The Laryngoscope

Effects of Short-term Electronic(e)-Cigarette Aerosol Exposure in the Mouse Larynx

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Objectives: The effects of electronic cigarettes (e-cigarettes) on the larynx are relatively unknown. This study examined the short-term effects of e-cigarette inhalation on cellular and inflammatory responses within the mouse laryngeal glottic and subglottic regions after exposure to pod-based devices (JUUL).

Methods: Male C57BL6/J mice (8–9 weeks) were assigned to control ($n = 9$), JUUL flavors Mint (JMi; $n = 10$) or Mango (JMa; $n = 10$). JUUL mice were exposed to 2 h/day for 1, 5, and 10 days using the inExpose inhalation system. Control mice were in room air. Vocal fold (VF) epithelial thickness, cell proliferation, subglandular area and composition, inflammatory cell infiltration, and surface topography were evaluated in the harvested larynges. Mouse body weight and urinary nicotine biomarkers were also measured. Chemical analysis of JUUL aerosols was conducted using selective ion flow tube mass spectrometry.

Results: JUUL-exposed mice had reduced body weight after day 5. Urinary nicotine biomarker levels indicated successful JUUL exposure and metabolism. Quantitative analysis of JUUL aerosol indicated that chemical constituents differ between JMi and JMa flavors. VF epithelial thickness, cellular proliferation, glandular area, and surface topography remained unchanged after JUUL exposures. Acidic mucus content increased after 1 day of JMi exposure. VF macrophage and T-cell levels slightly increased after 10 days of JMi exposures.

Conclusions: Short-term e-cigarette exposures cause minimal flavor- and region-specific cellular and inflammatory changes in the mouse larynx. This work provides a foundation for long-term studies to determine if these responses are altered with multiple e-cigarette components and concentrations.

Key Words: e-cigarette, inflammation, mouse model, mucus production, vocal fold epithelium.

Level of Evidence: N/A

***Laryngoscope*, 134:1316–1326, 2024**

INTRODUCTION

It is well known that cigarette smoke (CS) exposure drives the development of benign and malignant laryngeal diseases.[1–3] The mechanisms by which CS affects laryngeal cellular structure and function have been studied in animal models.[4–9] Laryngeal changes related to CS include increased cell proliferation, epithelial and basal cell hyperplasia, alterations in surface topography, changes in mucus gland morphology and composition, and changes in apoptosis cycles.

Electronic cigarettes (e-cigarettes) are lesser understood inhalational agents, with increasing use among youth.[10] E-cigarettes are battery-powered devices that heat and aerosolize a liquid mixture containing a vehicle (propylene glycol [PG] and vegetable glycerin), nicotine, and/or flavorings. Airway mucosal surfaces, including the larynx, are the first tissues to be exposed when the user inhales e-cigarette aerosol. Few studies have examined the direct effects of e-cigarette aerosol on the laryngeal mucosa and associated cell types. Exposure of human-engineered vocal fold (VF) mucosa to e-cigarette extract *in vitro* disrupted innate immune responses and induced epithelial remodeling.[11] E-cigarette extract also reduced human VF fibroblast viability to a cytotoxic level.[12] Preliminary *in vivo* studies have targeted mostly inflammatory responses in the laryngeal mucosa of rodents following e-cigarette exposure. A 1-month exposure in rats revealed no significant changes in epithelial distribution and inflammation,[13] while 4 months of exposure in mice induced elevated levels of the inflammatory cytokine, IL-4.[14]

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Additional supporting information may be found in the online version of this article.

Editor's Note: This Manuscript was accepted for publication on August 29, 2023.

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This study was supported by the National Institutes of Health, National Institute of Deafness and Other Communication Disorders (R21 DC016126 and R01 DC020176), National Heart Lung Blood Institute (R01 HL144388), and a New Century Scholars Research Grant from the American Speech-Language-Hearing Foundation.

The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Meeting Information: This work was presented at the American Laryngological Association's 2023 Annual Meeting at COSM in Boston, Massachusetts.

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DOI: 10.1002/lary.31043

Our long-term goal is to determine whether e-cigarette aerosol exposure contributes to laryngeal disease development. To gain a deeper insight, examining the effects of e-cigarette exposures in the larynx from shorter (~days-weeks) to longer (~months-years) time periods and establishing a timeline of disease progression is imperative. The early impact of e-cigarette exposures on underlying cellular and inflammatory processes in the larynx is currently unknown. The objective of this study is to investigate the short-term exposure effects of JUUL e-cigarette aerosol on the larynx after 1–10 days in a mouse model. Previously, we have successfully developed short-term CS-induced mouse laryngeal injury models.[4–6,15] We found that a similar experimental period can cause region-specific cellular changes within the larynx after CS exposures.[4] In the current study, we hypothesized that short-term e-cigarette exposure would also elicit region-specific cellular responses along with inflammation in the larynx within 1–10 days of exposure. Increased inflammatory cells and oxidative stress have already been reported in the lungs within 2 weeks after e-cigarette exposure, with changes observed as early as 3 days.[16–18] Moreover, we speculate that the possible outcomes from this study could be vital in furthering our understanding of e-cigarette-induced laryngeal disease onset and development. Therefore, to investigate this hypothesis, we examined the impact of two JUUL flavors, mint, and mango, on early cellular and inflammatory responses within the glottic and subglottic laryngeal regions. Specifically, we assessed VF epithelial thickness, VF cellular proliferation, submucosal gland area, mucus composition, inflammatory cell infiltration, and surface topography. We also report on the impact of e-cigarette exposure on animal body weight and nicotine metabolism and perform preliminary chemical characterization of e-cigarette aerosol.

METHODS

Animals

All experiments were conducted using male C57BL/6J mice, aged 8–9 weeks from Jackson Laboratory. Mice were housed in individual cages at the Stanford University Veterinary Service Center with normal food and water ad libitum. Body weight was examined regularly. Experimental procedures were approved and conducted in accordance with the guidelines of Stanford's Institutional Animal Care and Use Committee.

Experimental Design and JUUL Aerosol Generation

Mice were randomly assigned to one of three groups: room-air-exposed control, JUUL Mint (JMi), or JUUL Mango (JMa). Mice in the JUUL groups were placed in whole body exposure chambers and exposed to aerosol for 2 h/day (AM/PM) for 1 day ($n = 10$), 5 days ($n = 10$), and 10 days ($n = 10$). Control mice ($n = 9$) remained exposed to room air (Figure S1).

JUUL aerosol was generated using the inExpose system (SCIREQ Scientific Respiratory Equipment Inc., Montréal, Canada) equipped with a JUUL-compatible extension accessory. Exposure parameters were set as a puff volume of 70 mL, puff duration of 3.3–4 s, and puff frequency of 1 puff/min. Due to lack of standards for e-cigarette inhalation challenges, these parameters were designed to mimic human usage pattern, which includes deeper and longer puffs.[19] The mice received 120 puffs/day over 2 hours, similar to the average puff count of a human e-cigarette user.[20] We used pods with 5% nicotine (40 mg nicotine per pod).[21]

Animal Euthanasia, Tissue Collection and Processing

Mice had an intraperitoneal injection of Bromo-2′-deoxyuridine (BrdU; 100 mg/kg body weight; ab142567, Abcam, CA, USA) immediately after their final exposure at all time points. Two hours after BrdU administration, mice were euthanized by cervical dislocation under deep anesthesia by isoflurane. Harvested larynges were analyzed by histomorphometry, immunofluorescence (IF), immunohistochemistry (IHC), and scanning electron microscopy (SEM). Figure legends indicate the number of animals used for each analysis.

Urine was also collected via terminal bladder puncture. As previously described,[4] levels of urinary nicotine metabolites, cotinine and trans-3′-hydroxycotinine, were measured by Nicotine and Tobacco Product Assessment Resource (NicoTAR) at Roswell Park Comprehensive Cancer Center (Buffalo, NY, USA). The ratio between total urine trans-3′-hydroxycotinine levels to total urine cotinine levels, known as nicotine metabolite ratio (NMR), was also evaluated.

Chemical Characterization of JUUL Aerosol

JMi and JMa aerosol chemical characterization was performed using selective ion flow tube mass spectrometry (SIFT-MS, Syft) as described.[22] Levels of nicotine, propylene glycol, and aldehydes, including acrolein, formaldehyde, and acetaldehyde were measured (Table S1). Using the inExpose and a 100% charged JUUL device, three fresh JMi and JMa pods were each sampled into 0.5 L Tedlar gas sampling bags. Six 70 mL puffs were pumped into Tedlar bags directly connected to the output end of the inExpose puffing pump, resulting in an 80%–90% full bag capacity (Figure S2). Comparative controls included bags filled with room air. For statistical comparisons, analyte concentrations (ppb) were averaged across 15 ms for each chemical measured.

Evaluation of Laryngeal Mucosa by Histomorphometry

Larynges were fixed overnight at 4°C in 4% paraformaldehyde and transferred to 70% ethanol for paraffin embedding and sectioning by Stanford Animal Histology Services. Hematoxylin & Eosin (H&E) and Alcian blue/periodic acid Schiff (AB/PAS) staining were performed on 5 μm coronal sections to evaluate VF epithelial thickness, subglottic glandular area, and mucus composition, respectively. Images were captured with an AxioImager M1 microscope (Carl Zeiss, Gottingen, Germany) and blinded for analysis. Blinded images were analyzed using the same methodology as our previous investigations.[4,5]

Analysis of Cellular Proliferation by IF

IF staining was performed on unstained paraffin slides to detect proliferative (Ki67 and BrdU) and basal (p63) cells. Standard IF protocols and imaging were followed as previously described.[4,5] Positive nuclear stains (Ki67, BrdU, and p63) were counted across right and left mid-membranous VF regions of blinded images across a length of 400 μm using a custom Fiji macro. Outcomes were represented as % positive (number of positive cells over total number of nuclei present in traced area). Detailed information on antibodies and their dilutions is included in Table S2.

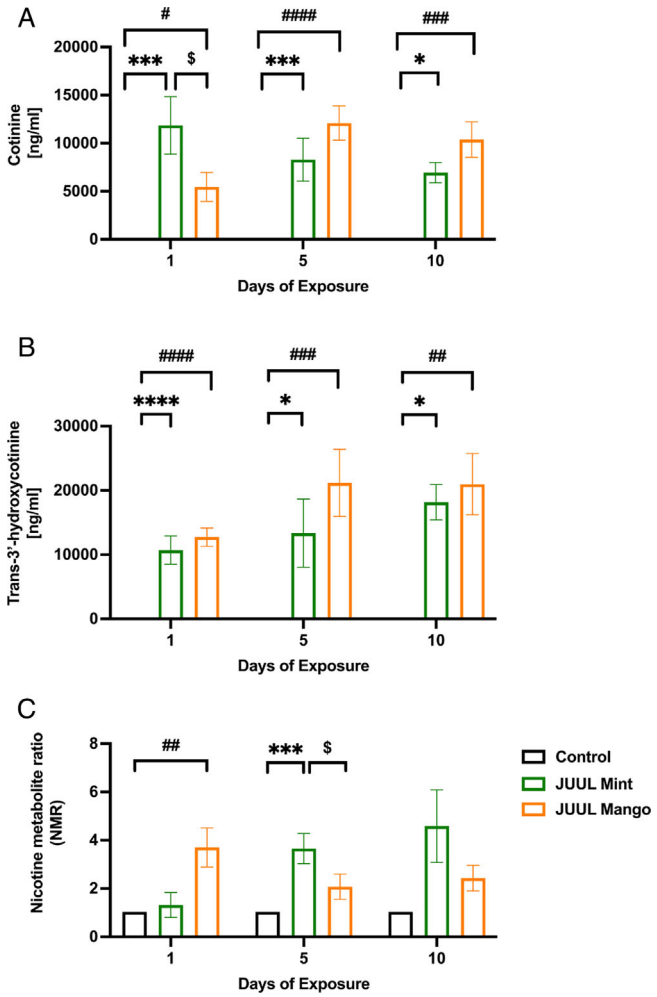

Fig. 1. Urinary nicotine metabolite levels. Mice exposed to JUUL Mint (JMi) and Mango (JMa) flavors had significantly higher levels of cotinine (ng/mL) (A) and trans-3′-hydroxycotinine (ng/mL) (B) when compared with the corresponding control group mice at days 1, 5, and 10. Cotinine levels of 1-day JMi-exposed mice were also significantly greater than 1-day JMa-exposed mice (A). The nicotine metabolite ratio (NMR) was significantly increased after JMa exposures after 1 day of exposure (C) relative to controls. NMR was also significantly elevated after 5 days of JMi exposure (C) compared with control and JMa-exposed mice. At day 1, $n = 8$ in control, $n = 5$ in JMi, and $n = 8$ in JMa groups. At day 5, $n = 10$ in control, $n = 7$ in JMi, and $n = 6$ in JMa groups. At day 10, $n = 4$ in control and $n = 9$ in JMi, and $n = 8$ in JMa groups. Bar graphs show the mean with standard error of the mean (SEM). * indicates significance between JMi and control. * indicates $p \leq 0.05$. *** indicates $p \leq 0.001$. **** indicates $p \leq 0.0001$. # significance between JMa and control. # indicates $p \leq 0.05$. ## indicates $p \leq 0.01$. ### indicates $p \leq 0.001$. #### indicates $p \leq 0.0001$. $ significance between JMi and JMa. $ indicates $p \leq 0.05$.

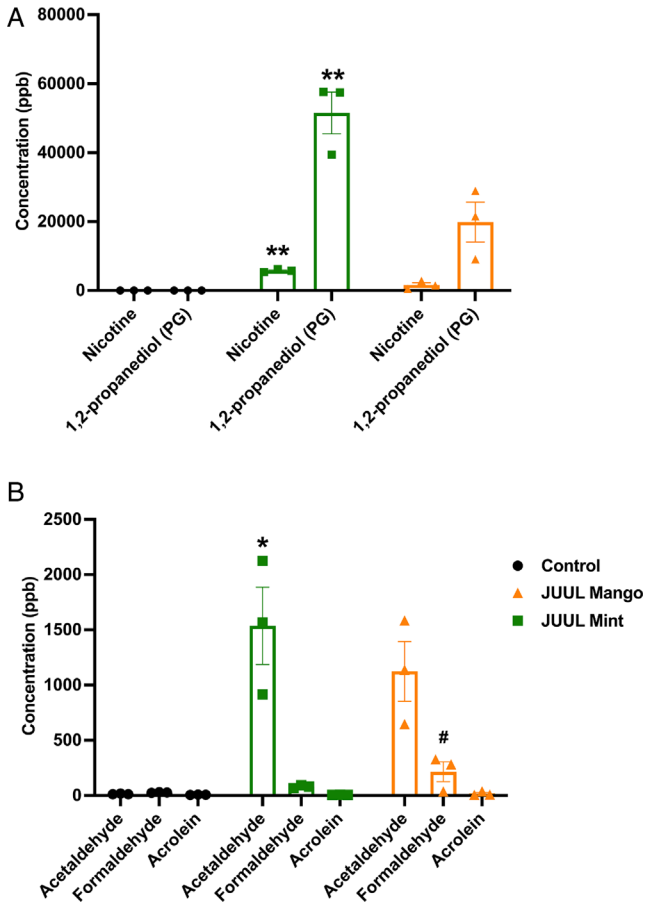

Fig. 2. Chemical characterization of JUUL aerosol. Nicotine, PG, and aldehydes were quantified in JUUL Mint (JMi) and Mango (JMa) aerosols using selective ion flow tube mass spectrometry (SIFT-MS). The concentration of all chemicals is represented as parts per billion (ppb). Chemicals such as nicotine (A), PG (A), and acetaldehyde (B) were significantly higher in JMi aerosols than in air controls. JMa aerosols contained significantly increased levels of formaldehyde (B) when compared with controls. Acrolein (B) levels were similar across all groups. n here indicates the number of Tedlar bags used for sample collection. $n = 3$ in control and both JUUL flavor groups at all time points. Bar graphs show the mean with standard error of the mean (SEM). * significance between JMi and control. * indicates $p \leq 0.05$. ** indicates $p \leq 0.01$. # significance between JMa and control. # indicates $p \leq 0.05$.

Assessment of Inflammatory Cell Infiltration

H&E stained laryngeal sections were evaluated blindly for early signs of inflammation, including infiltration of inflammatory cells, by a veterinary pathologist using an Olympus BX43 light microscope in the VF and subglottic regions. Also, myeloperoxidase/MPO + neutrophils, F4/80 + macrophages, and CD3 + T-cellular levels were quantified in the VF and subglottic regions using routine IHC performed by HistoWiz, Inc (histowiz.com), as previously described.[15]

SEM

Laryngeal tissues were processed for SEM according to our previous protocol.[4] Images of microprojections on VF epithelial surfaces and cilia in the subglottic regions were acquired by the FEI Magellan 400 XHR SEM at Stanford Nano Shared Facilities (SNSF). VF epithelial surface microprojection damage was visually examined by two blinded raters. In each VF sample, three images were taken at 25kx as technical replicates. In total, 12 images per experimental group were blindly rated. Images were categorically scored using our earlier protocol[4] and Blind analysis tool plugin in Fiji.

The VF epithelial surface microprojection density was also determined using the same images. Otsu algorithm on Fiji was used to objectively assess VF surface microprojection area coverage as previously performed in rabbits.[23]

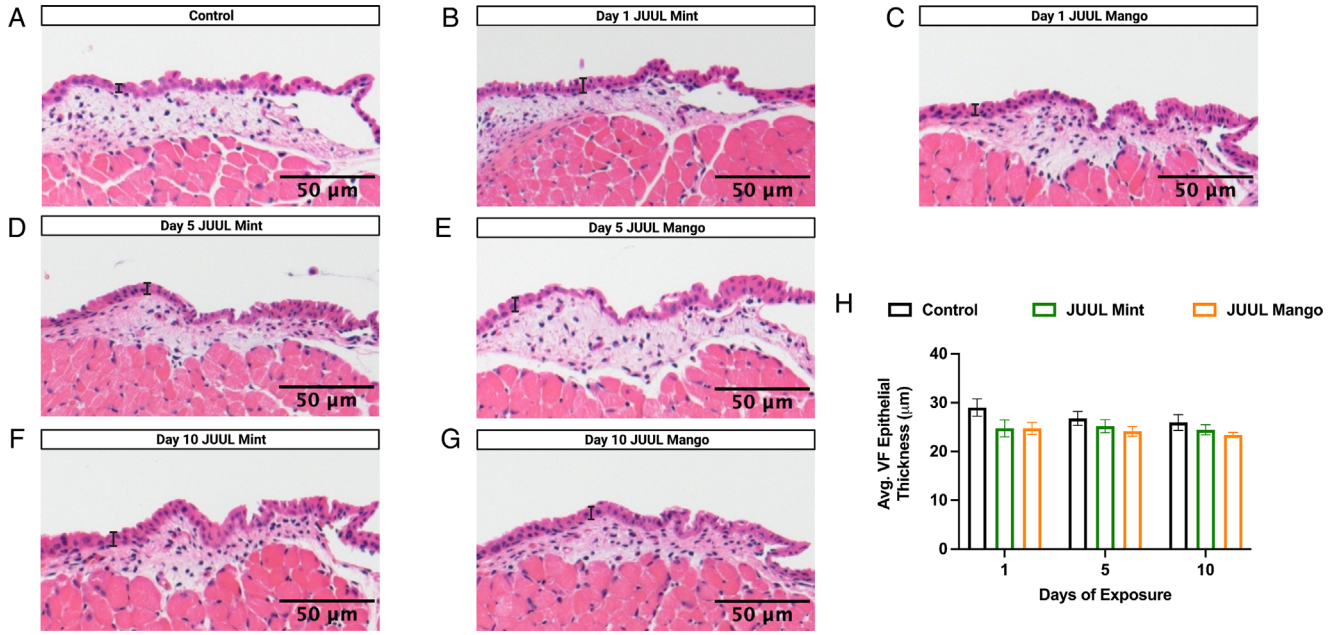

Fig. 3. VF epithelial thickness. JUUL Mint or Mango exposures did not alter VF epithelial thickness after 1 day (B, C, H), 5 days (D, E, H), and 10 days of exposure (F, G, H) in comparison with the control group mice (A, H). At day 1, $n = 5$ in control and both JUUL flavor groups. At days 5 and 10, $n = 6$ in control and both JUUL flavor groups. The symbol "I" indicates VF epithelial thickness. H&E stained images are at a magnification of 200×. Bar graphs show the mean with standard error of the mean (SEM).

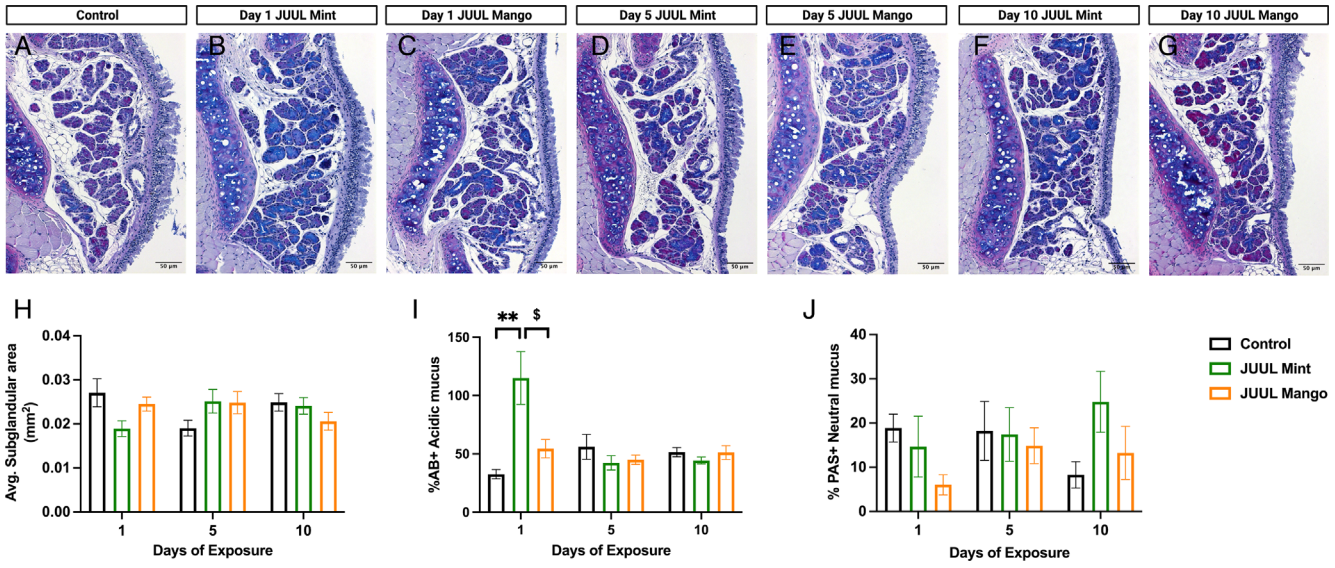

Fig. 4. Subglottic mucosal glandular area and mucus composition. The laryngeal subglottic mucosal area remained similar across all experimental groups at all time points (A–H). Acidic mucus content was significantly higher in JUUL Mint exposed mice (B, I) after 1 day of exposure compared with controls (A, I) and JUUL Mango exposed mice (C, I). Neutral mucus levels were similar across all the experimental groups (A–G, J). At day 1, $n = 5$ for control and both JUUL flavor groups. At day 5, $n = 6$ in control and both JUUL flavor groups. At day 10, $n = 6$ in the control and JMi groups; $n = 5$ in the JMa group. AB/PAS-stained images are at a magnification of 200×. Bar graphs show the mean with standard error of the mean (SEM). * indicates significance between JMi and control. ** indicates $p \leq 0.01$. $ indicates significance between JMi and JMa. $ indicates $p \leq 0.05$.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.3.1. Two-way analysis of variance (ANOVA) with Tukey's multiple comparison test as post hoc was performed for mice body weight computations. One-way ANOVAs followed by Tukey's multiple comparison test were used for nicotine metabolite, histological, and immunostaining analyses. Kruskal–Wallis test followed by post hoc uncorrected Dunn's multiple comparison test

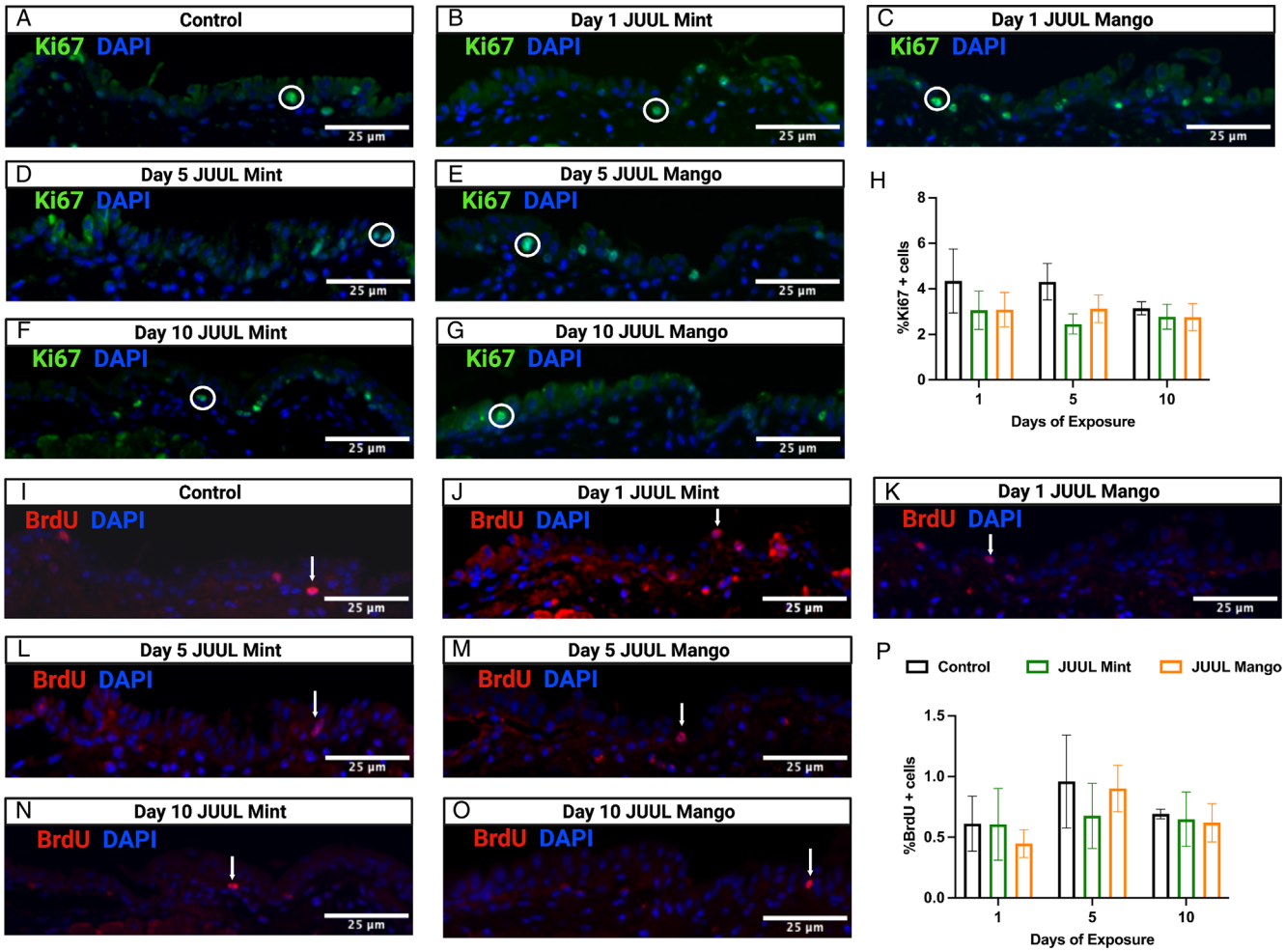

Fig. 5. VF epithelial Ki67- and BrdU-labeled cellular proliferation. JUUL Mint and Mango exposures had no significant changes to cellular proliferation as measured by Ki67 and BrdU immunofluorescent labeling after 1 day (B, C, H, J, K, P), 5 days (D, E, H, L, M, P), and 10 days of exposure (F, G, H, N, O, P) in comparison with the controls (A, H, I, P). At day 1, $n = 3$ in control and $n = 4$ in both JUUL flavor groups. At days 5 and 10, $n = 4$ in control and both JUUL flavor groups. White circles indicate Ki67-labeled cells in green. White single-headed arrows indicate BrdU-labeled cells in red. The nuclear stain is DAPI (blue). Immunofluorescent-stained images are at a magnification of 200×. Bar graphs show the mean with standard error of the mean (SEM).

was employed for JUUL aerosol chemical and SEM-based VF microprojection analyses. A p value ≤ 0.05 was considered statistically significant. Mean ± standard error of means (SEM) is depicted in all figures. p values and 95% confidence intervals for all statistically significant measurements are listed in Table S3.

For all measurements except urinary nicotine metabolite levels, JUUL aerosol chemical characterization and IHC, intra- and inter-rater reliability analysis was conducted on 20% of randomly selected samples. Specifically, two-way mixed-effects, absolute agreement intraclass correlation coefficients (ICC) were calculated using SPSS Statistics (Version 26, IBM) and interpreted per established.[24] ICC values were indicative of good to excellent consistency for intra- and inter-rater analyses (Table S4).

RESULTS

Effects of JUUL Aerosol Mouse Body Weight and Nicotine Metabolism

There was a significant interaction between exposure duration and exposure type for 5- and 10-day experimental groups (Table S3). Body weight of 5-day JMi-exposed mice remained significantly lower than the body weight gained by control mice starting from days 3 to 5 (Table S3; Figure S3A). Body weight of 5-day JMa mice was also significantly lower than the controls (Table S3; Figure S3A). 10-day JMi-exposed mice weighed significantly less than their respective controls starting from days 3 to 10 (Table S3; Figure S3B). Moreover, these mice weighed significantly less than the 10-day JMa-exposed mice at days 3, 5, and 6 (Table S3; Figure S3B). No significant differences were observed between 10-day JMa-exposed and control mice. Mice body weight was similar across the three groups at day 1.

Nicotine metabolism was estimated by measuring cotinine and trans-3′-hydroxycotinine. JMi exposures significantly increased cotinine and trans-3′-hydroxycotinine levels (Fig. 1A,B; Table S3) at days 1, 5, and 10. Cotinine levels in 1-day JMi-exposed mice were also significantly elevated as compared with 1-day JMa-exposed mice (Fig. 1A,B; Table S3). Mice exposed to JMa also had

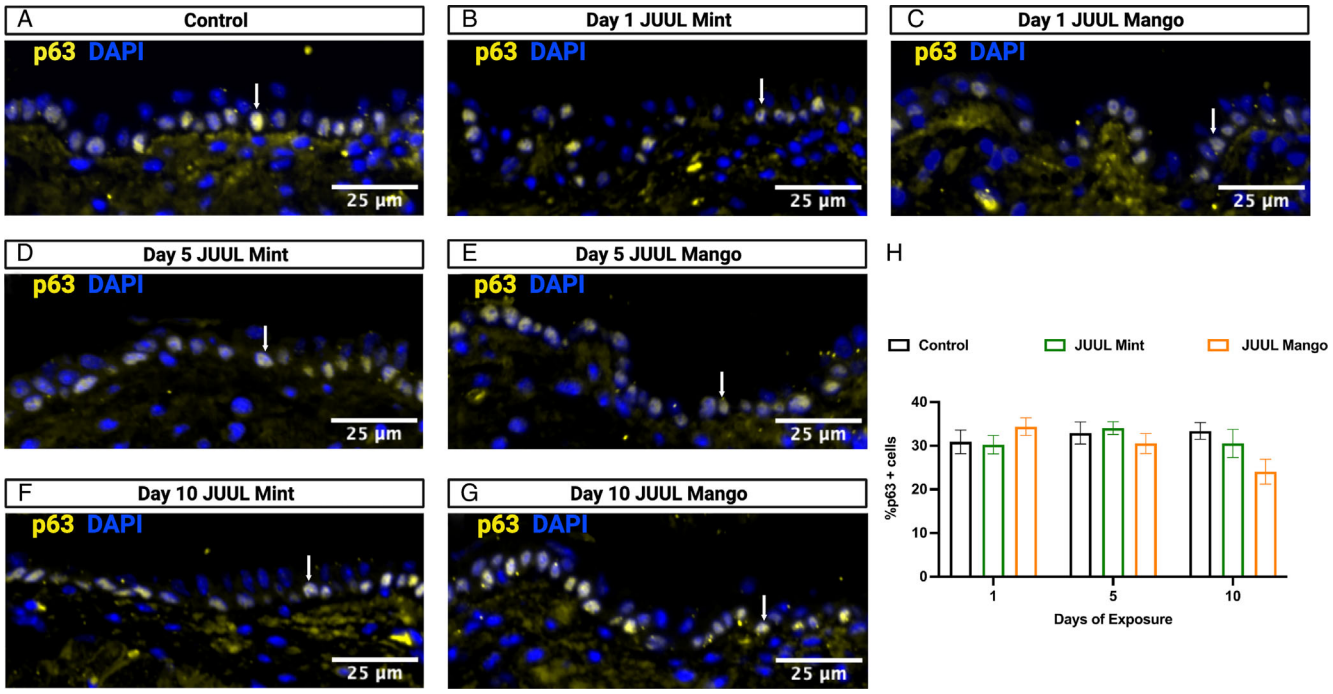

Fig. 6. VF epithelial p63-labeled basal cell proliferation. p63-labeled basal cell populations in the VF epithelium had no significant changes after JUUL Mint and Mango exposures at all time points when compared with the control group (A–H). At days 1 and 5, $n = 3$ in control and $n = 4$ in both JUUL flavor groups. At day 10, $n = 4$ in the control and both the JUUL flavor groups. White single-headed arrows indicate p63 labeled cells in yellow. The nuclear stain is DAPI (blue). Immunofluorescent-stained images are at a magnification of 200×. Bar graphs show the mean with standard error of the mean (SEM).

significantly higher levels of cotinine and trans-3′-hydroxycotinine (Fig. 1A,B; Table S3) at days 1, 5, and 10. An assessment of NMR following JMi exposure demonstrated a significant elevation only at day 5, compared with control and JMa-exposed groups (Fig. 1C; Table S3). JMa exposures significantly increased NMR compared with controls only at day 1 (Fig. 1C; Table S3). At day 10, NMR was comparable with controls after both flavor exposures (Fig. 1C).

Chemical Characterization of JUUL Aerosol

Commonly found e-cigarette constituents like nicotine, PG, and aldehydes were quantified in JMi and JMa aerosols using SIFT-MS. JMi aerosol had significantly higher levels of nicotine (Fig. 2A), PG (Fig. 2A), and acetaldehyde (Fig. 2B) relative to controls (Table S3). Formaldehyde (Fig. 2B; Table S3) was significantly increased in JMa aerosols than the controls. Acrolein levels (Fig. 2B) remained similar between all experimental groups.

Histomorphometric Evaluation of Laryngeal Mucosa

VF epithelial thickness measured using H&E was the same as the control after exposure to both flavors at all time points (Fig. 3A–H). Subglottic glandular hypertrophy was not observed after JMi or JMa exposures across all experimental groups when stained with AB/PAS (Fig. 4A–H). At day 1, JMi flavor significantly increased AB+ acidic mucus levels compared with control and JMa-exposed mice (Fig. 4A–C,I; Table S3; Figure S4). PAS+ neutral mucus levels remained unchanged after exposure to both flavors and at all time points (Fig. 4A–G, J).

Assessment of VF Cellular Proliferation

VF epithelial Ki67 (Fig. 5A–H) and BrdU (Fig. 5I–P) labeled cells remained comparable at all time points after both JUUL flavor exposures. Moreover, p63-labeled epithelial basal cells were also unaltered across all experimental groups (Fig. 6A–H).

Examination of Inflammatory Cell Infiltration in Laryngeal Mucosa

Blinded pathological rating of H&E slides showed no obvious signs of early inflammatory cell infiltration in the VF and subglottic regions. Immunohistochemical assessments of MPO + neutrophils in the VF and subglottic regions were similar to the controls at all time points after both JUUL flavor exposures (Fig. 7A,B; Figures S5 and S6). VF F4/80+ macrophage levels were similar across experimental groups at days 1 and 5 (Fig. 7C; Figure S7). At day 10, VF F4/80+ macrophage levels remained significantly elevated after JMi exposures when compared with JMa exposures, but not significantly higher than the corresponding control group (Fig. 7C; Table S3; Figure S7). In the subglottis, significantly lower levels of macrophages were observed after JMi exposures

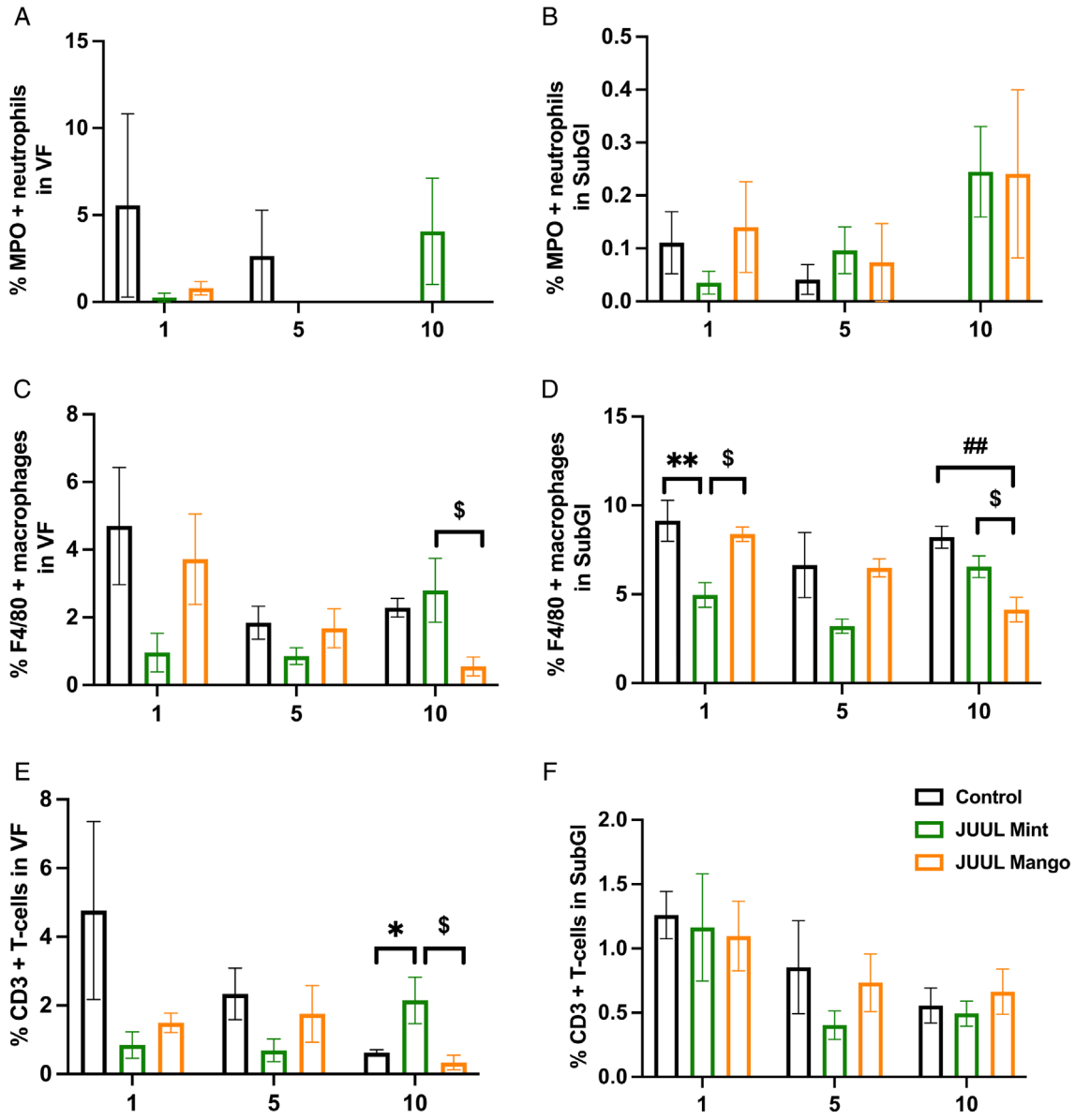

Fig. 7. Inflammatory cell infiltration in VF and subglottis. JUUL Mint (JMi) and Mango (JMa) aerosol exposures did not alter the levels of MPO+ neutrophils in the VF (A) and subglottic (B; SubGl) laryngeal regions. F4/80+ macrophage cellular levels were significantly higher in the VF regions after 10 days of JMi exposures (C) when compared with only the JMa aerosol-exposed group. Subglottic macrophage levels remained significantly lower in the JMi aerosol-exposed groups rather than the other two experimental groups at day 1 (D). Also, 10 days of JMa aerosol exposure significantly inhibited macrophage levels in the subglottis relative to the control and JMi groups (D). JMi exposures significantly increased the levels of CD3+ T cells in the VF at day 10 (E) when compared with the control and JMa groups. T-cell population was unchanged in the subglottis at all time points and after both JUUL flavor exposures (F). For MPO+ neutrophil analysis, $n = 5$ for the control and both JUUL flavor groups at day 1. At day 5, $n = 5$ in control and $n = 6$ in both the JUUL flavor groups. At day 10, $n = 5$ in the control and JMi groups and $n = 6$ in the JMa group. For F4/80+ macrophage analysis, $n = 5$ in control and $n = 5$ in both the JUUL flavor groups at day 1. At days 5 and 10, $n = 5$ in control, $n = 6$ in both the JUUL flavor groups. For CD3+ T-cell analysis, $n = 5$ in the control and both the JUUL flavor groups at day 1. At day 5, $n = 4$ in the control, $n = 5$ in the JMi, and $n = 6$ in the JMa groups. At day 10, $n = 5$ in the control and JMa groups and $n = 4$ for the JMi group. Bar graphs show the mean with standard error of the mean (SEM). * indicates significance between JMi and control. * indicates $p \leq 0.05$. ** indicates $p \leq 0.01$. # significance between JMa and control. ## indicates $p \leq 0.01$. $ indicates significance between JMi and JMa. $ indicates $p \leq 0.05$. $$ indicates $p \leq 0.01$.

at day 1 when compared with the control and JMa-exposed-mice (Fig. 7D; Table S3; Figure S8). Five days of both JUUL flavor exposures had no significant impact on subglottic macrophage levels across all groups (Fig. 7D; Figure S8). At day 10, JMa exposures significantly suppressed macrophage populations in the subglottis relative to control and JMi-exposed groups (Fig. 7D; Table S3; Figure S8).

In terms of the CD3+ T cells in the VF regions, no major alterations were observed between experimental groups at days 1 and 5 (Fig. 7E; Figure S9). An increased

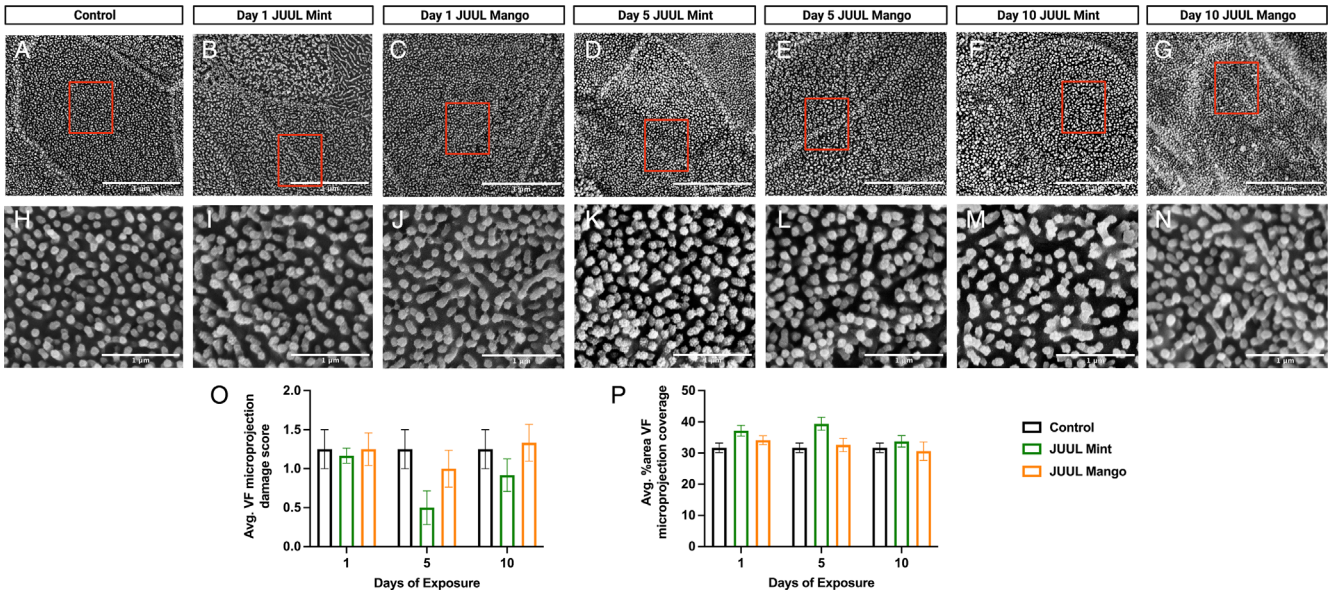

Fig. 8. Scanning electron micrographs of VF epithelial surface microprojections. JUUL Mint and Mango exposed mice did not exhibit any significant VF epithelial surface microprojection damage after 1 (B, C, I, J, O), 5 (D, E, K, L, O), and 10 days of exposure (F, G, M, N, O) relative to the control group mice (A, H, O), upon SEM. Also, the area covered by VF epithelial microprojections did not differ between experimental groups at all time points (A–N, P). For all groups, $n = 4$, where n indicates the number of right and left vocal folds examined. SEM images in the top panel (A–G) are at a magnification of 5kx with a horizontal field width (HFW) of 29.8 μm, and images in the bottom panel (H–N) are at a magnification of 25kx with an HFW of 5.97 μm. The red squares in 5kx images represent regions imaged at 25kx. Bar graphs show the mean with standard error of the mean (SEM).

cell CD3+ cell count was observed in the VF regions only after 10 days of JMi exposure in comparison with the other two groups (Fig. 7E; Table S3; Figure S9). Subglottic CD3+ T cells remained unchanged at all time points after both JUUL flavor exposures (Fig. 7F; Figure S10).

SEM

VF epithelial surface microprojections appeared structurally similar across all experimental groups (Fig. 8A–N). Visual examination of VF epithelial surfaces indicates that exposures to both JUUL flavors do not damage microprojections (Fig. 8O). Surface area coverage of microprojections also remained unchanged among experimental groups (Fig. 8A–N, P). Furthermore, SEM analysis showed no structural deformities in laryngeal subglottic cilia (Figure S11).

DISCUSSION

We evaluated the short-term e-cigarette inhalation effects of two different JUUL flavors on early cellular and inflammatory responses in the glottic and subglottic mouse larynx. Minimal flavor- and region-specific changes to cellular and inflammatory responses were observed across VF and subglottic laryngeal regions. In addition, we observed successful e-cigarette aerosol inhalation by mice, flavor-specific body weight alterations, and varying chemical constituents between flavors.

Following market trends, this study used JUUL pod-based devices containing 40 mg of nicotine.[21] As JUUL pods utilize nicotine salts rather than freebase nicotine, systemic nicotine delivery is faster and comparable with traditional smoking.[21,25] At all time points across experimental groups, nicotine metabolites were significantly elevated. These results suggest that JUUL mice experienced successful nicotine metabolism and exposure. The levels of these metabolites are greater than reported in a similar short-term CS study.[4] The nicotine content of a single 5% JUUL pod is like that of smoking at least one pack of cigarettes.[21] It is not surprising that nicotine biomarkers were detected at higher levels in this current study.

In humans, NMR is another marker of nicotine metabolism and cigarette consumption.[26] NMR was significantly increased only after 1 day of JMa and 5 days of JMi exposures. Contrastingly, we saw elevated NMR after 5 and 10 days of CS exposures in our short-term smoking model.[4] In terms of e-cigarettes, NMR is only recommended as a marker of nicotine metabolism.[27] It is inconclusive whether NMR can also directly be considered as a marker for e-cigarette consumption, due to its variable nicotine content.

An elevated NMR is also indicative of weight loss in smokers.[28] This was corroborated by the results for our previous short-term smoking model.[4] We saw NMR increases in the JMi-exposed group at day 5 and saw a corresponding body weight loss. However, this pattern was not evident in other groups, despite body weight reductions observed 10 days after JMi exposures. Evidence shows animal body weight loss post e-cigarette exposure with nicotine free e-cigarettes,[29–31] indicating e-cigarette-induced body weight loss may not entirely be nicotine-dependent. Since their emergence in the early

2000s into the US market,[32] there is limited information available about the effects of e-cigarettes on body weight in preclinical animal models and clinical studies. Currently, few reports in mice models show a decline in mice body weight ranging between shorter (14 days) and longer (12 weeks) exposures.[33–35] Most clinical studies show that human e-cigarette use is associated with obesity or the intention to lose or control weight.[36–38] However, the exact implications of e-cigarette use on body weight remain to be well-elucidated, specifically in the long term. The variations in human e-cigarette use, puffing patterns, dual usage with traditional cigarettes, type/generation of e-cigarette device used, and e-cigarette ingredients like with or without nicotine or flavorings make it further challenging to deduce the effects of e-cigarettes on body weight precisely. In this study, the overall reduction in body weight of JMi- or JMa-exposed mice at days 5 and 10 was minimal in comparison with baseline (day 1). Moreover, the body weights of the exposed mice stabilized closer to baseline around 8–10 days of exposure, but exhibited no weight gain, unlike the control mice. Hence, it is likely that the significant statistical differences observed between the JUUL-exposed and control mice are more related to the controls gaining weight. It is important that future studies continue to investigate the impact of e-cigarettes on body weight by taking into account a variety of factors associated with e-cigarette usage patterns. To begin, a possible approach for future in vivo studies may be to standardize exposure/usage duration patterns by following the guidelines established by the Organization for Economic Cooperation and Development (OECD) for acute (2 weeks),[39] subacute (4 weeks),[40] subchronic (13 weeks),[41] and chronic (12 months)[42] inhalation toxicities. These guidelines are extensively used for CS-related exposure studies and may help us to track body weight changes over a timeline *in vivo*. As an immediate effort to explore the effects of the JUUL, we plan to conduct our future *in vivo* studies to study the larynx and other exposure parameters, including body weight, based on these OECD exposure duration guidelines. This will further solidify our understanding of e-cigarette-related laryngeal modifications over time.

The SIFT-MS analysis of the two JUUL flavors revealed differences in nicotine, PG, and aldehyde levels in aerosolized e-liquid. Their concentrations in e-cigarette aerosols were statistically higher than room air. For JMi, there were higher concentrations of nicotine, PG, and acetaldehyde. JMa aerosol contained higher levels of formaldehyde. The significance of these chemicals lies in their potential toxic impact on the airway epithelium. To describe toxicity, minimum risk levels (MRLs) are used to indicate significant human exposure levels. MRL provides an estimate of the daily exposure level to a hazardous substance that is unlikely to cause adverse health effects for a particular period.[43] A chemical's potential for adverse effects increases as its concentration exceeds the MRL. Table S1 summarizes sample constituent concentrations, MRL where applicable, and potential clinical significance of these concentrations. Individual chemicals were not directly tested in the current study. Future analyses of individual e-cigarette aerosol constituents on cellular and inflammatory responses in the larynx will be guided by these preliminary findings.

Epithelial turnover depends on cell proliferation.[44] In response to injury, airway basal cells proliferate and generate either normally differentiated epithelium or altered histologic phenotypes.[45] We observed no differences in the number of proliferative and basal cells and VF epithelial thickness between e-cigarette and control mice. Similarly, short durations of CS exposure do not increase epithelial thickness but do increase cell proliferation and basal cell numbers.[4] In addition, removal of e-cigarette aerosol extract from human-engineered VF mucosa triggers increased basal cells.[11] A 4-week exposure to e-cigarettes also did not increase epithelial thickness or cell proliferation in rats.[13] Interestingly, short exposures to e-cigarette aerosols in engineered human oral[46] and nasal mucosa[47] reduces the number of proliferative cells. These findings suggest exposure to e-cigarettes and cigarettes may induce unique epithelial proliferative responses, and further studies on the larynx are needed.

The subglottic region of the larynx contains mucus-producing submucosal glands.[48] CS increases airway mucus production, including in the larynx.[5,9,49,50] However, laryngeal mucus production changes due to e-cigarette exposure are poorly understood. We observed no changes in submucosal gland area following e-cigarette exposure. However, the JMi group experienced a transient increase in acidic mucus composition following one day. The functional significance of this finding is unknown. There is evidence that menthol[51] and nicotine[52] may alter airway mucus properties. JMi aerosol had higher nicotine concentrations than air controls in this study. According to a previous report,[53] menthol is a dominant flavoring in JMi aerosol. Investigations are needed to determine how individual chemical constituents affect laryngeal mucus composition.

The effects of e-cigarette exposure on inflammatory responses have been well documented in lower airways and cardiovascular system.[54] In terms of the larynx, no inflammation was seen in a 4-week rat model.[13] Another study demonstrated that e-cigarette exposure for 16 weeks altered the inflammatory cytokine profile in mice.[14] However, to date there is almost no evidence about early inflammatory cell responders, including neutrophils, macrophages, T cells, and eosinophils in an e-cigarette-exposed larynx. Inflammatory cells are known to appear within seconds of contact with an external stimulus.[55,56] Previously, an increased macrophage count has been demonstrated within 3 and 14 days of e-cigarette exposure in the lungs.[16–18] Likewise, an influx of other cells like neutrophils, T cells, and eosinophils has also been observed in the lower airways after 2 weeks of e-cigarette exposure.[16] This study demonstrated increased T-cell levels post short-term e-cigarette exposure. Specifically, we observed increases only in the VF laryngeal region after 10 days of exposure to JMi aerosol when compared with air-exposed controls. Elevated macrophage levels were also seen after 10 days of JMi exposure relative to JMa exposures, but it was not significant compared with the air-exposed controls. Although significant, the increases in inflammatory

cells were small in magnitude and all specific to the JMi flavor and observed only in the VF. JMa elicited no influxes of inflammatory cells in both VF and subglottic regions. Interestingly, macrophages were suppressed in the subglottis after 10-day exposure to JMa, as well as 1-day exposure to JMi. These findings are indicative of the early inflammatory cell infiltration responses possibly being flavor- and region-specific. Also, these differential inflammatory cell responses may be attributed to specific chemicals within these JUUL flavors. Menthol is an example of a flavoring chemical associated with inflammation.[57,58] Considering that this chemical is a major flavoring component of JMi aerosol,[53] an increase in inflammatory cells after JMi exposures is not surprising, although the magnitude was small. Similar flavor-specific e-cigarette exposure-related inflammatory findings have been reported earlier in the lungs as well.[59] Subsequent research should explore these e-cigarette flavor-specific inflammatory cell changes in greater detail in the larynx and other airway regions.

Although flavor- and or region-specific inflammatory outcomes may have been evident in this study, it is to be noted that only a modest number of positive cells was detected by IHC. Furthermore, no major signs of inflammatory cell infiltration were observed by the blinded pathologist. Collectively, these results do not suggest a strong inflammatory cell response after short-term JMi and JMa exposures. Pathological inflammatory responses are time-dependent and longer-term observations may reveal substantial differences in inflammatory cell populations in an e-cigarette-exposed larynx and other airway regions. To our knowledge, this is the first study to evaluate the effects of e-cigarettes on early inflammatory cells in the larynx upon short-term inhalation exposures. Future studies should also evaluate the short- and long-term effects of e-cigarette exposures on laryngeal inflammation on a broader scale, including other cell types, cytokines, and pathways, to gain a comprehensive understanding.

Microprojections on the surface of superficial VF epithelial cells contribute to epithelial barrier integrity.[23] The superficial laryngeal epithelium is disrupted by even short-term CS exposures resulting in flattening of VF microprojections and shortening and destruction of subglottic cilia.[4] VF microprojections and subglottic cilia were not affected by e-cigarette exposure. This is consistent with data collected using a "frog palate paradigm", in which e-cigarette aerosol also did not disrupt epithelial cells or cilia.[60] This could be related to temperature. A burning cigarette reaches 950°C at its peak, whereas an e-liquid reaches 250°C at its peak during aerosolization.[61]

There are limitations to this study. Various combinations of e-cigarette devices, liquid compositions, and electrical power inputs are available to users. As a result, it is difficult to establish standards for e-cigarette inhalational testing. We examined two flavors of the popular e-cigarette, JUUL. The study was conducted before the U.S. Food and Drug Administration banned pod-based e-cigarette flavors.[62] While these flavors are no longer available, the e-liquid constituents are relevant across multiple product types. Finally, we focused on short-term exposure. Future studies will investigate the effects of chronic exposure to develop a timeline of potential pathophysiological changes in the larynx from e-cigarette exposure.

CONCLUSION

To our knowledge, this is the first study to demonstrate flavor- and region-specific changes to cellular and inflammatory responses in the larynx in a mouse model following short-term e-cigarette use. Our findings provide a foundation for continued investigation of laryngeal cellular and inflammatory changes along with underlying molecular mechanisms upon acute and chronic e-cigarette exposures using assorted products with varying flavors and chemical concentrations.

ACKNOWLEDGMENTS

We thank Dr. Nicolas Grillet from the Department of Otolaryngology, Stanford University, for his guidance with SEM sample preparation and imaging. We also thank Richard Chin from SNSF for SEM training and imaging. We acknowledge Kody Wasaka, Dayoung Kim, and Dr. Amirbahador Golchin Sani Masouleh for their assistance in IHC and data analysis contributions. We thank Dr. Kerriann Casey from the Department of Comparative Medicine, Stanford University, for her contributions in pathological rating of H&E slides. Research for this study was supported by the National Institutes of Health, National Institute of Deafness and Other Communication Disorders [R21 DC016126] and [R01 DC020176], and National Heart Lung Blood Institute [R01 HL114388]. Part of this work was performed at the SNSF, supported by the National Science Foundation under award ECCS-2026822. All figures were created using BioRender.com.

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