Cannabis smoke suppresses antiviral immune responses to influenza A in mice

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Abstract

Rationale Despite its increasingly widespread use, little is known about the impact of cannabis smoking on the response to viral infections like influenza A virus (IAV). Many assume that cannabis smoking will disrupt antiviral responses in a manner similar to cigarette smoking; however, since cannabinoids exhibit anti-inflammatory effects, cannabis smoke exposure may impact viral infection in distinct ways.

Methods Male and female BALB/c mice were exposed daily to cannabis smoke and concurrently intranasally instilled with IAV. Viral burden, inflammatory mediator levels (multiplex ELISA), lung immune cells populations (flow cytometry) and gene expression patterns (RNA sequencing) were assessed in the lungs. Plasma IAV-specific antibodies were measured *via* ELISA.

Results We found that cannabis smoke exposure increased pulmonary viral burden while decreasing total leukocytes, including macrophages, monocytes and dendritic cell populations in the lungs. Furthermore, infection-induced upregulation of certain inflammatory mediators (interferon-γ and C-C motif chemokine ligand 5) was blunted by cannabis smoke exposure, which in females was linked to the transcriptional downregulation of pathways involved in innate and adaptive immune responses. Finally, plasma levels of IAV-specific IgM and IgG1 were significantly decreased in cannabis smoke-exposed, infected mice compared to infected controls, only in female mice.

Conclusions Overall, cannabis smoke exposure disrupted host-defence processes, leading to increased viral burden and dampened inflammatory signalling. These results suggest that cannabis smoking is detrimental to the maintenance of pulmonary homeostasis during viral infection and highlight the need for data regarding the impact on immune competency in humans.

Introduction

Globally, an estimated 250 000 to 500 000 deaths are attributable to influenza infections, representing a significant health burden worldwide [1]. In healthy individuals, respiratory infections are often associated with acute presentation of moderate symptoms and immunopathology, ultimately leading to a full recovery with no long-term sequelae. More specifically, the pulmonary response to influenza A (IAV) infection requires the intact function of both the innate and adaptive immune systems, involving interferon (IFN) signalling and lymphocyte activation, in particular [2, 3]. Beyond healthy individuals, influenza infection is a risk factor for acute exacerbations of COPD and has a profound impact on patient morbidity and mortality [4]. Although tobacco smoking has long been known to increase susceptibility and severity of

respiratory viral infection [5, 6], the effect of cannabis smoke on the pulmonary antiviral response remains poorly understood. There is some evidence that cannabis users report increased incidence of respiratory symptoms associated with bronchitis and pneumonia [7, 8] as well as increased rates of hospitalisation and length of stay during respiratory infection compared to nonsmokers [9, 10]. Furthermore, clinical studies of HIV^+ men have shown that cannabis smoke exposure worsened respiratory infections [11–13] and one multivariate Mendelian randomisation study found that cannabis use disorder was associated with increased risk of chronic lower respiratory infections and asthma-related infections in the general population [10]. Therefore, in the wake of widespread legalisation, it is crucial that we understand to what extent cannabis smoking might impact the pulmonary host-defence mechanisms.

Despite the growing popularity of cannabis use worldwide, there are no data directly investigating the relationship between cannabis smoke exposure and lung immune response to viral infection. To date, studies using animal models have largely focused on the specific effects of cannabinoids, such as cannabidiol (CBD) and tetrahydrocannabinol (THC), on the inflammatory response *via* systemic administration (reviewed in [14]). Few human studies have explored the impact of cannabis smoking on lung infections, and those that exist were conducted in 1980–1990s, when the strains of cannabis and their cannabinoid contents differed dramatically from strains currently available for sale in Canada and the United States [15]. Given that smoke itself is an inflammatory insult, many assume that cannabis smoking will have similar immunomodulatory effects as tobacco smoking, where cigarette smoke exposure during influenza infection has long been known to increase pulmonary inflammation while worsening immunopathology and outcomes associated with viral infection in both pre-clinical models [16–19] and clinical studies [6, 20]. However, THC and CBD contained in cannabis smoke exhibit immunomodulatory effects and have been shown to dampen host-defence responses to viral infection when administered systemically (reviewed in [21]). For example, murine influenza infection studies found that oral THC administration suppressed cellular responses to IAV while leading to exacerbated immunopathology [22, 23]. Although smoke inhalation remains the most common route of cannabis use in most countries (*i.e.* >80% of Canadian cannabis users) [24, 25], there is little information on whether cannabis smoke affects the severity of respiratory tract viral infections. Therefore, new studies using modern and relevant cannabis compositions are required in order to better understand the effects of cannabis smoking on pulmonary antiviral responses.

In this pre-clinical study, we sought to explore the effects of acute cannabis exposure on the pulmonary immune response to influenza A infection. Using a whole-body exposure system, female mice were exposed to cannabis smoke over a 10-day period, where at day 5 mice were intranasally instilled with vehicle (PBS) or IAV (50 PFU (plaque-forming units)). We found that weight loss trajectory was significantly affected in infected mice exposed to cannabis smoke. Viral burden in the lungs approximately doubled in cannabis-exposed, IAV-infected mice compared to room air infected controls, which was associated with decreased total immune cells, macrophages, monocytes and dendritic cells (DCs) in the lungs. Furthermore, levels of certain immune mediators and expression of genes involved in key immune response pathways were downregulated in the lungs by cannabis smoke exposure during infection. Experiments were repeated in male mice, where similar, though less pronounced effects were observed: worsened weight loss and reductions in certain inflammatory cells and cytokines in the lung, but no significant effect on viral titre. In summary, cannabis smoke exposure suppressed aspects of the pulmonary immune response to IAV infection, leading to worsened viral load in the lungs.

Methods

Mouse cannabis smoke exposure and influenza infection

6- to 8-week-old female and male BALB/c mice were purchased from Charles River Laboratories (Montreal, QC, Canada) and all experimental procedures were approved by the animal research ethics board of McMaster University (#19-08-23). Mice were whole-body exposed using a SIU-24 (Promech, Vintrie, Sweden) to room air or to the smoke from six cannabis cigarettes (indica-dominant strain, 10–14% THC and 0–2% CBD) twice daily for 1 h as shown in figure 1. This strain was chosen as its cannabinoid content is an accurate representation of recreational cannabis products used in the consumer marketplace [26, 27]. On day 5, mice were intranasally inoculated with 35 µL containing 50 PFU mouse-adapted influenza A virus (A/FM/1/47-MA) or PBS vehicle. Details on animal protocol/euthanasia can be found in the supplementary material.

Viral burden

Viral burden was assessed using Madin–Darby canine kidney cell plaque assay as described previously [16] as well as *via* viral transcript quantification; details of the experimental methods can be found in the supplementary material.

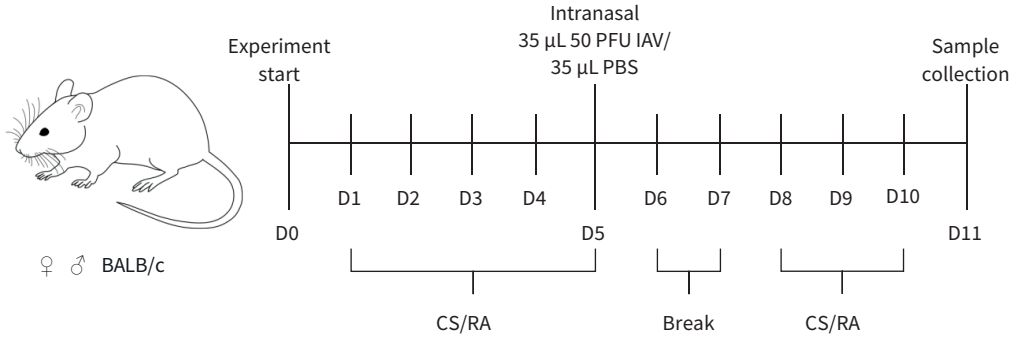

FIGURE 1 Animal exposure, infection and sample collection timeline. Female and male 6- to 8-week-old BALB/c mice were whole-body exposed to the smoke of six cannabis cigarettes (CS) or room air control (RA) twice per day for five consecutive days. Following smoke exposure on day 5 (D5), mice were intranasally inoculated with 50 PFU mouse-adapted (A/FM/1/47-MA) influenza A virus (IAV) or PBS control. Mice were given a 2-day break from cannabis smoke exposure following inoculation. Exposure protocols resumed on day 8 and continued until day 10 and animals were sacrificed on day 11. PFU: plaque-forming units.

RNA sequencing

Samples were sequenced using two lanes of a Novaseq S1 flow cell with 100 bp paired end reads to a minimum depth of 30 million reads per sample at The Centre for Applied Genomics at the Hospital for Sick Children (Toronto, ON, Canada). Heat maps show z-scores (calculated using the average expression for each gene) and functional enrichment analysis was performed using GOrilla online platform. Details on transcript quantification and analysis methods can be found in the supplementary material.

Flow cytometric analysis

Lung tissue was digested and fluorescently labelled using antibodies and flow cytometric gating strategy as described previously [28]. Detailed methods can be found in the supplementary material.

Immune mediator quantification

The remaining half of the left lobe was homogenised then supernatants were aliquoted and prepared for immune mediator quantification *via* mouse cytokine array/chemokine array 44-plex (Eve Technologies, Calgary, AB, Canada). Point-to-point semi-logarithmic analysis was applied to all immune mediator quantities.

Anti-IAV immunoglobulin ELISA

IAV-specific antibodies were measured in the plasma as described previously [16] and details can be found in the supplementary material.

Statistical analysis

GraphPad Prism 9 (v.9.5.1) was used for statistical analyses. The data are presented as mean±SEM. Two-way ANOVAs with Šidák's multiple comparisons test were used to compare the means of four experimental groups. When specified, unpaired two-tailed t-tests were performed to compare the means of two groups. Differences were considered statistically significant when $p \leqslant 0.05$.

Results

Cannabis smoke exposure alters weight loss and increases viral titre in female mice

As outlined in figure 1, mice were concurrently exposed to cannabis smoke and infected with mouse-adapted influenza A virus over a 10-day period. None of the infected mice displayed severe symptoms, beyond weight loss, nor reached a humane end-point over the course of the infection. In female mice, weight loss relative to starting weight was exacerbated in cannabis-exposed, infected mice at days 5–6 and there was a trend towards significantly worsened total weight loss over the entire post-infection period ($p=0.0595$) measured as the area under the curve (figure 2a and b). *Via* viral plaque assay, cannabis smoke exposure in IAV-infected mice led to a modest but significant increase in viral burden relative to infected controls (figure 2c). Similarly, IAV gene expression in the lung as assessed *via* bulk RNA sequencing (seq) was increased in cannabis smoke-exposed female mice compared to controls (figure 2d), suggesting viral clearance was impaired by cannabis smoke exposure.

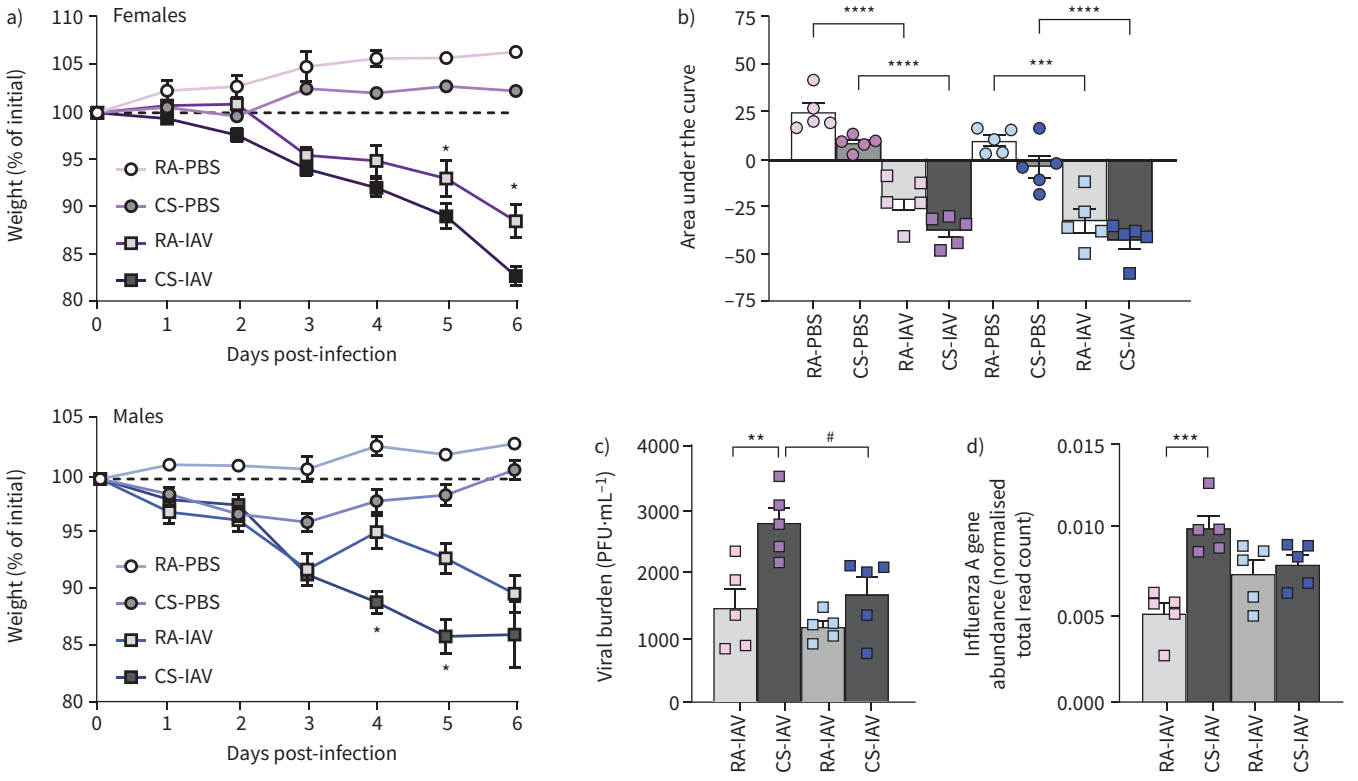

FIGURE 2 Cannabis smoke (CS) exposure alters weight loss and viral clearance following influenza A infection. a) Mice were weighed at the time of inoculation and continued to be weighed daily until sample collection. Significance asterisks indicate a significant difference between room air control (RA)-influenza A virus (IAV) and CS-IAV groups. b) Area under the curve assessed by setting 100% weight at day 0 post-infection (dotted line in a) as the baseline. Viral burden was quantified in lung tissue homogenate *via* c) Madin–Darby canine kidney plaque assay and d) viral gene expression was quantified in lung tissue *via* bulk RNA sequencing. Data represent mean+SEM; n=5 per group. *: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001, two-way ANOVA with Šidák's multiple comparisons test between all experimental groups; #: differences between males and females of same experimental group.

Parallel experiments were performed in a cohort of male mice, where weight loss relative to starting weight was similarly exacerbated in male cannabis-exposed, IAV-infected mice relative to room air, infected controls at days 4–5, although total weight loss measured as area under the curve was not significantly affected (figure 2a and b). Furthermore, in male mice, no change in viral burden or viral gene expression was observed cannabis-exposed, IAV-infected mice (figure 2c and d).

Cannabis smoke exposure suppresses lung immune responses to IAV

To examine how lung immune responses to IAV are influenced by cannabis smoke exposure, we assessed innate immune cell populations in the lung tissue *via* flow cytometry. In both male and female mice, cannabis smoke alone did not alter any of these immune cell populations, except a decrease in the proportion of DCs in female mice (figure 3a and b). Among infected female groups, total immune cells ($CD45^+$), macrophages, monocytes and DCs were significantly decreased by cannabis smoke exposure (figure 3a). Interestingly, when expressed as a fraction of total $CD45^+$ immune cells, no significant differences were observed between room air- and cannabis smoke-exposed mice infected with IAV (figure 3b). In male mice, immune cell populations were largely unaffected by IAV infection, where $CD45^+$, neutrophil, macrophage, monocyte and dendritic cell counts did not increase in infected groups and were lower than female mice (figure 3a and b). Furthermore, cannabis smoke exposure in IAV-infected mice had no significant effect on absolute immune cell population counts, while the proportion of monocytes was decreased in cannabis smoke-exposed, IAV-infected males compared to room air, infected controls (figure 3a and b).

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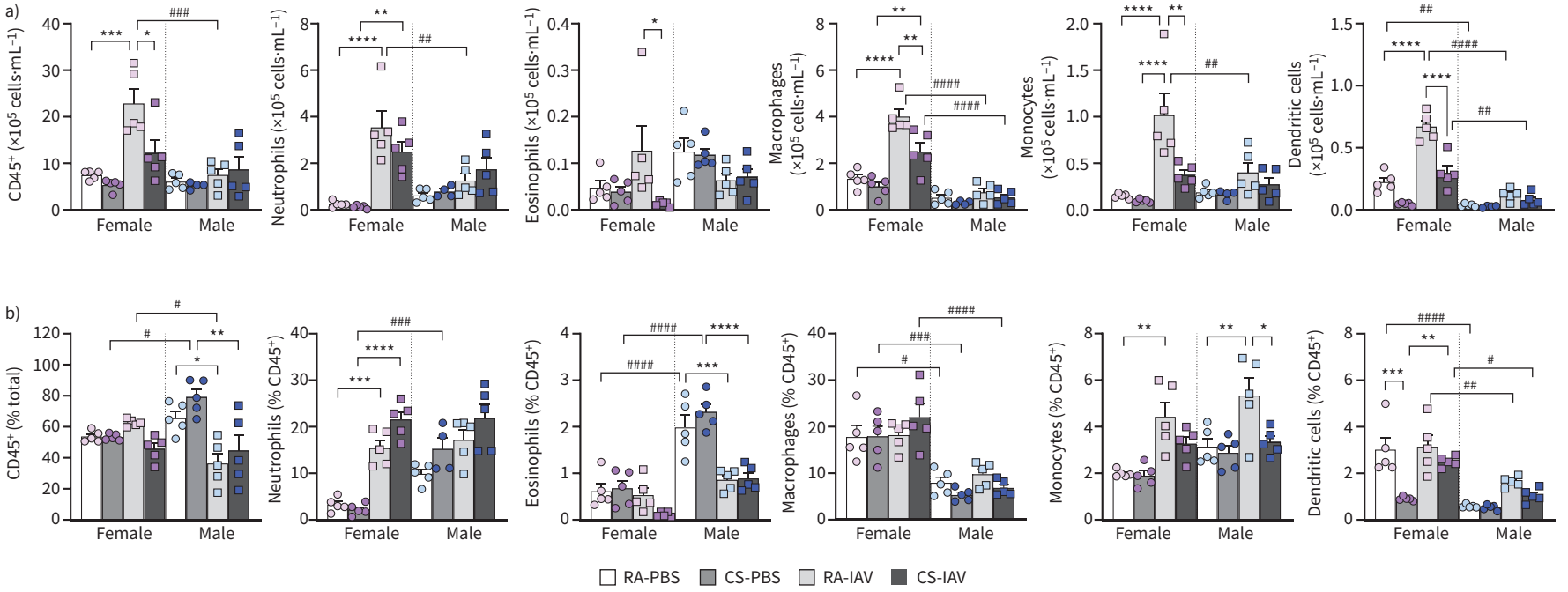

FIGURE 3 Concurrent cannabis smoke (CS) exposure and influenza A infection dampens inflammation in the lungs. Lung innate immune cell populations were quantified *via* flow cytometry. a) Absolute cell count (% multiplied by total cell count *via* haemocytometer using Türk's solution staining) and b) proportionality (%) were determined. Data represent mean+SEM; n=4–5 per group. *: p⩽0.05, **: p⩽0.01, ***: p⩽0.001, ****: p⩽0.0001, two-way ANOVA with Šidák's multiple comparisons test between all experimental groups; #: differences between males and females of same experimental group. RA: room air control; IAV: influenza A virus.

Since cannabis smoke reduced IAV-associated immune cell recruitment, we assessed its impact on pulmonary immune mediator levels *via* multiplex cytokine array. Firstly, no significant changes in cytokine levels were detected between uninfected room air and cannabis smoke-exposed mice of both sexes (supplementary tables S1 and S2). In the lungs of IAV-infected female mice, levels of IFN-γ (trend, p=0.0641), C-C motif chemokine ligand (CCL)5, interleukin (IL)-4 and IL-13 were decreased in cannabis smoke-exposed animals compared to room air mice, with no effect observed on IFN-β1, C-X-C motif chemokine ligand (CXCL)10, tumour necrosis factor (TNF)-α or IL-1β levels in the lungs (figure 4a), indicating that some, but not all, components of the antiviral response were suppressed and innate immune cytokines were largely unaffected. Principal component analysis (PCA) of all cytokines detected revealed that global immune mediator cluster separation was primarily driven by influenza infection (89% variance, p<0.001) rather than cannabis smoke exposure (2% variance, p=0.14), with no obvious separation between room air- and cannabis smoke-exposed groups infected with IAV (figure 4b). In male mice, several cytokines were reduced in cannabis smoke-exposed, IAV-infected animals compared to infected controls, including IFN-γ, TNF-α, CCL5, IL-1β, IL-4 (trend, p=0.0603) and IFN-γ-induced protein-10/CXCL10 (figure 4a). Overall, few sex-related differences were observed except increased TNF-α and IL-1β in infected room air-exposed males compared to females and immune-mediator PCA showed results similar to females with variance predominantly driven by IAV infection (63.4%) and to a lesser extent by cannabis smoke exposure (9.7%) (figure 4a and b).

Statistical analyses revealed that there was a significant interaction between cannabis smoke exposure and IAV infection on monocyte and DC counts, $CD45^+$ and neutrophil proportions, as well as IFN-γ, CCL5, IL-4 and IL-13 levels, probably due to the fact that cannabis smoke had little impact in uninfected mice, but had a profound effect in IAV-inoculated mice.

IFN-stimulated gene expression is modestly affected by cannabis smoke exposure

In order to explore whether cannabis smoking dampened classical antiviral pathways, we assessed the expression of IFN-stimulated genes (ISGs) *via* lung RNAseq. In females, 74 of the total 364 ISGs were significantly affected by exposure or infection, 41 of which were differentially expressed in infected cannabis smoke-exposed mice compared to infected room air-exposed controls. As can be observed in the figure 5a heatmap, expression of these genes was increased by infection and further upregulated by cannabis smoke exposure, such as *Cd163*, *Cyp1b1*, *Serpine1*, *Cxcl10* and *Trim21*, among others. However, a subset of genes that were upregulated by infection was downregulated by cannabis smoke exposure, including *Cd74*, *Arntl*, *Npas2*, *Ccna1* and *Cx3cl1* (figure 5a). Overall, PCA analysis of all ISGs revealed that changes in expression were driven by infection (59.8% variance, p<0.05) and by cannabis smoke exposure (8.9% variance, p<0.05), though less substantially (figure 5b). In contrast to the cytokine PCA plot, a separation between room air- and cannabis smoke-exposed groups infected with IAV was observed (figure 5b). For ISG expression analysis in male mice, 63 genes were significantly affected by exposure or treatment, 28 of which were differentially expressed in cannabis smoke-exposed mice compared to room air controls. In males, the majority of ISGs upregulated by infection were downregulated by cannabis smoke exposure compared to room air-exposed mice (*Arntl*, *Cd74*, *Aim2*, *Cd69*, *etc*.) with only a few ISGs being further upregulated by cannabis smoke exposure (*Cry1* and *Gpx2*) (figure 5a).

Cannabis smoking dampens pulmonary immune signalling and circulating IAV-specific antibody levels in females

Since ISG expression during IAV infection was only modestly affected by cannabis smoke exposure, we performed an untargeted characterisation of all differentially expressed genes. In females, we found that, of the 6175 genes upregulated by IAV infection in room air-exposed mice, 214 genes were downregulated by cannabis smoke exposure, with the top 100 genes by p-value fitting this suppression pattern shown in figure 6a. Gene ontology analysis of these 214 suppressed genes revealed that several inflammatory and immune pathways were dampened by cannabis smoke exposure in IAV-infected females, such as phagocytosis, innate immune response, humoral immune response, defence response to bacterium, and B-cell receptor signalling, among others (figure 6b). Conversely, we found that, of the 6175 IAV-upregulated genes, 425 were further potentiated by cannabis smoke exposure, exhibiting an exacerbation pattern (figure 7a). Interestingly, gene ontology analysis revealed that these genes were linked to few pathways; only intermediate filament organisation, intermediate filament-based process, and cytoskeleton organisation reached statistical significance (figure 7b). In male mice, we found that of the 5859 genes significantly upregulated by infection in room air-exposed mice, the 387 genes that were downregulated and the 66 genes that were further upregulated by cannabis smoke exposure were not significantly associated with any gene ontology pathways (only top 100 by p-value shown; supplementary figure S1).

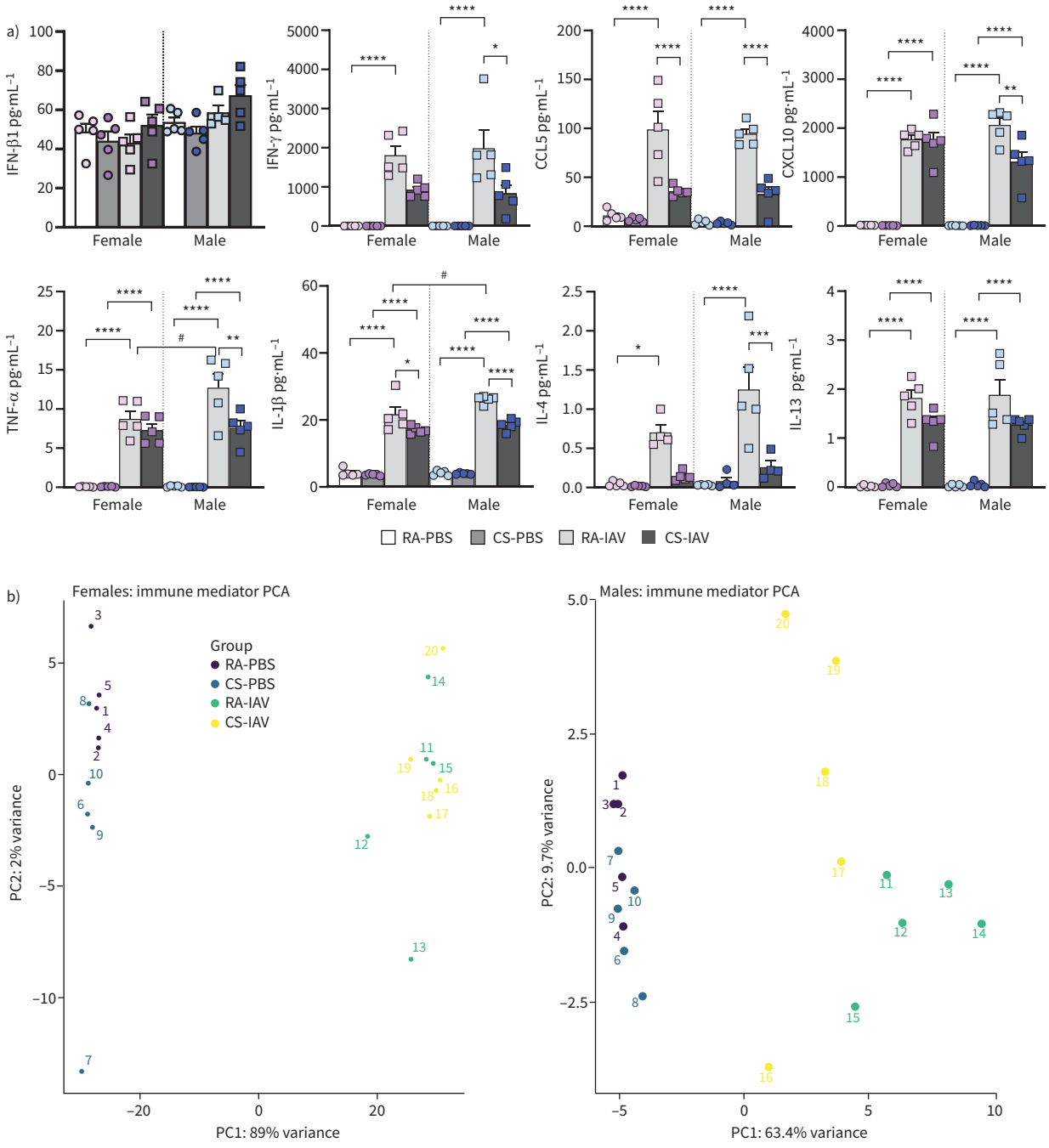

FIGURE 4 Cannabis smoke (CS) exposure suppresses antiviral immune mediator levels following influenza A infection. a) Immune mediators were quantified in lung homogenate *via* multiplex analysis and b) clustering was identified using principal component analysis (PCA) in females and males. Data represent mean+SEM; n=5 per group. *: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001, two-way ANOVA with Šidák's multiple comparisons test; #: differences between males and females of same experimental group. IFN: interferon; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; TNF: tumour necrosis factor; IL: interleukin; RA: room air control; IAV: influenza A virus.

In light of the observed decreases in both antigen-presenting cell populations and gene expression of several adaptive immune pathways, we wanted to assess whether cannabis smoke exposure significantly impaired their humoral response to IAV infection. Firstly, we measured IAV-specific antibodies in the circulation *via* ELISA. In female mice, we found that cannabis smoke exposure reduced circulating levels of anti-IAV IgM and IgG1 compared to infected controls, while IAV-specific IgG2a was unaffected

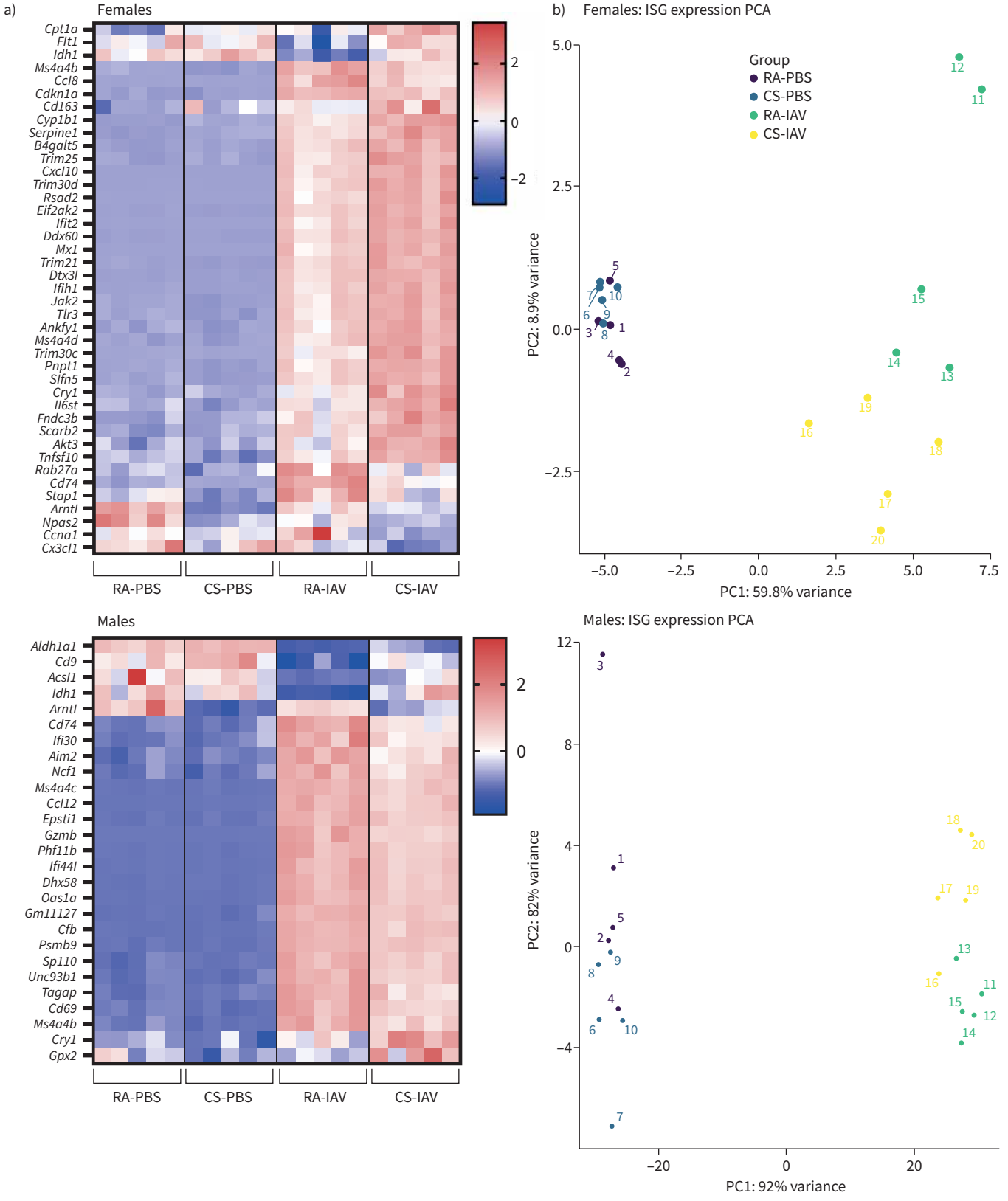

FIGURE 5 Cannabis smoke (CS) exposure modulates interferon-stimulated gene (ISG) expression following influenza A infection. a) Heatmaps and b) principal component analysis (PCA) of differentially expressed interferon-stimulated genes in the lungs as assessed *via* lung RNA sequencing in females and males, z-scores ranging from 3 (red) to −3 (blue). Data represent mean+SEM; n=5 per group. RA: room air control; IAV: influenza A virus.

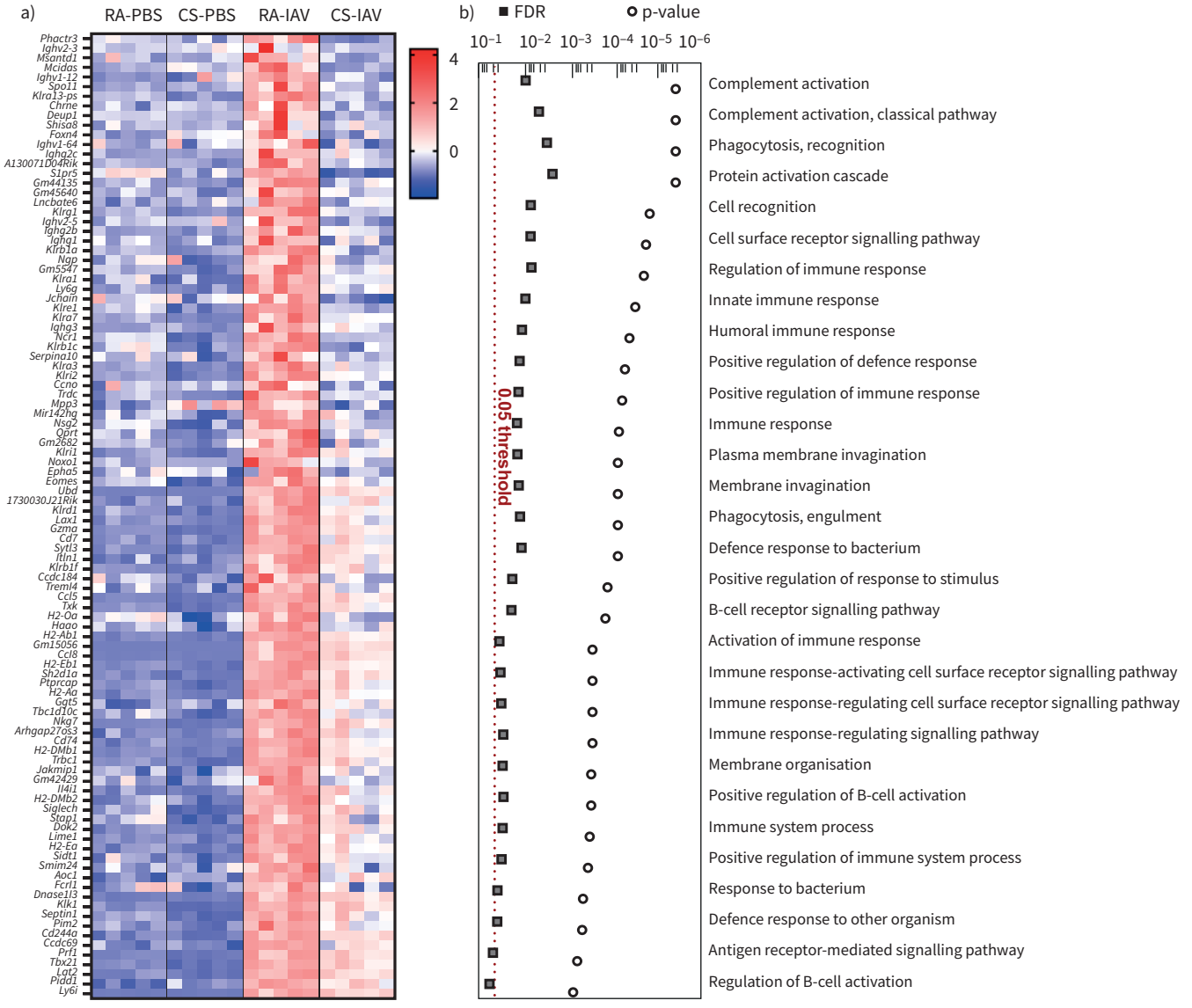

FIGURE 6 Cannabis smoke (CS) exposure suppresses influenza A virus (IAV)-associated upregulation of immune system pathways in the lungs of female mice. a) Heatmap of top 100 genes upregulated in IAV-infected mice and downregulated by cannabis smoke exposure as assessed *via* lung RNA sequencing, ordered by p-value, z-scores ranging from 4 (red) to −2 (blue). b) Gene ontology analysis of those differentially expressed genes. n=5 per group. FDR: false discovery rate. Significance at <0.05 (red dotted line). RA: room air control.

(figure 8a). In alignment with immune signalling data, cannabis smoke exposure had no significant impact on circulating levels of IgM, IgG1 or IgG2a in male infected mice and antibody levels in males were significantly lower than females, on the whole (figure 8a). We also assessed the surface expression of major histocompatibility complex class II (MHCII), an antigen-presenting peptide complex crucial to the activation of the adaptive immune response, in macrophages and dendritic cells *via* flow cytometry. In females, MHCII expression was reduced in macrophages and DCs from cannabis smoke-exposed, IAV-infected mice compared to room air controls, observed as a decrease in mean fluorescence intensity and as a leftward shift in the fluorescence histogram (figure 8b). Statistical analyses revealed that there was a significant interaction between cannabis smoke exposure and IAV infection on macrophage and DC MHCII expression in females. In male mice, no significant change in MHCII surface expression was observed in macrophages or DCs and overall expression in macrophages was significantly lower compared to all female groups (figure 8b). Taken together, these data strongly suggest that antigen presentation and humoral immune responses are suppressed by cannabis smoke exposure during IAV infection, even at such an early stage of the antiviral response.

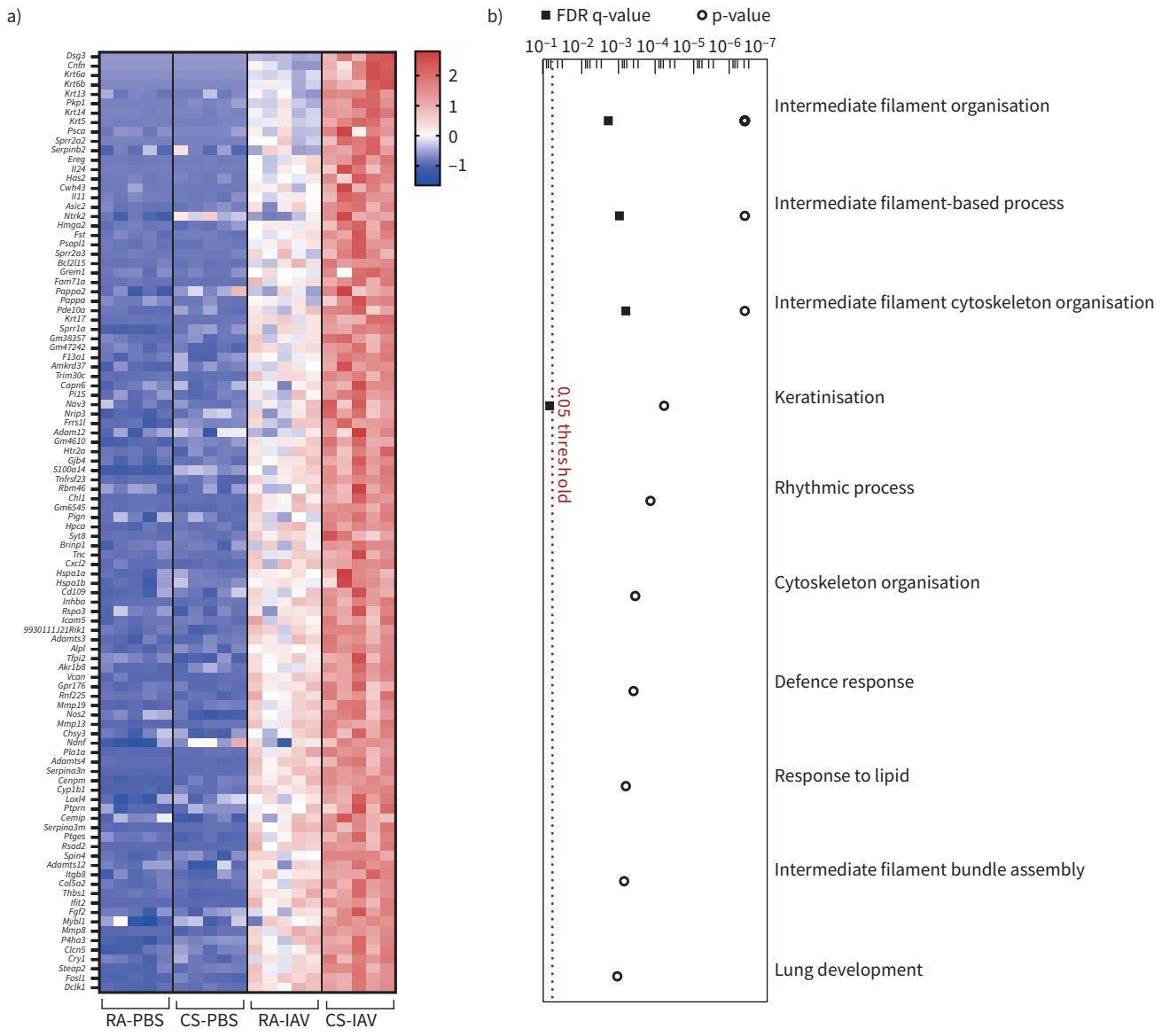

FIGURE 7 Cannabis smoke (CS) exposure potentiates several genes upregulated by influenza A virus (IAV) infection in the lungs of female mice. a) Heatmap of top 100 genes upregulated in IAV-infected mice and downregulated by cannabis smoke exposure as assessed *via* whole-lung RNA sequencing, ordered by p-value, z-scores ranging from 3 (red) to −1.5 (blue). b) Gene ontology analysis of those differentially expressed genes. n=5 per group. FDR: false discovery rate. Significance at <0.05 (red dotted line). RA: room air control.

Discussion

Despite the growing popularity of cannabis use and its recent legalisation in many countries around the world, there is little research looking into the direct effects of cannabis smoke inhalation on the response to respiratory infections. Not only are data limited, but updated models of cannabis smoke exposure are needed to investigate how lung health is affected by exposure to modern cannabis strains, which vary significantly from historical strains [15]. To begin to address this unmet need, we used a validated model of cannabis smoke exposure [28] and IAV infection to demonstrate that cannabis smoke exposure impairs viral clearance and suppresses key aspects of the immune response. We found that cannabis smoke exposure increased viral burden while decreasing pulmonary immune cell populations, reducing cytokine levels, downregulating the expression of immune response genes in the lungs as well as decreasing circulating IAV-specific antibodies, particularly in female mice. In all, our data suggest that cannabis smoke may negatively impact immune competence and also highlight the need for further investigation into the mechanisms through which cannabis smoke exposure affects antiviral responses.

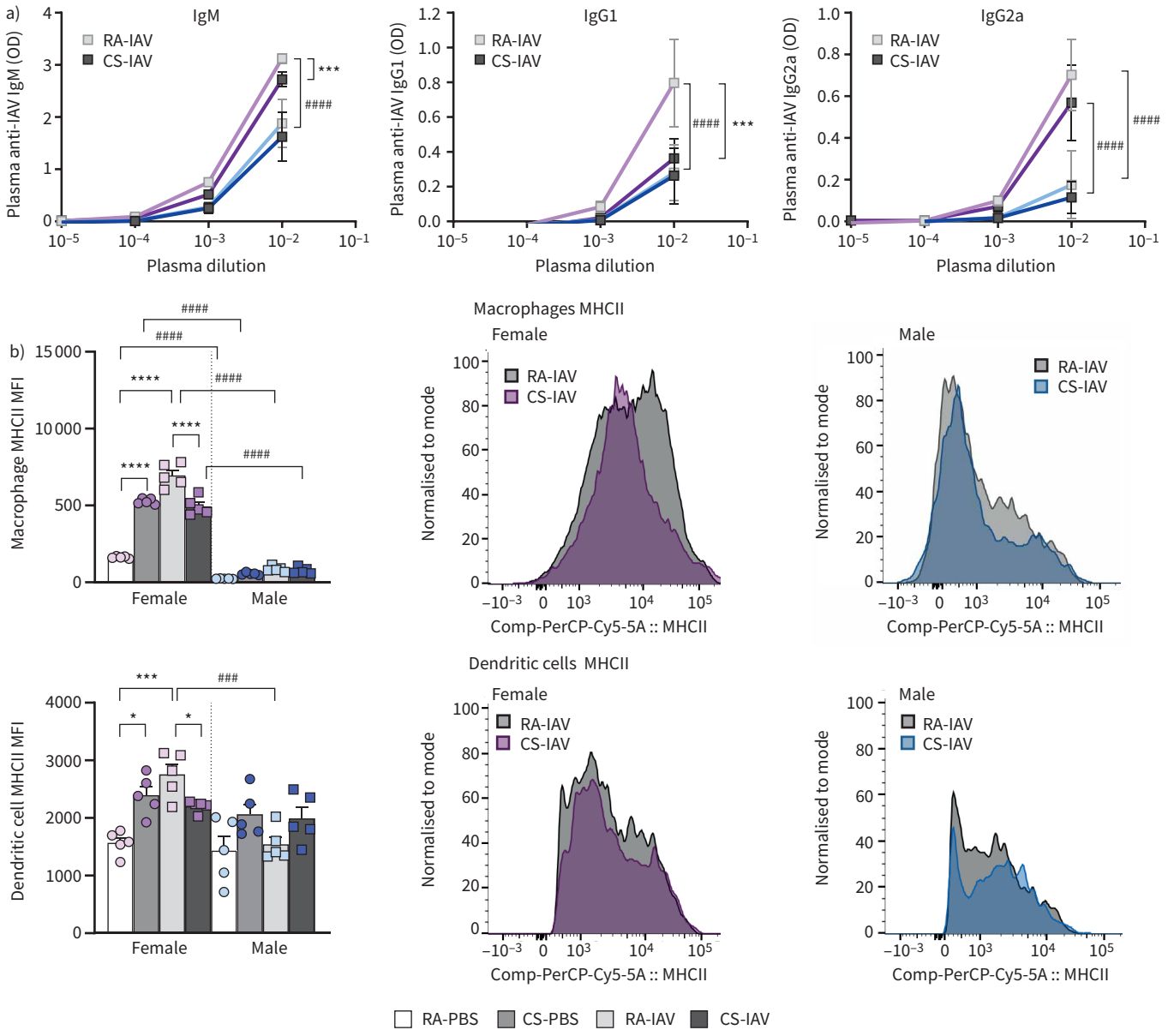

FIGURE 8 Cannabis smoke (CS) exposure reduces influenza A virus (IAV)-specific antibody levels in the plasma and antigen-presenting cell major histocompatibility complex class II (MHCII) expression. a) Levels of anti-IAV IgM, IgG1 and IgG2a in serially diluted plasma samples (10^{-2}–10^{-5}), expressed as optical density (OD) measured at 450 nm; ELISAs were run multiple times with representative data shown. b) Mean fluorescence intensity (MFI) of MHCII in macrophages and dendritic cells, represented as mean or histograms normalised to mode. Data represent mean±SEM; n=5 per group. *: p⩽0.05, ***: p⩽0.001, ****: p⩽0.0001 two-way ANOVA with Šidák's multiple comparisons test between all experimental groups; #: difference between males and females of same group.

A major finding from our study is that cannabis smoke exposure during IAV infection increases weight loss post-infection and worsens viral burden in the lungs of female mice. The increase in viral burden and viral gene abundance indicate that the antiviral immune response is compromised by cannabis smoke exposure, which aligns with the observed suppression of immune cell infiltration and signalling in the lungs. Firstly, we saw a reduction in total immune cells, specifically affecting macrophages and DCs, the primary antigen-presenting cells in the lungs. In addition, we found that macrophage and DC expression of the key antigen presenting complex MHCII was significantly decreased in IAV-infected mice exposed to cannabis smoke compared to their room air controls, suggesting that adaptive immune activation may be impaired. In the context of viral infection, previous research has shown that DCs and macrophages are key to the activation of the immune response, where depletion of this population can lead to compromised

immune signalling, reduced adaptive immune system activation, worsened weight loss and impaired viral clearance [29–31]. In accordance with the reduction in immune cell infiltration, we demonstrated that cannabis smoke exposure during IAV-infection was also associated with significant reductions in certain, but not all, cytokines (*i.e.* IFN-γ and CCL5). It is therefore plausible that cannabis smoke exposure during IAV infection interferes with beneficial immune cell recruitment and activation, thus impairing the homeostatic response to viral infection.

Interestingly, we found that, although male mice exhibited similar changes in weight loss and significant reductions in lung cytokine levels, the effects of both cannabis smoking and IAV infection on immune cell infiltration, inflammatory signalling, viral load and antibody production were less pronounced than what was observed in females. The differences in the response to IAV between sexes is not surprising, as previous studies have shown that female mice exhibit heightened pulmonary inflammatory responses to IAV while males exhibit reduced symptomology and recover more quickly from infection despite reduced immune activation [32–34]. In addition, a methodological factor that may have contributed to the observed sex differences is that IAV was administered in both experiments at the same PFU per mouse while their body weights were quite different at the time of inoculation: ∼18 g for females *versus* 22 g for males. Although sex hormones may also play a role, it is possible that much of the observed sex difference resulted from the fact that females received a higher relative dose of IAV than males, which makes direct comparisons between male and female data problematic.

Given that smoke itself is an inflammatory insult, it is often assumed that cannabis smoke leads to similar immunomodulatory effects as tobacco smoke, where tobacco smoke exposure has been shown to increase pulmonary inflammation and worsen immunopathology associated with IAV [16–18]. Some of our results align with the effects of tobacco smoking: worsened weight loss as well as similarities in gene expression changes, where tobacco smoke exposure further upregulated *Cxcl10* and *Ifit2* expression in neutrophils [19] similar to that which we observed in the pulmonary ISG expression data. Nevertheless, it is important to note the myriad ways in which cannabis smoke exposure distinctively impacts the antiviral response in our study. Unlike cannabis smoke exposure, cigarette smoke exposure in IAV-infected mice leads to decreased survival and heightened inflammatory responses: increased lung immune cells (total cells, neutrophils and monocytes) and elevated cytokine secretion (IL-6, macrophage inflammatory protein-2 and TNF-α), without affecting viral burden [16–19, 35, 36]). Instead, our cannabis smoke exposure results fit in many ways with the effects of systemic THC administration during IAV infection, although with some important differences. Oral THC administration did not affect symptomology and morbidity, but did decrease influenza-induced immune cell infiltration, bronchoalveolar lavage fluid IFN-γ levels, inflammation scores in the airways, and antigen-presenting cells in the lungs, such as DCs, monocytes and macrophages [22, 23, 37]. However, in contrast to the impact of cannabis smoke exposure, systemic THC administration reduced pulmonary neutrophilia, which led authors to suggest that disruption of neutrophil chemotaxis caused by THC underlies the impaired viral clearance [23]. These results emphasise that cannabis smoke exposure impacts antiviral responses in a complex manner, not simply driven by its nature as a combustion product, but also influenced by its cannabinoid content and their anti-inflammatory properties. Future studies using cannabinoid receptor knockout animals, strains of cannabis with high CBD content, or combustion-free cannabis delivery systems (*i.e.* vaping) would help to dissect the contribution of specific cannabinoids and to understand the role of combustion products in the observed effects of cannabis smoking.

Another interesting finding in our study is the dampening of type 2 T-helper cell (Th2)-associated immune pathways. In our uninfected mice exposed to cannabis smoke, we saw no significant changes in pulmonary eosinophilia or in Th2-associated cytokines. However, in the context of viral infection, cannabis smoke exposure led to trend towards reduced lung eosinophil counts and significant reductions in IL-4 and IL-13 levels. However, since eosinophil counts (<0.5% of immune cells) and Th2 cytokines levels (1–2 $pg \cdot mL^{-1}$) were already relatively low, it seems unlikely that these changes significantly contributed to the reduction in viral clearance. In contrast, many case reports have found that some heavy cannabis smokers or cannabis vape users develop acute eosinophilic pneumonia, associated with dyspnoea and ground-glass opacities in some cases [38, 39]. Although our data may suggest an important species difference in eosinophilic response to cannabis smoke exposure, it is also possible that the duration and dose of cannabis smoke exposure in our model is not high enough to induce Th2 immune responses. For example, one of the most dramatic cases of cannabis smoke-associated acute eosinophilic pneumonia was observed in a young man who reported smoking 20 joints the evening prior to being admitted to the hospital [39]. Nevertheless, it would be interesting to explore how cannabis smoking might affect Th2-driven pathologies, such as asthma, and explore long-term, high-dose cannabis smoking to better understand whether there are significant species differences in eosinophilic responses.

A limitation of our study is our focus on detailing the immunopathological consequences of cannabis smoke exposure during IAV infection instead of focusing on mechanistic questions using alternative approaches (*e.g.* cannabinoid receptor knockouts or pharmacological interventions [37, 40]). Nevertheless, through both targeted and untargeted transcriptomic analyses, we found that certain ISGs and pulmonary immune pathways, comprised of genes typically upregulated by IAV infection, were significantly downregulated by cannabis smoke exposure. In particular, many pathways implicated in the adaptive immune response (humoral immune response, B-cell receptor signalling pathway, *etc.*) were suppressed by cannabis smoke exposure. Supporting this hypothesis, we found that circulating levels of IAV-specific IgM and IgG1 were reduced in female mice exposed to cannabis smoke. Since IgG1 is crucial to mounting a response to soluble and membrane proteins of viral and bacterial origin [41–43] and IgG1 deficiencies are associated with recurrent infection [44], the reduction of circulating IgG1 by cannabis smoke exposure is particularly concerning. Therefore, it would be interesting to directly examine the impact of cannabis smoke exposure on adaptive immune competency by looking at the lymphocyte subtype populations and their activation in the lungs, by assessing antibody neutralisation activity, or by using mouse strains lacking lymphocytes (*i.e. Rag*-knockout animals). Furthermore, if there is evidence that cannabis smoke exposure dampens the humoral response, this could have important consequences on long-term immune protection from re-infection and the efficacy of vaccines. Another important caveat is that our study focused on a single time point post-infection, which limits the application of our findings to the more acute responses to IAV. To better understand the impact of cannabis smoking on the different stages of antiviral immune responses, addition of later time points (days 14–21 post-infection) in future studies will allow us to elucidate the effects on infection resolution as well as on antibody production and immunoglobulin class switching, as the peak in IgG1 and IgG2 antibody production occurs later than 6 days post-infection. Furthermore, exploring earlier windows of time in the response to infection (2–24 h) may be more useful to study viral replication and the IFN-driven, innate immune responses to virus.

In summary, our study represents an important first step towards a better understanding of the impact of cannabis smoking on respiratory infections. Ultimately, we found that many aspects of the pulmonary immune response to IAV were suppressed by cannabis smoke exposure, often in ways that were distinct from the known effects of either tobacco smoke exposure or oral cannabinoid administration. Nevertheless, there are still many questions that remain unanswered surrounding the mechanisms through which cannabis smoke exposure affects antiviral responses as well as the clinical relevance of our results. We believe strongly that cannabis smoking must be studied separately and extensively, especially in light of its growing popularity and the paucity of relevant data on its immunomodulatory effects.

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References

1 Paget J, Spreeuwenberg P, Charu V, *et al.* Global mortality associated with seasonal influenza epidemics: New burden estimates and predictors from the GLaMOR Project. *J Glob Health* 2019; 9: 020421.
2 van de Sandt CE, Kreijtz JH, Rimmelzwaan GF. Evasion of influenza A viruses from innate and adaptive immune responses. *Viruses* 2012; 4: 1438–1476.
3 Chen X, Liu S, Goraya MU, *et al.* Host immune response to influenza A virus infection. *Front Immunol* 2018; 9: 320.
4 Mallia P, Johnston SL. Influenza infection and COPD. *Int J Chron Obstruct Pulmon Dis* 2007; 2: 55–64.
5 Feldman AS, He Y, Moore ML, *et al.* Toward primary prevention of asthma. Reviewing the evidence for early-life respiratory viral infections as modifiable risk factors to prevent childhood asthma. *Am J Respir Crit Care Med* 2015; 191: 34–44.
6 Kark JD, Lebiush M, Rannon L. Cigarette smoking as a risk factor for epidemic A(H1N1) influenza in young men. *N Engl J Med* 1982; 307: 1042–1046.
7 Tetrault JM, Crothers K, Moore BA, *et al.* Effects of marijuana smoking on pulmonary function and respiratory complications: a systematic review. *Arch Intern Med* 2007; 167: 221–228.

8 Macleod J, Robertson R, Copeland L, *et al.* Cannabis, tobacco smoking, and lung function: a cross-sectional observational study in a general practice population. *Br J Gen Pract* 2015; 65: e89–e95.
9 Polen MR, Sidney S, Tekawa IS, *et al.* Health care use by frequent marijuana smokers who do not smoke tobacco. *West J Med* 1993; 158: 596–601.
10 Rosoff DB, Yoo J, Lohoff FW. A genetically-informed study disentangling the relationships between tobacco smoking, cannabis use, alcohol consumption, substance use disorders and respiratory infections, including COVID-19. *medRxiv* 2021; preprint [https://doi.org/10.1101/2021.02.11.21251581].
11 Caiaffa WT, Vlahov D, Graham NM, *et al.* Drug smoking, *Pneumocystis carinii* pneumonia, and immunosuppression increase risk of bacterial pneumonia in human immunodeficiency virus-seropositive injection drug users. *Am J Respir Crit Care Med* 1994; 150: 1493–1498.
12 Tindall B, Cooper DA, Donovan B, *et al.* The Sydney AIDS Project: development of acquired immunodeficiency syndrome in a group of HIV seropositive homosexual men. *Aust N Z J Med* 1988; 18: 8–15.
13 Newell GR, Mansell PW, Wilson MB, *et al.* Risk factor analysis among men referred for possible acquired immune deficiency syndrome. *Prev Med* 1985; 14: 81–91.
14 Klein TW, Friedman H, Specter S. Marijuana, immunity and infection. *J Neuroimmunol* 1998; 83: 102–115.
15 Colizzi M, Bhattacharyya S. Does cannabis composition matter? Differential effects of delta-9-tetrahydrocannabinol and cannabidiol on human cognition. *Curr Addict Rep* 2017; 4: 62–74.
16 Robbins CS, Bauer CM, Vujicic N, *et al.* Cigarette smoke impacts immune inflammatory responses to influenza in mice. *Am J Respir Crit Care Med* 2006; 174: 1342–1351.
17 Bauer CM, Zavitz CC, Botelho FM, *et al.* Treating viral exacerbations of chronic obstructive pulmonary disease: insights from a mouse model of cigarette smoke and H1N1 influenza infection. *PLoS One* 2010; 5: e13251.
18 Gualano RC, Hansen MJ, Vlahos R, *et al.* Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir Res* 2008; 9: 53.
19 McGrath JJC, Vanderstocken G, Dvorkin-Gheva A, *et al.* Cigarette smoke augments CSF3 expression in neutrophils to compromise alveolar-capillary barrier function during influenza infection. *Eur Respir J* 2022; 60: 2102049.
20 Cohen S, Tyrrell DA, Russell MA, *et al.* Smoking, alcohol consumption, and susceptibility to the common cold. *Am J Public Health* 1993; 83: 1277–1283.
21 Tashkin DP, Roth MD. Pulmonary effects of inhaled cannabis smoke. *Am J Drug Alcohol Abuse* 2019; 45: 596–609.
22 Buchweitz JP, Karmaus PW, Harkema JR, *et al.* Modulation of airway responses to influenza A/PR/8/34 by delta9-tetrahydrocannabinol in C57BL/6 mice. *J Pharmacol Exp Ther* 2007; 323: 675–683.
23 Buchweitz JP, Karmaus PW, Williams KJ, *et al.* Targeted deletion of cannabinoid receptors CB1 and CB2 produced enhanced inflammatory responses to influenza A/PR/8/34 in the absence and presence of delta9-tetrahydrocannabinol. *J Leukoc Biol* 2008; 83: 785–796.
24 United Nations Office on Drugs and Crime (UNODC). World Drug Report 2018. 2018. https://www.unodc.org/wdr2018/index.html.
25 Health Canada. The Canadian Cannabis Survey: Methodological Report. 2019. https://publications.gc.ca/site/eng/9.875402/publication.html.
26 Cash MC, Cunnane K, Fan C, *et al.* Mapping cannabis potency in medical and recreational programs in the United States. *PLoS One* 2020; 15: e0230167.
27 ElSohly MA, Chandra S, Radwan M, *et al.* A comprehensive review of cannabis potency in the United States in the last decade. *Biol Psychiatry Cogn Neurosci Neuroimaging* 2021; 6: 603–606.
28 Fantauzzi MF, Cass SP, McGrath JJC, *et al.* Development and validation of a mouse model of contemporary cannabis smoke exposure. *ERJ Open Res* 2021; 7: 00107-2021.
29 Waithman J, Mintern JD. Dendritic cells and influenza A virus infection. *Virulence* 2012; 3: 603–608.
30 GeurtsvanKessel CH, Willart MA, van Rijt LS, *et al.* Clearance of influenza virus from the lung depends on migratory langerin^{+}CD11b^{-} but not plasmacytoid dendritic cells. *J Exp Med* 2008; 205: 1621–1634.
31 Pribul PK, Harker J, Wang B, *et al.* Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. *J Virol* 2008; 82: 4441–4448.
32 Robinson DP, Lorenzo ME, Jian W, *et al.* Elevated 17β-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses. *PLoS Pathog* 2011; 7: e1002149.
33 Lorenzo ME, Hodgson A, Robinson DP, *et al.* Antibody responses and cross protection against lethal influenza A viruses differ between the sexes in C57BL/6 mice. *Vaccine* 2011; 29: 9246–9255.
34 Vom Steeg LG, Klein SL. Sex and sex steroids impact influenza pathogenesis across the life course. *Semin Immunopathol* 2019; 41: 189–194.
35 Wang J, Li Q, Xie J, *et al.* Cigarette smoke inhibits BAFF expression and mucosal immunoglobulin A responses in the lung during influenza virus infection. *Respir Res* 2015; 16: 37.
36 Bauer CM, Dewitte-Orr SJ, Hornby KR, *et al.* Cigarette smoke suppresses type I interferon-mediated antiviral immunity in lung fibroblast and epithelial cells. *J Interferon Cytokine Res* 2008; 28: 167–179.

37 Karmaus PW, Chen W, Crawford R, *et al.* Δ9-tetrahydrocannabinol impairs the inflammatory response to influenza infection: role of antigen-presenting cells and the cannabinoid receptors 1 and 2. *Toxicol Sci* 2013; 131: 419–433.

38 Chaaban T. Acute eosinophilic pneumonia associated with non-cigarette smoking products: a systematic review. *Adv Respir Med* 2020; 88: 142–146.

39 Chabot C, Gouat F. Rare finding of acute eosinophilic pneumonia associated with heavy cannabis use: a case-report. *Radiol Case Rep* 2022; 17: 3040–3042.

40 Kaplan BL, Lawver JE, Karmaus PW, *et al.* The effects of targeted deletion of cannabinoid receptors CB1 and CB2 on intranasal sensitization and challenge with adjuvant-free ovalbumin. *Toxicol Pathol* 2010; 38: 382–392.

41 Balkovic ES, Florack JA, Six HR. Immunoglobulin G subclass antibody responses of mice to influenza virus antigens given in different forms. *Antiviral Res* 1987; 8: 151–160.

42 Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 2014; 5: 520.

43 Renegar KB, Small PA Jr, Boykins LG, *et al.* Role of IgA *versus* IgG in the control of influenza viral infection in the murine respiratory tract. *J Immunol* 2004; 173: 1978–1986.

44 Jefferis R, Kumararatne DS. Selective IgG subclass deficiency: quantification and clinical relevance. *Clin Exp Immunol* 1990; 81: 357–367.