoking prevalence among people aged 15 and above was reported to be 17.5% in 2019 (Burki 2021). According to the journal Lancet, tobacco smoking has caused more than 200 million deaths worldwide per year, with annual economic expenditures exceeding US$1 trillion over the last 30 years (Collaborators 2021). China experienced the largest absolute rise in smoking-related mortality between 1990 and 2019 (from 1.5 million in 1990 to 2.4 million in 2019) (Collaborators 2021). In the United States, combustible cigarette smoking is a leading cause of preventable diseases and death, as well as one of the most important risk factors for premature death and morbidity worldwide

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(Centers for Disease and Prevention 2013; Creamer et al. 2019).

E-cigarettes, also known as electronic nicotine delivery systems (ENDS), were first introduced in 2007 as a novel nicotine product (O'Connor et al. 2022). E-cigarettes have a mouthpiece and e-liquid cartridge, an atomizer that vaporizes the cartridge fluid, and a battery that powers the atomizer (Breland et al. 2017). E-cigarettes, which are primarily made up of vegetable glycerin, propylene glycol, nicotine, and flavoring ingredients, have steadily grown in popularity as a smoking cessation aid or replacement in Europe and America (Breland et al. 2017; O'Connor et al. 2022). The usage of e-cigarettes is fast expanding elsewhere, and it is becoming increasingly popular among adult smokers (Advani et al. 2022). According to the Morbidity and Mortality Weekly Report (MMWR), e-cigarettes are now the second most popular tobacco product in the US, with 4.5% of adults using them (Cornelius et al. 2020). From a tobacco harm reduction (THR) perspective, e-cigarettes are one of the categories that may contribute to THR by allowing adult cigarette smokers who are unable or unwilling to quit to achieve satisfactory nicotine consumption while consuming fewer and significantly lower levels of harmful substances (Hendlin et al. 2019; Notley et al. 2018; Thomas et al. 2021). Researches of Lee et al. support the idea that e-cigarettes can significantly impact health challenges from smoking (Lee et al. 2022; Rodrigo et al. 2021).

E-cigarettes have different aerosol than combustible cigarettes, they do not contain tobacco tar and carbon monoxide. Some studies have shown that the use of e-cigarettes is significantly less harmful than the use of combustible cigarettes (Drope et al. 2017). Based on a review of 185 studies, UK's Public Health England estimates that e-cigarettes are at least 95% less harmful than cigarette smoking (McNeill et al. 2015). Because the e-liquids do not undergo high-temperature combustion, e-cigarette aerosol composition is simpler than that of cigarette smoke, with fewer toxic substances and low secondhand smoke hazards (George et al. 2019; Goniewicz et al. 2014; Landmesser et al. 2021; Margham et al. 2016; Park et al. 2022). Furthermore, reports suggest that they may aid in smoking cessation or at the very least limit conventional cigarette use (Martinez et al. 2021; Myers Smith et al. 2022). E-cigarettes produce inhalable aerosols that contain several components with potential toxicological and biological relevance to respiratory health, but at much lower levels than cigarette smoke (Margham et al. 2016; Mikheev et al. 2016). To test the toxicity of e-cigarette aerosol in vitro, researchers exposed various cells to it and found that e-cigarettes induced oxidative stress, pro-inflammatory cytokine production and cytotoxicity in THP-1 cells and BEAS-2B cells (Ma et al. 2021). Studies have compared the effects of cigarette smoke condensate (CSC) and e-cigarette aerosol condensate (ECAC) on human lung epithelial cells (BEAS-2B) at toxicological doses, and the results show that ECAC has a higher toxicological threshold than CSC. CSC but not ECAC significantly influences biological and transcriptomic effects (Wang et al. 2021). The secretory activity and ciliary beating of mucus-secreting cell cultures exposed to cigarette smoke were significantly reduced, whereas the effect on cells treated with e-cigarette aerosol was less pronounced (Aufderheide and Emura 2017). Similarly, human bronchial epithelial (HBE) cells exposed to cigarette smoke show transcriptome changes such as decreased expression of cell adhesion genes increased intracellular permeability, and increased expression of antioxidant and detoxification genes, such as MAPK signaling, cell cycle regulation, apoptosis, response to organic matter, and response to hypoxia, while the cigarette group showed more differential genes than the e-cigarette group (Shen et al. 2016). These in vitro studies imply that e-cigarette aerosols may minimize the risk of respiratory disease compared to cigarette smoke. However, a study showed that e-cigarette aerosol affects mouse macrophage phenotype, enhances lung fibrosis, and accelerates chronic obstructive pulmonary disease (COPD) progression (Han et al. 2021a). The study also showed that short-term exposure of mice to e-cigarette aerosols caused inflammation and oxidative responses (Been et al. 2022). Laura et al. reported that long-term (6 months) exposure to e-cigarette vapor promotes inflammation and airway damage. When compared with cigarette smoke, Alexander et al. found a decrease in lung function but no increase in the inflammatory response in mice exposed to e-cigarette aerosol after whole-body exposure. Combustible cigarettes had a more pronounced effect on lung function and inflammation in mice (Larcombe et al. 2017). However, unlike the way humans inhale smoke, in vitro experiments have mostly observed cells treated with smoke extracts or e-cigarette liquids. In some of the in vivo studies, standardized exposure doses and exposure procedures are rarely met simultaneously, and comprehensive comparison with cigarette smoke is lacking, so reliable conclusions cannot be obtained.

Despite the growing body of evidence supporting the relative safety of e-cigarettes, there are fewer studies comparing the toxicology between e-cigarette aerosol and cigarette smoke in terms of proteomics. To date, it is uncertain whether e-cigarettes are safe for long-term use. Based on preliminary findings, to further determine the effects of e-cigarette aerosol exposure (low and high doses) on the respiratory system of mice compared to cigarette smoke, we, therefore, conducted a subchronic inhalation toxicity study. The experiment was conducted for a total of 10 weeks as described previously (Lim and Kim 2014). Nicotine is a key ingredient in cigarettes and e-cigarettes and serves as the primary biological basis for mediating the biological effects of e-cigarettes (Bolt 2020), and studies have shown that nicotine causes addiction and leads to lung damage

(Garcia-Arcos et al. 2016; Herman and Tarran 2020). We chose nicotine levels as a reference, standardized nicotine dosage levels in e-cigarette and cigarette exposure, and studied the exposure consequences using a comprehensive toxicological approach combined with examination of physiology, pathology, and proteomics. We used a programmed smoking machine (3 s inhalation, 27 s between inhalations) and exposed mice orally and nasally to the corresponding smoke/aerosol to observe changes in airway, lung, inflammation levels and fibrosis levels in a C57BL/6 mouse model. Proteomic analyses were also incorporated into the comprehensive systemic toxicology assessment framework to reflect differences in the complex biological response mechanisms induced by ECA and CS.

Materials and methods

Cigarettes and e-cigarettes administration

Watermelon-flavored e-cigarettes "Fresh Red" (RELX, China; 3% nicotine; power, 6.5w) and cigarettes (commercially available brand, China; tar content, 10 mg; nicotine content, 1.0 mg per cigarette) were used in the experiments. ECA and CS exposures were normalized using nicotine content as a reference. E-cigarette aerosol or cigarette smoke was generated by a customized two-channel smoking machine (RuoFeiTe Tech., China). To determine the respective nicotine content, we stabilized the airflow at 110 mL/min and collected ECA and CS separately for 30 min using an aerosol sampler with a Cambridge filter (Whatman, UK), respectively. The average nicotine level of ECA and CS was quantified by high performance liquid chromatography. The results showed that the nicotine concentration was maintained at 0.1 mg/L. Nicotine doses for the animal studies were obtained with modifications based on the method of Larcombe et al. (2017). The ventilation in mice was 0.0217 L/min (Alexander et al. 2008). Based on this calculation, mice exposed to smoke for 60 min per day could achieve a nicotine dose of 6 mg/kg.

Animals and experimental protocol

32 Specific pathogen-free male C57BL/6 mice (8 weeks) were purchased from Guangdong Medical Laboratory Animal Centre (China). They were housed under 12/12 light/dark cycles and had ad libitum access to food and water. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Guangzhou Boji Medical Biotechnological Co., Ltd. Mice were randomized into four groups ($n = 8$ each), including the air-exposed group (Control), ECA low-dose group (ECAL, nicotine 6 mg/kg), ECA high-dose group (ECAH, nicotine 12 mg/kg), and CS-exposed group (CS, nicotine 6 mg/kg). The smoking machine was connected to an animal oral and nasal inhalation exposure system (Beijing Huironghe Technology Co., Ltd., China) under a standard protocol: draw for 3 s, 27 s blowing interval (Fig. 1). Mice were immobilized in a stationary tube attached to the exposure system and received a continuous stream of air or air mixed with ECA or CS. Mice were given fresh air every 30 min during the exposure period. Except for Control, mice in ECAL and CS were exposed to smoke for 1 h per day, while ECAH was exposed for 2 h per day. Mice were exposed to smoke/aerosol 5 consecutive days per week for 10 weeks. Continuous changes in body weight and survival of mice were observed at the same timepoint weekly.

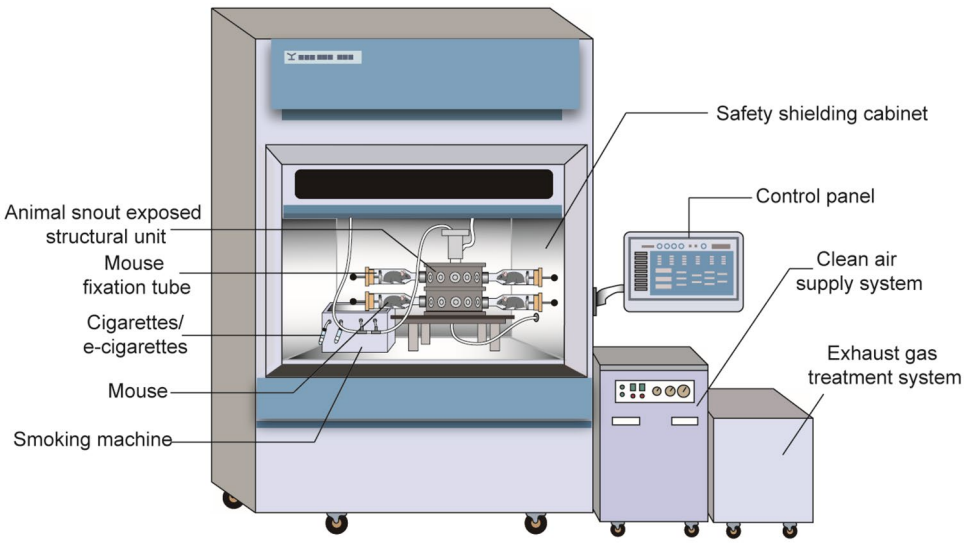

Fig. 1 Smoking machine and small animal snout inhalation exposure device. Both the smoking machine and the animal snout exposed structural unit were housed in a safety shielding cabinet. The smoking machine delivers the smoke/aerosol to the exposure system in a standard manner: draw for 3 s, 27 s interval, 55 mL draw volume. Mice were kept in the fixation tubes connected to the exposure units and received continuous air or air mixed with ECA or CS. The device was also equipped with a clean air supply system and an exhaust gas treatment system

Tracheal examination

After the exposure, the animals were sacrificed. The diameter and length of the trachea of mice were measured with a Vernier caliper, and three samples were collected from each group for histopathological examination. Tracheal pathology examination and scoring were performed by Servicebio (Wuhan, China).

Lung function measurement

The mice were placed in the unrestrained chamber of the EMKA pulmonary system (EMKA, France). The device was calibrated prior to use. Mice were placed in the testing chamber and allowed to stabilize for 5 min to acclimate to the environment, lung function indices such as enhanced pause (Penh), respiratory rate, 50% exhalation force (EF50), and ventilation per minute (MV) were examined and recorded under conscious awareness.

Cytokines and inflammatory mediators detection

Serum and bronchoalveolar lavage fluid (BALF) were collected for further investigation. Blood samples were collected from the retrobulbar venous plexus and the serum was obtained by centrifugation (1500 r/min, 15 min). The level of KC and granulocyte colony stimulating factor (G-CSF) in serum were tested with a protein chip (RayBiotech, USA). BALF was obtained from the left lung by repeated saline lavage and used for total leukocyte count measurement under a microscope. After centrifugation at 1500 rpm at 4 °C for 10 min, total BALF protein was assessed using Pierce™ BCA Protein Assay (Thermo Scientific, USA). The concentrations of IL-6 and TNF-α in BALF were analyzed by ELISA according to the manufacturer's instructions (ABclonal Technology, China).

Lung histopathology analysis

Hematoxylin–eosin (HE) and Masson staining

The entire lung was removed and weighed on an electronic balance to determine the lung coefficient (lung/body weight ratio). The right lung tissue samples were fixed in 10% formalin and paraffin sections were conducted. Routine HE and Masson staining were performed for morphologic assessment, with four samples measured, respectively, for each experimental group. Lung tissue sections were stained with HE and the same areas were photographed with a white light microscope (Nikon, Japan). Histological scoring was performed in a blinded manner referring to the Internationally Harmonized Nomenclature and Diagnostic (INHAND) Proposal, with severity scores ranging from 0 (no findings) to 4 (severe changes). Additional sections were stained with Masson to evaluate collagen deposition and fibrosis. Three fields of view were selected for each section, and Image-Pro Plus 6.0 (Media Cybemetics, USA) was applied to measure the percentage area of blue collagen fibril pixels in the imaged area, with the mean value calculated.

Transmission electron microscope (TEM)

The fixed mouse lungs were trimmed into samples of about 1 mm^3, fixed with electron microscope fixative for 2–4 h, and then fixed with 1% osmic acid for 2 h. The tissues were dehydrated in different concentrations of alcohol and acetone in turn for 15 min each time, and then embedded with an embedding medium to make 60–80 nm ultra-thin sections, which were then double-stained with uranium and lead, and finally observed under a transmission electron microscope, then the image acquired for analysis.

Proteomics analysis

Lung tissues were obtained and immediately frozen in liquid nitrogen. Each group provided three biological replicates for proteomic analysis by Lc-Bio Technologies (Hangzhou, China).

Tandem Mass Tag (TMT)-based quantitative proteomics

The concentration of proteins isolated from mouse lung tissue was determined using the BCA kit. Dithiothreitol, iodoacetamide, and trypsin were used to reduce, alkylate, and digest the isolated proteins, respectively. Then processed according to the tandem mass tag (TMT) kit's manufacturer's protocol. TMT-labeled peptides were combined and vacuum-dried, then mixtures were fractionated by high pH reversed-phase HPLC. The peptides were redissolved for LC–MS/MS Analysis. The Maxquant search engine (v.1.5.2.8) was used to analyze the proteomic data.

Screening and analysis of differentially expressed proteins (DEPs)

In differential expression analysis, fold change (FC) > 1.3 and P value < 0.05 were applied as screening criteria. An advanced volcano plot was drawn using the OmicStudio tools (https://www.omicstudio.cn/tool). Co-differentially expressed proteins were obtained using Wayne plots and heat maps were drawn (https://www.omicstudio.cn/tool).

Protein function and interaction analyses

Hierarchical clustering was performed according to different protein functional categories. The function of the identified

proteins was analyzed using gene ontology (GO) terms. Pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/kegg/mapper.html). Difference protein overall trend change was visualized by a heat map and a violin plot using the "Wu Kong" platform (https://www.omicsolution.com/wkomics/main/). After Cluster analysis, the different protein was imported into the STRING database to perform a PPI assay, then used the Cytoscape to draw the PPI network.

Network perturbation amplitudes (NPA) analyses

The molecular mechanism in the basic biology process in mouse pulsation is described by exposure to different smoke-induced protein changes in the collection of layered structured network models. This work used the "reason and effect" network model together with the NPA algorithm to calculate the value of the main node. Total quantitative protein was visualized by inflammation-related network perturbation amplitudes and barplot using NPA R-package according to the literature (Gonzalez-Suarez et al. 2016; Martin et al. 2019). We selected the musculus version "epithelial innate immune activation" model and then the Inflammatory Process Network (IPN), finally, biological mechanisms known to be linked and that can lead to toxicological responses were explored.

Western blotting

Lung tissue samples were mixed with RIPA lysis buffer (Beyotime, China) containing protease inhibitor mixture (Solarbio, China) and were homogenized in a bead mill type homogenizer (Omni International, UAS). Total protein was extracted from the tissue homogenate. To assess the expression of fibronectin (FN) and α-smooth muscle actin (α-SMA), we performed western blotting with a standard protocol as described previously (Wang et al. 2021). Anti-mouse α-SMA (ImmunoWay, USA), and anti-rabbit FN (Abcam, USA) were used.

Lung RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from lung tissue samples using RNAiso Plus (Takara, Japan) in a bead mill type homogenizer (Omni International, UAS). Quantification, reverse transcription, and RT-qPCR were performed as described previously (Wang et al. 2021). β-actin was used as an internal control, and the results were reported as the fold change of the test sample compared to Control. The primers for each gene were synthesized by Sangon Biotech (Shanghai, China), and the gene sequences are shown in Suppl. Table 2.

Statistical analysis

For all experiments, 7 to 8 mice were studied per group unless otherwise stated. All results were statistically processed by GraphPad Prism 8.0 software and expressed as means ± SEM of at least three independent experiments. The measurement data were analyzed using Ordinary one-way ANOVA with Tukey's multiple comparisons test. Count data were analyzed by Kruskal–Wallis test with Dunn's multiple comparisons test. P value < 0.05 was considered statistically significant.

Results

The variation of body weight and lung coefficients after exposure

Before the experiment, the mice in each group had a homogeneous weight distribution. Mice exposed to ECA and CS for 10 weeks gained slower bodyweight increases relative to Control, especially the mice in CS, but there were no significant differences between the four groups (Fig. 2a). CS exposure, but not ECA exposure, increased lung coefficients compared to Control (Fig. 2b). The results indicated that e-cigarette and cigarette exposure for 10 weeks did not significantly affect the body weight in mice, but cigarette smoke exposure resulted in elevated lung coefficients.

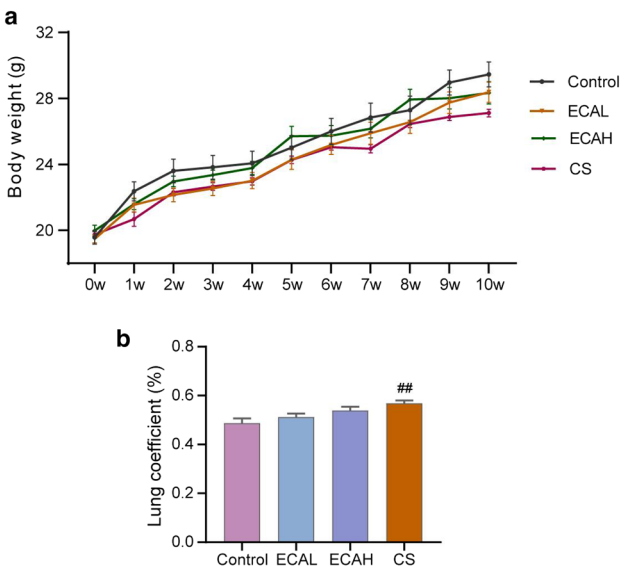

Fig. 2 Body weight and lung coefficients. **a** Curves of body weight changes in mice during 10 weeks of e-cigarette and cigarette inhalation (n = 7–8). Body weight of mice was measured at the same time-point each week. **b** Results of the lung coefficients were calculated based on the ratio of lung weight to body weight (n = 7–8). Data are represented as mean ± SEM. $^{\#\#}P < 0.01$ vs. Control

Morphological and histological changes of tracheal after exposure

The trachea is the primary part of the conducting airway and its pathological changes are closely related to respiratory diseases (Zepp and Morrisey 2019). Smoke inhalation injuries can lead to airway damage, which can induce life-threatening complications of airway obstruction (Enkhbaatar et al. 2016), so it is necessary to assess the respiratory system by the degree of tracheal pathology. By measuring tracheal changes in mice after smoke exposure, it was found that CS resulted in a smaller tracheal diameter but no change in length compared to Control. ECAL and ECAH did not cause observable tracheal morphological changes (Fig. 3a). Further pathological study by HE staining revealed that Control mice had intact tracheal mucosa epithelium covered with closely arranged epithelial cells. Both ECAL and ECAH exposures resulted in a small amount of inflammatory cell exudation from the tracheal lumen and submucosa, epithelial cell swelling, and cytoplasmic vacuolization. ECAH additionally had mucosal epithelium exfoliation. After CS exposure, epithelial cell detachment and swelling were significantly increased, and cilia were sparse. The submucosa was infiltrated with inflammatory cells and the cytoplasm was vacuolated (Fig. 3b). The pathology score in CS was higher than that in Control, while ECAL and ECAH were not statistically different. E-cigarette aerosol showed less pathological damage to the trachea than cigarette smoke at the same or even twofold dose (Fig. 3c).

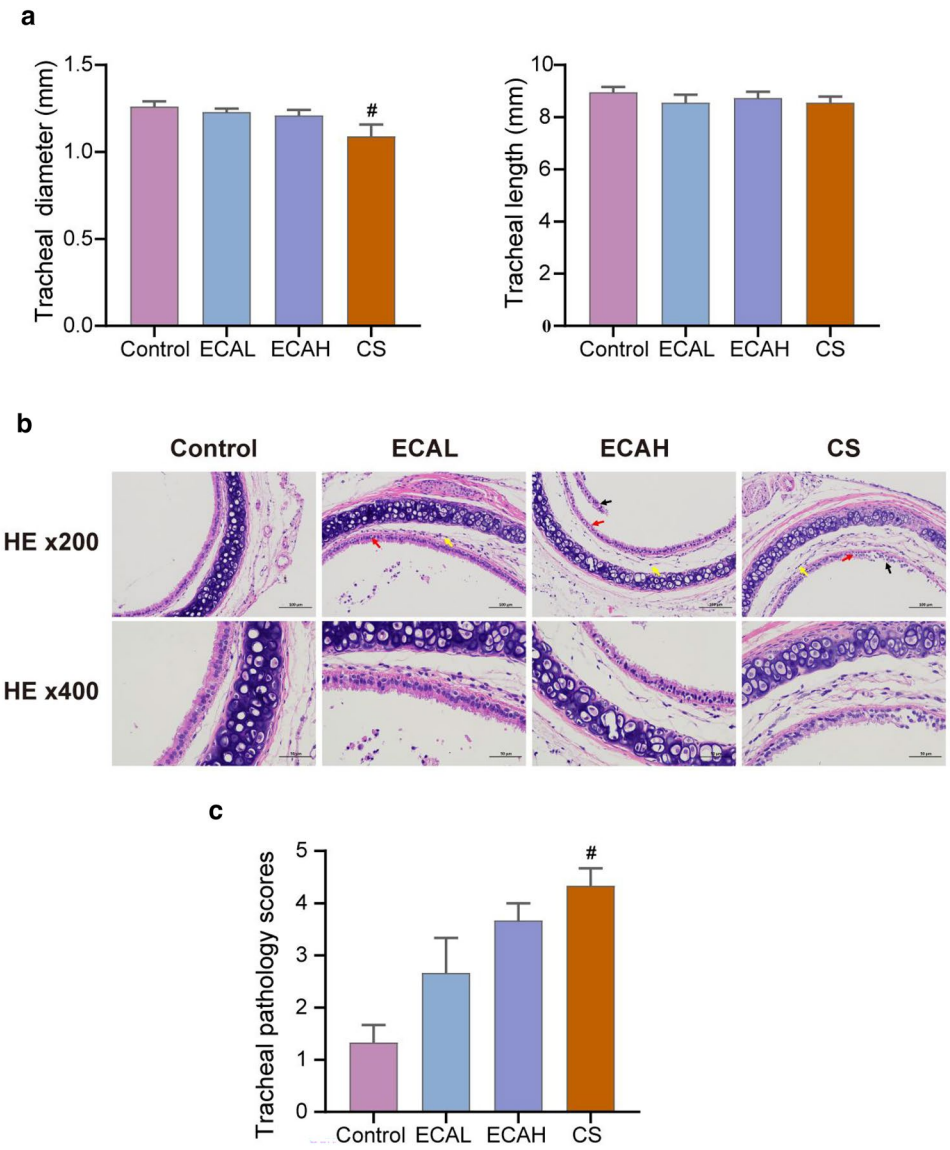

Fig. 3 Morphological and histological structure of trachea. **a** Changes in tracheal diameter and length in each group of mice were measured using vernier calipers after 10 weeks of smoke exposure ($n = 7$–8). **b** HE staining. Scale bar: 100 μm (first row), 50 μm (second row). Control tracheal mucosal epithelium was intact. ECAL and ECAH showed little inflammatory cell infiltration (yellow arrow), epithelial cell swelling, and cytoplasmic vacuolization (red arrow). ECAH also presented mucosal epithelial exfoliation (black arrow). CS showed more severe trachea damage and lesions ($n = 3$). **c** Referring to the INHAND recommendations, a four-level grading system was used for scoring, taking into account the degree of epithelial detachment, epithelial damage, and inflammation. Data are represented as mean ± SEM. $^{\#}P < 0.05$ vs. Control (color figure online)

CS has more severe effects on respiratory function changes than ECA

Cigarette smoking is linked to the cause of nearly all lung diseases—lung cancer, asthma, COPD, and fibrosis (Davis et al. 2022). Smoke causes abnormal lung function, so we measured changes in lung function to characterize the extent of lung damage in the animal. After 10 weeks of ECAL, ECAH, and CS exposure, significant changes in lung parameters of mice were detected by pulmonary function system (Fig. 4a). Penh, Respiratory rate, EF50, and MV were significantly increased in CS compared with Control, whereas no significant changes were observed after ECA exposure, such as ECAH only reduced MV. In addition, we found a

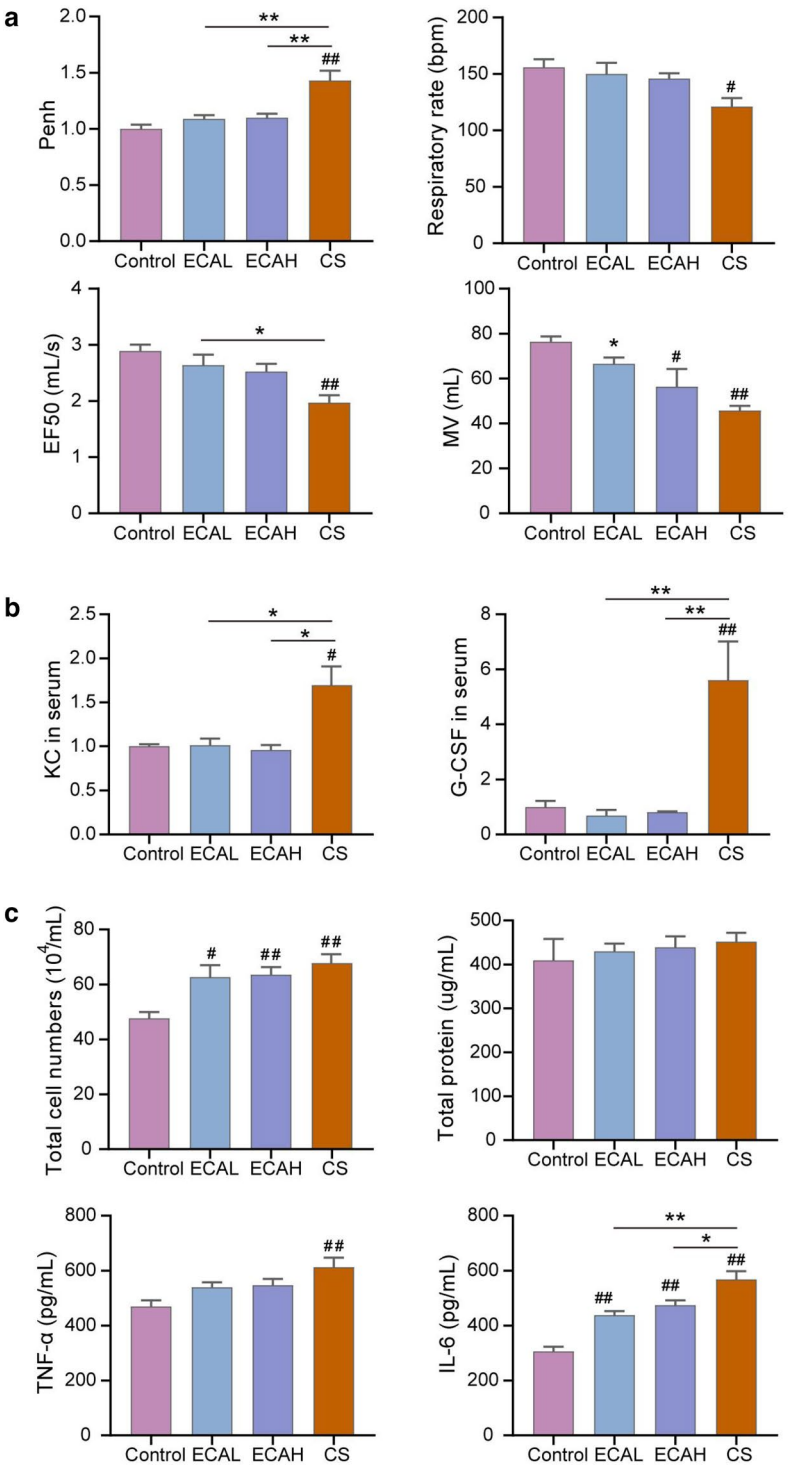

Fig. 4 Respiratory function and inflammatory responses detection. **a** EMKA pulmonary system was used to measure Penh, respiratory rate, EF50, and MV in mice to determine changes in pulmonary ventilation capacity. Penh was normalized to the Penh in Control group ($n=7$–8). **b** Inflammatory status in the serum. Levels of cytokines KC and G-CSF were measured by protein microarrays ($n=3$). **c** Levels of inflammation in BALF. Check the total leukocyte count and protein concentration in BALF, and analyze the concentration of IL-6 and TNF-α in BALF by ELISA ($n=7$–8). Data are represented as mean ± SEM. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Control. $^{*}P<0.05$, $^{**}P<0.01$ vs. CS

significant decrease in Penh after ECAL and ECAH exposure compared to CS. MV and EF50 in ECAL were significantly different from CS. These results suggested that CS more severely impaired lung function in mice compared to ECA in terms of ventilatory capacity under the experimental conditions used.

CS induced more inflammatory responses than ECA

Acute inflammatory responses protect the host from systemic infection and restore tissue homeostasis against injury or pathogens (Levy and Serhan 2014), and inflammation is a major hallmark of all chronic respiratory diseases (Komalla et al. 2020). To compare the severity of the inflammatory response in mice exposed to CS and ECA, inflammatory mediators and factors were detected in serum and BALF, respectively. Inflammatory factors in serum were measured by protein microarray, and the levels of KC and G-CSF were elevated after CS exposure compared to Control, while ECA exposure had no detectable effect. Compared with ECA exposure, KC was elevated nearly 0.5-fold and C-GCF was increased more than fourfold after CS exposure (Fig. 4b). The total cell numbers in BALF of mice exposed to CS and ECA were increased compared to Control, while the concentration of proteins did not change significantly. Both TNF-α and IL-6 were significantly increased in the BALF of the CS compared with Control. IL-6 was elevated in ECAL and ECAH, but not TNF-α. In addition, IL-6 levels were significantly lower after ECAL and ECAH exposure compared to CS exposure (Fig. 4c). Preliminary results suggest that both CS and ECA could trigger airway inflammation, but the pro-inflammatory effect of CS was much greater than that of ECA.

Higher histopathological scores for the lung in CS compared to ECA

To determine whether CS and ECA had an effect on lung pathology, stained sections and electron microscopic findings were comprehensively analyzed (Fig. 5a). Compared to Control, mice in ECAL had a small infiltration of inflammatory cells around the bronchi (black arrows). However, both ECAH and CS exposure resulted in increased focal infiltration of perivascular inflammatory cells (black arrows). CS additionally exhibited mild hemorrhage around alveoli and blood vessels (red arrows). TEM further showed that after e-cigarette and cigarette exposure, alveolar structures were damaged to varying degrees, with the collapse of the alveolar lumen and edema of endothelial cells (End) and type II epithelial cells. The lamellar vesicles (LB) were loosely structured and emptied. microvilli (Mv) were significantly sparse in the ECAH and CS, and a large number of collagen fibers (CF) were distributed. As seen simultaneously from the pathology scores (Fig. 5b), CS exposure resulted in higher scores compared to Control, with no significant effect of ECAL and ECAH exposure. Masson staining results revealed that both CS and EC exposure resulted in an increase in the percentage area of collagen compared to Control. However, the collagen area was relatively lower in the ECAL and ECAH compared to CS (Fig. 5c). TEM analysis coincided with the observation of HE staining and Masson staining. As shown in Fig. 5d, the expressions of Fn and α-SMA were significantly increased in lung tissue after cigarette smoking. The expression level of Fn was also increased to some extent in ECAH. However, with the same dose as CS, the fold change of Fn and α-SMA expression in ECAL was lower. The above results indicated that ECAL, ECAH, and CS exposure caused histological abnormalities in the lungs. The damage to lung pathology was more pronounced with CS compared to that of ECAL or ECAH.

Proteomic analysis of lung tissue in mice exposed to CS and ECA

Lung tissue from mice exposed to smoke for 10 weeks for proteomic analysis. Proteomic data quality analysis showed that the identified peptides had mass errors within 10 ppm and that the most numerous peptides consisted of 7–23 amino acids. Referring to these qualifying data, 3658 proteins were identified. Based on the criteria of P-value < 0.05 and fold change > 1.3, proteins that were significantly altered in mouse lung tissue were identified for each group of different smoke or aerosol exposures. Among them, 11 proteins were increased and 39 proteins were decreased in the ECAL (Fig. 6a and Suppl. Table 1), 21 proteins were increased and 91 proteins were decreased in the ECAH, 13 proteins were increased and 114 were decreased in CS, which with the highest number of protein changes. Venn diagrams (Fig. 6b) show that among these significantly different proteins, 8 proteins overlap, and they are Trappc5, Arfgef2, Lifr, Mettl26, F13a1, Apoc4, Clasp1, and Nck1. Interestingly, the level of Nck1 identified in ECA was not as high as in CS (Fig. 6c). For the mRNA expression levels of the overlap proteins Nck1, Lifr, Apoc4, and F131, the most significant changes were observed in the CS group compared to the control group, followed by ECAH. The changes in ECAL were not significant (Fig. 6d).

The subcellular localization of proteins (Suppl. Fig. 1a) showed that the DEPs were mainly concentrated in the cytoplasm, extracellular, mitochondria, nucleus, etc. Among them, 35% of the DEPs in CS were located extracellularly, which was more than that in ECA. Go secondary annotations indicated that the cellular process entries affected by differential proteins mainly involved cellular processes, biological regulation, metabolic processes, and responses to stimuli (Suppl. Fig. 1b). The results of GO enrichment analysis

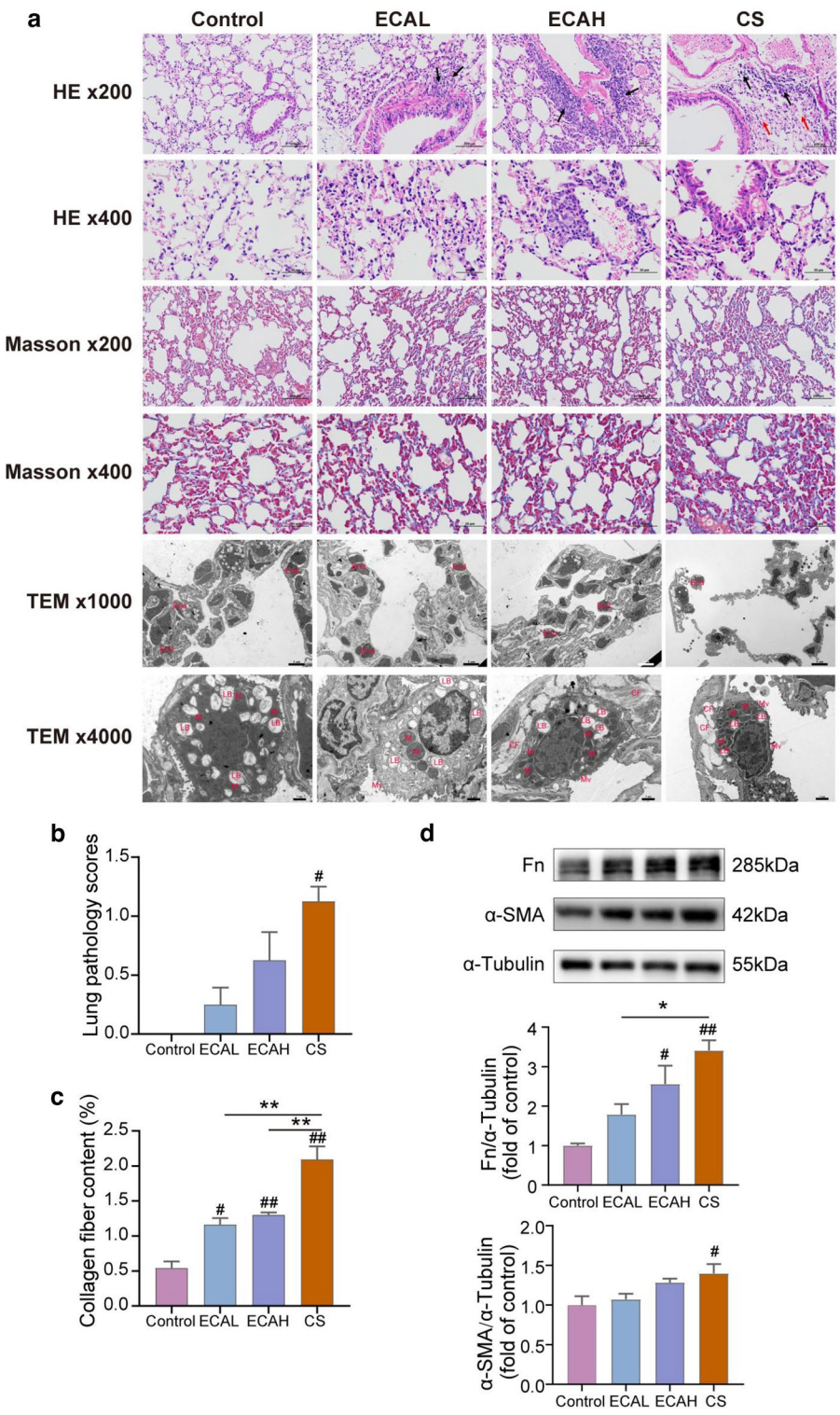

Fig. 5 Histopathological changes and scores. **a** After 10 weeks of exposure of male mice to air, ECAL, ECAH, or CS, lung tissues were taken and fixed in 10% formalin and paraffin sections were performed. Pathological processes were evaluated using routine HE staining ($n=4$), Masson staining ($n=4$), and TEM analysis ($n=3$). **b** Histological scoring was performed in a blinded manner according to INHAND. **c** Masson staining was used to calculate the area of interstitial lung fibrosis, determined as a percentage of blue staining over the area of the imaging area. **d** Expression of fibrosis marker proteins (Fn and α-SMA) in lung samples were examined by western blotting analysis ($n=3$). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Control. $^{**}P<0.01$ vs. CS

showed that more DEPs in the cigarette group regulate inflammatory and defense processes, such as positive regulation of immune response, regulation of defense response, regulation of cytokine production, and regulation of inflammatory response (Fig. 7a–c). However, fewer differential proteins in ECAL and ECAH were involved in immune and inflammatory responses, differential proteins in ECAL were mainly involved in cellular modification processes, while the differential proteins in ECAH mainly regulated defense responses and resistance to bacteria, in response to peptidases. KEGG pathway enrichment (Fig. 7d–f) showed that CS was enriched to many signaling pathways related to

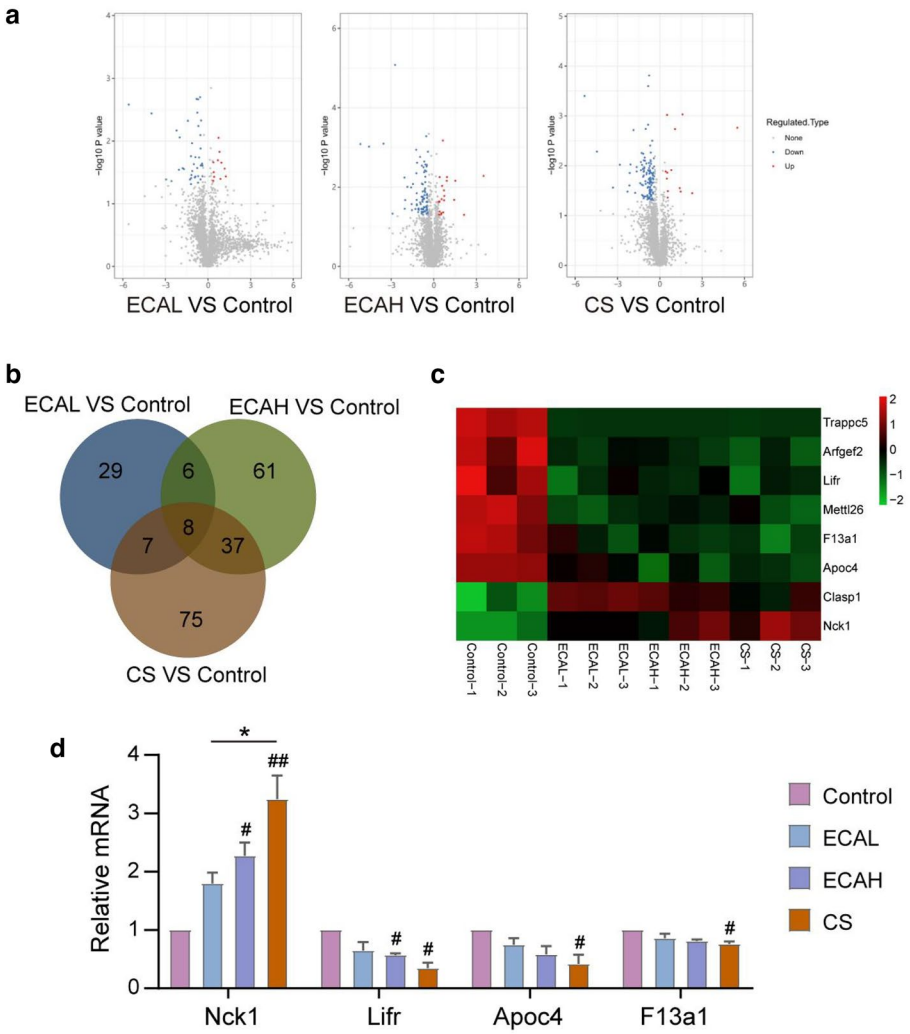

Fig. 6 Analysis of DEPs in lung tissue. **a** Volcano plot was drawn with the fold change transformed by Log2 as the horizontal axis and the logarithm of the *P*-value (-Log10) as the vertical axis. Red dots indicate significantly up-regulated proteins and blue dots indicate significantly down-regulated proteins. **b** Approximate relationship between DEPs in ECAL, ECAH, and CS groups was plotted using Venn diagrams. **c** Co-DEPs are shown by a heat map. Red represents relative up-regulation and green represents relative down-regulation. **d** RT-PCR was performed to detect the mRNA expression level of DEPs ($n=3$). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Control. $^{**}P<0.01$ vs. CS (color figure online)

inflammation, such as the HIF-1 signaling pathway, formation of extra-neutrophil trap, and COVID-19 related signaling pathway, JAK-STAT signaling pathway, pertussis, etc. At the same time, it is also enriched in signaling pathways related to different viral infections. In contrast, the differential proteins in ECAL were mainly enriched in human papillomavirus infection and the mTOR signaling pathway related to inflammation, while the differential proteins in ECAH were enriched in more inflammation-related signaling pathways, such as pertussis and neutrophil extracellular trap formation than that in ECAL, indicating that the differential proteins in CS were more concentrated in inflammation-related pathways, whereas e-cigarettes had less effect on these pathways.

We selected the differential proteins between CS and Control and plotted heat maps to examine the overall trends in the effects of e-cigarette aerosol and cigarette smoke on lung proteins (Fig. 8a). It can be seen intuitively that the differential proteins are divided into two categories. The violin plot suggests that the overall trend of ECAL and ECAH differential proteins was closer to the normal group as opposed to CS, indicating that the effect of cigarettes on these proteins was indeed more significant than that of e-cigarette exposures. The three up-regulated and three down-regulated proteins in the violin plot were verified by RT-PCR. As shown in Fig. 8b, the mRNA levels of Derl1, Rab32, and Ddx46 increased, and the mRNA levels of Trappc5, Dysf, and Arfgef2 decreased in CS. In contrast, in ECAH, only the mRNA levels of Dysf and Arfgef2 showed significant changes, while no significant changes were observed in ECAL. Then, we used NPA to further analyze (Fig. 9a) the perturbation amplitude of each group to the inflammatory network. The results showed that although the difference in the NPA coefficient of each group did not change much, the overall response of the CS to inflammation was more significant than that of ECAL, with more disturbances related to the inflammatory network. That also more firmly indicated that cigarette smoke did promote the body's inflammatory response to a certain extent than e-cigarette aerosol, which explains why their functional effects on the lungs were more

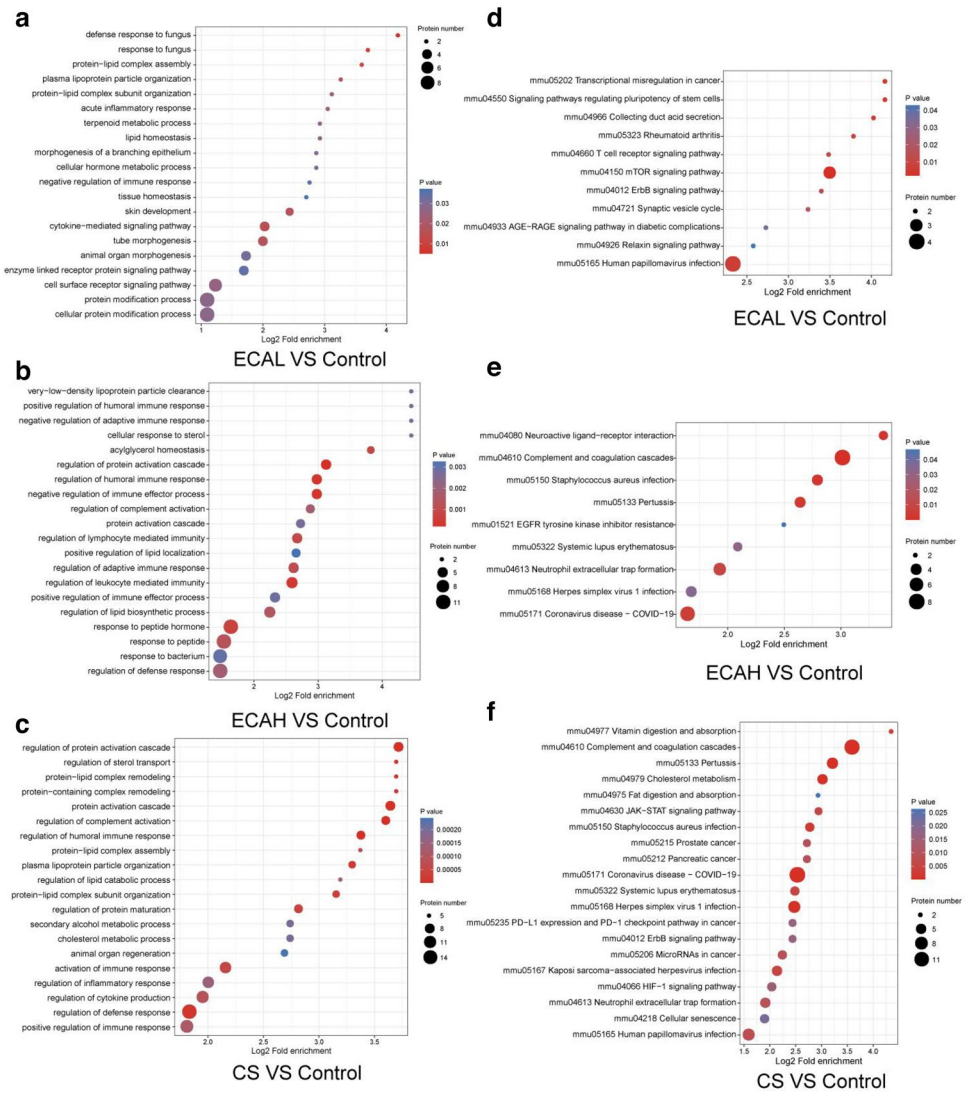

Fig. 7 GO enrichment analysis and KEGG pathway analysis of three groups vs. Control. **a**–**c** GO enrichment analysis of ECAL/ECAH/CS vs. Control, respectively. **d**–**f** KEGG pathway analysis of ECAL/ECAH/CS vs. Control, respectively

pronounced than e-cigarettes. The PPI network of all DEPs was established using STRING database and Cytoscape. We found that these differential proteins interact with each other and fell into two major clusters. CS and ECA similarly affected ribosomal protein (I) and complement system proteins (II) (Fig. 9b). Meanwhile, Apolipoprotein C-IV (Apoc4) and Coagulation factor XIII A chain (F13a1) were DEPs shared by CS and ECA (Fig. 9c).

Discussion

This study aimed to analyze the effects and potential mechanisms of e-cigarette aerosol and cigarette smoke on the respiratory system of mice under the experimental exposure protocol. Cigarette smoke showed greater alterations in airway pathology, lung function, inflammation, and lung histopathology than e-cigarette aerosol at the same nicotine dose and for the same duration of exposure. According to proteome analysis, the mechanisms of action of cigarettes and e-cigarettes in promoting the development of respiratory disease differed significantly.

E-cigarettes, which do not generate combustion products, are usually thought to be safer than combustible cigarettes (Ramamurthi et al. 2016). Switching to e-cigarettes may help with smoking cessation and smokers' health, and reduce the dangers of using combustible cigarettes (Beaglehole et al. 2019). E-cigarette use is usually a long-term process, and studies on the safety of e-cigarettes under long-term toxic dose exposure are necessary.

We used a non-invasive whole-body volumetric scanning method for awake animals that reflected the natural breathing pattern of mice. Respiratory parameters were used to assist in characterizing the lung function impairment in mice induced by smoke or aerosol exposure. Penh represents the variation in airway resistance and the degree of

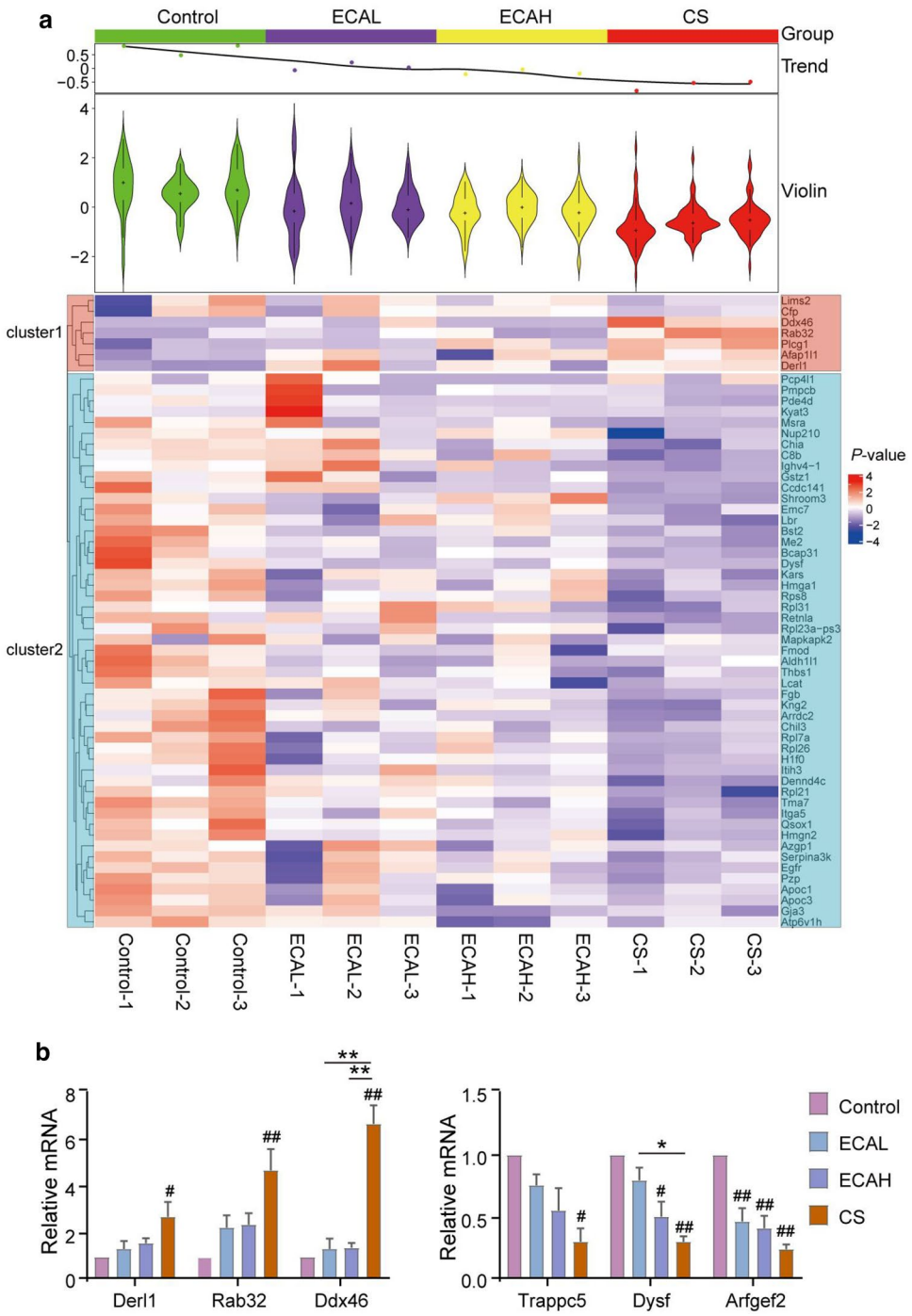

Fig. 8 Violin plots of DEPs. **a** Overall trend changes of the proteins were analyzed by heat and violin plots. **b** Three up-regulated and three down-regulated DEPs were validated by RT-qPCR. Data are presented as mean ± SEM of 3 individual experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Control. $^{**}P < 0.01$ vs. CS

bronchoconstriction and is calculated from peak expiratory flow, peak inspiratory flow, inspiratory time, and expiratory volume. Penh concentrates on indexes of airflow limitation and respiratory distress and is a recognized indicator of airway hyperresponsiveness (AHR) and lung function (Liu et al. 2019). In many studies observing animal lung disease models (Al-Shamlan and El-Hashim 2019; Csikós et al. 2020; Tham et al. 2021; Tian et al. 2019), Penh is mostly used as an indicator of observation to determine changes in airway response. In our study, a significant increase in Penh could be observed as a result of CS exposure, while no significant changes were observed after ECA exposure. Combined with other indices, such as respiratory rate and EF50, it appears to support the conclusion that CS affected lung function in mice more than ECAL and ECAH in this study.

G-CSF is a key regulator of neutrophil biology. G-CSF increases chemotaxis and migration of neutrophils and promotes phagocytosis of neutrophils. During stress

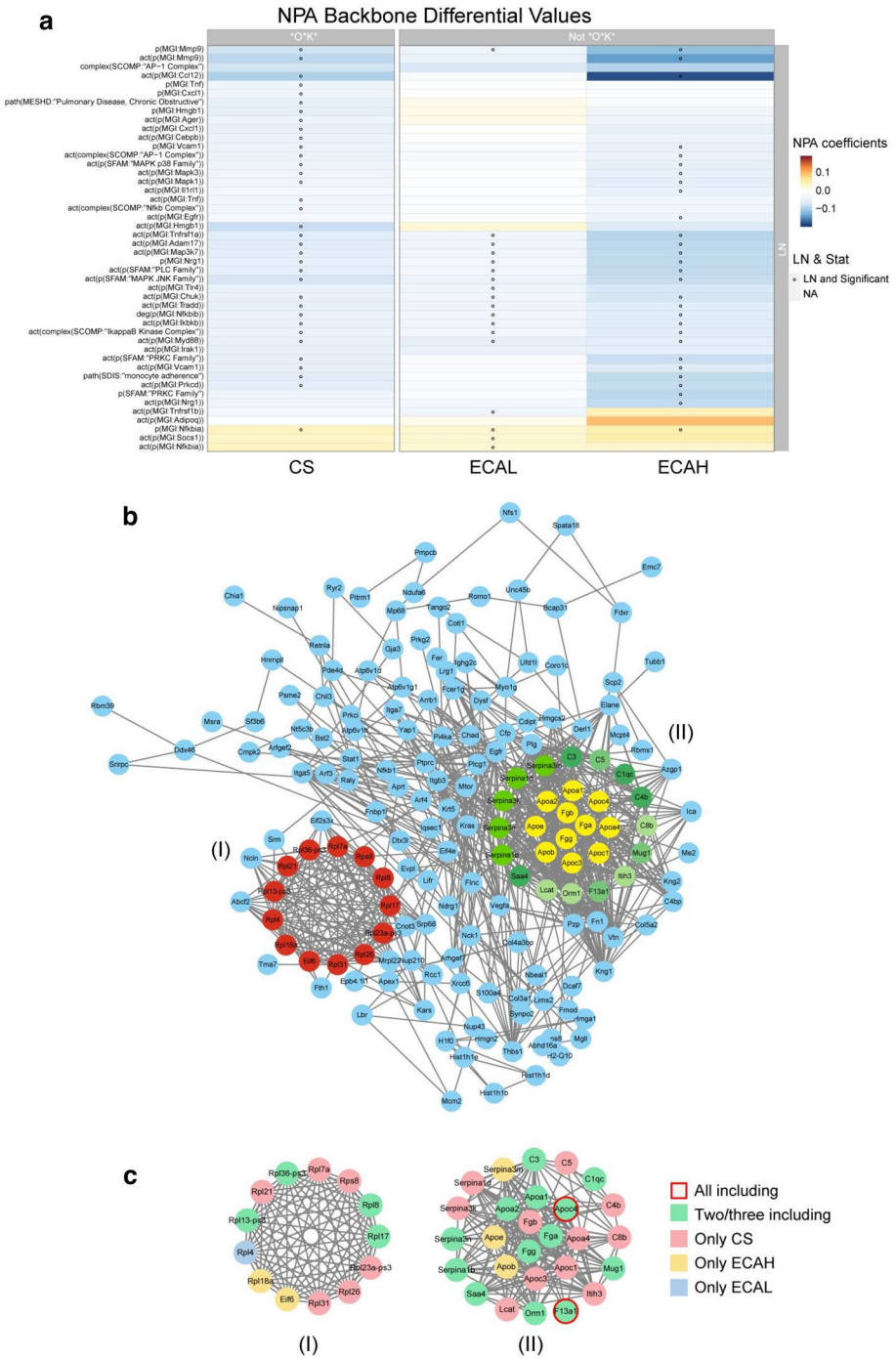

Fig. 9 NPA analysis and PPI analysis of DEPs. **a** Molecular mechanisms in mouse biological processes were characterized by exposure to different smoke-induced protein changes. The values of the major nodes were calculated using the NPA algorithm, and the total quantitative proteins were visualized by the magnitude of inflammation-related network perturbations and histograms. LN means leading nodes, if the actual NPA value lies above the 95% quantile of a null distribution, it is considered to be statistically significant and labeled as "O" or "K," respectively. **b**, **c** Protein–protein interaction network of the three groups of differential proteins, class I is a ribosomal protein (red), class II is a complement protein (green) and cholesterol transporter (yellow), and the red box in (**c**) is the three shared significantly different proteins (color figure online)

responses, such as infections, banded cells can be found in the peripheral blood in coordination with multiple cytokines such as G-CSF and have been used as a measure of inflammation(Mehta et al. 2015). To observe the effects of cigarette smoke and e-cigarette aerosol on inflammation in mice, we measured the serum G-CSF levels. Compared with ECAL and ECAH, GSF in CS was significantly higher than fourfold, and GSF may be an important factor involved in the inflammatory process in mice in smoke exposure models.

In vitro results in the literature have tentatively shown that e-cigarette aerosol exposure raises inflammation levels (Dusautoir et al. 2021; Lerner et al. 2015; Scott et al. 2018), but how the proinflammatory capability e-cigarettes are in vivo needs to be further elaboration. Constantinos et al. showed an increase in total cell count of BALF in mice exposed to e-cigarette aerosol or cigarette smoke for 4 weeks (Glynos et al. 2018). A study by Chad et al. found that acute e-cigarette smoke exposure resulted in elevated

levels of the pro-inflammatory mediator IL-6 in BALF (Lerner et al. 2015). Another study showed no change in BALF inflammation levels including total cell count and other inflammatory mediators with exposure to e-cigarette aerosol (Larcombe et al. 2017), but since the dose of exposure was not elucidated, it was not possible to determine whether the consistency of results was affected by different experiment condition. In our study, subchronic exposure to e-cigarette aerosols caused elevated levels of inflammation in BALF, which were more evident in CS. Total cell count and IL-6 were increased in BALF of mice exposed to ECA, but TNF-α was not elevated. In contrast, CS affected cell count, TNF-α and IL-6 levels in BALF, both cigarette smoke and e-cigarette aerosols were able to trigger an inflammatory response. Although our study was limited to total cells, total protein, IL-6, and TNF-α in BAIF, the results remained consistent with most studies. The assessment at the same nicotine dose is more informative due to the standardization of e-cigarette aerosol and cigarette smoke inhalation.

We employed the lung tissue samples from mice exposed to cigarette smoke or e-cigarette aerosols (low or high doses) for the proteome analysis, intending to compare the protein expression variations in lung produced by the two types of nicotine products. Monica Lee et al. used proteomic analysis to quantify 2611 proteins in lung samples from mice exposed to cigarettes and e-cigarettes. Compared to the control group, 204 proteins were significantly regulated in the cigarette group, but no significant protein changes were observed in the e-cigarette group (Lee et al. 2018). However, in our study, we found 127 and 112 significantly changed proteins in CS and ECAH, respectively, while ECAL had 50 significantly changed proteins. This may be due to the difference in exposure time and nicotine concentration. In both ECAL and ECAH, the number of differential proteins was lower than in CS compared to Control, which is consistent with previous research. Trappc5, Arfgef2, Lifr, Mettl26, F13a1, Apoc4, Clasp1, and Nck1 were identified as differential proteins shared by the three groups in the Venn diagram. This could be since both e-cigarettes and cigarettes contain the same substances, such as nicotine. Nck1 is an adapter protein that binds to IRAK-1, phosphorylates it, and then phosphorylates IB kinase (IKK) to translocate p65 to the nucleus, upregulating VCAM1, ICAM1, IL-1, IL-6, and other genes (Wines-Samuelson et al. 2020). Pro-inflammatory gene expression was upregulated in CS, while Nck1 was considerably upregulated, suggesting that cigarette smoke may increase endothelial cell inflammation. E-cigarette users had significantly higher levels of aldehyde detoxification and oxidative stress-related proteins associated with cigarette smoke, levels of innate defense proteins associated with chronic obstructive pulmonary disease, and Neutrophils and NET-related proteins in sputum compared to non-smokers, according to Reidel et al. (2018). KEGG pathway enrichment also shows that both ECAH and CS can activate neutrophil-related pathways, but CS undoubtedly activates more external stimuli-related pathways, such as the Hif-1 hypoxia signaling pathway (Meijer et al. 2012) and the JAK-STAT inflammatory and immune signaling pathway (Puigdevall et al. 2022). COVID-19 infection can result in pulmonary infection lesions and harm lung function (Siddiqui and Brightling 2021). We discovered that smoke exposure can activate the COVID-19 infection pathway, which is in line with the findings of Massey et al. (2022). Smoking worsens COVID-19 and has been identified as a risk factor for COVID-19 (World Health Organization 2020). Most of the DEPs were found extracellularly after exposure to nicotine smoke (about 35% in the cigarette group and about 26% in the high-dose e-cigarette group), which could be related to aerosol or smoke causing platelet and endothelial-derived extracellular vesicles increased (Mobarrez et al. 2020), which linked to Acute Coronary Syndrome (ACS) and Endothelial Dysfunction (ED) (Marei et al. 2022). Ddx46, one of the components of the small nuclear ribonucleoprotein complex, is raised 45-fold in cigarette exposure and is a target to treat pulmonary hypertension (Li et al. 2021a). After smoke exposure, KOG found increased protein post-translational modifications in DEPs in the lungs. Juan Wang et al. discovered that the thiol-oxidized proteome of rat lung tissue proteins showed the antioxidant defense response protein thioredoxin (THIO), ubiquitin-like modification activating enzyme 1 (UBA1), fatty acid synthase (FAS), and other protein thiol oxidative modifications were altered; however, the total amount of ubiquitinated proteins in the lungs of rats exposed to e-cigarettes did not increase significantly. Nevertheless, proteasome 20S activity was significantly increased (Wang et al. 2020), but our results showed that Ubiquitin fusion degradation protein 1 homolog (Ufd11) and E3 ubiquitin-protein ligase (Dtx3l) were significantly downregulated after cigarette and e-cigarette exposure, suggesting that smoke exposure affects protein ubiquitination degradation. E-cigarette aerosol causes mitochondrial DNA damage and Toll-like receptor 9 (TLR9)-mediated atherosclerosis according to previous studies (Li et al. 2021b), and KOG reveals that signal transduction processes are disrupted following smoking exposure.

We found a significant effect of smoke exposure on ribosomal associated proteins and these results are similar to the report that transcriptomic analysis identified differential ribosomal housekeeping genes in alveolar macrophages from patients with smoking-induced chronic obstructive pulmonary disease (Han et al. 2021b). In addition, studies have shown that nicotine treatments alter several immune pathways, such as the complement system

in the lung cancer cell line A549 (Navarrete-Perea et al. 2021), so we speculated that it was nicotine, one of the common components of CS and ECA, that affected the immune-related effects (Li et al. 2020).

Conclusions

Our findings revealed that both ECA and CS exposure had deleterious effects on the respiratory system and airways, as well as reductions in lung function using the animal model. E-cigarette aerosol, on the other hand, showed lower respiratory effects, decreased inflammatory responses, and less elevation of lung fibrosis indicators than those of combustible cigarette smoke at the same nicotine dose. Furthermore, proteomic analysis revealed that compared to combustible cigarette smoke, e-cigarette aerosols exposure resulted in fewer differentially expressed proteins and a smaller amount of disruption of inflammatory networks. The results that the cigarette group was enriched for more inflammatory pathways and that NCK1 was more significantly elevated after exposure to cigarette smoke compared to e-cigarette smoke suggest that inflammatory signaling in smoke exposure is an important regulator of atherosclerotic inflammation. Further investigation revealed that both combustible cigarettes and e-cigarettes had an observable effect on ribosomal proteins and complement system proteins, suggesting that smoke exposure may lead to impaired ribosome function thereby affecting the expression of other proteins and leading to reduced immunity, which requires further experimental validation. In conclusion, our findings indicated that e-cigarette aerosol appeared to be less hazardous to the respiratory system than that cigarette smoke at the same nicotine dose, providing further evidence to support human studies for e-cigarettes' relative safety.

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Author contributions Methodology: JC and ML; formal analysis and investigation: WY, LJ and HS; writing—original draft preparation: WY and XY; writing—review and editing: GH, KD and XJ; funding acquisition: PL. All authors have read and agreed to the published version of the manuscript.

Data availability Mass spectrometry proteomics data have been deposited in the iProX database (Project ID, IPX0004729000).

Declarations

Conflict of interest At the time of the research, Xuemin Yang, Kun Duan and Xingtao Jiang were employees of RELX Tech.

Ethical approval The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee of Guangzhou Boji Medical Biotechnological Co., Ltd.

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