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Laryngeal inflammatory response to smoke and vape in a murine model☆

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ABSTRACT

Purpose: To build a murine model for tobacco smoke and electronic cigarette vapor exposure to characterize the inflammatory and immune responses in the larynx.

Materials and methods: In this pilot study, twenty-four wild-type C57BL/6 mice were divided into four groups: smoke, vapor with nicotine, vapor without nicotine, and air only. Following daily exposure for 4 months, larynges were dissected and processed with cytokine detection arrays. Each laryngeal cytokine level between the four different groups was analyzed statistically by using statistical analysis software (SAS) to calculate the analysis of variance (ANOVA).

Results: IL-4 was the only cytokine found to achieve statistically significant different levels in this study, with elevated levels of IL-4 in the tobacco smoke and vapor with nicotine groups compared to the levels found in the vapor without nicotine and air only groups ($p = 0.0418$). While statistically non-significant, prominent findings revealed up-regulation of TGF-β2 and TGF-β3 in the smoke group, but near-normal levels of TGF-β2 and TGF-β3 and suppression of IL-10 in the vapor groups ($p > 0.05$).

Conclusion: The potential utility of the murine model is established for studying the inflammatory and immune effects of tobacco smoke and vapor on the mammalian larynx. IL-4 levels in mice larynges were significantly elevated in the tobacco smoke and vapor with nicotine groups.

1. Introduction

Laryngeal squamous cell carcinoma (SCC) accounts for 2%–3% of all malignant tumors and 90% of laryngeal cancers [1,2]. Smoking tobacco is the primary etiological agent for laryngeal dysplasia, and increases the risk of laryngeal cancer by 10 to 30 times more than in non-smokers [3,4]. Using electronic cigarettes, also known as vaping, provides an alternative mode of nicotine delivery by producing an inhaled aerosol instead of smoke [5]. While the effects of smoke on the larynx have been well-established, the effects of vape are unknown.

Approximately 60% of patients present with advanced laryngeal cancer at diagnosis [3]. However, literature has shown promising results for the early detection of head and neck cancer in patients using serum biomarkers [6,7]. Specifically, serum levels of IL-6, IL-8, IL-10, TGF-β and TNF-α levels have been found to be increased in patients with laryngeal SCC compared to healthy controls [8]. While cytokine serum levels have previously been studied, laryngeal cytokine levels in laryngeal SCC or pre-malignancy have not previously been described in the literature to the best of our knowledge. Similar to the idea behind potentially using serum biomarkers for the early detection of head and neck cancers, identification of elevated local inflammatory and immune biomarkers from laryngeal biopsies could serve as a prognostic factor for risk of future malignancy in patients. The goal of this pilot study is to demonstrate a murine model of tobacco smoke and electronic cigarette vapor exposure to characterize the inflammatory and immune responses in the mammalian larynx.

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This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine.

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2. Materials and methods

Following approval by our institutional animal care and use committee, twenty-four wild-type C57BL/6 mice were divided into four groups: tobacco smoke, vapor with nicotine, vapor without nicotine, and air only. In our pilot study, the number of animals required for significant cytokine expression was based on standard statistical methods using the InStat statistics program for the Macintosh. Based on prior work studying lungs in C57BL/6 mice exposed to tobacco smoke [9], the standard deviation for cytokine expression is expected be 2 compared to the air-only groups. The difference in cytokine expression between naive and exposed animals is anticipated to be 5 to 6 fold changes. Therefore to detect a 50% effect ($50\% \times 5 = 2.5$) with $p < 0.05$ and 90% power ($\alpha = 0.05$; $\beta = 0.10$), the required sample size is calculated as 5 to 6 mice per group.

Mice in the experimental groups were exposed to either active smoke from 4 cigarettes or vapor from an electronic cigarette, for a total of 31 min 40 sec per day, 5 days per week, for 16 weeks. A modified inhalation chamber (Buxco systems) was used to provide intermittent cycles of 3 s of tobacco smoke or vapor followed by 20 s of room air to mimic the actual puffing cycles of human smokers and prevent CO_2-induced asphyxiation [Fig. 1]. Doses of tobacco smoke in the mice were standardized by corresponding the serum cotinine, a metabolite of nicotine, levels in the mice to the average cotinine levels found in humans who smoke tobacco.

Following harvest, the larynges were dissected in their entirety from the mice under the Leica M60 stereo microscope, morselized in aggregate, filtered, and cultured in complete media overnight [Fig. 2]. The supernatants were pipetted to undergo cytokine analysis. Luminex T helper 17 (T_H17) and transforming growth factor β (TGF-β) 3-plex cytokine detection kits (EMD Millipore Corp., Billerica, MA) were run in the standard fashion to identify a total of 28 cytokines of potential interest. The Milliplex mouse T_H17 detection kit measured the concentration of 25 cytokines (IL-25/17E, GM-CSF, IFNγ, MIP-3a/CCL20, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-21, IL-22, IL-28B, IL-10, IL-23, IL-12p70, IL-27, IL-13, IL-15, IL-17a, IL-17F, IL-33, IL-31, TGF-β, TNF-α, CD40L). The Milliplex mouse TGF-β 3-plex kit measured the concentration of 3 cytokines (TGF-β1, TGF-β2, TGF-β3). The cytokine/chemokine array implements a system of fluorescent beads conjugated to capture antibodies for automated analysis. Statistical considerations and analysis were performed with ANOVA and SAS.

3. Results

IL-4 was significantly elevated in the tobacco smoke group and the vapor with nicotine group ($p = 0.0418$) [Fig. 3]. TGF-β2 and TGF-β3 were non-statistically significantly elevated in the tobacco smoke group. Vapor with and without nicotine both resulted in suppression of IL-10, although this was also not statistically-significant. Besides IL-4, concentration levels between the four groups for the remaining cytokines were all statistically non-significant, and concentration levels of 4 out of the 28 total cytokines analyzed were below the limit needed for measurement with the Luminex multiplex assay.

Regarding the murine model, 23 mice in the study survived the duration of the experiment, however one mouse in the cigarette group died prior to planned sacrifice presumably due to tar build-up in the exhaust tubing of the chamber. Cotinine levels were adequately found to gauge the intensity and duration of the exposures.

4. Discussion

Cigarette smoke has been shown to promote continuous T_H17 cell mediated lung inflammation through double-stranded cleavage of nuclear DNA, resulting emphysema in mice [9]. T_H17 cell differentiation

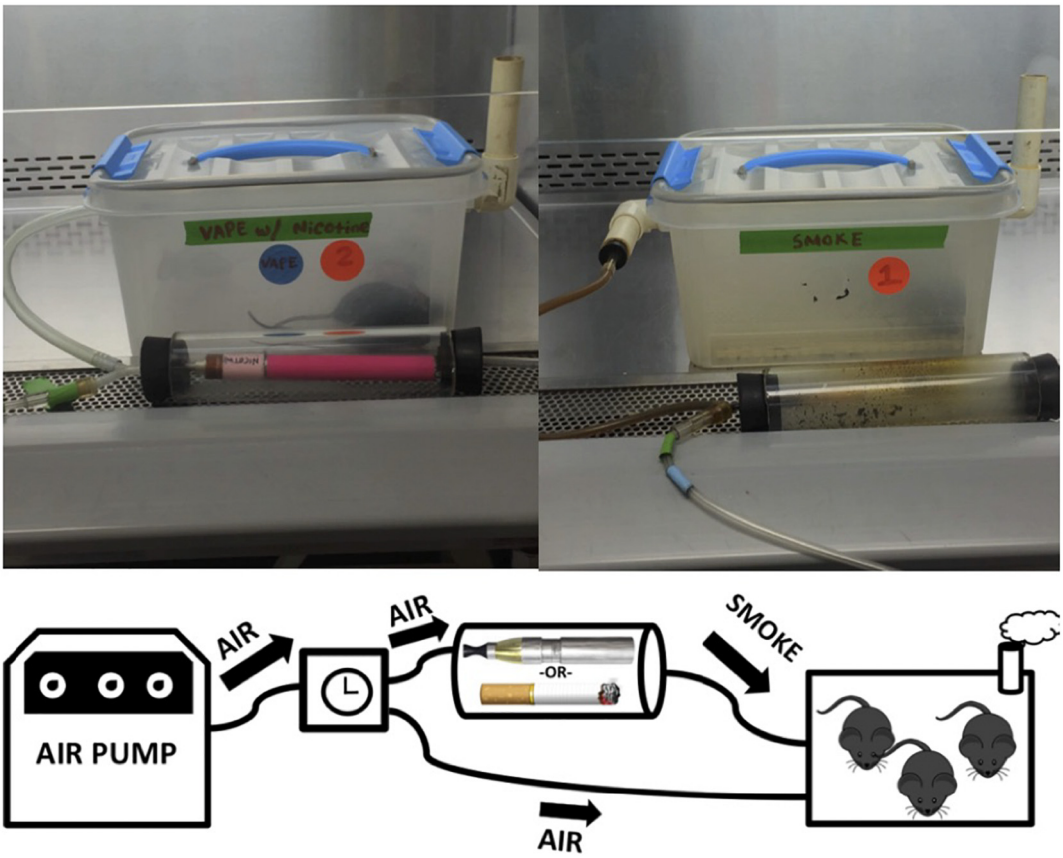

Fig. 1. Mice smoke and vape exposure set up.

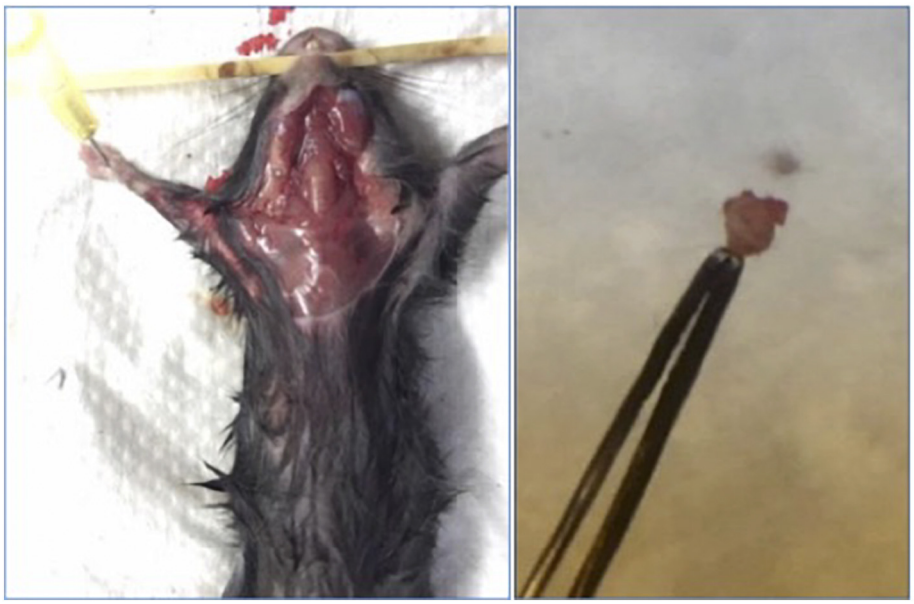

Fig. 2. Mouse larynx microdissection (left), Excised mouse larynx (right).

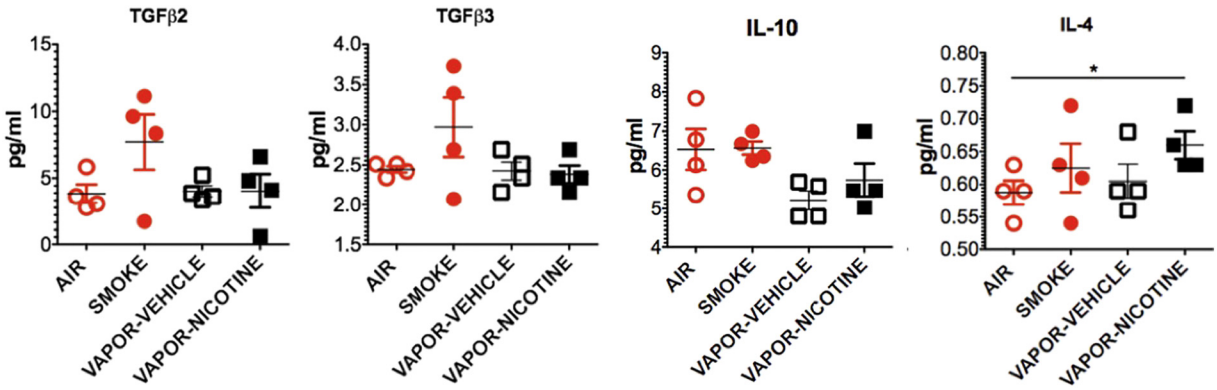

Fig. 3. TGF-B2, TGF-B3, IL-10, IL-4 levels.

from naïve T helper cells is induced by TGF-β and is inhibited by IL-4 [10]. Thus, the T_H17 and TGF-β cytokine detection kits were chosen for this study to analyze inflammatory and immune response to tobacco smoke and vapor exposure in mice larynges. The T_H17 cytokine detection kit included IL-4. In our study, the tobacco smoke could have induced T_H17 cell mediated laryngeal inflammation, upregulated by the non-statistically significantly elevated levels of TGF-β seen in the tobacco smoke group. IL-4, a key inflammatory marker of T_H2 cells, could have then increased in response to inhibit the TGF-β induced T_H17 cell mediated inflammation, explaining the significantly elevated levels of IL-4 in the tobacco smoke group. In this manner, TGF-β and IL-4 could have worked to respectively positively and negatively regulate T_H17 cell mediated inflammation promoted by tobacco smoke.

Interestingly, the TGF-β signaling pathway has tumor suppressor functions in healthy cells and early-stage cancer cells, and yet paradoxically also has tumorigenesis functions in late-stage cancer cells. Its tumor suppressor functions include cell-cycle arrest and apoptosis, while the tumorigenesis functions include suppression of immune and inflammatory processes and promotion of metastasis [11,12]. Since TGF-β2 and TGF-β3 levels were increased, although without significance, in the smoke group compared to the vape and air groups in our study, the increased TGF-β levels could reflect its tumorigenesis functions in the smoke group. Moreover, Elovic et al. found that IL-4 independently up-regulated TGF-β1 in eosinophils isolated from the peripheral blood of healthy human donors [13]. Therefore, although our samples were from larynges and not peripheral blood, significantly elevated levels of IL-4 in mice exposed to tobacco smoke could have directly resulted in the non-statistically significant upregulation of TGF-β in our study. Finally, IL-10 is an anti-inflammatory cytokine that inhibits the activity of T_H1 cells, NK cells, and macrophages during infection. The non-statistically significantly decreased IL-10 levels in both the vapor with and without nicotine groups in our study could suggest an inflammatory role of vapor from electronic cigarettes.

Caicedo-Granados et al. described a murine laryngeal carcinogenesis model using direct topical instillation of *N*-methyl-*N*-nitrosoruea, a highly labile carcinogen [14]. Although this is the only murine model for laryngeal cancer in the literature to date, it cannot be utilized fulfill our goal of studying the effect of tobacco smoke and electronic cigarette vapor on the mammalian larynx. To the best of our knowledge, our murine model is the first preclinical animal model used to study laryngeal inflammatory and immune responses after tobacco smoke and electronic cigarette vapor exposure. Although IL-4 was the only cytokine found to be significantly elevated in the tobacco smoke and vapor with nicotine groups, the limited statistical power in our present study is expected to be overcome with a larger sample size in subsequent studies.

5. Conclusion

Significantly elevated levels of IL-4 and upregulation of TGF-β,

while statistically non-significant, demonstrate the efficacy of the murine model for evaluating inflammatory, immune, and possibly carcinogenic effects of smoke and vape on the mammalian larynx. While tobacco smoke and vapor both appear to produce an inflammatory response in the murine model, further research with appropriate power is needed to reach statistical significance.

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