Acute effects of heated tobacco product (IQOS) aerosol-inhalation on lung tissue damage and inflammatory changes in the lungs

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ABSTRACT

Introduction: Emerging heated tobacco products (HTPs) were designed to reduce exposure to toxicants from combustible cigarettes (CS) by avoiding burning tobacco and instead heating tobacco. We studied the effects of short-term inhalation of aerosols emitted from HTP product called IQOS, on lung damage and immune-cell recruitment to the lungs in mice.

Methods: Numerous markers of lung damage and inflammation including albumin and lung immune-cell infiltrates, proinflammatory cytokines and chemokines were quantified in lungs and bronchoalveolar (BAL) fluid from IQOS, CS or air-exposed (negative control) mice.

Results: Importantly, as a surrogate marker of lung epithelial-cell damage, we detected significantly increased levels of albumin in the BAL fluid of both HTP and CS exposed mice compared to negative controls. Additionally, total numbers of leukocytes infiltrating the lungs were equivalent following both IQOS-aerosols and CS inhalation and significantly increased compared to air-exposed controls. We also observed significantly increased numbers of $CD4^+IL\text{-}17A^+$ T cells, a marker of a T cell immune response, in both groups compared to air controls; however, numbers were the highest following CS exposure. Finally, the numbers of $CD4^+ROR\gamma t^+$ T cells, an inflammatory T cell subtype expressing the transcription factor that is essential for promoting differentiation into pro-inflammatory Th17 cells, were significantly augmented in both groups compared to air-exposed controls. Levels of several cytokines in BAL were significantly elevated, reflecting a proinflammatory milieu.

Conclusions: Our study demonstrates that short-term inhalation of aerosols from IQOS generates damage and proinflammatory changes in the lung that are substantially similar to that elicited by CS-exposure.

Keywords: IQOS, Modified Risk Tobacco Products (MRTP), Lung Epithelial-cell Damage, Pulmonary Inflammation

Implications: Exposure of mice to heated tobacco product IQOS, one of the candidate modified-risk tobacco products (MRTPs), induces inflammatory immune-cell accumulation in the lungs and augments the levels of proinflammatory cytokines and chemokines in the bronchoalveolar lavage (BAL) fluid. Such an exacerbated pulmonary proinflammatory microenvironment accompanies with lung epithelial-cell damage in IQOS-exposed mice, suggesting a potential association with the impairment of lung function.

INTRODUCTION

Cigarette smoking is recognized to be a significant risk factor for the development of COPD, several cancers, cardiovascular and oral diseases.[1-5] Cigarette smoke (CS) triggers inflammation and other physiological changes in the airway epithelium causing pulmonary damage and suppression of both innate and adaptive immunity resulting in repeated infections.[6-9] Evidence from animal models shows that cessation of exposure to CS results in a reduction in pulmonary inflammation and at least partially restores immune function.[9] Pulmonary damage can result from a direct impact on lung tissue or an indirect effect as a consequence of the generation of a proinflammatory milieu comprising of cytokines and chemokines made by immune cells recruited to the lung.

In an effort to reduce tobacco-related harm and keep and expand their customer base, the tobacco companies attempted to develop "safer cigarettes" since the 1960s.[10] Recently, Philip Morris International (PMI) developed a heated tobacco product (HTP), also called 'Heat-not-Burn' (HnB) product, that is currently marketed under the brand name IQOS. With a sleek, technologically forward design, IQOS was initially launched in 2014 in Nagoya, Japan and Milan, Italy, and was gradually rolled out in other countries and was approved for sale in the USA in 2019.[11,12] On July 7th 2020, the FDA approved MRTP application for IQOS based on scientific studies that switching completely from conventional cigarettes to the IQOS system significantly reduces the body's exposure to harmful or potentially harmful chemicals.[13]

PMI has published several studies comparing the effects of exposure to aerosols from their HTP product to CS. *In vitro* studies conducted by PMI demonstrated that, at similar nicotine concentrations, HTP-aerosols elicited inflammatory processes and cellular stress responses

to a lower degree than CS.[14] Based on a study conducted in mice, long-term inhalation of aerosols from HTP had a lower impact on lung inflammation than exposure to CS.[15] These studies also demonstrated that switching exposures from chronic CS to HTP-aerosols considerably impeded the progression of CS-induced emphysematous and atherosclerotic changes.[15] PMI in a recently completed clinical study concluded that by switching from menthol cigarettes to menthol THS significant reduction in the exposure to harmful and potentially harmful constituents (HPHCs) were observed to levels approaching those detected in subjects who abstained from smoking, and this was associated with the overall improvements in biomarkers of potential harm.[16,17] However, an industry-independent study demonstrated that acute exposures to IQOS-aerosols impaired arterial flow-mediated dilation, a measure of vascular endothelial function, in a similar way to CS.[18] Furthermore, upon reviewing PMI's application to assess the toxicities associated with IQOS use, Moazed et al. concluded that there was evidence of severe pulmonary inflammation and immune toxicities in rats exposed to IQOS aerosols.[19] Their review further confirmed that among human users, there was no evidence of improvement in lung inflammation or pulmonary function in cigarette smokers who had switched over to IQOS. One of the major limitations of studies conducted by PMI was that they failed to evaluate measurements of lung-specific inflammatory immune responses in their human studies.

In this study, we report the impact of acute inhalation exposure to IQOS-aerosols and CS on pulmonary inflammation and lung damage as reflected by changes in immune cell infiltrates, the profiles of proinflammatory cytokines/chemokines in the BAL and in the levels of albumin in the BAL which served as a surrogate marker of lung epithelial-cell damage.

MATERIALS AND METHODS

Tobacco Products

IQOS devices (model 2.0) and HEETS Red Label inserts (Philip Morris International) were purchased from an IQOS flagship store in Toronto, ON, Canada. Reference tobacco cigarettes 3R4F were supplied by the Kentucky Tobacco Research & Development Center (University of Kentucky; Lexington, KY). Prior to testing, all HEETS inserts and tobacco cigarettes were conditioned at 22±1°C with a relative humidity of 60±3% for a period of 48 h following ISO 3402.[20] Both tobacco products used in the study (conventional tobacco cigarettes and Heetsticks for IQOS) were commercially available products containing different tobacco fillers. Both products were used as purchased without any modification as intended for consumer use. Reference tobacco cigarettes were light using an electric lighter, while the Heetsticks were placed in IQOS heater device (holder). Tobacco was not removed from cigarettes and placed in IQOS.

Mice

Eight week old female C57BL/6NCr mice were procured from Charles River and housed under specific pathogen-free conditions in the Department of Laboratory Animal Resources (Roswell Park Comprehensive Cancer Institute, Buffalo, NY) with light/dark cycle of 12/12 h. Number of animals per group in each experiment was n=10 (5 males and 5 females). All experiments involving mice were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Roswell Park Comprehensive Cancer Center (Buffalo, NY, USA) and complied with all state, federal, and NIH regulations.

Animal Exposure System

Mice were placed in a modified 15-liter exposure chamber (Vet Equip Inc.; Livermore, CA) with two sections separated with a 0.5cm steel wire mesh **(Supplemental Figure 1**). Each set of mice (male and female) were rotated once per day between cage positions to ensure uniform exposure to emissions from tobacco products. The exposure chamber was connected to a smoking machine using a Tygon tubing. During exposure to HTP, animal exposure chambers were connected to a vacuum-operated smoking machine developed in-house and customized for IQOS device (see **Supplemental Materials**). During exposure to CS, animal exposure chambers were connected to JB2090 automatic smoking machine (CH Technologies; Westwood, NJ).

Exposure Protocol

Mice were exposed for a total of 5h/day for 2 wks to emissions from 20 HEETS or 20 tobacco cigarettes. Each day, 20 series of puffs (12 puffs/series from IQOS and 8 puffs/series from tobacco cigarettes) were generated every 15 min following the Health Canada Intensive puffing regime of 55 ml puff every 30 sec. The puff durations for IQOS and tobacco cigarettes were 5 and 2 sec, respectively. IQOS device was activated manually before each series of puffs and placed in a charger during breaks between puff series. During intervals between individual puffs and breaks between puff series, animals were exposed to filtered air. Mice were euthanized 16 h after the final exposure.

Monitoring of Exposure Conditions

The concentration of particulates ($PM_{2.5}$) was constantly monitored during experiments using SidePak AM510 monitor (TSI; Shoreview, MN) connected directly to a sampling port in the exposure chamber. SidePak Buddy software[21] was used to analyze temporal and time-weighted average (TWA) $PM_{2.5}$ concentrations inside the exposure chambers. Calibration factors of 1.00 and 0.32 were used for IQOS and CS, respectively. We also collected air samples from inside exposure chambers to measure airborne nicotine concentration. Nicotine was sampled every day using an active sampling technique on XAD-4 sorbent tubes (SKC Inc.; Eighty Four, PA) with a flow rate of 1.7 l/min. Sorbent tubes were processed following NIOSH 2551 protocol[22] and nicotine was analyzed using gas chromatography (GC) as described in **Supplementary Materials**. Samples of animal fur and wipes of wall surface area inside the exposure chamber were also collected to measure potential deposition of nicotine. Detailed descriptions of the sampling procedures and analytical methods used are provided in **Supplementary Materials**. Temperature, atmospheric pressure and humidity sensors placed inside the exposure chamber were used for continuous monitoring of exposure conditions during experiments.

Analysis of Blood Samples

Blood samples were collected at the end of week 1 and 2 immediately after the exposure. Serum was separated and samples (100 µL) were analyzed for cotinine, a primary metabolite of nicotine, using liquid chromatography method with tandem mass spectrometry (LC-MS/MS) as described in **Supplementary Materials**.

Bronchoalveolar lavage (BAL) fluid and lung tissue collection and isolation of lung leukocytes

Mice were euthanized and tracheae were cannulated to collect BAL fluid by injecting 0.75 mL ice cold 1% BSA solution in PBS twice using IV catheters. Lungs were harvested and subjected to collagenase IV/DNase I digestion.[9] The resulting single-cell suspension was passed through a 40µm filter to remove debris and undigested tissue, then underlaid with Ficoll-Paque and centrifuged with brake off. Leukocytes at the interface were collected, washed and counted using trypan blue staining.

ELISA

Albumin levels in the BAL were quantified by ELISA using Bethyl Laboratories (Montgomery, TX, USA) reagents and plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB) from eBioscience Inc. (San Diego, CA, USA), and absorbance read at 450 nm as described previously.[9]

Flow cytometry

Immune cells were stained with cell type-specific antibodies (Supplementary Materials) for FACS analysis to determine the numbers and phenotype of various immune subsets as described previously.[9] Samples were acquired using LSRII-A flow cytometer (BD) and data were analyzed using FlowJo 10.03 software (Tree star Inc. OR, USA). Gating strategy followed was exactly as described previously [9] and summarized in the **Supplemental Figure 2**.

Multiplex cytokine/chemokine assay

Cytokine concentrations in the BAL were measured by performing Luminex multiplex cytokine/chemokine assay using Bio-Plex Pro Mouse Cytokine 23-plex Assay (BIORAD, #M2009RDPD, Lot# 64209360) following the manufacturer's instructions. The kit assayed for IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α. Data acquisition was performed on FLEXMAP 3D (Luminex Corp. Austin, TX, USA). During data analysis, limit of detection (LOD) was used for any data points with values <LOD.

Statistical analysis

Statistically significant differences between the mean values of different groups were determined by two-way ANOVA with Tukey's post-test comparisons by GraphPad Prism 8 software (GraphPad; La Jolla, CA). In each experiment, n=10 mice were used; 5 males and 5 females. The differences between the two groups were considered statistically significant when *p* values were <0.05.

RESULTS

Exposure Conditions

The exposure protocol used in our study resulted in comparable concentrations of particulates inside the chambers (197.0±47.0 μg/m^3 from IQOS vs. 269.0±94.0 μg/m^3 from CS; p>0.05; **Table 1**). Although significantly higher airborne nicotine concentrations were measured inside cages after exposure to IQOS compared to CS (432.5±260.8 vs. 174.7±106.2 μg/l; p<0.05; **Table 1**), exposure to both tobacco products resulted in comparable cotinine concentrations in the serum of exposed animals (29.5±19.7 vs. 35.6±17.8 ng/ml,

respectively; **Table 1**). Low levels of nicotine were detected on chamber walls after exposure to IQOS, while no nicotine was detected on chamber walls after exposure to CS. No nicotine was detected on animal fur after exposure to both tobacco products. Environmental conditions inside the chambers did not differ significantly except for slightly higher temperature recorded after exposure to IQOS (22.5±0.2°C vs. 21.7±0.5°C; $p<0.05$; **Table 1**).

Acute exposure to IQOS-aerosols or CS induces lung epithelial-cell damage

Albumin levels in the BAL of mice were quantified as a surrogate marker of lung epithelial-cell damage. We observed that acute exposures to IQOS-aerosols or CS induced significantly increased levels of albumin in the BAL of mice compared to air-exposed controls, indicating smoke/aerosol-induced lung epithelial-cell damage in mice (**Figure 1A**). However, lung epithelial cell damage was significantly higher in mice acutely exposed to CS than aerosols from IQOS. It is noteworthy that the albumin levels in the BAL after either IQOS-aerosol or CS inhalation were equivalent in mice of both sexes (**Supplemental Figure 3**), demonstrating that the lung damage induced to these exposures is not sex-biased.

Acute inhalation of IQOS-aerosols or CS augments pulmonary immune cell infiltration and modulates the numbers of innate immune cells in the lungs

Exposure of mice to emissions from IQOS and CS resulted in markedly augmented infiltration of total immune cells to the lungs compared to exposure to filtered air (**Figure 1B**). Immuno-phenotypic analysis of the infiltrating cells in the lungs revealed significantly increased numbers of neutrophils in mice following CS inhalation compared to both air and IQOS-aerosol inhalation (**Figure1B**). There was a marked reduction in the numbers of

macrophages in the lungs of mice as a result of IQOS-aerosol inhalation compared to air or CS exposures (**Figure 1B**).

We evaluated the role of sex on the differential responses to inhalation of IQOS-aerosols or CS and noted that acute exposure to CS induced higher immune-cell infiltration to the lungs of male compared to female mice. Sex did not have a differential impact on the total immune-cell infiltration to the lungs in mice exposed to IQOS-aerosols (**Supplementary Figure 4A**). Additionally, acute exposure to CS induced a greater accumulation of neutrophils when evaluated by sex (83,013 neutrophils/lung in males vs 29,570/lung in females, $p<0.01$) but did not influence the numbers of macrophages in the lungs of male vs female mice (**Supplementary Figure 4B,C**). Accumulation of neither neutrophils or macrophages in the lungs of mice exposed to IQOS-aerosols was impacted by sex (**Supplementary Figure 4B,C**).

Acute exposure to IQOS-aerosols or CS modulates adaptive immune cell accumulation in the lungs

Acute exposure of mice to CS but not aerosols from IQOS significantly enhanced the numbers of $CD4^+$ and $CD8^+$ T cells in the lungs as compared to exposure to filtered air (**Figure 1C**). In contrast, lower numbers of $CD19^+$ B cells were found in the lungs following inhalation of both IQOS aerosols and CS versus air-exposed mice (**Figure 1C**). Although acute IQOS exposure induced greater infiltration of T and B cells in male mice than female mice (9,849 $CD4^+$ T cells in males vs 4,863 cells in females; 24,709 $CD8^+$ T cells in males vs 2,604 cells in females; 10,946 $CD19^+$ B cells in males vs 5,124 cells in females), the differences

were not significant (**Supplemental Figure 4D-F**). Furthermore, infiltration of T and B cells to the lungs in response to acute exposure to CS was equivalent in mice of both sexes (**Supplemental Figure 4D-F**).

Pro-inflammatory cell accumulation in the lungs following acute exposure to IQOS-aerosol or cigarette smoke

We assessed the influence of acute exposure to IQOS-aerosols and CS on the accumulation of inflammatory T cells and found exposure to both IQOS-aerosols and CS induced increased infiltration of $CD4^+IL\text{-}17A^+$ T cells into the lungs compared to air exposed controls, however the response was higher in the CS group (**Figure 1C**). Acute exposure to both IQOS-aerosols and CS equivalently enhanced the infiltration of $CD4^+ROR\gamma t^+$ T cells (**Figure 1C**). The abundance of these inflammatory T cells is strongly associated with several diseases having inflammation as an underlying component (**Table 3**). There were no sex specific differences seen in infiltration of $CD4^+IL\text{-}17A^+$ and $CD4^+ROR\gamma t^+$ T cells in the lungs (**Supplementary Figure 4G,H**).

IQOS-aerosols and CS induce proinflammatory cytokines and chemokines in the BAL of mice following acute exposure

We observed that the levels of various cytokines and chemokines associated with inflammation were augmented after exposure to IQOS-aerosols and CS. Acute exposure to IQOS-aerosols elevated the levels of Th1 cytokines IL-2, IFN-γ, TNF-α, Th2 cytokines IL-13, IL-5 and Th17-related cytokine IL-17A in the BAL (**Table 2 and depicted with histograms in Supplementary Figure 5**). Furthermore, IQOS-aerosol exposure significantly elevated the levels of various inflammation-associated chemokines in the BAL of exposed mice. Levels of

RANTES, KC and Eotaxin, the signature chemokines of an inflammatory milieu were markedly augmented in the BAL of mice exposed to IQOS-aerosols (**Table 2 and Supplementary Figure 5**). We also detected elevated levels of, macrophage inflammatory proteins (MIP) MIP-1α and MIP-1β that belong to the family of chemotactic cytokines known as chemokines and are crucial for immune responses towards infection and inflammation in the BAL of mice exposed to IQOS-aerosols, nevertheless the differences were not statistically significant (**Table 2 and Supplementary Figure 5**)**.** Overall, the levels of proinflammatory cytokines and chemokines induced were similar following both IQOS and CS exposures. Association of various proinflammatory cytokine/chemokine biomarkers with various lung pathologies is given in **Table 3**.

DISCUSSION

We performed a side by side comparison of the potential impact of IQOS-aerosol and CS exposure on the recruitment of immune cell infiltrates to the lung, the levels of cytokines and chemokines and lung epithelial-cell damage using our pre-clinical animal model of aerosol and cigarette smoke inhalation. Our findings reveal that acute exposure to IQOS-aerosols induces a proinflammatory microenvironment comprising of inflammatory immune cell accumulation as well as multiple proinflammatory cytokines/chemokines in the lungs. Furthermore, this acute inhalatory exposure to IQOS-aerosols induced a proinflammatory lung microenvironment which promotes lung damage. These proinflammatory changes induced by IQOS-aerosols could potentially lead to further outcomes including increased susceptibility to respiratory infections and suppressed responses to prophylactic

vaccinations, conclusions which can be drawn from our previous studies on tobacco smoking and immunity.[7,9]

Although short-term inhalation of IQOS-aerosols, like CS, augmented total immune-cell infiltration to the lungs of mice, nevertheless we did not observe significant increase in the total numbers of neutrophils and macrophages. These findings corroborate with PMI's report on a mouse model of long-term exposure to CS and IQOS which demonstrated a lower impact of IQOS-aerosols on the absolute numbers of neutrophils and macrophages as compared to CS exposed mice.[15] Recent *in vitro* studies have demonstrated that IQOS exposure is as detrimental as CS and concluded that inhalation of IQOS-aerosols has the potential to induce oxidative stress and inflammation, increase infections, airway remodeling and initiate epithelial- mesenchymal transition (EMT)-related changes in the airways of users of these devices.[23] IQOS-aerosol induced detrimental effects could be potentially be attributed to high exposure to nicotine that was comparable to exposure achieved from CS. It is also possible that other toxicants emitted from IQOS could cause the observed detrimental effects. Tobacco inserts used in IQOS devices have been shown to emit numerous toxicants, including respiratory irritants and cardiovascular toxicants like tobacco-specific nitrosamines (TSNAs), metals, volatile organic compounds (VOCs), phenolic compounds, polycyclic aromatic hydrocarbons (PAHs), as well as minor tobacco alkaloids and organic solvents, although typically at levels lower than those found in CS. [24-27] Additionally, inhalation risks of large doses of humectants used in IQOS and other HTP products, e.g. propylene glycol and vegetable glycerin, are currently not well characterized.

Our observation that exposure to CS but not emissions from IQOS caused a significant enhancement in the numbers of $CD4^+$ and $CD8^+$ T cells in the lungs is supported by an early

study which showed higher numbers of these cells in the BAL of mice exposed to CS than IQOS-aerosols.[15] However in the present study, the pro-inflammatory nature of IQOS-aerosols is highlighted by our observation of the increased numbers of $CD4^{+}IL\text{-}17A^{+}$ and $CD4^{+}ROR\gamma t^{+}$ T cells in addition to increased total immune cells that infiltrated the lungs of IQOS-aerosol exposed mice similar to CS-exposed mice. The abundance of these cells has been strongly associated with diseases having inflammation as an underlying factor (**Table 3**). However, we also acknowledge that the chemical composition of IQOS emissions is different than CS exposure and this difference might play a major role in the differential accumulation or recruitment of various immune cells in the lungs after exposure. Our conclusion of a pro-inflammatory lung microenvironment induced by IQOS is further supported by the differential changes in the profiles of inflammatory cytokines and chemokines induced in the BAL. While proinflammatory cytokines such as TNF-α, IFN-γ, and KC were significantly increased, cytokines related to allergic asthma conditions, such as IL-5 and IL-13 were also increased in the BAL fluid, indicating Th2 differentiation. Further, the elevated levels of IL-9, RANTES, and Eotaxin suggest the potential for increased mast cells, eosinophilia, and mucin upregulation. Th1 cytokines, particularly IFN-γ, acting in concert with Th2 cytokines, suggest allergic inflammation in the lung with acute exposure to IQOS. Our cytokine data are consistent with the flow cytometric analysis showing augmented numbers of inflammatory T cells in the lungs of mice exposed to IQOS-aerosol, implicating elicited inflammatory response and immune regulation.

In contrast, PMI studies reported an abundance of pro-inflammatory cytokines and chemokines in the BAL of mice that inhaled cigarette smoke, but not in the BAL of mice that inhaled IQOS aerosols.[15] Additionally, in our study, the elevated levels of albumin

quantitated in the BAL as a surrogate marker of lung epithelial-cell damage substantiate the inflammatory milieu induced by the inhalation of IQOS aerosol. These detrimental effects could be due to the fact that IQOS-aerosol contains harmful constituents.[25] A review of the data from PMI's application submitted to the FDA reported that biomarkers of potential harm in human smokers including measures of inflammation, oxidative stress, and lung function were not detectably different after inhalation of IQOS-aerosol compared to CS.[19,28]

Limitations of our study include the lack of experiments to demonstrate the long-term effect of IQOS aerosol-inhalation on pulmonary inflammation and immune dysfunction, and that differences could result from experiments done using nose-only exposure systems. It is also possible that long-term exposure may reveal different results. Furthermore, we recognize that our study is limited to a one-time point investigation following 2-week IQOS-aerosol inhalation and lacks the histopathological evaluation of lung tissue following the exposures.

In conclusion, we have demonstrated that IQOS-aerosol inhalation is detrimental, recruiting proinflammatory cells to the lung, inducing a microenvironment mediated by proinflammatory cytokines and chemokines and lung epithelial-cell damage. Since sustained smoke, allergic or environmental-triggered inflammation induces airway remodeling that plays a pivotal role in airflow limitation and lung damage in disorders like asthma and COPD,[29-32] long-term inhalation of IQOS-aerosols could potentially also be detrimental to pulmonary health. Importantly, alterations in the lung epithelial cells and immune impairment after long term exposure to IQOS aerosols may be severe, thereby warranting more extensive investigations before IQOS could be confidently considered as a safe alternative to combustible cigarette smoking.

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Declaration of Interest:

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[**Note**: References 51-66 are available as supplementary material.]

FIGURE LEGENDS:

Figure 1:

(**A**) Acute exposure to IQOS aerosols or CS induces lung epithelial-cell damage. At the end of the exposures, mice were euthanized, BAL harvested and the levels of albumin in the BAL were quantified by ELISA as described in Materials and Methods. (**B-C**) Acute exposure to IQOS aerosols or smoke from combustible cigarettes (CS) modulates airway immune cell infiltration. Total number of leukocytes, $CD11b^{+}Ly6G^{+}$ neutrophils and $CD11b^{+}Ly6G^{-}CD68^{+}$ macrophages (**B**), and numbers of $CD4^{+}$ T cells, $CD8^{+}$ T cells and $CD19^{+}$ B cells and $CD4^{+}IL17A^{+}$ and $CD4^{+}ROR\gamma t^{+}$ inflammatory T cells (**C**) in the lungs of mice exposed to air, IQOS aerosols or CS were determined by flow cytometry using specific markers and following a gating strategy as described previously [9] and shown in **Supplemental Figure 2**. Data are mean±SE. *p≤0.05, **p≤0.01, ***p≤0.005 ****p≤0.0001, two-way ANOVA with Tukey's post-test comparisons by GraphPad Prism 8 software (GraphPad; La Jolla, CA). In each experiment n=10 mice Were used.

Table 1:

Exposure conditions inside chambers recorded during experiments (Mean±Std.Dev).

Note: * represents a significant difference between IQOS and CS exposure conditions (p=<0.05). All results are for n=10 (ten samples collected every day), except for serum cotinine which are for n=20 (ten mice serum samples per week). <LOQ = below limit of quantitation.

Table 2:

Two-week exposure to IQOS- aerosols or CS augments inflammatory cytokine/chemokine profile in the BAL fluid. Levels of various inflammation-associated cytokines and chemokines in the BAL of mice after 2 wks of exposure to air, IQOS aerosols or CS were quantified by Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (BioRad USA; Cat.# M60009RDPD) as described in Materials and Methods. Data are mean±SE. *p≤0.05, **p≤0.01, ***p≤0.001; IQOS and CS vs Air, or †p≤0.05 IQOS vs CS. Ordinary one-way ANOVA with Tukey's post-test comparisons by GraphPad Prism 8 software (GraphPad; La Jolla, CA). In each experiment n=10 mice were used.

Table 3: Biomarkers of lung pathologies having inflammation as a causal factor. Table depicts various cellular and secretory biomarkers and their association with lung diseases having pulmonary inflammation as an underlying factor.

Table 1. Exposure conditions inside chambers recorded during experiments (Mean±Std.Dev).

		Heat-not-Burn (HnB) product, IQOS	**Tobacco Cigarette**
Particulates $PM_{2.5}$ ($\mu g/m^3$)	Time-Weighted Average/Day	197±47	269±94
Airborne Nicotine (µg/l)	Average/Day	**432.5±260.8***	174.7±106.2
Serum Cotinine (ng/ml)	Week 1 and 2	29.5±19.7	35.6±17.8
Nicotine Deposited on Chamber Walls ($\mu g/cm^2$)	Average/Day	**8.0±2.2***	<LOQ
Nicotine Deposited on Animal Fur ($\mu g/cm^2$)	Average/10 Mice	<LOQ	<LOQ
Atmospheric Pressure (mBar)	Average/Day	991.2±9.1	993.5±2.7
Temperature (ºC)	Average/Day	**22.5±0.2***	21.7±0.5
Relative Humidity (%)	Average/Day	70.5±2.9	66.0±7.1

Table 2: Levels of various cytokines or chemokines (pg/mL) in the BAL fluid of mice exposed to Air, IQOS or CS aerosols for 2 weeks. Data are mean±SE.

Cytokine/ Chemokine (pg/mL)	Air	IQOS	CS
IL-2	1.19±0.14	2.79±0.28 ***	2.32±0.21 **
IFN-γ	24.60±4.94	**72.53±12.02** **	54.92±9.14
TNF-α	32.87±18.50	**157.94±32.64** *	147.15±31.13 *
IL-6	0.67±0.09	0.92±0.088	1.06±0.11 *
IL-9	26.50±4.10	**133.35±14.18** **	136.36±15.42 **
IL-13	21.50±3.76	**43.62±2.75** ***	27.91±2.39 **
IL-5	1.48±0.25	3.84±0.37 ***	2.74±0.14 *
IL-17A	4.22±1.29	8.94±1.68	6.98±1.60
G-CSF	9.25±1.94	8.71±0.99	15.92±1.91 * (†)
RANTES	13.39±2.79	**44.31±6.12** **	30.12±3.62 *
KC	83.29±14.54	**352.6±53.11** ***	284.27±29.75 **
Eotaxin	83.38±19.77	**386.81±62.90** ***	257.45±28.22 *
MIP-1α	11.16±1.82	18.61±3.04	32.27±5.11 **(†)**
MIP-1β	17.51±5.52	**121.08±31.55** *	141.13±33.29 *

Table 3: Various cellular and secretory biomarkers associated with pulmonary inflammation and disease.

	Cellular and secretory markers associated with lung inflammation and pulmonary diseases		
	Biomarker	**Clinical relevance**	**References**
1	$CD4^+IL\text{-}17A^+$ T cells	Respiratory infection, inflammation, lung adenocarcinoma	8, 9, 33
2	$CD4^+ROR\gamma t^+$ T cells	Respiratory infection, inflammation, Autoimmune diseases, IBD	8, 9, 34
3	IFN-γ	Respiratory infection, inflammation, Influenza virus infection, tuberculosis	35-37
4	TNF-α	Respiratory viral infection, COPD	38-40
5	IL-17A	Respiratory infection, inflammation, IBD, rheumatoid arthritis, psoriasis, COPD	8, 9, 41-43
6	MIP-1a	Respiratory infection, RSV infection and lung inflammation	44-46
7	MIP-1β	RSV infection, inflammation	44, 47, 48
8	IL-6	COPD, Respiratory infection, Asthma	40, 49
9	RANTES	RSV infection, Asthma, Sarcoidosis	50, 51
10	IL-2	COPD, Asthma, respiratory tract infection	52, 53
11	IL-5	Allergic Asthma, COPD	52, 54
12	KC	COPD, Lung inflammation	55-57
13	Eotaxin	COPD, Asthma	58, 59
14	G-CSF	COPD, Cystic Fibrosis	60, 61
15	IL-13	Respiratory infection, inflammation, asthma	62-66

Figure 1