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Not all vaping is the same: differential pulmonary effects of vaping cannabidiol versus nicotine

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ABSTRACT
Rationale Vaping has become a popular method of inhaling various psychoactive substances. While evaluating respiratory effects of vaping have primarily focused on nicotine-containing products, cannabidiol (CBD)-vaping is increasingly becoming popular. It currently remains unknown whether the health effects of vaping nicotine and cannabinoids are similar.
Objectives This study compares side by side the pulmonary effects of acute inhalation of vaporised CBD versus nicotine.
Methods In vivo inhalation study in mice and in vitro cytotoxicity experiments with human cells were performed to assess the pulmonary damage-inducing effects of CBD or nicotine aerosols emitted from vaping devices.
Measurements and main results Pulmonary inflammation in mice was scored by histology, flow cytometry, and quantifying levels of proinflammatory cytokines and chemokines. Lung damage was assessed by histology, measurement of myeloperoxidase activity and neutrophil elastase levels in the bronchoalveolar lavage fluid and lung tissue. Lung epithelial/endothelial integrity was assessed by quantifying BAL protein levels, albumin leak and pulmonary FITC-dextran leak. Oxidative stress was determined by measuring the antioxidant potential in the BAL and lungs. The cytotoxic effects of CBD and nicotine aerosols on human neutrophils and human small airway epithelial cells were evaluated using in vitro air–liquid interface system. Inhalation of CBD aerosol resulted in greater inflammatory changes, more severe lung damage and higher oxidative stress compared with nicotine. CBD aerosol also showed higher toxicity to human cells compared with nicotine.
Conclusions Vaping of CBD induces a potent inflammatory response and leads to more pathological changes associated with lung injury than vaping of nicotine.

INTRODUCTION
Electronic vaping products emerged in the late 1990s as an alternative mode of cannabis use. Cannabis vaporisers were typically large devices that heated dried cannabis herb to the point of cannabinoid vaporisation. In early 2000s, smaller portable vaporisers emerged as 'e-cigarettes' and have become a popular mode of nicotine administration.[1] E-cigarettes heat nicotine in a solution rather than from dried tobacco leaf. Recently, vaporisers in the cannabis market have followed a similar transition, with greater use of liquid cannabis extracts.[2–7]

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Vaping cannabinoids using electronic devices is a growing trend, however, the pulmonary health effects of inhalation of cannabis oil are not well understood.

WHAT THIS STUDY ADDS
⇒ This study showed that vaping cannabinoid induces more potent inflammatory responses in the lung, greater oxidative stress and leads to augmented pathological changes associated with lung injury than vaping of nicotine.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ This study provides insights to why potential respiratory health consequences of vaping cannabis may be more serious than those of vaping nicotine, emphasising the importance of collecting detailed information on the type of substance used by patients in their vaping products.

Aerosols emitted from vaping products contain not only psychoactive substances like nicotine and cannabinoids (primarily tetrahydrocannabinol (THC) and cannabidiol (CBD)), but also respiratory toxicants (eg, formaldehyde, acrolein, benzaldehyde).[8–11] Many chemical constituents involved in vaping nicotine and cannabinoids are similar, and others are very different, lending importance to considering these issues in the context of understanding respiratory consequences of vaping both substances. As an example, solvents used in nicotine and cannabinoid-containing vaping products can be different, due to the lipophilic properties of cannabinoids.[12] Vitamin E acetate was identified as an additive in THC-containing vaping products and played a significant role in the 2019 outbreak of e-cigarette and vaping-associated lung injury (EVALI).[13,14]

A limited number of studies on respiratory effects of vaping have primarily focused on nicotine-containing products. In vitro studies suggest that vaping nicotine can activate immune cells and impair some of their key functions.[15] Animal studies showed that exposure to nicotine from e-cigarettes adversely affect immunological responses.[16,17] Human observational studies have shown that vaping nicotine suppresses aspects of the innate immune system in nasal epithelial

Table 1 Comparison of chemicals detected in vaping products (unheated liquids) used in in vivo and in vitro exposure experiments

Chemicals	CBD-Vape (CalmVape from The Kind Group)	Nic-Vape (Juul by Juul Labs)
CBD	43.9 mg/mL	Not detected
$\Delta{-}9$ Tetrahydrocannabinol	Not detected	Not detected
Nicotine	Not detected	48.9 mg/mL
Solvent	MCT (medium chain triglycerides) – mixture of caprylic acid (C8), capric acid (C10), and lauric acid (C12)	Mixture of propylene glycol and vegetable glycerin
Flavourings and terpenes	Amylene hydrate (*pungent*); Benzyl alcohol (*chemical, fruity cherry, almond, balsamic, bitter*); Carvone (*sweet, minty, spearmint, carvone, caraway*); Linalol (*citrus, orange, lemon, floral, waxy, aldehydic, woody*); Caryophyllene (*spicy, clove, woody, nut skin, powdery, peppery*); Octanoic acid (*rancid, soapy, cheesy, fatty, brandy*); Decanoic acid, methyl ester (*fatty, oily, fruity*); Octanoic acid, methyl ester (*green, fruity, waxy, citrus, aldehydic and fatty*); Caryophyllene oxide (*dry, woody, cedar, old wood, carrot, ambrette*); Cherry propanol (*fruity, cherry, sweet, hay-like with cereal and bread like nuances*); Cis-carveol (*caraway*); Ethanone, 1-(4-methylphenyl)- (*sweet, creamy, fruity, cherry and heliotropine-like*)	Amylene hydrate (*pungent*); 2-Propanol, 1,1'-oxybis- (*alcoholic*); Benzaldehyde, 3,4-dimethoxy- (*sweet, creamy, vanilla*); Azolidine (*fishy*); Triethyl citrate (*mild fruity wine*); Butyrolactone (*milky, creamy with fruity peach like afternotes*)
Other additives	Not detected	Benzoic acid (combines with nicotine to create nicotine salt (*nicotine benzoate*))

CBD, cannabidiol; MCT, medium chain triglycerides.

cells.[18] [19] Epidemiological studies have reported associations between nicotine vaping and chronic respiratory conditions (chronic cough, bronchitis, asthma).[20–24] Since research on the respiratory and immunological health outcomes associated with cannabis use has exclusively focused on smoked cannabis,[25–31] it is currently unknown whether the health effects of vaping nicotine and cannabinoids are similar. This study aimed to compare, in a side-by-side format, the impact of acute inhalation of vaporised cannabinoid versus nicotine.

MATERIALS AND METHODS
Methods used are described in greater detail in online supplemental file.

Vaping products
We used two commercial vaping products, one containing CBD and the other containing nicotine (abbreviated in figures as CBD-vape and Nic-vape). The CBD-containing pod was Calm-Vape from The Kind Group LLC and the nicotine-containing pod was Juul by Juul Labs. Both products were purchased online in USA in November 2020. CalmVape pods were labelled as containing 50 mg/mL of CBD dissolved in a mixture of medium chain triglycerides (MCT) and had natural flavour. Juul was labelled as containing 5.0% nicotine dissolved in a mixture of propylene glycol (PG) and vegetable glycerin (VG) and Virginia Tobacco flavour. We tested unheated and heated liquids as well as emissions generated from both products using fully-validated and previously published chromatography–mass spectrometry assays.[32] Primary ingredients identified in the liquids from the two products are listed in table 1. Detailed list of chemicals identified in heated solutions, including yields of four potentially toxic carbonyl compounds (formaldehyde, acetaldehyde, acetone and acrolein) in emitted aerosols are provided in online supplemental figures E1–E4 and online supplemental tables E1 and E2.

Mice
Six-week-old C57BL/6NCr male and female mice were procured from Charles River Laboratory (Wilmington, Massachusetts, USA) and housed under specific pathogen-free conditions with light/dark cycle of 12/12 hours. The number of animals per exposure group was n=10 (5 males and 5 females, except for

Nic-vape which contained 5 males and 4 females; one female mouse was very small in size and needed to be euthanised before study completion). All experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee and complied with all state, federal and NIH regulations.

Animal exposure conditions
Aerosols from vaping products were produced using an e-cigarette aerosol generator described previously.[14] [33] The Juul device was used to aerosolize both the products. Animals were exposed in a modified 15 L induction chamber each day to a total of 20 puffs generated over 1 hour (1 puff every 3 min), 5 days/week for 2 weeks. Each puff had a volume of 55 mL and was aerosolized over 3 s duration. Aerosols from each vaping product were generated using identical puffing protocol intended to mimic vaping behaviour of experienced nicotine vapers.[34] Due to lack of publications describing vaping behaviour among CBD vapers, we followed the same puffing protocols for both products. Although we did not measure airborne CBD and nicotine inside animal exposure chambers, we have estimated based on volume of liquid vaporised per day, CBD and nicotine concentration in liquids, aerosol and air flow rates that animals were exposed on average to 20.5 mg/m^3 CBD and 22.8 mg/m^3 nicotine. Control animals were exposed to filtered air using the same exposure protocol.

In vitro exposure conditions
Cells used in in vitro experiments were directly exposed to freshly generated puffs in a closed exposure system where air–liquid interface (ALI) chambers were kept inside a 37°C incubator. For ALI experiments, we used the same closed-system vaping devices and refill liquid formulations as used for in vivo exposure experiments described above. Details of the method are provided in online supplemental file.

Assessment of pulmonary inflammation
Pulmonary inflammation was scored by flow cytometry, histochemistry, and by quantifying levels of cytokines and chemokines (online supplemental figure E5)[14] [35] [36] and complete details are provided in online supplemental file.

Assessment of lung damage

Lung epithelial/endothelial integrity was assessed by quantifying protein levels by BCA, and systemic to bronchoalveolar space albumin leak by ELISA using BAL samples and bronchoalveolar to systemic leak by measuring plasma fluorescence 1 hour after intratracheal instillation of fluorescent probe. Neutrophil elastase (NE) levels,[37 38] were measured in BAL and lung tissue using a NE ELISA kit from R&D systems (Cat. #DY4517-05) following manufacturer's protocol. Myeloperoxidase activity (MPO) was measured by calorimetric assay in the BAL and lung tissue[39] using MPO assay kit from Abcam (Cat. #ab105136) using manufacturer's instructions. Oil Red O stain was used to visualise lipid-laden alveolar macrophages.[14] Assay details are given in online supplemental file. Histological evaluations in sections of lungs were graded by a veterinary pathologist as described previously.[40 41]

Measurement of oxidative stress in mice BAL and lungs

Acute inflammatory responses rapidly overwhelm antioxidant systems to promote lung injury.[42–44] Oxidative stress was determined by measuring the antioxidant potential in the BAL and lung lysates as described in online supplemental file.

In vitro cytotoxicity tests

Human small airway epithelial cells (hSAECs) from LONZA and purified human neutrophils were directly exposed to freshly generated CBD and nicotine aerosols in air–liquid interface cultures. Exposure protocol and methodological details are in online supplemental material. Cytotoxicity was measured using trypan blue-dye exclusion, neutral red dye uptake and Annexin V-FITC apoptosis assays as described in online supplemental file.

Measurement of NE levels in PMN culture media

NE levels in the aerosol-exposed PMN cell culture conditioned media after recovery period were quantified by ELISA kit from R&D systems (Cat. #DY4517-05) following manufacturer's instructions.

FITC-dextran permeability assay

Paracellular permeability, across a monolayer of aerosol-exposed human SAECs from ALI cultures, was performed to assess barrier integrity and details are described in online supplemental file.

Statistical analysis

Statistically significant differences between the mean rank values of different exposure groups (CBD, nicotine and air controls) were determined by performing Kruskal-Wallis's non-parametric test. P values were corrected for multiple testing using the 'two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli' false discovery rate (FDR) method and the differences between two groups were considered statistically significant at $p < 0.05$ when FDR was set at $Q < 0.1$. We also evaluated if there were differences between male versus female mice in the responses to inhalation of CBD and nicotine aerosols in comparison with air. All statistical analyses were carried out using GraphPad Prism V.9.3.1 software (GraphPad; La Jolla, California, USA).

RESULTS

Exposure to CBD aerosol resulted in greater accumulation of innate and adaptive immune cells in lungs compared with nicotine exposure

Total immune cell infiltrate was significantly higher in the lungs of mice following inhalation of CBD aerosols than nicotine or filtered air (figure 1A). Seven of the 10 subsets of immune cells studied were significantly more impacted following CBD exposure compared with nicotine. Immunophenotypic analysis revealed a statistically significant infiltration of $CD11b^+Ly6G^+$ neutrophils in the lungs of mice following inhalation of CBD-aerosols (14 488 vs 3674 neutrophils in air, $p < 0.05$) as well as following nicotine aerosol-exposure (15 410 vs 3674 neutrophils in air, $p < 0.001$) (figure 1B). Total numbers of $CD11b^-CD11c^+Siglec-F^+$ alveolar macrophages were significantly reduced following CBD-Vape or Nic-Vape inhalation as compared with air exposed mice (15 965 cells in CBD and 18 834 cells in Nic-Vape vs 43 465 cells in air ($p < 0.05$)) (figure 1C). Inhalation of both CBD and nicotine aerosols resulted in significantly lower numbers of pulmonary interstitial $CD11b^-CD11c^+CD206^+$ macrophages as compared with air-exposed control mice (11 460 vs 47 319 cells for CBD-Vape, $p < 0.0001$) and 27 727 vs 47 319 cells for Nic-Vape, $p < 0.05$). The reduction in the numbers of pulmonary interstitial macrophages was significantly greater following inhalation of CBD aerosols compared with nicotine (11 460 cells in CBD-vape vs 27 727 cells in Nic-Vape, $p < 0.05$) (figure 1D). $CD11b^-CD11c^+arginase-1^+$ macrophages were significantly reduced following inhalation of both CBD-Vape and Nic-Vape compared with air-exposed control (13 450 vs 44 009 for CBD-Vape ($p < 0.001$), and 24 280 vs 44 009 for Nic-Vape ($p < 0.05$)) (figure 1E). However, the reduction in the numbers of $CD11b^-D11c^+arginase-1^+$ macrophages was significantly more following inhalation of CBD aerosols compared with Nic-Vape (13 450 cells in CBD-vape vs 24 280 cells in Nic-Vape ($p < 0.05$). The number of $CD19^+$ B cells were not statistically different (figure 1F). Following inhalation of CBD aerosols, the numbers of $CD8^+$ and $CD4^+$ T cells in the lungs were, respectively, 3.3-fold ($p < 0.001$) and 5.6-fold ($p < 0.0001$) higher than following nicotine inhalation (figure 1G,H). $CD4^+IL-17A^+$ T cells were not altered in the lungs following CBD-Vape or Nic-Vape exposures as compared with air control (figure 1I). $CD4^+ROR\gamma t^+$ T cells, expressing the master transcription factor essential for the differentiation into proinflammatory Th17 cells, were significantly increased following inhalation of CBD aerosols as compared with both nicotine (11 983 vs 2015; $p < 0.001$) and air-exposure (11 983 vs 5887; $p < 0.05$) (figure 1J). Furthermore, CBD aerosols resulted in markedly increased infiltration of $CD4^+Foxp3^+$ regulatory T cells into the lungs compared with nicotine (4401 cells in CBD-Vape vs 1688 cells in Nic-Vape; $p < 0.001$) (figure 1K). There were no statistically significant differences observed between male and female mice concerning the infiltration of any of the innate and adaptive immune cells, regardless of the different exposure conditions (online supplemental figure E6A–K).

Relatively more Oil Red O-positive lipid-laden macrophages were detected in BAL following CBD-aerosol inhalation compared with nicotine aerosol (0.66 vs 0.32; $p < 0.05$) or air-exposure (0.66 vs 0.15; $p < 0.001$) (figure 2A). Lung tissue sections of air-breathing mice contained rare (typically 1–2 positive cells in entire lung lobe) lipid-containing, Oil Red O-positive intra-alveolar macrophages (online supplemental figure E7A). In contrast, lungs from CBD and nicotine exposed animals regionally contained one or more Oil Red O-positive macrophages within multiple alveolar lumina that were often adjacent to one another (online supplemental figure E7B,C), with no obvious differences found in males versus females.

Histological examination of H&E-stained lung tissue sections from filtered air breathing control mice showed air-filled alveolar lumina bounded by thin alveolar walls (figure 2B). In contrast, peribronchiolar and/or intrabronchiolar, perivascular, alveolar infiltrates and interstitial infiltrates of lymphocytes,
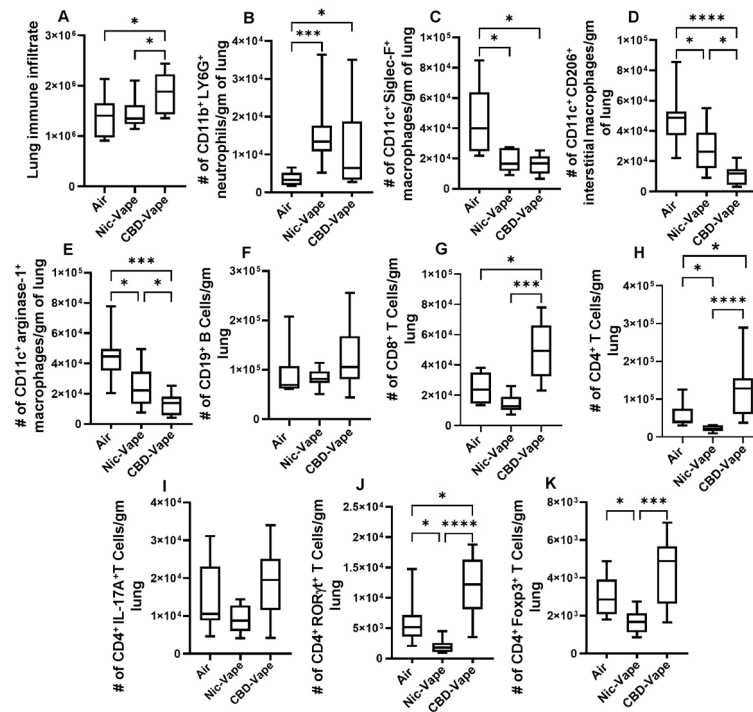
Figure 1 Impact of acute exposure to CBD or nicotine aerosols on pulmonary immune-cell infiltration. Total number of leucocytes (A), CD11b⁺Ly6G⁺ neutrophils (B), CD11b⁻ CD11c⁺Siglec-F⁺ macrophages (C), CD11b⁻ CD11c⁺CD206⁺ macrophages (D), CD11b⁻ CD11c⁺arginase⁺ macrophages (E) and numbers of CD19⁺ B cells (F), CD8⁺ T cells (G), CD4⁺ T cells (H), CD4⁺IL17A⁺ (I) and CD4⁺RORγt⁺ inflammatory T cells (J) and CD4⁺FOXP3⁺ T cells (K) in the lungs of mice exposed to air, nicotine, or CBD aerosol were determined by flow cytometry using specific markers and following a gating strategy as described previously and shown in online supplemental figure 1. Data are shown as box plots with whiskers at min and max. Difference between two groups is considered significant at p<0.05, statistical significance of the difference between two groups is indicated with symbols *p<0.05; ; ***p<0.001; ****p<0.0001after performing non-parametric Kruskal-Wallis test with false discovery rate (FDR) correction for multiple comparisons by GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). In each exposure condition, n=10 mice (5 males+5 females) (n=9 (5 males+4 females) for Nic-Vape) were used. CBD, cannabidiol.

macrophages and granulocytes were the predominant finding in the CBD and nicotine exposed mouse lungs (figure 2C–E). Small focal lesions and occasionally larger and more regionally extensive focal lesions were noted. Lesions were found primarily near terminal bronchioles and often subpleural. The frequency and severity of lesions was greater following CBD aerosol inhalation compared with nicotine. The male mice showed a greater frequency of most lesions as compared with female mice following inhalation of both CBD and nicotine.

CBD aerosol had a stronger modulatory effect on cytokine levels than nicotine aerosol

We found that CBD aerosol-inhalation significantly augmented the levels of cytokines IL-5, IL-6 and G-CSF in the BAL compared with both nicotine and air exposures (p<0.01) and significantly enhanced the levels of chemokine KC compared with air-control only (p<0.001) (figure 3A–D). Levels of IL-2 were significantly lower following CBD and Nic-vape aerosol-exposures compared with air (p<0.05) (figure 3E). IL-10 and IFN-γ levels were significantly reduced only after CBD aerosol-exposure compared with air (p<0.05) (figure 3F,G). IL-1α levels were not significantly different, though there was a trend for the values to be lower following exposure to CBD-Vape aerosols (figure 3H). There were no statistically significant differences observed between male and female mice in the levels of these cytokines or chemokines (online supplemental figure E8A–H and online supplemental table E3).

Exposure to CBD aerosols resulted in more lung endothelial damage than exposure to nicotine aerosol

Total protein levels in the BAL were elevated following the inhalation of CBD aerosols when compared with air controls (435 µg/mL vs 287 µg/mL; p<0.01) (figure 4A, left panel). Additionally, serum albumin levels leaking into the BAL were markedly increased following CBD aerosol inhalation when compared with both nicotine aerosol (70 303 ng/mL vs 32 741 ng/mL in Nic-Vape; p<0.01) and air inhalation (70 303 ng/mL vs 26 042 ng/mL in air control; p<0.0001) (figure 4B, left panel). Systemic leak of FITC-dextran from the lungs into the plasma was markedly higher following CBD aerosol inhalation than Nic-vape aerosol (469.9 ng/mL vs 227.6 ng/mL in Nic-Vape; p<0.01) or air exposures (469.9 ng/mL vs 157.5 ng/mL in air control; p<0.0001) (figure 4C, left panel). Furthermore, FITC-dextran leak following Nic-vape aerosol exposure was not significantly different when compared with air control (227.6 ng/mL vs 157.5 ng/mL). There were no statistically significant differences observed in the levels of these markers when comparing male with female mice following any of the exposures (figure 4A–C, right panels and online supplemental table E3).

It is known that elastase activity in inflammatory diseases increases and correlates with the levels of elastase proteins and neutrophil infiltrates as the disease progresses.[45 46] NE levels in the BAL were markedly augmented following inhalation of CBD aerosols (1.8-fold vs air; p<0.001) and Nic-Vape aerosols (1.42-fold vs air; p<0.01) (figure 5A, left panel). The levels of NE measured in lung tissue
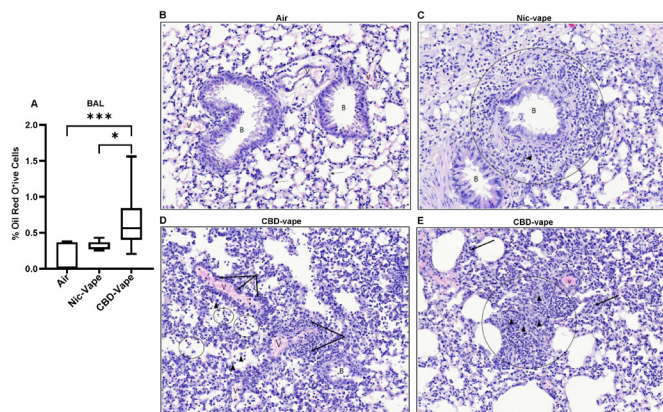
Figure 2 Inflammatory changes in the lungs following inhalation exposure to CBD and nicotine aerosols. At the end of the exposures, mice were euthanised, trachea cannulated to collect BAL in 1% FBS in PBS and lungs harvested. (A) BAL cells cyto-spun on glass slides were stained with 0.5% Oil Red O solution and Oil Red O-positive cells were counted as described in methods. Results are depicted as box plots with whiskers at min and max. Difference between two groups is considered significant at $p < 0.05$ and indicated with the symbols *$p < 0.05$; ***$p < 0.001$ by performing non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons using GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). In each experiment n=10 (5 males+5 females) for CBD-vape and air exposure and n=9 (5 males+4 females) for Nic-vape. (B–E) Left lung lobes from all mice were embedded, sectioned and H&E stained as described in methods. (B) (Air): Image of histologically unremarkable lung from air-breathing control mouse showing air-filled alveolar lumina bounded by thin alveolar walls (arrows). H&E, ×20. (C) Nic-vape: Peribronchiolar lymphocytic, macrophagic (arrowhead) granulocytic infiltrate (circle). H&E, ×20 magnification. B=bronchiolar lumen. (D, E) CBD-vape: Perivascular infiltrates composed of mononuclear (lymphocytes and macrophages) and granulocytic infiltrates (arrows). Intra-alveolar granulocytes (circles) and macrophages (arrowheads) were also present. H&E, ×20 magnification. BD, cannabidiol; B, bronchiolar lumen; FDR, false discovery rate; PBS, phosphate-buffered saline; V, vessel lumen.

were significantly increased following CBD-Vape as compared with both Nic-Vape (1.3-fold; $p < 0.01$) and air (1.41-fold; $p < 0.001$) (figure 5B, left panel). There were no statistically significant differences observed in NE levels between male and female mice, measured either in the BAL or lung tissues (figure 5A,B, right panels and online supplemental table E3).

We detected higher MPO activity in lung tissues following inhalation of CBD aerosols compared with nicotine aerosols (~2 fold; $p < 0.05$) and air (~8.44 fold; $p < 0.0001$) (figure 6A). BAL MPO activity following CBD and nicotine aerosol-inhalation was equivalent, but greater than air controls ($p < 0.01$) (online supplemental figure E9A). There were no statistically significant differences observed in MPO activity between male and female mice, either in lung tissue or in the BAL (figure 6B; online supplemental figure E9B and online supplemental table E3).

Exposure to CBD and nicotine aerosols decreased pulmonary antioxidant potential

The total antioxidant capacity was markedly decreased in both lung tissue ($p < 0.01$ vs air) and BAL ($p = 0.001$ vs air) following inhalation of CBD aerosols (figure 7A; online supplemental figure E10A). However, following nicotine aerosol-exposure it was significantly reduced only in the BAL ($p < 0.01$ vs air)
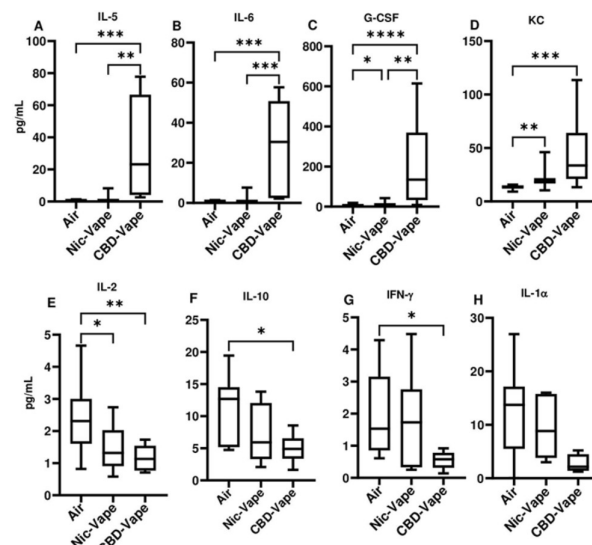
Figure 3 Modulation of the inflammatory cytokine/chemokine milieu in the BAL fluid following inhalation of CBD and nicotine aerosols. Levels of various inflammation-related cytokines and chemokines in the BAL (A–H) of mice after 2 weeks of exposure to air, Nic-Vape aerosols or CBD-Vape aerosols were quantified using MILLIPLEX MAP Kit as described in materials and methods. Data are shown as box plots with whiskers at min and max. Non-parametric Kruskal-Wallis test with FDR correction for multiple comparison was performed to see if statistically significant differences exist between two groups using GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). Difference between two groups is considered significant at $p < 0.05$ and are indicated with symbols *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. In each experiment n=10 for CBD-vape and air exposure (5 males+5 females) and n=9 (5 males+4 females) for Nic-Vape. CBD, cannabidiol.

compared with air (online supplemental figure E10A). We did not observe any statistically significant differences in the antioxidant potential between male and female mice for each exposure group, either in lung tissue or in the BAL (figure 7B; online supplemental figure E10B and online supplemental table E3).

CBD aerosol was more toxic than nicotine aerosol to hSAECs and disrupted their epithelial barrier integrity

We observed that when human SAECs were exposed in vitro to CBD aerosols for 1 hour, epithelial cell morphology was markedly disrupted (figure 8A,B vs 8C). Cell death in hSAECs was significantly increased only following CBD aerosol-exposure when compared with air (41% vs 12.5% in air control; $p < 0.05$) (figure 8D,E). Even though the cell death following CBD-Vape exposure was higher compared with exposure to nicotine aerosols, it, however, did not reach statistical significance (41% vs 16% in Nic-Vape). Additionally, exposure to CBD aerosols diminished the epithelial barrier integrity of human SAECs compared with air by 2.1-fold ($p < 0.05$) and while the exposure to nicotine aerosols also showed an increased trend, it was not significantly different compared with air-control (1.7-fold decrease in Nic-Vape vs air control) (figure 8F).

CBD aerosols but not nicotine aerosols induced apoptotic cell death in purified human neutrophils, but both enhanced the release of NE

Acute exposure to CBD aerosols induced marked cell death in purified human neutrophils (44.5% cell death following CBD-Vape vs 14% in air control; $p < 0.0001$) and (44.5% vs 21% cell

Bhat TA, et al. Thorax 2023;78:922–932. doi:10.1136/thoraxjnl-2022-218743
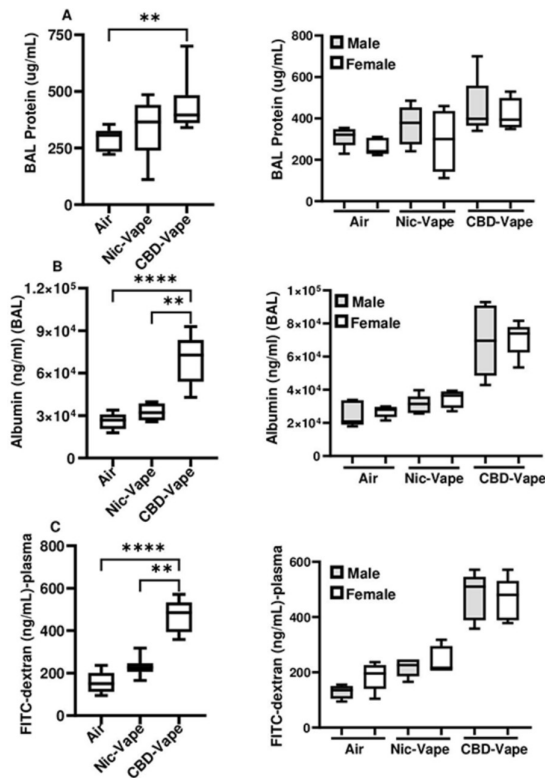
Figure 4 Markers of lung damage induced after inhalation exposure to CBD and nicotine aerosols. At the end of the exposures, mice were euthanised, BAL harvested and the levels of (A) total proteins and (B) albumin in the BAL were quantified as described in the Materials and Methods section. (C) Levels of FITC-dextran leaking into plasma were quantified. Left panels in each figure represent data from Males+Females combined, while right panels represent data as male versus females. Results are shown as box plots with whiskers at min and max. Differences between groups is considered significant at p<0.05 and are indicated as symbols **p<0.01, ****p<0.0001, calculated after performing non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons by employing GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). In each experiment n=10 (5 males+5 females) for air and CBD-vape exposures and n=9 (5 males+4 females) for Nic-Vape group. CBD, cannabidiol.

death in Nic-Vape; p<0.001) (figure 9A). CBD aerosol-induced neutrophil cell death was mainly due to increased apoptosis compared with air (29% vs 10% in air control; p<0.0001) and compared with Nic-Vape (29% vs 12% in Nic-Vape; p<0.001) (figure 9B). Furthermore, both CBD and nicotine aerosols lead to enhanced accumulation of NE levels in the neutrophil cell culture media as compared with air control (2-fold increase in CBD-Vape; p<0.001and 1.45-fold increase in Nic-Vape; p<0.05) (figure 9C). However, the levels were significantly higher following CBD versus nicotine exposure (CBD-Vape 1.4-fold higher than Nic-Vape; p<0.05) (figure 9C). Pictures of human neutrophils after ALI exposures and 24-hour recovery period are provided in online supplemental figure E12. Importantly, an aliquot of purified neutrophils that were incubated in media (unexposed) for the duration of the experiment showed low cell death (~11%) that was equivalent to values noted in neutrophils exposed to air (~14%) in the ALI chambers (figure 9A), nor was apoptosis or increased NE levels induced (figure 9B,C).
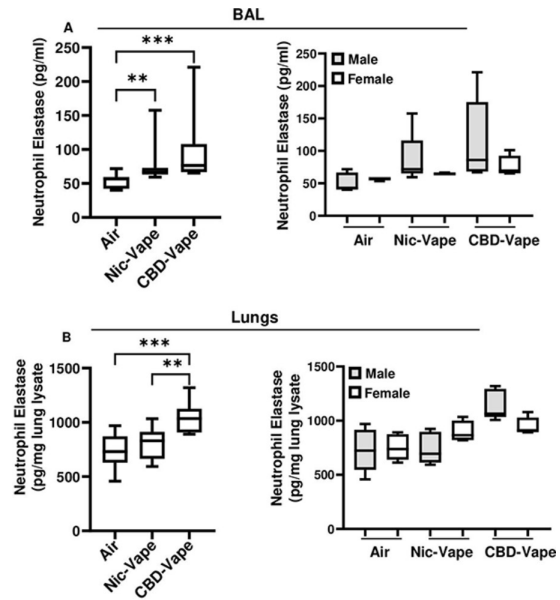

Figure 5 Levels of neutrophil elastase in lungs measured after exposure to CBD and nicotine aerosols. At the end of the 2-week exposures, mice were euthanised, BAL and lungs harvested, and tissue lysates prepared. (A, B) NE levels in the BAL and lung tissue lysates were quantified by ELISA. Left panels in each figure show data as males+females combined, while right panels represent data as male versus Females. Results are shown as box plots with whiskers at min and max. Non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons was used to see if statistically significant differences exist between two groups using GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). Difference between two groups was considered significant at p<0.05 and are indicated with symbols **p<0.01, ***p<0.001. In each experiment n=10 (5 males+5 females) for air and CBD-vape exposures each and n=9 (5 males+4 females) for Nic-Vape. CBD, cannabidiol; NE, neutrophil elastase.

DISCUSSION

Our findings revealed that detrimental effects on immune system and lung damage after inhalation exposure to cannabinoid-containing vaping product were more severe than after exposure to aerosols from a nicotine-containing vaping device. We have uncovered the harmful effect of vaporised CBD impacting pulmonary immune homeostasis using a mouse model of vaping and in vitro experiments with human cells. Our studies revealed that CBD vaping induces a proinflammatory pulmonary microenvironment, leading to marked accumulation of inflammatory immune cells exhibiting enhanced activity of tissue-damaging factors like MPO and NE and leading to induction of lung injury via processes that might include oxidative stress.

Although several studies have examined the respiratory effects of vaping nicotine, to our knowledge, this is the first report that demonstrates that even short-term exposure to vaporised CBD alters the inflammatory milieu in the lung, leading to lung damage. It is important to note that we used CBD-containing vaping products devoid of THC, to eliminate any potential influence of THC on pulmonary effects. Due to legal restrictions in accessing vaping products containing THC, we were unable to conduct experiments that would also compare the effects of THC vaping. A single recent study reported that CBD in vaping products can be considered as a precursor of THC, thus compounding the problem further by inducing CBD-independent, THC-mediated effects related to general use of cannabis vaping products.[47] Furthermore,
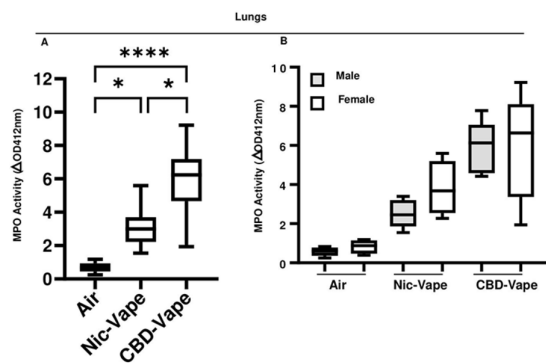
Figure 6 Augmentation of the myeloperoxidase (MPO) activity in lungs after exposure to CBD and nicotine aerosols. (A, B) We measured MPO activity in lung tissue lysates of nicotine or CBD aerosol-exposed animals using MPO assay kit from Abcam as described in detail in the supplemental material section. Left panel depicts data as males+females, while right panel represents data as male versus females. Data shown as box plots with whiskers at min and max. Non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons was performed to see if statistically significant differences exist between two groups using GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). Difference between two groups was considered significant at $p<0.05$ and is indicated with the symbols *$p<0.05$, ****$p<0.0001$. In each experiment n=10 mice for air and CBD-vape exposures each (5 males+5 females) and n=9 for Nic-Vape group (5 males+4 females). CBD, cannabidiol; FDR, false discovery rate.

any respiratory toxic effects of vaping could potentially be exacerbated by the presence of other constituents of vaping products, including different solvents (MCT in CBD product and PG:VG in nicotine products), different flavours and terpenes present in both products as revealed by product analysis. Numerous potential degradation byproducts were detected in both heated solutions,
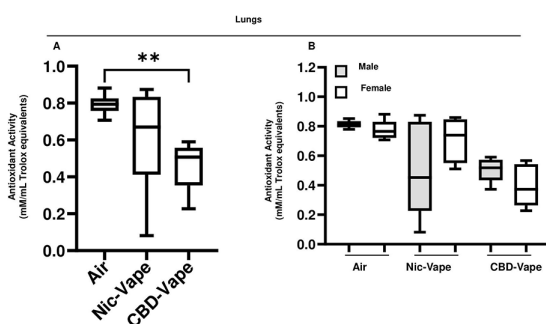

Figure 7 Changes in antioxidant potential in the lung following exposure to CBD and nicotine aerosols. At the end of the exposures to nicotine or CBD aerosols, mice were euthanised and lung tissue lysates were prepared. (A, B) Total antioxidant levels in lung tissue lysates were quantified using Cayman's antioxidant assay kit (Cat# 709001) as described in detail in online supplemental material section. Left panel depicts data as males+females combined, while right panel represents data as male versus females. Data shown as box plots with whiskers at min and max. Non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons was used to see if statistically significant differences exist between two groups using GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). Difference between two groups was considered significant at $p<0.05$ and is indicated with the symbol **$p<0.01$. In each experiment, n=10 mice for air and CBD-vape exposures each (5 males+5 females) and n=9 for Nic-Vape group (5 males+4 females). CBD, cannabidiol; FDR, false discovery rate.
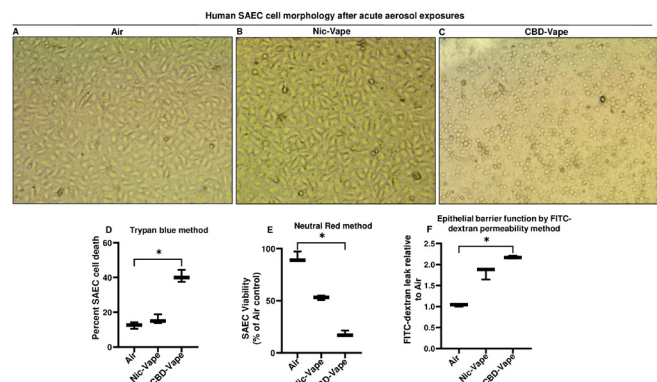
Figure 8 Epithelial cell morphology, cytotoxicity and epithelial barrier integrity of human small airway epithelial cells (SAEC) following exposure to CBD and nicotine aerosols. Human SAECs (0.5 million cells/culture insert in a 6-well plate format) were cultured in complete growth media overnight and next day directly exposed at the air–liquid interface (ALI) to 110 puffs of air, nicotine (50 mg/mL nicotine) or CBD (50 mg/mL)-containing aerosols as described in methods. All aerosols were generated in a 55 mL puff volume as described in the Methods section. At the end of the exposures, cells were removed from the ALI chamber and incubated in complete growth media for 24 hours at 37°C. After recovery period ended, cells were first imaged (A–C) at ×200 in a phase-contrast microscope (Olympus IX73) and then cell toxicity assays using (D) trypan blue method (measured as percent cell death) or (E) neutral red uptake method (depicted as percent of air control) were performed. For (F) FITC-dextran permeability assay, 1 million human SAECs/culture insert were cultured overnight and next day directly exposed at the ALI to 110 puffs of air, nicotine (50 mg/mL) or CBD (50 mg/mL)-containing aerosols. On completion of the exposures, FITC-dextran at 10 mg/mL concentration was added to the apical chamber and incubated for 2.5 hours. samples were harvested from the basolateral chamber and fluorescence measured using Exci485/Emi528 wavelengths in a synergy H1 hybrid plate reader (BioTek). The concentration of FITC-dextran in the basolateral chamber was calculated using a standard curve. Difference between two groups was considered significant at $p<0.05$ and is indicated with the symbol *$p<0.05$, calculated using non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons by GraphPad prism V.9 software (GraphPad; La Jolla, California, USA). Results are depicted as box plots with whiskers at min and max from three independent experiments each performed in triplicate as described in online supplemental methods. CBD, cannabidiol; FDR, false discovery rate; SAECs, small airway epithelial cells.

suggesting that both products are susceptible to high temperatures. However, higher levels of carbonyl compounds were detected in CBD-containing aerosol, suggesting that CBD vaping product used in our study may have been more susceptible to thermal degradation compared with nicotine product. This may be due to differences in chemical composition of two solutions and/or differences in vaporisation conditions inside vaping devices.

Numerous markers of inflammation and pulmonary damage measured in our study were consistently higher after exposure to CBD-containing vaping product than nicotine-containing vaping device. For example, we report that inhalation of aerosolized CBD vape oil caused a much stronger enhancement in the numbers of both CD4^{+} and CD8^{+} T cells, and high numbers of neutrophils in the lungs. A similar profile of increased neutrophils and modulation of CD4^{+} and CD8^{+} T cells was seen in the lung of a patient who had vaped cannabis oil causing lung injury and respiratory failure.[3] A tight nexus exists between inflammatory T cells, neutrophil-mobilising factors and neutrophil recruitment in pulmonary
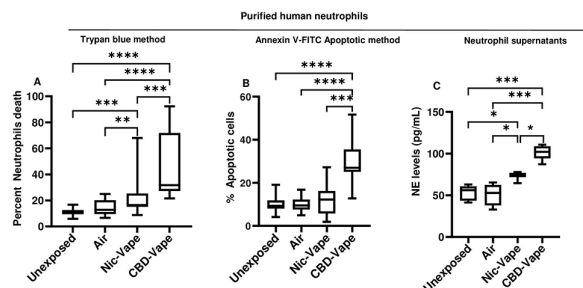
Figure 9 Apoptotic cell death in purified human neutrophils and levels of neutrophil elastase (NE) released after exposure to CBD and nicotine aerosols. Human neutrophils purified from whole blood were seeded at 0.8 million cells/culture insert in a 6-well plate format and immediately exposed to 110 puffs of Air, nicotine (50 mg/mL nicotine) or CBD (50 mg/mL)-containing aerosols at the air–liquid interface in a 55 mL puff volume as described in the Methods section. At the end of the exposures, cells were removed from the ALI chamber and recovered in complete growth media for 24 hours. Next, cell death was measured using (A) trypan blue dye-exclusion assay and (B) annexin V-FITC apoptosis as mentioned in detail in the Methods section. Immediately after isolation, an aliquot of the purified neutrophils was kept in complete medium in a CO_2 incubator as negative 'unexposed controls' for the entire duration of the assay, to estimate the extent of cell death, induction of apoptosis, or NE levels in the absence of any exposure. (C) Levels of NE were quantified in conditioned media harvested after recovery period ended by performing NE ELISA as discussed in the Methods section. Data are depicted as box plots with whiskers at min and max from independent experiments each performed in triplicate. For apoptosis assay: average data from n=5 donors per group. For trypan blue method: average data from unexposed control n=6 donors, air control and Nic-Vape n=7 donors, and CBD-Vape n=8 donors. For NE assay average data from n=6 donors per group). Difference between two groups was considered significant at p<0.05 which is indicated with the symbols *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 calculated using non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons by GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). ALI, air–liquid interface; CBD, cannabidiol; FDR, false discovery rate.

inflammatory disorders.[48] An increased inflammatory milieu in the lungs after exposure to CBD aerosols could orchestrate accumulation and/or activation of neutrophils in the bronchoalveolar space directly, via release of specific neutrophil-mobilising factors like IL-6, G-CSF and KC (CXCL1) or indirectly via activation of resident lung macrophages and epithelial cells.[49 50] It is also possible that CBD aerosols could damage lung epithelium and initiate a process of neutrophil recruitment and activation via mechanisms including local tissue damage.[51] Indeed, we demonstrate that CBD aerosols damage epithelial integrity and induce cell death in hSAECs, suggesting that CBD aerosols could directly induce lung-tissue damage and activate DAMPs to mediate pulmonary inflammation.

Neutrophil infiltration and NE-induced lung-tissue destruction is known to mediate cigarette smoke-induced pulmonary damage and compromised epithelial barrier integrity.[52–56] Neutrophils after homing to the lung exhibit an activated phenotype and perpetuate the inflammatory process,[57] so we reasoned that augmentation of the numbers of neutrophils accumulating in the lungs of mice exposed to CBD aerosols (14 488 vs 3674 in air) might have biological consequences due to their activation status. These granulocytic cells are the source of two important factors, myeloperoxidase and NE that are involved in microbicidal activity and pulmonary remodelling. The enhanced levels of MPO, as observed in our study, are considered a significant inflammatory and oxidative stress marker in several diseases including lung injury.[39 58] MPO protein released

from activated neutrophils acts as an autocrine modulator, exhibiting proinflammatory cytokine-like properties to induce PMN activation, in a manner that is independent of MPO catalytic activity.[59] Thus, in the light of these reports and our present findings, we posit that chronic CBD aerosol-inhalation could induce a strong proinflammatory microenvironment that could be more detrimental to pulmonary homeostasis and might further aggravate existing lung diseases. Neutrophil infiltration to the lungs after CBD aerosol-inhalation might possibly be induced by the upregulation of soluble chemokines like KC, and thus could be responsible for enhanced levels of NE found in the BAL and lung tissue. Since CBD aerosol-exposure induced the release of NE from human neutrophils in vitro, this supports the conclusion that CBD aerosols have the potential to directly activate neutrophils in vivo and augment pulmonary inflammation in users. As the levels of NE and associated activity increase during neutrophil-mediated inflammatory responses,[46] we thus only measured the levels of NE as an index of its activity. Additionally, the detrimental impact of vaping CBD could likely be exacerbated by impaired antioxidant systems as we observed, where factors like CBD-Vape-induced MPO could play critical roles.[39 58]

$CD4^+ROR\gamma t^+$ T cells, which express the master transcription factor essential for the differentiation into proinflammatory Th17 cells, were augmented and predict a proinflammatory microenvironment induced by CBD aerosols. Increased induction of $CD4^+FoxP3^+$ T regulatory cells (Tregs) might be one mechanism by which CBD vaping could induce immunosuppression. Tregs suppress activation, proliferation and cytokine production of $CD4^+$ and $CD8^+$ T cells and are implicated in suppressing dendritic cells via downregulating costimulatory molecules.[60] Tregs express the IL-2 receptor α-chain, which facilitates IL-2 consumption and thus could deprive T effector cells of IL-2 availability, contributing to T effector cell suppression.[61] Thus, decreased IL-2 levels in the BAL due to CBD aerosol-induced augmentation of Tregs could mediate T cell dysfunction and suppression of adaptive immunity in the lungs, increase susceptibility to respiratory infections and induce poor outcomes to prophylactic vaccination.

We observed a significant reduction in the number of lung interstitial macrophages after exposure to CBD. Siglec-F[+], typically considered an eosinophil marker, is highly expressed in murine alveolar macrophages, and when used in combination with CD11c, provides the most accurate identification of alveolar macrophages in the mouse lung. The significant reduction in the numbers of $CD11b^-CD11c^+Siglec-F^+$ alveolar macrophages observed in mice exposed to CBD is supported by previous studies.[62] An earlier study reported that EC exposure in mice induced alveolar macrophage dysfunction associated with poor pathogen uptake, with concomitant enhancement of pulmonary pathogen burden that correlated with delayed recovery time and increased mortality after infection.[16] A marked decrease in the numbers of anti-inflammatory pulmonary arginase-1[+] M2 macrophages following CBD aerosol-inhalation could further contribute to dysregulated pulmonary immune homeostasis and damage.[63] The heightened proinflammatory microenvironment detected in the lungs following CBD inhalation could be partly attributed to the reduced numbers of anti-inflammatory M2 pulmonary macrophages. Diminished numbers of macrophages could significantly impact pulmonary responses to respiratory infections, which can be compounded by dysfunctional macrophage-phagocytosis. Thus, exposure to aerosols from nicotine-containing electronic cigarettes induced a reduction in phagocytosis of nontypeable *Haemophilus influenza* (NTHI) by macrophages, largely due to the presence of e-cigarette flavourings.[64] Since, common flavourings are also used for CBD formulations, it raises the possibility that CBD aerosol-induced macrophage dysfunction could be intensified with the addition of various flavouring chemicals.

Table 2 Comparison of various markers of study outcomes after exposure to CBD-containing vaping product (CBD-vape) and nicotine-containing vaping product (Nic-vape)

Study outcomes	Markers	Results CBD-Vape vs Nic-Vape
1. Lung damage	Total protein levels in BAL	Equivalent
	FITC-dextran levels in blood	More in CBD-Vape (****)
	Albumin levels in BAL	More in CBD-Vape (****)
	NE levels in BAL	Equivalent
	NE levels in lungs	More in CBD-Vape (**)
	MPO activity in BAL	Equivalent
	MPO activity in lungs	More in CBD-Vape (**)
2. Inflammatory markers	IL-6	More CBD-Vape (***)
	IL1alpha	Equivalent
	G-CSF	More in CBD-Vape (**)
	IL-5	More in CBD-Vape (**)
	KC	Equivalent
	IL-2	Equivalent
	IL-10	Equivalent
	IFN-γ	Equivalent
3. Immune cells	Total lung immune infiltrate	More in CBD-Vape (*)
	# of Neutrophils	Equivalent
	# of CD8$^+$ T cells	More in CBD-Vape (***)
	# of CD4$^+$ T cells	More in CBD-Vape (****)
	# of CD19$^+$ B cells/gm lung	Equivalent
	# of CD4$^+$ IL-17A$^+$T cells/gm lung	Equivalent
	# of CD4$^+$ RORgt$^+$ T cells/gm lung	More in CBD-Vape (****)
	# of CD4$^+$ Foxp3$^+$ T cells/gm lung	More in CBD-Vape (***)
	# of CD11c$^+$ Siglec-F$^+$ macrophages/gm of lung	Equivalent
	# of CD11c$^+$ CD206$^+$ interstitial macrophages/gm of lung	Less in CBD-Vape (*)
	# of CD11c$^+$ Arginase-1$^+$ macrophages/gm of lung	Less in CBD-Vape (*)
3. Histology	Granulocytes	Frequency and severity greater in CBD-Vape
	Macrophages	Frequency and severity greater in CBD-Vape
	Lymphocytes	Frequency and severity greater in CBD-Vape
	Lipid-containing, intra-alveolar macrophages	Equivalent
4. Oxidative stress	Total antioxidant levels in lungs and BAL	Equivalent
5. Small airway epithelial cell toxicity	Cell death	Higher but not significant
	Epithelial barrier integrity disruption	Higher but not significant
6. Human neutrophil dysfunction	Higher but not significant	More in CBD-Vape (***)
	Apoptotic cell death	More in CBD-Vape (***)
	NE release in condition media	More in CBD-Vape (*)

Non-parametric Kruskal-Wallis test with FDR correction for multiple comparisons was performed to see if statistically significant differences exist between CBD-Vape vs Nic-Vape for various parameters measured in the study using GraphPad Prism V.9 software (GraphPad; La Jolla, California, USA). The difference between two groups was considered significant at p<0.05 obtained when FDR was set at Q<0.1 and are indicated with the symbols.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
CBD, cannabidiol; FDR, false discovery rate; NE, neutrophil elastase.

The presence of lipid laden intra-alveolar macrophages observed in cells recovered from the BAL of mice after CBD aerosol-inhalation in our study corroborates with a previous report that showed the onset of exogenous lipoid pneumonia in a patient who vaped cannabis oils and had lipid laden lung macrophages.[65] As exogenous lipoid pneumonia with lipid-laden lung macrophages is a hallmark of EVALI, it thus suggests that inhalation of CBD vape oil might not be risk-free and could lead to lung injury in cannabis vapers. The cases presented in EVALI clinical reports suggested a stronger aetiological link between cannabis vaping and respiratory failure compared with nicotine vaping.[65–68] In contrast to our earlier studies in mice that inhaled VEA (the likely cause of EVALI), in this

study, we did not observe the presence of foamy macrophages.[14] While the overall risks for pulmonary complications associated with vaping may be lower compared with smoking, vaping appears to pose a respiratory health risk, especially in long-term cannabis vapers.

In summary, our study clearly establishes that a proinflammatory milieu in the lungs induced by CBD aerosol-inhalation was greater than that induced by nicotine aerosols, and this was reflected by pulmonary barrier integrity disruption and lung damage (summarised in table 2). This suggests that cannabis vaping could potentially lead to more severe outcomes, including increased susceptibility to respiratory infections, poor responses to

prophylactic vaccinations and worsening of symptoms in patients with underlying pulmonary inflammatory diseases.[16–19 35 36 69 70] We did not observe any visible changes in animal behaviour during or after the exposures, nor weight changes compared with air control (online supplemental figure E11). In this study, nicotine aerosol-exposure did not induce the levels of various cytokines/chemokines in the BAL to the same extent when compared with a previous study.[71] Reasons for this discrepancy could be that these authors measured the transcript levels of various cytokines in lung homogenates following nicotine-aerosol exposures, and transcript levels sometimes do not translate to changes when measured at the protein levels as done by us. Furthermore, cytokine profile and immune system modulation are dependent on the specific e-liquid, method of aerosol generation/exposure duration, and flavouring chemicals added. While cannabis has proven health benefits in pain management, sleep, relieving the symptoms of chemotherapy-induced nausea/vomiting in cancer patients and in patients experiencing seizures,[72–76] there is simply a lack of robust evidence about cannabis safety when delivered from vaping products. In this regard, our study is novel and identifies the role of CBD aerosol-inhalation in inducing pulmonary inflammation and lung damage.

Since our study used animal and in vitro exposure models, several limitations need to be considered when extrapolating results presented to real-life exposure in humans. One limitation of this study is its focus was only on short-term exposure. The outcome of long-term chronic exposure to CBD-aerosols, and their impact on responses to respiratory infection and/or prophylactic vaccination are of importance. Our animal exposure methodology was based on whole-body exposure system and nose-only exposure may perhaps be more suitable to simulate exposure of experienced vapers. Despite using whole-body exposure system, we verified that deposition on animal fur (and cage walls) did not contribute to ingestion of particulates by animal grooming and thereby influence the toxicity observed. We used a limited number of mice per exposure group; it is likely that larger numbers of mice could have further strengthened our study conclusions. Although the in vitro ALI model provides an inhalation exposure-specific approach for performing the biological study on health effects related to use of vaping products, extrapolating data from in vitro studies to human risks remains hypothetical. Future observational and experimental studies with regular users of CBD and nicotine-containing vaping products are needed to confirm our findings. Finally, in our experiments, animals and cells were exposed to aerosols from both products generated in an identical way. However, users of cannabis-based vaping products may use these products in a very different way than nicotine vapers (eg, less frequently). As data from observational studies among cannabis vapers emerge, potential differences in product use patterns observed in realistic conditions should be taken into consideration when simulating animal and in vitro exposure in laboratory settings. Future studies should investigate respiratory effects of vaping products containing a wide spectrum of cannabinoids, including THC.

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