Toxicology Reports 11 (2023) 40–57

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Increased oxidative stress responses in murine macrophages exposed at the air-liquid interface to third- and fourth-generation electronic nicotine delivery system (ENDS) aerosols

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ARTICLE INFO

Handling Editor: Prof. L.H. Lash

Keywords:
Electronic nicotine delivery system (ENDS)
Electronic-cigarette
Air-liquid interface (ALI)
in vitro toxicity
Freebase nicotine
Nicotine salt

ABSTRACT

Background: New fourth generation electronic nicotine delivery system (ENDS) devices contain high levels of nicotine salt (up to 60 mg/mL), whose cellular and molecular effects on immune cells are currently unknown. Here, we used a physiologically-relevant in vitro air-liquid interface (ALI) exposure model to assess the toxicity of distinct ENDS, a 3rd-generation electronic-cigarette (e-cig) and two 4th-generation ENDS devices (JUUL and Posh Plus).

Methods: Murine macrophages (RAW 264.7) were exposed at the ALI to either air, Menthol or Crème Brûlée-flavored ENDS aerosols generated from those devices for 1-hour per day for 1 or 3 consecutive days. Cellular and molecular toxicity was evaluated 24 h post-exposure.

Results: 1-day of Menthol-flavored JUUL aerosol exposure significantly decreased cell viability and significantly increased lactate dehydrogenase (LDH) levels compared to air controls. Further, JUUL Menthol elicited significantly increased reactive oxygen species (ROS) and nitric oxide (NO) production compared to air controls. Posh Crème Brûlée-flavored aerosols displayed significant cytotoxicity – decreased cell viability and increased LDH levels –after 1- and 3-day exposures, while the Crème Brûlée-flavored aerosol produced by the 3rd-generation e-cig device only displayed significant cytotoxicity after 3 days compared to air controls. Further, both Posh and third-generation e-cig Crème Brûlée flavored-aerosols elicited significantly increased ROS plus high levels of 8-isoprostane after 1 and 3 days compared to air controls, indicating increased oxidative stress. Posh and third-generation e-cig Crème Brûlée flavored-aerosols elicited reduction in NO levels after one day, but elicited increase in NO after 3 days. Genes in common dysregulated by both devices after 1 day included α_7*nAChR, Cyp1a1, Ahr*, *Mmp12,* and *iNos*.

Conclusion: Our results suggest that ENDS Menthol and Crème Brûlée-flavored aerosol exposures from both 3rd- and 4th-generation ENDS devices are cytotoxic to macrophages and cause oxidative stress. This can translate into macrophage dysfunction. Although 4th-generation disposable ENDS devices have no adjustable operational settings and are considered low-powered ENDS devices, their aerosols can induce cellular toxicity compared to air-exposed control cells. This study provides scientific evidence for regulation of nicotine salt-based disposable ENDS products.

1. Introduction

Currently, over 2 million 12–17-year-old along with more than 8 million adults use electronic nicotine delivery system (ENDS) devices in the Unites States (U.S.), fueling concerns over the health-related consequences of vaping [9,76]. ENDS, also known as electronic cigarettes (e-cigs), are battery-operated devices that deliver an inhalable heated aerosolized mixture of nicotine, flavoring compounds, and solvents [vegetable glycerin (VG), propylene glycol (PG)]. It has been more than a decade since the release of ENDS in the U.S. While previous generations of ENDS originated as low-voltage/low-powered devices, they now have evolved to include larger and more powerful devices with

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https://doi.org/10.1016/j.toxrep.2023.06.008
Received 3 December 2022; Received in revised form 6 June 2023; Accepted 14 June 2023
Available online 20 June 2023

customizable features, along with the availability of more than 16,000 appealing e-liquid flavors, and several nicotine formulations [90]. Presently, ENDS are categorized as either open (high-powered) or closed (low-powered) system devices based on operational settings [56]. The 3rd generation e-cig models are open-system, since they offer the ability to adjust voltage (3–8 V) and wattage (10–53 W) settings, plus atomizer resistance coils (0.15–1.5 ohm), which in turn regulates the internal temperature of the device [1]. Vaping e-liquids at high temperatures/voltage settings can generate increased levels of hazardous carbonyl compounds such as formaldehyde, acetaldehyde, and acrolein, that have been shown to play a major role in a number of respiratory disorders often observed in cigarette smokers [16,42,94]. Although all models of e-cigs have the capacity to generate various amounts of carbonyl compounds, due to thermal decomposition of e-liquid ingredients, mainly PG and VG, the use of 3rd generation e-cigs for sub-ohm vaping is particularly dangerous due to the capacity of these models to operate at higher voltage/wattage settings [68,89,94]. Moreover, these devices can produce fine and ultrafine particles that are small enough to deposit deep within the lower airways [57,61,65].

In contrast, the introduction of 4th generation "pod-style" devices has dramatically changed the landscape of the ENDS market due to increased popularity of the JUUL, closed-system device. Unlike 3rd generation e-cig devices that are open-system and self-modifiable, JUUL and other fourth-generation ENDS devices are temperature-regulated, with no modifiable settings that are either one-time use disposable devices or function with disposable pods. The JUUL device operates with a 1.6-ohm atomizer coil and a power output of ~ 8 W [95]. These operational settings allow for the production of smaller discrete aerosols compared to several other 3rd generation e-cig models. Another unique feature of fourth generation devices is the use of nicotine salt formulation. The use of nicotine salt allows the delivery of higher levels of nicotine (35 or 59 mg/mL) without causing throat irritation, compared to previous generations of e-cigs that use freebase nicotine (3–36 mg/mL) [33,52].

Currently, the majority of e-cig users use 3rd or 4th generation ENDS devices, with 4th generation devices being the most popular [53,100]. Thus far, only one study [36] has evaluated the influence of 3rd or 4th generation ENDS devices on immunological responses in the respiratory tract by using human sputum samples [36]. In this study, it was found that 3rd generation and 4th generation e-cig users had more macrophages per mg of sputum (544 vs 505 macrophages/mg sputum, respectively) compared to non-vapers (469 macrophages/mg sputum) [36]. These authors also observed that users of 4th generation devices had considerably lower levels of IFN-γ, MCP-1, and VEGF, compared to 3rd generation e-cig users [36]. Collectively these authors suggested that use of 4th generation e-cig devices may result in immunosuppressive responses due to nicotine salt. Thus, differential pulmonary responses can occur based on ENDS model type (3rd versus 4th generation).

Further, in line with the results from Hickman et al. [36], emerging evidence suggests that ENDS aerosols affect immune function of alveolar macrophages, which are the main resident cell types in the lungs, which act as key protective cells, part of the first line of defense against inhaled toxicants [27,105]. Moreover, macrophages are the most dominant cell type of the immune system, and play a major role in respiratory system homeostasis, the resolution of inflammatory responses and tissue repair mechanisms [62]. Immune homeostasis depends heavily on the function of activated macrophages and their ability to produce reactive oxygen species (ROS) to maintain the respiratory environment [34]. Oxidative stress signaling through the production of ROS and nitric oxide (NO) has been suggested to play a role in macrophage polarization based on inflammatory and anti-inflammatory cellular responses [97]. Moreover, imbalances between the production of ROS and NO lead to oxidative stress, immunosuppressive responses, and cellular damage [11]. We and others have reported that e-cig exposures increase oxidative stress levels in macrophages and promotes the production of reactive oxygen species (ROS) production [106,64,79,85]. Additionally, in vitro and in vivo studies have shown that e-cig aerosol exposures reduce macrophage phagocytosis and dysregulate genes related to inflammation [101,17,40,85,92]. Overall, these results highlight that ENDS aerosol can negatively impact macrophage morphology, as well as function.

E-liquid flavors are comprised of a complex mixture of flavoring chemicals; however, all flavors among brands are not created equal. A study by Omaiye et al., found that flavoring chemicals including ethyl maltol and menthol in e-liquids, can reach a cumulative concentration exceeding 10 mg/mL [70,71]. This is an important finding since common flavorings such as vanillin (aldehyde) and ethyl maltol (alcohol), found in JUUL pod liquids, including Crème Brûlée, have been found to be cytotoxic and to increase oxidative stress levels in vitro [41,63,71]. Menthol is a widely used flavoring chemical that provides a cooling sensation and is used in tobacco products to hide the harshness of nicotine [25,74,98]. Menthol was found to be cytotoxic to bronchial epithelial cells [31,50,71,84,88] and macrophages [64] in vitro. Recently, several studies reported that the cytotoxic effects of ENDS aerosols are linked to the presence of favoring chemicals [3,24,30,38,41,50,64,67,73,72,71,87]. Additionally, flavoring agents in e-liquids have been reported to cause pulmonary toxicity, respiratory irritation, reduced lung function [20,47,60], and exacerbate respiratory conditions, including asthma [17,35,54,93]. Collectively these findings suggest that ENDS-flavored e-cig aerosols may affect immune and respiratory homeostasis.

Currently, few data exist regarding whether exposures to Menthol or Crème Brûlée-flavored e-cig aerosols are detrimental to macrophage homeostasis. In the present study, we investigated the effects of Menthol and Crème Brûlée-flavored aerosols in vitro on mouse macrophages (RAW 246.7) exposed at the air-liquid interface (ALI) using either a 3rd generation e-cig device, JUUL (pod-based) or Posh Plus (disposable) 4th generation devices. Crème Brûlée, was one of JUUL's top 3 'most' popular flavors in 2018, and is still a flavor available among other e-cig brands [5]. Menthol is one of the most commonly used flavors among pod-style 4th generation devices [13,45]. Overall, with the increasing popularity of flavored e-liquids and the usage of 4th generation "Juul-like" devices among youth, it is imperative to investigate cellular and molecular effects of aerosols generated from these ENDS devices to help guide future regulations of flavorings, freebase, and nicotine salt-based e-cig products.

2. Methods

2.1. ENDS aerosol production and chemical analysis

Flavored e-liquids containing freebase nicotine were purchased from online retailers: The Vape Mall (Wentzville, MO) for Crème Brûlée-flavored (30 mg/mL, 30PG/70VG) and EC Blends (Salem, OR) for Menthol-flavored (36 mg/mL, 40PG/60VG) e-liquids, respectively. These e-liquids were used in the tank-style third generation ENDS device. We used the highest freebase nicotine concentration available in the flavored-e-liquids, along with specific PG/VG ratios, to be comparable to high nicotine salt levels (~50 mg/mL) and the solvent ratios (70/30) contained in 4th generation ENDS. Also, the high levels of nicotine in e-liquids chosen for this experiment, mimics the nicotine exposures of heavy smokers (>1 pack of cigarettes per day) [103]. For the 4th generation devices, we used 1) Menthol favored JUUL pods (3% and 5% nicotine salt) purchased directly from JUUL labs (San Francisco, CA) to ensure authenticity; and 2) Posh Plus disposable vape pens (60% nicotine salt), in Crème Brûlée flavor, which were purchased from InLine Vape LLC (Warren, MI).

Aerosol generation from 3rd generation ENDS was performed as previously described in Noël et al. [69]. In brief, we used the automated 3rd generation e-cig Scireq® Inexpose vaping system (Montreal, QC, Canada), which is composed of a Joyetech eVic VTC mini mod connected to a SCIREQ e-cigarette aerosol generator. The e-cig device was

operated using a 0.15 Ω atomizer coil and operated at 3.0 V. We used a puffing topography regime of 3 s puffs every 30 s, with a puff volume of 55 mL, as recommended by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) CRM N° 81 parameters [18]. For the 4th generation devices, either a JUUL rechargeable pod-based device (JUUL Labs) or a Posh Plus disposable device were used for aerosol generation. As previously described in Pinkston et al. [79], the 4th generation devices were connected to a programmable peristaltic pump (Cole-Parmer Masterflex L/S) with 1 in. diameter tygon tubing. We used a standard vaping topography profile of 5 s duration every 30 s with a 30 mL puff volume. Overall, this study was design to use representative vaping topography profiles for both types of ENDS devices. Therefore, we conducted in vitro experiments at the ALI for two generations of ENDS devices that were used under similar vaping topography profiles.

Following a published procedure [26], we carried out aerosol characterization of selected solvents and nicotine levels for the 3rd and 4th generation devices by collecting 10 puffs of the ENDS aerosols on 44-mm Cambridge filter pads employing a 1 L/min loading regimen. Thus, we used a targeted approach to determine the levels of selected chemicals in the ENDS aerosols. Quantification of nicotine, PG and glycerin was performed by using gas chromatography with a flame ionization detector (GC-FID) method. Carbonyl compounds were analyzed by collection of 10 puffs of ENDS aerosols in silica gel sorbent tubes containing 2,4-dinitrophenylhydrazine (DNPH) with a loading regime of 1 L/min, followed by analysis using the EPA method TO-11A, based on high performance liquid chromatography (HPLC). Fourth generation devices contain nicotine salt which consists of free base nicotine to which an organic acid, usually benzoic acid is added [10,78,82]. To quantify JUUL and Posh Plus aerosols for organic acid concentrations, both devices were connected to the peristaltic pump followed by direct connection to a fritted glass impinger containing an aerosol trapping solution consisting of 30 mL acidified 2,4-dinitrophenylhydrazine. Organic acids analyses were performed by ion chromatography. All ENDS aerosol samples described above were collected at the Louisiana State University (LSU) School of Veterinary Medicine (SVM) Inhalation Research Facility, followed by overnight shipping on dry ice to Enthalpy Analytical, LLC for subsequent chemical analyses.

2.2. Cell culture

RAW 246.7 cells (murine macrophage cell line; ATCC TIB-71) were used to conduct in vitro ENDS aerosol exposures. Since our laboratory uses both in vitro and in vivo models to assess the toxicity of various ENDS products, we used mouse macrophages to obtain data that are directly comparable with our in vivo data from mice exposed to ENDS aerosols by inhalation. Further, we previously reported that mouse macrophages behave similarly to human cells following in vitro air-liquid interface (ALI) exposures to Crème Brûlée flavored JUUL aerosols [79]. RAW cells were maintained in T-75 tissue culture flasks using DMEM Hams F-12 medium with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin. Once cells reached about 80–90% confluence, macrophages were seeded onto 24 mm transwells with a 0.4-μm pore size polyester membrane insert (Catalog #3450, Corning Incorporated, Corning, NY; 4.2 cm^2 cell culture area/insert) in 6-well plates, at a seeding density of ~0.5–1.0 × 10^6 cells/insert. One day after seeding, apical media was removed, with media maintained only on the basolateral side. Macrophages were exposed to ENDS aerosols for either 1 or 3 consecutive days. Cells between passages 4 and 13 were utilized for experiments.

2.3. Cell exposure to ENDS aerosols at the ALI

Table 1 highlights the experimental study design for these in vitro ALI experiments. As previously described in Noël et al. [68] and Pinkston et al. [79], our ALI exposure system allows for direct ENDS aerosol exposure as a more physiological representation of how cells are exposed within the lung, compared to submerged cell culture methods. Our customized ALI system (Vitrocell Systems GMBH; Waldkirch, Germany) has a 6/4 stainless steel exposure module for 4 × 6 well/24 mm diameter inserts. This system is connected to an aerosol distribution system. Our in vitro exposure system allows cell inserts to be exposed at the ALI to ENDS aerosols or to medical grade compressed air (control). The module for ENDS-exposed cells contained 4 chambers (or wells), of which 3 wells were dedicated to direct exposure of macrophages grown on transwells to ENDS aerosols, diluted with medical grade compressed air. One well was utilized for real-time measurement of aerosol deposition by a Quartz Crystal Microbalance (QCM; Vitrocell). The module that was utilized for air control contained 3 wells in which cells were exposed to medical grade compressed air. Directly before ALI exposure, wells of the modules were filled with 3 mL of complete culture media. Transwells containing macrophages were then transferred to the Vitrocell exposure modules. Culture media was only contained on the basolateral side of the transwell to maintain the cells in normal culture conditions during experimentation. The air control and ENDS exposure modules were then connected to a water bath to maintain the cell temperature at 37 °C.

The 3rd generation ENDS exposure system (Scireq®) was connected with 1-inch diameter tygon tubing to the aerosol distribution system of the Vitrocell ALI exposure system. To generate the Menthol JUUL or Crème Brûlée-flavored Posh Plus aerosols, the 4th generation devices were connected to a programmable peristaltic pump (Cole-Parmer Masterflex L/S) with 1 in. diameter tygon tubing, and directly connected to the Vitrocell® exposure system.

All exposures were conducted for 1 h to mimic actual patterns of 4th generation ENDS usage (~120 puffs per day), which results in the consumption of 1 JUUL pod [2,69,96]. Exposures to ENDS aerosol (n = 3 transwell inserts) and medical grade compressed air (n = 3 transwell inserts) were conducted in parallel. Immediately after the exposure, transwells were removed from the ALI modules, returned to the 6-well

Table 1
In vitro air-liquid interface experimental study design.

Cell type	E-liquid flavor	Nicotine concentration	Nicotine chemical form	Treatment/Type of ENDS device	Exposure duration
Murine macrophages	—	—	—	Exposure to medical grade compressed air	1 day
Murine macrophages	Menthol	3% (~35 mg/mL)	Nicotine salt	JUUL (4th generation)	1 day
Murine macrophages	Menthol	5% (~50 mg/mL)	Nicotine salt	JUUL (4th generation)	1 day
Murine macrophages	Menthol	36 mg/mL	Free base nicotine	E-cig 3rd generation	1 day
Murine macrophages	—	—	—	Exposure to medical grade compressed air	1 day
Murine macrophages	Crème-Brulée	6% (~60 mg/mL)	Nicotine salt	Posh Plus (4th generation)	1 day
Murine macrophages	Crème-Brulée	30 mg/mL	Free base nicotine	E-cig 3rd generation	1 day
Murine macrophages	—	—	—	Exposure to medical grade compressed air	3 days
Murine macrophages	Crème-Brulée	6% (~60 mg/mL)	Nicotine salt	Posh Plus (4th generation)	3 days
Murine macrophages	Crème-Brulée	30 mg/mL	Free base nicotine	E-cig 3rd generation	3 days

ENDS: electronic nicotine delivery system.
Each exposure condition was assessed during 3 independent experiments, each conducted with 3 technical replicates.

plates, and then placed back into the incubator at 37 °C for 24 h prior to collection for analysis. Each experiment was repeated 3 independent times each with 3 technical replicates.

2.4. Scanning electron microscopy (SEM) analysis

SEM was used to view the surface morphology of the macrophages after exposure to ENDS Crème Brûlée-flavored aerosols. All chemicals and materials for SEM were purchased from Electron Microscopy Sciences (Hatfield, PA). Cell fixation and imaging were performed as previously described [79]. In brief, cells were washed (apical and basolateral surfaces) with PBS following the 24-hour post-ALI recovery period. After washing, cells within the transwell were fixed with 1.25% (v/v) glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, followed by washing the apical and basal chambers with 0.1 M sodium cacodylate with 5% sucrose, followed by post-fixation procedures. Next, transwell membranes were detached completely from the insert before undergoing a series of dehydration steps with ethanol. Membrane samples (with the previously fixed cells) were further dehydrated by incubation in hexamethyldisilizane before placement in a desiccator to dry overnight. Detached membranes were mounted on aluminum stubs before analysis on a FEI Quanta 3D dual beam scanning electron microscope (Phillips/FEI). Images were taken at an accelerating voltage of 5 kV.

The following four assays were all carried out as previously described in Pinkston et al. [79].

2.5. Cell viability

Trypan blue staining was used to assess cell viability 24 h after exposure. Viability was evaluated using a TC20 automated cell counter (catalog #1450102, Bio-Rad Laboratories, Hercules, CA). Duplicate readings of each sample were taken. Based on the significance of the cell viability results, in this manuscript, we refer to exposures under cytotoxic conditions, when the viable cell count, as measured by the Trypan blue exclusion assay, was significantly different between ENDS exposed-cells and medical grade compressed air-exposed cells (controls). This is in opposition to under non-cytotoxic conditions, defined as no significant difference between the viable cell count of ENDS-exposed cells and controls.

2.6. Lactate dehydrogenase (LDH) cytotoxicity assay

The extracellular release of LDH was measured in the basolateral culture media at 24 h post-exposure to ENDS aerosol. The assay was carried out according to manufacturer's directions (CyQuant, LDH cytotoxicity assay kit, catalog # C20300, Invitrogen, Thermo Fisher Scientific, Waltham, MA). In brief, 50 μL of cell culture medium removed from the basal side of the Transwell, was combined with LDH assay reaction mixture, followed by stop solution in a 96-well plate after the 30-minute incubation period. The absorbance was read at 490/680 using a spectrophotometer (Tecan Infinite 2000, Tecan Group Ltd, Mannedorf, Switzerland). For each sample, the cell medium absorbance readings were normalized to the cell count. Absorbance values for the air control groups were set at 100%. Samples were run in duplicate.

2.7. Reactive oxygen species (ROS) quantification assay

Extracellular ROS production in the basolateral cell culture media was measured using OxyBURSTGreen H2HFF-BSA (catalog # D2935, Invitrogen, Thermo Fisher Scientific, Waltham, MA) fluorogenic reagent at 24 h post-exposure to ENDS aerosol. The assay was performed following the manufacturer's instructions. Fluorescence was measured spectrophotometrically (TECAN infinite 2000; excitation: 488 nm, emission: 530 nm). For each sample, the fluorescence was normalized to the cell count. Fluorescence values for the air control groups were set at 100%. All samples were run in duplicate.

2.8. Griess assay

Extracellular nitrite/nitric oxide (NO) release 24-hours after ENDS exposure was quantified within the cell culture media through photometrical detection of NO with a Griess reagent kit (catalog #30100, Biotium, Fremont, CA) in accordance with the manufacturer's protocol. The optical density (OD) of each sample was measured at 548 nm using a spectrophotometer (TECAN infinite 2000). For each sample, the absorbance was normalized to the cell count. The absorbance values for the air control groups were set at 100%. All samples were run in duplicate.

2.9. 8-isoprostane ELISA

Extracellular release of 8-isoprostane 24-hours post exposure to ENDS aerosols, was quantified within the basolateral cell culture media using an 8-Isoprostane Express ELISA Kit (catalog # 516360; Cayman Chemical; Ann Arbor, MI), according to the manufacturer's protocol, with the addition of butylated hydroxytolune (BHT) at the time of sample collection. The absorbance of samples was measured using a spectrophotometer (TECAN infinite 2000) between the wavelengths of 405 – 420 nm. Absorbance values were analyzed and calculated using the MyAssays online software program (myassays.com). All samples were run in duplicate.

2.10. RNA isolation and real-time quantitative reverse-transcriptase PCR analyses

Collected cells from each ENDS exposure group and air controls were pelleted and stored at − 80°C until analyzed. Technical replicates from each experiment were pooled, and total RNA isolation was performed using the Qiagen RNeasy Mini Kit (Cat # 74136, Qiagen, USA). Quantification of mRNA was performed spectrophotometrically (260/280 nm ratio, NanoDrop 1000, Thermo Scientific). The Bio-Rad iScript cDNA synthesis kit (Cat # 1708890, Bio-Rad Laboratories Inc, Hercules, CA, USA) was used for mRNA conversion, followed by amplification using a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). To investigate gene expression, quantitative real-time PCR (qRT-PCR) was performed using cDNA from macrophages with Taqman pre-developed primer-probe sets (Applied Biosystems, University Park, IL, USA). The reaction volumes used were 25 μL, with 40 reaction cycles for each gene using the Applied Biosystems 7300 Real-Time PCR System. The comparative cycle threshold ($\Delta\Delta C_T$) method was used to determine relative gene expression. Hypoxanthine guanine phosphoribosyltransferase (*Hprt1*) was used as a housekeeping gene to normalize the expression of each gene. Results are reported as fold change over control [$(2^{-\Delta\Delta CT})$]. A fold-change $> |1.5|$ was considered significant.

2.11. RT^2 profiler PCR array

To analyze gene expression from the e-cig and Posh Plus 3-day Crème Brûlée-flavored aerosol macrophage experiments, we used a PCR array (Qiagen, catalog #PAMM-034Z) according to the manufacturer's instructions, which was specific for the expression of 84 genes related to the TH-1 and TH-2 pathway responses. In Brief, 0.5 μg of total RNA was reverse transcribed with the RT^2 First Strand Kit (Qiagen, catalog # 330401) according to the manufacturer's directions. Each cDNA sample was mixed with RT^2 SYBR Green qPCR Master mix (Qiagen Catalog # 330503). A 25 μL reaction mixture was added to each of the PCR Array plate that contained pre-dispensed gene-specific primer sets. The plate was analyzed using Applied Biosystems model 7300 real-time cyclers. Gene expression and fold change were calculated using the ΔΔ*C*t method, using Qiagen's online PCR Array analysis software program. Δ*C*t data were calculated and normalized using the average geometric mean of the following housekeeping genes: *Hsp90ab1*, *Gusb*, *Actb*, and

B2m ($n = 3$ independent samples per group).

2.12. Ingenuity pathway analysis

Gene expression data from RT-PCR and the RT^2 Profiler PCR array were used to investigate associated gene networks and biological pathways utilizing the Ingenuity Pathway Analysis (Qiagen, Ingenuity Systems, Redwood City, CA, USA) web-based bioinformatics application software program. Only significantly dysregulated genes (fold-change > |1.5|) were considered for the analysis.

2.13. Statistical analysis

Statistical analysis for all biological outcomes were performed using GraphPad Prism 9 Software (GraphPad Software, San Diego, CA). Since the main function of an ENDS device is to deliver nicotine, we used the 'equivalent nicotine-delivery capacity' of the device as a normalization factor, to compare selected biological outcomes between ENDS devices of a different generation using e-liquids of a same flavor. Thus, for ENDS devices comparison purposes, we normalized our results to the estimated total nicotine exposure per treatment condition. This was defined as the concentration of nicotine in ENDS aerosol in μg/puff multiplied by the total number of puffs (120) generated during a 1-hour exposure, which equals the estimated total nicotine exposure. Selected biological outcomes were then expressed as ratios to 1000 μg of nicotine. A student's t-test was performed to compare results between ENDS aerosol groups and their respective air controls. A one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test was used when testing differences between 3 or more ENDS groups. Results are presented as mean ± standard error of the mean (SEM). Results were considered statistically significance at $p < 0.05$.

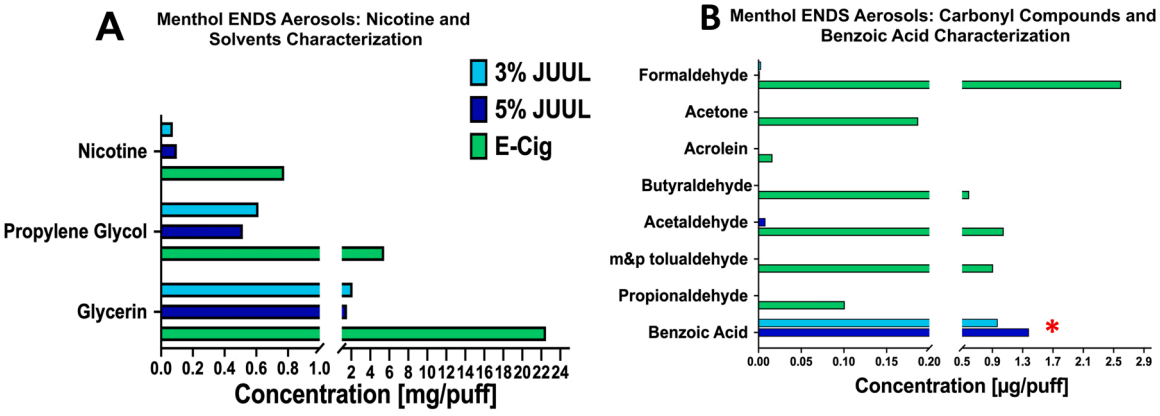
3. Results

3.1. Menthol-flavored aerosols from a 3rd generation e-cig device produce high levels of solvents and carbonyls

Using a targeted approach, we analyzed the chemical profiles (nicotine, solvents, carbonyls) of Menthol-flavored aerosols generated from JUUL 3% and 5% nicotine salt pods, as well as from a freebase nicotine containing e-liquid (36 mg/mL), aerosolized through a 3rd-generation e-cig device (Fig. 1A and 1B). Levels of benzoic acid within the aerosol of JUUL pods were also tested due to its nicotine salt formulation (Fig. 1B). Using a similar vaping topography profile for all ENDS devices, we found that JUUL 3% and 5%- pod aerosols contained 0.0735 mg/puff and 0.0987 mg/puff, of nicotine, respectively, while e-cig aerosol had 0.776 mg/puff of nicotine. For solvents, 3rd generation e-cig aerosol had high levels of PG (5.46 mg/puff) and VG (22.5 mg/puff), whereas JUUL 3% contained 0.614 mg/puff of PG and 2.13 mg/puff of VG, and JUUL 5% had 0.515 mg/puff of PG and 1.55 mg/puff of VG. For the JUUL devices, we found that the 3% nicotine pods contained lower levels of benzoic acid (0.97 μg/puff) compared to 5% pods (1.38 μg/puff). Upon analyzing the carbonyl compounds, we found that JUUL 3% and 5% contained trace amounts of formaldehyde, acrolein and acetaldehyde (Fig. 1). This is in contrast with e-cig Menthol flavored aerosol, which contained high levels of carbonyls, including m&p tolualdehyde (0.91 μg/puff), acetaldehyde (1.05 μg/puff), and formaldehyde (2.60 μg/puff) (Fig. 1). Overall, our results for the chemical profile of the ENDS aerosols, generated under the specific operational settings used in this study, reveal that Menthol-flavored ENDS aerosols generated from either a JUUL or 3rd-generation e-cig devices produce different chemical profiles.

3.2. 1-day ALI Menthol-flavored JUUL aerosol exposure reduces cell viability and increases LDH levels as well as oxidative stress levels in murine macrophages compared to air-exposed control cells

We investigated the effect of 1-day ENDS Menthol-flavored ALI aerosol exposure on murine macrophages. The dosimetry of the aerosol resulted in an average dose of 10.7 μg/cm^2 ± 2.3 for JUUL 3% nicotine salt pods, 15.4 μg/cm^2 ± 4.0 for JUUL 5% nicotine salt pods and 34.7 μg/cm^2 ± 7.7 for 3rd generation e-cig Menthol after 1-day of exposure, as measured by the QCM (Fig. 1C). We found that Menthol-flavored JUUL exposure in both nicotine concentrations, decreased cell viability ≥ 30% compared to air controls. E-cig Menthol-exposure resulted in a non-statistically significant reduction in cell viability



C Aerosol Deposition on Macrophages

Aerosol deposited mass Dosimetry	
3% JUUL	10.7 μg/cm² ± 2.3
5% JUUL	15.4 μg/cm² ± 4.0
E-cig	34.7 μg/cm² ± 7.7

Fig. 1. Menthol-flavored aerosols from a 3rd generation e-cig device, produces high levels of nicotine, solvents and carbonyls. Aerosols generated from a JUUL device using 3% and 5% nicotine salt pods, as well as a 3rd generation e-cig device were tested for (A) solvent (PG and VG) and nicotine concentrations; (B) carbonyl chemical production. Benzoic acid levels were also tested for JUUL only* ; (C) the average deposited dose per cell insert/sample measured in μg/cm^2 of ENDS aerosols by a quartz crystal microbalance (QCM) for 1-day ALI exposures. Aerosol dosimetry data represent the mean ± SD.

compared to air controls. Only JUUL 5% and e-cig increased LDH levels ≥ 50% compared to air controls (Fig. 2A and 2B). Further, JUUL 3% and 5% Menthol, along with e-cig Menthol, elicited ≥ 50% increase in 8-isoprostane, a marker of oxidative stress, indicative of lipid peroxidation. Both 3% and 5% JUUL pods along with e-cig resulted in ≥ 20% increased levels of extracellular ROS production compared to air controls (Fig. 2C and 2D), while only JUUL 5% Menthol and e-cig Menthol resulted in increased levels of nitrogen species production compared to air controls (Fig. 2E). Increased levels of ROS and NO are indicative of macrophage activation [28]. Collectively, these results suggest that ENDS Menthol-flavored aerosols are cytotoxic and induces extracellular oxidative stress responses compared to air-exposed control cells.

3.3. *Menthol-flavored ENDS aerosols dysregulates genes involved in oxidative stress and biotransformation responses compared to air-exposed control cells*

We investigated the regulation of genes that play a role in inflammation, biotransformation and oxidative mechanisms using qRT-PCR (Fig. 3). Macrophages exposed to JUUL Menthol-flavored aerosol had different gene expression patterns between the 3% and 5% nicotine salt pods compared to air controls. JUUL 3% pods downregulated the expression of 6 out of the 10 inflammation and oxidative stress-related genes, including *α7nAChR*, *Nos2*, *Tgfβ*, *Hmox1*, *Ahr, and Nqo1,* compared with JUUL 5%, that upregulated the same genes. Only JUUL 5% Menthol caused increased expression of *Mmp12*, related to airway remodeling, along with the downregulation of the *Il-6* gene, that plays a role in cellular inflammation. Compared to air controls exposure to 3rd-generation e-cig aerosol resulted in the same gene expression pattern, related to inflammation and oxidative stress, as the 5% JUUL pod, as indicated by the upregulation of 7 genes (*α7nAChR*, *Nos2*, *Tgfβ*, *Hmox1*, *Ahr, Nqo1, Mmp12)*, along with the downregulation of *Il-6*. These results suggest that macrophages exposed for 1-day to Menthol-flavored ENDS aerosol containing either 5% of nicotine salt, from JUUL, or freebase nicotine, from 3rd generation e-cig devices, compared to air control cells, increase oxidative stress signaling without induction of inflammation. This may translate into impaired respiratory immunity.

3.4. *Based on estimated total nicotine exposure, menthol-flavored JUUL aerosols lead to increased toxicity compared to 3rd generation e-cig aerosols*

Using 'equivalent nicotine-delivery capacity' of the device, we normalized the results obtained from the different ENDS devices (3% and 5% JUUL and third-generation e-cig) for selected biological outcomes presented in Fig. 2, allowing for toxicity comparison of devices based on nicotine exposure (Fig. 4). After normalization to nicotine exposure, 1-day ALI exposure to 3% and 5% JUUL aerosols caused a significant increase in extracellular release of LDH, 8-isoprostane, ROS, and NO species levels compared to 3rd generation e-cig aerosols (Fig. 4). These data suggest that nicotine salt may induce greater cellular effects in murine macrophages than free base nicotine.

3.5. *Crème Brûlée aerosols produced from a 3rd generation e-cig device produce high levels of nicotine, solvents and carbonyls*

In addition to analyzing responses from Menthol-flavored aerosols, we also analyzed the chemical profiles (organic acid, nicotine, solvents, carbonyls; Fig. 5) of Crème Brulée-flavored aerosol for both the Posh Plus 4th-generation vape pen disposable device containing 60 mg/mL

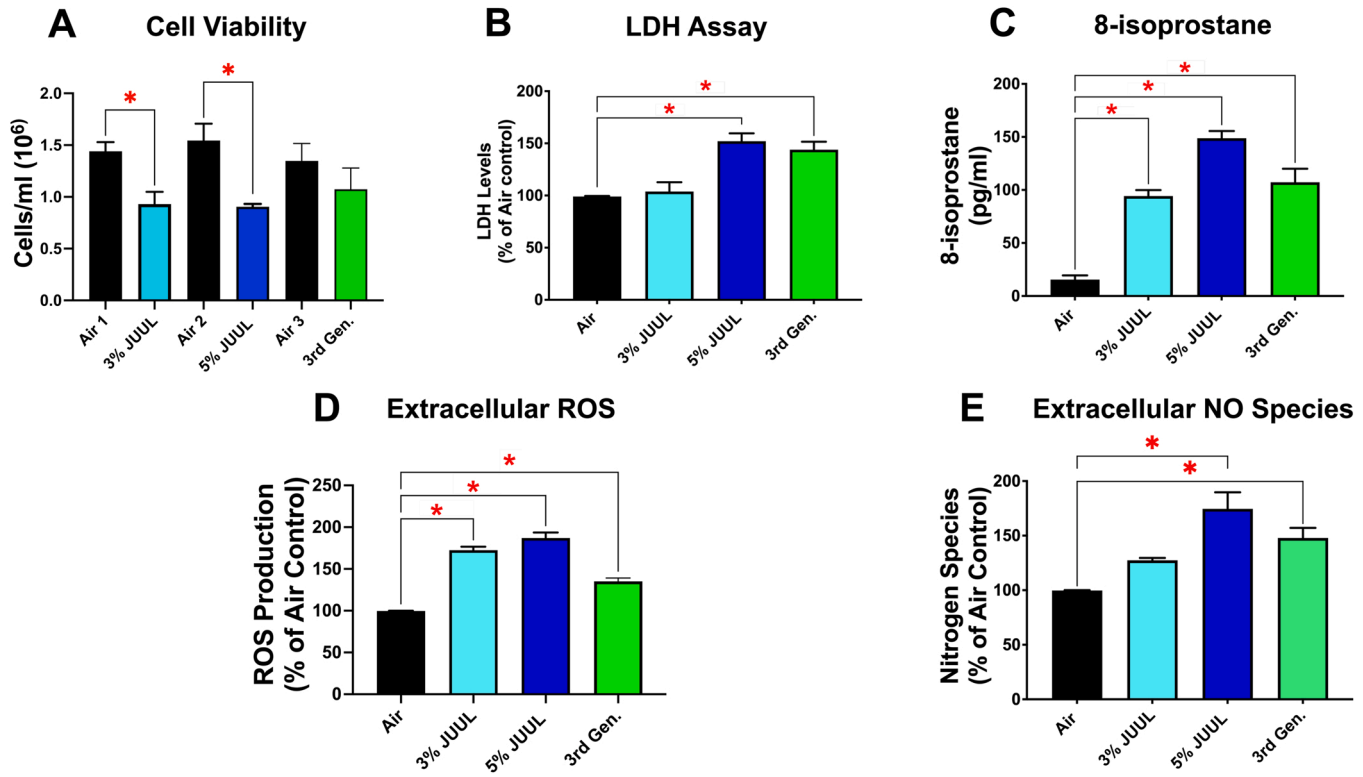


Fig. 2. 1-day ALI Menthol-flavored JUUL aerosol exposure reduces cell viability and increases LDH levels as well as oxidative stress levels in murine macrophages compared to air controls. One-day ALI exposure to ENDS aerosols causes (A) a significant reduction in cell viability for JUUL in both 3% and 5% nicotine salt concentrations compared to its respective air control as measured using trypan blue exclusion; (B) causes a significant increase in extracellular release of LDH in JUUL 5% and e-cig groups compared to the air control; (C) leads to a significant increase in extracellular 8-isoprostane levels compared to air; (D) increased extracellular ROS species production in all three groups compared to air; and (E) increased NO species production in JUUL 5% and e-cig groups compared to air controls. A student's t-test was performed to compare results between ENDS aerosol groups and their respective air controls. Data represent the mean ± SEM for n = 3 independent experiments, each with 3 technical replicates per group. * $p < 0.05$ statistically different from air controls.

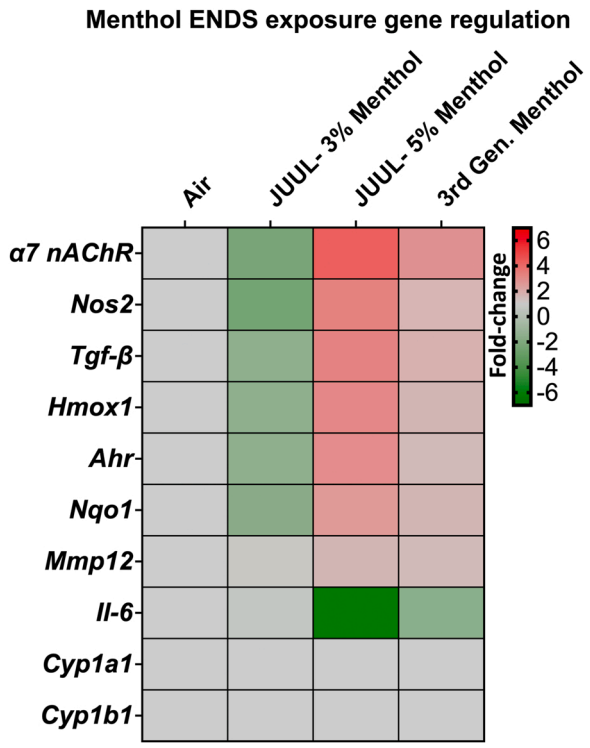


Fig. 3. Menthol-flavored ENDS aerosols dysregulates genes involved in oxidative stress and biotransformation responses compared to air-exposed control cells. A heatmap displaying device and nicotine form specific expression patterns of dysregulated genes by the JUUL and e-cig Menthol-flavored aerosol exposures. Data is presented as fold-change compared to respective air-control group. Samples from each experiment were pooled for RNA collection, and data is representative of 3 independent experiments performed in triplicate. Fold-changes $> |1.5|$ were considered significant. Red denotes up-regulation and green denotes down-regulation.

nicotine salt, which is higher than the JUUL 5% nicotine salt pods. We also used a 3rd-generation e-cig device with Crème Brûlée-flavored e-liquid, containing 30 mg/mL of freebase nicotine, which was the highest nicotine level commercially available in this flavor. As expected, Posh Plus aerosols contained nicotine (0.28 mg/puff), PG (3.06 mg/puff) and VG (4.28 mg/puff). For the carbonyl compounds, the Posh aerosol contained low levels of acetaldehyde (0.01 μg/puff), formaldehyde (0.02 μg/puff), diacetyl (0.03 μg/puff) along with trace amounts of acrolein and acetaldehyde. Since Posh Plus contains nicotine salt, we also tested for organic acids, specifically benzoic acid. We found that Posh Plus Crème Brûlée aerosol contains moderate levels of benzoic acid (4.27 μg/puff). Using a similar vaping topography profile for all ENDS devices, in contrast to Posh Plus, e-cig Crème Brûlée-flavored aerosols contained high levels of nicotine (0.50 mg/puff), PG (5.28 mg/puff) and VG (1.71 mg/puff). For the carbonyl compounds, the e-cig aerosol contained high levels of acetaldehyde (3.88 μg/puff), formaldehyde (13.0 μg/puff) and acrolein (9.32 μg/puff). In addition, e-cig aerosols also contained high levels of 2-butanone (3.6 μg/puff), benzaldehyde (0.93 μg/puff) and acetone (5.72 μg/puff). Overall, Posh Plus aerosol contained high levels of benzoic acid, while e-cig aerosol contained high levels of carbonyls, both of which can have detrimental effects on immune cell function.

3.6. Crème Brûlée-flavored ENDS aerosols affect the surface morphology of murine macrophages compared to air-exposed control cells

To investigate the effect of short-term ENDS Crème Brûlée-flavored aerosol exposure on murine macrophages, we exposed RAW 246.7 cells for 1 or 3 consecutive days to the Posh Plus or a 3rd generation e-cig device. The dosimetry of the aerosol resulted in an average dose of 75.2 μg/cm^2 ± 17 for Posh and 34.6 μg/cm^2 ± 14 for e-cig after 1-day of exposure and 70.2 μg/cm^2 ± 7 for Posh and 29.5 μg/cm^2 ± 14 respectively after 3-days of exposure, as measured by the QCM (Fig. 5 C). Macrophages have wave-like external dorsal membrane ruffles that act as sensors to engulf extracellular particles and pathogens [91]. They also have external lamellipodia (sheet-like projections) and filopodia (finger-like projections) that play critical roles in cell migration, cell-to-cell communication, and pathogen recognition [91]. In comparison to air control cells, macrophages exposed to Posh Plus seem to exhibit detrimental effects and cell surface structural changes (Fig. 6). In Fig. 6A, macrophages exposed to Posh Plus and e-cig are observed to have their extracellular surface projections still intact after 1-day of aerosol exposure, in comparison to air controls. After 3 consecutive days (1 hr/day) of ALI exposure, however, Posh Plus causes cell surface structural changes, in which dorsal membrane ruffles, as well as external lamellipodia and filopodia are absent (Fig. 6B). These results suggest that Posh Plus crème brulée changes the overall cell's appearance after 3 days of exposure compared to air controls. These effects may impair overall macrophage function.

3.7. 1-day ALI Crème Brûlée-flavored ENDS aerosol exposure alters levels of oxidative stress in macrophages under cytotoxic and non-cytotoxic conditions compared to air-exposed control cells

After 1 day of aerosol exposure, we observed that Posh Plus aerosol is cytotoxic, as indicated by a $\geq$ 50% significant decrease in cellular viability (Fig. 7A), and by a corresponding significant increase in extracellular release of LDH compared to air controls (Fig. 7B). In terms of oxidative stress, we found increased levels of 8-isoprostane for both devices compared to controls (Fig. 7C); however, only Posh Plus aerosol induced increased extracellular ROS levels, while e-cig exposure resulted in a $\geq$ 30% reduction in ROS levels compared to controls (Fig. 7D). Also, we observed decreased NO levels in both devices compared to controls (Fig. 7E). Collectively, these results indicate that short-term ALI exposures to Crème Brûlée-flavored ENDS aerosols can affect the redox state of macrophages.

3.8. Crème Brûlée-flavored ENDS aerosols dysregulate genes involved in cellular biotransformation and oxidative stress compared to air-exposed control cells

At the gene expression level (Fig. 8), we investigated the same genes that were tested for Menthol, to observe whether Crème Brulée-flavored aerosols would display similar patterns of gene dysregulation in both devices compared to air-exposed control cells. We found that both Posh Plus and 3rd generation e-cig aerosol exposures elicit similar patterns of dysregulated genes, with the upregulation of 5 common genes (*Mmp12*, *Tgfβ*, *Cyp1a1*, *Ahr, and α7nAChR*) that play key roles in oxidative stress responses. Both devices also exhibited downregulation of 2 common genes (*Il-6, Nos2*). These genes coincide with results from Fig. 7 that show decreased levels of NO species production post ALI exposure. Overall, our data suggest that 1-day ALI Crème Brulée-flavored aerosol exposure alters levels of oxidative stress in macrophages, including at the transcriptional level, through dysregulation of oxidative stress-related genes.

3.9. 3-day ALI Crème Brûlée-flavored ENDS aerosol exposure is cytotoxic and increases levels of oxidative stress in macrophages compared to air-exposed control cells

After exposures that lasted 1 h/day for 3 consecutive days, we observed that macrophages exposed at the ALI to Posh and e-cig Crème Brûlée-flavored aerosols displayed significant cytotoxicity marked by decreased cell viability ($\geq$ 50%) (Fig. 9A) and increased LDH levels ($\geq$ 150%) (Fig. 9B) in both devices compared to air controls. Moreover,

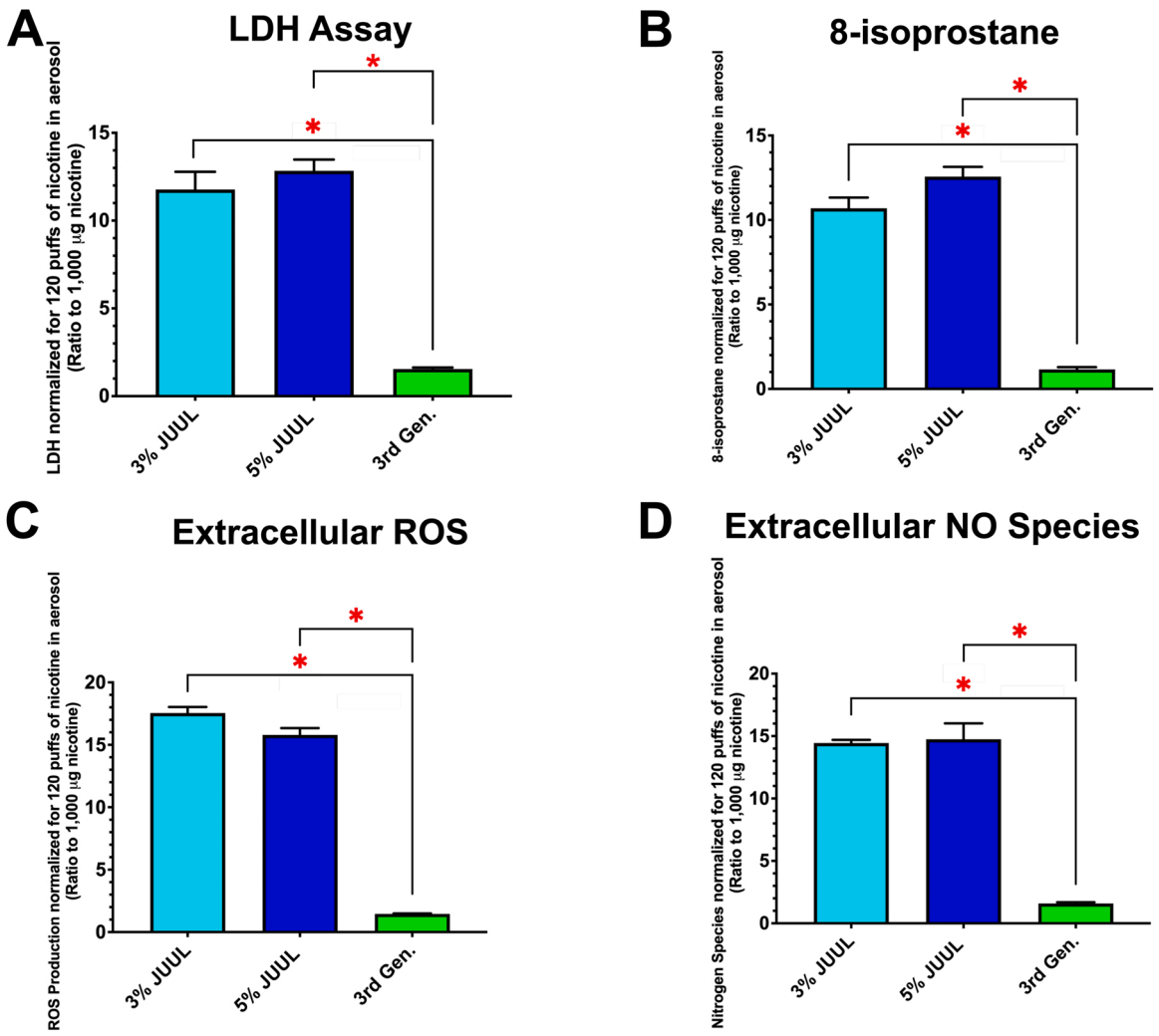


Fig. 4. After normalization to the estimated total nicotine exposure, menthol-flavored JUUL aerosols lead to increased toxicity compared to 3rd generation e-cig aerosols. We estimated the nicotine content for each condition based on the 'equivalent nicotine delivery capacity'. Based on the results in Fig. 1C, the results from Fig. 2 were normalized to the estimated total nicotine exposure in aerosol and are expressed as ratios to 1000 μg of nicotine, allowing for toxicity comparison of devices based on nicotine exposure. One-day ALI exposure to 3% and 5% JUUL aerosols causes (A) a significant increase in extracellular release of LDH, (B) a significant increase in extracellular 8-isoprostane levels, (C) a significant increase in extracellular ROS species production; and (D) a significant increase in NO species production, compared to 3rd Generation e-cig aerosols. One-way ANOVA, followed by a Tukey's post-hoc test, was used to compare results between ENDS aerosol-exposed cell groups. Data represent the mean ± SEM for n = 3 independent experiments, each with 3 technical replicates per group. * $p < 0.05$ statistically different.

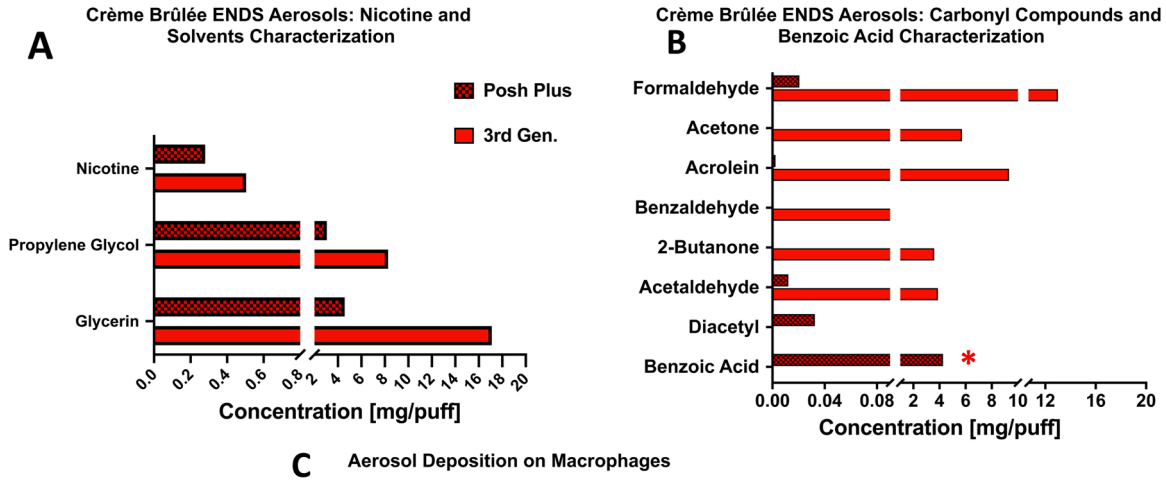
both Posh and e-cig Crème Brûlée-flavored aerosols elicited high levels of 8-isoprostane (≥150 pg/mL; Fig. 9C), including a ≥ 50% increase in ROS and NO levels compared to air controls (Fig. 9D and E), indicating increased oxidative stress. Together, the data from Fig. 7 & 9 show a time-course response regarding increased redox imbalances in macrophages that are exposed at the ALI to Crème Brûlée-flavored ENDS aerosols.

3.10. Based on estimated total nicotine exposure, crème-brûlée-flavored Posh Plus aerosols lead to increased toxicity compared to 3rd generation e-cig aerosols

Using the concept of 'equivalent nicotine-delivery capacity' of the devices, after normalizing the results from Figs. 7 and 9 to nicotine exposure, for the Posh Plus aerosols, we found a significant exposure-response relationship (1-day vs. 3-day) for all outcomes evaluated: extracellular release of LDH, 8-isoprostane, ROS, and NO (Fig. 10). After three-day of exposure, the release of extracellular LDH and NO was significantly increased following the exposure to the Posh Plus aerosols compared to the 3rd-Generation e-cig aerosols (Fig. 10). Overall, similarly to menthol-flavored ENDS aerosols (Fig. 4), our results indicate that Crème Brûlée flavored ENDS aerosols generated by a 4th-generation device are more detrimental to murine macrophages than aerosols generated by a 3rd-generation devices (Fig. 10).

3.11. Posh Plus Crème Brûlée flavored aerosols display a mixed Th1/Th2 transcriptomic response and upregulate genes associated with oxidative stress and allergy/asthma in murine macrophages compared to air-exposed control cells

Using a PCR array containing 86 genes that play a role in Th1 and Th2 mediated responses, we found that Posh aerosol dysregulated 31 genes compared to the air-exposed control cells, while only 15 genes were dysregulated by the 3rd-generation e-cig aerosol compared to air controls (Fig. 11). Posh Plus aerosol exposure resulted in the upregulation of genes associated with Th2-type allergy and asthma responses, including *Il-13ra, IL-4ra, IL-12b*, and *Il-18*. (Fig. 11). We also analyzed 7 genes related to oxidative stress responses and extracellular matrix remodeling by qRT-PCR. All 7 genes (*Ahr, Cyp1a1, Nos2, Hmox1, Nqo1, α7nChR, Mmp12*) were upregulated for Posh Plus aerosol compared to



C Aerosol Deposition on Macrophages

Aerosol deposited mass 1-day exposure Dosimetry		Aerosol deposited mass 3-day exposure Dosimetry	
Posh Plus	75.2 μg/cm² ± 17	Posh Plus	70.2 μg/cm² ± 7
E-cig	34.6 μg/cm² ± 14	E-cig	29.5 μg/cm² ± 14

Fig. 5. Crème Brûlée aerosols produced from a 3rd generation e-cig device produces high levels of nicotine, solvents and carbonyls. Aerosols generated from a Posh Plus disposable device using 6% nicotine salt, as well as a 3rd generation e-cig device were tested for (A) solvent (PG and VG) and nicotine concentrations; (B) carbonyl chemical production. Benzoic acid levels were also tested for Posh Plus only* ; (C) the average deposited dose of ENDS aerosols measured in μg/cm^2 using a QCM for 1 day and 3 days ALI exposures. Aerosol dosimetry data represent the mean ± SD.

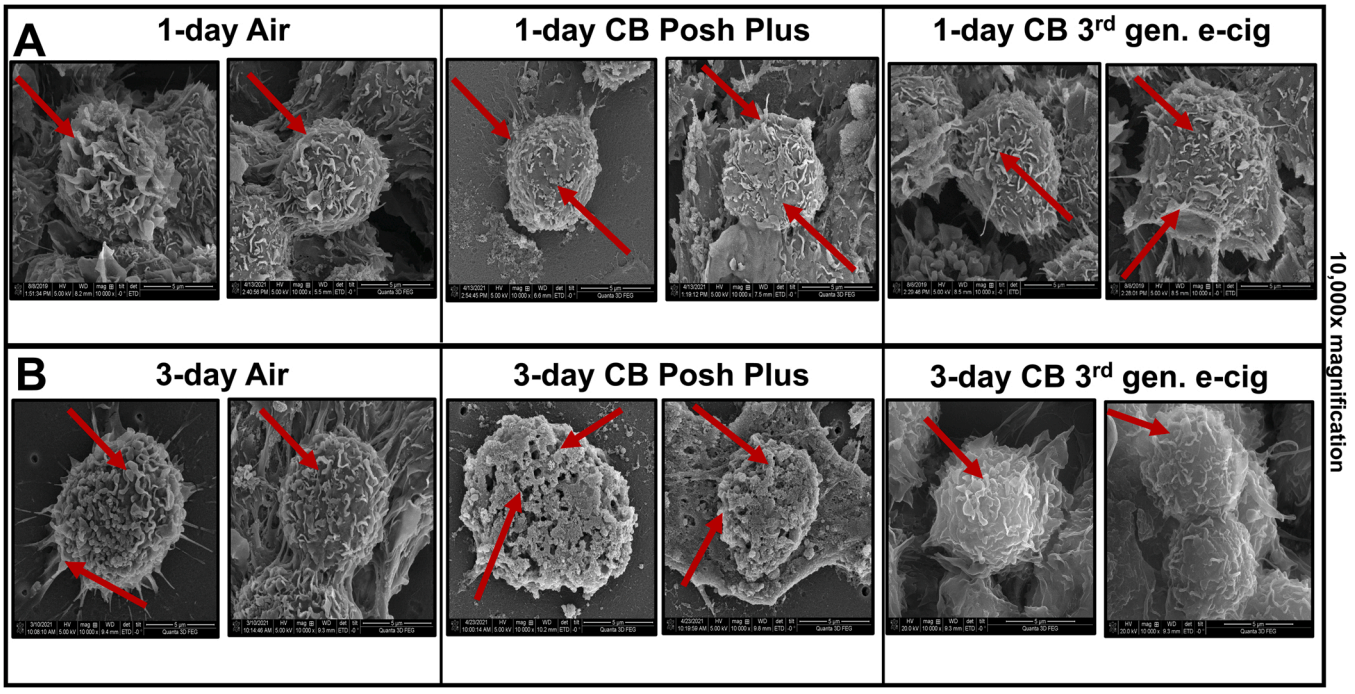


Fig. 6. Crème Brûlée (CB)-flavored ENDS aerosols affect the surface morphology of murine macrophages compared to controls. ENDS Crème Brûlée-flavored aerosol exposure alters cellular surface morphology on cells exposed to Posh Plus (A) after 1 day and (B) after 3 consecutive days of aerosol exposure compared to e-cig exposed cells as indicated by SEM analysis. Changes in external features of the macrophages exposed to CB-flavored ENDS compared to respective Air controls are indicated by red arrows pointing to cellular external structures (dorsal membrane ruffles and lamellipodia/filopodia). Images were taken at 10,000x magnification.

control cells. The comparison of the air-exposed cells with the 3rd-generation e-cig aerosol exposed cells revealed the same pattern of expression except for the α7nChR gene, which was not changed.

To better understand the molecular mechanisms associated with the exposures to ENDS aerosols in Crème Brûlée flavor, we conducted IPA analysis using the expression of dysregulated genes from Fig. 11. IPA analyses revealed that 3-day ENDS exposure is involved with several pathways related to macrophage function, pulmonary injury, oxidative stress, and xenobiotic metabolism (Fig. 12 A-B). For 3rd generation e-cig and Posh Plus, the most notable canonical pathways included: macrophage classical activation signaling, Th1 and Th2 activation, production of NO and ROS in macrophages, *Nrf2* mediated oxidative stress responses, and xenobiotic metabolism signaling. Taken together, our results suggest that Posh Plus and e-cig ENDS aerosols affect similar

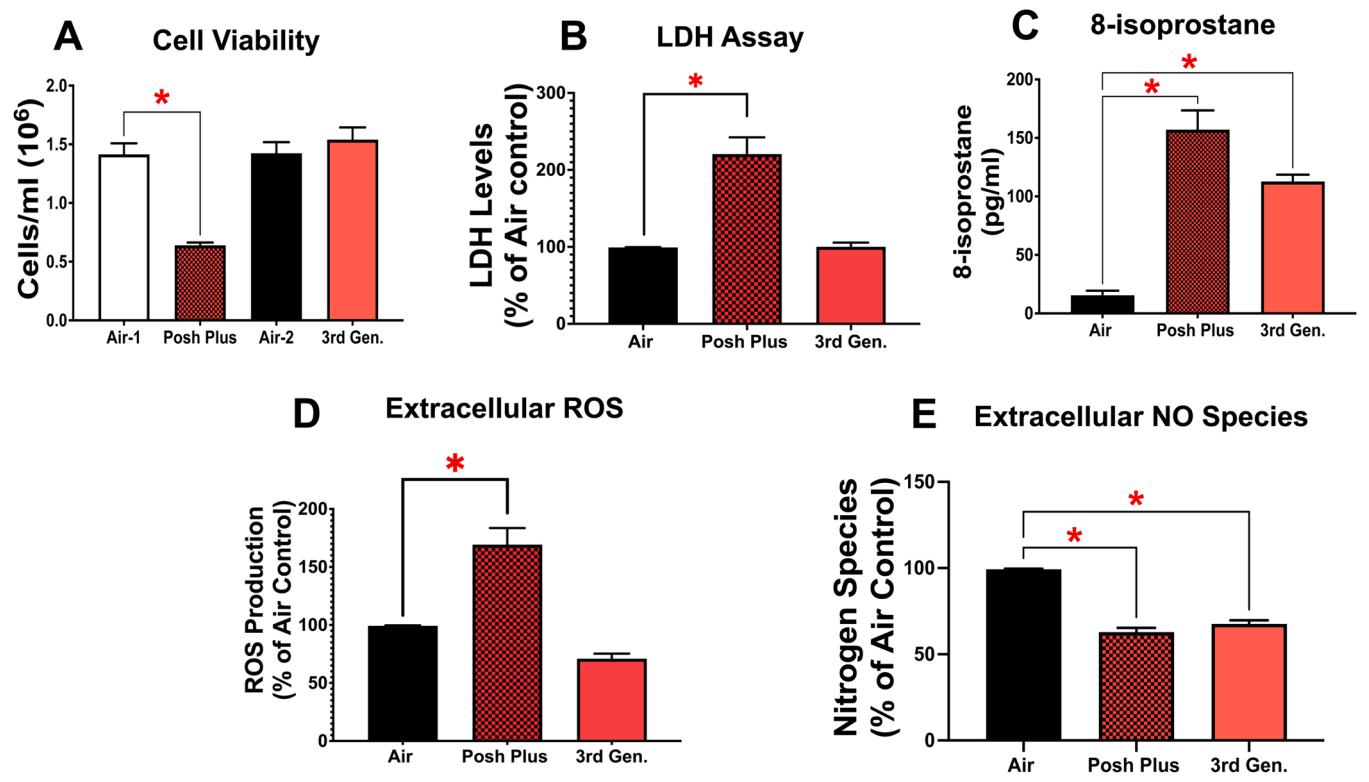


Fig. 7. 1 day ALI Crème Brûlée-flavored ENDS aerosol exposure alters levels of oxidative stress in macrophages under cytotoxic and non-cytotoxic conditions, compared to air controls. One day ALI exposure to 4th generation ENDS Crème Brûlée-flavored aerosols (A) significantly reduces cell viability for Posh Plus as measured by Trypan Blue Exclusion (B) is cytotoxic as indicated by a significant increase in extracellular release of LDH in Posh Plus; (C) Posh Plus and E-cig Crème Brûlée 1-day ALI exposure increases 8-isoprostane levels; (D) increases ROS in Posh Plus and decreases levels in e-cig; (E) reduces NO species levels in both devices. A student's t-test was performed to compare results between ENDS aerosol groups and their respective air controls. Data represent the mean ± SEM for n = 3 independent experiments, each with 3 technical replicates per group. * $p < 0.05$ statistically different from air controls.

signaling pathways within macrophages (Fig. 11 & 12).

4. Discussion

Recent reports indicate that ENDS aerosols have detrimental effects on respiratory immune responses, including impaired macrophage function [101,17,39,40,63,64,85,92]. To our knowledge, we are the first to report in vitro responses based on similar vaping topography profiles of 3rd and 4th generation ENDS devices in Crème Brûlée and Menthol flavors. In the present study, we employed a physiologically relevant in vitro ALI exposure model to assess the immunotoxicity of Menthol and Crème Brûlée-flavored aerosols produced by ENDS devices from 2 distinct generations. We used a 3rd generation e-cig, and two 4th generation devices (JUUL pod-based and Posh Plus disposable) to expose murine macrophages (RAW 246.7) to these ENDS aerosols for 1-hour for 1 day or for 1-hour for 3 consecutive days. After normalization to total estimated nicotine exposure, our results show that murine macrophages are more sensitive to Menthol and Crème Brûlée-flavored aerosols produced by a 4th generation device compared to exposure from a 3rd generation device after 1-day of exposure (Figs. 4 and 10). One-day exposure to Menthol-flavored aerosols was cytotoxic and affected oxidative metabolism, as measured by ROS, 8-isoprostane and NO in both 3rd generation and JUUL (3% and 5% nicotine salt; Fig. 2) devices compared to air-exposed control cells. Posh Plus aerosol short-term exposure (1-day) was cytotoxic, increased levels of oxidative stress and seems to lead to macrophage activation compared to controls (Fig. 7), whereas exposure to e-cig seems to suppress signaling involved in macrophage function compared to air-exposed cells (Fig. 7). Moreover, exposure to Crème Brûlée-flavored aerosols for 3-days produced from both devices was cytotoxic, and increased levels of oxidative stress, accompanied by increased ROS, 8-isoprostane and NO levels compared to controls (Fig. 9). At 3-days, Posh Plus was detrimental to macrophages. These effects also were supported at the molecular level with both devices dysregulating the expression of genes related to xenobiotic metabolism and oxidative stress, as well as to allergy and asthma (Figs. 11 & 12). Overall, our results indicate that flavored aerosols combined with nicotine from a 3rd or 4th generation device, have suppressive effects on macrophage inflammatory responses, and additionally show that this occurs while oxidative stress pathways are stimulated.

The design and power output of ENDS devices can have a major influence on aerosol generation, as well as on the production of chemical constituents that are emitted into the aerosol [89,94]. The concentration of nicotine salt in JUUL pods (3% −35 mg/mL, 5% −59 mg/mL) [43] and Posh Plus (6%−60 mg/mL), is higher than that found in traditional cigarettes (~2 mg/stick) or other freebase nicotine containing e-liquids (3–36 mg/mL) [44,7,8]. When testing the levels of nicotine within the aerosol for the 4th generation devices, we found that JUUL 3% and 5% nicotine salt concentrations for Menthol pods produced lower levels (0.0735 mg/mL and 0.0987 mg/mL, respectively) than Posh Plus (0.280 mg/mL) (Fig. 1A & 5A). The levels of nicotine in JUUL coincide with ranges previously reported (up to~170 μg/puff) [24,56,79,81,95]. Nicotine salt formulations contain benzoic acid, a respiratory irritant. Although from the same ENDS generation, we found that Posh Plus contained higher levels of benzoic acid (4.27 μg/puff) compared to JUUL 3% (0.97 μg/puff) and 5% (0.0987 μg/puff) (Fig. 1B & 5B). Upon analyzing concentrations of aerosolized freebase nicotine generated by a 3rd-generation e-cig device, we found that nicotine levels for Menthol (0.776 mg/puff) were higher compared to Crème Brûlée (0.504 mg/puff) (Fig. 1A & 5A). Although we observed that aerosol concentrations of nicotine salt are lower than freebase nicotine, previous findings indicate that nicotine salt has a higher rate of absorption [24,

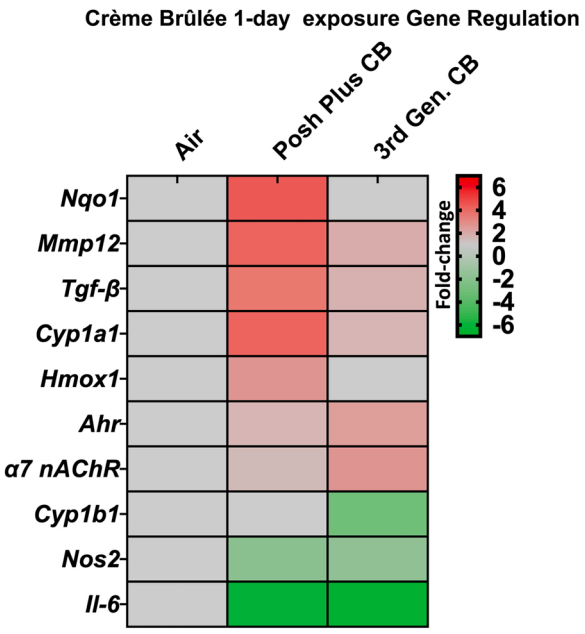


Fig. 8. Crème Brûlée-flavored ENDS aerosols dysregulate genes involved in cellular biotransformation and oxidative stress compared to air-exposed cells. A heatmap displaying the regulation of genes involved in oxidative stress, inflammation, and airway remodeling. Data is presented as fold-change compared to respective air-control group. Samples from each experiment were pooled for RNA collection, and data is representative of 3 independent experiments performed in triplicate. Fold-changes $> |1.5|$ were considered significant. Red denotes up-regulation and green denotes down-regulation.

32,71]. Our results show that under similar vaping topography profiles, the Posh Plus device generates a denser cloud with a higher deposited dose at the surface of the cells than the ENDS aerosols produced by third-generation devices (Fig. 5C). Together, our current findings suggest that for the same e-liquid flavor, even though concentrations of aerosolized nicotine are lower when produced by a 4th-generation ENDS device, operating at low power, as compared to a 3rd-generation device, operating at high power, a phenomenon previously reported by others [37,100], the cellular macrophage toxicity is greater following exposure to 4th-generation ENDS aerosols (Figs. 4 and 10). These data therefore imply that the ENDS aerosol toxicity may be driven by the presence of benzoic acid, and thus the nicotine chemical form, i.e., salt versus freebase, used in the e-liquid.

In addition, decomposition of solvents and flavorings during the heating of the e-liquids through the ENDS device, generates hazardous carbonyl chemicals, including acrolein and formaldehyde [24,89]. It was previously demonstrated that carbonyl emissions are lower when generated by closed-system devices (low power) and higher when generated by open system devices (high power) [4,67,89]. Our results (Fig. 1B & 5B) are in line with these findings, as we observed that carbonyl concentrations measured in Menthol- and Crème Brûlée-flavored aerosols produced from the 3rd generation device were much higher compared to both JUUL and Posh Plus 4th generation devices with the same flavor (Fig. 1B & 5B). Also, Crème Brûlée e-liquid aerosolized through the 3rd generation device produced higher levels of carbonyls than Menthol (Fig. 5B). Menthol is a simpler flavor usually composed of ≤ 4 flavoring chemicals, whereas Crème Brûlée-flavored e-liquids have a combination of vanillin and ethyl maltol along with ≥ 6 additional constituents, therefore, Crème Brûlée flavor is a more complex mixture of flavoring chemicals [30,48,71]. These findings suggest that production of high concentrations of carbonyl compounds generated from a 3rd generation ENDS device is flavor specific (Fig. 1B & 5B).

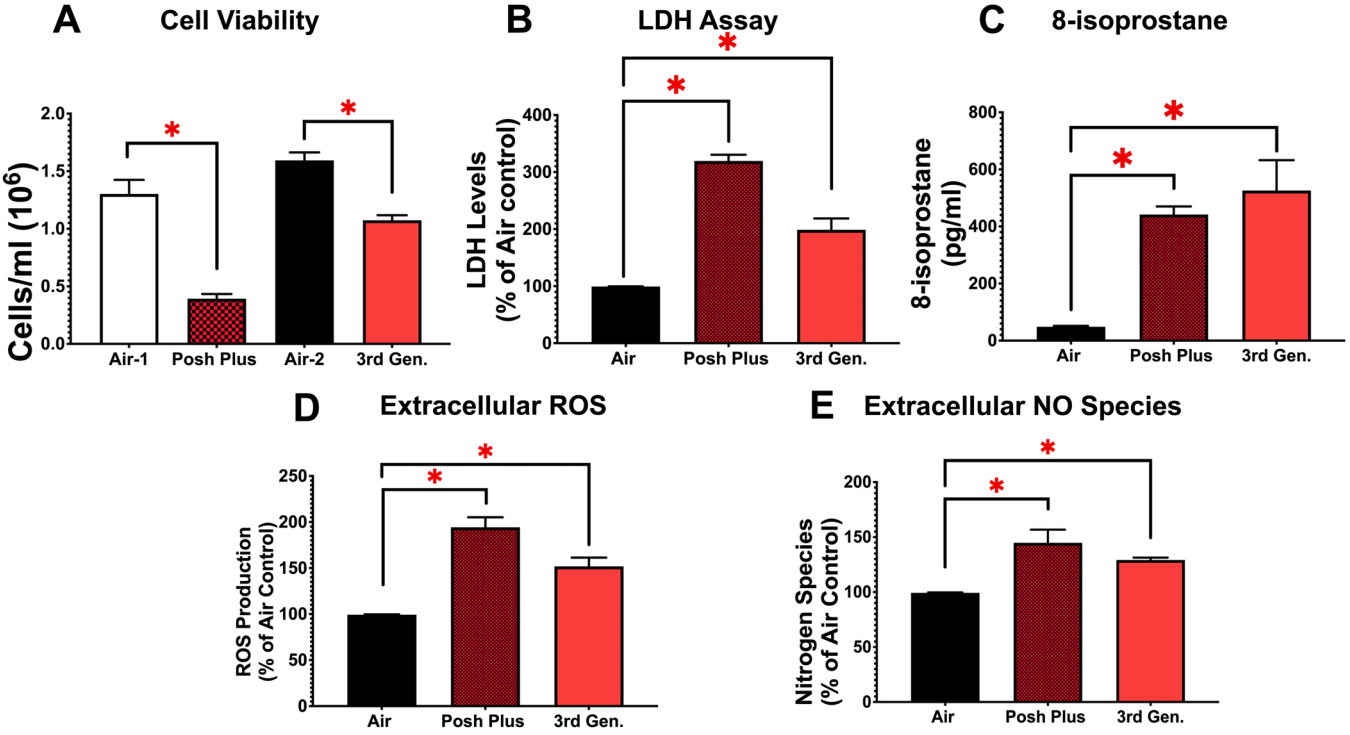


Fig. 9. 3-day ALI Crème Brûlée-flavored ENDS aerosol exposure is cytotoxic and increases levels of oxidative stress in RAW macrophages, compared to air controls. Posh Plus and E-cig Crème Brûlée (A) significantly reduced cell viability; (B) and were cytotoxic as indicated by extracellular release of LDH; (C) increased extracellular levels of 8-isoprostane, (D) increased extracellular ROS production, and (E) increased NO species levels in both ENDS devices. Each experiment was repeated 3 independent times, each with 3 technical replicates. A student's t-test was performed to compare results between ENDS aerosol groups and their respective air controls. Data represent the mean ± SEM for n = 3 independent experiments, each with 3 technical replicates per group. * $p < 0.05$ statistically different from air controls.

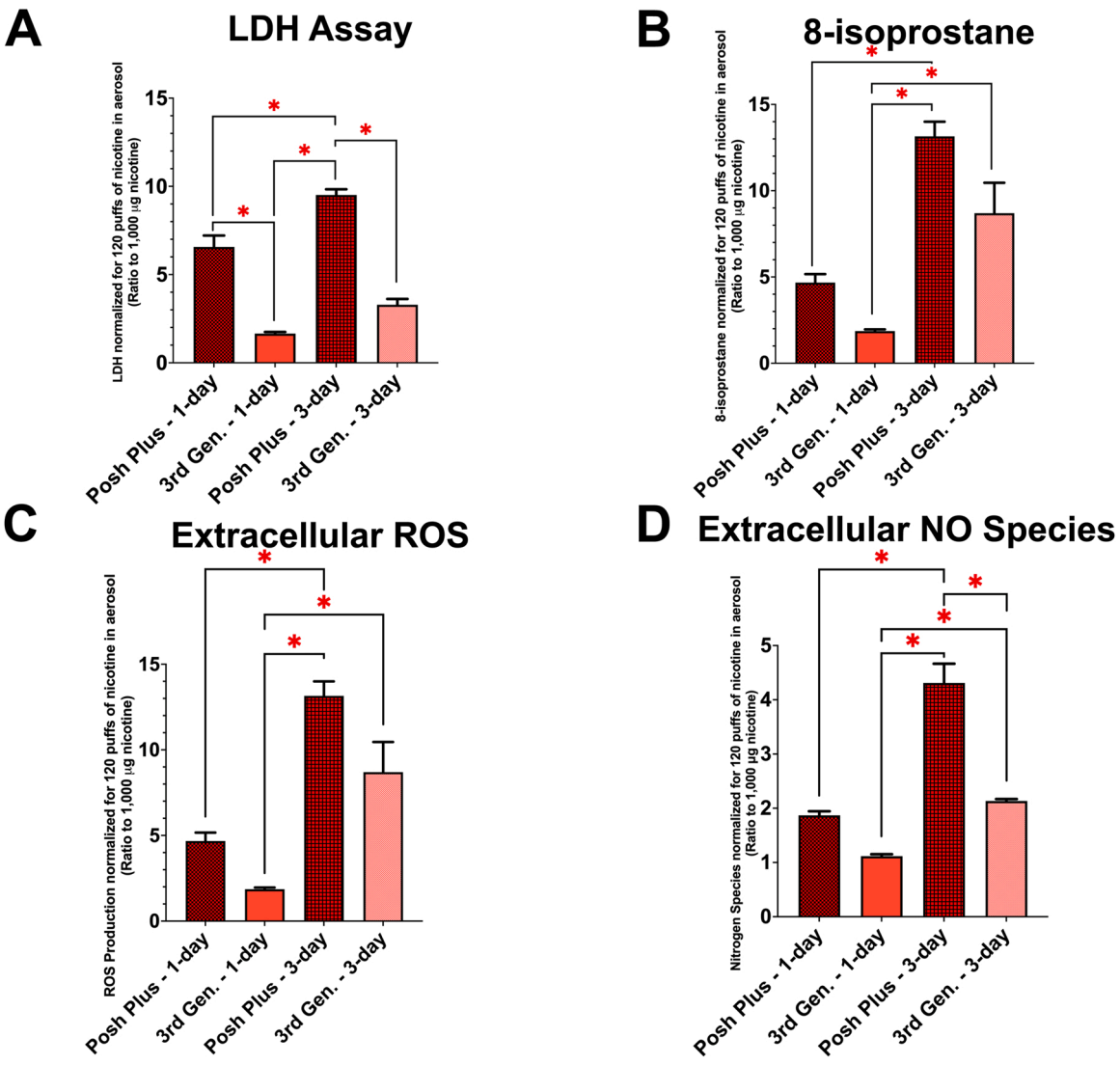


Fig. 10. After normalization to the estimated total nicotine exposure, Crème-Brûlée-flavored Posh Plus aerosols lead to increased toxicity compared to 3rd generation e-cig aerosols. Using the concept of 'equivalent nicotine-delivery capacity' of the devices, we normalized the results obtained from the different ENDS devices (Fig. 7 & 9) on a nicotine exposure basis. For the Posh Plus aerosols, we found a significant exposure-response relationship (1-day vs. 3-day) for all outcomes evaluated: extracellular release of LDH (A), extracellular levels of 8-isoprostane (B), extracellular ROS species production (C), and extracellular NO species production (D). After three-day of exposure, the release of extracellular LDH (A) and NO species (D) was significantly increased following the exposure to the Posh Plus aerosols compared to the 3rd-Generation e-cig aerosols. One-way ANOVA, followed by a Tukey's post-hoc test, was used to compare results between ENDS aerosol-exposed cell groups. Data represent the mean ± SEM for n = 3 independent experiments, each with 3 technical replicates per group. * $p < 0.05$ statistically different.

Moreover, for the same flavor, after normalization to nicotine exposure, we found heightened in vitro toxicity due to exposure to ENDS aerosols produced by 4th-generation ENDS compared to 3rd-generation ENDS (Figs. 4 and 10). These data also re-emphasize that ENDS aerosol carbonyl content may not be the main contributor to the aerosol toxicity, but rather the nicotine chemical form (free base vs. salt) may play a major role within our in vitro exposure system.

Further, we previously reported the effects of 1-day JUUL Crème Brulée-flavored aerosol exposures on macrophages exposed at the ALI [79]. Although similar vaping topography settings were used in our previous study [79] and in the current study, the deposited dose of Posh Plus Crème Brûlée aerosol at the surface of the cells was much higher than we what previously reported for JUUL Crème Brûlée aerosol (75.2 µg/cm^2 ± 17 vs. 15.8 µg/cm^2 ± 0.17, for Posh Plus and JUUL aerosols, respectively) after 1-day (Fig. 5 C; and [79]). Thus, even though these ENDS aerosols were produced by two 4th-generation devices using the same Crème Brûlée flavor and the same vaping topography, there is nearly a 5-fold difference in the ALI deposited dose generated by the Posh Plus device. ALI exposures allow cells to be exposed to all the ENDS aerosol components, including the particulate and gas phases [49,75,104]. In these studies, we evaluated the chemical profiles of the ENDS aerosols (Fig. 5; and [79]) and showed that, with the exception of benzoic acid concentration, there were minimal differences in terms of concentrations for nicotine, solvents, and carbonyls, while the total particulate matter deposited at the surface of the cells, which was determined by the microbalance, showed notable differences (Fig. 5 C; and [79]). Thus, for the same flavor tested (Crème Brûlée), with two devices from the 4th-generation, one re-usable and one disposable, the ENDS aerosols showed similar chemical profiles with distinct amount of aerosol droplets/particulates produced (Fig. 5C; [79]). The reason for this difference is currently unknown but may be associated with the Posh Plus device having different operational settings (atomizer resistance, battery voltage, and power) and/or different solvent ratios than JUUL devices, enabling the production of larger and more dense aerosols. As the popularity of 4th generation disposable devices continue to increase among youth, more research is needed to

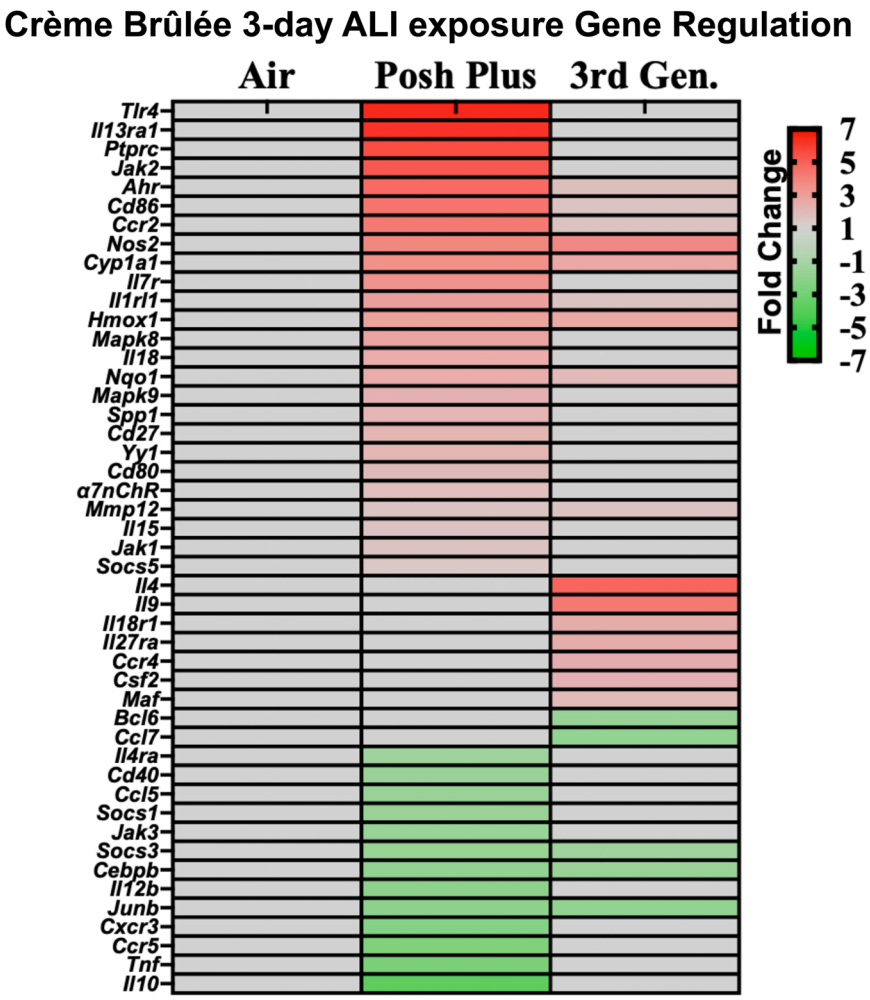
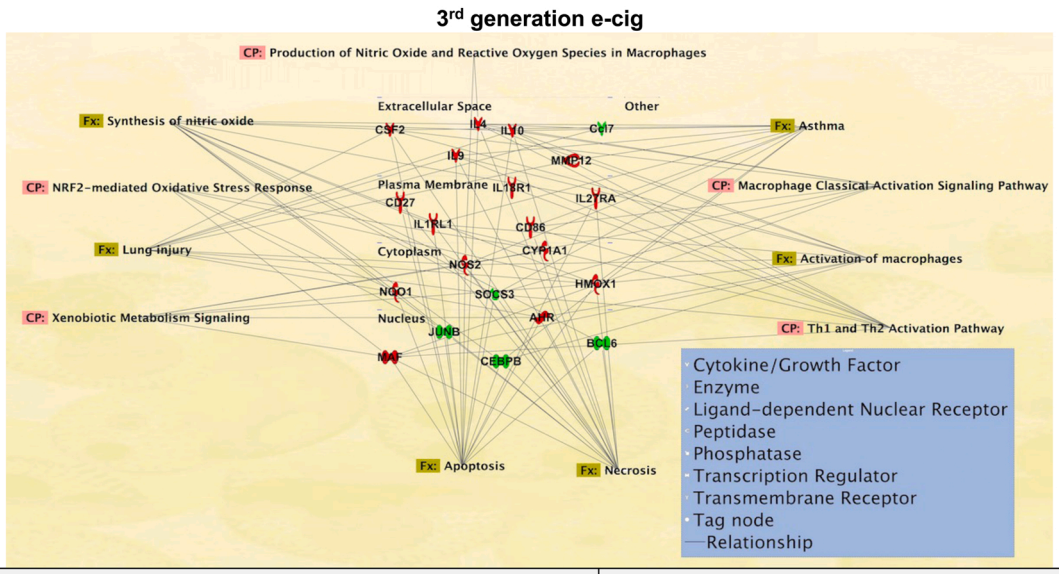


Fig. 11. Posh Plus Crème Brûlée-flavored aerosols display a transcriptomic Th1/Th2 response and upregulates genes associated with oxidative stress and allergy/asthma in murine macrophages, compared to air-exposed cells. A heatmap displaying transcriptional expression of genes related to Th1 and Th2 mediated responses, along with additional genes related to oxidative stress. Data compiled from 3 independent experiments each performed in triplicate. Fold-changes > |1.5| were considered significant. Red denotes up-regulation and green denotes down-regulation.

investigate on ENDS aerosols generated from newer 4th generation devices.

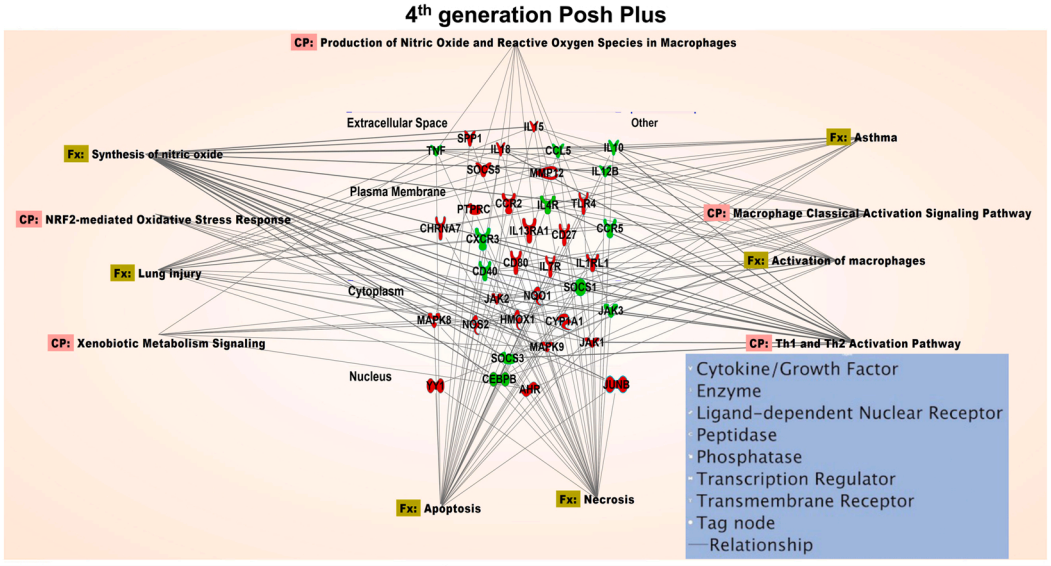
In terms of in vitro toxicity, for 1-day Menthol ALI exposure, we observed that JUUL 3% and 5% nicotine salt pods resulted in significantly reduced cell viability compared to air controls (Fig. 2A). Cell viability was also reduced by 3rd gen e-cig Menthol exposure, although this effect was not significant compared to controls (Fig. 2A). Only JUUL 5% and 3rd generation e-cig aerosols exhibited significantly elevated LDH levels compared to air-exposed cells (Fig. 2A, B). The extracellular release of LDH suggests that these Menthol aerosol exposures mainly resulted in necrotic cell death, possibly due to plasma membrane disruption [14]. This is supported by our results of reduced cellular viability/increased membrane permeability, as evidenced by the trypan blue exclusion assay results for the JUUL 5% aerosol (Fig. 2A). As for the significant reduction in cell viability without significantly increasing extracellular levels of LDH following JUUL 3% Menthol exposure compared to controls (Fig. 2A, B), this may be due to apoptotic rather than necrotic cell death, the former occurring without loss of membrane integrity [23,46]. These results are in accordance with previous studies using either nicotine free Menthol-flavored e-liquid extract or flavorless e-cig vapor condensate on macrophages, which have reported cytotoxicity, occurring either through necrotic cell death, or the occurrence of both necrosis and apoptosis [64,85,86]. Thus, our results (Fig. 2) along with the above published data [64,85,86] suggest that macrophages could undergo different modes of cell death following exposures to ENDS aerosols, including those containing Menthol flavorings. Although ROS/NO both play important roles in macrophage homeostasis at low levels [15,21], production of ROS/NO in higher quantities can cause oxidative stress. Also, we found that Menthol ENDS exposure from both devices increased extracellular production of 8-isoprostane compared to controls (Fig. 2C). 8-isoprostane is a biomarker of oxidative stress that measures lipid peroxidation of arachidonic acid present within the cell membrane phospholipid bilayer [15]. JUUL 3% Menthol downregulated two genes involved in ROS (*Hmox1* and *Nqo1*) compared to air-exposed cells (Fig. 3). These two genes are induced by oxidative stress and play key roles in protecting the cell from oxidative stress damage. Thus, our results for JUUL 3% Menthol suggest that this ENDS aerosol inhibits the antioxidant protective roles of the protein-coding genes *Hmox1* and *Nqo1* (Fig. 3). It is important to bear in mind that these gene expression results reflect the levels of intracellular antioxidant defense, while this contrasts with the elevated ROS levels measured in the cell media (Fig. 2D), which mirrors extracellular ROS levels. These results could be explained by the fact that JUUL 3% Menthol significantly increased cytotoxicity compared to the air controls (Fig. 2A) and that cell death is a potent inducer of extracellular ROS [97]. Overall, these results suggest that the increased extracellular ROS measured in the media of the macrophages exposed to JUUL 3% Menthol (Fig. 2D) is secondary to the increased cytotoxicity (Fig. 2A) following this exposure, rather than a direct effect on the intracellular ROS levels (Fig. 3) from the viable cells. In addition to oxidative stress related genes, we evaluated the expression of 10 genes related to nicotine and xenobiotic metabolism, inflammation, and airway remodeling (Fig. 3). JUUL 5% Menthol and 3rd generation e-cig Menthol compared to air-exposed cells, upregulated 7 genes, including *α7nChR* (4.3 vs. 2.8-fold, respectively), which confirms high level of nicotine exposure, while only downregulating *Il-6,* which suggests that inflammatory pathways are weakly stimulated.

A



Canonical pathway (CP)	Associated genes
Production of nitric oxide and reactive oxygen species in macrophages	IL4; NOS2.
Macrophage classical activation signaling pathway	IL4; IL10; CSF2; CD86; NOS2; SOCS3; MAF.
Th1 and Th2 activation pathway	IL1R1L1; IL4; IL9; IL10; IL18R1; IL27RA; CD86; SOCS3; MAF.
Xenobiotic metabolism signaling	CYP1A1; AHR; HMOX1; NQO1; NOS2; MAF.
NRF2-mediated oxidative stress response	CYP1A1; HMOX1; NQO1; JUNB; MAF.

B



Canonical pathway (CP)	Associated genes
Production of nitric oxide and reactive oxygen species in macrophages	JAK1; JAK2; JAK3; MAPK8; MAPK9; NOS2; TLR4; TNF.
Macrophage classical activation signaling pathway	CCL5; CD40; CD80; IL10; IL12B; IL13RA; IL15; IL18; JAK1; JAK2; NOS2; SOCS1; SOCS3; TLR4; TNF; YY1.
Th1 and Th2 activation pathway	CCR5; CD40; CD80; CXCR3; IL1RL1; IL4R; IL10; IL12B; IL18; JAK1; JAK2; JAK3; SOCS1; SOCS3.
Xenobiotic metabolism signaling	AHR; CYP1A1; HMOX1; MAPK8; MAPK9; NOS2; NQO1; TNF.
NRF2-mediated oxidative stress response	CYP1A1; HMOX1, JUNB; MAPK8, MAPK9, NQO1.

Fig. 12. A-B: Ingenuity pathway analysis reveals that 3-day Posh Plus and e-cig aerosol exposures are associated with networks of genes that affects macrophage function, xenobiotic metabolism, and pulmonary diseases. Gene interaction networks altered by 3-day ENDS aerosol exposure are correlated with several pathways that impact macrophage function and pulmonary health outcomes. CP: canonical pathway; Fx: gene-to-function relationship.
[Reprinted with permission from QIAGEN Silicon Valley.].

Downregulation of *Tgf-β*, coincides with this result since this gene regulates anti-inflammatory responses in macrophage function [83]. Upregulation of oxidative metabolism genes *(Nqo1, Hmox1, and Nos2)* suggests that JUUL 5% and 3rd generation e-cig Menthol exposure, compared to controls, results in little or no inflammation while oxidative stress pathways are stimulated.

After 3-days of exposure, 3rd generation Crème Brulée e-cig aerosol became toxic to macrophages compared to controls, while Posh Plus

induced significant toxicity starting at 1-day compared to air-exposed cells (Figs. 7A-B, 9A-E). Through further qualitative investigation of macrophage morphology evaluated via SEM, we observed that compared to air control cells, macrophages after 1 and 3 days of exposure to Posh Plus exhibited damage to the external surface (Fig. 6). Macrophages are sentinel cells that continually scan the extracellular environment for antigens and nutrients. Damage to macrophage structures is shown in Fig. 6, which suggest that Posh Plus may impair overall macrophage function. Several studies have reported impairment of macrophage phagocytic activity due to ENDS exposure [101,17,40,85,92], and provides further support for this finding. In addition, Crème Brûlée flavor contains ethyl maltol and vanillin, which are often used in sweet and dessert flavored e-liquids [48,71]. Several studies have reported vanillin and ethyl maltol to be detrimental to cellular function and structure, including cytotoxicity, oxidative stress, inflammation, along with barrier dysfunction in lung cells [3,30,63,87]. This suggests that Crème Brûlée flavored ENDS aerosols are detrimental to macrophages and other lung cells, and may lead to impaired pulmonary homeostasis.

Additionally, we found that 3-days of exposure to Posh Plus and 3rd generation e-cig Crème Brûlée, compared to air controls, resulted in increased production of ROS/NO as well as increased production of 8-isoprostane (Fig. 9). Similarly to the results of the 1-day exposure, these data are also supported by the dysregulation of the genes *Nos2*, *Hmox1* and *Nqo1*, compared to air-exposed cells (Fig. 11). These data are in line with other studies that have shown that ENDS exposures cause oxidative stress responses in lung cells [29,51,63,64,68,79,85]. Moreover, our results for increased levels of 8-isoprostane in vitro coincide with a study conducted by Carnevale et al. [12] showing that people who use ENDS devices have increased biomarkers of oxidative stress/injury, including 8-isoprostane in blood [12]. Collectively, our data suggest that disruption of oxidative metabolism in macrophages plays a major role in Crème Brûlée-flavored ENDS aerosol toxicity, and this may lead to lung injury with prolonged exposure.

It is well-known that macrophages have a high level of plasticity, which enables them to quickly polarize to Th1/M1 classically activated (proinflammatory) phenotype or Th2/M2 alternatively activated (anti-inflammatory/pro-fibrotic phenotype), following exposures to environmental stressors [11]. Several studies have reported contradictory results suggesting that ENDS exposure can result in the induction of a pro-inflammatory response [30,51,63,64,85], while others suggest no inflammation, but rather, in some cases, even suppression [101,36,6,58,59,66]. These differential responses may be ENDS aerosol flavor specific. We found that after 3-days, Posh Plus aerosol upregulated 18 genes and downregulated 13 genes (fold-change range: 6.4 to −3.8) related to Th-1 and Th-2 pathways, compared to controls, while comparison of air-exposed cells with 3rd generation e-cig aerosol showed upregulation of 10 genes and downregulation of 5 genes (fold-change range: 3.69 to −2.2) related to these same pathways (Figs. 11–12). IPA results further highlighted the involvement of the Th1 & Th2 canonical pathways (Figs. 11–12). It was previously reported that M1 and M2 macrophages remain in a mixed population under normal conditions, and a shift to either polarized state could lead to the development of injury or disease [97]. From this study, it is difficult to determine whether the murine macrophages exposed to the Crème Brûlée-flavored ENDS aerosols generated from both devices polarized into a M1 or M2 phenotype, as the gene expression results did not display a clear direction for either polarization (Fig. 11 & 12). Nonetheless, collectively, these results suggest that prolonged exposures to Crème Brûlée-flavored ENDS aerosols may be detrimental to lung homeostasis through an imbalance of M1-M2 polarization; a phenomenon that is often related to pathological lung conditions, including fibrosis [55].

Finally, a recurring effect among a majority of the ENDS aerosol exposures conducted in this study includes the finding that in our in vitro exposure system, the macrophages exposed at the ALI exhibited signs of oxidative stress, without any significant sign of inflammatory responses (Figs. 1 to 12). This was unexpected, as oxidative stress and inflammation are two linked biological processes induced by environmental insults/injury, and are usually present together [99]. While dysregulated cellular redox homeostasis can initiate the inflammatory signaling cascade [99], exposures to ENDS aerosols have previously been shown to suppress immune responses and inflammation [101,36,6,58,59,66], as seen in our study (Figs. 2 to 12). Hence, the potential mechanisms of the ENDS aerosols toxicity observed in the current study may involve 'oxinflammation', a pre-pathological condition featuring pro-oxidant responses that correlate with sub-clinical (or low-grade) inflammatory responses [99]. This pre-disease state is due to the dysfunction of the inflammatory response regulatory negative feedback network, which in turn, with the constant exposure to the environmental stressor, results in increased oxidative stress/redox signaling, and thus a pro-oxidant environment, while only generating sub-clinical or low-grade inflammation [99]. The chronic presence of a pro-oxidant milieu and of sub-clinical inflammation that evades the inflammation resolution stage, due to dysfunctional negative feedback regulation, has been shown to characterize several diseases, including cardiovascular diseases and breast cancer [99,102,22,77,80]. Whether ENDS aerosols generated from our in vitro model system affected the inflammatory response regulatory negative feedback network in macrophages is currently unknown, but future investigation is merited to better understand the mechanisms by which ENDS aerosols induce pulmonary toxicity.

Limitations.

Although our study has many strengths, including: 1) the characterization of the ENDS aerosols for nicotine, PG, VG, as well as for a selection of carbonyls and organic acids; in addition to 2) determining the deposited dose of ENDS aerosols at the surface of the cells (μg/cm^2); plus 3) establishing a dose-response relationship for JUUL 3% and JUUL 5%, as well as an exposure-response relationship (or repeated daily exposures, 1 day versus 3 days, for Crème Brûlée-flavored ENDS); and 4) using the gene expression for the nicotinic receptor as a biomarker of cellular nicotine exposure; our study has a few limitations. Even though ENDS devices have common features, including the operational parts of the device and e-liquid ingredients, specific ENDS device characteristics, as well as those of the e-liquid use, will vary from device-to-device, and therefore lead to variable quantity of nicotine emitted by each device [37]. Thus, the use of a common normalizing factor between those ENDS devices is difficult to establish, even if 'equivalent nicotine-delivery capacity' of the device is used as a normalizing factor. It was previously shown that high power ENDS devices (e.g., third generation e-cig device), deliver significantly more nicotine to users in comparison to low power ENDS devices (e.g., JUUL device) [37,100]. Further, for low-powered ENDS devices, the nicotine apportioning between the gas and the particulate phases of the aerosol was 40% and 60%, respectively, whereas for high-powered ENDS devices, 95% of the delivered nicotine was in the particulate phase [49]. Although we characterized the chemical profiles and the total particulate matter deposited at the surface of the cells, since ALI exposes cells to both the gas phase as well as the particulate phase of the ENDS aerosol, and nicotine is present in both of those phases [49], we cannot establish with certainty the amount of nicotine that was delivered to the cells within our in vitro exposure system. This would have required additional analyses of droplet sizes and the nicotine concentration within those droplets by chromatography techniques, which exceeded the scope of this toxicological study. Thus, these facts make the use of a common normalizing factor, based on the equivalent nicotine delivery capacity of the device, a complex issue. However, our results comparing the various ENDS aerosols to their respective air controls, are valid and informative, as they provide valuable insight on how a vaping product could affect cellular and molecular responses compared to abstinence.

Furthermore, the concept of puffing frequency, which is associated with e-liquid concentration as well as e-cig device operational settings, is linked to compensatory puffing behavior. It was previously reported

that ENDS users compensate by changing their vaping topography by taking longer and deeper puffs when exposed to low levels of nicotine (6 mg/mL) compared to higher levels (24 mg/mL of nicotine) [19]. As expected, using standard vaping topography profiles, as usually done in experimental settings, does not take into account compensatory puffing behavior, which leads to different nicotine delivery to the ENDS user. Because compensatory vaping behavior is subjective, the concept of puffing frequency is hard to capture within a standardized experiment, but may impact real-world ENDS toxicity. In addition, as expected, an in vitro model using a mono-culture of macrophages will not recapitulate the entire range of lung responses, since it does not include the interactions of the various cells from the lung epithelium, which all contribute to the defense and response mechanisms of the lungs. Overall, the results presented in this study may not be generalizable to all types of ENDS devices, including those studied herein. These data may be flavor-, cell type- (murine macrophages), as well as exposure condition-specific.

In summary, by employing a physiologically relevant in vitro ALI system to expose macrophages to 3rd or 4th generation ENDS aerosols, produced under similar vaping topography profiles, we found, for two specific flavors analyzed, Menthol and Crème Brûlée, that cellular responses to both 3rd and 4th generation ENDS aerosols are detrimental to macrophages. After normalization to total nicotine exposure, our results suggest that differential effects between 3rd and 4th generation ENDS may be driven by nicotine salt, as suggested by a study in humans [36]. Thus, although 4th-generation disposable ENDS devices have no adjustable operational settings (low power devices), they can induce cellular toxicity. It is possible that the combination of ethyl maltol, vanillin flavorings, high levels of nicotine salts plus solvents from Posh Plus led to the physiological responses that we observed after 1 and 3-days of exposure (Figs. 6–12). In addition, we found that using simpler flavorings, e.g. Menthol, composed of ≤ 4 flavoring chemicals, is less detrimental to cellular function of macrophages, compared to complex flavoring mixtures like Crème Brûlée, composed of ≥ 6 flavoring chemicals [30,48,71]. More research is needed to better understand the respiratory health impact of vaping products from the third and fourth generations of ENDS.

Funding

Research reported in this publication was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health under Award Number K01HL149053 (AN) (Career Development Award, NHLBI and FDA Center for Tobacco Products, CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or FDA CTP. This research was also supported by Louisiana State University (LSU), School of Veterinary Medicine Start Up Funds to AN, and by a grant to AP from the Louisiana Governor's Biotechnology Initiative GBI-BOR#013.

CRediT authorship contribution statement

RP, AP and AN designed the study. Sample preparation and assays were carried out by RP. RP conducted data analysis and was assisted by AN. The manuscript was drafted by RP and revised/edited by AN. All authors approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alexandra Noel reports financial support was provided by National Institutes of Health.

Data Availability

Data will be made available on request.

Acknowledgement

The authors thank Dr. Xiao Wu, Mr. William M. Selig, Ms. Blaire Holliday, and Mrs. Zakia Perveen of the Louisiana State University School of Veterinary Medicine for their excellent technical assistance.

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