CANNABIS SMOKE EXPOSURE MODELS AND IMMUNOMODULATION

DEVELOPING AND VALIDATING A NOVEL *IN VITRO* SMOKE EXPOSURE MODEL AND INVESTIGATING THE INNATE IMMUNOLOGICAL IMPACT OF CANNABIS SMOKE EXPOSURE ON PRIMARY HUMAN BRONCHIAL EPITHELIAL CELLS

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TITLE: Developing and validating a novel *in vitro* smoke exposure model and investigating the innate immunological impact of cannabis smoke exposure on primary human bronchial epithelial cells

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LAY ABSTRACT

Despite its recent legalization in Canada, cannabis smoke has been understudied and a lack of evidence exists to inform legislative policies, medicinal and recreational usage. Due to a lack of relevant ways to study cannabis smoke in a lab setting, it is difficult to accumulate literature around its impacts in the lungs. Here, we addressed this gap by engineering and validating a novel model to expose lung cultures to cannabis smoke. In addition, we investigated its impact on the immune response. Our findings suggest exposure to cannabis smoke alters the immune functions of these cells. We also found that in response to a viral mimetic stimulus, cell cultures pre-exposed to cannabis smoke exhibited impaired immune responses. Our novel model to expose cell cultures to cannabis smoke creates a foundation for future researchers to investigate environmental insults, such as cannabis smoke, in the context of respiratory health and infectious disease.

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 as well as medicinal 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 (HBECs).

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 HBECs 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.

HBECs exposed to cannabis smoke using IVES showed changes in cell morphology and disruption of barrier function without significant cytotoxicity. Cannabis smoke elevated interleukin-1 (IL-1) family cytokines and elevated CYP1A1 and CYP1B1 expression relative to control. These findings validate IVES to have an effect in HBECs at a molecular level following cannabis smoke exposure. In addition, HBECs stimulated with a viral mimetic, Poly I:C, challenge following cannabis smoke exposure showed a suppression of key antiviral cytokines.

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

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In Sanskrit, there is an adage - *Maatha, Pitha, Guru, Deivam* or "unconditional reverence to your mother, father, teachers and God." I would like to affirm that in my life and throughout my master's degree, these personalities have been my support and guidance.

My parents fled here to Canada as refugees fleeing a civil war. They faced countless struggles, indescribable failure, and humiliation to give my brother and I an opportunity to spread our wings devoid of strings. To my family, I am grateful for every sacrifice you have made, and every lesson learned. I am eternally in debt to you all.

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LIST OF ABBREVIATIONS AND SYMBOLS

ALI – Air liquid interface
AMP – Adenosine monophosphate
ATP – Adenosine triphosphate
CB1 – Cannabinoid receptor type 1
CB2 – Cannabinoid receptor type 2
CBD – Cannabidiol
COPD – Chronic obstructive pulmonary disease
CYP1A1 – Cytochrome P450 Family 1 Subfamily A Member 1
CYP1B1 – Cytochrome P450 Family 1 Subfamily B Member 1
FEV – Forced expiratory volume
GM-CSF – Colony Stimulating Factor 2
G-CSF – Colony Stimulating Factor 3
HBECS – Human bronchial epithelial cells
IL-1 – Interleukin 1
IL-1α – Interleukin 1 alpha
IL-1β – Interleukin 1 beta
IL-18 – Interleukin 18
IL-1Ra – Interleukin 1 receptor antagonist
IVES – *In vitro exposure system*
LDH – Lactate 12hydrogenase
MIP-1β – C-C Motif Chemokine Ligand 4
Poly I:C – polyinosinic:polycytidylic acid
TEER – Transepithelial electrical resistance
THC – Delta-9-tetrahydrocannabinol
TLR3 – Toll-like receptor 3
TNFa – Tumour necrosis factor alpha
TXNIP – Thioredoxin interacting protein

DECLARATION OF ACADEMIC ACHIEVEMENT

The research and data presented in this thesis are the cumulative efforts of myself, Abiram Jonathan Chandiramohan, and other colleagues and collaborators in the Hirota laboratory and the Firestone Institute for Respiratory Health. I significantly contributed to all aspects of the data contained in this thesis including, but not limited to, project design, experimental execution, data collection, data interpretation, and manuscript development. These have been done under the direct supervision of Dr. Jeremy Hirota. This work was directly supported by members of Hirota laboratory – Quynh T. Cao, Dr. Mohammadhossein Dabaghi, Nicholas Tiessen, Mary Stewart, Jenny P. Nguyen and Yechan Kim – who assisted in sample collection and processing and experimental execution. The aforementioned members of the Hirota laboratory are affiliated with McMaster University. RNA analysis was performed by Dr. Jennifer A. Aguiar and Dr. Andrew C. Doxey from the University of Waterloo. Primary human bronchial epithelial cell samples were provided by Dr. Nathan Hambly, Mr. Daniel Vermunt, Dr. Nima Makhdami and Dr. Gerard Cox.

The research presented in this thesis has been prepared in one manuscript for which I share primary authorship with Dr. Mohammadhossein Dabaghi. It is entitled "Development and validation of an open-source, disposable, 3D printed *in vitro* environmental exposure system for Transwell culture inserts" and has been published in the European Respiratory Journal Open.

CHAPTER 1: INTRODUCTION

1.1 Cannabis

1.1.1 Historical Significance and Current Cannabis Use Trends

The earliest accounts of cannabis (*Cannabis sativa, Cannabis indica*) are traced back over 5000 years ago to modern-day Romania (1). In addition to its recreational use, which owes its popularity to its psychoactive properties, the earliest recorded use of cannabis in medicine is in 400 A.D (2). However, its use medically and religiously is estimated to have begun before that in 1000 B.C in various regions in Asia including India, Tibet, and China (3). As it made its way westward, its potential medical uses were exploited and patients were often treated with it for numerous reasons such as an analgesic, anticonvulsant, anti-inflammatory agent, and to treat psychiatric concerns (3). Upon its popularity in Western medicine, over 100 scientific studies were published outlining some of its potential uses to treat patients. However, since the active components of cannabis were not yet isolated, its potency and efficacy in treatment varied as a function of the sample's origins, method of preparation and other such factors (4). Therefore, the results were often not replicable.

Despite its early origins in ancient society, cannabis has endured throughout history to the present day. Its popularity and prevalence have only partially been owed to its recent legalization in Canada when Bill C-45 (the Cannabis Act) was passed in 2018 (5). Its legalization has nonetheless been highly contested. On the side of pro-legalization, the argument has revolved around the ability for federal legislation to strictly regulate who is able to secure and use cannabis, increasing the likelihood that youth and children will not be exposed to it (6). On the contrary, proponents of anti-legalization argue that the extensive potential harms of use, especially to children and youth brain development, outweigh the potential for stringent regulation (6). As an example, the Canadian Medical Association Journal stated, "Cannabis legalization fails to protect Canada's youth" and concluded that "if Parliament truly cares about the public health and safety of Canadians, especially our youth, this bill will not pass" (5, 6).

Irrespective of merits awarded to either side of the legalization debate, cannabis usage in recent times has increased significantly over the last two decades. In 2004, cannabis was consumed in 12.2% of men and 6.6% of women in Canada (7). Before legalization in 2017, cannabis prevalence increased to 18.7% of men and 11.1% of women (7). Following legalization, in 2020, Health Canada reported 21.1% of men and 18.4% of women over the age of 15 having used cannabis at least once in the past 3 month (8). Legalization may have only expedited this process by means of making regulated products more accessible for public consumption.

1.1.2 Routes of Administration

Adverse health impacts of cannabis have been correlated with the route of administration that the user uses to consume the substance (9). Smoking cannabis has remained the most prevalent route of administration in 90% of consumers (10, 11). This preference was consistent across all age groups (9-11). Despite it being the preferred route of administration, smoking cannabis has been associated with a slew of acute and chronic respiratory symptoms including coughing, wheezing, sputum production, and bronchitis (9, 12). Moreover, clinical parameters such as FEV and vital capacity of the lungs have also been shown to decline with prolonged exposure to cannabis smoke (9, 13). Interestingly, however, some of these respiratory symptoms have been shown to subside with decreased usage or later abstinence in stark contrast to tobacco smoke (9, 14). More serious implications on a user's health have also been studied extensively. Case studies link lung bullae, emphysema, pneumothoraces, and other forms of chronic lung injuries with prolonged use (9, 15-16). As expected, practices wherein cannabis is consumed through smoke are associated with higher levels of carbon-monoxide exposure (17).

1.2 Lung Physiology, Lung Immunity and Cannabis Smoke

1.2.1 Physiology of the Respiratory Epithelium

The lungs are 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 environmental insults, including allergens, viruses, bacteria, and air pollutants (18). The pseudostratified airway epithelial cells that line the lungs have evolved to mitigate the risks from external insults by providing a tight physical and immunological barrier (19–21). 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 attacks (22, 23). Airway epithelium dysfunction has been strongly implicated in the pathogenesis of many airway diseases including asthma and COPD (24–26). Importantly, environmental insults capable of damaging the airway epithelium are also risk factors for these same respiratory diseases (27–30).

1.2.2 Active Cannabinoids and Immune Modulators

The presence of endocannabinoid receptors on immune cells throughout the body indicate that they may play an important role in immune modulation. Exogenous cannabinoids tetrahydrocannabinol (THC) and cannabidiol (CBD) have been shown to bind to the G-Protein coupled endocannabinoid receptors, CB1 and CB2, and have the potential to inhibit adenylyl cyclase activity (31). By virtue, this binding can inhibit the conversion of ATP to cyclic AMP (31). This inhibition can then go on to have a slew of impacts on the functions of the cell due to the reduced production of this secondary messenger, cyclic AMP (31). Moreover, exogenous cannabinoids such as THC and CBD, amongst others, have been shown to influence the activities of the immune response. For example, one study found that THC induced apoptosis in macrophages and T cells in *in vitro* rodent models (32). Another study showed that naive murine lymphocytes underwent increased apoptosis in response to THC upon the activation of these cells as THC downregulated CB2 receptors on the cell surfaces (32, 33). It is important to note that in other studies, THC and other cannabinoids were shown to play a protective role as they prevented apoptosis in non-transformed cells of the central nervous system through CB1 and CB2 mediated pathways (33).

Cannabis compounds have been shown to have anti-inflammatory as well as pro-inflammatory functions in airway epithelial cells (34, 35). In one study, cytokines that initiated and resolved inflammation through human T-cell, B-cell, and CD8+ mediated pathways were dysregulated in the presence of THC (36). The study concluded that THC has potential pro-inflammatory and anti-inflammatory effects depending on the cell population used (36). This variability and lack of understanding of the precise role that cannabinoids play in the regulation, or dysregulation, of the immune response warrants the need to study it further. Recent studies have garnered some potential applications for cannabinoids in preventing inflammatory cytokine storms. Specifically, a study assessing the impact of phytocannabinoids, such as THC, were suggested to have an anti-inflammatory effect which would reduce the severity of the cytokine storm that would otherwise drive mortality in viral infections such as SARS-CoV-2 (37). Another study found that CBD inhibited SARS-CoV-2 replication and promoted host immunity in airway epithelial cells (38). As these studies suggest, the potential therapeutic uses of phytocannabinoids, or potential dysregulation of the immune response, warrants further investigation to inform future cannabis-related legislation, recreational use, and therapeutics.

1.2.3 Common Respiratory Viruses and Impact on Healthcare

Respiratory viruses have historically presented impressions on patients, healthcare workers, the healthcare system, and the economy. The current COVID19 pandemic is an example of the detrimental impact that a respiratory virus can have. To date, the SARS-CoV-2 virus has infected approximately 359 million people and has led to the death of approximately 5.62 million people worldwide. It was estimated to be the third leading cause of death in 2020 causing a deficit of over $3.3 trillion which accounts for about 15% of the United States' GDP (39, 40). According to an estimate by The American Hospital Association, the COVID19 pandemic has cost $202.6 billion in revenue for American healthcare to date (39).

Even prior to the COVID-19 pandemic, other prevalent respiratory viruses such as the influenza virus were responsible for seasonal infections amongst a major part of the population. Influenza has been categorized as the 8th infectious agent that is detrimental to the healthcare system (41, 42). Furthermore, older adults (> 65 years old), in whom comorbidities are more prevalent, are at higher risk for fatal complications from influenza and for requiring hospitalization during infections (41, 43-45). In addition, the seasonal flu has presented socio-economic burdens in communities such as absenteeism from work and has aggravated pre-existing inequalities in healthcare access and hospitalizations (45-46).

As recreational, as well as medical, use of cannabis increases in the community, its impact on the respiratory defense system and respiratory immune response in the context of viral exposures needs to be understood.

1.2.4 Tobacco Smoke and Cannabis Smoke in the Context of Respiratory Illness

Tobacco smoking, a direct environmental insult to the airway epithelium, remains common despite its well-described effects on respiratory health (47–51). Like tobacco smoke, cannabis smoking is also a direct environmental attack to the lungs. As a result of progressive legislation on a global scale, cannabis consumption has been on the rise, where 90% of consumers prefer smoking as a route of delivery (52, 53). Tobacco and cannabis smoke exposure induce similar clinical features and are associated with increased prevalence of coughing, wheezing, and chest tightness (54–56). Tobacco and cannabis smoke exposure also present divergent clinical features, with tobacco smokers more likely to develop lung cancer relative to cannabis smokers (57, 58). This observed divergence may be due to the presence of phytocannabinoids with anti-inflammatory properties unique to cannabis (59–62). 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.

1.3 *in vitro* Models of Cannabis Smoke Exposure

1.3.1 Critical Assessment of Current *in vitro* Models

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 (63, 64). The majority of *in* vitro models use tobacco smoke extracts in submerged epithelial monolayer cultures in a miscible liquid format (65–67). 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, 68). Smoke extract exposures do not entirely reflect whole smoke exposure as the latter generates heat and water-insoluble hydrocarbon combustion products (54, 69-71). Additionally, submerged monolayer cell culture designs do not entirely reflect the *in-situ* airway environment where a pseudostratified airway epithelium is exposed to inhaled air on an apical side while attached to a basement membrane on the basal side. To more accurately reflect the *in-situ* environment, airway epithelial cells can be cultured on Transwell inserts under air–liquid interface (ALI) culture conditions to create a pseudostratified tissue structure (72, 73). 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 (72, 73). The strengths and limitations of popular smoke-generating machines have been characterized extensively (74–77). Commercially available smoking machines offer high throughput designs and can 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 (77). Some devices are directly amenable to multiple exposures beyond smoke, including environmental toxins, gases, therapeutic aerosols, aerosolized pathogens, and other volatile compounds (74–77). The throughput and automation benefits of these environmental exposure systems are offset by some important limitations (77–81). From an operations perspective, these are capital intensive closed systems that cannot be customized, limiting accessibility to specialized research facilities and field participants (77). From a technical perspective, limitations may include potential air and smoke mixing inefficiencies, aging of smoke in mixing vessels, and turbulence that is inconsistent with lung physiology (77). 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.

1.3.2 Impact of Cannabis Smoke Extract on Epithelial Barrier Function, Immune Profile, and Transcriptional Regulation

As aforementioned, current *in vitro* models of cannabis exposure have relied on cannabis smoke extract in studies assessing their impact on key functional parameters such as epithelial barrier function, cytokine immune profiles, and transcriptomic profiles. Previously, our lab showed that cannabis smoke extract, like tobacco smoke extract, impaired epithelial barrier function and dysregulated immune cytokine and transcriptional responses in Calu-3 cell lines (35). Our work showed that cannabis smoke can be harmful to respiratory function (35). Whether other major constituents of mainstream cannabis smoke such as heat and combustion products present in recreational exposure differentially impact the protective functions of primary HBECs is yet to be elucidated.

Previous studies have shown the IL-1 family of inflammatory cytokines such as IL-1α, IL-1β, IL-18, and IL-1Ra to be elevated in tobacco smokers (82-86). Given the similarities between tobacco and cannabis smoke (35), we measured these 4 IL-1 family cytokines as a metric for inflammation in HBECs exposed to cannabis smoke through IVES. In addition, previous studies have shown that cellular detoxification genes CYP1A1 and CYP1B1 are upregulated in the airway epithelium cigarette smokers (35, 87-89). Therefore, we assessed the expression of these genes as a metric of any oxidative stress that HBECs underwent due to the cannabis smoke exposure throughout our study.

1.4 Aims and Research Objectives of the Current Study

1.4.1 PART 1: Introduction of a Novel *in vitro Exposure System (IVES):* Central Aim, Research Objectives, Hypotheses

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 solution to create an open-source, disposable, *in vitro* exposure system that is widely accessible without requiring specialized environmental exposure

infrastructure. Additive manufacturing has been utilized in various fields to realize complex 3D constructs with resolution at the micron level. In contrast to conventional manufacturing technologies, additive manufacturing is accessible and independent of specialized 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 optimize 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 HBECs. We hypothesize that HBECs exposed to cannabis smoke through IVES will exhibit impairment of epithelial barrier function, potentiation of IL-1 family cytokines, and potentiation of oxidative stress genes, CYP1A1 and CYP1B1. Our 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 Transwell wells. IVES can also be customized to accommodate more than four Transwell inserts. However, upon increased exposure chambers added to IVES, further validation studies would need to be performed to ensure uniform flow distribution and shear stress distributions across all chambers. Finally, IVES is amenable to dilution of smoke with room air. This feature will allow IVES to be adapted for smoke concentration–response studies where room air can dilute smoke.

1.4.2 PART 2: Using *IVES* to Elucidate the Immunological Impact of Cannabis Smoke on the Airway Epithelium in the Context of Viral Mimetic Stimulation: Central Aim, Research Objectives, Hypotheses

In PART 1, we validate IVES as a legitimate model to study cannabis smoke exposure *in vitro*. In this current section, we will continue to utilize the validated exposure model to expose primary HBECs to cannabis smoke. Following exposures, we will study the impact of prior, acute cannabis smoke exposure on the cell's immune response to a viral mimetic, polyinosinic:polycytidylic acid (Poly I:C). Poly I:C is a synthetic double-stranded viral RNA analog. It is recognized through the toll-like receptor 3 (TLR3) pathway

(90, 91). Stimulation with Poly I:C has been shown to closely mirror viral infections by increasing inflammatory cytokine production and mucus production (90, 91). In addition, Poly I:C impairs epithelial barrier function similar to that which occurs during viral infection in airway epithelial cells (90, 91). In previous studies, Poly I:C was shown to induce a robust immune response in primary HBECs (90-92). In particular, it elevated expression of inflammatory cytokines such as IL-6, IL-8, TNFa and antiviral cytokines such as CXCL10 and RANTES (90-92). In the current study, we investigated the impact of cannabis smoke exposure on the immune profile induced by Poly I:C in primary HBEC cultures at ALI. We hypothesized that prior cannabis smoke exposure would impair and suppress antiviral cytokine production resulting from Poly I:C immune stimulation. In addition, we hypothesized that cannabis smoke exposure would augment inflammatory cytokine production. Finally, we hypothesized that prior cannabis smoke exposure would further impair epithelial barrier function than impairment due to Poly I:C stimulation alone.

1.4.3 PART 3: Understanding the Role of Dose-Response of Cannabis Smoke and Cell Culture Media Supplements on Immune Profiles to Improve IVES: Central Aim, Research Objectives, Hypotheses

In PART 1 and PART 2, we validate the IVES model to expose primary HBECs to cannabis smoke and furthermore, applied the model to study cannabis smoke in the context of viral mimetic exposure-induced immune profiles. In the current section, we further refine our exposure model for future cannabis or other environmental exposure studies by performing dose-response experiments to identify an optimized dose of cannabis smoke for our model that minimizes cell lifting while still inducing an immunological effect.

In addition, a recent study outlining curated protocols for cell culture techniques and assay adaptations in the context of respiratory infections suggests that infection responses in ALI cultures with and without Pneumacult ALI Basal Media supplements are considerably different (93). Cell culture signaling pathways in the context of infection can be impacted in the presence or absence of media supplements (93). Due to manufacturer constraints and privacy, the composition of Pneumacult media supplements is largely unknown. Heparin and hydrocortisone are the only confirmed constituents in the supplements (94). However, hydrocortisone, a glucocorticoid, has been shown to have anti-inflammatory and immunosuppressive effects *in vitro* (95). This constituent may affect the immune response of HBECs to cannabis smoke and subsequent viral mimetic stimulation and therefore, warrants a need to be characterized to inform future studies using IVES. Therefore, we sought to investigate the differential immune response of

HBECs to cannabis smoke through IVES in the presence and absence of Pneumacult ALI Basal Media supplements.

CHAPTER 2: MATERIALS AND METHODS

2.1 *IVES* Development

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 optimized at 3 mm through multiple iterations to minimize obstruction with organic combustion by-products. Exposure chamber size was designed based on the dimension of a Transwell insert for 24 well-plate (VWR, Pennsylvania, USA; catalog 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. In the airway, nasal conchae create turbulence in the inhaled air (96). However, distal portions of the bronchi are governed by laminar flow (96). Therefore, we structured IVES such that HBECs would be exposed to indirect smoke and not 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 IVES design, whereas Transwells from different manufacturers with different dimensions may need independent validation.

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.

2.2 Fluid Dynamic Modeling

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:

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

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

where ρ is the air density, $\vec{\vartheta}$ represents the velocity vector field, p shows the pressure, $\underline{\tau}$ 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 mL·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.

2.3 Cell Culture Protocol

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 (summary of patient demographic data is provided in **Table 1**) undergoing routine clinical procedures, and plated into T25 flasks containing PneumaCult Ex-Plus Basal medium (StemCell Technologies, Vancouver, British Columbia, Canada; catalog number 05040) with PneumaCult Ex-Plus 50× supplement, 0.01% hydrocortisone stock solution (StemCell Technologies, Vancouver, British Columbia, Canada; catalog

number 7925) and 1% antibiotic-antimycotic (Thermo Fisher Scientific, Mississauga, Ontario, Canada; catalog 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 approximately 14 days after seeding, 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, Vancouver, British Columbia, Canada; catalog 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, Vancouver, British Columbia, Canada; catalog number 7980) to support development and differentiation of a pseudostratified 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. Experiments were performed between day 14 and day 15 of ALI culture. For supplement-free experiments from PART 3, twelve hours prior to the start of experiments, experimental conditions were fed basally with supplement/supplement-free (without PneumaCult-ALI 10× supplement, PneumaCult-ALI Maintenance 100× Supplement) ALI media. Supplement-free media was continued to be used throughout the course of the experiment in experimental conditions. Control conditions continued to be fed with media containing the supplements listed above.

Given that the research objectives of our study are solely to introduce a novel way to study cannabis smoke exposure, we justify the use of patient samples. Our study does not focus on the specific impacts that cannabis smoke has on healthy vs. disease state donor samples.

Table 1. Demographic data for patient primary human bronchial epithelial cells used for validation studies in Part 1. Patient samples were taken from consented patients through bronchoscopies. N/A indicates unavailable data.

Patient #	Age	Sex	Diagnosis	Smoking Status	Pack-Year History
1	81	Female	Lung mass	Ex-smoker	15 pack-year
2	47	Male	Sarcoidosis	Non-smoker	N/A
3	87	Female	Lung nodule	Ex-smoker	N/A
4	57	Female	Lung mass	Non-smoker	N/A
5	62	Female	Sarcoidosis	Non-smoker	N/A

2.4 Cannabis Cigarette Preparation

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.

2.5 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).

2.6 Cannabis 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% CO2 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 (97–102). This procedure optimized through multiple iterations for our model, consisted of one puff of smoke separated by three puffs of fresh room air to mimic the behavior 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 mL·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% CO2 humidified incubator. Twenty-four hours later, basal and apical medium was collected, spun down at 7500×*g* and 4°C for 15 min and supernatants were stored at −80°C for subsequent quantification of cytokines and assessment of cytotoxicity.

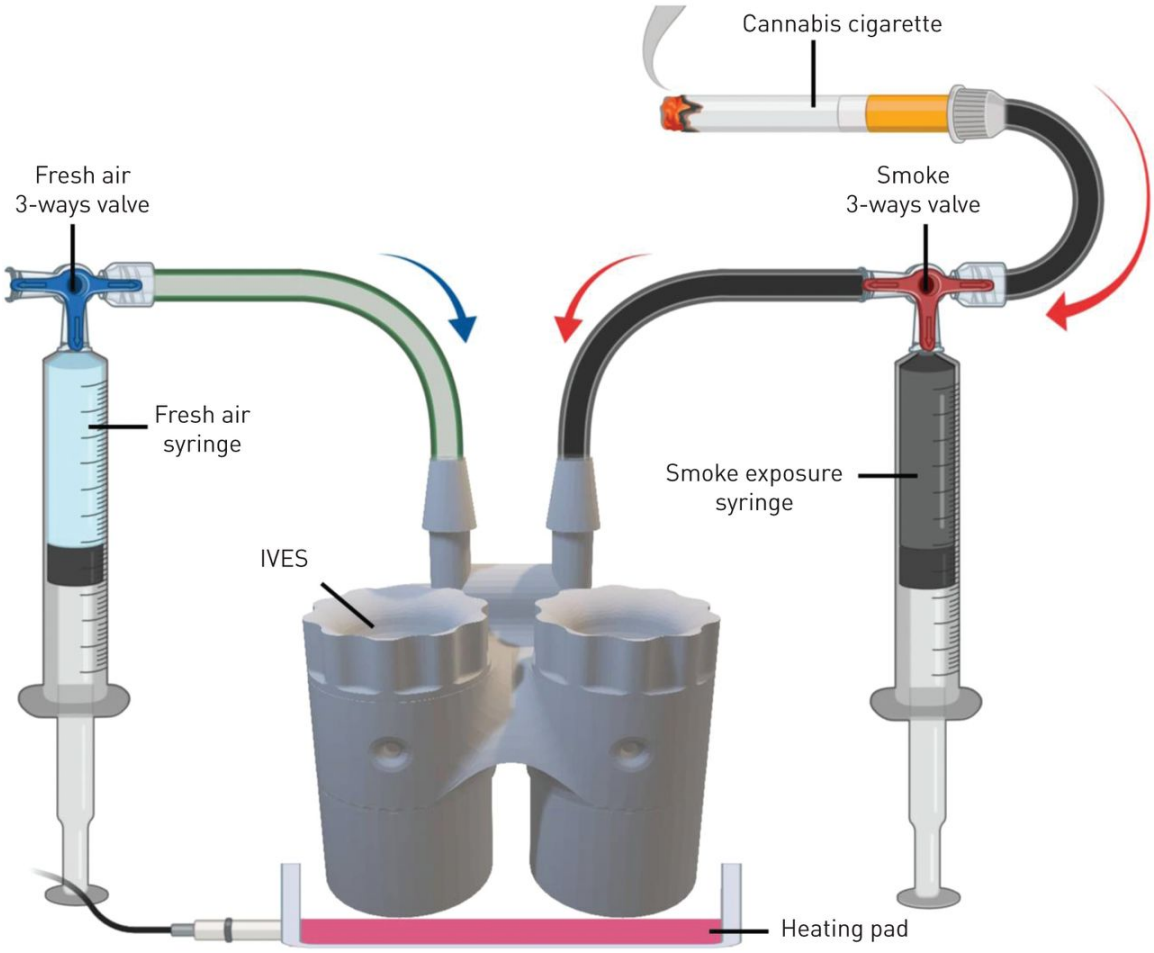

Figure 2. Schematic depicting the *in vitro* exposure system (IVES) connected to air and smoke sources. A three-way valve connects the cannabis cigarette to IVES through a 50-mL syringe. Another three-way valve connects room air to IVES. Smoke is drawn through the smoke exposure syringe and expelled 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).

2.7 Immune Response Stimulation with a Viral Mimetic, Poly I:C

Two hours post-cannabis smoke exposure, cells were stimulated apically and basally with 10ug/ml of Poly I:C. 10ug/ml of Poly I:C has previously been shown in our lab to stimulate a robust immune response in primary HBECs. Twenty-four hours post stimulation, apical and basal media was collected, centrifuged at 7500×*g* and 4°C for 15 min and supernatants were stored at −80°C for subsequent quantification of cytokines and assessment of cytotoxicity.

2.8 Cytokine Quantification

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

2.9 Transcriptomic Analysis and Quantifications

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).

2.10 Processing of Raw Microarray Data

Raw intensity values from the Clarion 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) (103). The single channel array normalization (SCAN) method was used to obtain log2-transformed normalized expression values with the SCAN.UPC R package (version 2.26.0) (104), with annotation data from the Bioconductor project (version 3.9) (105).

2.11 Cell Cytotoxicity

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

2.12 Statistical Analyses

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. Bonferroni correction was performed following an ANOVA to assess differences in antiviral cytokine expression. Differences in inflammatory cytokine expression were assessed using paired t-tests. A p-value <0.05 was considered statistically significant.

2.13 Exposure Protocol for Dose-Response Experiments (PART 3)

Cannabis smoke exposures were performed as explained above using the modified Foltin puff procedure. Cells were exposed to smoke from one full cannabis cigarette smoked to completion one of three cigarette weights - 0.09g, 0.18g, or 0.375g.

2.14 Exposure Protocol for Supplement-Free Experiments (PART 3)

Cannabis smoke exposures were performed as explained above in the modified Foltin puff procedure. Cells were exposed to smoke from one full cannabis cigarette smoked to completion weighing 0.18g.

CHAPTER 3: RESULTS

PART 1 Introduction of a Novel *in vitro* Exposure System (IVES)

3.1 Validation of *IVES*: IVES Fluid Dynamic Modeling

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 in velocity was recognized by changing the mesh size from finer to extra fine. A defined mesh resolution was chosen to run all simulations for fluid dynamic modeling (**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 mL·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 1.0–1.5 µm·s−1 and generated shear stresses (<<1 Pa).

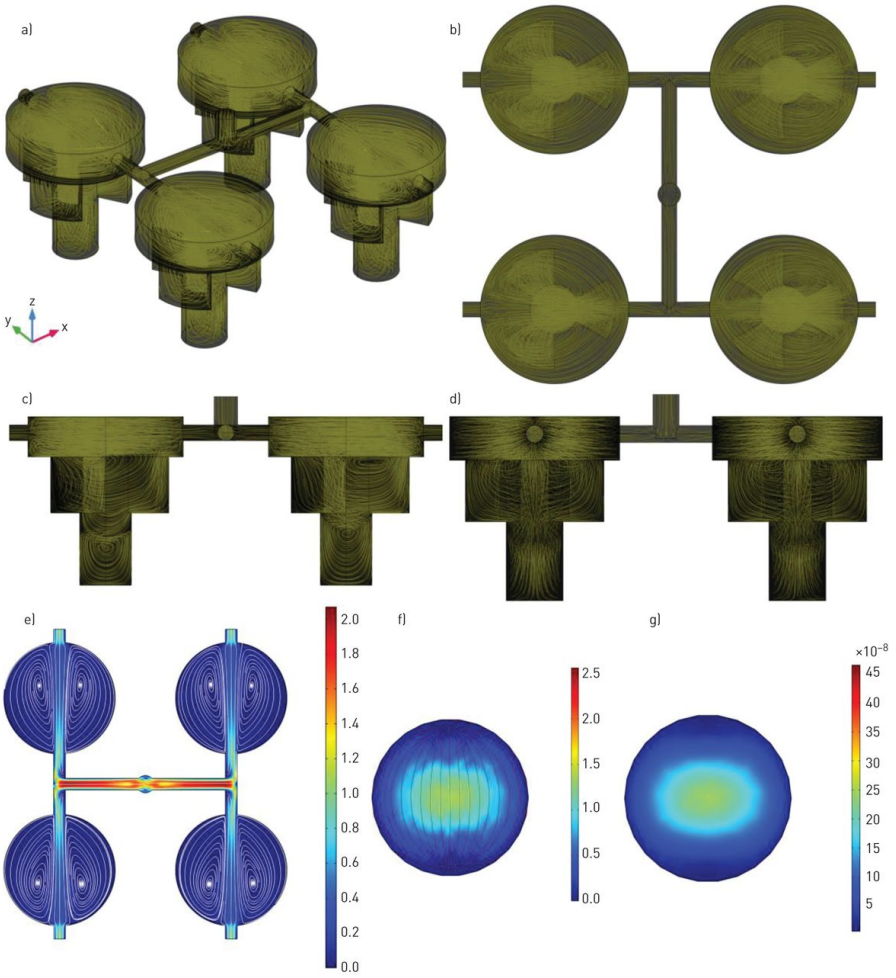

Figure 3. Quantitative simulation for *in vitro* exposure system (IVES) using COMSOL Multiphysics with air used as the gas of interest for simulation. a) A three-dimensional view of the IVES with air flow streamlines showing vortices in IVES and how gases distribute. **b)** Top view with gas flow streamlines. **c** and **d)** The side views with gas flow streamlines. **e)** The top view of the velocity profile (m·s−1) for the modeled gas presenting a uniform flow distribution among all four exposure chambers with a gentle velocity decrease. **f)** The velocity profile (µm·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.

Internal gas diffusion inside a chamber was quantified with CO2, a product of combustion, as the gas of interest with an assumption of 1 mol·L−1 at the inlet with normal diffusion in the air (CO2 as the gas of interest was assumed to be transported in air by diffusion and convection). For this part of the simulation, mass transport of CO2 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 modeling (the gas flow at the inlet was assumed to be 1.75 mL·s−1). The simulation was studied dependent on time, and the CO2 diffusion was modeled over 5 s, which was the time of exposure. It was assumed that the CO2 concentration at the outlet was zero (the boundary condition for CO2 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 CO2 in the chamber, Fick's law was used as described below:

$$\frac{\partial C_{CO_2}}{\partial t} = D_{CO_2}\left(\frac{\partial^2 C_{CO_2}}{\partial x^2} + \frac{\partial^2 C_{CO_2}}{\partial y^2} + \frac{\partial^2 C_{CO_2}}{\partial z^2}\right) \quad 3$$

where C_{CO_2} is the CO_2 concentration in the smoke exposure chamber and D_{CO_2} (D_{CO_2} =0.16 cm2·s−1) (106) is the diffusion coefficient of CO_2 molecules in the chamber.

The concentration distribution of CO2 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 CO2 concentration to become uniform in the entire chamber. 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.

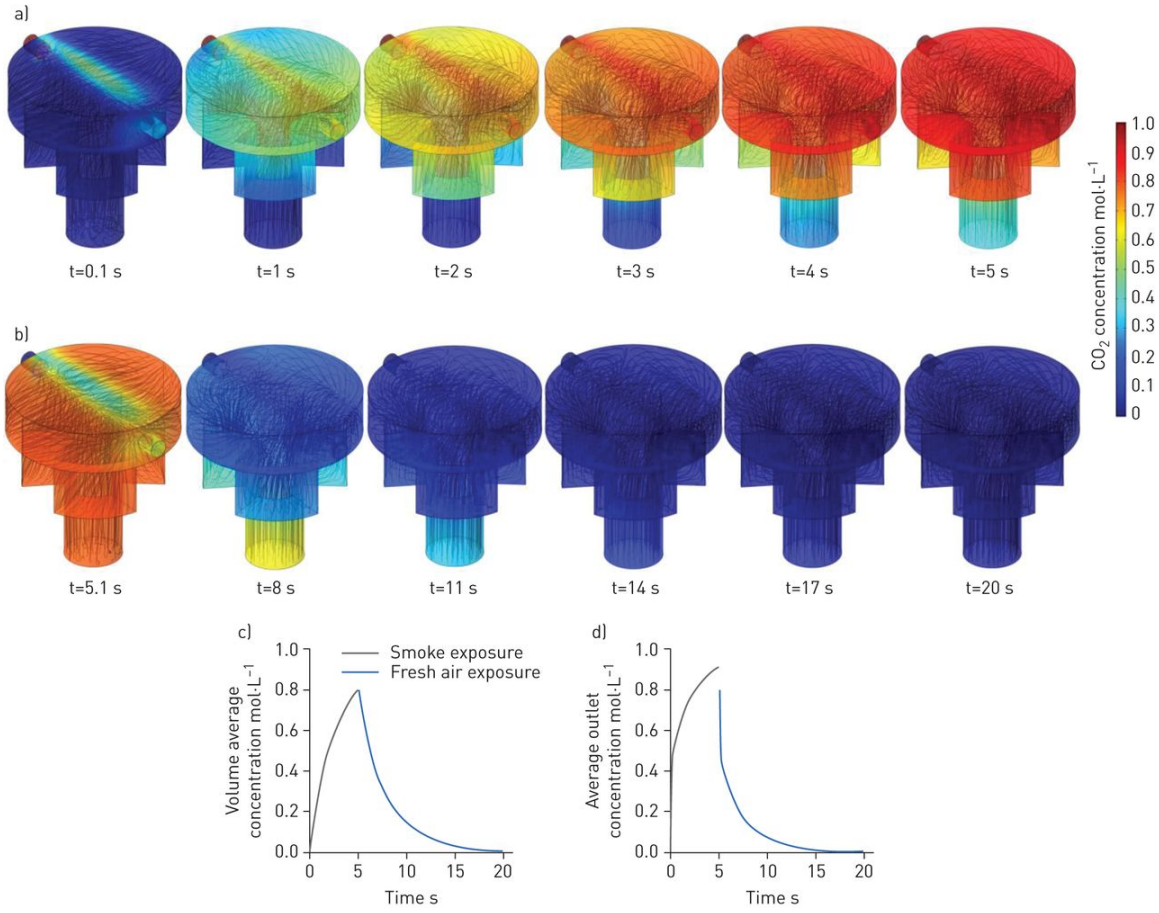

Figure 4. Three-dimensional quantitative modeling results of the gas (CO2) concentration distribution in an *in vitro* exposure system chamber. a) real-time CO2 concentration distribution over 5 s exposure of smoke (initial CO2 concentration modeled at 1.0 mol·L−1) showing a gentle gas distribution in 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).

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

Following quantitative modeling, 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**.

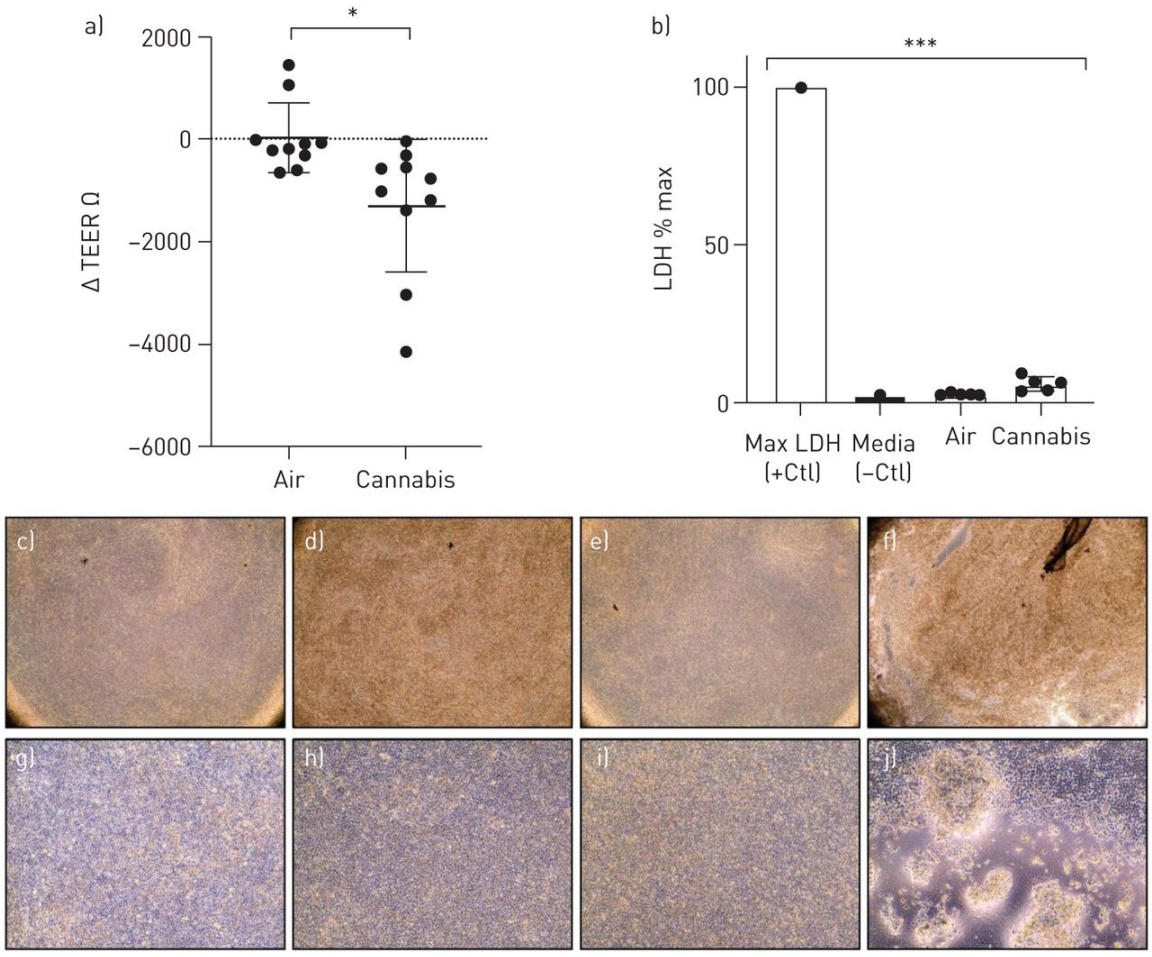

Figure 5. Epithelial barrier function, cell cytotoxicity, and morphology of HBECs after smoke exposure. a) Change in transepithelial electrical resistance (TEER) from baseline after room air *versus* whole cannabis smoke exposure. Analyzed with paired t-test, p=0.029, n=10. b) Lactate dehydrogenase (LDH) expression as a proportion of maximal LDH release, analyzed *via* ANOVA and Tukey's *post hoc* test. Shows representative microscopy (×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 (×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). *: $p<0.05$; ***: $p<0.001$.

3.3 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 (82–84). We therefore analyzed the differential expression of selected IL-1 cytokine family members, IL-1α, IL-1β, 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-1α ($p=0.054$) 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-1α, IL-18 and IL-1Ra were elevated in 5 of 5 samples (100%), whereas IL-1β ($p=0.296$) was elevated in 4 of 5 samples (80%) (**Figure 6**).

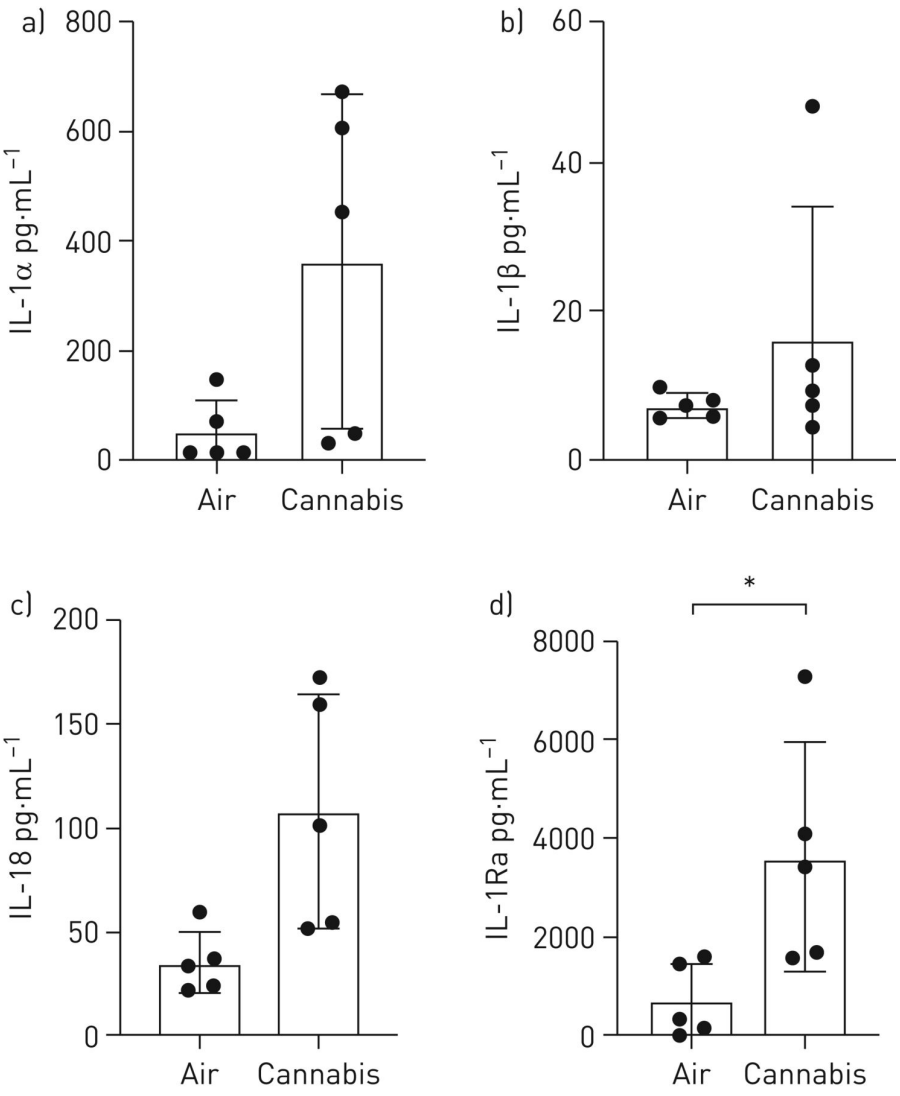

Figure 6. Interleukin IL-1 cytokine family member quantification in apical washing of primary human airway epithelial cells exposed to air or whole cannabis smoke. a) IL-1α, p=0.054; b) IL-1β, p=0.296; c) IL-18, p=0.064; and d) IL-1R antagonist (IL-1Ra), p<0.05 (analyzed *via* paired t-tests). *: p<0.05.

We have demonstrated that cannabis smoke extract suppresses CXCL10 and CCL5 in Calu-3 epithelial cells under submerged monolayer conditions (35). We therefore analyzed CXCL10 and CCL5 levels following cannabis smoke exposure in IVES with primary human airway epithelial cells. We observed no significant changes in both CXCL10 and CCL5 expression (**Figure 7**). On a donor basis 4 of 5 (80%) donor samples displayed suppression of CXCL10 ($p=0.110$) and 0 of 5 (0%) donor samples displayed suppression of CCL5 ($p=0.252$).

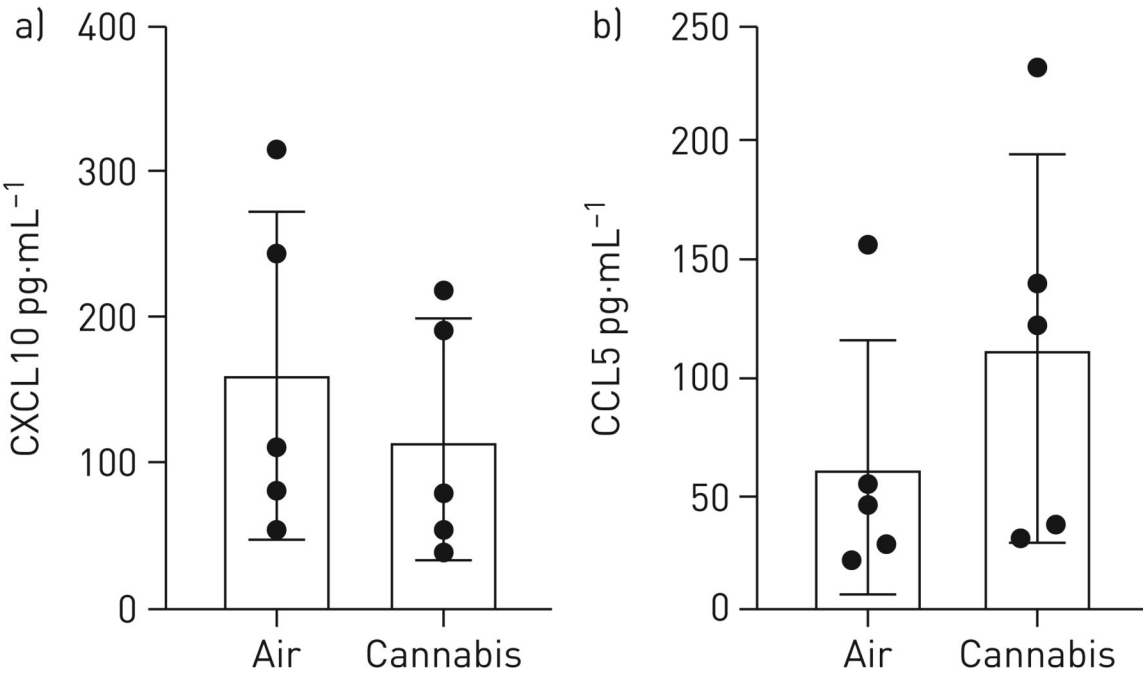

Figure 7. Antiviral cytokine quantification in apical washing of primary human airway epithelial cells exposed to air or whole cannabis smoke. a) CXCL10, p=0.110; and **b)** CCL5, p=0.252 (n=5, analyzed *via* paired t-tests).

3.4 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 (18, 35). 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 (107, 108). 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**).

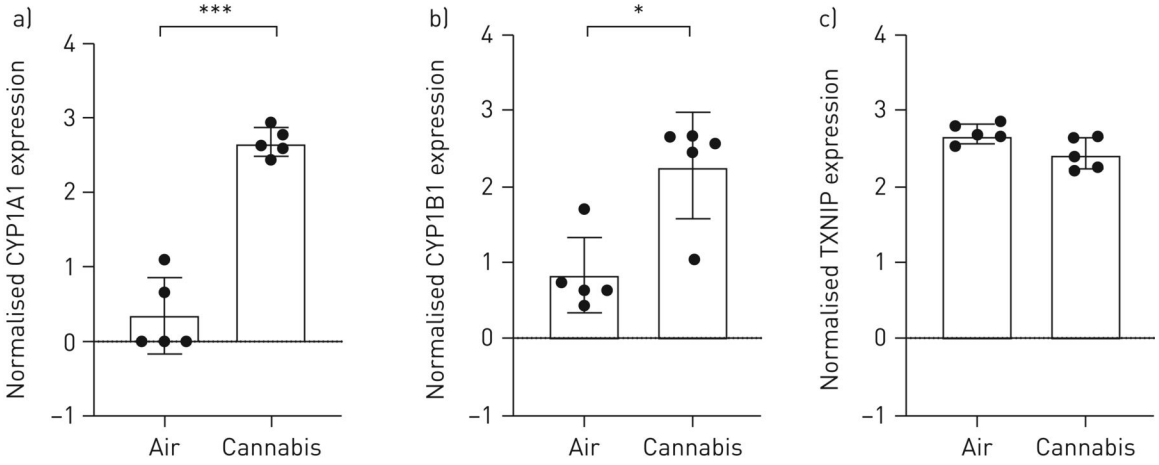

Figure 8. Selected oxidative stress genes expressed in primary human airway epithelial cells exposed to air or whole cannabis smoke. a) CYP1A1, p<0.001; **b)** CYP1B1, p<0.05; and **c)** TXNIP, p=0.058 (n=5, analyzed *via* paired t-tests). *: p<0.05; ***: p<0.001.

PART 2: Using IVES to the Elucidate Immunological Impact of Cannabis Smoke on the Airway Epithelium in the Context of Viral Mimetic Stimulation

3.5 Prior cannabis smoke exposure suppresses cytokines involved in antiviral responses to Poly I:C

In the previous section, we showed IVES to be a way to study cannabis smoke exposure *in vitro*. Following this, we aimed to apply our newly validated model in the context of cannabis smoke exposure and viral infections in primary HBECs. We exposed cell cultures at ALI to smoke from one cannabis cigarette (0.7g) smoked to completion. Two hours post-smoke exposure, we stimulated cells with Poly I:C (10 ug/ml) apically and basally for optimal stimulation. Twenty-four hours post stimulation, apical and basal media was collected and cytokines were quantified using a 42-plex cytokine array. Cells that were exposed to cannabis smoke prior to viral mimetic stimulation showed a suppression of key antiviral cytokines as compared to cells exposed to room air and stimulated with Poly I:C. In particular, antiviral cytokines such as CXCL10, TNFa, GM-CSF, IL-10, IL-6, IL-9, G-CSF, IL-7, and MIP-1β showed reduced expression in response to Poly I:C in the experimental group (**Figure 9**). These cytokines have been implicated in viral immunity in the airway epithelium and in recruitment of other immune cells to the site of infection (42-50). P-values for the significance of cytokines suppression by cannabis smoke exposure are given here: CXCL10 ($p=0.238$), TNFa ($p=0.091$), GM-CSF ($p=0.052$), IL-10 ($p=0.501$), IL-6 ($p=0.217$), IL-9 ($p=0.054$), G-CSF ($p=0.063$), IL-7 ($p=0.332$), and MIP-1β ($p=0.246$) (analyzed via ANOVA with Bonferroni correction).

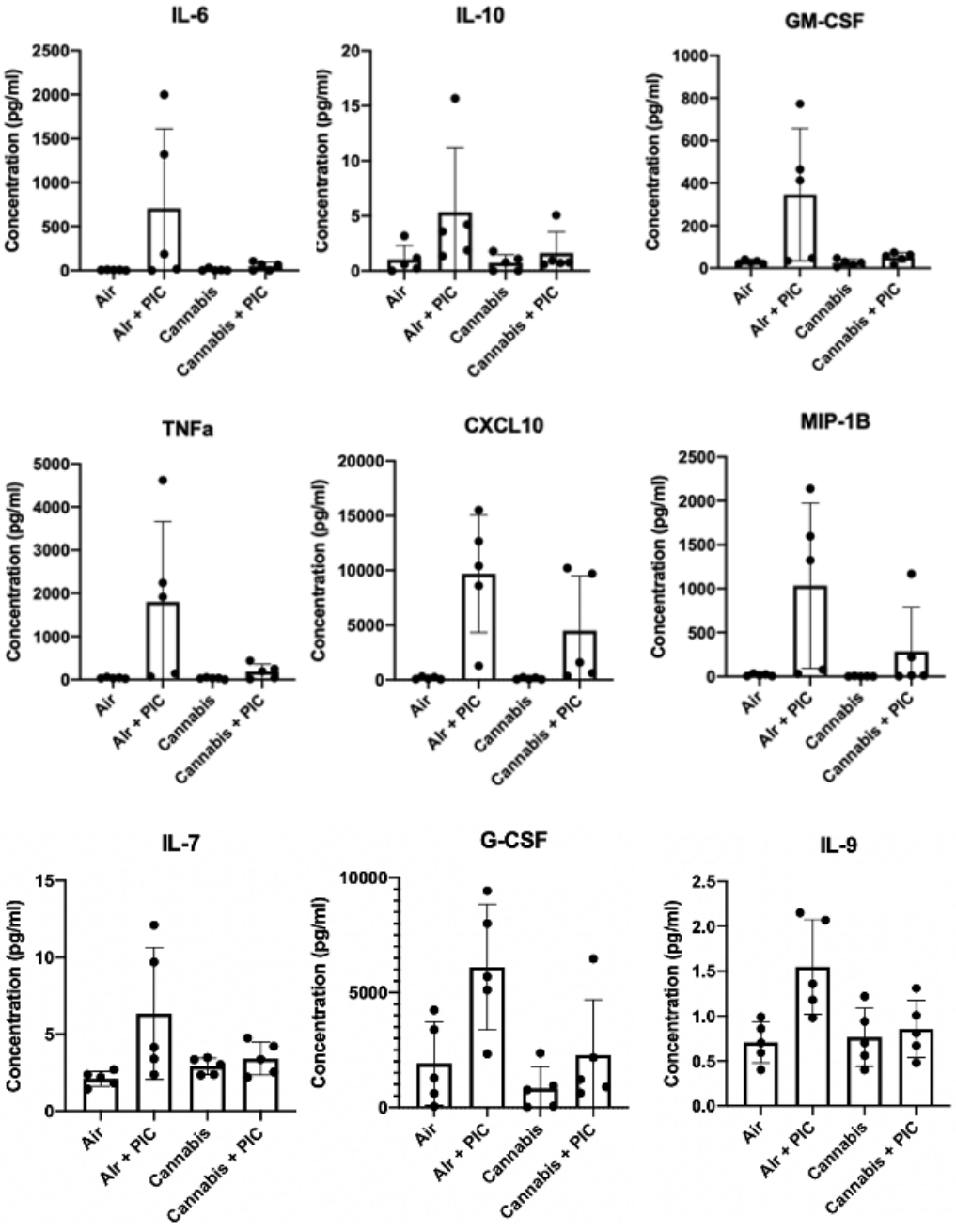

Figure 9. Antiviral cytokine expression in the apical media of primary human bronchial epithelial cells. Data is following cannabis smoke exposure from one cannabis cigarette (0.7g) smoked to completion and with viral mimetic stimulation two hours after smoke exposure. Analyzed via ANOVA with Bonferroni correction.

3.6 Epithelial barrier function was not further impaired by viral mimetic stimulation after cannabis exposure

Epithelial barrier function was tested through TEER measurements prior to smoke exposure and 24 hours following viral mimetic stimulation (**Figure 10**). Primary HBECs exposed to cannabis smoke and stimulated with Poly I:C showed a significant ($p=0.015$) impairment of epithelial barrier function compared to primary HBECs exposed to room air alone. Primary HBECs exposed to cannabis smoke experienced a decreased epithelial barrier function compared to primary HBECs exposed to room air ($p=0.055$). However, viral mimetic stimulation with Poly I:C alone did not significantly impair epithelial barrier function ($p=0.767$). In addition, primary HBECs exposed to cannabis smoke and later stimulated with Poly I:C also did not show significant impairment of epithelial barrier function ($p>0.999$) when compared to HBECs exposed to cannabis smoke alone. Our data suggests that acute cannabis smoke exposure prior to viral mimetic stimulation does not augment the barrier impairing effect of Poly I:C on the integrity of the airway epithelium. P-values were determined via an ANOVA and Bonferroni correction.

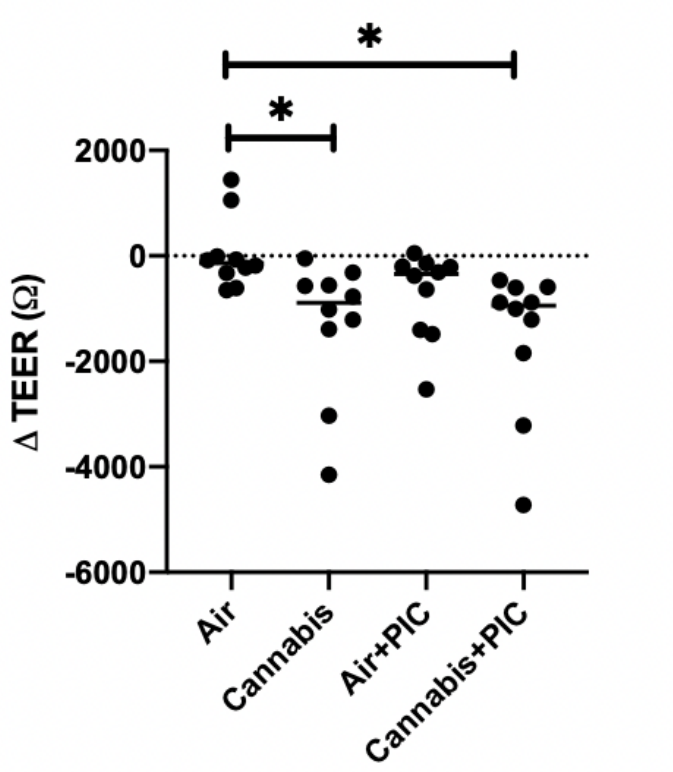

Figure 10. Transepithelial electrical resistance as a measure of epithelial barrier function in primary human bronchial epithelial cells. Data is following cannabis smoke exposure from one cannabis cigarette (0.7g) smoked to completion and with viral stimulation two hours after smoke exposure. Analyzed via ANOVA with Bonferroni correction.

PART 3: Understanding the Role of Dose-Response and Cell Culture Media Supplements on Immune Profiles to Improve IVES

3.7 Primary human bronchial epithelial cells displayed dose-dependent qualitative cell lifting

In PART 2, we utilized IVES to expose primary HBECs at ALI to cannabis smoke prior to assessing how this exposure would impact the cells' immune response and epithelial barrier functionality to Poly I:C. The experiments in PART 1 and PART 2 were conducted using 0.7g of cannabis product smoked to completion. Although this dose of smoke was directly comparable to research grade tobacco cigarettes of equivalent weight and commonly used in tobacco smoke research, qualitative assessment of the morphology of our primary HBEC cultures indicated that this dose was highly potent. Cells exposed to 0.7g of cannabis smoke showed rounded phenotyping along with lifting from the cell culture transwell insert membrane typical of apoptosis. To determine an optimized dose of cannabis smoke which would minimize cell lifting yet maintain an immunological effect, we performed a dose-response experiment (n=3) and assessed cell lifting qualitatively and IL-8 induction. HBECs were exposed to cannabis smoke from one cigarette of either 0.09g, 0.18g or 0.375g of cannabis product. Qualitatively, we found that the highest dose where cell lifting was not observed after cannabis smoke exposure was 0.18g (representative images provided in **Figure 11**). In addition, induction of IL-8 at this dose was comparable to IL-8 expression at 0.09g and 0.375g doses (**Figure 12**). Given this data, we suggest that 0.18g of cannabis product smoked to completion is the optimal dose which balances cell lifting with immune response in our model. Consequently, we use 0.18g of cannabis product for future experiments.

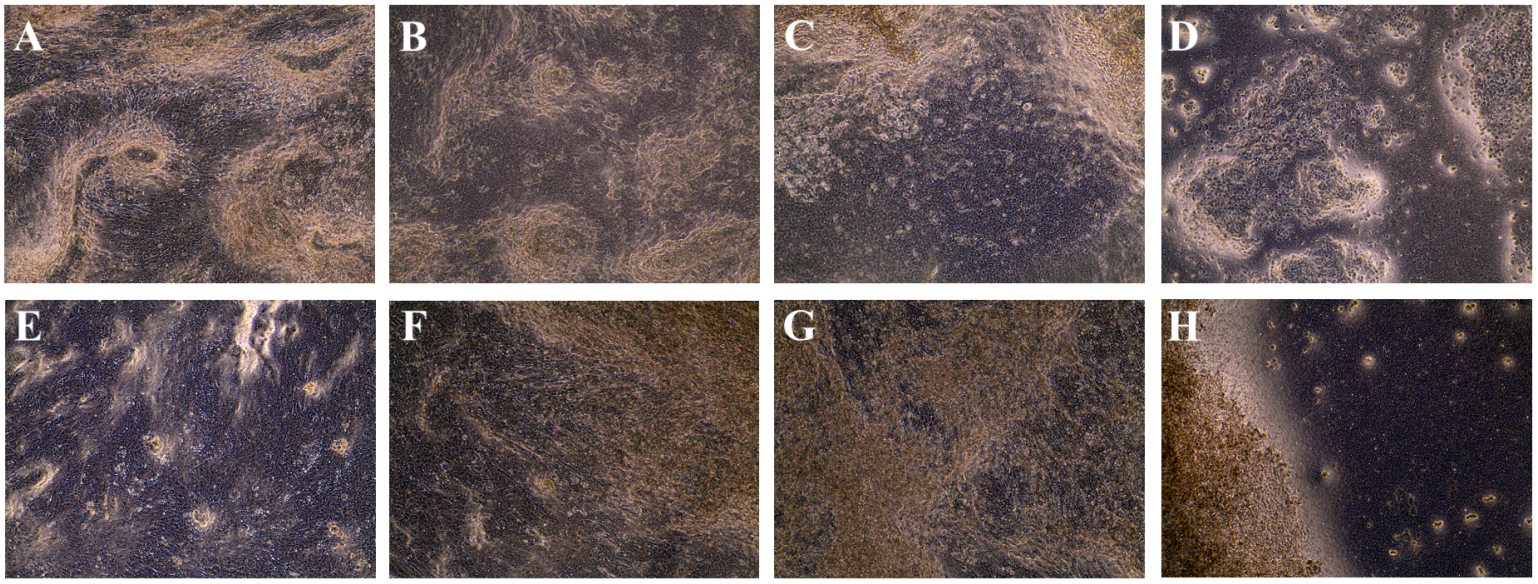

Figure 11. Post cannabis smoke exposure dose response microscopy at 10x magnification. a) Patient 1 - Control **b)** Patient 1 - 0.09g cannabis product **c)** Patient 1 - 0.18g cannabis product **d)** Patient 1 - 0.375g cannabis product **e)** Patient 2 - Control **f)** Patient 2 - 0.09g cannabis product **g)** Patient 2 - 0.18g cannabis product **h)** Patient 2 - 0.375g cannabis product. Each cigarette was smoked to completion.

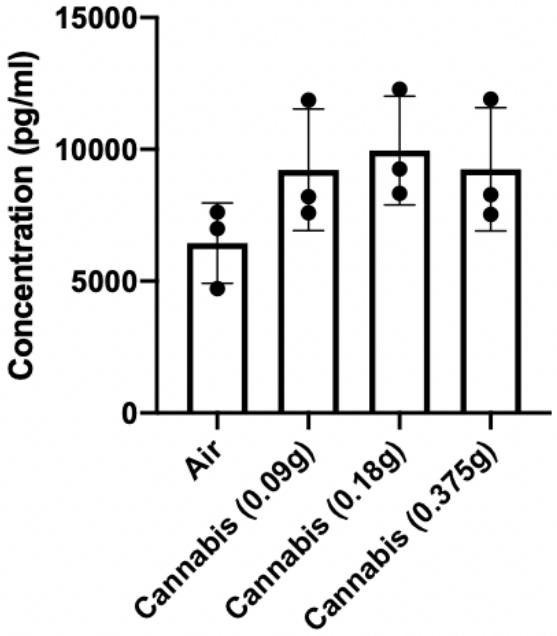

Figure 12. Post-cannabis smoke exposure IL-8 expression. Data is in the apical media of primary human bronchial epithelial cells (n=3) as a function of cannabis dose. Analyzed via ANOVA with Bonferroni correction.

3.8 Primary human bronchial epithelial cells in supplement-free media showed reduced expression of cannabis-induced IL-1 family inflammatory cytokines

As aforementioned, we assessed the impact of media supplements on HBEC immune profiles in our exposure model. HBECs (n=5) were either supplement-fed or supplement-starved 12 hours prior to and during the experiment. Cells were exposed to 0.18g of cannabis product smoked to completion and media was quantified for immune cytokines. Concentrations of IL-1 family cytokines - IL-1α, IL-1β, IL-18, and IL-1Ra - were measured in supplement-free cells and supplement-fed cells after exposure to cannabis smoke (**Figure 13**). IL-1α was significantly elevated in supplement-fed HBECs exposed to cannabis smoke compared to supplement-fed HBECs exposed to room air ($p=0.004$). IL-1α was not significantly elevated in supplement-free HBECs exposed to cannabis smoke compared to supplement-free HBECs exposed to room air ($p=0.606$). IL-1α was significantly elevated ($p=0.042$) in supplement-fed HBECs exposed to cannabis smoke compared to supplement-free HBECs exposed to cannabis smoke. IL-1β was elevated in supplement-fed HBECs exposed to cannabis smoke compared to supplement-fed HBECs exposed to room air ($p=0.099$). IL-1β was not significantly elevated in supplement-free HBECs exposed to cannabis smoke compared to supplement-free HBECs exposed to room air ($p=0.925$). IL-1β was elevated ($p=0.099$) in supplement-fed HBECs exposed to cannabis smoke compared to supplement-free HBECs exposed to cannabis smoke. IL-18 was elevated in supplement-fed HBECs exposed to cannabis smoke compared to supplement-fed HBECs exposed to room air ($p=0.083$). IL-18 was not significantly elevated in supplement-free HBECs exposed to cannabis smoke compared to supplement-free HBECs exposed to room air ($p=0.801$). IL-1Ra was significantly elevated in supplement-fed HBECs exposed to cannabis smoke compared to supplement-fed HBECs exposed to room air ($p<0.001$). IL-1Ra was not significantly elevated in supplement-free HBECs exposed to cannabis smoke compared to supplement-free HBECs exposed to room air ($p=0.109$).

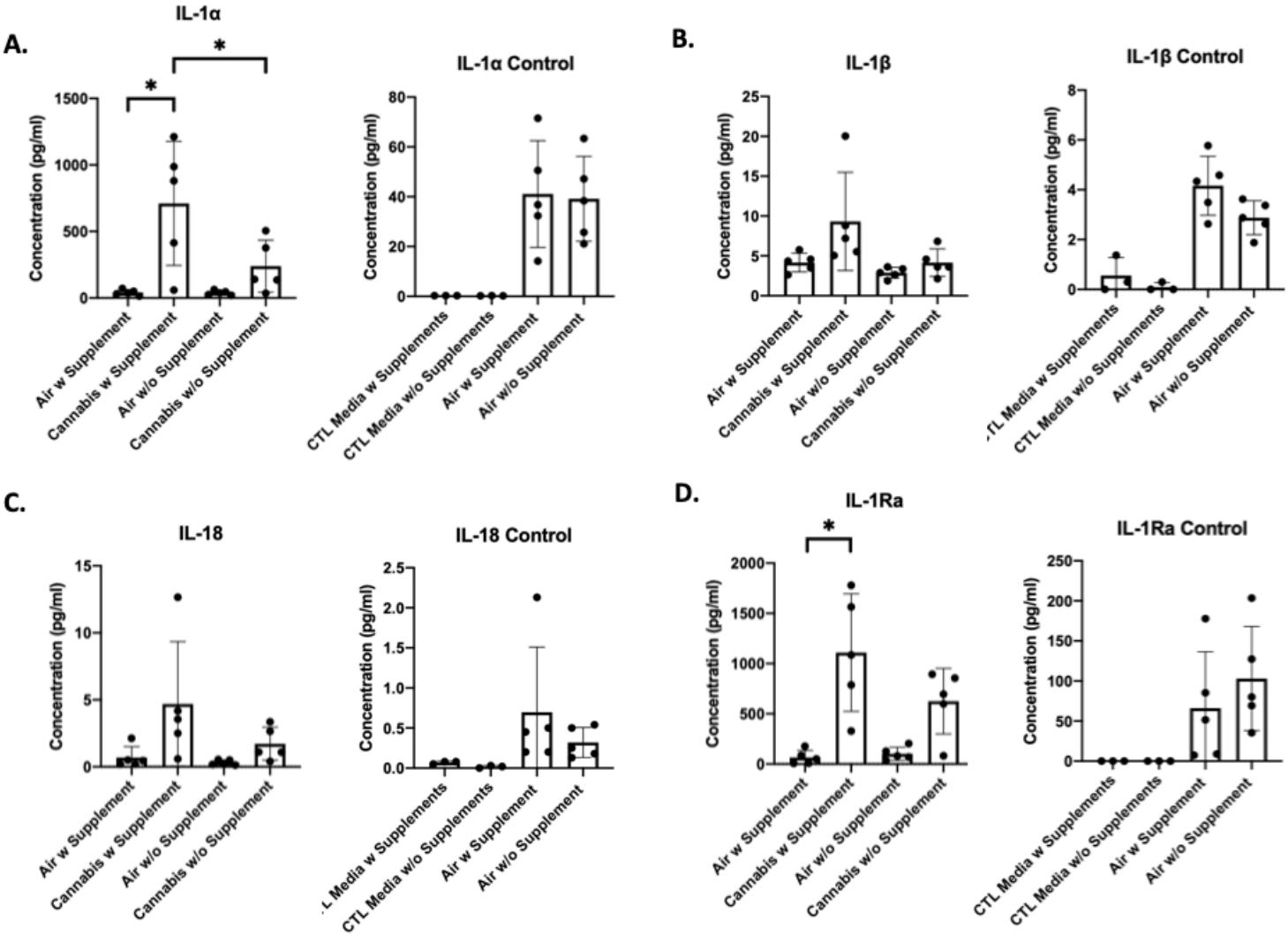

Figure 13. IL-1 family cytokines expression in the presence or absence of cell culture media supplements. Baseline expression in the presence and absence of cell culture is also shown as controls. **a)** IL-1α **b)** IL-1β **c)** IL-18 **d)** IL-1Ra. Analyzed via ANOVA with Bonferroni correction.

CHAPTER 4: DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSION

4.1 Discussion

The US Center for Disease Control declares that cigarette smoking is the leading preventable cause of death in the United States (109). Adding to the pressing concern is the relatively new increase in global legalization and practice of smoking cannabis (110-112). Notably, evidence suggests that cannabis smoke consists of differential components relative to tobacco smoke, which may function as immunomodulatory agents (59, 113, 114). 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 (115–119), we utilized 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 optimize 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 modeling 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 CO2 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 co-exposures 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 (22). Human airways rely on the latter, tight and adherens junctions between cells at the cells of the apical border, to prevent inhaled pathogens and other insults from causing harm to the airways (22). In smokers, the epithelium is found to be dysfunctional and abnormally differentiated, leaving a higher risk for viral and bacterial infection (22). 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 (120). Previous studies have implicated oxidative stress brought on by cigarette smoke with the disruption of epithelial barrier function (121). 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 (122-124). 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 suggests that cannabis smoke, similar to tobacco smoke, is able to compromise barrier function. Furthermore, the data establishes that monitoring epithelial cell barrier function measurements following exposures using IVES are possible.

Cytokines are signaling 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 signaling 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 (54, 60, 125–129). 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 (58). 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-1α, IL-1β and IL-18 expression, following cannabis smoke exposure relative to control, findings that are similar to observations made in other studies with tobacco smoke (82–84). These results indicate an inflammatory response induced by cannabis smoke characterized by IL-1 family cytokines, which may share downstream consequences with tobacco smoke that include neutrophilia driven by an IL-1R–IL-17 axis (130). 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 inhibiting IL-1α and IL-1β (131). In cannabis smoke however, IL-1Ra expression has been shown to be elevated owing to the unclear immunomodulatory features of cannabis active components (57, 132, 133).

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 defense 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 analyzed 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 (134–136). 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 (137–139). Consequently, we analyzed the expression of CYP1A1, CYP1B1 and TXNIP in primary human epithelial cells exposed to cannabis smoke using IVES. We chose these 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 modeling molecular changes in human airway epithelial cells exposed to whole cannabis smoke.

In a physiological context, the path that environmental air takes in the lungs can be separated into two functionally different regions - the conducting zone and the respiratory zone (22). Air that passes through the nostrils encounters the conduction zone of the lungs where turbulence and mucociliary action push the air into the nasal walls trapping allergens, other pollutants and pathogens in the mucus (22). Although mucus produced by goblet cells in epithelium and ciliary action together work to filter environmental air, pathogens trapped in the mucus can settle through the mucus and contact the airway epithelial cells that form the physical barrier between the outside environment and the living system (22). To avoid invasion by the pathogens that contact the epithelium, the proper regulation and rapid response of the immune defense system in HBECs are crucial. Resilience and susceptibility to environmental pathogens including allergens, pollutants, bacteria, and viruses are mediated in part by the integrity of the physical barrier between adjacent cells of the epithelium, antimicrobial peptides secreted by the epithelial cells, and by the immune cytokines that help to regulate and recruit a coordinated immunological defense against them (22). Disruption or dysregulation of these components would leave the living system prone to environmental insults including infections, (22). Our data in PART 1 has suggested that following cannabis smoke exposure, HBECs experienced a significant impairment in epithelial barrier function. We reasoned that this would render the epithelial barrier compromised and allow more viruses to circumvent the tight junctions between the cells. We hypothesized that this increased permeability to viral pathogens would in turn further impair barrier function through virus-induced injury. However, our data suggests that viral stimulation following cannabis smoke exposure did not further impact epithelial barrier function significantly. For a potential explanation, we turn to the mechanism by which Poly I:C enters the epithelial cell. Poly I:C induces an immune response in airway epithelial cells through TLR3-mediated pathways (140-148). TLR3 is majorly responsible for recognizing dsRNA oligonucleotide patterns (140-148). Poly I:C activation of this receptor has been implicated in a significant decrease in epithelial barrier function in airway epithelial cells through a protein kinase D-mediated breakdown of apical junctions in airway epithelial cells (140-148). However, tobacco cigarette smoke has been shown to inhibit activation of TLR3 by disrupting TLR3 cleavage (140-148). Consequently, it is possible that cannabis smoke similarly disrupted TLR3 cleavage and as a result, prevented Poly I:C activation of TLR3 (140-148). In this case, downstream activation of protein kinase D would have been disrupted and resulting disassembly of apical junctions in the HBECs would not have occurred as intensely (140-148). This is a possible explanation for why co-exposure to cannabis smoke and Poly I:C did not impair epithelial barrier function significantly more than cannabis smoke exposure alone.

In addition to disruption of the epithelial barrier, viral pathogens are met with cascades of immunological defenses in the form of various immune cells, cytokines and

chemokines that induce inflammation and antiviral pathways (146). Adaptive immune cascades involving T-cell and B-cell recruitment rely on well-regulated and early innate immune responses in the airway epithelium (144, 146). Disrupting the early non-specific, innate immune responses may delay or disrupt the recruitment of more aggressive adaptive immune functionality (142, 144, 146). Our data in PART 2 suggests that prior exposure to cannabis smoke suppresses key antiviral cytokines - CXCL10, TNFa, GM-CSF, IL-10, IL-6, IL-9, G-CSF, IL-7, and MIP-1β - in response to Poly I:C, which have been implicated in resilience and susceptibility to viral infections. Specifically, IL-9 and G-CSF showed significantly reduced expression in response to Poly I:C following cannabis smoke exposure. These immune markers have been shown to be crucial in certain forms of viral disease. For example, G-CSF in the airway epithelium has been shown to prolong and increase the survival of bloodborne neutrophils which, in turn, have been shown to be crucial for recovery from severe influenza infections (148). As our data suggests that these cytokines are downregulated following cannabis smoke exposure, it follows that cannabis smoke exposure may increase the susceptibility and severity of respiratory viral infections. A potential mechanism by which this antiviral suppression occurs may be through inhibition of the TLR3 receptor which is responsible for activating transcription factors IRF3/7, NF-kB, and activator protein 1 (91, 140, 141). These in turn regulate the production of type 1 IFNs, proinflammatory cytokines and chemokines, respectively (91, 140, 141). Tobacco cigarette smoke has been shown to inhibit TLR3 activation by disrupting TLR3 cleavage (142). Therefore, it is likely that cannabis smoke has a similar effect on TLR3 inhibition. Whether these effects are transient with cannabis smoke exposure or prolonged remains to be elucidated and would inform usage of cannabis product in the context of viral pathogens, pandemics, and respiratory immunopathology.

As aforementioned, in PART 1 and 2, HBECs in our cannabis exposure experiments were exposed to 0.7g of cannabis product smoked to completion. Due to its only recent legalization, cannabis cigarettes for the purpose of research and development have not been standardized. Therefore, a standardized cannabis cigarette, analogous to the Kentucky research grade tobacco cigarette (0.7g of tobacco product) for research purposes, does not exist and has not been well-defined. However, we saw it reasonable to perform our experiments by standardizing our doses by comparable weight of the active product to the Kentucky research grade tobacco cigarette. This dosage was only meant to serve as a starting point for analysis and experimentation. We recognize that multiple factors may influence whether this dosage was optimal for our model of cannabis smoke exposure. Qualitatively, we noted that there were differences in the number of Foltin Puff cycles needed for completion of the cigarette, the combustibility of the cigarette, and phenotype of smoke (thick or thin white smoke) which were dependent on the experimenter who rolled the cigarette, the level of tightness cannabis product upon packing in the cigarette, and moisture content of the product. Future studies should aim

to standardize cannabis cigarettes so that cannabis exposure can be well-defined in *in vitro* models. In addition, despite LDH assay data suggesting that cell cytotoxicity in HBECs exposed to cannabis smoke was insignificant, qualitative assessment of cell cultures following cannabis smoke exposure showed that portions of the cell population lifted off the Transwell® culture insert. Therefore, to determine the optimal dose of cannabis smoke that would reconcile between cell lifting and inducing an optimal immune response in HBECs, we performed a dose-response analysis. Three doses of 0.09g, 0.18g, and 0.375g of cannabis product were smoked to completion. HBECs showed a dose-dependent increase in cell lifting. Qualitatively, 0.18g of cannabis product was determined to be the maximum dose at which no cell lifting was observed 24 hours post-exposure. However, 0.18g of cannabis smoke still induced an immune response as measured by IL-8 expression in HBECs. IL-8 was chosen as a metric for immune response to cannabis smoke in this experiment as it has been shown to be elevated in human bronchial epithelial cells after exposure to cigarette smoke (149). There was no significant difference between IL-8 expression between 0.18g and 0.375g of cannabis product. As a result, we suggest that 0.18g of cannabis product is the optimal dose for our exposure system.

In addition to identifying the optimal dose of cannabis smoke for our exposure system, we recognized the role cell culture media constituents may have on the immunology of HBECs (93, 150-154). Cell culture media supplement formulation methods and constituents are often proprietary and therefore, are difficult to assess rigorously. However, it has previously been identified that the nature of cell culture media can impact the proliferation, expansion, and accuracy of function in *in vivo* systems of cells (150-154). Moreover, cell culture media and its micronutrients contained within have been shown to impact immunity (150-154). As a result, we assessed the impact of supplement-free and supplement-fed cell media culture in the context of cannabis smoke to determine if it played a role in our system. In response to 0.18g of cannabis smoke, HBECs in the presence of supplements showed an increase in IL-1 family cytokines - IL-1α, IL-1β, IL-18, and IL-1Ra - as expected. These cytokines have been implicated in acute inflammatory processes in the innate immune response. However, HBECs in the absence of supplements showed a reduced sensitivity to cannabis smoke. IL-1 family cytokines expression was impaired. In particular, HBECs showed a statistically significant suppression of IL-1α in response to cannabis smoke as compared to in the presence of supplements in the culture media. Taken together, this data suggests that the impact of cell culture media constituents on immune profiles in HBECs cannot be ignored and future studies must be conducted to define their immune impact in HBECs, determine their clinical relevance, and increase the accuracy of our cannabis exposure model.

4.2 Limitations of Study

Firstly, whether the reduced TEER in cell cultures exposed to cannabis smoke was due to impairment of cellular junctions, cultures lifting off the surface of the insert due to a highly potent dose (0.7g) of cannabis smoke, or both, remains a limitation of our data. Secondly, we have performed these experiments using a diseased patient demographic and not healthy airway epithelial cells. Whether or not disease state impacts the immune function of airway epithelial cells in response to cannabis smoke remains to be elucidated. This present study does not focus on the specific immunological impact of cannabis smoke on airway epithelial cells and instead, focuses on introducing and applying a novel method to study cannabis smoke *in vitro*. Therefore, we justify the use of disease state cells in this way.

4.3 Future Directions

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 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 standardized product that has 0.7 g per cigarette. We recognize 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, co-exposure 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 addition, the experiments outlined in this present study were conducted using a THC-dominant strain of cannabis smoke. Whether the strain, and thereby the composition of active compounds, differentially impacts

immune profiles in HBECs is yet to be determined. Therefore, future studies should consider using *IVES* to assess the impact of THC-dominant, CBD-dominant, and THC/CBD-dominant strains of cannabis smoke on immune profiles in the context of antiviral immunity. Finally, our model is easily amenable to other routes of administrations of cannabis such as vape. Future studies intending to assess whether immunological differences exist between cannabis smoking and cannabis vaping can utilize IVES to investigate.

4.4 Conclusion

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. In addition, we have applied IVES to assess the immunological impact of prior cannabis smoke exposure on antiviral immunity. 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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