Development and validation of an open-source, disposable, 3D-printed *in vitro* environmental exposure system for Transwell culture inserts

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ABSTRACT Accessible *in vitro* models recapitulating the human airway that are amenable to study whole cannabis smoke exposure are needed for immunological and toxicological studies that inform public health policy and recreational cannabis use. In the present study, we developed and validated a novel three-dimensional (3D)-printed *in vitro* exposure system (IVES) that can be directly applied to study the effect of cannabis smoke exposure on primary human bronchial epithelial cells.

Using commercially available design software and a 3D printer, we designed a four-chamber Transwell insert holder for exposures to whole smoke. COMSOL Multiphysics software was used to model gas distribution, concentration gradients, velocity profile and shear stress within IVES. Following simulations, primary human bronchial epithelial cells cultured at the air–liquid interface on Transwell inserts were exposed to whole cannabis smoke using a modified version of the Foltin puff procedure. Following 24 h, outcome measurements included cell morphology, epithelial barrier function, lactate dehydrogenase (LDH) levels, cytokine expression and gene expression.

Whole smoke delivered through IVES possesses velocity profiles consistent with uniform gas distribution across the four chambers and complete mixing. Airflow velocity ranged between 1.0 and 1.5 $\mu\text{m}\cdot\text{s}^{-1}$ and generated low shear stresses ($\ll 1$ Pa). Human airway epithelial cells exposed to cannabis smoke using IVES showed changes in cell morphology and disruption of barrier function without significant cytotoxicity. Cannabis smoke elevated interleukin-1 family cytokines and elevated CYP1A1 and CYP1B1 expression relative to control, validating IVES smoke exposure impacts in human airway epithelial cells at a molecular level.

The growing legalisation of cannabis on a global scale must be paired with research related to potential health impacts of lung exposures. IVES represents an accessible, open-source, exposure system that can be used to model varying types of cannabis smoke exposures with human airway epithelial cells grown under air–liquid interface culture conditions.

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Introduction

The lung is responsible for gas exchange between the outside world and the human body. During ventilation, the lungs are in contact with the external environment and exposed to air that may contain a variety of pathogens, including allergens, viruses, bacteria, and air pollutants, including organic combustion by-products [1]. The pseudo-stratified airway epithelial cells that line the lungs have evolved to mitigate the risks from external insults, providing a tight physical and immunological barrier [2–4]. It contributes to coordinated immune responses, including mucus production and airway surface-lining fluid secretion, cytokine and chemokine secretion for local and systemic immune cell recruitment, and antimicrobial protein secretion in response to environmental assaults [5, 6]. Airway epithelium dysfunction has been strongly implicated in the pathogenesis of many airway diseases including asthma, COPD, and pulmonary fibrosis [7–9]. Importantly, environmental insults capable of damaging the airway epithelium are also risk factors for these same respiratory diseases [10–13].

Tobacco smoking, a direct environmental insult to the airway epithelium, remains common on a global scale, despite well-described effects on respiratory health [14–18]. Similar to tobacco smoke, cannabis smoking is also a direct environmental attack to the lungs. As a result of progressive legalisation on a global scale, cannabis consumption has been on the rise, where 90% of consumers prefer smoking as a route of delivery [19, 20]. Tobacco and cannabis smoke exposure induce similar clinical features such that both environmental exposures are associated with increased prevalence of coughing, wheezing, chest tightness, and risk for developing COPD [21–23]. Tobacco and cannabis smoke exposure also present divergent clinical features, with tobacco smokers more likely to develop lung cancer relative to cannabis smokers [24, 25]. This observed divergence may be due to the presence of phytocannabinoids with anti-inflammatory properties unique to cannabis [26–29]. The uncertainty surrounding the health effects of cannabis smoke and discrepancies with the known negative impacts of tobacco smoke warrants further investigation to inform government policies, recreational practices and cultivation strategies in an era of broadening acceptance for legal and open markets.

In vitro smoke exposure models using human airway epithelial cells have been crucial for tobacco smoke research and will likely be important for cannabis smoke research [30, 31]. A proportion of available smoke exposure research, primarily with tobacco, has been established through submerged monolayer culture designs in which smoke extract has been used to expose cells [32–34]. Our group has recently applied these methods to Calu-3 cells with cannabis smoke extract exposure and observed an induction of a proinflammatory cytokine response and suppression of antiviral cytokines [35, 36]. Smoke extract exposures do not entirely reflect whole smoke exposure as the latter generates heat and water-insoluble hydrocarbon combustion products [21, 37–39]. Additionally, submerged monolayer cell culture designs do not entirely reflect the *in situ* airway environment where a pseudo-stratified airway epithelium is exposed to inhaled air on an apical side while attached to a basement membrane on the basal side. To more accurately model the *in situ* environment, airway epithelial cells can be cultured on Transwell inserts under air–liquid interface (ALI) culture conditions to create a pseudo-stratified tissue structure [40, 41]. Transwell inserts of airway epithelial cells under ALI can be used for whole smoke exposures using advanced systems that model inhalation and exhalation patterns of humans [40, 41]. The strengths and limitations of popular smoke-generating machines have been characterised extensively [42–45]. Commercially available smoking machines offer high throughput designs and are able to accommodate multiple cigarettes or cell culture plates that can be exposed with independent syringes, allowing for control over the parameters of each exposure under automated conditions [45]. Some devices are directly amenable to multiple exposures beyond smoke, including environmental toxins, gases, therapeutic aerosols, aerosolised pathogens, and other volatile compounds [42–45]. The throughput and automation benefits of these environmental exposure systems are offset by some important limitations [45–49]. From an operations perspective, these are capital intensive closed systems that cannot be customised, limiting accessibility to specialised research facilities and field participants [45]. From a technical perspective, limitations may include potential air and smoke mixing inefficiencies, ageing of smoke in mixing vessels, and turbulence that is inconsistent with lung physiology [45]. Collectively, the development and validation of a low-cost *in vitro* environmental exposure system for Transwell culture inserts of ALI cultured airway epithelial cells will broaden the ability of researchers to perform essential research related to cannabis smoke exposure and lung health.

To address existing constraints with environmental exposure systems and related research, we propose that additive manufacturing, such as three-dimensional (3D) printing, can function as a disruptive solution to create an open-source, disposable, *in vitro* exposure system that is widely accessible without requiring specialised environmental exposure infrastructure. Additive manufacturing has been utilised in various fields to realise complex 3D constructs with resolution at the micron level. In contrast to conventional manufacturing technologies, additive manufacturing is accessible and independent of specialised

technologies or personnel, with widely available commercial 3D printers able to rapidly generate functional designs based on computer aided design (CAD) files. Additive manufacturing technologies have accelerated prototyping steps while reducing costs, effectively enabling researchers with limited design training to optimise novel designs independent of historical manufacturing constraints. Importantly, commercially available and capital accessible additive manufacturing technologies are sufficient for the medium throughput production that is required at a standard research laboratory scale.

In the present study, we develop and validate an open-source, disposable, 3D-printed *in vitro* environmental exposure system for Transwell culture inserts and apply to study the effect of whole cannabis smoke on primary human bronchial epithelial cells (HBECs). Our *in vitro* exposure system (IVES) is widely accessible due to the open-source CAD file, amenability to commercially available 3D printers, and design for widely used Transwell culture inserts. We validate our model with cannabis smoke laying a foundation for additional modalities, including tobacco smoke and vaping products. IVES also gives the capacity for four Transwell inserts to be exposed to the same environmental exposure, providing medium throughput, while reducing variability that may occur with independent exposures for separate Transwells. Finally, IVES is amenable to dilution of smoke with room air for adaptation to concentration–response studies. Using cannabis smoke as an exposure of current public health importance, we validate IVES with primary HBECs and demonstrate an impact of the exposure on epithelial barrier function, interleukin (IL)-1 family cytokine expression and expression of genes involved in cellular detoxification.

Materials and methods

System design

Autodesk Inventor 2018 software was used for all designs. The IVES units were 3D printed with a FormLabs Form 2 printer using clear resin (RS-F2-GPCL-04, MA, USA). IVES contains four Transwell exposure chambers, two inlets, four outlets, and four chamber caps (figure 1). Each inlet is distributed to each of the four exposure chambers. Inlet diameter was optimised at 3 mm to minimise obstruction with organic combustion by-products. Exposure chamber size was designed based on the dimension of a Transwell insert for 24 well-plate (VWR, catalogue number: 29442-082). In addition, the location of the inlet and outlet of each chamber was designed such that Transwell inserts would experience indirect exposures of turbulent air. Each chamber had an outlet for exhaust from the chamber. Threaded caps for the exposure chambers created seals that eliminated exhaust through this path. IVES has been validated using Corning Transwell inserts. Distinct Transwell inserts of similar dimensions will be adaptable to the

FIGURE 1 Three-dimensional schematic view of the *in vitro* exposure system. As shown, air or exposure (e.g. smoke) delivered to the inlet is equally distributed across the four exposure chambers which house the Transwell inserts. Circulated air or exposure (e.g. smoke) exits passively through the outlet in each exposure chamber. Scale bar=1 cm.

IVES design, whereas Transwells from different manufacturers with different dimensions may need independent validation. The IVES design file is deposited in the online supplement as a .stl file format.

Fluid dynamic modelling

During design phases, a quantitative simulation of fluid dynamics was performed using COMSOL Multiphysics software to model the gas distribution and concentration gradients in the IVES unit. To model the velocity profile and shear stress, the entire fluidic path from the merged inlet to the chambers and the outlets was included. The main purpose of this simulation was to ensure that the gas was equally distributed among all chambers with minimal shear stress experienced at the surface of Transwell inserts. Air was used as the gas of interest in this part of the simulation, under the assumption that air was behaving as a Newtonian fluid. The governing equations used in the simulation as follows:

$$\rho \nabla \cdot (\vec{\vartheta} \vec{\vartheta}) = -\nabla p + \nabla \cdot \vec{\tau} + \rho \vec{g} + S_r \quad (1)$$

$$\nabla \cdot (\rho \vec{\vartheta}) = 0 \quad (2)$$

where ρ is the air density, $\vec{\vartheta}$ represents the velocity vector field, p shows the pressure, $\vec{\tau}$ expresses the stress tensor field, $\vec{g}$ is the gravitational force, and S_r shows any possible external source term. The acceleration forces, including both the local and the conventional forces, are expressed on the left side of equation 1, and the forces created by pressure gradient and viscosity are described on the right side of the equation. A no-slip condition on the walls was assumed for the entire IVES unit, with the model assuming no back-flow. A gas flow rate of $7 \text{ mL}\cdot\text{s}^{-1}$ was used as the boundary condition and the gas flow direction was set to be normal to the inlet and outlets. Equation 2 expresses the mass conservation for the exposure unit. In the simulation, initial values were set to zero. Then, the simulation was studied under a steady-state condition.

Cell culture

All studies using human cells were approved by the Hamilton Integrated Research Ethics Board (approval: 5305T). Primary HBECs were isolated from a bronchial brushing from consented subjects undergoing routine clinical procedures, and plated into T25 flasks containing PneumaCult Ex-Plus Basal medium (StemCell Technologies; catalogue number 05040) with PneumaCult Ex-Plus 50× supplement, 0.01% hydrocortisone stock solution (StemCell Technologies; catalogue number 7925) and 1% antibiotic-antimycotic (Thermo Fisher Scientific; catalogue number 15240062). Once cultures achieved ~80% confluence, cells were passaged at a density of 50 000–100 000 cells per insert into polyester Transwell inserts for exposure experiments. Cells were fed with 200 μL and 750 μL of PneumaCult Ex-Plus Basal medium in the apical and basal compartments respectively. Once cultures reached 100% confluency, apical medium was removed, and culture inserts were fed from the basal side to bring cultures to ALI (day 0 of ALI). Following 24 h, culture inserts were fed from the basal compartment with 750 μL of PneumaCult-ALI Basal medium (StemCell Technologies; catalogue number 05001) with PneumaCult-ALI 10× supplement, PneumaCult-ALI Maintenance 100× Supplement, 1% antibiotic-antimycotic, 0.5% hydrocortisone stock solution and 0.2% heparin solution (StemCell Technologies; catalogue number 7980) to support development and differentiation of a pseudo-stratified epithelial culture (day 1 of ALI). Transwell cultures were fed from the basal compartment and a PBS wash was performed on the apical compartment every other day. Exposure experiments were performed between day 14 and day 15 of ALI culture.

Cannabis material and cigarette manufacturing

A Kentucky research-grade cigarette (Lot:3R4f) contains ~0.7 g of dried tobacco leaves and has been used extensively for *in vitro* studies. Using research-grade tobacco cigarettes as a reference, cannabis cigarettes with ~0.7 g of dried cannabis flower (~10% THC, 0% CBD; *Purple Sun God*, lot: 00117 (b161)) were manufactured with RAW rolling papers and cardboard filters. Cannabis was purchased from the Ontario Cannabis Store with a Health Canada-approved research license.

Epithelial barrier function assessment

Transepithelial electrical resistance (TEER) was measured using a Millicell ERS-2 Voltohmmeter (EMD Millipore, Etobicoke, Ontario, Canada) to quantify epithelial barrier function according to manufacturer's directions. TEER was measured before and 24 h after exposures (air or smoke).

Exposure protocol

In a biosafety cabinet, 800 μL of PneumaCult-ALI medium was added into each individual chamber of the IVES unit. Transwell inserts were transferred into the IVES and left in a 37°C , 5% CO_2 humidified

incubator to equilibrate for 10 min before exposures. In a fume hood, exposures were performed with a 50-mL syringe connected to the fresh air inlet of the IVES through a PVC Tygon tube (length: 32 cm, lumen diameter: 2 mm) and a three-way valve (figure 2). Another 50-mL syringe was connected to the smoke exposure inlet on the IVES through a PVC Tygon tube (length: 32 cm, lumen diameter: 2 mm) and a three-way valve. For the experiments with cannabis, a cigarette was placed into another PVC Tygon tube (length: 32 cm, lumen diameter: 8 mm), with the opening sealed with parafilm and connected to the three-way valve. The dose was administered according to a modified version of the Foltin puff procedure [50–55]. This procedure, optimised for our model, consisted of one puff of smoke separated by three puffs of fresh room air to mimic the behaviour of human smoking patterns. Each puff of smoke or room air was 35 mL in volume and was perfused over the Transwell insert at a rate of $7 \text{ mL}\cdot\text{s}^{-1}$. Between each puff, the cells were undisturbed for 10 s. Each experimental group received one cigarette smoked to completion using this regimen. Cells were exposed to freshly generated smoke outside of an incubator in a fume hood on a 37°C heating bed until the 0.7 g hand-rolled cannabis cigarette was entirely combusted (~ 20 min). Control conditions received as many puffs of room air as the corresponding experimental condition received smoke under the same regimen. Following exposure, inserts were immediately transferred to a new plate with 600 μL of PneumaCult-ALI medium in the basal compartment. Plates were then transferred to a 37°C , 5% CO_2 humidified incubator. Twenty-four hours later, basal and apical medium was collected, spun down at $7500\times g$ and 4°C for 15 min and supernatants were stored at -80°C for subsequent quantification of cytokines and assessment of cytotoxicity.

Quantification of cytokines

Cell culture medium was collected and spun down at $7500\times g$ for 15 min at 4°C . Apical supernatants were subsequently analysed *via* a 42-plex multiple cytokine array (Eve Technologies, Calgary, Alberta, Canada).

FIGURE 2 A schematic depicting the *in vitro* exposure system (IVES) connected to air and smoke sources. A three-way valve connects the cannabis cigarette to IVES through a 50-mL syringe. Another three-way valve connects room air to IVES. Smoke is drawn through the smoke exposure syringe and expelled with predetermined rate and volume into IVES. Room air is introduced with the fresh air syringe in a similar fashion. A heating pad positioned below IVES maintains the experimental system at 37°C [figure generated with BioRender].

Cell membrane integrity and cytotoxicity

A CYTOQUANT lactate dehydrogenase (LDH) Cytotoxicity Assay kit (Thermo Fisher Scientific; catalogue number C20301) was used according to the manufacturer's guidelines with positive controls used to indicate maximal LDH release.

RNA isolation, purification and transcriptomic analysis

Following collection of apical and basal media 24 h after exposure, total RNA was extracted and isolated using an RNeasy Kit (QIAGEN, Toronto, Ontario, Canada). Cells in each Transwell were lysed in 100 μL of RLT Isolation Buffer with 1% β -mercaptoethanol (2Me) (v/v) and stored at -80°C . Subsequently, RNA was purified using the manufacturer's protocol and quantified *via* Nanodrop200. cDNA was prepared and underwent transcriptomic analysis using Clariom S Microarray chips (Thermo Fisher Scientific, Mississauga, Ontario, Canada).

Processing of raw microarray data

Raw intensity values from the Clariom S microarray experiment were imported into the R statistical language environment (version 3.6.1; R Core Team, 2019). Probe definition files were obtained from the Brainarray database (version 24) [56]. The single channel array normalization (SCAN) method was used to obtain $\log_2$ -transformed normalised expression values with the SCAN.UPC R package (version 2.26.0) [57], with annotation data from the Bioconductor project (version 3.9) [58].

Statistical analysis

All statistical tests were performed on GraphPad Prism 8 version 8.3.0 (GraphPad Headquarters, San Diego, CA, USA). A paired t-test was performed to assess significance between the differences in epithelial barrier function between the control group and experimental group. An ANOVA followed by a Tukey's *post hoc* test was performed to assess differences in cell cytotoxicity following smoke exposure. Differences in cytokine expression were assessed using paired t-tests. A p-value <0.05 was considered statistically significant.

Results

IVES fluid dynamic modelling

A mesh analysis was conducted to determine the finest mesh resolution. The mesh size varied from coarse to extra fine, and the mesh size was chosen to be finer as no significant change was recognised by changing the mesh size from finer to extra fine. A defined mesh resolution was chosen to run all simulations for fluid dynamic modelling (figure 3) with a 3D view of velocity streamlines presented (figure 3a–d). The purpose of the streamlines is to show vortices in IVES after air exposure with a flow rate of $7 \text{ mL}\cdot\text{s}^{-1}$. Quantitative values for the velocity profile were consistent with a uniform gas distribution with complete mixing (streamlines) (figure 3e). In addition, the velocity profile (figure 3f) and the shear stress profile (figure 3g) at the Transwell insert growth area location was simulated, investigating the impact of the exposure to the cells growing on this surface. Airflow velocity at an approximation of cell location was $1.0\text{--}1.5 \mu\text{m}\cdot\text{s}^{-1}$ and generated shear stresses ($<1 \text{ Pa}$).

Internal gas diffusion inside a chamber was quantified with CO_2 , a product of combustion, as the gas of interest with an assumption of $1 \text{ mol}\cdot\text{L}^{-1}$ at the inlet with normal diffusion in the air (CO_2 as the gas of the interest was assumed to be transported in air by diffusion and convection). For this part of the simulation, mass transport of CO_2 was coupled with the laminar flow study, which was used to model the velocity profile in IVES. As the gas was uniformly distributed among all four exposed chambers (figure 3), only one chamber was considered in the modelling (the gas flow at the inlet was assumed to be $1.75 \text{ mL}\cdot\text{s}^{-1}$). The simulation was studied dependent on time, and the CO_2 diffusion was modelled over 5 s, which was the time of exposure. It was assumed that the CO_2 concentration at the outlet was zero (the boundary condition for CO_2 concentration at the outlet was assumed to be zero). A full cycle of smoke–fresh air was studied, and the cycle included one smoke exposure for 5 s (the initial concentration was set to zero; it was assumed that there was no CO_2 in the chamber at the beginning) and one continuous fresh air exposure for 15 s (the initial concentration was taken from the final average volumetric concentration of the smoke exposure after 5 s resting based on the experimental set-up). To model the mass transport of CO_2 in the chamber, Fick's law was used as described below:

$$\frac{\partial C_{\text{CO}_2}}{\partial t} = D_{\text{CO}_2} \left(\frac{\partial^2 C_{\text{CO}_2}}{\partial x^2} + \frac{\partial^2 C_{\text{CO}_2}}{\partial y^2} + \frac{\partial^2 C_{\text{CO}_2}}{\partial z^2} \right) \quad (3)$$

where C_{CO_2} is the CO_2 concentration in the smoke exposure chamber and D_{CO_2} ($D_{\text{CO}_2}=0.16 \text{ cm}^2\cdot\text{s}^{-1}$) [59] is the diffusion coefficient of CO_2 molecules in the chamber.

FIGURE 3 Quantitative simulation for *in vitro* exposure system (IVES) using COMSOL Multiphysics with air used as the gas of interest for simulation. a) A three-dimensional view of the IVES with air flow streamlines showing vortices in IVES and how gases distribute. b) Top view with gas flow streamlines. c and d) The side views with gas flow streamlines. e) The top view of the velocity profile ($\text{m}\cdot\text{s}^{-1}$) for the modelled gas presenting a uniform flow distribution among all four exposure chambers with a gentle velocity decrease. f) The velocity profile ($\mu\text{m}\cdot\text{s}^{-1}$) at the close approximation to the surface where the cells were cultured. g) The shear stress (Pa) profile at the location that the cells were cultured. It should be noted that both air and smoke inlets are merged into a larger duct which is only shown in this figure.

The concentration distribution of CO_2 in the chamber over 5 s of exposure is presented in figure 4a with streamlines representing the concentration gradient in the chamber. After each smoke exposure, there was a 10-second resting time to allow the CO_2 concentration to become uniform in the entire chamber.

FIGURE 4 Three-dimensional quantitative modelling results of the gas [CO₂] concentration distribution in an *in vitro* exposure system chamber: a) real-time CO₂ concentration distribution over 5 s exposure of smoke (initial CO₂ concentration modelled at 1.0 mol·L⁻¹) showing a gentle gas distribution in the exposure chamber; b) real-time CO₂ concentration distribution over 5 s exposure of fresh air (the initial CO₂ concentration was the final CO₂ concentration in the exposure chamber from the previous smoke exposure and it was assumed that there was no CO₂ in the fresh air exposed to the chamber); c) volume average concentration of CO₂ in the chamber for one smoke–fresh air cycle would lead to a drop in CO₂ concentration in the chamber back to zero; and d) average outlet concentration of CO₂ after one smoke–fresh air cycle confirming that exposure kinetics were sufficient to reach a repeatable smoke–air exposure cycle (zero concentration at the outlet).

Therefore, the initial concentration for the fresh air exposure was calculated from the previous step and assumed to be uniform in the entire chamber, as seen in figure 4b. Figure 4b also shows the rate of change and uniformity of the CO₂ concentration in the chamber during fresh air exposure. Figure 4c and d show the volumetric average CO₂ concentration of the chamber and the average CO₂ concentration at the outlet of the chamber, respectively. The simulation results confirmed that the CO₂ concentration in the chamber reached zero after the first fresh air exposure, suggesting that the cells would experience a similar pattern for each cycle, thereby the size of the smoke exposure chamber was small enough to let a fast and uniform diffusion occur in the chamber. This means that the cells would experience the same gas concentration in each cycle.

Impact of *in vitro* whole cannabis smoke exposure on airway epithelial cell viability and barrier function

Following quantitative modelling, we next applied Ives for cannabis smoke exposure experiments with multiple biological readouts of relevance to epithelial cell biology. We measured TEER before and after fresh, whole cannabis smoke exposure on primary HBECs. We compared the change in TEER (Δ TEER) before and after exposure between cultures that received room air or smoke. Our results suggest that

individual donor cultures exposed to cannabis smoke in IVES experienced a decrease in epithelial barrier function as compared to air-exposed controls ($p < 0.05$) (figure 5a). The decline in epithelial barrier function was not associated with any increase in LDH, a measure of cell membrane integrity and cell viability (figure 5b) suggesting that cell cytotoxicity was minimally impacted by our model.

Following whole smoke exposure, cell cultures exhibited qualitative changes in morphology. Qualitative analyses revealed higher incidences of cell lifting, areas of patchiness and a circular shape of the cells relative to controls. A representative microscopic image reflecting these notable changes is shown in figure 5.

Impact of in vitro whole cannabis smoke exposure on airway epithelial cell immune responses

IL-1 cytokine family members are elevated in the context of tobacco smoke exposure to airway epithelial cells and lung tissue [60–62]. We therefore analysed the differential expression of selected IL-1 cytokine family members, IL-1 α , IL-1 β , IL-18, IL-1Ra, in our model of cannabis exposure using IVES, as we have demonstrated significant overlap between airway epithelial cell responses to these two exposures in a submerged monolayer culture system [35]. Trends for elevations in IL-1 α ($p = 0.054$), IL-1 β ($p = 0.296$) and IL-18 ($p = 0.064$) were observed and a significant elevation of IL-1Ra ($p < 0.05$) in five individual donor samples ($n = 5$) following cannabis smoke exposure relative to room air. On a donor basis, IL-1 α , IL-18 and IL-1Ra were elevated in 5 of 5 samples (100%), whereas IL-1 β was elevated in 4 of 5 samples (80%) (figure 6).

We have demonstrated that cannabis smoke extract suppresses CXCL10 and CCL5 in Calu-3 epithelial cells under submerged monolayer conditions [35]. We therefore analysed CXCL10 and CCL5 levels following cannabis smoke exposure in IVES with primary human airway epithelial cells. We observed no significant changes in both CXCL10 or CCL5 expression (figure 7). On a donor basis 4 of 5 (80%) donor

FIGURE 5 a) Change in transepithelial electrical resistance (TEER) from baseline after room air *versus* whole cannabis smoke exposure. Analysed with paired t-test, $p = 0.029$, $n = 10$. b) Lactate dehydrogenase (LDH) expression as a proportion of maximal LDH release, analysed *via* ANOVA and Tukey's *post hoc* test. Shows representative microscopy ($\times 40$) of c) Transwell inserts with human bronchial epithelial cells (HBECs) prior to room air exposure, d) Transwell with HBECs after room air exposure, e) Transwell with HBECs prior to whole cannabis smoke exposure, and f) Transwell with HBECs after whole cannabis smoke exposure [product weight of 0.7 g]. Representative microscopy ($\times 100$) of g) Transwell inserts with HBECs prior to room air exposure, h) Transwell with HBECs after room air exposure, i) Transwell with HBECs prior to whole cannabis smoke exposure, and j) Transwell with HBECs after whole cannabis smoke exposure [product weight of 0.7 g]. *: $p < 0.05$; ***: $p < 0.001$.

FIGURE 6 Interleukin (IL)-1 cytokine family member quantification in apical washing of primary human airway epithelial cells exposed to air or whole cannabis smoke. a) IL-1 α , $p=0.054$; b) IL-1 β , $p=0.296$; c) IL-18, $p=0.064$; and d) IL-1R antagonist (IL-1Ra), $p<0.05$ (analysed via paired t-tests). *: $p<0.05$.

samples displayed suppression of CXCL10 ($p=0.110$) and 0 of 5 (0%) donor samples displayed suppression of CCL5 ($p=0.252$).

In vitro whole cannabis smoke exposure induces expression of genes involved in cellular detoxification

We have demonstrated that tobacco and cannabis smoke extract exposures with Calu-3 epithelial cells cultured under submerged monolayer conditions results in elevations in gene expression for CYP1A1 and CYP1B1, both of which function as phase II detoxification enzymes [3]. Additionally, thioredoxin interacting protein (TXNIP) has been shown to inhibit the antioxidative effect of thioredoxin, resulting in an accumulation of reactive oxygen species and cellular stress [63, 64]. We therefore determined the gene expression level of CYP1A1, CYP1B1 and TXNIP in primary human airway epithelial cells following cannabis smoke exposure with IVES. We demonstrate that cannabis smoke exposure results in robust induction of both gene transcripts in 5 of 5 (100%) donor samples for CYP1A1 ($p<0.001$), 5 of 5 (100%) donor samples for CYP1B1 ($p<0.05$) and suppression of TXNIP in 4 of 5 (80%) donor samples ($p=0.058$) (figure 8).

Discussion

The US Center for Disease Control declares that cigarette smoking is the leading preventable cause of death in the United States [65]. Adding to the pressing concern is the relatively new increase in global

FIGURE 7 Antiviral cytokine quantification in apical washing of primary human airway epithelial cells exposed to air or whole cannabis smoke. a) CXCL10, $p=0.110$; and b) CCL5, $p=0.252$ ($n=5$, analysed via paired t-tests).

FIGURE 8 Selected oxidative stress genes expressed in primary human airway epithelial cells exposed to air or whole cannabis smoke. a) CYP1A1, $p < 0.001$; b) CYP1B1, $p < 0.05$; and c) TXNIP, $p = 0.058$ ($n = 5$, analysed via paired t-tests). *: $p < 0.05$; ***: $p < 0.001$.

legalisation and practice of smoking cannabis [66–68]. Notably, evidence suggests that cannabis smoke consists of differential components relative to tobacco smoke, which may function as immunomodulatory agents [26, 69, 70]. To determine shared or divergent consequences of cannabis smoke on lung health, relevant experimental data must be generated to inform policy and decision making at the personal and population level. Frequently used low-cost *in vitro* models of smoke exposure may not adequately reflect the realities of smoke delivery *in situ*, while advanced smoke exposure model systems, more physiologically relevant, are often expensive and inaccessible for many research groups. To address these constraints and the need for accessible, relevant, *in vitro* experimental systems, we sought to develop and validate a novel smoke exposure model applicable to various modalities through the conception of IVES.

To capture the benefit of exposing cells directly to smoke while overcoming the shortcomings of the current smoke exposure systems, which are bulky, expensive or lacking in adaptation to custom experimental set-ups [71–75], we utilised 3D-printing technology to design and develop a versatile smoke exposure system that can be easily scaled up or down. Initially, the IVES was designed and fabricated as a single-chamber exposure unit. The purpose of this step was to optimise the size of the chamber considering various criteria such as basal liquid volume, accessibility to Transwell insert and the basal medium, and the fluidic path of smoke exposure. Then, the final design was scaled up to the current IVES as proposed in this work (figures 1 and 2). The fluidic dynamic modelling conducted in this study confirmed that the design of IVES and the exposure protocol were well designed to expose cells in a repeatable fashion. Figure 3 shows a uniform gas flow distribution in IVES with indirect gas exposure to cells, which was critical in creating a stress-free exposure to cells. Additionally, mass transfer results from the simulation revealed that the length of fresh air exposure was sufficient to zero CO_2 concentration (figure 4). This *in vitro* model can be easily redesigned for different applications and purposes. The size of IVES can be changed to fit various sizes of Transwell inserts or more exposure chambers can be integrated with IVES. Moreover, a fluidic path can be added to the basal side of the chambers to create a dynamic flow in the liquid compartment of the system. Overall, the proposed exposure system in this work is inexpensive, easy to use, easy to fabricate and amenable to multiple experimental designs, including smoke, vaping and coexposures with pathogens.

The mucociliary escalator, secreted antimicrobial products and paracellular permeability mediated by intracellular junctions collectively serve to establish the barrier function between the external and internal environments [5]. Human airways rely on the latter, tight and adherens junctions between cells at the apical border, to prevent inhaled pathogens and other insults from causing harm to the airways [5]. In smokers, the epithelium is found to be dysfunctional and abnormally differentiated, leaving a higher risk for viral and bacterial infection [5]. To determine how IVES delivery of cannabis smoke impacted epithelial barrier function, TEER was measured in 10 individual donor primary HBECs prior to and following cannabis smoke exposure. Using IVES as a model for cannabis smoke exposure using primary HBECs, our data suggest that a compromised barrier may be observed in human cannabis smokers. The lungs rely on the formation and strict regulation of a mechanical barrier established by airway epithelial cells [76]. Previous studies have implicated oxidative stress brought on by cigarette smoke with the disruption of epithelial barrier function [77]. Other studies have found that cigarette smoke increases the permeability of human airways disrupting the balance between external fluids and macromolecules through altered regulation of multiple tight junction and adherens junction proteins [78–80].

Despite extensive studies of airway epithelial cell barrier function and tobacco smoke exposure, few data are available for cannabis smoke exposure. Using submerged monolayer cultures of Calu-3 cells, we were able to demonstrate that cannabis smoke condition medium was able to reduce barrier function, as assessed by TEER, a response shared with tobacco smoke-conditioned medium [35]. In the present study, we extended our published findings by interrogating how whole cannabis smoke impacted barrier function in primary human airway epithelial cells grown under ALI conditions. Our data confirm that cannabis smoke, similar to tobacco smoke, is able to compromise barrier function. Furthermore, the data establish that monitoring epithelial cell barrier function measurements following exposures using IVES are possible.

Cytokines are signalling molecules crucial to the proper innate and adaptive immune function. They perform a host of essential duties ranging from mitigating viral, bacterial, fungal infections to signalling a cascade of other immunomodulatory agents responding to allergens in the air. Airway inflammation associated with changes in cytokines that regulate immune function is present in both cannabis and tobacco smokers resulting in clinical presentation of coughing, wheezing and the onset of asthma and COPD [21, 27, 81–85]. Notably, the IL-1 family of cytokines has been implicated in acute inflammatory processes as well as linked to cytokine balance disruption in cigarette smokers [25]. IL-1 family cytokine expression at the protein level was assessed in our model, following cannabis smoke exposure in five individual patient donor samples. Our experiments with five independent donors show trends for an increase in IL-1 α , IL-1 β and IL-18 expression, following cannabis smoke exposure relative to control, findings that are similar to observations made in other studies with tobacco smoke [60–62]. These results indicate an inflammatory response induced by cannabis smoke characterised by IL-1 family cytokines, which may share downstream consequences with tobacco smoke that include neutrophilia driven by an IL-1R–IL-17 axis [86]. We also observed a significant elevation of IL-1Ra following smoke exposure. Notably, other studies have found a suppression of IL-1Ra expression in tobacco smokers, which works by antagonising IL-1 α and IL-1 β [87]. In cannabis smoke however, IL-1Ra expression has been shown to be elevated owing to the unclear immunomodulatory features of cannabis active components [24, 88, 89].

Our published *in vitro* data using Calu-3 airway epithelial cells under submerged monolayer culture conditions suggest that cannabis smoke extract-conditioned medium attenuates expression of antiviral pathways important in host defence in human airway epithelial cells [35, 36]. To explore the possibility that whole cannabis smoke exposure of primary human airway epithelial cells grown under ALI-culture conditions behaved similarly, we analysed CXCL10 and CCL5 levels as previously performed [35, 36]. The current data show negligible changes from the baseline of CXCL10 and CCL5 in primary HBECs. This suggests inherent differences in the model and/or use of primary cells at ALI when compared to cell lines in submerged monolayers. Moreover, cytokines have been shown to not be expressed differentially following cigarette exposure alone; rather, they require viral/viral mimetic challenges prefacing expression [90–92]. It will be relevant for future studies to induce CXCL10/CCL5 through viral/viral mimetic challenges to assess the impact of cannabis smoke on these antiviral cytokines.

Previously, we have shown that aryl hydrocarbon receptor-induced genes associated with oxidative stress, CYP1A1 and CYP1B1, are significantly induced in Calu-3 cells exposed to cannabis and tobacco smoke extracts [35]. Similarly, we have shown that TXNIP was reduced [35]. Other studies have shown similar results of increased oxidative stress, as indicated by dysregulated expression of these same genes in various types of smoke exposure such as tobacco, polycyclic aromatic hydrocarbons, incense smoke and cannabis smoke [93–95]. Consequently, we analysed the expression of CYP1A1, CYP1B1 and TXNIP in primary human epithelial cells exposed to cannabis smoke using IVES. We chose these particular genes because of their robust induction in our Calu-3 cell model with cannabis smoke-conditioned medium. A significant elevation was observed in CYP1A1 and CYP1B1, while a negative trend was observed in TXNIP following smoke exposure, validating IVES for modelling molecular changes in human airway epithelial cells exposed to whole cannabis smoke.

The aim of our study has been to validate and introduce an open-access, disposable, easy-to-use method for whole smoke exposure to airway epithelial cells grown on Transwell inserts. The dosing parameters used in our study served as a means to reliably evoke a cannabis induced effect. To incorporate aspects of human smoke exposure studies we used a modified Foltin puff procedure. In this procedure used for clinical research, human participants are asked to inhale smoke for 5 s, hold for 10 s and then exhale. We used these durations to define the durations for inhalations, exhalations and periods of rest. To determine the volumes for the inhalations and exhalations, we used the ISO 3308 routine analytical cigarette-smoking machine guideline. To determine the mass of cannabis, we aligned with the Kentucky research-grade cigarette, a standardised product that has 0.7 g per cigarette. We recognise that these conditions represent a single possible combination that does not provide concentration–response outcomes that may be impacted by length of time for combustion, frequency and volumes of inhalation/exhalation, and mass of cannabis combusted. Despite our single set of conditions, we validate IVES for equal distribution of smoke

across the four exposure chambers, equal distribution of smoke pressure over the base of the Transwell insert, minimal shear stress across the growth area, a capacity for the human airway epithelial cells to respond to smoke at a cellular and molecular level. Future studies can expand applications for the validated IVES to explore different concentrations, durations of smoke, period of smoke, coexposure with pathogens or introduction of vaping technologies. Additional experiments could be performed to define a no-observed effect level for studies with a toxicology focus.

In this study, we have outlined and validated the developmental parameters and flow simulation and streamlined the exposure protocol to measure epithelial barrier function, cytokine expression and gene expression. We have applied it to an exposure study assessing the impact of cannabis smoke exposure of primary HBECs. IVES shows stark similarities between existing models and promises the ability to generate needed relevant data to inform public health policy and individual user practices.

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