CANNABIS SMOKE AND RESPIRATORY IMMUNOMODULATION

INVESTIGATING THE *IN VIVO* EFFECTS OF CANNABIS SMOKE EXPOSURE ON LUNG INNATE IMMUNITY

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LAY ABSTRACT

Cannabis is widely used for recreational and medical purposes. Smoking is the most popular method to consume the drug among users. However, little is understood about the effects of cannabis smoke on lung health, despite evidence suggesting that it may lead to negative health outcomes. To address this gap in knowledge, we developed two unique mouse models of cannabis smoke exposure. Using these models, we explored the effects of cannabis smoke on lung immune responses in healthy and influenza infected mice. Our findings suggested that lung immunity is altered following cannabis smoke exposure. Additionally, we found that overall health was worsened during influenza infection in cannabis smoke-exposed mice. This effect was associated with weakened viral immunity in the lungs. The models we developed and the findings using it thus far create the foundation for future studies on cannabis smoke and lung health.

ABSTRACT

Cannabis is widely used for recreational and medicinal purposes. Inhalation of cannabis smoke is the predominant method of drug consumption, exposing the lungs to THC and CBD, as well as a plethora of toxic combustion products. Clinical observations suggest that cannabis smoking contributes to the development of respiratory symptoms and may play a role in the pathogenesis of inflammatory lung disease. However, the association between cannabis smoke, dysregulated pulmonary immunity, and the development of lung disease is inconclusive. To improve our understanding of this relationship, we developed novel mouse models to investigate the effect of cannabis smoke exposure on lung immunity.

Using compositionally relevant cannabis strains, we established a mouse model of cannabis smoke exposure and validated that it delivers cannabis smoke by measuring cannabis smoke-associated metabolites in the blood. In our initial lung immune characterization, we demonstrated that acute cannabis smoke exposure induces modest changes to innate immune cellularity in the airways and lung tissue. Specifically, lung macrophage subpopulations were proportionally altered following smoke exposure. As well, we demonstrated that lung disease-associated mediators, including MDC, TARC, and VEGF, were dysregulated in cannabis smoke-exposed lung tissue.

In addition to our initial characterization, we established a first-of-its-kind concurrent cannabis smoke exposure and influenza infection model. Using this model, we demonstrated that cannabis smoke exposure exacerbates weight loss following influenza infection. These increases in weight loss corresponded with dysregulated cellular responses and immune mediator expression. Cell types involved in early innate immune signaling, such as macrophages and dendritic cells, were significantly affected by concurrent exposure and infection. Additionally, anti-viral mediators. including IFNγ, IP-10, RANTES, and TNFα, were decreased in cannabis smoke-exposed, infected lung tissue.

Collectively, we defined two novel models of cannabis smoke exposure that can be leveraged in future investigations on the inflammatory effects and associated health outcomes of cannabis smoke.

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Lastly, I would like to acknowledge the mice who gave their lives in my study and others like it. Humanity owes a great debt to those animals whose existence were defined by a sacrifice in which they had no part in deciding. While I dream of a day where we progress beyond the need to impart the worst aspects of human health on undeserving recipients, for now, I remain eternally grateful for all that they have done for us.

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LIST OF ABBREVIATIONS AND SYMBOLS

A/FM/1/47-MA	Influenza A/Fort Monmouth/1/1947-mouse adapted
ACK	Ammonium-chloride-potassium lysing buffer
AM	Alveolar macrophage
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
CB_1	Cannabinoid receptor type 1
CB_2	Cannabinoid receptor type 2
CBD	Cannabidiol
COHb	Carboxyhemoglobin
COPD	Chronic obstructive pulmonary disease
cRPMI	Complete RPMI media
CS	Cannabis smoke
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DEAE	Diethylethanolamine
DMEM	Dulbecco's modified eagle medium
dpi	Days post infection
EDTA	Ethylenediaminetetraacetic acid
FEV_1	Forced expiratory volume, 1 second
FVC	Forced vital capacity
GABA	Gamma-aminobutyric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I.N.	Intranasal
IAV	Influenza A virus
IFNβ-1	Interferon beta 1
IFNγ	Interferon gamma
IL	Interleukin
IM	Interstitial macrophage
IM1	Interstitial macrophage 1
IM2	Interstitial macrophage 2
IM3	Interstitial macrophage 3
MCP-5 / CCL12	Chemokine (C-C motif) ligand 12
MDC / CCL22	Chemokine (C-C motif) ligand 22
MDCK	Madin-Darby canine kidney cells
MEM	Minimum essential media
MIP-2 / CXCL2	Chemokine (C-X-C motif) ligand 2

MIP-3β / CCL19	Chemokine (C-C motif) ligand 19
MNC	Mononuclear cells
Mo-AM	Monocyte-derived alveolar macrophage
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PMC	Polymorphonuclear cells
RA	Room air
RANTES / CCL5	Chemokine (C-C motif) ligand 5
Res-AM	Tissue-resident alveolar macrophage
SEM	Standard error of the mean
TARC / CCL17	Chemokine (C-C motif) ligand 17
TCN	Total cell number
THC	Delta-9-tetrahydrocannabinol
THC-COOH	11-Nor-9-carboxy-delta-9-tetrahydrocannabinol
TNFα	Tumour necrosis factor
TPCK	Tosyl phenylalanyl chloromethyl ketone
TPM	Total particulate matter
VEGF	Vascular endothelial growth factor
α	Alpha
β	Beta
γ	Gamma

DECLARATION OF ACADEMIC ACHIEVEMENT

The research presented in this thesis are the cumulative works of myself and various colleagues. I significantly contributed to all aspects of this thesis, including project conceptualization, experimental design, sample collection and processing, data interpretation, and knowledge sharing under the supervision of Dr. Jeremy Hirota and Dr. Martin Stampfli. Directly supporting this work conceptually and technically were the members of the Stampfli laboratory – Joshua McGrath, Steven Cass, Danya Thayaparan, Peipei Wang, and Joanna Kasinska – who assisted with animal work, sample collection and processing, and data interpretation. Blood metabolite analysis was performed by Dr. Stephen Hill from the McMaster University Medical Centre Core Laboratory as well as Dr. Tracey Campbell and Nicola Henriquez from the McMaster University Centre for Microbial Chemical Biology. Dr. Sam Afkhami from the McMaster Immunology Research Centre performed influenza administrations and provided expertise. Bioinformatic analysis was performed by Jennifer Aguiar and Dr. Andrew Doxey from the University of Waterloo.

The research presented in this thesis has been prepared into two manuscripts for publication in which I hold primary authorship. The first, titled “Development and validation of a mouse model of contemporary cannabis smoke exposure” has been accepted for publication in European Respiratory Journal Open Research. The second, tentatively titled “Cannabis smoke exposure modulates respiratory immunity and suppresses immune responses to influenza infection in mice”, is currently being prepared for submission. In addition, insight from this project informed the preparation of an auxiliary manuscript titled “Expression of endocannabinoid system components in human airway epithelial cells: impact of sex and chronic respiratory disease status”, which has been published in European Respiratory Journal Open Research. I share primary authorship on this publication with Jennifer Aguiar from the University of Waterloo.

CHAPTER 1: INTRODUCTION

1.1. Cannabis

Cannabis (*Cannabis sativa, Cannabis indica)* is a mood-altering drug that is widely used for its psychoactive and remedial effects. It is the most consumed drug worldwide with an estimated 192 million users – a number which is expected to increase due to trends in recreational legalization as well as innovations in medical applications[1]. The drug is comprised of over 400 biologically active compounds with ~25% of these being cannabinoids, most notably delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD)[2]. These constituents are thought to play distinct roles in a variety of physiological processes including neurocognition, appetite stimulation, pain alleviation, and immunity[3–8]. While cannabis can be consumed in a variety of ways, the most common route of administration is inhalation via the combustion of cannabis cigarettes[9,10]. Research efforts exploring the effects of cannabis use often center around the therapeutic potential of the drug, emphasizing the medicinal properties of specific cannabinoids. However, research which delineates the potential adverse effects of cannabis is insufficient and does not adequately inform users of potential health consequences, particularly those associated with cannabis smoke exposure. Given the growing popularity of cannabis, it is imperative to fully understand the implications of its use on health and disease, as it becomes more prevalent among recreational and medicinal users.

1.1.1. Physiological Effects and Cannabinoids

Cannabis has a wide variety of effects on nearly every organ system in the body. The recreational popularity of cannabis is driven largely by the psychoactive effects of the drug[11]. The characteristic euphoria or "high" induced by cannabis consumption comes with feelings of intoxication, as well as decreased anxiety, tension, depression, and alertness, and increased sociability, creativity, mindfulness, and relaxation[8]. As well, cannabis can induce perceptual changes that can make visual, audio, and emotional stimuli more intense or enjoyable. Cannabis also impairs cognitive and psychomotor functions, leading to deficiencies in short-term memory, concentration, reaction time, and motor coordination[12]. While the psychotropic nature of cannabis makes it attractive recreationally, the analgesic, sedative, and anxiolytic effects of the drug encourage its use as a clinician- or self-prescribed therapeutic for body pain, insomnia, and mental health disorders, among others. However, despite these effects being well documented by users, the exact

relationship between cannabis use and these perceived remedial outcomes is not well understood. Current prevailing thought in the field is that specific cannabinoids drive the different medicinal properties associated with cannabis use and that strain composition determines the strength of these effects[13]. Among the dozens of cannabinoids which have been identified, THC and CBD are both the most prevalent and well understood constituents, yet more research is necessary to fully understand their roles in the physiological effects of cannabis.

THC is the most abundant cannabinoid present in cannabis and has been the principal focus of research since its identification in 1964[14]. It is the primary constituent that drives the psychoactive effect of cannabis consumption[15]. Investigations have shown that THC causes its psychoactive effects by binding to cannabinoid receptor type 1 (CB_1) receptors in the central nervous system, but also exerts effects by binding CB_1 receptors in the lungs, liver, kidneys, and sparsely throughout other organs[16]. Evidence suggests that CB_1 receptors modulate the release of neurotransmitters such as dopamine, glutamate, and gamma-aminobutyric acid to alleviate overactive neuronal responses to stimuli such as pain and other inflammatory processes[17]. The binding and subsequent activation of these receptors in the brain by THC has been reported to generate a reduction of negative traits such as anxiety and impulsivity, while increasing feelings of euphoria and happiness[18,19]. CB_1 receptors found in other tissues have been reported to reduce feelings of pain and irritation upon activation, such as in the gastrointestinal system[20]. THC also activates cannabinoid receptor type 2 (CB_2) receptors, which are primarily found on leukocytes and in the spleen, but are expressed all throughout the body[21]. The activation of CB_2 receptors on leukocytes has been implicated to have a variety of immunosuppressive and anti-inflammatory effects. The binding of CB_2 receptors by exogenous agonists, such as THC, has been shown to downregulate cytokine release, reactive oxygen species production, along with immune cell migration and recruitment[22]. As well, studies using CB_2 deletion models have demonstrated increased cellular infiltration, particularly by neutrophils and macrophages, increased inflammatory cytokine production, and exacerbated tissue damage upon inflammatory stimulation[23]. Taken together, the evidence suggests that THC does not only modulate psychotropic processes but may also have profound effects pertaining to pain and inflammation which could contribute to its therapeutic potential.

CBD is the second most abundant cannabinoid in cannabis and has received particular attention for its proposed medicinal value. While it is considered a primary cannabinoid, CBD does not induce psychoactive effects, even at high

concentrations. Instead, the popularity of CBD as a potential treatment is due to its perceived remedial benefit by users without any of the intoxicating effects of typical cannabis use. However, whether CBD has therapeutic effects and the mechanism in which these effects are induced is not yet fully elucidated. CBD does not have a strong affinity for CB_1 or CB_2 receptors, though it still affects neurotransmitter modulation and receptor activation[24]. Current research suggests that CBD functions as an allosteric modulator of receptors in the endocannabinoid system, leading to altered cannabinoid binding[25]. As well, CBD has been shown to be a cannabinoid reuptake inhibitor, leading to the accumulation of cannabinoids in the brain and other tissues[26]. Consequently, CBD is proposed to modulate analgesic and anxiolytic processes by facilitating enhanced or inhibited endo- and exogenous cannabinoid effector function[27]. While the current understanding of CBD supports its use as a potential therapeutic, further research is necessary to assess the effectiveness and potential drawbacks of the compound.

1.1.2. Prevalence

Cannabis is currently the most popular drug in the world, with an estimated 192 million recreational and medicinal users worldwide according to the United Nations' World Drug Report released in 2017[1]. North America and Europe alone are thought to consume 35% of all cannabis sold in the world, with most of this use being considered recreational and/or illicit. The largest demographic are between the ages of twenty one and thirty five and are considered to be lower middle class[28]. Medicinal cannabis use has grown in prevalence in recent years, as clinical observations and individual experiences support the potential remedial effect of the drug on diseases such as multiple sclerosis, cancer, arthritis, inflammatory bowel syndrome, insomnia, and depression[29]. Medicinal cannabis usage makes up 17% of all cannabis consumption in the US, with this proportion expected to grow as stigma erodes and therapeutic applications become more available and better understood[30]. While some medicinal users consult clinicians, most users self-prescribe cannabis to treat ailments without professional advice. These individuals, combined with recreational users, represent the vast majority of all cannabis users which is of particular concern, given that they may not be adequately informed about the risks of cannabis consumption.

1.1.3. Routes of Administration

Cannabis can be consumed in a variety of ways with different effects based on the route of administration. Cannabis can be consumed via inhalation by burning the dried flower in a cigarette, through a hand pipe, or through a water pipe. It can

also be consumed via ingestion through its incorporation in a variety of food and drink products. Recent trends among cannabis consumers involve the vaporization of oils made from cannabis products and inhalation using a vape or similar tool. Consuming cannabis through combustion and subsequent inhalation is the most popular recreational mode of use in North America[10]. 90% of cannabis consumers report using combustion methods, with users stating that their most common combustion method was via cannabis cigarettes or hand pipe[9]. Vaporizing cannabis products has become more prevalent among cannabis users, potentially due to the general association between smoking and adverse health outcomes. Studies have suggested that the growing prevalence of 'cannavaping' is due to a belief that vaping is a safer alternative to combustion methods, but this rationale remains to be fully supported[31]. Despite this sentiment, vaping remains less prevalent, as high cost, reduced availability, enjoyment of smoking, and public stigma exist as barriers to entry[32]. Inhalation methods such as combustion and vaporization are popular due to the predictability and fast onset of psychoactive effects, a benefit that is drastically reduced by other methods of consumption such as ingestion[33]. As such, inhalation will likely continue to be the predominant way that cannabis is consumed recreationally, and the barriers to vaporizing leaves traditional combustion methods as the most popular inhalation-based approach for the foreseeable future.

1.2.Cannabis Smoke and Respiratory Health

Inhalation of combusted cannabis smoke is the most common method of drug consumption. Smoking results in the quickest delivery of the biologically active components of cannabis, making it the most attractive method for consuming the drug. However, smoke inhalation directly exposes the lungs to harmful chemical compounds and combustion by-products which can have detrimental affects on respiratory health. Much like the smoke of tobacco cigarettes, cannabis smoke contains a myriad of different lung irritants, mutagens, and carcinogens, including tar, ammonia, hydrogen cyanide, and nitrogen oxide[34]. As well, the incomplete combustion of plant matter, such as cannabis, leads to the production and subsequent inhalation of carbon black, a known instigator of lung disease[35]. Further exacerbating exposure to these compounds are the behavioural differences in how cannabis is typically smoked. Cannabis smokers typically inhale more deeply and hold the smoke within their lungs for longer than tobacco smokers[8]. Additionally, cannabis smokers typically do not use professionally manufactured filters in their cigarettes. Consequently, smoking a cannabis cigarette has been shown to result in a three-fold increase in inhaled tar relative to a tobacco cigarette[36]. Given these differences, it is estimated that smoking three cannabis

cigarettes a day is equivalent to smoking twenty tobacco cigarettes in terms of exposure to harmful combustion by-products. Thus, the risk of acute and chronic respiratory distress as a result of cannabis smoking is exceptionally high.

1.2.1. Pulmonary Symptomology

Chronic cannabis smoke exposure has been associated with increased respiratory symptoms and altered lung function. Cannabis smokers report increased incidence of cough, sputum production, shortness of breath, wheezing, and chest tightness[37–39]. The prevalence of cough, sputum production, and wheezing has been shown to be reduced in those who quit cannabis smoking, but shortness of breath remained prevalent[40]. Lung function is typically measured via spirometry by assessing the ratio of forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC)[41]. Individuals with progressive lung diseases, such as chronic obstructive pulmonary disease (COPD), are characterized by reduced FEV_1/FVC ratios, largely driven by decreases in FEV_1 due to obstruction in the airways[42]. To date, cannabis smoking has not been strongly associated with reduced FEV_1/FVC, unlike tobacco smoking[43]. While some studies investigating lung function in chronic smokers observed a lack of change in FEV_1/FVC[44–47], others demonstrated reductions of up to 2% in cannabis smokers[38,40,48,49]. Notably, while respiratory symptoms decline after quitting, studies which observed reductions in FEV_1/FVC observed that this decrease persisted in previous cannabis smokers[50]. The exact relationship between cannabis smoking and declines in lung function is inconclusive, however, the association between chronic cannabis smoke exposure and respiratory symptomology remains clear.

1.2.2. Lung Disease and Pathology

The association between cannabis smoking and the development of lung disease is currently ambiguous. At this time, no definitive association between cannabis smoking and lung cancer has been found, despite evidence suggesting cannabis smokers are at an increased risk[51]. Similarly, there has been no observed relationship between cannabis smoke and the development of COPD[52]. However, evidence suggests that cannabis smoking leads to proximal airway injury, such as erythema, edema, and mucous hypersecretion, as well as inflammatory modulation in the bronchial mucosa[52–55]. Additionally, cannabis smoking has been strongly correlated with chronic bronchitis, with even asymptomatic smokers presenting with central airway inflammation[53]. The evidence of respiratory symptomology, potential changes in lung function, and airway injury supports that cannabis smoke is detrimental to lung health, despite a lack of conclusive associations with some

types of lung disease. Evidently, observations thus far suggest that cannabis smoking has profound effects on respiratory immunity which result in lung damage and pathology.

1.3. Cannabis Smoke and Lung Immunity

The lungs are constantly exposed to microbes and irritants, and thus requires robust immune surveillance and protection. Immunity within the airways and lungs is comprised of a complex network of physical barriers and innate and adaptive immune mechanisms. While all these components play an essential role in maintaining lung homeostasis, the innate immune compartment is of particular importance due to its role as the first line of defense against environmental insults. Innate immunity in the lungs is made up of epithelial cells and a variety of leukocytes, signaling molecules, and soluble peptides that carry out numerous functions including phagocytosis, antigen uptake and presentation, cell recruitment and activation, and the induction of apoptosis[56]. These effector functions help maintain lung homeostasis by clearing apoptotic cells and debris. As well, they are critical for preventing pathogen colonization and enabling the downstream activation of long-term adaptive immunity. As such, compromised innate immunity in the lungs can lead to vulnerability to microbial infection and exacerbated immunopathology, with severe dysregulation resulting in the development of chronic lung disease.

1.3.1. Macrophages & Monocytes

Macrophages are the most abundant mononuclear leukocyte in the lungs and serve as the primary phagocytes in the airways and lung tissue. Alveolar macrophages (AMs), which make up around 95% of leukocytes in healthy airways, act as the main sentinel cell in the lungs, by identifying and phagocytosing invading microbes and apoptotic cells[57]. Additionally, AMs can initiate immune cell recruitment and activation upon recognition of damage- and pathogen-associated molecular patterns (DAMPs, PAMPs) by releasing pro-inflammatory signaling molecules such as IL-1α, IL-1β, TNFα, IL-6, and IL-8. Recent advances in cell phenotyping using animal models have identified lung macrophage subtypes with different origins and effector functions. Tissue-resident alveolar macrophages (Res-AMs) are of embryonic origin, are self-renewing, and are thought to maintain lung homeostasis through various protective and reparative functions[58]. Upon depletion due to environmental or pathological insult, Res-AMs are replenished by the infiltration and differentiation of circulating monocytes, with these populations identified as monocyte-derived alveolar macrophages (Mo-AMs). Additionally,

interstitial macrophages (IMs) are a monocyte-derived lung macrophage population found in the lung interstitial space. Three subtypes of IMs have been identified based on cell surface markers (IM1s, IM2s, IM3s)[59,60]. It is not currently well understood how each of these monocyte-derived populations differ in function, phenotype, and composition following inflammatory stimulation. However, recent evidence suggests that Res-AMs have an intact phagocytic capacity and produce a moderate inflammatory response upon microbial challenge, while Mo-AMs, IM1s, IM2s, and IM3s have reduced phagocytic capabilities and heightened inflammatory mediator expression[59]. Importantly, specific lung macrophage subpopulations have been associated with the development of lung pathologies in mice. For instance, Mo-AMs have been shown to contribute to the pathogenesis of bleomycin-induced lung fibrosis[61,62]. Furthermore, lung IMs have been implicated to promote tobacco cigarette smoke-induced emphysema and mucus hypersecretion[63]. These findings highlight the necessity of further research to better understand the role of lung macrophages and specific macrophage subpopulations in maintaining lung homeostasis.

1.3.2. Neutrophils

Neutrophilic infiltration is a major component of host immune responses to pathogens. Following microbial stimulation, neutrophils are recruited from the pulmonary vasculature into the lung tissue and airways via signaling by macrophages and epithelial cells[64]. Upon migration and activation, neutrophils exert several anti-microbial mechanisms including the production of cytotoxic peptides, the release of oxygen radicals, and phagocytosis. The massive influx of neutrophils and localized inflammatory response that they produce lead to the clearance of pathogenic microbes and infected cells. Following infection resolution, activated neutrophils undergo apoptosis and are cleared by macrophages via efferocytosis[65]. The programmed cell death of neutrophils following infection is essential, as prolonged and exacerbated neutrophilia can lead to sustained inflammation, resulting in excessive bystander cell death and tissue damage. Defects in neutrophil migration, effector function, and apoptosis can have severe consequences and can lead to the pathogenesis of lung disease. For example, COPD is often characterized by chronic exacerbated neutrophilia, with animal models demonstrating that this response may be mediated by tobacco smoke-induced deficiencies in macrophage immune signaling and subsequent neutrophil chemotaxis[66,67]. While neutrophils remain as a necessary proponent of localized inflammation, their potency and pathological potential must be carefully balanced.

1.3.3. Dendritic Cells

Dendritic cells (DC) are key antigen presenting cells in the lungs and are potent inducers of adaptive immunity[68]. DCs are positioned beneath the lung epithelium, with their dendrites protruding into the airway luminal space. At steady state, DCs sample the respiratory mucosa for antigens, innocuous or otherwise. Upon activation, DCs transport the antigen back to the mediastinal lymph nodes for subsequent cytotoxic T cell stimulation, PAMP-recognition dependently[69]. Along with being adept inducers of adaptive immunity, DCs also have phagocytic capabilities, allowing them to directly influence microbial clearance. Given their role in facilitating T cell stimulation, defects in DC function have profound consequences on anti-microbial immunity. Anti-viral responses are particularly dependent on DC antigen presentation, so compromised DC function or reduced cellularity can lead to insufficient adaptive immune cell mobilization and exacerbated viral pathology[70]. The position of pulmonary DCs at the innate-adaptive immunity interface underscore their importance in lung immune responses.

1.3.4. Eosinophils

The migration and activation of eosinophils due to parasitic or microbial infiltration is critical in inducing acute inflammatory responses. The main effector function of eosinophils involves degranulation and the release of various cytotoxic peptides, reactive oxygen species, cytokines, and lipid mediators[71]. Much like neutrophils, eosinophils are short lived granulocytes which are cleared via efferocytosis upon inflammatory resolution. Pathologically, eosinophilia is associated with the excessive inflammation and tissue damage in response to allergen exposure in individuals with allergic asthma[72]. As well, eosinophilic inflammation is prevalent in a subset of COPD patients independent of asthma, with elevated eosinophilia being observed during COPD exacerbation[73]. While research studying the contribution of eosinophils to chronic inflammation is still ongoing, it remains clear that eosinophils have great pathological potential and warrant consideration when investigating respiratory disease.

1.3.5. Cannabis Smoke and Lung Innate Immunity

Long term exposure to cannabis smoke has been associated with inflammatory pathologies in the lungs, however, the exact relationship between cannabis smoke exposure and its impact on lung innate immunity has yet to be fully explored. Cannabis smokers have been observed to have significant histopathological evidence of pulmonary inflammation, damage to the respiratory

mucosa, and increased bronchitis index scores, which includes measures of erythema, edema, and airway mucous secretion[53]. However, conflicting evidence about the contribution of specific lung immune cells conceals how cannabis smoke may induce these downstream pathologies. For example, increased lung macrophages and exacerbated neutrophilia have been observed in some investigations of cannabis smoke-induced pulmonary inflammation, but not in others[53,74]. Similarly, data from the few existing rodent models have demonstrated inconsistent effects of cannabis smoke on lung immune cell infiltration and function. Macrophage cellularity, mediator production, and phagocytic capacity, for instance, have been shown to be exacerbated in some studies, but suppressed in others[75,76]. Critically, many of the investigations using animal models are from the previous century and thus lack many of the insights gained from the tobacco smoking literature regarding smoke-induced dysregulated immunity and lung disease. Most importantly, these studies, as well as historical clinical observations, may be limited in relevancy due to the nature of cannabis itself.

It is unclear what factors are contributing to these contrasting effects within the cannabis smoke literature; however, certainly obfuscating things is the evolving landscape surrounding cannabis use and potency. The historically illicit nature of cannabis and social stigma around drug use potentially complicates clinical studies which rely on the accurate reporting of cannabis consumption. As well, the composition of commonly available cannabis strains has varied significantly over time, with modern commercially available strains containing THC or CBD concentrations exceeding 15%, compared to the less than 5% observed in cannabis from the past[77,78]. The lack of standardized cannabis products prior to legalization in many countries further complicates the reporting of composition by users and in research studies. Consequently, early clinical studies or animal investigations of cannabis smoke exposure may not accurately reflect the health implications of modern cannabis use. This inconclusiveness, in conjunction with the conflicting evidence observed to date, supports the need for further investigations characterizing the mechanisms by which cannabis smoke contributes to innate immunity dysregulation and the development of lung pathology.

1.4. Cannabis Smoke and Viral Lung Infection

The lungs are exposed to a myriad of viral microbes, such as influenza viruses, rhinoviruses, coronaviruses, and respiratory syncytial viruses, that result in the pathogenesis of upper and lower respiratory tract conditions including rhinosinusitis, bronchitis, and pneumonia. In healthy individuals, these conditions are often associated with acute presentation of moderate symptoms and

immunopathology, but full recovery is the typical outcome. Dysregulated immunity in the lungs can lead to increased susceptibility to these viral pathogens, resulting in exacerbated disease and aggravated inflammation that can worsen existing conditions. Influenza, for example, is one of the most common viral infections during acute exacerbations of COPD and is thought to have a profound effect on patient morbidity and mortality[79]. Given these detrimental effects, it is imperative to understand the risk factors that predispose individuals to viral lung infection. Environmental smoke exposure has long been shown to increase susceptibility and severity of viral infection in the respiratory tract[80–82]. However, the effect of cannabis smoke specifically is poorly understood and has yet to be sufficiently assessed. The potential immunomodulatory nature of cannabis smoke supports the hypothesis that exposure may adversely affect host responses to viral infection. Particularly, impairments of innate immune cell recruitment and function can have drastic ramifications on the effectiveness and efficiency of the overall host immune response.

1.4.1. Lung Innate Immune Responses to Influenza A Virus

Influenza A viruses (IAV) are contagious pathogens which induce severe lower respiratory tract infections and conditions, including bronchitis and pneumonia. They contribute to the estimated 250 000 to 500 000 deaths worldwide attributable to influenza infection and present a significant global health burden[83]. Host responses to influenza A infection involve the rapid mobilization of innate immune cells and subsequent activation of adaptive immunity[84,85]. Upon infiltration, IAVs infect epithelial cells lining the respiratory mucosa and hijack their transcriptional machinery to replicate and disseminate. Following infection, epithelial cells release factors to induce the transcription of interferons and pro-inflammatory cytokines to recruit and activate innate effector cells. Virally infected cells are identified via the recognition of PAMPs by pathogen recognition receptors, such as toll-like receptors, present on the surface of recruited innate immune cells. As the primary sentinel cell in airways, AMs play an important role in viral clearance by phagocytosing infected cells and further inducing the migration of other inflammatory immune cells, such as neutrophils and DCs. A massive influx of neutrophils following IAV colonization is responsible for elevating local inflammation and further supporting viral clearance. Importantly, upon recognition of virally infected cells, DCs undergo phenotypic changes to a T cell stimulatory profile, resulting in the activation of antigen-specific adaptive immunity. The combination of innate and adaptive host responses leads to the limiting of viral spread, viral clearance, and infection resolution. Given the importance of the innate

immune compartment in facilitating early responses and mobilizing the adaptive arm, defects in innate immune cell recruitment or function can have profound effects on host response effectiveness, immunopathology, and overall health outcomes following IAV infection.

1.4.2. Cannabis Smoke and Influenza A Infection

The effect of cannabis smoke on innate immune responses to IAV infection is insufficiently understood. Cannabis smokers have been reported to have increased incidence of respiratory symptoms associated with bronchitis and pneumonia[37]. As well, evidence suggests that cannabis smokers have increased rates of hospitalization and length of stay due to respiratory infection compared to non-smokers[86]. However, to date, there exists no studies which investigate the direct relationship between cannabis smoke exposure, dysregulated lung immunity, viral infection, and health outcomes. Given this omission, findings from animal models of tobacco smoke and cannabinoid exposure provide valuable insight into the potential effect of cannabis smoke on responses to IAV. Tobacco cigarette smoke has been demonstrated in animal models to exacerbate innate inflammatory responses following IAV infection, leading to heightened disease severity, excessive morbidity, and mortality[87–89]. This exacerbation is characterized by increased mononuclear cellularity, neutrophilia, and pro-inflammatory mediator expression in the lungs and airways[87,90]. Conversely, findings from concurrent THC administration and influenza infection animal studies suggest that THC suppresses cellular responses to IAV, but still leads to elevated immunopathology[91,92]. Given that smoke itself is an inflammatory insult, expectations in the field are that cannabis smoke will have similar immunomodulatory effects as tobacco smoke, but the proposed opposing effects of THC must not be overlooked. Thus, currently available data do not fully reflect the chemical profile or composition of cannabis smoke, necessitating the need for full investigations on the impact of cannabis smoke on viral responses.

1.5. Central Aim, Research Objectives and Hypotheses

1.5.1. Central Aim

Clinical observations suggest that cannabis smoking contributes to the development of respiratory symptoms and may play a role in the pathogenesis of inflammatory lung disease. However, the association between cannabis smoke, dysregulated lung immunity, and the development of lung disease is inconclusive. While animal models have been used to begin delineating this relationship, their relevancy is limited due to changes in the composition of modern cannabis strains.

This thesis sought to address this knowledge gap by establishing a novel mouse model of cannabis smoke exposure using commercially available and compositionally relevant cannabis strains. As well, given the lack of studies which investigate the effects of cannabis smoke exposure on immune responses to viral infection, a novel model of concurrent cannabis smoke exposure and influenza A infection was adapted. Using these models, we sought to characterize the effects of acute cannabis smoke exposure on pulmonary innate immune components at baseline and in response to influenza infection. The findings of this thesis build the foundation for future characterizations and mechanistic investigations of cannabis smoke's effect on respiratory immunity.

1.5.2. Research Objectives

The specific objectives of this thesis project were:

- To establish and validate a novel mouse model of cannabis smoke exposure.
- To characterize the *in vivo* effects of cannabis smoke exposure on lung immune cell populations and immune mediators.
- To investigate the *in vivo* effects of cannabis smoke exposure on morbidity and lung immune responses following influenza infection.

1.5.3. Hypotheses

Overarching Hypothesis: We hypothesize that cannabis smoke exposure modulates immune responses at baseline and in the context of viral challenge.

Specific Hypothesis #1: Cannabis smoke exposure induces an inflammatory environment in the lung tissue by expanding pro-inflammatory cell populations and increasing inflammatory immune mediator production at baseline.

Specific Hypothesis #2: Concurrent cannabis smoke exposure and influenza infection leads to increased morbidity driven by an exacerbated inflammatory response to viral challenge.

CHAPTER 2: MATERIALS AND METHODS

2.1. Animals

Six- to eight-week-old male and female BALB/c mice were purchased from Charles River Laboratories (Quebec, Canada). Mice were housed in the McMaster Central Animal Facility with *ad libitum* access to food and water and subjected to a 12-hour lighting cycle. All experimental procedures were approved by the Animal Research Ethics Board of McMaster University.

2.2. Cannabis Cigarette Preparation

Cannabis cigarettes were prepared using cannabis purchased from the Ontario Cannabis Store (Ontario, Canada) and Cannalogue (Ontario, Canada). All strains used were indica dominant and contained 10 – 14% THC and 0 – 2% CBD. Cigarettes were hand-rolled by grinding dried cannabis flower, packing into king size Premier cigarette tubes (R.J. Reynolds Tobacco Company, USA), and twisting off the end to seal the cigarette (**Figure 1A**, **1B**). Each cigarette contained 0.84 (± 0.06) grams of cannabis. Prior to running the smoke exposure protocol, the filter of each cigarette was removed, and the cannabis was packed towards the closed end (**Figure 1C**).

2.3. Cannabis Smoke Exposure

Prior to cannabis smoke exposure, mice were acclimatized to restrainers over three days. Mice were whole-body exposed using a Smoke Inhalation Unit 24 (Promech, Sweden) (**Figure 1D**) to the smoke of six cannabis cigarettes twice daily for one day, four days, or eight days total, as noted in the text. Mice were given a three hour break in between daily exposure sessions. Control mice were sham-exposed to room air.

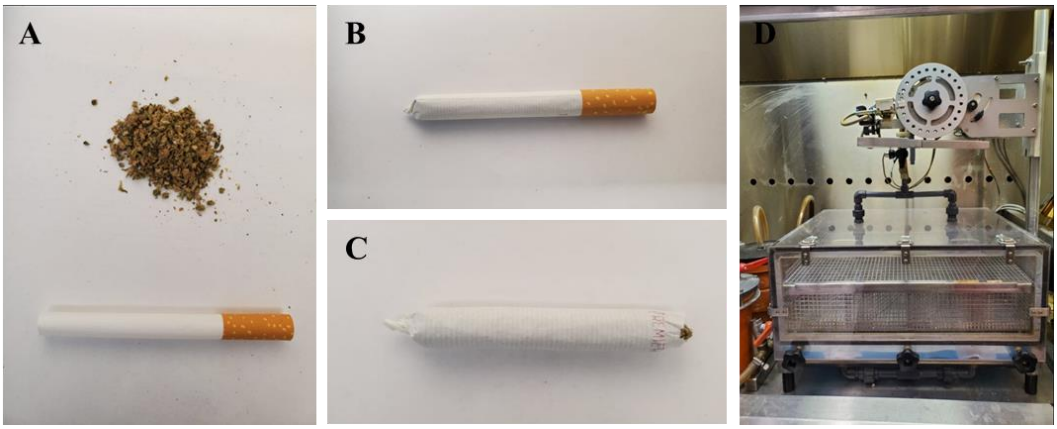

Figure 1. Cannabis cigarette preparation and smoke exposure system. Cannabis cigarettes are prepared by (A) grinding 0.84 (± 0.06) grams of dried cannabis flower (10 – 14% THC, 0% CBD), (B) packing into king size empty cigarette tubes and twisting off the end to seal the cigarette. (C) The filter of each cigarette is removed, and cannabis is packed towards the sealed end of the cigarette prior to smoke exposure. (D) Cannabis cigarettes are loaded into the cigarette holder on a Smoke Inhalation Unit 24 system. Experimental animals are placed in the lower chamber. Cannabis smoke and fresh air are pumped into the chamber following a computerized protocol attached to the system.

2.4. Influenza A Virus Delivery

Mice were whole-body exposed to cannabis smoke or room air for five days (**Figure 2**). Three hours following the second smoke exposure on the fifth day, smoke and room air exposed mice were intranasally inoculated with 35 µL of PBS containing 50 plaque-forming units (PFU) of mouse-adapted influenza A virus (A/FM/1/47-MA) or PBS vehicle. Mice were given a two-day break from cannabis smoke exposure following inoculation. Exposure protocols resumed following the break for three days. In total, mice were exposed to eight days of cannabis smoke exposure. Samples were collected on day six post-infection, day eleven of the entire protocol. Weight measurements and endpoint monitoring were completed daily at the same time following influenza A or vehicle inoculation.

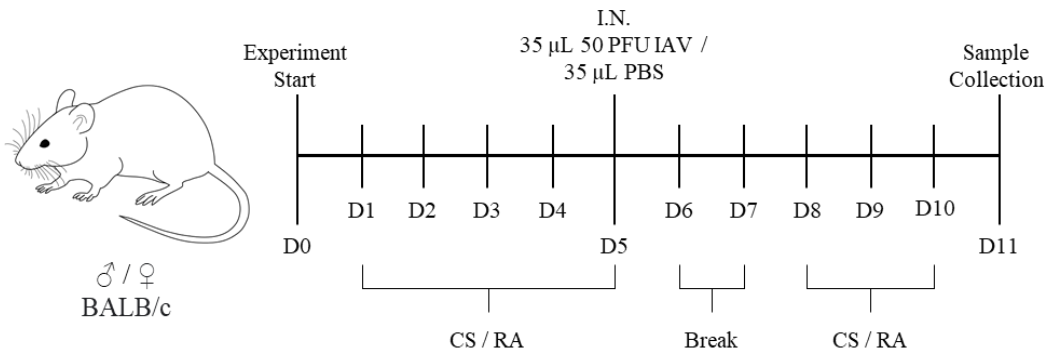

Figure 2. Experimental exposure, intranasal inoculation, and sample collection timeline. Male and female six- to eight-week-old BALB/c mice were acclimatized to smoke exposure restrainers prior to initiation of experiment. Mice were whole-body exposed to the smoke of six cannabis cigarettes (CS) or room air control (RA) twice per day for five days. Following smoke exposure on day 5, mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or phosphate saline buffer (PBS) control. Mice were given a two-day break from cannabis or room air exposure following inoculation. Exposure protocols resumed on day eight and continued until day ten. Samples were collected on day 11.

2.5. Total Particulate Matter

Total particulate matter was measured in the smoke exposure system using a Flowmeter 4045 (TSI Incorporated, USA) and paper filters. Six cannabis cigarettes were placed in the exposure system and burned using the same settings as the experimental smoke exposure procedure. Smoke and air outflow was measured during the burning of the first cigarette using the Flowmeter 4045. Filters were placed and subsequently removed one at a time in the outflow tubing during the burning of cigarettes one, three, and five. Following the exposure, the filters were weighed and TPM was derived by dividing the average change in filter mass by the average change in outflow multiplied by four.

2.6. Carboxyhemoglobin and Cannabinoids

Fifteen or 60 minutes following the second smoke exposure session on day 1, whole blood was collected from smoke exposed and control mice using capillary tubes via retro-orbital bleed. Heparinized capillary tubes were used to collect whole blood from the opposite eye. Carboxyhemoglobin in whole blood samples was quantified via CO-oximetry by the McMaster University Medical Centre core laboratory. Plasma was collected by incubating and centrifuging heparinized whole blood samples. Samples were prepared for THC, THC-COOH, and CBD quantification via mass spectroscopy by the McMaster University Centre for Microbial Chemical Biology. Briefly, samples were treated with cold acetonitrile containing 1% (v/v) formic and 50 ng/mL of internal standard mixture. Next, HPLC grade water was added to the samples, which were then injected into an Agilent 1290 Infinity II HPLC coupled to an Agilent 6495C iFunnel QQQ mass spectrometer (Agilent, California, USA). Separated analytes were eluted and introduced to the mass spectrometer. Quantitation of each cannabinoid was based on the peak area measurement measured by the mass spectrometer. Pooled blank mouse plasma from RA mice was used as matrix background and was processed using the same protocol. Standards of THC, THC-COOH, and CBD were purchased (Sigma Aldrich Canada, Ontario, Canada), prepared in the matrix background, and processed using the same protocol as the samples.

2.7. Cell Counts and Differentials

Whole lungs and trachea were excised from the chest cavity. Bronchoalveolar lavage (BAL) was collected by cannulating the trachea, tying off the right lung lobe, instilling the left lobe with 250 µL of cold sterile phosphate buffered saline (PBS), and re-aspirating the fluid. Two sequential instillations were performed in this manner and combined. BAL samples were centrifuged, supernatant removed, and resuspended in 130 µL of PBS. Cell counts were performed on the resuspended samples via haemocytometer and light microscopy.

Remaining samples were used for cell differentials via cytospins. BAL samples were centrifuged onto pre-wet microscope slides and then stained using Hema-3 fixative and solutions. Mononuclear and polymorphonuclear cell populations were identified and imaged using Zen microscopy imaging software (Zeiss International, Germany). Cell differentials were multiplied by cell counts to derive differential cell counts.

2.8. Flow Cytometry

Middle, inferior, and post-caval lung lobes were excised and collected in cRPMI (RPMI containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine). Lobes were incubated for one hour at 37°C in 10 mL of cRPMI with 1500 units of 260 units/mg type 1 collagenase (Worthington Biochemical, Cat. #LS004194). Following incubation, lobes were pressed through a 35 µm filter, centrifuged, and supernatant was removed. Samples were then treated with Ammonium-Chloride-Potassium (ACK) lysis buffer for one minute, diluted with PBS, and centrifuged. Following centrifugation, samples (now single cell suspensions) were resuspended in 1% bovine serum albumin supplemented with 2 mM EDTA. Single cell suspensions were plated, centrifuged, treated with blocking solution, and stained with fluorophore-labelled antibodies. Remaining single cell suspensions were used for cell counts using a haemocytometer, Türk's solution, and light microscopy. Stained samples were assessed for immune cell population composition and quantity using a BD LSRFortessa Flow Cytometer (BD Biosciences, Canada).

The following antibodies were used for flow cytometric analysis (**Figure 3**): LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific, Cat. #L34967), CD45-Alexa Fluor 700 (BioLegend, Cat. #103127), B220-APC-Cy7 (BioLegend, Cat. #103211), CD3e-APC-Cy7 (BioLegend, Cat. #100329), EpCAM-APC-Cy7 (BioLegend, Cat. #118217), Ly6G-BV785 (BioLegend, Cat. #127645), CD11b-PE-Dazzle 594 (BioLegend, Cat. #101255), CD64-PE-Cy7 (BioLegend, Cat. #139313), MerTK-APC (BioLegend, Cat. #151507), SiglecF-PE (BioLegend, Cat. #155505), CD11c-BV650 (BioLegend, Cat. #117339), CD24-BV421 (BioLegend, Cat. #101825), MHCII-PerCP-Cy5.5 (BioLegend, Cat. #107625), and Ly6C-BV510 (BioLegend, Cat. #128033).

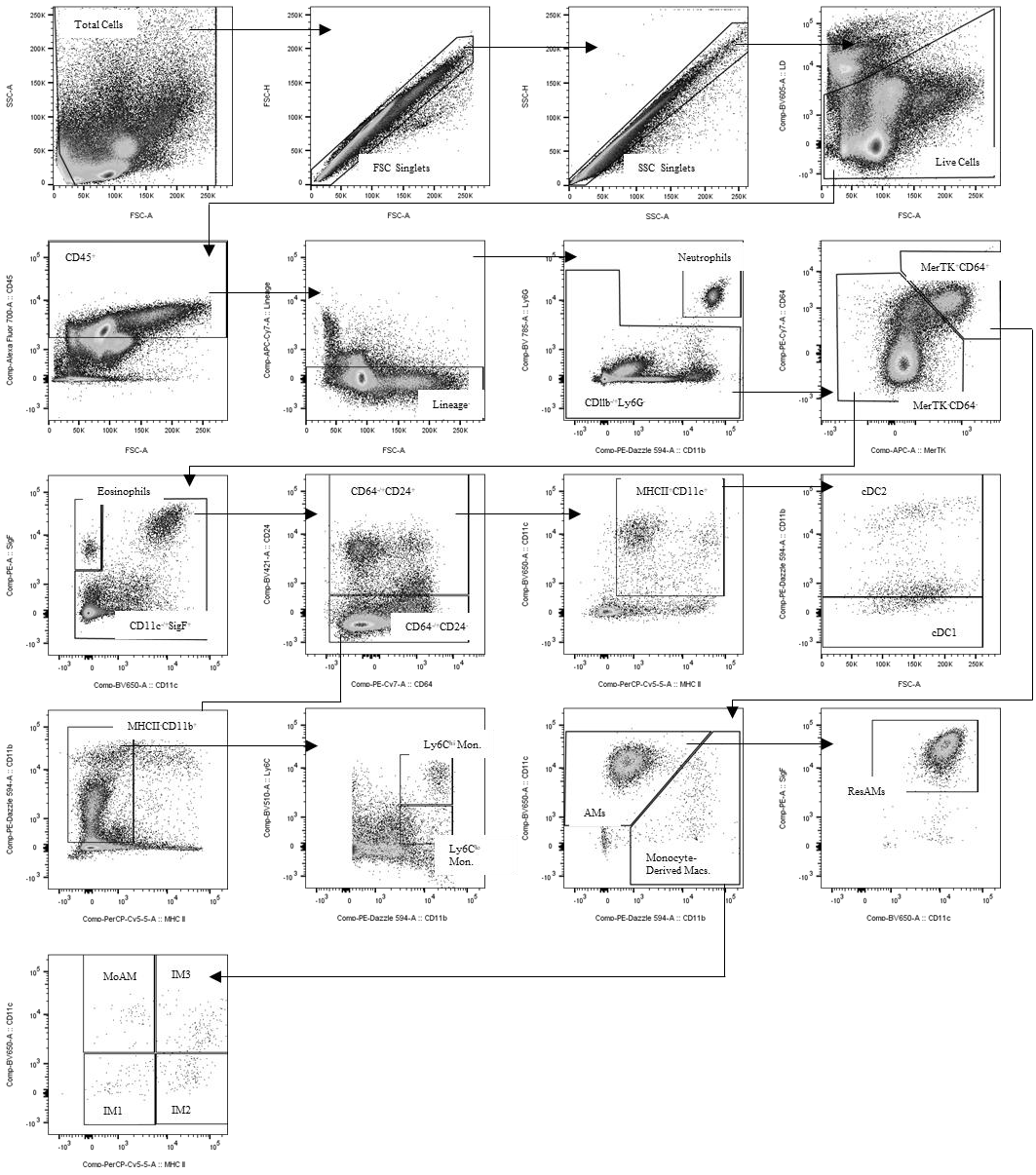

Figure 3. Lung tissue innate immune cell flow cytometry gating strategy. Gating was determined based on population identification and fluorescence-minus-one controls. Gating strategy generated using lung tissue collected from a room air exposed female control mouse. Lineage gate contains $B220^{+}$, $CD3e^{+}$, and $EpCAM^{+}$ cell populations.

2.9. Immune Mediators

Left lung lobes were collected in PBS and homogenized. Lung tissue homogenate was centrifuged, and the supernatant was collected. Supernatant was prepared for immune mediator quantification via Mouse Cytokine Array / Chemokine Array 44-plex (Eve Technologies, Calgary, Alberta, Canada). Prior to submission, samples were inactivated via exposure to 2000 µJ of ultraviolet radiation for one minute. Point to point semi-logarithmic analysis was applied to all immune mediator quantities. Principal Component Analysis (PCA) of samples using all 45 cytokines was performed using the 'prcomp' function (default settings) from the stats package in R (v. 3.6.2) (RCoreTeam, 2017) and was subsequently plotted with the ggplot2 package in R (v. 3.3.3)[93].

2.10. Plaque Assays

Left lung lobes were homogenized in PBS, centrifuged, and supernatant was collected. Madin-Darby Canine Kidney (MDCK) cells were cultured in DMEM and plated in 6-well plates. Sample supernatants were serially diluted in prepared "flu media" containing 90% 2X Minimum Essential Media (MEM), 3% 7.5% sodium bicarbonate, 2% 200 mM L-glutamine, 2% 10 mg/mL penicillin streptomycin, 2% 1M HEPES, and 1% 35% bovine serum albumin. Flu media was diluted 1:1 with distilled water and supplemented with 1 µg/mL TPCK-trypsin prior to serial dilutions. Plated MDCK cells were washed with PBS and diluted samples were transferred onto their corresponding wells. Plates were incubated for 60 minutes at 37°C for one hour with agitations every 15 minutes. Following incubation, media was removed, and wells were washed with PBS. Subsequently, plaque media containing a 1:1 dilution of 1% OXOID agar and flu media supplemented with 1 µg/mL TPCK-trypsin and 1% DEAE was added to each well. Plates were incubated at room temperature to allow the plaque media to solidify, and then were incubated for 48 hours at 37°C. Following incubation, cells were fixed with 4% paraformaldehyde and solidified plaque media was removed with running water. Plates were incubated for 20 minutes with crystal violet and then gently washed with PBS. Viral titre was quantified by counting visible plaques in each well.

2.11. Statistical analysis

GraphPad Prism 9 (GraphPad Software Inc., USA) was used for statistical analyses. The data was expressed in terms of mean and standard errors of the mean (SEM). Statistical outliers were removed using the ROUT method with a Q coefficient of 1%. Two-way ANOVAs with Tukey's multiple comparisons test were used to compare the means of four experimental groups. Unpaired t-tests were used to compare the means of two infected groups for viral titre analysis. Unpaired t-tests were performed to compare the means of two groups within the same sex.

The significance of variance between PCA groups was determined by a PERMANOVA performed using the 'adonis' function (default settings) from the vegan R package (v. 2.5-6)[94]. Differences were considered statistically significant when $p \leq 0.05$.

CHAPTER 3: RESULTS

3.1. Cannabis Smoke Exposure

3.1.1. Combustion, Inhalation, and Systemic Distribution

We first sought to establish a mouse model of cannabis smoke exposure using a Smoke Inhalation Unit 24 which has previously been used in tobacco smoke exposure studies (**Figure 1D**). Six cannabis cigarettes were selected for each exposure session based on preliminary experiments to determine tolerance. Combustion was quantified by total particulate matter (TPM) and carried out in a single smoke exposure session. During exposure, the average TPM concentration within the exposure chamber was 698.9 (SD = 66.1) µg/L. Cannabis smoke-exposed mice had reduced levels of activity and ventilation rates. Upon removal from the chamber, mice appeared lethargic and hunched but returned to normal activity levels and posture within one hour. Despite these behavioural changes, cannabis smoke exposure was well tolerated. For comparative purposes, these changes in behaviour are also observed in our well-characterized tobacco cigarette smoke models using the same exposure system[95].

Following removal from the smoke exposure chamber, blood was collected within 15 minutes for quantification of metabolites of combustion and phytocannabinoids. A parallel experiment was carried out with blood collection 60 minutes following cannabis smoke exposure. Carboxyhemoglobin (COHb), a metabolite produced when hemoglobin is bound by carbon monoxide inhaled from combustion, was significantly elevated in both male and female smoke exposed mice (**Figure 4A**). In samples collected 60 minutes post-exposure, COHb levels returned to baseline in both males and females (**Figure 5A**). To validate phytocannabinoid delivery through smoke exposure, plasma samples were analyzed via mass spectroscopy (**Figure 4B**). Plasma THC and THC-COOH were both present in all male and female smoke exposed mice compared to controls, which had no traces of either cannabinoid detected. CBD was not detected in any tested samples, consistent with the composition of the selected cannabis strains (reported as 10 – 14% THC and 0 – 2% CBD). In plasma samples collected 60 minutes post exposure, THC was detected in 1/5 males and 1/5 females, while THC-COOH was detected in 3/5 males and 3/5 females (**Figure 5B**). Plasma samples with detectable THC and THC-COOH had levels only slightly above the lower level of detection for the analysis. Similar to the earlier assessment, CBD was undetected in all samples at 60 minutes post-exposure.

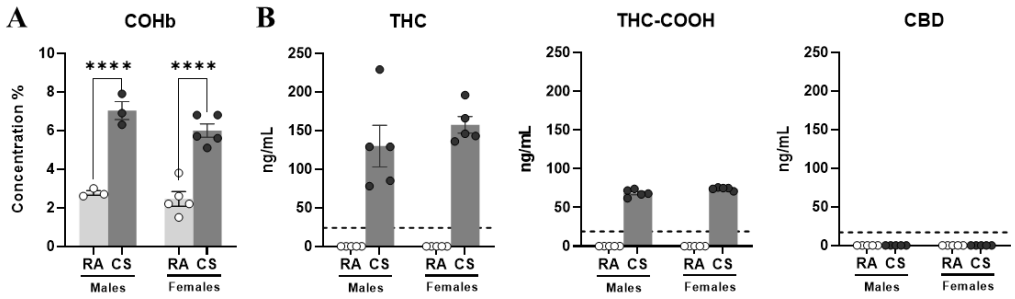

Figure 4. Cannabis smoke exposure leads to elevated plasma carboxyhemoglobin, THC, and THC-COOH at 15 minutes post-exposure. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice in a day, three hours apart. Whole blood was collected via retro-orbital bleed within 15 minutes following the second exposure session. (A) Carboxyhemoglobin (COHb) percentage was quantified via CO-oximetry. (B) Plasma tetrahydrocannabinol (THC), cannabidiol (CBD), and carboxy-tetrahydrocannabinol (THC-COOH) were quantified via mass spectroscopy. Dotted lines represent the limit of detection for specific cannabinoid. Data points at 0 ng/mL represent values below the limit of detection in cannabinoid analysis. Data represent mean ± SEM; n = 3 – 5/group; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. ****P ≤ 0.0001, unpaired t-test within each sex in COHb analysis.

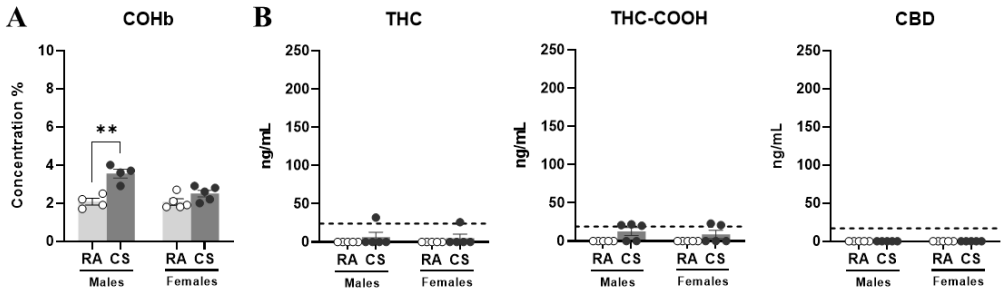

Figure 5. Plasma carboxyhemoglobin, THC, and THC-COOH return to baseline 60 minutes following cannabis smoke exposure. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice in a day, three hours apart. Whole blood was collected via retro-orbital bleed 60 minutes following the second exposure session. (A) Carboxyhemoglobin (COHb) percentage was quantified via CO-oximetry. (B) Plasma tetrahydrocannabinol (THC), cannabidiol (CBD), and carboxy-tetrahydrocannabinol (THC-COOH) were quantified via mass spectroscopy. Dotted lines represent the limit of detection for specific cannabinoid. Data points at 0 ng/mL represent values below the limit of detection in cannabinoid analysis. Data represent mean ± SEM; n = 3 – 5/group; *P ≤ 0.05, **P ≤ 0.01, unpaired t-test within each sex in COHb analysis.

3.1.2. Lung Immune Cell Populations

Once the exposure model was established and validated, we began to investigate the effect of cannabis smoke exposure on various aspects of respiratory immunity. Specifically, we first assessed how cannabis smoke alone affects the composition of immune cells and immune mediators in the lungs using a four-day model. Cytospins used to quantify immune populations within the airways, while populations in whole lung tissue were analyzed by flow cytometry. Total immune cellularity was significantly increased in the airways of female cannabis smoke-exposed mice compared to control (**Figure 6**). Differential cell analysis revealed that this increase was driven by an expansion of mononuclear cells; no polymorphonuclear cells were present in any of the female samples. Total immune cellularity and mononuclear cell quantities did not significantly change in the airways of male cannabis smoke-exposed mice.

Flow cytometric analysis of cannabis smoke-exposed lung tissue further demonstrated modulation of immune cell populations (**Figure 7**). While the proportion of total $CD45^+$ cells in the lungs remained unchanged in cannabis-exposed animals, regardless of sex, specific immune cell populations were altered. In male cannabis smoke-exposed mice, the proportion of macrophages were significantly increased; neutrophils, dendritic cells, and eosinophils were unchanged; and monocytes were significantly decreased. In female cannabis smoke-exposed mice, the proportion of eosinophils were significantly increased; total macrophages, neutrophils, and monocytes were unchanged; and dendritic cells were significantly decreased. No changes in total cell count were observed for any of the quantified populations (**Figure 8**).

Specific lung macrophage subpopulations have been suggested to be important in the development of disease-associated lung inflammation[60,62,96]. We therefore included complementary cell markers in our flow cytometric analysis to identify relevant subtypes (**Figure 9**). In male cannabis smoke-exposed mice, tissue-resident alveolar macrophages, monocyte-derived alveolar macrophages, and IM1s were significantly increased while IM2s and IM3s were unchanged as a proportion of total leukocytes, compared to control. Conversely, in female cannabis smoke-exposed mice, only the proportion of IM1s were significantly increased; all other subpopulations were unchanged. Of the quantified subpopulations, Mo-AMs in the male cannabis-exposed mice were the only population observed to have an elevation in total cellularity, with the remaining populations going unchanged (**Figure 10**).

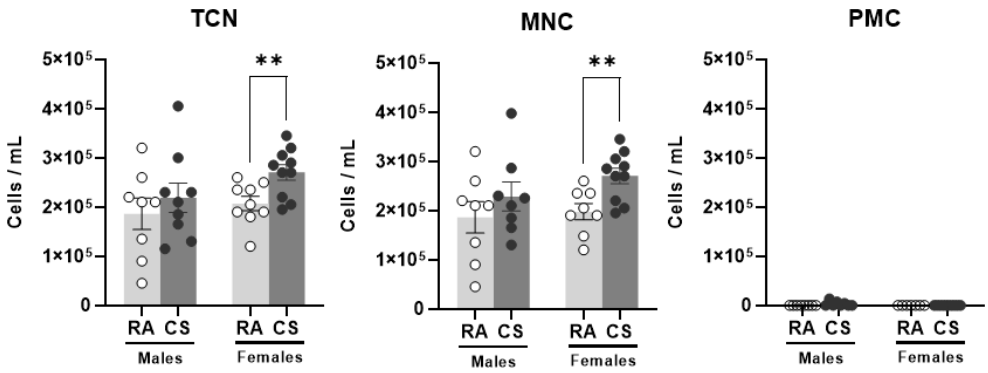

Figure 6. Cannabis smoke exposure modulates immune cellularity in the airways. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice a day for four days. BAL total cell number (TCN), mononuclear cells (MNC), and polymorphonuclear cells (PMC) were determined via haemocytometer and cytospin differentials. Data represent mean ± SEM; n = 8 – 10/group, data pooled from two identical experiments; *P ≤ 0.05, **P ≤ 0.01, unpaired t-test within each sex.

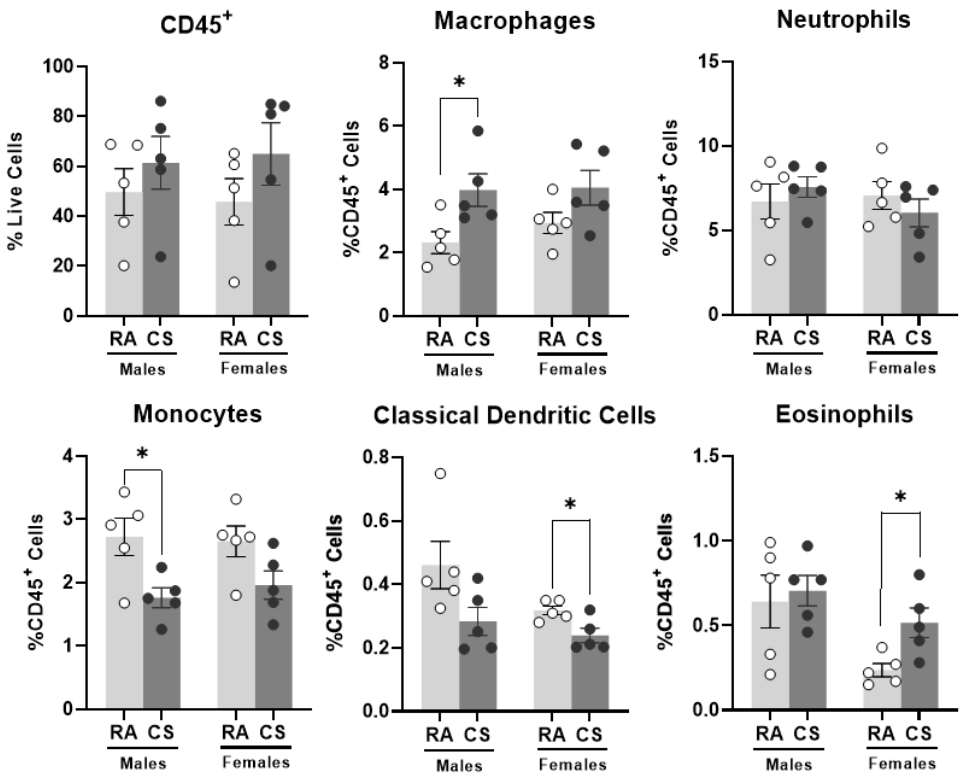

Figure 7. Cannabis smoke exposure modulates the <u>proportional composition</u> of innate immune cell populations in the lungs. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice a day for four days. Lung innate immune cell populations were quantified via flow cytometry. Proportionality was determined via haemocytometer. Data represent mean ± SEM; n = 5/group; *P ≤ 0.05, unpaired t-test within each sex.

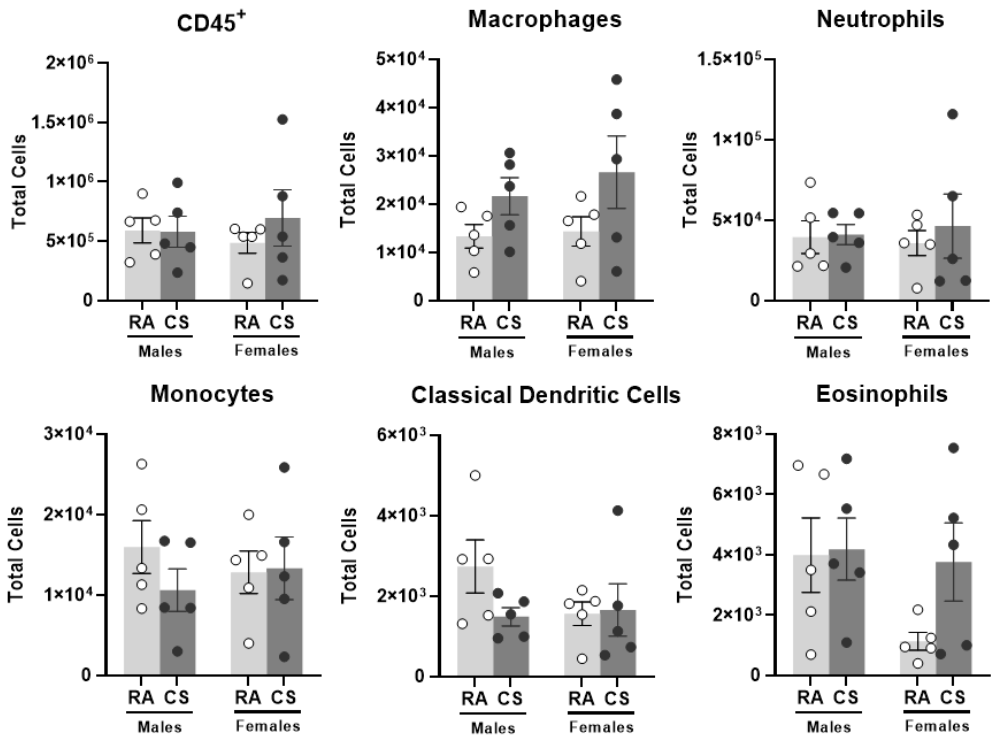

Figure 8. Cannabis smoke exposure has no effect on <u>total innate immune cellularity</u> in the lungs. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice a day for four days. Lung innate immune cell populations were quantified via flow cytometry. Total cell count was determined via haemocytometer, Türk's solution staining, and light microscopy. Data represent mean ± SEM; n = 5/group; *P ≤ 0.05, unpaired t-test within each sex.

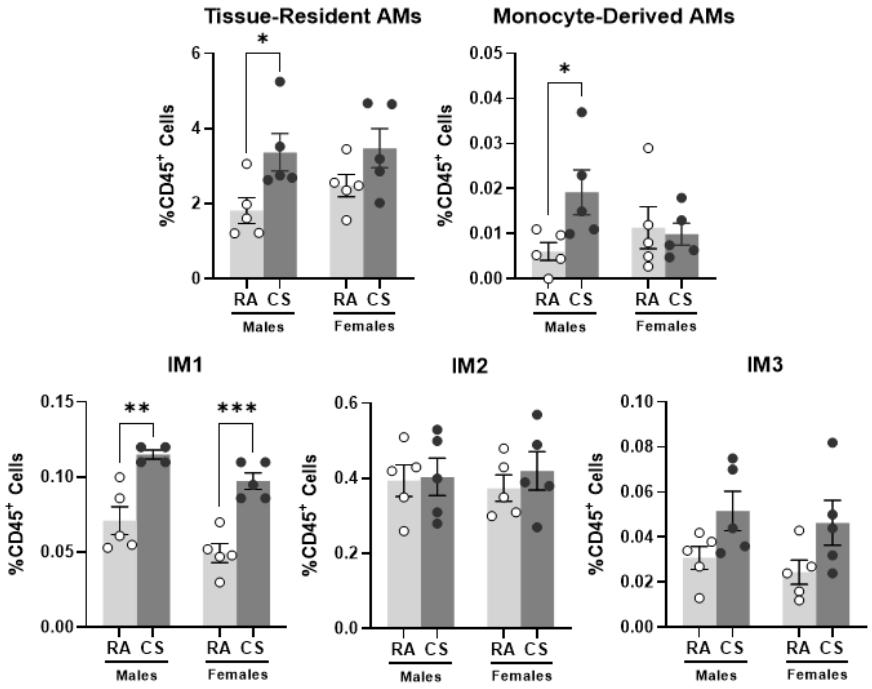

Figure 9. Cannabis smoke exposure modulates the <u>proportional composition</u> of macrophage subpopulations in the lungs. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice a day for four days. Macrophage subpopulations were quantified via flow cytometry. Proportionality was determined via haemocytometer. AM – Alveolar Macrophage, IM – Interstitial Macrophage. Data represent mean ± SEM; n = 4 – 5/group; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, unpaired t-test within each sex.

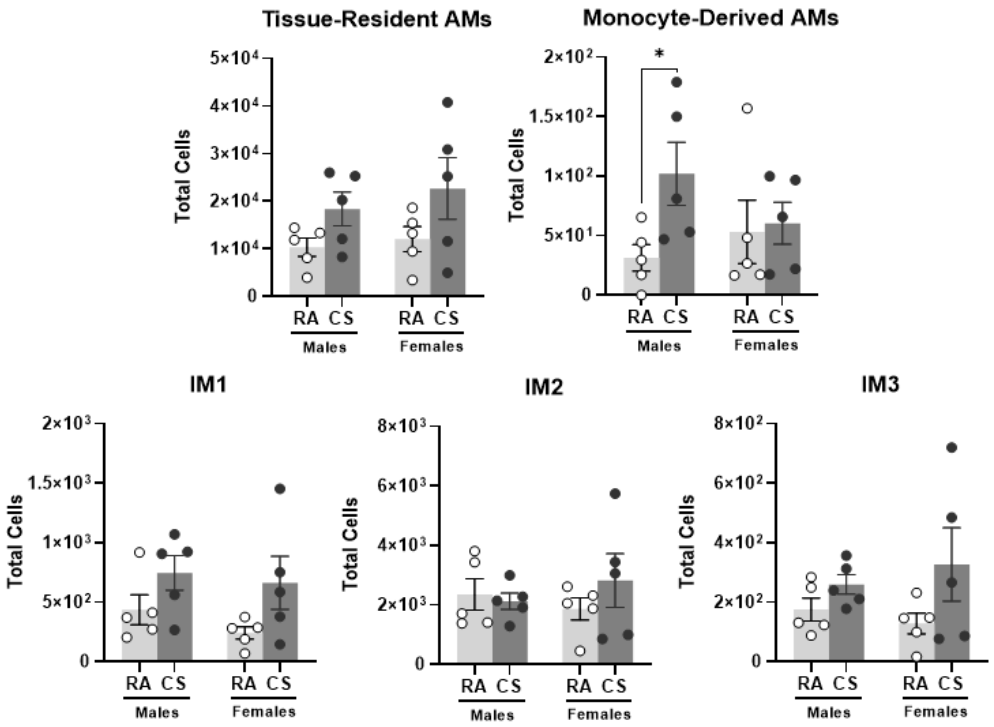

Figure 10. Cannabis smoke exposure has no effect on macrophage subpopulation <u>total cellularity</u> in the lungs. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice a day for four days. Macrophage subpopulations were quantified via flow cytometry. Total cell count was determined via haemocytometer, Türk's solution staining, and light microscopy. AM – Alveolar Macrophage, IM – Interstitial Macrophage. Data represent mean ± SEM; n = 4 – 5/group; *P ≤ 0.05, unpaired t-test within each sex.

3.1.3. Lung Immune Mediators

To further characterize cannabis smoke exposure-induced changes to the lung immune environment, we quantified immune mediators in the lung tissue using a 44-plex cytokine array. Detectable macrophage-associated immune mediators such as MCP-5 (CCL12), MIP-2 (CXCL2), and MIP-3β (CCL19) were unchanged, while macrophage-derived chemokine (MDC) was significantly increased in male smoke exposed mice (**Figure 11A**). IL-1α, an interleukin-1 family cytokine which has been demonstrated to be involved in smoke-associated inflammation[97], was also unchanged in cannabis smoke exposed mice compared to control (**Figure 11B**). Anti-viral cytokines, which are typically involved in early innate immune signaling in response to viral infection and have been proposed to be suppressed by cannabis smoke exposure[98,99], such as IFNβ-1, IP-10 (CXCL10), and RANTES (CCL5), were unchanged in cannabis smoke exposed mice compared to control (**Figure 11C**). Additional cytokines including TARC (CCL17) in the males and vascular endothelial growth factor (VEGF) in the females were significantly increased compared to room air control, with trends conserved across sexes (**Figure 11D**). All other mediators assessed are presented with their concentrations, level of detection, cannabis smoke to room air ratio, and P value in **Table 1**.

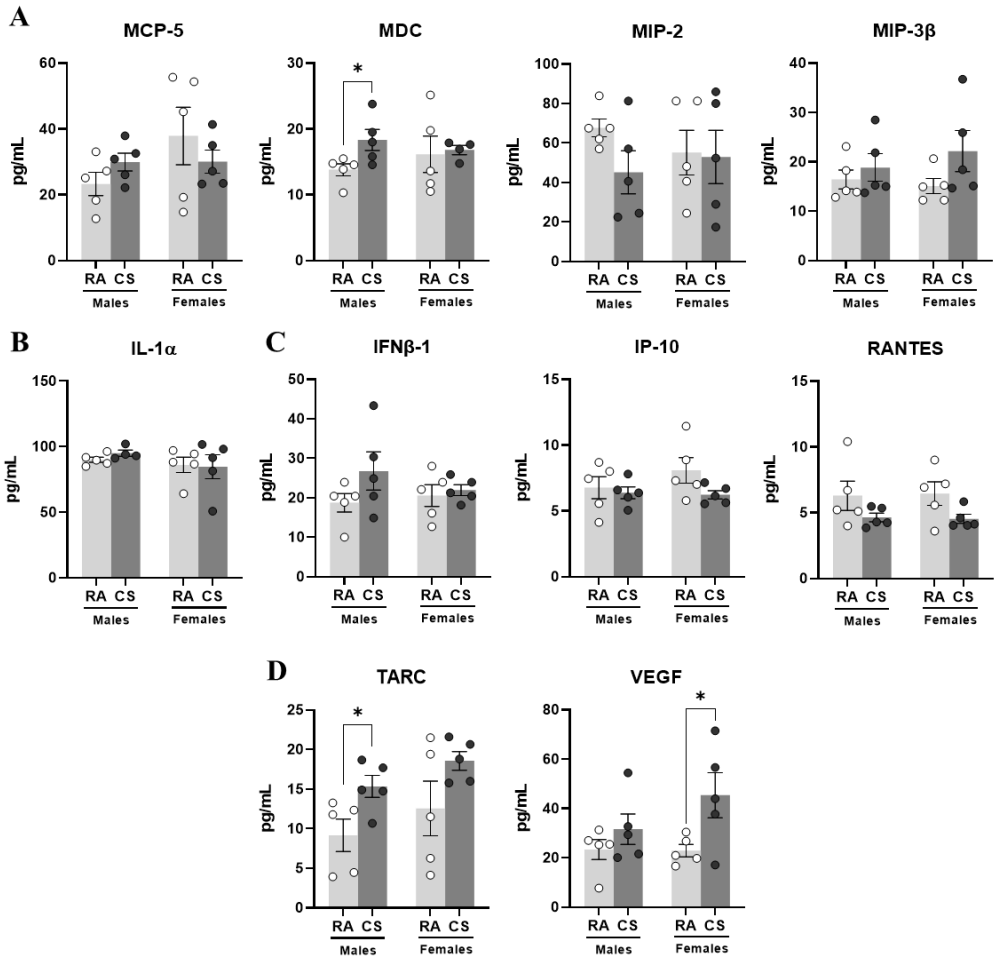

Figure 11. Impact of cannabis smoke exposure on immune mediators in the lungs. Male and female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of 6 cannabis cigarettes (CS) twice a day for four days. Immune mediators were quantified via multiplex analysis (Eve Technologies). Shown mediators are those with quantities above the analysis' lower level of detection and are associated with (A) macrophage cell signaling, (B) smoke-associated inflammation, or (C) anti-viral signaling. (D) Other immune mediators with significant differences above the lower level of detection are also shown. Data represent mean ± SEM; n = 5/group; *P ≤ 0.05, unpaired t-test within each sex.

Table 1. Multiplex analysis of immune mediator expression in the lungs of male and female cannabis smoke exposed mice. Experimental and lower level of detection (LLD) values are represented in pg/mL. **Bolded** factors have quantities above the LLD and contain statistical significance in at least one sex. n = 5/group.

		Males				Females			
Factor	**LLD**	**RA (SEM)**	**CS (SEM)**	**CS:RA Ratio**	**P value**	**RA (SEM)**	**CS (SEM)**	**CS:RA Ratio**	**P value**
6Ckine/Exodus2	4.00	15711.00 (3801.00)	18142.00 (1530.00)	1.15	0.57	21084 (3706.00)	14610 (430.00)	0.69	0.12
Eotaxin	0.40	33.74 (4.98)	42.18 (2.56)	1.25	0.17	41.49 (5.80)	42.11 (2.58)	1.01	0.93
EPO	8.00	0.87 (0.05)	0.94 (0.08)	1.08	0.61	0.83 (0.03)	0.84 (0.06)	1.01	0.87
Fractalkine	8.00	75.68 (15.25)	80.21 (10.72)	1.06	0.81	64.19 (13.62)	69.15 (9.74)	1.08	0.77
G-CSF	0.50	1.00 (0.18)	0.79 (0.08)	0.79	0.37	1.06 (0.13)	1.54 (0.50)	1.45	0.38
GM-CSF	10.90	2.26 (0.17)	2.42 (0.10)	1.07	0.43	2.23 (0.06)	2.05 (0.09)	0.92	0.12
IFNβ-1	10.00	18.74 (2.33)	26.79 (4.86)	1.43	0.17	20.58 (2.75)	21.93 (1.36)	1.07	0.67
IFNγ	0.40	4.37 (0.19)	4.87 (0.30)	1.11	0.15	3.63 (0.80)	4.19 (0.60)	1.15	0.59
IL-1α	10.30	90.03 (1.94)	95.09 (2.40)	1.06	0.14	86.10 (5.79)	84.71 (9.15)	0.98	0.90
IL-1β	5.40	1.25 (0.18)	2.94 (0.20)	2.35	<0.01	1.67 (0.51)	1.37 (0.48)	0.82	0.68
IL-2	0.60	6.06 (0.81)	10.32 (2.16)	1.70	0.10	6.28 (0.60)	6.43 (1.20)	1.02	0.91
IL-3	0.70	0.51 (0.01)	0.48 (0.01)	0.94	0.17	0.49 (0.02)	0.50 (0.02)	1.02	0.79
IL-4	0.20	0.46 (0.01)	0.48 (0.01)	1.04	0.14	0.47 (0.01)	0.47 (0.01)	1.00	>0.99
IL-5	0.30	0.45 (0.01)	0.50 (0.06)	1.11	0.40	0.46 (0.01)	0.43 (0.01)	0.93	0.14
IL-6	0.40	0.47 (0.06)	0.58 (0.09)	1.23	0.34	0.50 (0.05)	0.41 (0.01)	0.82	0.16
IL-7	1.40	1.36 (0.20)	1.97 (0.56)	1.45	0.32	1.51 (0.16)	1.68 (0.30)	1.11	0.64
IL-9	17.30	7.50 (1.90)	9.74 (3.16)	1.30	0.56	9.05 (1.80)	8.53 (1.37)	0.94	0.82
IL-10	1.20	0.43 (0.04)	0.47 (0.08)	1.09	0.62	0.66 (0.12)	0.47 (0.16)	0.71	0.36
IL-11	10.00	2.24 (0.24)	2.39 (0.31)	1.07	0.71	2.74 (0.38)	2.39 (0.18)	0.87	0.42
IL-12p40	3.90	1.61 (0.95)	3.52 (2.20)	2.19	0.45	0.61 (0.33)	0.08 (0.06)	0.13	0.17
IL-12p70	4.80	2.10 (0.11)	1.78 (0.10)	0.85	0.06	1.89 (0.06)	1.79 (0.08)	0.95	0.38
IL-13	33.30	1.28 (0.03)	1.31 (0.02)	1.02	0.35	1.30 (0.02)	1.30 (0.04)	1.00	0.88
IL-15	7.40	5.41 (0.59)	4.78 (0.69)	0.88	0.51	3.88 (0.37)	4.75 (0.49)	1.22	0.20

Table 1 continued.

		Males				Females			
Factor	**LLD**	**RA (SEM)**	**CS (SEM)**	**CS:RA Ratio**	**P value**	**RA (SEM)**	**CS (SEM)**	**CS:RA Ratio**	**P value**
IL-16	1.00	3232.00 (184.00)	3034.00 (243.80)	0.94	0.54	3255.00 (458.70)	3148.00 (316.00)	0.97	0.85
IL-17	0.20	0.51 (0.01)	0.54 (0.03)	1.06	0.29	0.54 (0.02)	0.52 (0.02)	0.96	0.41
IL-20	21.00	2.75 (0.46)	2.54 (0.17)	0.92	0.69	3.07 (0.44)	4.18 (0.19)	1.36	0.07
IP-10	0.10	6.77 (0.84)	6.38 (0.44)	0.94	0.69	8.09 (0.97)	6.24 (0.31)	0.77	0.11
KC	1.80	12.20 (1.63)	13.99 (1.67)	1.15	0.46	10.71 (2.53)	7.27 (2.55)	0.68	0.37
LIF	0.50	0.55 (0.02)	0.48 (0.03)	0.87	0.11	0.54 (0.03)	0.47 (0.01)	0.87	0.06
LIX	22.10	3.43 (0.12)	3.57 (0.38)	1.04	0.74	3.53 (0.31)	3.73 (0.39)	1.07	0.70
MCP-1	6.70	1.92 (0.13)	1.98 (0.37)	1.03	0.89	2.43 (0.10)	1.83 (0.08)	0.75	<0.01
MCP-5	1.00	23.29 (3.54)	29.92 (2.71)	1.28	0.17	37.85 (8.74)	30.06 (3.53)	0.79	0.43
M-CSF	3.50	0.19 (0.01)	0.21 (0.04)	1.11	0.71	0.38 (0.06)	0.14 (0.02)	0.37	0.02
MDC	**2.00**	**13.80 (0.91)**	**18.34 (1.61)**	**1.33**	**0.04**	**16.15 (2.76)**	**16.81 (0.71)**	**1.04**	**0.84**
MIG	2.40	18.33 (2.97)	23.17 (4.27)	1.26	0.38	32.97 (8.60)	31.00 (4.00)	0.94	0.84
MIP-1α	7.70	2.00 (0.35)	3.97 (1.22)	1.99	0.16	2.15 (0.30)	1.76 (0.28)	0.82	0.38
MIP-1β	11.90	0.38 (0.05)	0.28 (0.06)	0.74	0.24	0.31 (0.05)	0.43 (0.13)	1.39	0.39
MIP-2	30.60	67.61 (4.53)	45.16 (10.98)	0.67	0.10	55.15 (11.34)	52.94 (13.53)	0.96	0.90
MIP-3α	5.00	1.05 (0.07)	1.35 (0.25)	1.29	0.29	1.09 (0.12)	1.09 (0.10)	1.00	0.97
MIP-3β	8.00	16.44 (1.91)	18.87 (2.79)	1.15	0.49	15.07 (1.53)	22.17 (4.17)	1.47	0.15
RANTES	2.70	6.29 (1.12)	4.65 (0.33)	0.74	0.20	6.46 (0.90)	4.53 (0.34)	0.70	0.08
TARC	**2.00**	**9.15 (2.04)**	**15.34 (1.40)**	**1.68**	**0.04**	**12.56 (3.45)**	**18.56 (1.18)**	**1.48**	**0.14**
TIMP-1	2.00	223.70 (23.69)	251.60 (20.44)	1.12	0.40	190.60 (43.24)	207.50 (9.30)	1.09	0.71
TNFα	1.70	0.11 (0.01)	0.11 (0.01)	1.00	0.60	0.11 (0.01)	0.11 (0.01)	1.00	0.85
VEGF	**0.10**	**23.36 (4.07)**	**31.63 (6.17)**	**1.35**	**0.30**	**22.90 (2.50)**	**45.39 (9.13)**	**1.98**	**0.04**

3.2. Concurrent Cannabis Smoke Exposure and Influenza Infection

3.2.1. Morbidity and Viral Titre

To investigate the effects of cannabis smoke on influenza-associated health outcomes and immune responses, male and female BALB/c mice were concurrently exposed to cannabis smoke and infected with mouse-adapted influenza A virus (**Figure 2**). Weight loss relative to starting weight was exacerbated in male cannabis-exposed, infected mice relative to room air infected controls, at days 4 and 5 (**Figure 12A**). Weight loss relative to starting weight was also exacerbated in female cannabis-exposed, infected mice at days 5 and 6 (**Figure 12B**). Total weight loss over the entire post-infection period was unchanged between cannabis smoke-exposed infected mice and infected controls, in both males and females. None of the infected mice displayed significant symptoms beyond weight loss or reached endpoint over the course of the infection.

Following concurrent cannabis smoke exposure and influenza A infection, viral titre in the lung tissue at 6 days post-infection (dpi) was quantified. No change in viral titre was observed in male cannabis-exposed, infected mice compared to infected controls (**Figure 13A**). However, female cannabis-exposed, infected mice had a near two-fold elevation of viral titre relative to infected controls (**Figure 13B**). Virus was not detectable in uninfected control groups (data not shown).

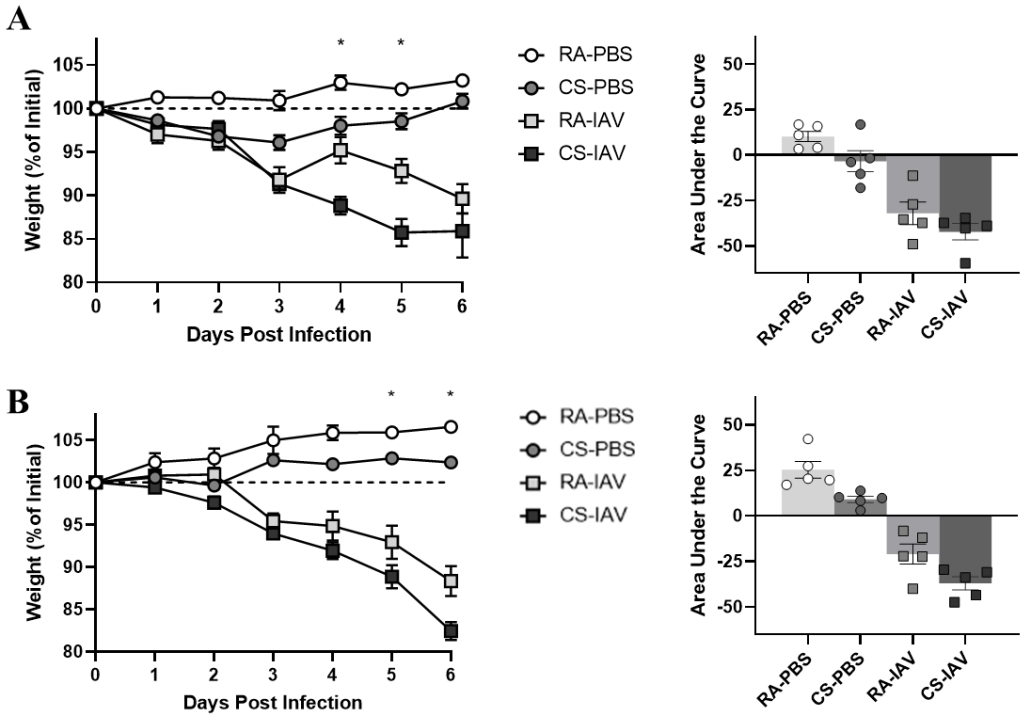

Figure 12. Cannabis smoke exposure alters weight loss following influenza A infection. (A) Male and (B) female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice per day for five days. Following exposure, mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or phosphate buffered saline (PBS) vehicle. After a two-day break, exposure protocols resumed for three days and samples were collected on the fourth day. Mice were weighed at the time of inoculation and continued to be weighed daily until sample collection. Significance asterisks indicate a significant difference between RA-IAV and CS-IAV groups. Area under the curve assessed by setting 100% weight at 0 days post infection (dotted line) as the baseline. Data represent mean ± SEM; n = 5/group; *P ≤ 0.05, two-way ANOVA with Tukey's multiple comparisons test between all experimental groups.

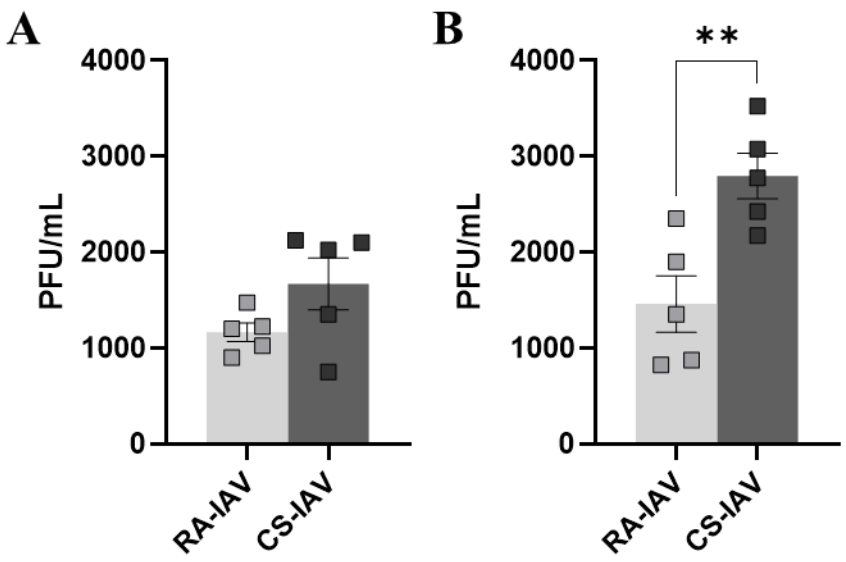

Figure 13. Cannabis smoke exposure has minimal effect on viral titre in the lungs at 6 days post-influenza A infection. (A) Male and (B) female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice per day for five days. Following exposure, mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or phosphate buffered saline (PBS) vehicle. After a two-day break, exposure protocols resumed for three days and samples were collected on the fourth day. Viral titre was quantified in lung tissue homogenate via plaque assay using Madin-Darby Canine Kidney (MDCK) cells. No viral plaques were detected in uninfected groups. Data represent mean ± SEM; n = 5/group; *P ≤ 0.05, **P ≤ 0.01, unpaired t-test between experimental groups.

3.2.2. Lung Immune Cell Populations

To examine how lung immune responses to influenza infection are influenced by cannabis smoke exposure, we first quantified immune cell populations in the lung tissue. Flow cytometric analysis revealed moderate modulation of lung innate immune cell populations in cannabis-exposed, infected mice at 6 days post-infection. In male mice, the proportion of monocytes and classical dendritic cells were decreased compared to infected controls, while $CD45^+$ cells, macrophages, and neutrophils remained unchanged (**Figure 14A**). Conversely, in female mice, the proportion of $CD45^+$ cells decreased, neutrophils increased, and macrophages, monocytes, and classical dendritic cells remained unchanged, relative to infected controls (**Figure 14B**). Total cellularity was unchanged between male cannabis-exposed and control infected groups (**Figure 15A**). However, total $CD45^+$ cells, macrophages, monocytes, and classical dendritic cells were decreased in female cannabis-exposed, infected mice (**Figure 15B**). Cannabis smoke alone did not alter any of the studied populations in either sex, except for classical dendritic cells, which were decreased proportionally and totally in female cannabis smoke-exposed mice.

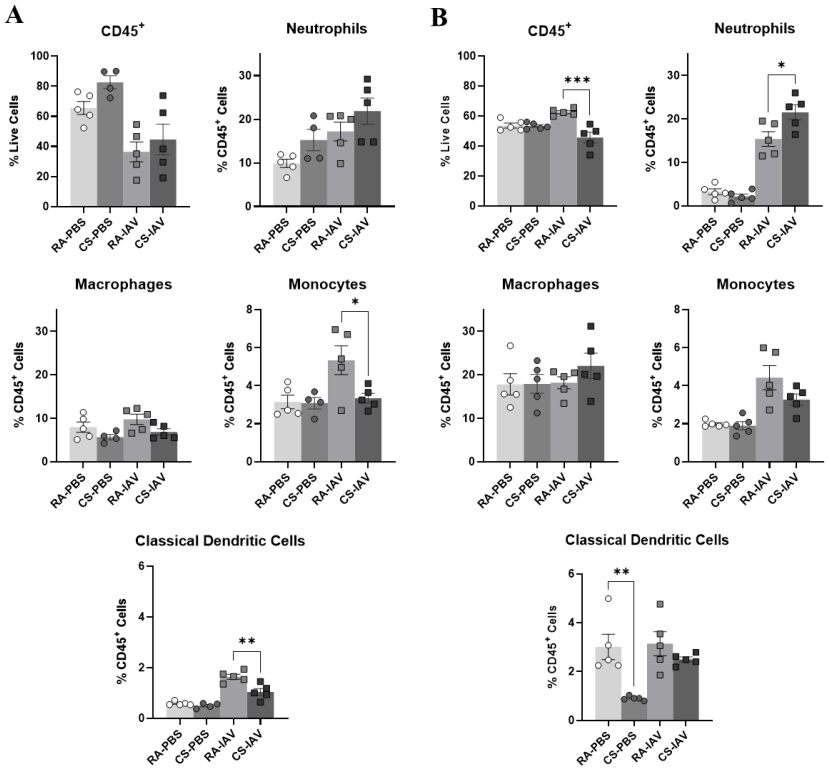

Figure 14. Concurrent cannabis smoke exposure and influenza A infection modulates the <u>proportionality</u> of innate immune cell populations in the lungs. (A) Male and (B) female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice per day for five days. Following exposure, mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or phosphate buffered saline (PBS) vehicle. After a two-day break, exposure protocols resumed for three days and samples were collected on the fourth day. Lung innate immune cell populations were quantified via flow cytometry. Proportionality was determined via haemocytometer, Türk's solution staining, and light microscopy. Data represent mean ± SEM; n = 4 – 5/group; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, two-way ANOVA with Tukey's multiple comparisons test between all experimental groups; only significance within inoculation groups shown.

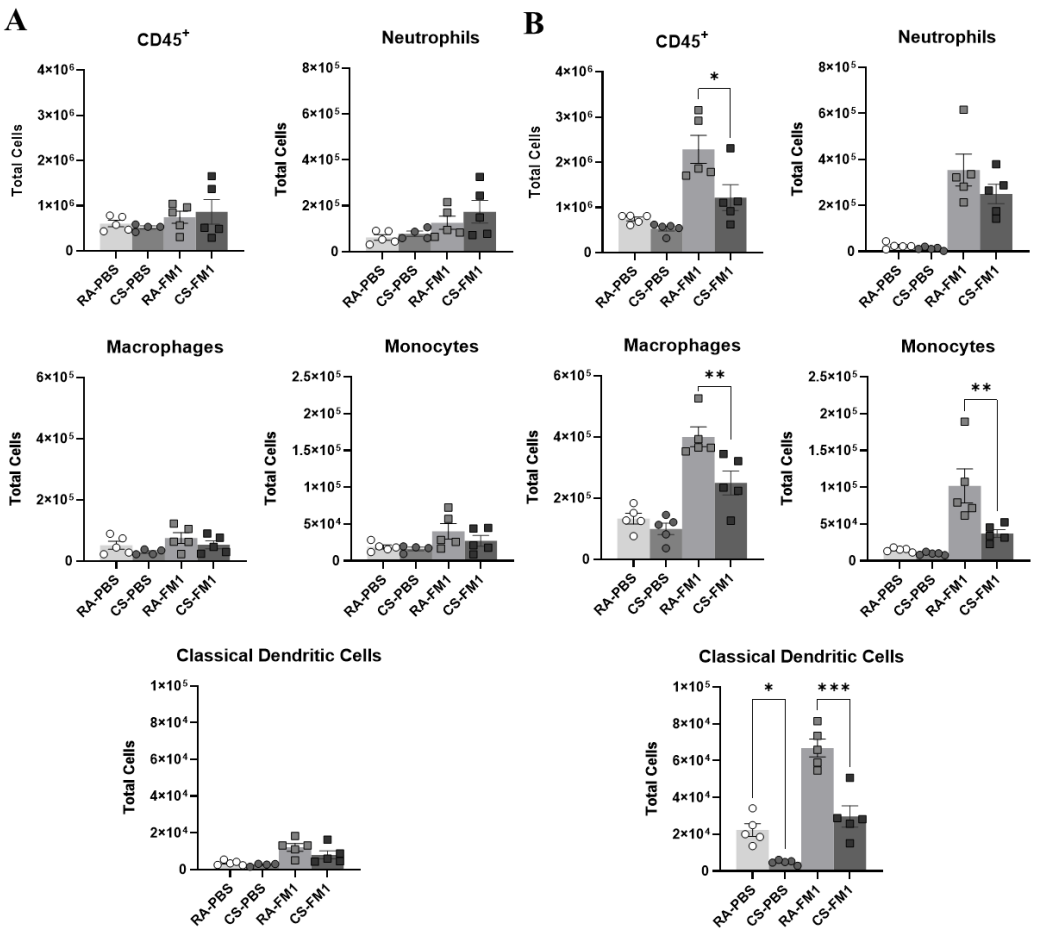

Figure 15. Concurrent cannabis smoke exposure and influenza A infection modulates <u>total innate immune cellularity</u> in the lungs. (A) Male and (B) female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice per day for five days. Following exposure, mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or phosphate buffered saline (PBS) vehicle. After a two-day break, exposure protocols resumed for three days and samples were collected on the fourth day. Lung innate immune cell populations were quantified via flow cytometry. Total cell count was determined via haemocytometer, Türk's solution staining, and light microscopy. Data represent mean ± SEM; n = 4 – 5/group; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, two-way ANOVA with Tukey's multiple comparisons test between all experimental groups; only significance within inoculation groups shown.

3.2.3. Lung Immune Mediators

To assess whether cannabis smoke exposure affects immune signaling following influenza infection, we quantified immune mediators in the lung tissue using a 44-plex cytokine array. Principle component analysis revealed that global immune mediator cluster separation was primarily driven by influenza infection (males – $P < 0.001$, females – $P < 0.001$) rather than cannabis smoke exposure (males – $P = 0.63$, females – $P = 0.14$) (**Figure 16**). Within infected mice, cluster separation between room air and cannabis-exposed mice was insignificant in both sexes (males – adjusted $P = 1.00$, females – adjusted $P = 0.708$). In addition, immune mediator quantification revealed significant suppression of anti-viral signaling molecules in cannabis-exposed, infected mice of both sexes (**Figure 17**). In male cannabis-exposed, infected mice, IFNγ, IP-10, RANTES, and TNFα were decreased compared to infected controls. Similarly, IFNγ and RANTES were decreased in female cannabis-exposed, infected mice. No changes were detected between uninfected room air and cannabis smoke exposed mice. All other mediators assessed are presented with their concentrations, level of detection, cannabis smoke to room air ratio, and P value in **Table 2** and **Table 3**.

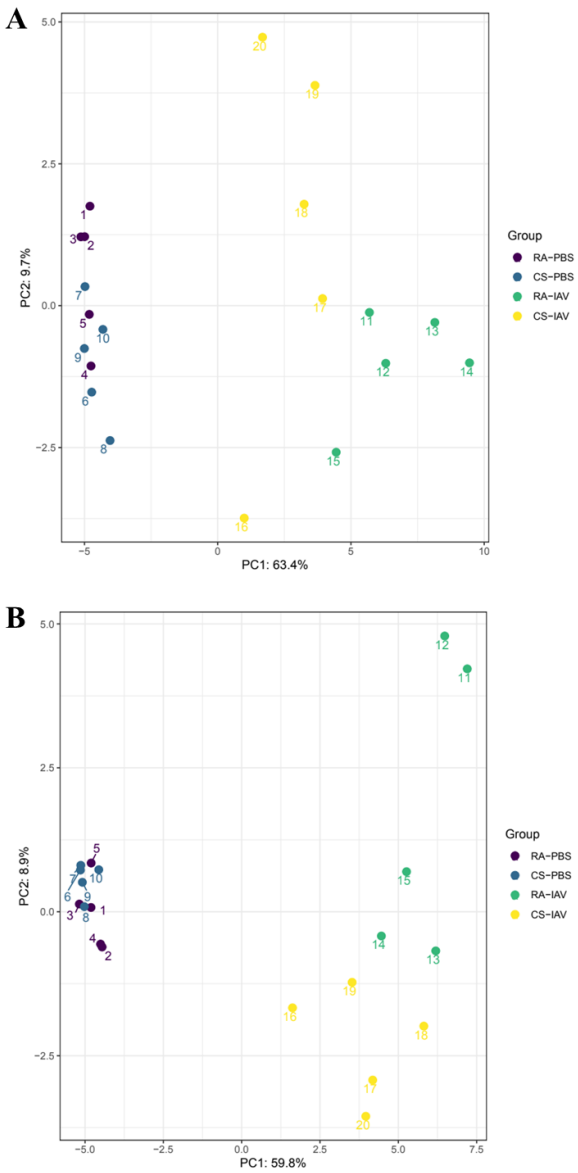

Figure 16. Cannabis smoke exposure minimally impacts global immune mediator clustering following influenza A infection. (A) Male and (B) female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice per day for five days. Following exposure, mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or phosphate buffered saline (PBS) vehicle. After a two-day break, exposure protocols resumed for three days and samples were collected on the fourth day. Immune mediators were quantified via multiplex analysis (Eve Technologies). Global immune mediator clustering was identified using principal component analysis (PCA). n = 5/group.

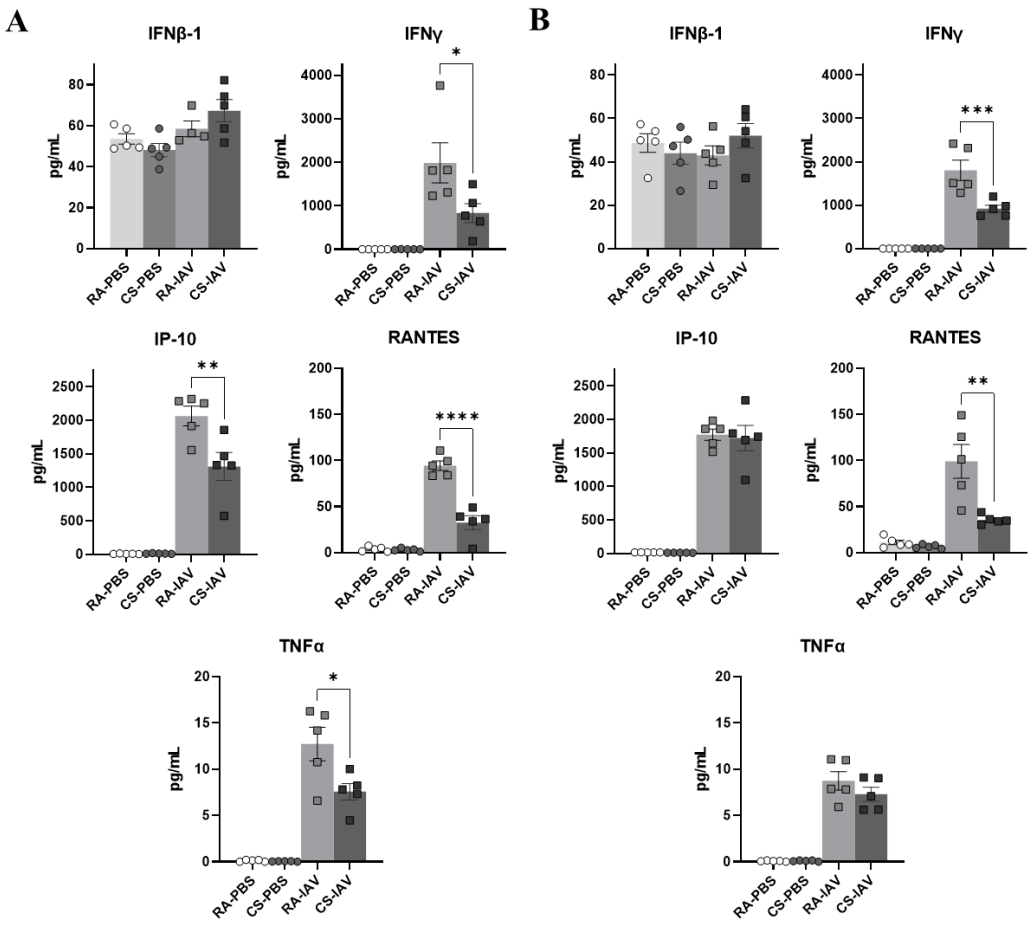

Figure 17. Cannabis smoke exposure suppresses influenza A virus-induced anti-viral immune mediator expansion in the lungs. (A) Male and (B) female six- to eight-week-old BALB/c mice were exposed to room air (RA) or the smoke of six cannabis cigarettes (CS) twice per day for five days. Following exposure, mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or phosphate buffered saline (PBS) vehicle. After a two-day break, exposure protocols resumed for three days and samples were collected on the fourth day. Immune mediators were quantified via multiplex analysis (Eve Technologies). Data represent mean ± SEM; n = 5/group; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, two-way ANOVA with Tukey's multiple comparisons test between all experimental groups; only significance within inoculation groups shown; statistical outliers removed (ROUT, Q = 1%)

Table 2. Multiplex analysis of immune mediator expression in the lungs of male room air and cannabis smoke exposed mice with concurrent influenza A infection. Experimental and lower level of detection (LLD) values are represented in pg/mL. **Bolded** factors have quantities above the LLD and contain statistical significance in at least one vehicle or virus infected pairing. n = 5/group, two-way ANOVA with Tukey's post-hoc test, outliers removed (ROUT, Q = 1%).

Factor	LLD	RA-PBS (SEM)	CS-PBS (SEM)	CS:RA Ratio	P value	RA-IAV (SEM)	CS-IAV (SEM)	CS:RA Ratio	P value
6Ckine/Exodus2	4.00	1027.00 (77.70)	861.30 (188.30)	0.84	0.81	725.80 (51.50)	560.80 (127.30)	0.77	0.86
Eotaxin	0.40	39.85 (6.02)	55.14 (6.23)	1.38	0.45	119.70 (10.41)	92.89 (4.14)	0.78	0.07
EPO	8.00	-	-	-	-	-	-	-	-
Fractalkine	8.00	86.51 (11.10)	64.94 (7.21)	0.75	0.21	44.59 (5.77)	48.26 (3.27)	1.08	0.98
G-CSF	0.50	0.54 (0.11)	1.13 (0.22)	2.09	>0.99	675.60 (94.21)	651.50 (136.50)	0.96	>0.99
GM-CSF	10.90	1.08 (0.19)	1.44 (0.25)	1.33	>0.99	18.59 (0.80)	24.72 (3.80)	1.33	0.16
IFNβ-1	10.00	53.57 (2.51)	48.12 (3.24)	0.90	0.75	58.47 (3.86)	67.33 (5.49)	1.15	0.44
IFNγ	**0.40**	**1.44 (0.27)**	**2.80 (0.24)**	**1.94**	**>0.99**	**1986.00 (460.60)**	**832.60 (218.20)**	**0.42**	**0.03**
IL-1α	10.30	36.95 (2.11)	48.79 (6.55)	1.32	0.40	113.60 (6.55)	96.01 (4.09)	0.85	0.12
IL-1β	**5.40**	**4.12 (0.32)**	**3.96 (0.10)**	**0.96**	**>0.99**	**26.12 (0.52)**	**18.48 (0.76)**	**0.71**	**<0.01**
IL-2	0.60	2.93 (0.20)	3.65 (0.48)	1.25	0.73	2.41 (0.44)	2.30 (0.71)	0.95	>0.99
IL-3	**0.70**	**0.06 (0.03)**	**0.16 (0.05)**	**2.67**	**0.99**	**3.32 (0.14)**	**2.07 (0.55)**	**0.62**	**0.03**
IL-4	**0.20**	**0.03 (0.01)**	**0.08 (0.05)**	**2.67**	**>0.99**	**1.25 (0.29)**	**0.26 (0.08)**	**0.21**	**<0.01**
IL-5	**0.30**	**0.36 (0.08)**	**1.01 (0.47)**	**2.81**	**0.99**	**24.13 (2.93)**	**15.49 (1.22)**	**0.64**	**0.01**
IL-6	0.40	1.22 (0.23)	1.84 (0.38)	1.51	>0.99	833.60 (67.65)	654.70 (65.76)	0.79	0.07
IL-7	1.40	0.82 (0.14)	1.26 (0.23)	1.54	0.44	1.00 (0.18)	0.80 (0.23)	0.80	0.89
IL-9	17.30	8.07 (2.60)	10.36 (2.38)	1.28	0.86	15.03 (0.31)	12.47 (2.23)	0.83	0.82
IL-10	**1.20**	**0.23 (0.14)**	**0.34 (0.16)**	**1.48**	**>0.99**	**77.72 (9.24)**	**31.38 (3.98)**	**0.40**	**<0.01**
IL-11	10.00	-	-	-	-	117.90 (27.58)	115.40 (31.38)	0.98	>0.99
IL-12p40	3.90	9.95 (1.25)	16.54 (2.02)	1.66	0.13	8.33 (1.12)	7.62 (1.12)	0.91	0.99
IL-12p70	4.80	0.62 (0.10)	0.27 (0.07)	0.44	>0.99	8.23 (2.66)	3.98 (1.03)	0.48	0.19
IL-13	33.30	0.02 (0.01)	0.05 (0.02)	2.50	>0.99	1.88 (0.30)	1.27 (0.07)	0.68	0.06
IL-15	7.40	2.83 (0.52)	3.17 (0.74)	1.12	0.99	5.80 (1.39)	3.68 (0.80)	0.63	0.39

Table 2 continued.

Factor	LLD	RA-PBS (SEM)	CS-PBS (SEM)	CS:RA Ratio	P value	RA-IAV (SEM)	CS-IAV (SEM)	CS:RA Ratio	P value
IL-16	1.00	269.90 (49.36)	165.60 (30.58)	0.61	0.42	213.30 (47.07)	266.70 (57.27)	1.25	0.85
IL-17	**0.20**	**0.51 (0.09)**	**0.78 (0.27)**	**1.53**	**0.93**	**3.68 (0.21)**	**1.56 (0.52)**	**0.42**	**<0.01**
IL-20	21.00	-	-	-	-	-	-	-	-
IP-10	**0.10**	**9.10 (1.85)**	**12.31 (2.06)**	**1.35**	**>0.99**	**2064.00 (146.50)**	**1311.00 (207.8)**	**0.64**	**<0.01**
KC	1.80	18.65 (1.41)	19.90 (0.30)	1.07	>0.99	472.70 (22.87)	336.60 (68.09)	0.71	0.07
LIF	**0.50**	**0.12 (0.02)**	**0.15 (0.02)**	**1.25**	**>0.99**	**26.43 (4.60)**	**15.57 (2.63)**	**0.59**	**0.05**
LIX	22.10	-	-	-	-	22.23 (1.05)	17.00 (7.09)	0.76	0.63
MCP-1	6.70	9.21 (2.28)	12.68 (0.49)	1.38	>0.99	958.20 (116.90)	715.00 (79.62)	0.75	0.11
MCP-5	1.00	16.40 (2.82)	12.51 (0.76)	0.76	>0.99	617.50 (107.2)	360.90 (126.9)	0.58	0.15
M-CSF	**3.50**	**0.52 (0.14)**	**0.45 (0.04)**	**0.87**	**>0.99**	**11.54 (1.52)**	**6.83 (1.01)**	**0.59**	**0.01**
MDC	2.00	8.23 (1.00)	10.18 (1.73)	1.24	0.79	11.72 (1.98)	11.12 (0.57)	0.95	0.99
MIG	2.40	26.36 (3.61)	23.96 (5.42)	0.91	>0.99	1684.00 (134.30)	1266.00 (255.90)	0.75	0.21
MIP-1α	7.70	12.87 (1.41)	17.54 (0.51)	1.36	>0.99	414.80 (54.28)	261.80 (64.34)	0.63	0.09
MIP-1β	**11.90**	**1.11 (0.45)**	**1.96 (0.53)**	**1.77**	**>0.99**	**1246.00 (167.40)**	**625.30 (150.90)**	**0.50**	**0.01**
MIP-2	30.60	9.56 (4.20)	23.30 (10.95)	2.44	0.52	99.14 (6.20)	80.66 (4.31)	0.81	0.28
MIP-3α	5.00	0.22 (0.05)	0.42 (0.10)	1.91	0.87	1.38 (0.28)	1.09 (0.23)	0.79	0.71
MIP-3β	8.00	11.03 (1.07)	9.42 (1.33)	0.85	>0.99	54.48 (6.99)	40.18 (6.54)	0.74	0.20
RANTES	**2.70**	**3.84 (1.19)**	**3.10 (0.68)**	**0.81**	**>0.99**	**94.38 (5.11)**	**32.54 (7.51)**	**0.34**	**<0.01**
TARC	2.00	6.50 (1.43)	5.91 (0.94)	0.91	0.99	4.55 (1.61)	8.29 (1.11)	1.16	0.22
TIMP-1	2.00	358.50 (67.55)	295.80 (14.15)	0.83	>0.99	19334.00 (2152.00)	21803.00 (3026)	1.13	0.78
TNFα	**1.70**	**0.11 (0.04)**	**0.04 (0.01)**	**0.36**	**>0.99**	**12.73 (1.81)**	**7.57 (0.90)**	**0.59**	**0.01**
VEGF	0.10	3.86 (0.73)	5.46 (1.13)	1.41	0.64	2.28 (1.09)	2.26 (0.70)	0.99	>0.99

Table 3. Multiplex analysis of immune mediator expression in the lungs of female room air and cannabis smoke exposed mice with concurrent influenza A infection. Experimental and lower level of detection (LLD) values are represented in pg/mL. **Bolded** factors have quantities above the LLD and contain statistical significance in at least one vehicle or virus infected pairing. n = 5/group, two-way ANOVA with Tukey's post-hoc test, outliers removed (ROUT, Q = 1%).

Factor	LLD	RA-PBS (SEM)	CS-PBS (SEM)	CS:RA Ratio	P value	RA-IAV (SEM)	CS-IAV (SEM)	CS:RA Ratio	P value
6Ckine/Exodus2	4.00	916.30 (99.54)	473.50 (94.14)	0.52	0.08	517.80 (145.90)	401.20 (81.59)	0.77	0.86
Eotaxin	0.40	63.22 (9.64)	79.11 (6.66)	1.25	0.83	140.30 (23.40)	109.90 (4.35)	0.78	0.39
EPO	8.00	-	-	-	-	-	-	-	-
Fractalkine	8.00	42.94 (4.59)	42.00 (3.03)	0.98	>0.99	32.63 (4.391)	24.55 (0.88)	0.75	0.47
G-CSF	0.50	0.50 (0.04)	0.56 (0.10)	1.12	>0.99	744.30 (40.71)	797.50 (60.40)	1.07	0.73
GM-CSF	**10.90**	**0.50 (0.12)**	**0.55 (0.07)**	**1.10**	**>0.99**	**14.29 (1.46)**	**19.25 (0.87)**	**1.35**	**<0.01**
IFNβ-1	10.00	48.68 (4.28)	43.96 (5.08)	0.90	0.90	42.99 (4.36)	52.08 (5.55)	1.21	0.56
IFNγ	**0.40**	**2.08 (0.34)**	**1.90 (0.33)**	**0.91**	**>0.99**	**1805.00 (234.50)**	**923.10 (82.54)**	**0.51**	**<0.01**
IL-1α	10.30	33.64 (1.82)	31.96 (1.10)	0.95	0.97	103.60 (3.39)	101.60 (3.28)	0.98	0.95
IL-1β	5.40	4.19 (0.51)	3.66 (0.12)	0.87	0.99	21.68 (2.29)	17.04 (0.50)	0.79	0.06
IL-2	0.60	3.53 (0.17)	4.29 (0.09)	1.22	0.06	2.51 (0.19)	1.85 (0.28)	0.74	0.12
IL-3	0.70	0.04 (0.02)	0.04 (0.01)	1.00	>0.99	1.44 (0.39)	0.93 (0.23)	0.65	0.42
IL-4	**0.20**	**0.04 (0.01)**	**0.02 (0.01)**	**0.50**	**0.98**	**0.70 (0.10)**	**0.14 (0.03)**	**0.20**	**<0.01**
IL-5	**0.30**	**0.39 (0.06)**	**0.19 (0.03)**	**0.49**	**>0.99**	**45.84 (17.89)**	**13.46 (3.06)**	**0.29**	**0.04**
IL-6	0.40	1.03 (0.16)	0.94 (0.12)	0.91	>0.99	822.70 (171.1)	754.60 (86.43)	0.92	0.96
IL-7	1.40	0.72 (0.16)	0.53 (0.13)	0.74	0.61	0.26 (0.08)	0.38 (0.06)	1.46	0.89
IL-9	17.30	6.74 (1.06)	14.58 (1.76)	2.16	0.05	9.43 (2.34)	6.68 (2.33)	0.71	0.75
IL-10	1.20	0.18 (0.07)	0.16 (0.03)	0.88	>0.99	88.33 (4.19)	62.11 (14.70)	0.70	0.11
IL-11	10.00	-	-	-	-	84.46 (21.13)	94.15 (22.14)	1.11	0.97
IL-12p40	3.90	8.18 (1.04)	11.24 (1.48)	1.37	0.27	3.27 (0.75)	3.32 (1.15)	1.02	>0.99
IL-12p70	4.80	0.44 (0.14)	0.21 (0.09)	0.48	0.89	2.10 (0.45)	2.72 (0.28)	1.30	0.34
IL-13	**33.30**	**0.01 (0.01)**	**0.04 (0.02)**	**4.00**	**>0.99**	**1.82 (0.16)**	**1.31 (0.13)**	**0.72**	**0.02**
IL-15	7.40	1.51 (0.25)	1.06 (0.13)	0.70	0.83	2.40 (0.62)	1.78 (0.28)	0.74	0.65

Table 3 continued.

Factor	LLD	RA-PBS (SEM)	CS-PBS (SEM)	CS:RA Ratio	P value	RA-IAV (SEM)	CS-IAV (SEM)	CS:RA Ratio	P value
IL-16	1.00	251.00 (35.98)	139.40 (22.46)	0.56	0.07	132.50 (30.42)	108.90 (26.13)	0.82	0.94
IL-17	**0.20**	**0.44 (0.03)**	**0.52 (0.55)**	**1.18**	**0.99**	**1.60 (0.41)**	**0.84 (0.06)**	**0.53**	**0.04**
IL-20	21.00	-	-	-	-	-	-	-	-
IP-10	0.10	17.71 (0.47)	11.78 (0.59)	0.67	>0.99	1772.00 (83.87)	1721.00 (189.00)	0.97	0.99
KC	**1.80**	**28.74 (4.17)**	**16.15 (1.41)**	**0.56**	**0.99**	**404.20 (38.81)**	**276.20 (37.94)**	**0.68**	**0.02**
LIF	0.50	0.06 (0.02)	0.10 (0.02)	1.67	>0.99	22.31 (2.05)	26.11 (4.18)	1.17	0.66
LIX	22.10	-	-	-	-	-	-	-	-
MCP-1	6.70	13.90 (0.72)	12.00 (1.38)	0.86	>0.99	1093.00 (249.20)	965.30 (102.5)	0.88	0.91
MCP-5	1.00	19.45 (1.28)	14.48 (1.59)	0.74	>0.99	406.90 (109.90)	305.70 (63.45)	0.75	0.68
M-CSF	3.50	0.83 (0.13)	0.61 (0.17)	0.73	>0.99	8.79 (1.67)	8.40 (2.25)	0.96	>0.99
MDC	**2.00**	**9.28 (0.53)**	**6.77 (0.97)**	**0.73**	**0.33**	**12.14 (1.18)**	**7.20 (1.23)**	**0.59**	**0.02**
MIG	**2.40**	**20.38 (1.82)**	**8.30 (1.78)**	**0.41**	**>0.99**	**2462.00 (484.80)**	**879.10 (38.15)**	**0.36**	**<0.01**
MIP-1α	7.70	12.39 (2.24)	14.78 (1.59)	1.19	>0.99	568.30 (139.60)	354.00 (26.81)	0.62	0.19
MIP-1β	11.90	1.49 (0.94)	0.40 (0.16)	0.27	>0.99	1659.00 (394.20)	865.60 (60.63)	0.52	0.05
MIP-2	30.60	15.52 (4.74)	16.19 (2.69)	1.04	>0.99	111.30 (11.83)	90.22 (3.36)	0.81	0.16
MIP-3α	**5.00**	**0.52 (0.06)**	**0.42 (0.04)**	**0.81**	**0.97**	**1.96 (0.20)**	**0.74 (0.24)**	**0.38**	**<0.01**
MIP-3β	8.00	10.73 (0.61)	9.74 (0.60)	0.91	>0.99	42.99 (3.17)	36.42 (4.89)	0.85	0.42
RANTES	**2.70**	**11.15 (2.43)**	**6.69 (0.93)**	**0.60**	**0.99**	**98.98 (18.31)**	**35.81 (2.24)**	**0.36**	**<0.01**
TARC	2.00	7.90 (1.57)	8.96 (1.51)	1.13	0.94	4.93 (1.16)	4.59 (0.85)	0.93	>0.99
TIMP-1	2.00	281.70 (11.18)	376.70 (29.79)	1.34	>0.99	21992.00 (2643.00)	18496.00 (3141.00)	0.84	0.63
TNFα	1.70	0.07 (0.02)	0.08 (0.03)	1.14	>0.99	8.74 (1.00)	7.29 (0.77)	0.83	0.40
VEGF	0.10	9.89 (1.86)	9.22 (1.11)	0.93	0.97	2.65 (0.46)	2.51 (0.52)	0.95	>0.99

CHAPTER 4: DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSION

4.1. Discussion

The increasing recreational and medicinal use of cannabis has brought to light an absence of experimental evidence pertaining to potential pulmonary health risks. Given that our limited knowledge is based on historical data where cannabis compositions significantly varied, we sought to understand the effect of inhaled cannabis smoke using a well-controlled system and modern, compositionally-relevant cannabis strains[77,78]. We demonstrated that cannabis smoke exposure resulted in systemic distribution of cannabinoids, the detection of which rapidly degraded. Lung immune profiling revealed consistent patterns between male and female mice, with changes in monocyte and macrophage cell populations and immune mediators, such as macrophage-derived chemokine (MDC), TARC (CCL17), and vascular endothelial growth factor (VEGF). As well, we demonstrated that concurrent cannabis smoke exposure and influenza infection leads to altered immunopathology, characterized by altered immune cell infiltration and suppressed anti-viral signaling. Collectively, our findings create a modernized foundation for further exploration of different strains of cannabis, exposure protocols, and interaction with pathogens important in lung health and disease.

The development of this cannabis smoke exposure model was based heavily on existing models of tobacco smoke exposure that have been successfully used in respiratory investigations, among others[67]. Cannabis provides unique challenges in terms of model development compared to tobacco due to the variety of strain options and lack of controlled, research-grade cigarette products available. As such, we opted to establish our model using indica-dominant strains with THC levels of 10 – 14% and CBD levels of 0 – 2 % due to the popularity of this composition among recreational users[77]. At the time of this report, no licensed cannabis retailers in Canada manufacture consistent cannabis cigarettes on a scale large enough to facilitate research. Thus, we developed an in-house method to produce such cigarettes using ground dried cannabis (**Figure 1**). As a result, our model is the first of its kind to use commercially available, compositionally relevant cannabis in a high-capacity exposure system utilizing consistent cigarettes. Previous models are limited in relevance to modern, real world use due to the legality of cannabis and changes in popular strain compositions over time. In addition, our model characterization involved the assessment of both male and female mice, given the growing body of evidence that sex differences play an integral role in cannabis

metabolism and cannabinoid signaling[100–102]. With these experimental variables implemented, our cannabis smoke exposure system effectively models real-world cannabis use patterns while respecting the need for reliability in a research environment.

Our cannabis smoke exposure model was effective at delivering cannabis smoke to mice. Within the exposure chamber, mice displayed common symptoms of smoke exposure seen in our previous tobacco studies but tolerated smoke exposure well[67,95]. While studies have demonstrated that cannabis smoke increases locomotion in rats[103], our model has the opposite effect, potentially due to the opposing effects of prolonged time in the smoke-filled exposure chamber. TPM in the chamber was seen to be at similar levels to those seen in tobacco smoke studies using the same system[67,95], which may also contribute to the behavioural changes we observed.

In our post-exposure analysis, we found elevated levels of COHb, THC, and THC-COOH across the sexes that decreased 60 minutes post exposure (**Figure 4**, **5**). In human cannabis smokers, plasma levels of THC and CBD dose-dependently rise immediately following smoke inhalation and subsequently fall upon smoking cessation[104,105]. This decline corresponds with a slow increase in plasma THC-COOH, as THC is metabolised[105]. While only trace amounts of plasma THC are typically detected after an hour following cessation, plasma THC-COOH can remain elevated for multiple days following administration, making it easily quantifiable in diagnostic settings[15]. The trends we observed in COHb, THC, CBD and THC-COOH at the 15 minute time point correspond with those seen in humans immediately following high THC, low CBD cannabis smoke inhalation and cessation. The decline in COHb and THC at 60 minutes correspond with expectations, however, the fall in plasma THC-COOH does not match human pharmacokinetics. Importantly, plasma samples from the two timepoints were taken from two separate exposures with different experimental animals. As such, variations between exposure sessions could result in differences in the amount of cannabis smoke delivered. This potential effect would be diminished in longer protocols that involve multiple days of exposure to balance out any single session variability. Future quantifications of plasma cannabinoids should prioritize tracing metabolite levels from the same animals over time to clarify these dynamics. Nonetheless, we confirmed that our model system resulted in the systemic delivery of cannabinoids and smoke-derived products, at a magnitude equivalent with human smokers.

Maintaining immune homeostasis in the lungs is essential to support positive respiratory health outcomes. Previous studies have demonstrated a strong link between tobacco smoke exposure, chronic pulmonary inflammation, and the development of COPD[106] – a relationship not yet fully explored in terms of cannabis smoke. Instigating this relationship is a smoke-induced modulation of immune cell populations and mediators leading to a chronic state of lung inflammation and tissue damage. In particular, innate immune cell populations such as lung macrophages and neutrophils have been implicated to be the main effector cells in the development of smoke-associated lung inflammation[67,97,107]. Given this evidence, we initiated our characterization of cannabis smoke-induced alterations to respiratory immunity by investigating whether cannabis smoke exposure alters immune cell populations.

Our findings show that four days of cannabis smoke exposure modulated innate immune cell populations in the airways and lung tissue (**Figure 6**, **7**, **8, 9, 10**). Specifically, we found that macrophages were increased in the airways of female smoke-exposed mice and proportionally increased in the lung tissue of male smoke-exposed mice. These increases were observed in conjunction with decreases in monocyte populations, suggesting that monocyte recruitment, activation, and differentiation may be specifically affected by cannabis smoke exposure. In addition, our analysis of lung macrophage subpopulations demonstrates a potential effect on monocyte-derived subtypes that have been associated previously with heightened inflammatory profiles and may contribute to the development of lung pathologies[60,62,96]. The lack of change in total cell counts within these populations suggests that our cannabis smoke exposure protocol mildly affects the composition of innate immune populations in the lungs, but not to a degree high enough to affect overall cellularity. In addition, our data shows that four days of cannabis smoke exposure did not lead to any changes in neutrophils in the airways or lung tissue. While the current tobacco smoke literature suggests that exacerbated neutrophilia in the lungs is a prominent contributor to smoke-associated chronic inflammation, increases in macrophage quantity and phenotypic changes are thought to be the driver of exacerbated neutrophilic infiltration into smoke-exposed lung tissue[107]. Therefore, our findings are consistent with the hypothesis that acute cannabis smoke exposure may be inducing early macrophage-associated immunomodulation that characterizes tobacco smoke-associated chronic inflammation.

Along with immune cell dysregulation, elevated pro-inflammatory cytokine levels have been associated with the induction of chronic lung inflammation and the development of COPD[108]. Additionally, tobacco and cannabis smoke has been

suggested to modulate immune mediators involved in anti-viral signaling[98,99]. Our immune mediator analysis demonstrates a subtle modulation of the detected cytokines and chemokines in the lung tissue after four days of cannabis smoke exposure (**Figure 11**, **Table 1**). Of the detected mediators, our findings indicated increases of MDC, TARC, and VEGF in both sexes. MDC and TARC are both macrophage-associated mediators which have been previously demonstrated to be increased by tobacco smoke exposure and are associated with the pro-inflammatory environment observed in the lungs of patients with COPD[109]. VEGF, which is highly expressed in the lung epithelium, has been shown to be reduced by tobacco smoke[110]. Reductions of VEGF are associated with loss in endothelial integrity and emphysema development in the pathogenesis of COPD[111]. Conversely, elevated VEGF has been observed in the sputum of individuals with bronchitis and has been associated with airflow limitation[112]. In addition, mediators previously implicated in tobacco smoke investigations, such as IL-1α, were not altered. As well, other macrophage and monocyte associated mediators as well as anti-viral signaling molecules were undetected or unchanged. Cumulatively, our observations suggest that acute cannabis smoke exposure alters the expression levels of some pro-inflammatory and disease-associated mediators but does not share the full effects of tobacco smoke. These findings, along with the observed changes to immune cell populations, support the hypothesis that cannabis smoke is not innocuous in affecting lung immunity and that more considerable modulation may be induced at higher doses or longer exposures times.

Our initial characterization demonstrated compelling evidence that cannabis smoke exposure modulates lung immunity. While our findings were modest, the data supported further examination of how the observed cannabis smoke-induced immunomodulation can contribute to overall adverse health outcomes. Given the clinical evidence that cannabis smoking may lead to exacerbated symptomology and increased incidence of lung infection[37,86,113], we adapted our model to include a viral infection challenge, to assess indications of increased immunopathology and to study the impact of cannabis smoke on lung host responses. Our concurrent exposure model (**Figure 2**) was developed using influenza A virus, a common respiratory virus associated with severe lung infection, particularly in individuals with compromised lung immune integrity[114]. Supporting its use is the myriad of *in vivo* tobacco smoke exposure studies which use influenza A virus as a model pathogen[87], allowing for a simplified means of comparison between the two smoking insults. As well, studies investigating the impact of inhaled cannabinoids alone on immune responses to influenza provide additional opportunity for outcome comparison[92,115], particularly given the composition of the cannabis strains used in

our investigation. These comparisons are particularly imperative due to lack of cannabis smoke and viral infection models that currently exist. By combining the findings of our novel concurrent cannabis smoke exposure and influenza infection model with parallel investigations in the tobacco and cannabinoid literature, we can effectively demonstrate and further predict the health consequences of cannabis smoke-induced lung immunomodulation.

Previous *in vivo* investigations have demonstrated that combined tobacco smoke exposure and influenza infection leads to exacerbated immunopathology[87]. Mice administered influenza A virus and subsequently smoked exposed have been shown to have dose-dependent increases in weight loss, dehydration, and hypomobility relative to unexposed infected controls[87,116]. Our findings demonstrated excessive weight loss on specific days in both male and female cannabis-exposed mice (**Figure 12**), indicating heightened immunopathology. Conversely, no significant signs of morbidity were observed beyond weight loss in any of the infected mice. While this lack of symptomology does not reflect expectations set by the tobacco smoke literature, it does correspond with findings from concurrent influenza infection and THC administration studies[92,115]. Taken together, our observations correspond with expectations that cannabis smoke exposure worsens health conditions following influenza A infection. Additional studies which manipulate the dosage and length of infection are necessary to validate the observed trends in immunopathology.

Viral burden in infected lung tissue is a marker of pathogen replication. Excess viral replication can increase severity and duration of infection[117]. Our findings show that cannabis smoke exposure led to elevated viral burden in the lung tissue of female infected mice on day six post-infection by approximately two-fold (**Figure 13**). While this increase was modest, it suggests that there may be some aspect of compromised immunity involved in viral clearance. However, our findings correspond with the viral burden seen in investigations using tobacco smoke models[87]. Despite this, studies which quantify viral RNA following either tobacco smoke exposure or THC administration demonstrate that both insults lead to elevated viral surface protein transcript expressison[88,92,115], suggesting that gene-level quantification may further elucidate the effect of cannabis smoke on viral clearance.

Immune cell infiltration and activation is one of the primary effector responses induced by viral infection. Particularly, innate immune cells such as macrophages, neutrophils, and dendritic cells are responsible for early phagocytosis, signaling molecule production, and antigen uptake and

presentation[84]. If these cell populations are compositionally or functionally compromised, the effectiveness of early and induced immune responses to viral infection may be severely diminished. Previous tobacco smoke studies have shown that smoke exposure exacerbates immune cell infiltration in the lungs following influenza infection, leading to excessive inflammation, tissue damage, and immunopathology[87]. Conversely, THC administration has been shown to decrease influenza-induced immune cell infiltration and inflammation in the airways[92,115]. Using our model, we demonstrated that cannabis smoke exposed infected mice had altered lung immune cell compositions compared to unexposed infected controls (**Figure 14**). Heightened neutrophilia during infection has been shown to contribute to excessive inflammatory changes associated with lung disease, such as exacerbated mucus hypersecretion and emphysema, resulting in increased immunopathology and worsened health outcomes[118,119]. In addition, suppression of antigen-presenting cells such as dendritic cells or monocytes has been shown to contribute to compromised immune signaling and activation, leading to deficient viral clearance and prolonged disease[70]. These trends seen within affected cell types, such as neutrophils and dendritic cells, match those seen in concurrent tobacco smoke and influenza studies[87]. Interestingly, our uninfected control groups did not reflect the same trends observed in our baseline characterization study. These differences may be attributed to the length of the exposure protocol from each investigation; the baseline study involved four days of smoke exposure while the concurrent infection study involved a total of eight days of smoke exposure. As such, the proportionality changes in immune cellularity we observed may be dose- and time-dependent. These findings support the need for further characterizations using longer smoke exposure protocols that more adequately model real world smoker conditions.

In addition, we observed decreases in total cellularity in the lungs of female cannabis smoke-exposed, infected mice (**Figure 15**). Overall decreases in leukocytes suggest reduced immune cell infiltration following viral infection and may be indicative of a compromised host response[120]. Specifically, depletions in lung macrophages have been associated with increased viral load and dampened early innate signaling[121]. Monocyte infiltration and differentiation into the lung tissue during viral infection is critical in replenishing depleted macrophage and dendritic cell populations[122–124]. Given that we observe decreased monocyte cellularity in conjunction with decreased macrophages and dendritic cells, it is plausible that these effector populations are being depleted but are not being adequately replenished due to compromised monocyte recruitment. Additional lineage tracing studies which concurrently investigate monocyte infiltration and

differentiation are necessary to clarify this relationship. Such assessments would allow us to better understand the mechanisms behind cannabis smoke-induced dysregulated host responses.

Immune mediators serve an essential function in anti-viral responses by mediating immune cell infiltration and directly acting upon virally infected cells[125]. We demonstrated that cannabis smoke does not significantly alter immune mediator clustering between cannabis smoke infected mice and unexposed infected controls (**Figure 16**). However, our data suggests that key anti-viral signaling molecules, including IFNγ, IP-10, RANTES, and TNFα, are attenuated in one or both sexes at day six post-infection (**Figure 17**, **Table 2**, **3**). While our analysis lacks the resolution to determine if these findings are the result of suppressed mediator production or increased cellular uptake, it does suggest that the overall inflammatory profile in the lungs is different compared to unexposed counterparts. Maintaining inflammatory homeostasis is critical in ensuring an adequate immune response to viral infection. As demonstrated in tobacco smoke exposure as well as THC administration studies, suppression or exacerbation of immune signaling can lead to detrimental inflammatory outcomes[87,92,115]. Consequently, our findings support the hypothesis that cannabis smoke exposure may be mediating the course of immunopathology by decreasing anti-viral signaling and attenuating subsequent immune cell infiltration and viral clearance. However, additional investigation is necessary to further elucidate the downstream effects of attenuated anti-viral signaling as a consequence of cannabis smoke exposure. Cumulatively, our concurrent cannabis smoke exposure and influenza infection model demonstrated that acute cannabis smoke exposure dysregulates host immune responses and exacerbates viral immunopathology.

Updated models of cannabis smoke exposure are needed to investigate how modern cannabis strains, which significantly vary from historical strains, impact lung health and disease. To address this unmet need, we characterized a novel model of smoke exposure, focusing on metabolic analysis for phytocannabinoids, and lung immune profiling, in both male and female mice. Our model system recapitulates the pharmacokinetic changes observed in human cannabis smokers, providing evidence that our exposure protocol replicates real-world dynamics. Cannabis exposure resulted in innate immune cell and mediator changes that are proposed to contribute to chronic inflammation and are associated with the development of lung pathology. In addition, we defined a novel model of concurrent cannabis smoke exposure and influenza A infection to demonstrate the effects of cannabis smoke on health outcomes and immune responses following

viral infection. We demonstrated that cannabis smoke exposure can mediate immunopathological disease progression and alter immune cell composition and mediator expression in response to influenza infection. Collectively, our results define two validated modern cannabis smoke exposure models essential for studying the relationship between cannabis consumption and respiratory health.

4.2. Future Directions

The data presented in this thesis represent the development, validation, and initial application of two novel models of cannabis smoke exposure. Using these models, we established that cannabis smoke, alone and with influenza infection, is not innocuous and may have profound effects on lung immunity and overall respiratory health outcomes. While our findings begin to define the relationship between cannabis smoke and pulmonary immune homeostasis, additional investigations are necessary to further substantiate our observations. To complement our concurrent cannabis smoke and influenza infection findings, we are in the process of analysing gene-level changes to lung transcript expression via bulk RNAseq. This analysis will provide greater depth of understanding of how cannabis smoke affects immune signaling but will also allow us to identify altered genes beyond immunity that may be relevant to cannabis smoke exposure or cannabinoid signaling. As well, this study will allow us to quantify viral RNA to bolster our viral burden data and to solidify trends seen in other exposure systems.

Our study focused on innate immunity, as evidence suggests that this compartment is the earliest affected by smoke exposure. However, the adaptive immune arm remains critical in maintaining lung homeostasis and has been shown to be altered due to cannabis smoke exposure[75]. As such, investigations into T cell and B cell mediated protection are essential to fully understand how cannabis smoke affects total lung immunity. Additionally, while our study took an exploratory approach to define cell populations and immune mediators of interest, further analysis of the functional consequences of cannabis smoke on particular cell types, such as macrophages and neutrophils, is essential. Assessing whether cannabis smoke exposure leads to altered phagocytic, signaling, or antigen uptake capacity would help elucidate potential mechanisms by which cannabis smoke affects lung immunity.

We utilized a high THC, low CBD strain due to its popularity among recreational users. However, there is a growing trend of users who are seeking out CBD high strains due to their perceived medicinal and therapeutic effects. Repeating our investigations using a high CBD strain would allow for additional health risks to be identified and for direct comparison between strains with different compositions. This would enable us to identify how each constituent affects each

inflammatory readout. Theoretically, this would allow us to assess which outcomes are the result of smoke exposure itself, and which are unique to specific constituents of the smoke. Further supplementing this study would be the incorporation of pulmonary THC and CBD administration into our model system for comparison. In addition to the cannabis itself, we also can manipulate the length of the exposure protocol and the number of cigarettes burned in a single exposure session. As such, we are able to develop models of acute and chronic exposure to best reflect real world usage patterns among cannabis smokers. Importantly, longer term exposure protocols can be used to effectively model the conditions which can lead to the pathogenesis of chronic lung diseases such as COPD. In addition, many of our observations were sex-dependent, further supporting the need for future investigations to include both sexes in their design. Characterization studies using these updated conditions would greatly contribute to our understanding of the relationship between cannabis, the smoke it produces, and adverse health outcomes.

4.3. Conclusion

While the field of cannabis research remains exciting and filled with therapeutic potential, understanding how cannabis use can contribute to adverse health outcomes is of utmost importance. Given the growing popularity of cannabis smoking among recreational and medicinal users, it is imperative to delineate the risks factors associated with chronic cannabis smoke exposure. The current state of the cannabis smoke and respiratory health literature is insufficient in adequately defining the effects of cannabis smoke on the development of lung pathology and disease. The findings and conclusions presented in this thesis are an important first step in establishing a framework for future investigation into this increasingly important public health issue.

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