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RESEARCH ARTICLE | *Electronic Cigarettes: Not All Good News?*

Comparison of the effects of e-cigarette vapor with cigarette smoke on lung function and inflammation in mice

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Glynos C, Bibli SI, Katsaounou P, Pavlidou A, Magkou C, Karavana V, Topouzis S, Kalomenidis I, Zakynthinos S, Papapetropoulos A. Comparison of the effects of e-cigarette vapor with cigarette smoke on lung function and inflammation in mice. *Am J Physiol Lung Cell Mol Physiol* 315: L662–L672, 2018. First published August 9, 2018; doi:10.1152/ajplung.00389.2017.—Electronic cigarettes (e-cigs) are advertised as a less harmful nicotine delivery system or as a new smoking cessation tool. We aimed to assess the in vivo effects of e-cig vapor in the lung and to compare them to those of cigarette smoke (CS). We exposed C57BL/6 mice for either 3 days or 4 wk to ambient air, CS, or e-cig vapor containing *1*) propylene glycol/vegetable glycerol (PG:VG-Sol; 1:1), *2*) PG:VG with nicotine (G:VG-N), or *3*) PG:VG with nicotine and flavor (PG:VG-N+F) and determined oxidative stress, inflammation, and pulmonary mechanics. E-cig vapors, especially PG:VG-N+F, increased bronchoalveolar lavage fluid (BALF) cellularity, Muc5ac production, as well as BALF and lung oxidative stress markers at least comparably and in many cases more than CS. BALF protein content at both time points studied was only elevated in the PG:VG-N+F group. After 3 days, PG:VG-Sol altered tissue elasticity, static compliance, and airway resistance, whereas after 4 wk CS was the only treatment adversely affecting these parameters. Airway hyperresponsiveness in response to methacholine was increased similarly in the CS and PG:VG-N+F groups. Our findings suggest that exposure to e-cig vapor can trigger inflammatory responses and adversely affect respiratory system mechanics. In many cases, the added flavor in e-cigs exacerbated the detrimental effects of e-cig vapor. We conclude that both e-cig vaping and conventional cigarette smoking negatively impact lung biology.

cigarette smoking; electronic cigarettes; lung hyperresponsiveness; lung inflammation; lung mechanics

INTRODUCTION

Smoking is the leading cause of morbidity and mortality in men and women and for this reason, smoking prevention and cessation strategies have tremendous potential for improving overall public health (4). In recent years, electronic cigarettes (e-cigarettes or e-cigs) are increasingly advertised as a reduced-risk nicotine product and an attractive alternative smoking cessation tool (23, 24, 35). In fact, part of the medical community believes that they can be used as a harm-reduction strategy for smokers. E-cigs have become rapidly popular worldwide (6, 35), although their effectiveness as a smoking cessation tool has not been rigorously proven yet (29, 36). Currently, their effects on human health have not been adequately addressed (6, 11, 36). Although some negative short-term health effects have already been shown (6, 20, 36), altogether there is still paucity of reliable data regarding long-term exposure effects.

E-cigs are battery-powered devices, which do not contain or burn natural tobacco. They consist of a rechargeable battery, a heater, and a refillable cartridge with liquids, usually consisting of propylene glycol, vegetable glycerol, nicotine, and flavorings (6, 11). When the battery-powered heater is activated, it heats the solution to produce a vapor containing various heat-produced ingredients of variable concentration, which are inhaled by the user. Quality control of e-cigs among various brands has been a matter of controversy, raising concerns about their safety profile and their toxicity (12, 35, 46, 47). The United States Food and Drug Administration has indicated that e-cigs contain a number of toxins and carcinogens (i.e., nitrosamines, diethylene glycol) (8). Recently, there have been attempts to regulate the market of the e-cig devices (6, 8). As experiment-based scientific knowledge is still largely lacking in the field, it is crucial to vigorously assess e-cig toxicity, safety, and health effects.

The smoke of conventional cigarettes contains more than 4,000 chemicals with multiple immunomodulatory and other effects on the lungs (9, 33). Compared with their effects, e-cig vaping is advertised as less harmful. Based on the few published in vitro or in vivo studies, e-cig vapor seems to have adverse effects on both cultured cells and on experimental animals (reviewed in 6, 11). E-cig vapor induces inflammation, augments the development of allergic airway inflammation in asthma models, suppresses the host defense, and triggers effects associated with chronic obstructive pulmonary disease-like tissue damage (26, 30, 42). In humans, clinical manifes-

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tations such as acute eosinophilic pneumonia and lipoid pneumonia (31, 43) have been related to e-cig use. It has also been shown recently that acute exposure to e-cig vapor increases aortic stiffness, blood pressure (16, 23), and airway resistance (45) whereas conversely, it decreases airway conductance (34) in healthy subjects and changes the slope of phase III curve in asthmatic smokers (34). It should be stressed that all the above-mentioned studies focus on the acute pathophysiological effects of the e-cig vapor with regard to the respiratory and cardiovascular function. Thus, the potential longer-term effects of e-cig vapor exposure in animals and humans still remain unexplored and unknown.

The aim of the present study was to determine the effects of both acute (3-day) and subchronic (4-wk) exposure of mice to components of vaporized e-cig liquids on the respiratory functional parameters and inflammatory responses and to compare them side-by side to those of air and classic cigarette smoke (CS) exposure, using a well-established animal model (10).

MATERIAL AND METHODS

Animals. Sex disparity in response to chronic smoke exposure has been observed in animal models (5). In the present study we used only male mice for two reasons. First, it is estimated that men smoke nearly five times as much as women worldwide (21). Second, limiting our studies to male mice would be expected to reduce variability allowing the use of a smaller number of animals that conforms with the 3Rs principle (replacement, refinement, and reduction) in humane animal research. Because of the abundance of information on C57BL6 and its susceptibility to lung injury, this strain was chosen. Eight-to-twelve-week-old male C57BL/6 wild-type Pasteur Institute (Athens, Greece) or Fleming Institute (Vari, Greece) mice, weighing 16–24 g, were exposed for 3 days or 4 wk to air (medical air grade), CS, or e-cig vapor from *1*) propylene glycol/vegetable glycerol (PG:VG-Sol; 1:1), *2*) PG:VG with nicotine (PG:VG-N; 18 mg/ml), or *3*) PG:VG-N+F with flavor (PG:VG-N+F; tobacco blend) (Fig. 1*E*). A partial chemical characterization of the tobacco blend flavor (Nobacco American Tobacco) has been previously published (14). It should be mentioned that this product is not a tobacco extract. Mice were maintained in standard conditions under a 12-h:12-h light-dark cycle and provided a standard diet and chlorinated tap water ad libitum. All procedures were in accordance to the European Union Directive for care and use of laboratory animals and were approved by the competent Regional Veterinary Service and the ethical committee of Evangelismos Hospital.

CS and e-cig vapor exposure. The apparatus used in our study is shown in Fig. 1, *A* and *B*, it exposes the entire body of animals to the treatment applied. The apparatus has been described before (10, 18) and has been used by us in recent studies (10, 18). A pump connected with five syringes was used to create positive/negative pressure cycles to drive flow of the smoke or vapor to a chamber where the animals were kept. The chamber volume was 7,500 cm^3, and the flow of medical air into the chamber was between 1.5 and 2 l/min. The puff volume was 20 ml. In the chamber a smoke/air ratio of 1:6 was obtained. All of these parameters were identical between the CS and e-cig exposure. The e-cig exposure was performed using the same system as for the CS exposure; the only change was the use of an adaptor that held the e-cig in place because of its different diameter. Three different experimental series of air, CS, and e-cig vapor exposures were performed for the acute study, and two experimental series were performed for the subchronic study. Control mice were exposed to medical air. For CS exposure, five reference cigarettes (3R4F, University of Kentucky; Fig. 1*C*) were used, whereas for vaping five eRoll devices (Joye Technology) were employed. The eRoll (Fig. 1*D*) is a first generation e-cig device (39) and was chosen as it was among the most widely used in Greece when the study was initiated. The smoke and vapor from all five cigarettes or e-cigs was directed toward the chamber were the animals were. Mice were exposed to CS or e-cig 4 times a day with 30-min smoke-free intervals for 3 days or 4 wk. For the CS treatment, 15 puffs were drawn per session, which sufficed to burn the entire conventional cigarette. The eRoll cartridge holds 0.4 ml of liquid and contains a chromium coil. Eight puffs/min for two minutes (i.e., a total of 16 puffs) were drawn during each session, with 4 sessions being used per day with 30-min intervals. The animal whole-body exposure lasted 7 min in each session. Our protocol uses less than half of the total amount of the 0.4 ml in the cartridge. The cartridge was replaced after every vaping session, i.e., 4 times per day. This procedure avoided overheating of the chromium coil. To eliminate the metal decay that could be relevant to the long-term treatment, we changed the chromium coil every second week. Lack of overheating was empirically confirmed by regular e-cig users who vaped the eRoll device at the same rate of 16 puffs over a 2-min period and reported no heating or change in vapor taste.

Respiratory system mechanics. The function of the respiratory system of mice after 3 days or 4 wk exposure to CS or e-cig vapor was estimated with the use of the forced oscillation technique and by performing static pressure volume curves, as previously described (19). Following 3-day or 4-wk treatment, the animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). An anterior cervical incision was performed, and the animals were tracheostomized below the level of banding with a tracheal cannula (20-gauge). The animals were connected to a small animal ventilator (Scireq, Montreal, Canada) and ventilated with a 7 ml/kg tidal volume, 150 breaths/min, and the end expiratory pressure was set to 3 cmH_2O. Following 3 min of ventilation, an intraperitoneal injection of succinylcholine (8 mg/kg) was performed to cease spontaneous breathing, and after 1 min, three forced oscillation perturbations were performed with 1-min intervals, to estimate lung mechanics. A static pressure volume curve was also constructed following 1 min of ventilation after the last oscillation perturbation. Results from repeated measures in every animal were averaged. Prior to measurements (30 s) the lung volume history was once standardized by one inflation to total lung capacity, as estimated by airway opening pressure at 30 cmH_2O. During ventilation, the heart rate was monitored to ensure adequate depth of anesthesia.

Forced oscillation technique. The forced oscillation perturbation consists of a pseudorandom waveform of low frequencies (0.5–19.75 Hz) applied for 8 s with a peak-to-peak volume of 3 ml/kg. Pressure and volume data are recorded, and the impedance of the respiratory system is calculated using the Fast Fourier transformation. Impedance (Z) is then fitted to constant phase model, where Zrs is the baseline measurement of Z: $Zrs(f) = Rn + i2\pi fI + (G - iH)/(2\pi f)\alpha$, where Rn is the Newtonian resistance of the airways, i is the imaginary unit, f is the frequency, I is the inertance of the gas in the airways, G represents tissue viscance (viscous dissipation of energy), H represents tissue elasticity, and α can be calculated through the equation $\alpha = (2/\pi)\arctan(H/G)$. Data were accepted only when the coefficient of determination (fit of the model) was more than 0.9. For static pressure volume curve, static pressure volume curves of the respiratory system were performed by gradually inflating and deflating the lungs with a total volume of 40 ml/kg at 7 steps each. The static compliance of the respiratory system was estimated by the slope of the midlinear part in the expiratory limb of the pressure volume curve. Hysteresis (area between inspiratory and expiratory limb) was automatically calculated (FlexiVent software) (44).

Airway hyperresponsiveness. Twenty-four hours after the 3-day CS exposure or e-cig vapor, mice were anesthetized, tracheostomized, paralyzed, and ventilated with Flexivent (SCIREQ Scientific Respiratory Equipment, Inc., Montreal, Canada). After baseline Zrs, methacholine (2.5, 10, 20, 40, 60 mg/ml) or saline were delivered (Aeroneb; SCIREQ) for 10 s. Afterwards, a 2-s forced oscillation perturbation (1–20 Hz) was performed every 10 s for 3 min. Before measurements and before every aerosol delivery, the volume history

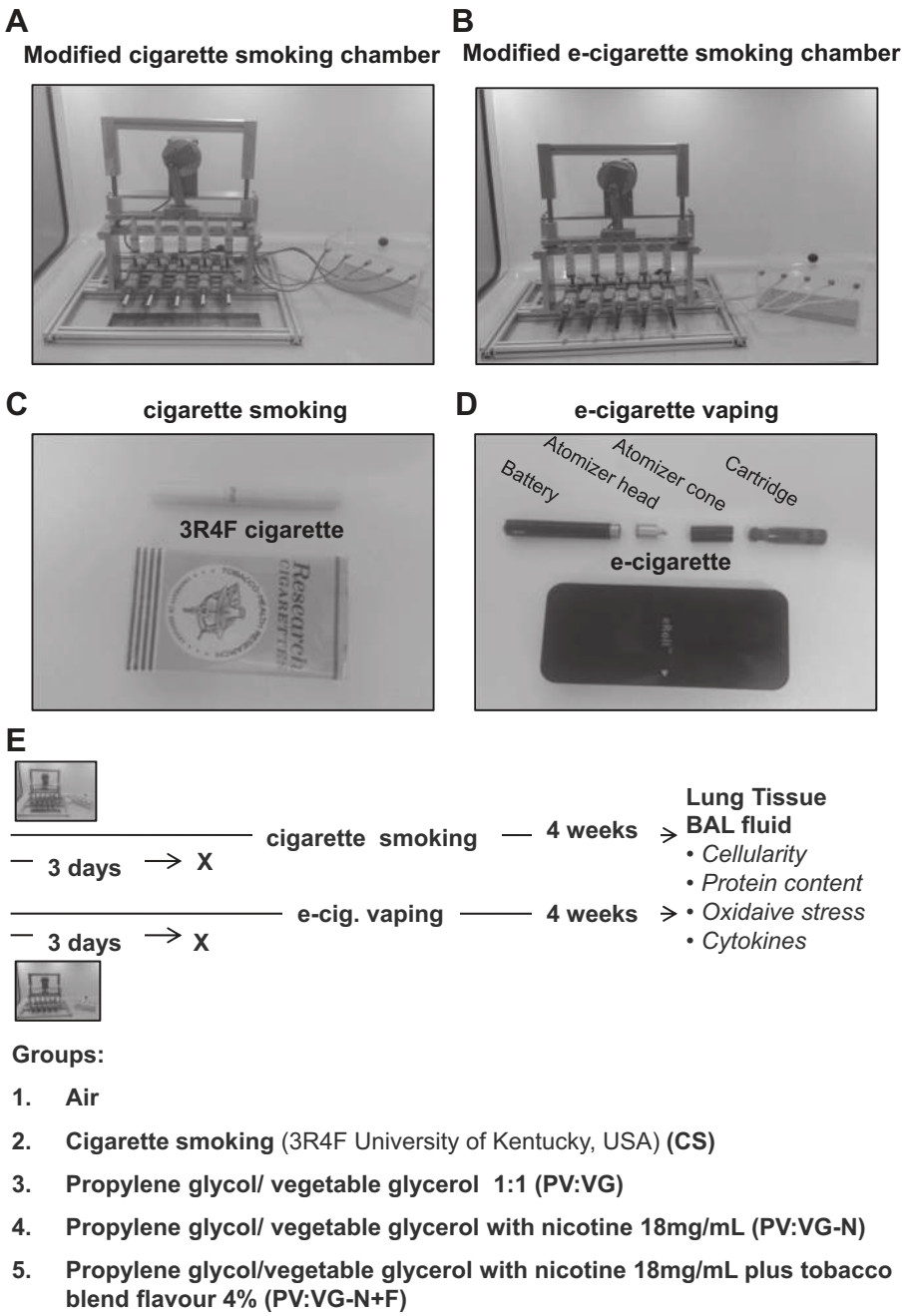

Fig. 1. Equipment and treatment protocol. *A*: proprietary modified chamber used for both CS and e-cigarette (e-cig) vapor exposure of mice. *B*: schematic representation of the protocol of exposure to CS and e-cig vapor. BAL fluid, bronchoalveolar lung fluid; CS, cigarette smoke.

of the lung was established with two 6-s deep inflations to a pressure limit of 30 cmH_2O. Measurements of Zrs were fit with the constant phase model, where Rn is the Newtonian resistance of the airways, G represents tissue resistance, and H represents tissue elasticity. After each dose of methacholine, model parameters were expressed as % ratio of the baseline (41, 44).

Bronchoalveolar lavage fluid. The animals were euthanized by exsanguination (vena cava dissection) following anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally 24 h after the last exposure to air, CS, or e-cig vapor (Fig. 1*E*). After exsanguination, the trachea was cannulated with a 20-gauge plastic catheter. Lungs were lavaged by infusing 1 ml warm saline, three sequential times. The recovered bronchoalveolar lavage fluid (BALF) was centrifuged; cells were collected and resuspended in PBS. Differential BALF cell counts were performed on Giemsa-stained cytospins, and percentages of eosinophils lymphocytes, neutrophils, and macrophages were determined. Protein concentration was measured in the BALF using the Lowry method, employing bovine serum albumin as a standard.

Lung histology. Following bronchoalveolar lavage, the left lung was harvested from mice and fixed using a 4% formaldehyde solution. The tissue was embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin using standard methods. Two pathologists blinded for treatment evaluated the histopathological findings in the lung. A scoring system to grade the degree of lung inflammation has been used based on the following histological features: *1*) capillary congestion, *2*) intra-alveolar hemorrhage, *3*) interstitial neutrophil infiltration, *4*) intra-alveolar neutrophil infiltration, and *5*) focal thickening of alveolar membranes. A scale from 0 to 3 for each feature will be used (0: absence, 1: mild, 2: moderate, 3: most severe) (32).

Immunohistochemistry. Mouse lung paraffin sections 0.5 μm thick were applied to positive electrical charge-coated slides and left at

55°C to remove paraffin excess. Sections were deparaffinized by two consecutive treatments (5 min each) with xylene. Rehydration was performed with graded ethanols (90, 80, and 70%) for 4 min each. Antigen retrieval was subsequently performed by boiling the sections with 1× Target Retrieval Solution pH 6.0 (Dako, Athens, Greece) in a steamer for 30 min. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 15 min at room temperature. Primary antibody mouse monoclonal Mucin 5AC (clone 45M1, PierceThermo Scientific) diluted 1:50 (vol/vol) in REAL Antibody Diluent (Dako) was applied to sections and incubated for 30 min at room temperature. Immunostaining reaction was developed using REAL EnVision Detection System Peroxidase/DAB$^+$ Rabbit/Mouse (Dako) incubated for 30 min at room temperature. Washes were performed using Tris-buffered saline-tween 20 buffer for 10 min. Immunoreactivity was detected using DAKO REAL DAB$^+$ Chromogen reagent for 5 min. Sections were counterstained with hematoxylin, dehydrated, mounted, and examined. Sections from which the primary antibody was omitted served as negative control. Immunohistochemistry slides were evaluated by light microscopy, and the immunosignal was scored using a semiquantitative scoring system as previously described (1). An intensity score was assigned representing the estimated average intensity of positive staining cells. The staining intensity was classified into 4 scales scored as negative (0), weak (1+), moderate (2+), and intensive (3+) (1).

Determination of oxidative stress in the lung and the BALF. Biomarkers of oxidative stress were determined by measuring malondialdehyde (MDA) and protein carbonylation in the lung tissue. For MDA measurement, lung tissue samples were pulverized and then minced in a small volume of ice-cold 20 mm Tris·HCl buffer (pH 7.4) in a 1:10 wt/vol ratio and homogenized. After centrifugation at 3,000 *g* for 10 min at 4°C, the clear homogenate supernatant was used for biochemical assay. For the determination of MDA, 0.65 ml of 10.3 mmol/l *N*-methyl-2-phenyl-indole in acetonitrile was added to 0.2 ml of tissue sample. After vortexing for 3–4 s, 0.15 ml of 15.4 mol/l methanesulfonic acid was added, and samples were mixed well, closed with a tight stopper, and incubated at 45°C for 40 min. The samples were then cooled on ice, centrifuged, and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve, made with standard MDA solutions (from 2 to 20 nmol/ml), was also run for quantitation. Measurements were performed in triplicate. MDA levels were expressed as μmol/mg protein (3). For protein carbonylation, a modification of the technique of Levine et al. (27), based on spectrophotometric measurement of 2,4-dinitrophenylhydrazine (DNPH) derivatives of protein carbonyls, was used to quantify protein carbonyl content in the lung or the BALF of the mice. Briefly, 100 μl of the homogenized lung tissue or of the BALF was incubated either with 500 ml DNPH or 2 mol/l HCl for 1 h at room temperature. The samples were then reprecipated with 600 μl 20% trichloroacetic acid, incubated for 5 min on ice, and subsequently extracted with ethanol/ethyl acetate (1:1, vol/vol), 3 times at 11,000 *g* for 10 min at 4°C. The pellets were carefully drained and dissolved in 6 mol/l guanidine solution in HO. The difference between the spectra of the DNPH-treated sample and the HCl control was determined at 360 nm, and the results are expressed as nmol protein carbonyl/mg protein using a molar extinction coefficient of 22,000 mol/l. Protein concentration was determined using the Lowrey assay.

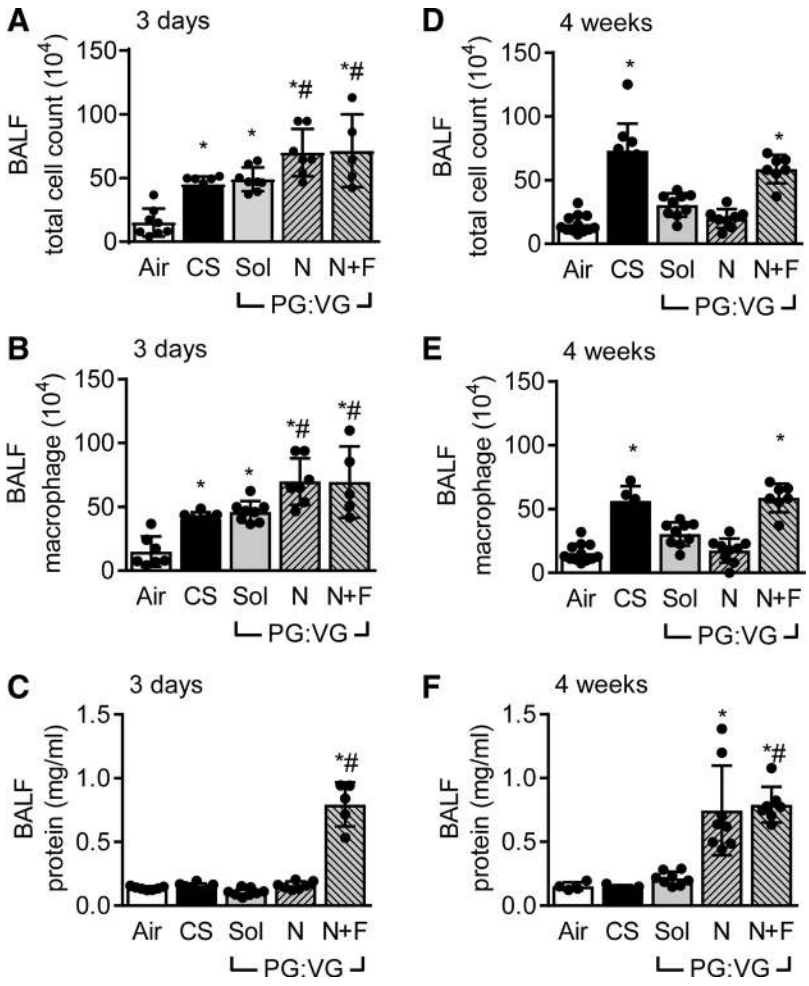

Fig. 2. Effects on BALF cellularity and protein content. Following an acute 3-day and a subchronic 4-wk exposure to CS and e-cig vapor, BALF was obtained, and following centrifugation total cellularity was determined (*A* and *D*). Differential counts, to assess cell type-specific contribution (*B* and *E*) were performed on Giemsa-stained cytospins. Cell-free protein was determined in the supernatant (*C* and *F*). Values are expressed as mean ± SD; $n = 5$–10 mice; *$P < 0.05$ vs. air, #$P < 0.05$ vs. CS. BALF, bronchoalveolar lung fluid; CS, cigarette smoke; PG:VG-Sol, propylene glycol/vegetable glycerol; PG:VG-N, propylene glycol/vegetable glycerol with nicotine; PG:VG-N+F, propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor.

Determination of cytokine in the BALF. Cytokines levels (TNF-α, IL-1β, IL-6) in the BALF were measured in 100 μl BALF using the manufacturer's protocol (HS Quantikine; R&D Systems, Minneapolis, MN). To determine the tissue cytokine levels, lungs were homogenized as for the MDA measurements, and TNF-α, IL-1β, and IL-6 were measured in the supernatant corresponding to similar amounts of protein (2 mg protein).

Statistical analysis. Results are presented as means ± SD of the number of indicated observations. Statistical analysis was performed with Sigma Stat software (SPSS 11.5, Chicago, IL) using nonparametric tests for continuous variables (Kruskall-Wallis, Mann-Whitney *U*-test). Differences were considered significant when $P < 0.05$.

RESULTS

BALF cellularity and protein content. Total cell counts in BALF of mice exposed to e-cig vapor for 3 days were increased in all groups compared with air-breathing mice, mainly because of macrophage influx. BALF cellularity in CS-exposed mice was also increased compared with air-breathing mice, because of macrophage influx (90% of total cell count) and to a lesser extent to neutrophils (7.1% of total cell count) (Fig. 2, *A* and *B*). BALF cell-free protein content was increased only in the PG:VG-N+F group ($P = 0.001$), compared with air-breathing mice (Fig. 2*C*).

The results were different after 4 wk of exposure to CS or e-cig vapor (Fig. 2, *D–F*). Total BALF cell count was elevated only in the CS ($P = 0.0001$) and PG:VG-N+F ($P = 0.0001$) groups, again mainly because of macrophage influx. These results indicate that neither the PG:VG vehicle or nicotine addition to e-cigs affected these parameters at 4 wk of vaping; however, the addition of flavor to nicotine-containing e-cigs is

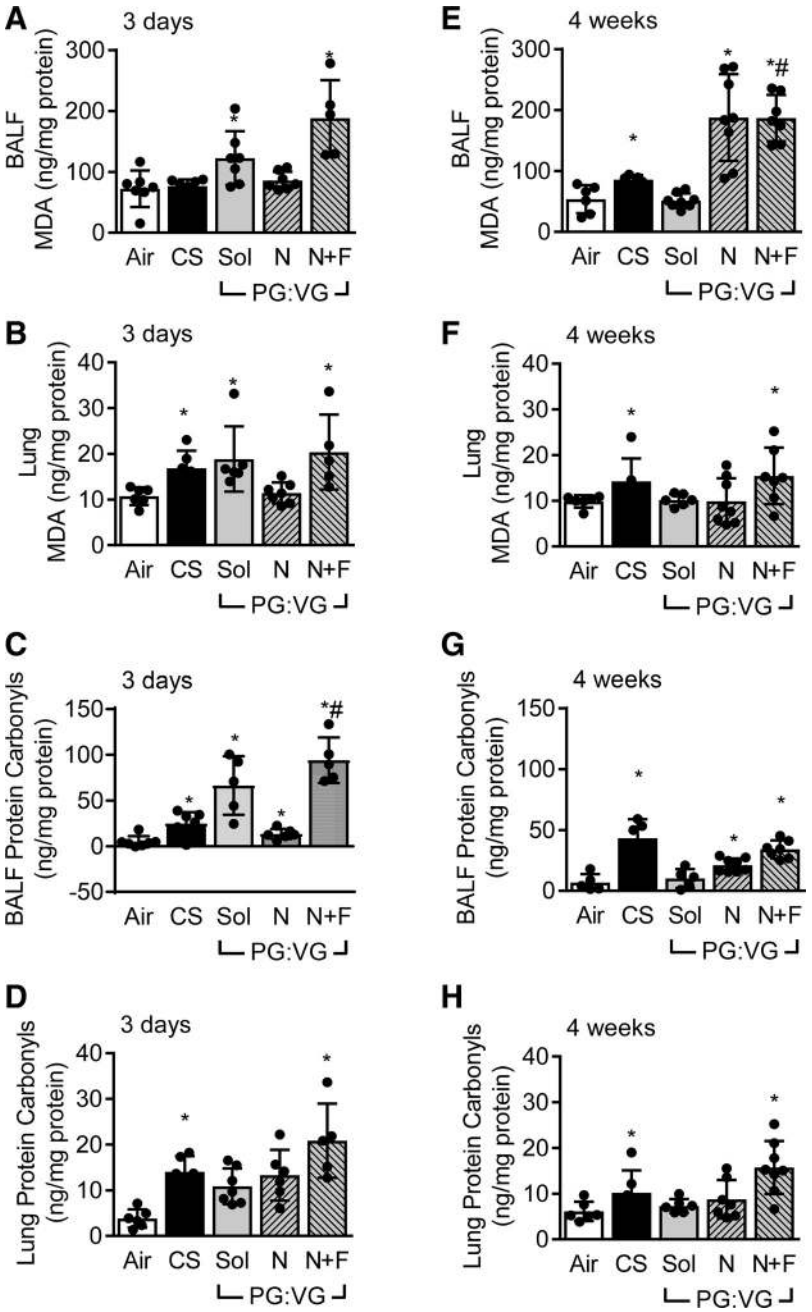

Fig. 3. Determination of markers of oxidative stress following smoking and vaping. Following an acute 3-day and a subchronic 4-wk exposure to CS and e-cig vapor MDA (*A*, *B*, *E*, *F*) and protein carbonyl (*C*, *D*, *G*, *H*) oxidative markers were determined in the BALF (*A*, *C*, *E*, *G*) and lung homogenate (*B*, *D*, *F*, *H*).Values are expressed as mean ± SD; $n = 5–8$ mice; *$P < 0.05$ vs. air, #$P < 0.05$ vs. CS. BALF, bronchoalveolar lung fluid; CS, cigarette smoke; MDA, malondialdehyde; PG:VG-Sol, propylene glycol/vegetable glycerol; PG:VG-N, propylene glycol/vegetable glycerol with nicotine; PG:VG-N+F, propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor.

capable of significantly inducing macrophage influx into the BALF. BALF cellularity of CS mice also increased because of macrophage (87.5% of total cell count) and neutrophil (8.2% of total cell count) influx (Fig. 2, *D* and *E*). BALF protein content was elevated in the PG:VG-N ($P = 0.014$) and PG:VG-N+F ($P = 0.003$) groups only, compared with air-breathing mice (Fig. 2*F*), suggesting that exposure to nicotine through inhalation of e-cig vapor but not through CS exposure can increase BALF protein content at this time point; in this case, addition of flavor to the e-cig had no further effect.

Markers of oxidative stress in the BALF. A 3-day exposure to CS or e-cig vapor resulted in *1*) increased levels of MDA in the PG:VG ($P = 0.016$) and PG:VG-N+F ($P = 0.03$) and *2*) increased protein carbonyls in all cigarette-/e-cig-exposed groups compared with air-breathing mice (Figs. 3, *A* and *C*) [CS mice ($P = 0.013$), PG:VG-Sol ($P = 0.03$), PG:VG-N ($P = 0.022$), and PG:VG-N+F ($P = 0.004$)].

Four-week exposure to CS or e-cig vapor resulted in *1*) increased levels of MDA in CS ($P = 0.011$), PG:VG-N ($P = 0.002$), and PG:VG-N+F ($P = 0.003$) groups, *2*) increased levels of protein carbonyls in BALF in CS ($P = 0.009$), PG:VG-N ($P = 0.019$), and PG:VG-N+F ($P = 0.004$) groups compared with air-breathing mice (Fig. 3, *E* and *G*). The only group that did not show an increase in BALF protein oxidation markers at 4 wk relative to air was the one exposed to PG:VG.

Markers of oxidative stress in lung tissue. Three-day exposure to CS or e-cig vapor resulted in *1*) increased MDA levels observed in the CS ($P = 0.006$), PG:VG-Sol ($P = 0.004$), and PG:VG-N+F ($P = 0.011$) groups and *2*) increased lung protein carbonyls in the CS ($P = 0.004$) and PG:VG-N+F ($P = 0.004$) compared with air-breathing mice in lung tissue (Fig. 3, *B* and *D*). From these results, one can conclude that the nicotine in the e-cig vapor seems to mitigate the effect of PG:VG-Sol, whereas the additional inclusion of flavor reverses this effect. This pattern is broadly reminiscent of the pattern in oxidation markers seen in BALF (Fig. 3, *A* and *C*).

Four-week exposure to CS or e-cig vapor resulted in *1*) increased MDA levels only in the CS and PG:VG-N+F groups and *2*) similarly, increased protein carbonyls levels in CS ($P = 0.037$) and in PG:VG-N+F ($P = 0.005$) groups (Fig. 3, *F* and *H*). Again, as seen at 3 days of exposure, the addition of flavor to the nicotine-containing e-cigs seemed to elevate the oxidation markers in the lung tissue to levels similar to CS.

Levels of proinflammatory cytokines in lung homogenates. Exposure to CS or e-cig vapor for 3 days resulted in increased levels of IL-1β and IL-6 in the PG:VG-N+F group only (IL-1β, $P = 0.047$ and IL-6, $P = 0.047$, Fig. 4, *B* and *C*) without affecting TNF-α levels (Fig. 4*A*). Following 4 wk of exposure to either CS or to e-cig vapor, in agreement with the 3-day exposure, TNF-α levels remained unchanged (Fig. 4*D*). However, in contrast to the shorter exposure where IL-1β and IL-6 were significantly increased only in the PG:VG-N+F group, in this longer exposure levels of these two cytokines were significantly elevated (IL-1β, IL-6, $P = 0.047$) only in the CS-exposed mice (Fig. 4, *E* and *F*).

Lung histopathology and Muc5a immunohistochemistry. Exposure of mice for 3 days to CS in comparison to air or e-cig vapor resulted in a pronounced focal thickening and interstitial inflammation. E-cig exposure failed to produce statistically significant changes in the combined score (Fig. 5*A*). The effects of inhaling CS was equally pronounced after 4 wk of exposure (Fig. 5*B*). Moreover, it seems that the increase in the score is more prominent at 3 days versus 4 wk. Muc5ac protein was homogenously detected in the apical surface of bronchial epithelial cells. CS, PG:VG-Sol, and PG:VG-N+F groups exhibited a pronounced Muc5ac production in the airways of mice upon 3-day exposure compared with air-exposed mice (Fig. 6).

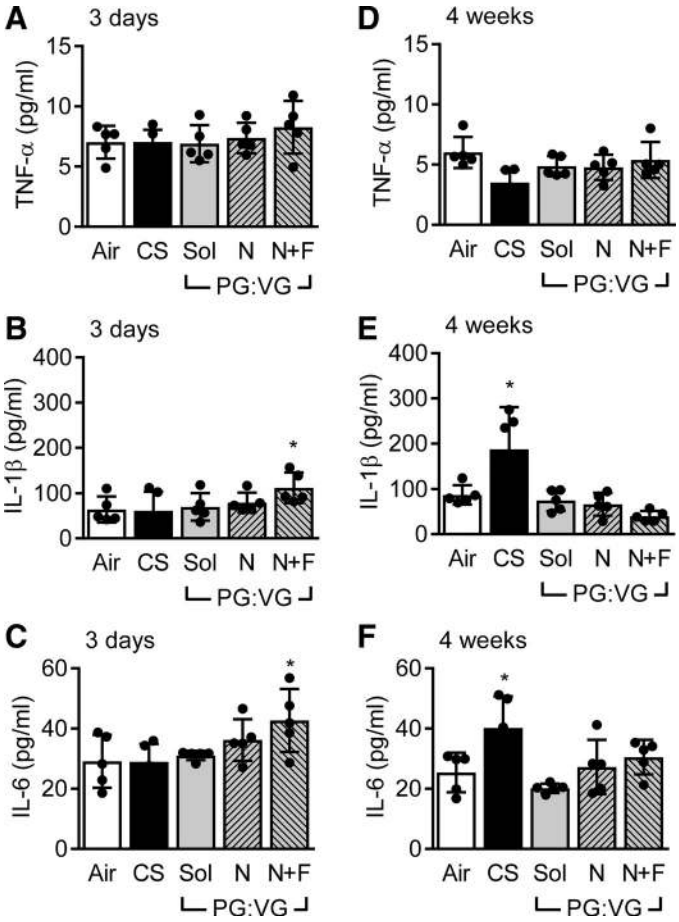

Fig. 4. Determination of markers of inflammation following smoking and vaping. Following an acute 3-day and a subchronic 4-wk exposure to CS and e-cig vapor, cytokines were measured using commercial kits in lung homogenates. *A* and *D*: TNF-α; *B* and *E*: IL-1β; *C* and *F*: IL-6. Values are expressed as mean ± SD, $n = 5$ mice; *$P < 0.05$ vs. air. CS, cigarette smoking; IL, interleukin; PG:VG-Sol, propylene glycol/vegetable glycerol; PG:VG-N, propylene glycol/vegetable glycerol with nicotine; PG:VG-N+F, propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor; TNF-α: tumor necrosis factor-α.

Measurements of respiratory system mechanics following CS or e-cig vapor inhalation. In addition, we assessed the functional consequences of CS in mice, so we determined airway resistance, tissue elasticity, and static compliance in the various groups. Surprisingly, after 3 days of CS or e-cig vapor exposure, all three parameters were significantly changed only in the PG:VG-Sol group. This group presented increased airway resistance ($P = 0.004$) and tissue elasticity ($P = 0.001$) and decreased static compliance ($P = 0.001$) compared with either the air-exposed mice or to the CS-inhaling group ($P = 0.005$ vs. CS for airway resistance; $P = 0.001$ for tissue elasticity, and $P = 0.001$ for decreased compliance) (Fig. 7, *A*–*C*). After a 4-wk exposure to CS the observed pattern was

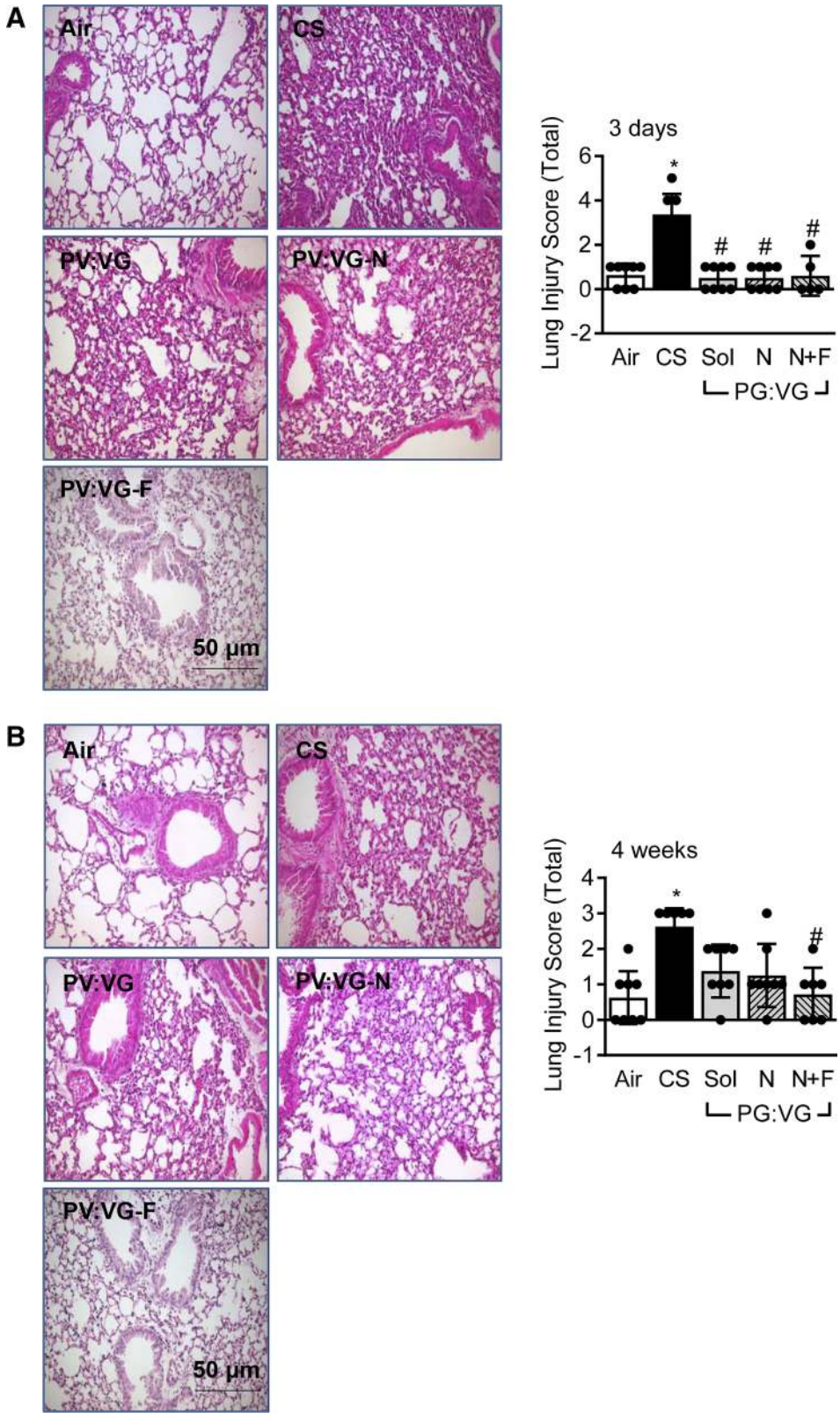

Fig. 5. Lung histological changes are evident after smoking but not after vaping. Following an acute 3-day (*A*) and a subchronic 4-wk (*B*) exposure to CS and e-cig vapor, the left lung was obtained and histopathology was performed on hematoxylin-eosin-fixed sections according to Murao et al. (26).Values are expressed as mean ± SD; $n = 5–8$ mice; $*P < 0.05$ vs. air, $\#P < 0.05$ vs. CS. CS, cigarette smoke; PG:VG-Sol, propylene glycol/vegetable glycerol; PG:VG-N, propylene glycol/vegetable glycerol with nicotine; PG:VG-N+F, propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor.

different; only the CS-exposed mice presented significantly altered functional parameters.

Determination of airway hyperresponsiveness. To test for changes in pulmonary reactivity to a standard experimental airway stimulus, we challenged mice that had previously undergone a 3-day exposure to the same five inhaling regimes to various doses of methacholine. Only two groups, the CS group and the PG:VG-N+F vapor-exposed group,

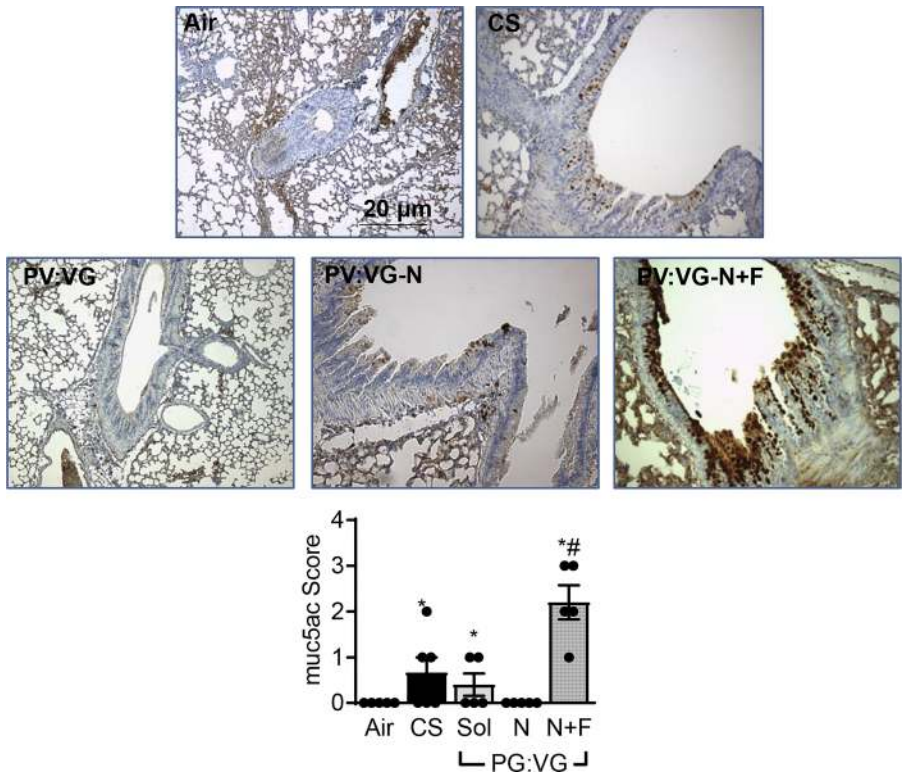

Fig. 6. Changes in lung Muc5ac following smoking and vaping. After 3 days of exposure to CS or e-cig vapor, mouse lung paraffin sections were stained using an anti-Mucin 5ac antibody. IHC slides were evaluated by light microscopy and the immune signal was scored using a semiquantitative scoring system as previously described (1). Values are expressed as mean ± SD; $n = 5–6$ mice; $*P < 0.05$ vs. air, $\#P < 0.05$ vs. CS. CS, cigarette smoke; IHC, immunohistochemistry; PG:VG-Sol, propylene glycol/vegetable glycerol; PG:VG-N, propylene glycol/vegetable glycerol with nicotine; PG:VG-N+F, propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor.

exhibited increased airway hyperresponsiveness to increasing doses of methacholine compared with air-exposed mice (Fig. 8, *A* and *B*).

DISCUSSION

The recent advent of e-cigs and their rapidly increasing use among smokers puts pressure on the scientific community to generate well-controlled preclinical and clinical studies of pulmonary function to compare side-by-side the effects of "classical" CS to the effects of vapors from e-cigs. Up to now, there is only a limited number of in vitro studies on human and murine pulmonary epithelial cell lines, fibroblasts, and stem cells [(2, 15, 37) studies reviewed in (6)] that have addressed the acute toxicological profile of refill liquid contents of e-cigs. In a recent study of Scheffler et al. (38), direct exposure of primary bronchial epithelial cells to e-cig vapor containing glycerol/propylene glycol induced oxidative stress that was less pronounced compared with the stress induced by conventional CS. In addition, the effect of chemicals found in the e-cig flavors has also been shown to be responsible for toxic effects on pulmonary fibroblasts (2, 7). Recent studies have addressed the pulmonary effects of e-cigs in animal models in vivo (17, 22, 26, 42), and although they are very informative, they are characterized by either use of a single time of exposure, by focus on inflammatory and immune responses alone in the absence of addressing functional lung mechanics, and lastly, only occasionally do they address the effect of specific e-cig components, such as the widely used vehicle PG:VG or added flavors. To complete this gap in our in vivo study in mice, we *1*) varied the length of exposure to either CS and e-cig vapor and *2*) we examined the effects of nicotine and flavorings added to the main "vehicle" in e-cigs (PG:VG) on inflammatory markers and functional parameters of the exposed lungs.

Pulmonary irritation and other effects of propylene glycol (a major constituent of e-cig fluid vehicle) have been noted in humans (6, 20). In agreement with these observations, our study showed that the propylene glycol-containing vehicle (PG:VG-Sol) was able to increase BALF cellularity, induce oxidative modifications, raise epithelial Muc5a production, and negatively impact lung mechanics at 3 days of exposure. These effects were mostly absent following exposure to PG:VG for 4 wk, suggesting a transient respiratory irritation by the vehicle that subsides upon further exposure.

As far as nicotine content in e-cig liquids and its biological effects are concerned, most previous studies have generally reported a "nicotine effect," usually by comparison to "air," without being able to distinguish whether the observed changes are because of the e-cig liquid vehicle, the nicotine, or the flavors, if present. In our experiments, nicotine addition was found to exert variable effects either aggravating, causing no change or ameliorating the detrimental effects of e-cig vapor depending on the parameter and time point studied. E-cig toxicity when tested in vitro with embryonic and adult cells was not attributed to nicotine but correlated with the number and concentration of chemicals used to flavor fluids (2). Only one in vivo study, that of Garcia-Across et al. (17), has shown clear nicotine-dependent effects on mouse airway hyperactivity, mucin production, distal airspace enlargement, cytokine and protease expression, and impaired ciliary beat frequency.

Hundreds of flavors have been proven safe as food additives but there are no data about their impact to the respiratory system, and it is well known that the route of exposure plays a significant role in the response to xenobiotics. In vitro as well as human studies have revealed potential hazards of e-cig vapor because of their flavoring components (17, 20, 26, 40). In our study, tobacco-flavored, nicotine-containing e-cig vapor

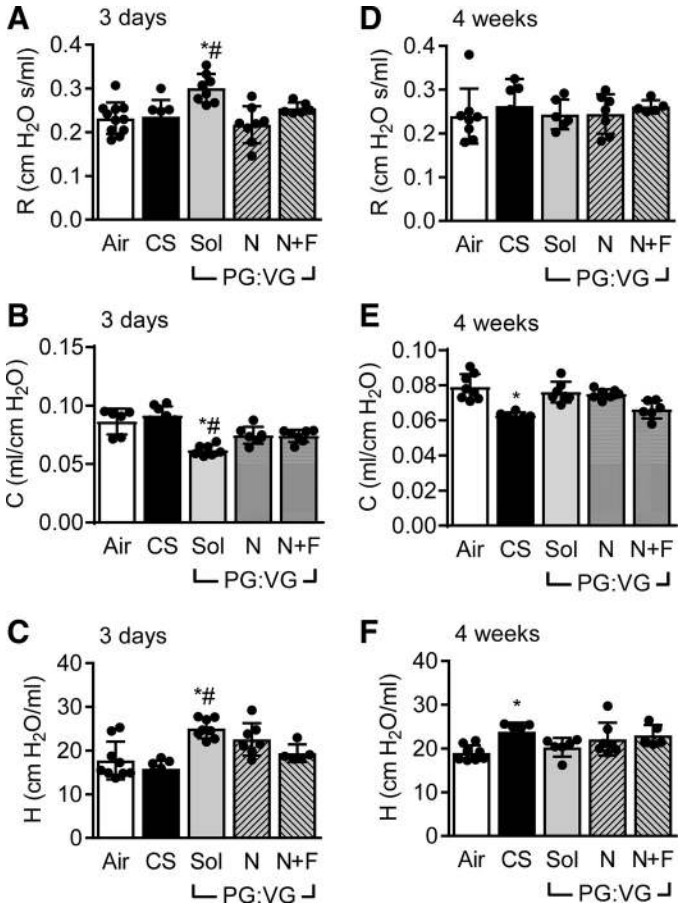

Fig. 7. Changes in respiratory system mechanics following smoking and vaping. Mouse lung function was measured using a forced oscillation before the onset of cigarette exposure (baseline) and following exposure of mice for either 3 days or 4 wk to CS or e-cig vapor. Parameters determined include R (*A* and *D*), C (*B* and *F*), and H (*C* and *E*). Values are expressed as mean ± SD; $n = 5$–12 mice $^*P < 0.05$ vs. air, $\#P < 0.05$ vs. CS. C, static compliance; CS, cigarette smoke; H, tissue elasticity; PG:VG-Sol, propylene glycol/vegetable glycerol; PG:VG-N, propylene glycol/vegetable glycerol with nicotine; PG:VG-N+F, propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor; R, airway resistance.

(PG:VG-N+F) showed significant effects in BALF cellularity and protein content after both 3 days and 4 wk of exposure. Notably, the determination of the two oxidative markers (MDA, carbonyls) showed that the flavor contained in e-cig vapor allowed for a marked effect in both the BALF and the lung; in the latter case, the effect was not shown by nicotine-alone e-cig exposure (PG:VG-N). Along the same lines, at the 3-day exposure, again among the various e-cig exposure regimes, only the PG:VG-N+F-exposed mice showed increases in IL-6 and IL-1β. Last, PG:VG-N+F exposure alone among e-cig-inhalation regimes was able to exacerbate the methacholine response, as did CS. Thus, in many of the parameters studied herein, the hazardous effects noted after exposure to e-cig vapors were exacerbated or persisted longer when flavoring was added to the refill liquid. Given the thousands of flavors available world-wide, meticulous examination of these liquids and their components to prove which ones are safe would be a daunting task.

Comparison of the effects of CS versus e-cig vapor yielded interesting results. Both short-term (3-day) and long-term (4-wk) exposure to e-cig vapor resulted in an increase in BALF cellularity, BALF protein, and oxidative stress that were more pronounced in the e-cig group or equal between e-cigs and CS. In line with our observations, Lerner et al. (26) reported that exposure of mice to e-cigs diminished lung glutathione levels. Levels of inflammatory cytokines exhibited a different pattern compared with BALF cellularity and oxidative stress. After 3 days of exposure, only PG:VG-N+F increased the levels of IL-1β and IL-6 in the lung, whereas after 4 wk the effect of PG:VG-N+F was absent and only CS elevated IL-1β and IL-6 in the lung. Our observations are in accordance with the study of Lerner et al. (26) where acute (3 days) exposure to e-cig containing both nicotine (16 mg) and classic tobacco flavor elicited an increase in the levels of proinflammatory mediators, such as IL-6, in the BALF. In a different study where mice were exposed for 2 wk to e-cig vapor (42), although macrophage influx in the BALF was seen, IL-6 levels in the BALF were lower than in mice that were exposed to air. This discrepancy may be related to the different time of exposure between the two studies and to the different compartment analyzed (BALF in their work vs. lung homogenate herein). It should be noted that in the work of Sussan et al. (42) it is not entirely clear whether the exposure of these mice was to flavored or unflavored e-cigs.

To examine further the potential harmful effect on the airways and compare CS and e-cig effects, we measured mucin production. After 3 days of exposure CS, PG:VG-Sol, and PG:VG-N+F exposure showed higher levels of Muc5ac in the

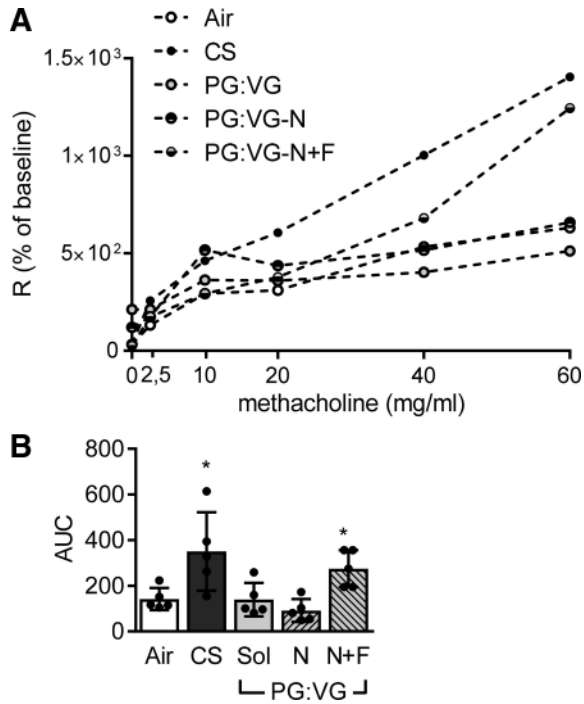

Fig. 8. Airway hyperresponsiveness in animals exposed to cigarette smoke or e-cig vapor. Airway responsiveness mice was assessed before and after methacholine challenge (2.5, 10, 20, 40, 60 mg/ml), 24 hours following a 3-day exposure to room air, CS, or e-cig vapor. *A*: airway resistance (R) as percent increase of baseline in response to methacholine. *B*: area under curve (AUC) for R. Values are expressed as mean ± SD; $n = 5$ mice; $^*P < 0.05$ vs. air. CS, cigarette smoke; PG:VG-Sol, propylene glycol/vegetable glycerol; PG:VG-N, propylene glycol/vegetable glycerol with nicotine; PG:VG-N+F, propylene glycol/vegetable glycerol with nicotine and tobacco blend flavor.

airways compared with air-exposed mice. Of note, among mucins, Muc5ac is a predominant gel-forming mucin induced in allergic murine lungs (13), suggesting a fundamental effector role in airway hyperreactivity. Our finding regarding Muc5ac in mice exposed to CS and PG:VG-N+F correlates with the observation that these groups of mice also exhibited increased airway reactivity after methacholine challenge. In line with this, Lim et al. (28) reported that e-cig vapor exacerbated the allergy-induced asthma symptoms in mice, although in this latter model the e-cig liquid was not vaporized but was instilled intratracheally. Complementing our findings, Garcia-Arcos et al. (17) also reported that long-term exposure to nicotine-containing e-cig vapor increased airway hyperreactivity to methacholine.

Our results provide evidence for impairment of functional lung parameters in mice after short-term exposure to e-cig vapors. Interestingly, resistance, elastance, and compliance were only affected in the PG:VG-Sol group. Larcombe et al. (25) have also reported decrements in parenchymal lung function at both functional residual capacity and high transrespiratory pressures after exposure to e-cig vapors. The above-mentioned preclinical data are in agreement with human studies; Vardavas et al. (45) have shown that short-term exposure to e-cig vapor can increase the impedance and peripheral airway flow resistance, suggesting that airway constriction because of e-cig use is a result of the irritant effects of propylene glycol. After more prolonged exposure to e-cig vapors, the effect on pulmonary mechanics disappeared. At this time point, pulmonary mechanics were adversely affected in CS-exposed mice.

To study the effects of CS or e-cig vapors on lung structure we used histology and determined inflammatory cell intra-alveolar and interstitial recruitment, congestion, and induced intra-alveolar and interstitial edema. Interestingly, we found a high lung injury score only in CS-exposed mice both after 3 days or 4 wk of exposure, compared with e-cig vapor- or air-exposed mice. Our data are aligned with the evidence of the less toxic effect of e-cig vapor compared with tobacco smoke (37), especially regarding the loss of lung integrity in mice (22), albeit e-cig vapor exposure clearly promotes pulmonary inflammatory effects (6).

In summary, we have shown that all ingredients of e-cig refills, including the vehicle PG:VG, can induce lung inflammation and cause changes in respiratory mechanics. These effects were exacerbated by the addition of flavoring to the e-cig. The observed detrimental effects in the lung upon e-cig vapor exposure in animal models highlight the need for further investigation of safety and toxicity of these rapidly expanding devices worldwide.

GRANTS

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

A. Papapetropoulos conceived and designed research; C.G., S.-I.B., A. Pavlidou, C.M., and V.K. performed experiments; C.G., S.-I.B., C.M., V.K., and A. Papapetropoulos analyzed data; C.G., P.K., S.T., I.K., S.Z., and A. Papapetropoulos interpreted results of experiments; C.G. and S.-I.B. prepared figures; C.G., P.K., S.T., and A. Papapetropoulos drafted manuscript; I.K. and S.Z. edited and revised manuscript; C.G., S.-I.B., P.K., A. Pavlidou, V.K., S.T., I.K., S.Z., and A. Papapetropoulos approved final version of manuscript.

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