Immune modulation by chronic exposure to waterpipe smoke and immediate-early gene regulation in murine lungs

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ABSTRACT

Objective We investigated the effects of chronic waterpipe (WP) smoke on pulmonary function and immune response in a murine model using a research-grade WP and the effects of acute exposure on the regulation of immediate-early genes (IEGs).

Methods WP smoke was generated using three WP smoke puffing regimens based on the Beirut regimen. WP smoke samples generated under these puffing regimens were quantified for nicotine concentration. Mice were chronically exposed for 6 months followed by assessment of pulmonary function and airway inflammation. Transcriptomic analysis using RNAseq was conducted after acute exposure to characterise the IEG response. These biomarkers were then compared with those generated after exposure to dry smoke (without water added to the WP bowl).

Results We determined that nicotine composition in WP smoke ranged from 0.4 to 2.5 mg per puffing session. The lung immune response was sensitive to the incremental severity of chronic exposure, with modest decreases in airway inflammatory cells and chemokine levels compared with air-exposed controls. Pulmonary function was unmodified by chronic WP exposure. Acute WP exposure was found to activate the immune response and identified known and novel IEG as potential biomarkers of WP exposure.

Conclusion Chronic exposure to WP smoke leads to immune suppression without significant changes to pulmonary function. Transcriptomic analysis of the lung after acute exposure to WP smoke showed activation of the immune response and revealed IEGs that are common to WP and dry smoke, as well as pools of IEGs unique to each exposure, identifying potential biomarkers specific to WP exposure.

INTRODUCTION

Waterpipes (WPs), also known as hookah, have been used for centuries in the Eastern Mediterranean region and parts of Asia and Africa.¹ However, the popularity of WP smoking has seen a dramatic surge in recent years in the USA and abroad, particularly among young adults.^{2,3} In the USA alone, the prevalence of WP use among youths increased from 7% to 12% between 2010 and 2015.⁴ Moreover, despite successful legislative bans on indoor smoking in many countries, most indoor smoking legislation exempts or does not enforce these bans on WP establishments.⁵

Thus, WPs represent an emerging alternative tobacco product that may pose significant risks to public health, particularly among young individuals.

Contrary to common misconception, serious health effects are related to WP use. Epidemiological studies have shown that WP smoking is associated with increased risks of cardiovascular diseases,⁶ cancer (lung, oesophageal, head and neck, and gastric),^{7–10} metabolic syndrome¹¹ and chronic obstructive pulmonary disease (COPD).¹² An additional health risk that is of particular concern for young adults is nicotine dependence, and similar to any other tobacco product is represented in WP smokers by substance addiction behaviours.^{13–19} In agreement with the health risks identified to date, analytical examination of the components in WP smoke include a mixture of chemicals with known or suspected disease-causative agents that closely resemble those found in cigarette smoke (CS), and in many cases at higher concentrations.^{20,21} Indeed, WP is estimated to deliver to cells and tissues a similar group of toxicants that are known to dysregulate cell molecular mechanisms, including DNA repair and methylation.^{22–26} Herein, we investigated the effects of lung health using a murine model by analysing inflammation and pulmonary function after chronic WP exposure for 6 months. On the basis of the scarcity of papers published on this topic, the exposure length of 6 months is rarely studied. Thus, the current study unveiled the modulation of immunological response after chronic WP exposure.

Biomarkers of tobacco use are predictors of molecular changes that precede toxicological health outcomes.²⁷ These biomarkers provide information to policymakers during the assessment of health risks associated with new and modified tobacco products.²⁸ Several biomarkers exist for conventional tobacco products; however, biomarkers specific for WP, smokeless tobacco products or other modified-risk tobacco products have not been well established.^{29,30} We report, for the first time, the differential expression of immediate-early genes (IEG) upregulated in the lung after acute exposure to WP and dry smoke (with no water in the bowl). The IEGs identified in this study are potential biomarkers of exposure to WP.

METHODS

WP smoke

WP smoke was generated via a research-grade WP fabricated by Battelle as described previously.³¹

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Table 1 Waterpipe puffing regimens

Regimen	Low	Modified Beirut	High	Beirut ³²
Puff volume (mL)	300	530	750	530
Puff duration (s)	3	3	3	2.6
Puff interval (s)	18	18	18	17
Puffs per session	171	171	171	171
Session duration (min)	60	60	60	61
Mean puff flow (L/min)	6	10.6	15	12.2
Total volume (L)*	51.3	90.6	128.25	90.6

*Total volume per session.

Before each exposure, 1400 mL fresh water was added to the WP bowl tank, and 12–13 g shisha was added to the head. Subsequently, the head was covered with aluminium foil, and a lit 40 mm charcoal (Al Fakher) was placed on top. At 10 min postlighting, the WP was puffed according to specific regimens using a connected peristaltic pump. After 40 min, the charcoal was replaced by an identical charcoal. Dry smoke was generated using a setting similar to that for WP smoke, without the addition of water to the bowl. The shisha products used are commercially available Blue mint (product 1, a minted product) and Exotic Pirate's Cave (product 2) from Starbuzz. A diagram of the WP setting is provided in online supplementary figure 1.

Puffing regimens

WP smoke was generated with one of three different puffing regimens. The regimens were based on the Beirut puffing regimen.³² For this study, a modified Beirut regimen was executed with a peristaltic pump (table 1). Additionally, 'high' and 'low' puffing regimens were generated, as described in table 1.

Mouse exposure

The source and strain of mice (C57BL/6J, female mice, 6–7 weeks old and 19 g average weight) used in all experiments were purchased from Jackson Laboratory. WP smoke was generated as described in table 1 and was mixed with filtered dilution air at a flow rate of 10 L/min. Animals were exposed to WP smoke for 1 hour/day, 5 days/week for 6 months in a nose-only exposure chamber (Jaeger-Baumgartner). Body weight measurements used a digital plate balance to monitor weight change at 1, 2, 4 and 6 months in the mice used for chronic exposure to product 2. Online supplementary table 1 shows the sample number (n) and the exact duration for each experiment.

TPM collection and nicotine measurements

For the analysis of the nicotine content of WP smoke, we used a previously described method with few modifications.³³ Briefly, total particulate matter (TPM) of WP smoke was collected (product 2) on a Cambridge filter pad (CFP) (47 mm diameter PSI borosilicate glass depth media, 1–2 µm) located inside an aluminium housing hooked to the mouse exposure chamber. Product 1 was not measured. For nicotine analysis of the mainstream smoke, the CFP was sampled for 30 min at a high puffing regimen, without the dilution air injection and with the peristaltic pump for sampling set at 2 L/min. To obtain a good signal to noise ratio for the mass spectroscopic analysis, we concentrated two more exposures to the same CFP using the following procedure. The CFP was left in the aluminium housing equilibrated to room conditions overnight, and the exposure was repeated for two additional 24-hour periods. Three replicates (n=3) were collected for the analysis as described above. Gravimetric

analysis was performed to determine the TPM masses by calculating the weight difference of the CFP before and after smoking. The average (±SD) TPM per exposure for nicotine analysis was 3339 (715) mg/m³, and the mean weight difference of the CFP was 482 (141) mg. The TPM was then extracted with 20 mL of extraction solution (isopropyl alcohol containing approximately 0.1 mg/mL anethole internal standards) with gentle shaking at 160 revolutions per minute (rpm) for 30 min. Extracts were analysed for nicotine using gas chromatography-flame ionisation detection. Calibration curves were constructed with 10 different analyte concentrations ranging from 0.004 to 1.0 mg/mL. Quantification was determined using area ratios of the analyte-to-internal standard with a linear, least-squares fit to the calibration curve. Nicotine mass ratio refers to the total nicotine divided by the total suspended particle (TSP), as previously defined.³⁴

TSP collection

For all exposures, TSP was collected daily to a CFP throughout the entire 60 min exposure using a peristaltic pump that sampled the exposure chamber at 0.4 L/min. For the acute exposure, TSPs were sampled for 5 days in the mouse exposure chamber according to the same experimental conditions used for the acute 1-hour exposures. TSP data are presented in table 2.

Airway inflammation

Mice were euthanised approximately 24 hours after the final WP exposure. Bronchoalveolar lavage (BAL) was performed, and cell-free supernatants were used for cytokine analysis, as described previously.^{35 36} Total cell counts from BAL were quantified via an automatic cell counter (Adam MC, Bulldog Bio) and differential cell counts were determined via microscopic evaluation of cytopsin preparations (Thermo Fisher) according to standard cytological criteria.³⁷ Total cell counts in the air control groups of product 1 and product 2 did not differ statistically. Online supplementary table 2 contains statistics and biological replicas.

Cytokine and chemokine detection

Inflammatory cytokines were quantified in BAL samples using a chip assay based on Luminex Technology (Luminex Corporation) and Milliplex panel kit MCYTOMAG-70K (MilliporeSigma); this allowed the simultaneous testing of 32 analytes. The standard protocol of Milliplex panel was employed, with a few exceptions. Briefly, 15 µL of BAL sample was used, and the incubation period was increased to 16 hours in a cold room. Samples were randomly positioned on a 96-well plate. The Curiox (Curiox Biosystems) wash system was used on plates, as reported previously.^{38 39}

Lung function

Mice were anaesthetised with a ketamine (90 mg/kg)-xylazine (18 mg/kg) mixture. Once sedated, tracheostomy was performed, and an 18-gauge cannula was inserted. Diffusing capacity for carbon monoxide (CO) was then measured using the single-breath method as described.^{40 41} For pressure-volume (PV) curves, after diffusion factor for carbon monoxide (DFCO) was measured, mice were connected to a flexiVent ventilator as described previously.⁴² DFCO measurement is an indication of lung function similar to diffusion capacity of the lung for carbon monoxide (DLCO). This diffusing capacity metric is dimensionless, varying between 0 and 1, where 0 reflects no CO uptake and 1 reflects complete CO uptake. Sampling pure alveolar gas is not feasible in the mice. Sampled gas includes the small

Table 2 TSP recorded during exposure experiments in the mouse nose-only exposure chamber

Regimen	Product 1 TSP* (mg/m ³)±SD	Product 2 TSP* (mg/m ³)±SD
Chronic exposure (6 months)		
Low	330±064	317±35
Modified Beirut	644±141	521±40
High	827±155	743±76
Acute exposure (1 hour)		
WP	ND	560±133
Dry smoke†	ND	654±307

ND means product 1 was not tested.

*Mean TSPs of daily measures taken through each 1-hour exposure session according to product and puffing regimen.

†Mean TSP for WP smoke generated without water in the bowl (dry smoke). TSP includes all particulates and water from the smoke. For WP mainstream smoke, we found that water weight accounts for 57% (±13, SD; n=4) of the total filter weight, which was used as a correction factor for nicotine quantification.

TSP, total suspended particle; WP, waterpipe.

amount of gas found in the anatomical dead space that changes the quantitative values compared with DLCO. However, because the volume of the dead space is relatively constant in a group of mice that are biologically similar, it does not alter the ability to detect changes in gas diffusion in a host of lung pathologies. Changes in DFCO have been reliably measured in mice with fibrosis, emphysema, cystic fibrosis, influenza and acute lung injury.^{40–41} Quasi-static PV curves were performed as previously reported.^{42–43} Because mouse lungs never reached a true maximal lung volume,⁴³ we opted to define lung capacity (V_{35}) as the volume from the second inflation curve at 35 cm H₂O, a pressure beyond which all mouse strains show progressive stiffening. Quasi-static compliance of the respiratory system was computed from the PV relationships as the slope of the deflation limb between 3 and 8 cm H₂O, which is where the curves are most linear. Lung function was tested in mice exposed to product 2 for 6 months, as described above. The following were the replicates per regimen (n): filtered air (18), low (19), modified Beirut (20) and high (20).

RNAseq analysis

We built an index sequence for STAR using the Gencode M13 reference.⁴⁴ A total of 50600 genes were identified. Before sequence alignment, we applied trim_galore (V.0.4.3) with the cutadapt package (V.1.12). We mapped raw sequencing reads to the mouse reference genome (mm10) using STAR aligner and calculated the raw count using feature Counts package. To test reproducibility and examine outlier samples, we conducted principal component analysis before conducting a differentially expressed gene (DEG) analysis. We generated a gene-by-sample matrix of reading counts that were analysed using edgeR after removing unwanted variation (RUVg).⁴⁵ The output of this analysis is a set of genomic regions that are significantly different between the experimental groups. Correction for multiple hypothesis testing was accomplished using Benjamini-Hochberg false discovery rate (FDR) with FDR <0.05 as the cut-off. Due to the small number of biological replicates (n=3, WP and Air; n=2, dry smoke), we used the limma-based edgeR method to determine DEGs.⁴⁶

Acute exposure

Individual mice were exposed to product 2 for precisely 1 hour and then were euthanised immediately via carbon dioxide

inhalation. Blood was collected using cardiac puncture. The lung was rinsed using phosphate buffer saline (PBS) similar to the procedure in BAL. Systemic perfusion was performed through a cardiac puncture injection of 30 mL of PBS. The lungs were collected and fast frozen. The elapsed time from euthanasia to lung collection for each mouse was around 6 min. The frozen lung was later homogenised via a Freezer Mill (Spex 6775), using two cycles of 5 s. The ground samples were not allowed to thaw until RNA extraction, which was performed using the Qiagen AllPrep DNA/RNA Mini Kit. Purified RNA was sent to Novogene for RNAseq procedures using an Illumina platform to a standard of 40 million reads and specific strand libraries. RNAseq analysis with Ingenuity Pathway Analysis (IPA) (Qiagen) used data of DEGs filtered by log₂ fold change at a cut-off of ±1.2, and pathway analysis filtered by direct experimental evidence from experiments in lung tissue, lung cells, cancer lung cells, immune cells, fibroblast, fibroblast cancer, immune cells and cancer immune cells. Online supplementary table 1 shows the sample number (n) and the exact date of the experiment (n=5, filtered air, WP and dry smoke).

RESULTS

Chronic exposure to WP smoke does not affect weight gain

The puffing regimens selected for the 6-month exposure were generally well tolerated by mice. In the low regimen group, one animal died during WP exposure from product 2 over the study period (online supplementary table 1). After 6 months of exposure to product 2, animal body mass was 23.0 g (±0.5 SD), a finding consistent with data provided by the supplier. Linear regression of weight gain (product 2) showed that all groups gained weight at similar rates when an experimental error is considered (online supplementary figure 2 and online supplementary table 3).

Nicotine exposures were similar to those of WP users

The Beirut puffing regimen was selected as it is the average smoking topography of WP use observed in humans smoking WP in a café environment in Lebanon,³² and agrees with subsequently conducted research.⁴⁷ The high and low regimens were designed to flank the Beirut regimen. The TSPs from the three regimens were between 300 and 800 mg/m³ (table 2), and each regimen showed similar values for both products 1 and 2, without significant differences in the TSPs. Nicotine quantification of the WP smoke from product 2 was measured to serve as a marker of exposure and a reference to estimate nicotine in product 1. We obtained the fraction of nicotine from the TPM collected during three consecutive exposures of WP smoke without any dilution air and computed the mass ratio (table 3; the procedure is described in detail in the Methods section).

The product of the nicotine mass ratio and the mass collected on the filter during exposure, corrected for water incorporated from the smoke (57%), was used to determine the nicotine delivered during exposure to product 2 (table 4). We estimated the exposure to nicotine in product 1, assuming similar nicotine

Table 3 Nicotine content in WP mainstream*

	Nicotine (mg)	TPM (mg)	Mass ratio
Mean	3.8	608.4	0.006
±SD	0.5	32	0.001

*Nicotine quantification in WP smoke from filter pads: n=3 for product 2; product 1 was not measured.

TPM, total particulate matter; WP, waterpipe.

Table 4 Total nicotine measured by exposure in each puffing regimen

Shisha	Nicotine per regimen (mg) $\pm$ SD*		
	Low	Modified Beirut	High
Product 1	0.41 $\pm$ 0.36	1.4 $\pm$ 0.20	2.5 $\pm$ 0.12
Product 2	0.39 $\pm$ 0.17	1.1 $\pm$ 0.09	2.3 $\pm$ 0.09

*SD calculated by error propagation. Estimated values for product 1 based on nicotine analysis of product 2.

concentrations in products 1 and 2. The mass of nicotine in WP smoke diluted with the filtered air used for mice during the exposure was between 0.4 and 2.5 mg, agreeing with those values reported in other studies, which showed that the nicotine supplied during a typical smoking session is between 0.32 and 7.8 mg.^{48–50}

Chronic exposure decreases lymphocyte counts in BAL fluid

We investigated whether the puffing regimens elicit a dysregulation of the immune cells. Hence, a quantitative analysis of the number of inflammatory cells, including lymphocytes, macrophages and neutrophils, was conducted using differential cell counts of BAL. The BAL samples examined for cell counts of mice exposed for 6 months showed that the number of lymphocytes showed a tendency to decrease according to the intensity of the puffing regimen for products 1 and 2, and in most cases were significantly lower than the filtered air controls (figure 1A,D). In contrast to lymphocytes, macrophages remained unchanged compared with air controls for

both products 1 and 2 (figure 1B,E). In most cases, the high variability in the number of neutrophils masked meaningful declines (figure 1C,F). Lack of a robust immune response, with plausible immunosuppression, was observed at 6 months for products 1 and 2.

Chronic exposure decreases CXCL9, CXCL10 and CCL11 in BAL fluid

To further understand the decreased immune response after chronic WP exposure, we quantified a broad array of cytokines/chemokines in the cell-free BAL fluid from the mice that showed immunosuppression after 6 months of WP smoke using product 2. Luminex-based analysis of 32 cytokines revealed that across the panel tested, the levels of most cytokines remained unchanged after 6 months of exposure. However, C-X-C Motif Chemokine Ligand 9 (CXCL9 or MIG), C-X-C Motif Chemokine Ligand 10 (CXCL10 or IP-10) and C-C Motif Chemokine Ligand 11 (CCL11 or eotaxin) were significantly reduced for some of the exposure regimens, without a marked correlation with the puffing regimens (figure 2). CXCL10 and CXCL9 are chemokines produced in response to CS. They bind to receptors that are mainly expressed on T lymphocytes, powerfully attracting other cells from the innate and adaptive immunity.^{51–54} CCL11 attracts eosinophils that are important for the development of lung airway hyper-responsiveness in asthma and allergies.⁵⁵ The current results showed minimal stimulation of the immune response, with key cytokines for inflammation during CS exposure significantly reduced in the exposure to product 2.

Figure 1 Quantification of inflammatory cells in bronchoalveolar lavage from mice exposed for 6 months to three regimens of waterpipe mainstream smoke. Ratios indicate the relative changes in the normalised cell counts (cells/mL) for each regimen using filtered air control (Air) as reference. Shown are the results of two shisha products: (A–C) product 1; (D–F) product 2. Beirut: modified Beirut; Air: filtered air control group. Error bars represent SE. We assumed an equal variance in the distribution to apply a Student's t-test with an alpha level set to 0.05. The p values above the bars denote significant changes. Online supplementary table 2 reports the total cell counts, normalised cell counts, number of replicas and the percentage distribution in the group.

Figure 2 Postchronic exposure analysis of cytokine concentration in bronchoalveolar lavage fluid of murine lungs. No other molecule in the test showed a significant difference. Air: air-exposed control group; Beirut: modified Beirut. Depicted are the results of product 2. Product 1 remained untested. For each group, error bars represent SE for $n=3$. Student's t -test, assuming equal variance for the distributions and alpha level of 0.05, was used to compare each group with air control. CXCL9: C-X-C Motif Chemokine Ligand 9; CXCL10: C-X-C Motif Chemokine Ligand 10; CCL11: C-C Motif Chemokine Ligand 11. Above the bars are p values. ND, below the lower limit of detection.

Chronic exposure does not affect lung function

In mouse models of CS-induced emphysema, emphysematous lesions are indirectly monitored by the deterioration in pulmonary function. The analysis of lung function of mice exposed to product 2 for 6 months showed that lung function is normal as the PV data showed no significant changes to filtered air control mice (online supplementary figure 3).

Acute exposure has significant effects on IEG expression

To identify genetic biomarkers of WP exposure, we used RNAseq analysis to monitor the expression of genes in the lungs after a 1-hour acute exposure to product 2. Furthermore, we determined whether these biomarkers of exposure differed based on the addition of water to the bowl to generate dry smoke. Acute exposures to mainstream WP and dry smoke were similar in TSP to the modified Beirut regimen; however, we found that water accounts for ~60% of the measured weight in WP smoke (see the Methods section). Values previously reported in comparable experiments showed that water content in dry smoke accounts for $\sim 0.56\times$ relative to mainstream WP.⁵⁶ Therefore,

based on the TSP corrected for water, we estimated that dry WP smoke is $\sim 33\%$ higher in dry mass. Our results showed that the strongest exposure, based on the number of IEGs upregulated, is dry smoke (141 genes, compared with 49 genes for WP; see figure 3A and online supplementary table 4), which has no means to filter the water-soluble smoke constituents or attenuate smoke temperature. We also found a set of common genes that are differentially expressed in the two acute exposures when compared with the filtered air controls (figure 3A-B). The common genes are potential biomarkers of exposure, while those that show a graded response between dry smoke > WP > air may serve as biomarkers for the strength of exposure. The results indicate that activation or inhibition of genes is enhanced by dry smoke, which is the most harmful exposure as it shows the largest change in gene expression as well as the largest number of genes regulated between the two groups.

Among the DEGs that are common to all experimental groups are those previously identified as IEGs (figure 3B). We observed that CD69 was significantly induced; CD69 is a marker of T cell activation and is considered to be an early response to

A

B

IEGs common to WP and dry bowl	Symbol	WP (F.C.)	Dry (F.C.)
Known IEGs			
CD69 molecule	Cd69	3.1	5.4
dual specificity phosphatase 2	Dusp2	3.0	5.6
apolipoprotein L domain containing 1	Apold1	3.9	9.9
Novel IEGs			
CD19 molecule	Cd19	2.4	3.0
CD79a molecule	Cd79a	2.4	3.1
cell death-inducing DFFA-like effector a	Cidea	-2.7	-11.9
Fc fragment of IgE receptor II	Fcer2a	2.6	3.2
Fc fragment of IgM receptor	Fcmr	3.3	4.4
2'-5'-oligoadenylate synthetase 3	Oas3	2.4	3.7
paired box 5	Pax5	2.3	2.9
uncoupling protein 1	Ucp1	-17.3	-90.4

Figure 3 Gene regulation of acute exposure to waterpipe (WP) smoke in murine lungs. Analysis of the lung transcriptome of mice exposed for 1 hour to mainstream WP, dry WP (dry smoke) or filtered air control group. The analysis produced differentially expressed genes (DEG) data sets. The DEGs are cut-off at ± 1.2 log₂ fold change relative to filtered air control (see details in the Methods section). Depicted is a Venn diagram (A) to represent the relationship of DEGs produced by the WP and dry WP acute exposures. Panel (A) key: brown shade, shared genes between WP and dry WP data sets; green, genes unique to WP data; red, genes unique to dry WP data. The analysis of the shared genes revealed known and novel immediate-early genes (IEGs) with potential to become biomarkers of exposure. Panel (B) shows the gene names and the fold changes (FC) relative to filtered air control of known and novel IEGs. The experiment used product 2 only (product 1 remains untested). $n=3$, WP and filtered air control; $n=2$, dry smoke.

lymphocyte-mediated inflammation activated by CS.^{57,58} Dusp2 (PAC1) is a dual threonine/tyrosine phosphatase of the Mitogen-activated Protein (MAP) kinases, crucial for the signal transduction function of immune cells.^{59–62} Another previously identified IEG is *Verge* or *APOLD1*, which displays modulatory functions of vascular endothelium associated with the development of hypertension in smokers.^{63,64} The results indicate that exposure to WP and dry smoke regulates genes known to be IEGs with functions related to the activation of inflammatory and cardiovascular responses.

In addition, a set of genes were identified as novel IEGs that were common among all exposures (figure 3B). The function of the novel IEGs is primarily focused on B cells. *Pax5* is a transcriptional regulator that is essential for the commitment of a common lymphoid progenitor towards a B cell lineage.^{65–67} *CD79a* (*Igα*) is an integral member of the B cell receptor complex (BCR) that is necessary for the transduction of intracellular signals on antigen binding.^{68,69} *Fcεr2a* (*CD23*, isoform 'a') is an IgE receptor that is required in B cells for antigen presentation to T cells.^{70,71} Two modulators of B cell immunity are also novel IEGs. *CD19* amplifies BCR signal transduction, and *Fcμr* enhances survival of activated B cells and modulates BCR signal.^{64,72–75} Other common IEGs are described in online supplementary data 1. The results suggest that certain genes are novel IEGs and are potential genetic biomarkers of exposure to tobacco products. Moreover, the function of IEGs suggests a strong immune response to acute WP exposure.

Based on the results of the comparative analysis of the RNAseq data, a unique set of genes for each experimental group were identified (figure 3A). Therefore, the transcriptomic analysis of IEGs can potentially assign toxicological effects to a specific exposure based on unique changes in gene expression after acute exposures. Simultaneous analysis of the RNAseq data set for the two exposures using IPA canonical pathway analysis separated the two exposures by clusters of genes and defined the activity status of the pathway based on z-scores. This analysis found that WP has an inhibitory effect on 'GP6 Signaling' and 'LXR/RXR' pathways (online supplementary tables 5 and 6) and consists of downregulated genes that increase inflammation (fibrinogen, collagen, laminin) and upregulated genes that suppress inflammation (interleukin-1-receptor-2).^{76–79} In contrast, dry smoke activates the 'PKCθ Signaling in T Lymphocytes' pathway (online supplementary table 7), which includes genes that function in calcium signalling (*CACNA1L*, *CACNA2D4*),^{80–82} cell development (*Rac2*),⁸³ signal transduction (*NFKBID*),^{84–86} antigen presentation (*H2-Q6*, *H2-Ob*)⁸⁷ and antigen receptor function (*Cd3d*, *CD28*),^{88–91} which are of particular relevance for T cell activation and function. These results indicate a distinctive toxicological signature for each exposure at the level of gene regulation.

DISCUSSION

The results from our study indicate that there was no significant increase in inflammatory cells and no decrease in pulmonary function after exposure to WP smoke for 6 months. The 6-month exposure length is rarely tested using puffing regimens that are similar to those observed in WP users.⁴⁸ Nonetheless, based on nicotine content, our modified Beirut and high regimens are true representatives of habitual chronic WP smoke usage in humans, while the low regimen is a representative of WP smoking with an electric heat source.⁵⁰ Nicotine in product 2 was used as a reference to estimate nicotine in product 1 (a menthol product), based on a study that showed nicotine in

flavoured shisha products is lower than its unflavoured counterpart.⁹² In our study, a low number of replicas of product 1 limited some experiments (BAL, pulmonary function), and in other cases the design of the experiment was developed after the analysis of product 1 exposure (body weight). Furthermore, some techniques (RNAseq, Milliplex) are expensive to perform in our laboratory. Therefore, the discussion is mainly focused on results achieved with product 2, in instances where product 1 is unavailable.

All puffing regimens, including the higher regimen, were well tolerated in mice as no physiological deterioration was evident in the pulmonary function tests, whereas weight remained unchanged. Previous population studies demonstrated a complex association between smoking and weight, and to date a consensus has not been reached on the cause or directionality of the effect.^{93–95} However, controlled studies using rodents showed that CS and nicotine exposures are associated with weight loss.^{96–98} Moreover, mice exposed to CS of similar TSP concentrations as what were quantified in the current WP study created emphysematous lesions and compromised pulmonary function, leading to the development of COPD.^{99–102} Therefore, our results may be constrained due to the length of exposure and the characteristics of the smoke mixture. However, compared with CS exposures of similar TSP, the WP puffing regimens tested caused less physiological effects. Policymakers should be aware that further research is needed to evaluate the risks of developing or exacerbating COPD, emphysema and cancer.

Nevertheless, exposure to WP presented plausible health risks at the cellular level. The moderate decreases in lymphocyte counts in BAL and in the chemokine concentration observed in some puffing regimens are indicators of immune suppression. Therefore, immune suppression in the chronic WP exposure is plausible and agrees with previously documented nicotine and CO-related effects of CS,^{103–107} mediated by molecular mechanisms, including blockage of T cell activation^{108–110} and direct inhibition of eosinophil function.¹¹¹ The suppressive effect of the immune response may be associated with the length of the exposure, as previously noted in a study that revealed inflammation in response to shorter exposures to WP.^{112–114} Indeed, we observed an inflammatory effect by acute exposure. Further molecular studies will determine if the immune modulation by chronic exposure to WP is similar to exposure to CS.^{115–118} For example, a test for the compromised function of macrophages as previously reported in the context of CS is needed.¹¹⁹ Future experiments will clarify the clinical relevance of the immune suppression by WP, for example, using animal models developed in the laboratory to determine bacterial and viral clearance.^{120,121} A limitation of our work is the use of female mice only. Previous research showed gender-specific health effects and gender differences in the prevalence of tobacco use.^{114,122,123} Nevertheless, our results imply that WP users may experience an increased risk of developing severe lung infections and cancer, analogous to what is experienced by cigarette users. Future experiments will inform public health policies aimed to disseminate this and other information regarding health risks associated with WP use.^{124–126}

In our research, we identified IEGs in murine lungs expressed after acute exposure to dry smoke and WP mainstream smoke from product 2. Previous research showed that traditional tobacco exposure biomarkers measured immediately after acute exposure in humans were sensitive to detect toxicity differences between tobacco products, in a model dubbed 'boost' measurement paradigm.¹²⁷ In support of the 'boost' paradigm at the gene regulation level, we observed that the activation of IEGs is rapid, as 1 hour of exposure is sufficient for gene regulation.^{128–130}

The robust differences support the finding that the IEG response is attractive for transcriptional biomarker discovery.¹³¹ The harmfulness of exposure, based on the number of IEGs per group, follows the trend, dry smoke > WP > air. Such trend demonstrates that dry smoke is most toxic, while water in the WP reduces some combustion components in smoke and decreases smoke temperature compared with conventional cigarette combustion.^{132 133} Recently, an innovative semiquantitative analysis showed that water filters a minute class of toxicants from mainstream WP smoke and instead water favoured the nucleation of particulates of facile transmembrane transport.^{134 135} However, in our study, the strong agreement between the degree of toxicity and the total number of IEGs may be related to a decrease in temperature and/or lower concentration of the toxicants in mainstream smoke.

The transcriptomics analysis revealed several IEGs common to all exposures, as well as gene expression profiles that characterise each type of exposure, thereby indicating the specificity of the genetic response. Contrary to the immune suppression experienced in the lungs following chronic exposure, the function of common IEGs are, in most cases, transmembrane receptor mediators of immune response, similar to the known physiological and genetic changes observed after acute exposure to CS.^{106 136 137} The group of common novel IEGs were found to be strongly associated with B cell immunity. In contrast, a group of genes with functions in T cell immunity are exclusively

upregulated in dry smoke exposure, whereas genes with inhibitory function on lung inflammation were found in response to WP exposure. Therefore, IEGs display the potential to differentiate between distinct exposures. In addition, we showed that the magnitude of the modulation of IEGs is generally positively correlated with the harmfulness of the exposure, similar to that observed in the transcriptomics of rats exposed to CS.¹³⁸ Therefore, IEGs showed specificity and dynamic range that are desirable characteristics in a biomarker.

The present research on IEGs after acute WP exposure is the first of its kind and addresses the need for genetic biomarkers that are specifically tailored to WP use.¹³⁹ We adopted previous approaches to show that IEGs may potentially quantify and predict harmful effects in humans, known as biomarkers of potential harm, based on the magnitude and direction of expression.^{140–142} Furthermore, IEG testing in the nasal epithelium and gingiva (the gums) is a promising and non-invasive alternative with clinical relevance.^{143 144} These results emphasise the importance of policies that support the research and development of IEGs as novel non-traditional biomarkers of newly modified tobacco products.

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Competing interests None declared.

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Ethics approval This study was approved by the Animal Care and Use Committee (IACUC) at Johns Hopkins University. All animal experiments (methodology and procedures) were performed according to the recommendations and guidelines of the IACUC.

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What this paper adds

What is already known on this subject

- Health risks are associated with exposure to waterpipe (WP) smoke in animal models and in vitro experiments.
- Prior results revealed an inflammatory response due to short-term exposures.
- WP smoke contains a larger concentration of toxicants than cigarette smoke, and users believe that water in the WP bowl has a filtering effect.
- Recent evidence suggests that analysis of genes by RNAseq may become a tool for biomarker discovery.

What important gaps in knowledge exist on this topic

- The health consequences of chronic WP use are not well understood.
- In tobacco regulatory science, a current focus is to identify biomarkers of exposure that are specific to WP and other newly modified tobacco products.
- The immediate expression of genes after an acute dose of WP smoke has never been analysed.

What this paper adds

- We used a standardised WP exposure model that is similar to WP use by humans.
- Herein, we report immune suppression that occurs after chronic exposure to WP.
- Additionally, we showed that global gene regulation is modified by acute exposure to WP and suggest immune cell activation.
- The magnitude of gene regulation is associated with the severity of acute exposure.
- Our results indicate that whole-cell transcript analysis (transcriptome) could be considered an indicator of exposure (biomarker) with specificity to WP and other tobacco products.

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