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ORGAN TOXICITY AND MECHANISMS

Differential inflammatory profile in the lungs of mice exposed to cannabis smoke with varying THC:CBD ratio

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Abstract

Cannabis contains cannabinoids including Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC causes the psychoactive effects of cannabis, and both THC and CBD are thought to be anti-inflammatory. Cannabis is typically consumed by inhaling smoke that contains thousands of combustion products that may damage the lungs. However, the relationship between cannabis smoke exposure and alterations in respiratory health is poorly defined. To address this gap in knowledge, we first developed a mouse model of cannabis smoke exposure using a nose-only rodent inhalation exposure system. We then tested the acute effects of two dried cannabis products that differ substantially in their THC–CBD ratio: Indica-THC dominant (I-THC; 16–22% THC) and Sativa-CBD dominant (S-CBD; 13–19% CBD). We demonstrate that this smoke exposure regime not only delivers physiologically relevant levels of THC to the bloodstream, but that acute inhalation of cannabis smoke modulates the pulmonary immune response. Cannabis smoke decreased the percentage of lung alveolar macrophages but increased lung interstitial macrophages (IMs). There was also a decrease in lung dendritic cells as well as $Ly6C^{intermediate}$ and $Ly6C^{low}$ monocytes, but an increase in lung neutrophils and $CD8^+$ T cells. These immune cell changes were paralleled with changes in several immune mediators. These immunological modifications were more pronounced when mice were exposed to S-CBD compared to the I-THC variety. Thus, we show that acute cannabis smoke differentially affects lung immunity based on the THC:CBD ratio, thereby providing a foundation to further explore the effect of chronic cannabis smoke exposures on pulmonary health.

Keywords Cannabis · THC · Cannabinoid · Inflammation · Macrophage · Cytokines

Introduction

Cannabis, commonly referred to as marijuana, is the most widely used psychoactive drug, with approximately 150 million users worldwide (Joshi et al. 2014). Cannabis contains of over 400 chemicals, of which at least 100 are cannabinoids. Cannabinoids are secondary metabolites produced in the trichomes of the female inflorescence and are thought to impart most of the anecdotal health benefits of cannabis use. The two most abundant cannabinoids are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Booth and Bohlmann 2019) and most commercial cannabis varieties are defined by their THC/CBD ratio. Cannabinoids modulate a variety of physiological processes such as neurocognition, appetite, pain, and immunity (Baron 2018; Costiniuk and Jenabian 2019; Nader and Sanchez 2018). THC is responsible for the psychoactive effects of cannabis whereas both THC and CBD are thought to have anti-inflammatory properties.

Cannabis is the second most smoked product after tobacco (Hoffman et al. 1975), and is commonly inhaled via a joint or water pipe. Like tobacco smoke, cannabis smoke contains at least 33 carcinogens (e.g., polycyclic aromatic hydrocarbons) and other toxicants that could damage the lung and impair gas exchange (Schwartz 2017). Unlike for

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tobacco smoke though, where the adverse respiratory effects are well-established, there are gaps and inconsistencies in the literature regarding the lung health effects of cannabis use, largely owing to issues in legality or concomitant tobacco use by human study subjects. Evidence does support that cannabis smoke exposure is associated with cough, wheeze, sputum production and dyspnea (Ghasemiesfe et al. 2018). Smoking cannabis may also increase the risk of severe bronchitis, although there is conflicting evidence that this leads to chronic obstructive pulmonary disease (COPD), an obstructive lung disease known to be caused by tobacco smoke and other inhaled pollutants (Gracie and Hancox 2021). Cannabis smoke may also affect physical (e.g., mucociliary clearance) and immunological defense mechanisms (Chatkin et al. 2017). Support for the latter comes from studies showing that the number of macrophages are higher in the lungs of cannabis smokers vs. non-smokers (Tashkin et al. 2002). In many of these studies, the cannabis contained THC levels below 5% and outcomes focused on pulmonary morphology (Fleischman et al. 1979, 1975; Rosenkrantz and Braude 1974). Recently, two studies reported that mice which received whole-body-exposure to cannabis smoke exhibited lung inflammation (Fantauzzi et al. 2021; Helyes et al. 2017). Overall, these results are suggestive that cannabis smoke may be deleterious to the lungs.

Despite cannabis legalization in a growing number of countries, and enthusiasm for its possible therapeutic properties, our understanding of the potential lung health consequences of inhaled cannabis smoke remains inconclusive. This is further complicated by the enormous variation in cannabis strains available today, with over 800 different cultivars with distinct chemical profiles (de la Fuente et al. 2020). Consequently, previous studies utilizing cannabis strains may not reflect outcomes of current cannabis use, particularly given that the average concentration of THC in commercially available strains can now exceed 15%. To our knowledge, there are no studies comparing the effects of different cannabis cultivars on lung health, including those that are CBD-dominant. These are gaps in knowledge though, that can be addressed using preclinical exposure models. However, and unlike for tobacco, there are no standardized cannabis smoke exposure protocols for preclinical models, protocols that would allow for comparison between studies. We recently published a standardized cannabis smoke extract protocol for in vitro exposures that delivers cannabinoids to the cell culture media (Aloufi et al. 2022). Herein, we have developed a mouse model of cannabis smoke exposure using a nose-only rodent inhalation exposure system. We chose a nose-only exposure (as opposed to whole body) to limit non-respiratory exposures (i.e., ingestion via grooming behavior) and thus be more representative of human use. We then used this methodology to test the effects of two varieties of cannabis with vastly different levels of THC and CBD. Not only did our smoke exposure regime deliver physiologically relevant levels of cannabinoids to the bloodstream, but inhalation of cannabis smoke modulated the lung immune landscape typified by altered immune cell composition and cytokine profiles. Cannabis smoke from both cannabis cultivars increased pulmonary inflammation. Overall, these results suggest that even an acute cannabis smoke exposure affects lung inflammation and thus may have implications for habitual users.

Materials and methods

Mice

Ten-week-old male and female C57BL/6 mice were bred and maintained in the Research Institute of the McGill University Health Centre (RI MUHC). Mice were given ad libitum access to food and water. Cages were housed within a temperature-controlled vivarium on a 12-h light/dark cycle. All animal procedures were approved by the McGill University Animal Care Committee and were carried out in accordance with the guidelines of the Canadian Council on Animal Care Committee.

Preparation of cannabis cigarettes

Cannabis cigarettes were prepared using cannabis purchased from the *Société québécoise* du *cannabis (SQDC)* online store (Quebec, Canada). Two different varieties of cannabis were used in this study: (1) Indica-THC dominant (I-THC) with 16–22% THC and 0–0.1% CBD (#688,083,002,311) and (2): Sativa-CBD dominant (S-CBD) with 0.1–2% THC and 13–19% CBD (#694,144,000,219). Cannabis joints were hand-rolled by grinding the dried cannabis flower and packing into classic 1 1/4 Size rolling paper (RAW). Each cannabis cigarette contained 0.5 ± 0.05 g of cannabis. A slim unrefined cellulose filter (RAW) was added to the end of each cannabis cigarette.

Cannabis smoke exposure protocol

Mice were exposed to two cannabis cigarettes twice daily for 3 days using a nose-only rodent inhalation InExpose system (SCIREQ, Montreal, Canada). On the third day of exposure, mice were exposed only once in the morning and subsequently killed 1 h after the last exposure. The puff profile was set to deliver three puffs/ minute with 35 mL puff volume and a 2 s puff duration. There was a 3-h break in between the daily exposure sessions. Control mice were exposed to room air and manipulated in an identical fashion.

Organ collection and cell isolation

Mice were anesthetized with 0.7 ml intraperitoneal injection of Avertin (2,2,2-tribromoethanol, 250 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) and euthanized. The trachea was cannulated, and two bronchoalveolar lavages (BAL; 2 *1 ml of Roswell Park Memorial Institute (RPMI) 1640; Thermo Fisher) were performed to collect cells present in the airways. Airway cells obtained from the BAL were centrifuged and resuspended; lysis of red blood cells in the BAL fluid was performed using red blood cell lysis buffer ACK (ammonium–chloride–potassium; Thermo Fisher). Lungs were removed and placed in RPMI. Lung tissue was cut into small pieces and digested with collagenase IV (Sigma) at a concentration of 150 units per ml and incubated at 37 °C for 1 h. After that, lung homogenates were obtained by gently rubbing organs over 70 µm nylon mesh. The nylon mesh was washed twice with RPMI 1640, and the lung homogenates were centrifuged at 400*g* for 5 min. Lysis of red blood cells was performed using 0.5 ml of ACK. Spleens were also collected and homogenates obtained by gently rubbing organs over 70 µm nylon mesh, followed by centrifugation and lysis of red blood cells by ACK lysis buffer.

Flow cytometry

Aliquots of 10^5 BAL cells or 10^6 lung/spleen cells were seeded into round-bottom 96-well plates. Cells were then labeled with viability dye eFluor™ 506 (AmCyan)-65–0866-14 (Invitrogen) at a concentration of 1:1000 for 30 min (4 °C). Subsequently, the cells were washed with PBS supplemented with 0.5% BSA (HyCloneTM) and 2 mM EDTA (Invitrogen) and incubated with anti-CD16/32 antibody (BD Biosciences) for 20 min at 4 °C at a concentration of 0.5:70 in PBS containing 0.5% BSA and 2 mM EDTA at 4 °C for 20 min. Cells were then incubated with fluorochrome-tagged antibodies at a concentration of 0.5:70 at 4 °C for 30 min. Antibodies for the myeloid panel were: PerCP/Cy5.5 anti-CD64 (clone X54-5/7.1), BV421 anti-MERTK (clone 2B10C42), Alexafluor-488 anti-CD11b (clone M1/70), PE-Cy7 anti-Ly6G (clone 1A8), APC-Cy7 anti-CD11c (clone N418), PE anti-Ly6c (HK1.4) and APC anti-MHCII (clone M5/114.15.2). The gating strategy utilized for flow cytometry of myeloid cells is based on Gibbings et al. (Gibbings et al. 2017). Antibodies for the adaptive panel were: FITC anti-CD3 (clone17A2), PE anti-CD4 (clone GK1.5) and APC anti-CD8 (clone 53–6.7); all antibodies were purchased from BioLegend, except for the anti-CD3 antibody which was obtained from Invitrogen. Then, cells were washed with PBS containing 0.5% BSA and 2 mM EDTA and fixed using IC Fixation buffer (Invitrogen) at 4 °C for 20 min. Finally, cells were washed and resuspended in PBS with 0.5% BSA and 2 mM EDTA. Fluorescence compensation for each fluorochrome was performed using beads to ensure that the fluorescence detected was derived from the fluorochrome being measured. Additionally, the fluorescence minus one (FMO) control was done by staining samples with all the fluorophores in each panel, minus one of them to help determine where the gates should be set. At the end, data were immediately acquired using the FACSCanto II flow cytometer (BD Biosciences) and data were analyzed by FlowJo software (version 10.2).

THC ELISA

Whole blood was collected at the time of killing into 1.5 ml Eppendorf tubes containing 100 ul of EDTA. Plasma was then collected by centrifugation. THC was analyzed using the Neogen Corporation THC ELISA according to the manufacturer's recommendations. The absorbance was read at 450 nm within 15 min by infinite TECAN (M200 pro, TECAN, CA) and quantification performed using a standard curve as we have described (Aloufi et al. 2022).

Cytokine analysis

Cytokines were quantified in BAL fluid using the Mouse Cytokine Array/Chemokine Array 44-plex (Eve Technologies, Calgary, AB, Canada).

Statistical analysis

Using GraphPad Prism 6 (v. 6.02; La Jolla, CA, USA), statistical analysis was performed using unpaired *t* tests ($\alpha = 0.05$) or for groups of more than two via analysis of variance (ANOVA) with Bonferroni–Holm correction for multiple comparisons.

Results

Validation of the preclinical cannabis smoke exposure protocol

We aimed to develop a preclinical model of cannabis smoke exposure using a nose-only inhalation rodent exposure system to deliver physiologically relevant levels of cannabinoids to the blood. To achieve this, two cannabis cigarettes from THC-dominant (I-THC) or CBD-dominant (S-CBD) varieties of cannabis were used for each exposure session and combustion was assessed by percentage of particle density (% PD). During the cannabis smoke exposure, the % PD within the exposure chamber varied between 0.5 and 1%, and mice returned to normal activity within 1 h after the exposure session. Immediately after the last exposure on day 3, blood was collected for quantification of Δ^9-THC–COOH

levels using a qualitative ELISA assay; we previously generated a standard curve for quantification (Aloufi et al. 2022). As expected, mice exposed only to air room air had no detectable THC. In contrast, plasma from cannabis-exposed mice had a significant increase in blood Δ^9-THC–COOH when the I-THC dominant variety was used; there was significantly less THC in the blood of mice exposed to the Sativa-CBD dominant variety (Fig. 1). The concentration of THC in the mice exposed to I-THC is similar to that reported in the blood of human cannabis smokers (Perez-Reyes et al. 1981). Together, our results confirm that the cannabis smoke exposure regime used in this study delivers physiological levels of cannabinoids to the blood of mice.

Acute cannabis smoke exposure modifies immune cell populations in the lungs and BAL

Next, we sought to comprehensively profile the effects of acute cannabis smoke exposure on respiratory immune cell populations both in the BAL and lungs (Gibbings et al. 2017). For this, we used two flow cytometry panels to identify the myeloid (Fig. 2) and lymphoid (Fig. 3) populations. First, analysis of cells from the lungs demonstrated that the percentage of total live cells in the lungs remained unchanged in cannabis-exposed animals (Fig. 4A), suggesting negligible effects on immune cell survival. However, the percentage of macrophages (MerTK$^+$, CD64$^+$ cells) in the lungs was significantly decreased in mice exposed to both I-THC and S-CBD varieties compared to air-exposed mice; there was no difference in lung macrophages based on the THC:CBD ratio (Fig. 4B). This decrease in macrophages

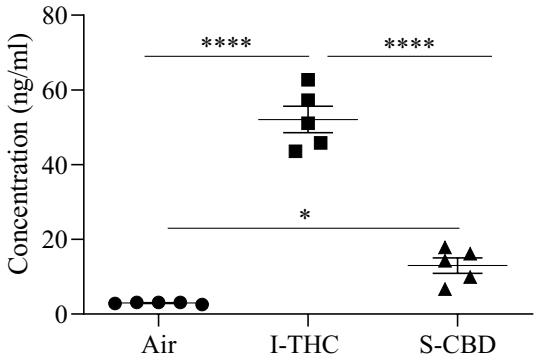

Fig. 1 Acute cannabis smoke exposure increases plasma Δ^9-THC–COOH concentration. Mice were exposed to room air or the smoke of 2 cannabis cigarettes from Indica-THC dominant (I-THC) and Sativa-CBD dominant (S-CBD) twice in a single day. Plasma Δ^9-THC–COOH was measured via ELISA in the blood. Mice exposed to the THC-dominant and CBD-dominant cannabis smoke have more Δ^9-THC–COOH compared to mice exposed to air (****$p < 0.0001$ and *$p = 0.0334$, respectively). There was significantly more blood levels of Δ^9-THC–COOH in mice exposed to the THC dominant versus the CBD dominant. Data represent mean ± SEM; $n = 5$ per group

was reflected by a decrease in tissue-resident macrophages (MerTK$^+$, CD64$^+$, CD11b$^-$, CD11c$^+$ cells) in mice exposed to both I-THC and S-CBD varieties compared to air group (Fig. 4C); interestingly, the percentage of interstitial macrophages (IMs) (MerTK$^+$, CD64$^+$, CD11b$^+$, CD11c$^-$) was significantly increased by I-THC and S-CBD smoke exposure (Fig. 4D). Notably, the percentage of neutrophils in mice exposed to smoke from both the I-THC and S-CBD varieties was also significantly higher compared to air-exposed mice (Fig. 4E).

In the same flow cytometry panel, we also evaluated different subsets of monocytes that can differentiate into macrophages or dendritic cells (DCs). While there was no change in the total percentage of lung monocytes (MerTK$^-$, CD64$^{low/-}$, CD11c$^-$, MHCII$^{int/-}$, CD11b$^+$, Ly6G$^-$, Ly6C$^{+/intermediate/-}$ cells) in response to acute cannabis smoke inhalation (Fig. 4F), there were significant differences in monocyte subsets as distinguished by the levels of Ly6C (Fig. 4G–I). Here, the percentage of Ly6C^{high} monocytes was significantly increased in mice exposed to I-THC and S-CBD varieties compared to air group (Fig. 4I). However, the percentage of Ly6C^{low} monocytes was significantly decreased in mice exposed to cannabis smoke exposure compared to air group. Ly6C^{intermediate} monocytes were significantly less in mice exposed to I-THC and S-CBD varieties compared to air group. Finally, there was a significant decrease in the percentage of pulmonary DCs (MerTK$^-$, CD64$^{low/-}$, CD11c$^+$, MHCII$^+$) in mice exposed to cannabis smoke compared to air group (Fig. 4J).

We also evaluated for changes in lymphoid cells in the lungs of exposed mice, and in particular differences in CD4$^+$ and CD8$^+$ T cells, since cannabinoids can module T cell numbers via alterations in proliferation and survival in vitro (Devi et al. 2022; Wu et al. 2008). However, the impact of cannabis smoke on the number of T cell subsets in the lungs in vivo is not known. Our data show that there was an overall increase in the percentage of CD3$^+$ cells in mice exposed to I-THC and S-CBD compared to air group (Fig. 5A). There was no change in the percentage of CD4 + T cells in response to acute cannabis smoke exposure (Fig. 5B). There was a significant increase in the percentage of CD8$^+$ T cells in the lungs in response to inhalation of cannabis smoke from both varieties compared to the air-exposed mice (Fig. 5C).

In addition to immune profiling of the lung tissue, we also performed separate analysis on cells in the airways. However, inhalation of cannabis smoke had varying effects on the immune cell composition in the BAL. The percentage of total live immune cells in the BAL was significantly decreased in mice exposed to cannabis smoke compared to those exposed only to room air (Fig. 6A). Further analysis revealed that there were no changes in the percentage of total macrophages (Fig. 6B) or macrophage subpopulations (Fig. 6C, D) in the BAL of mice exposed to both the

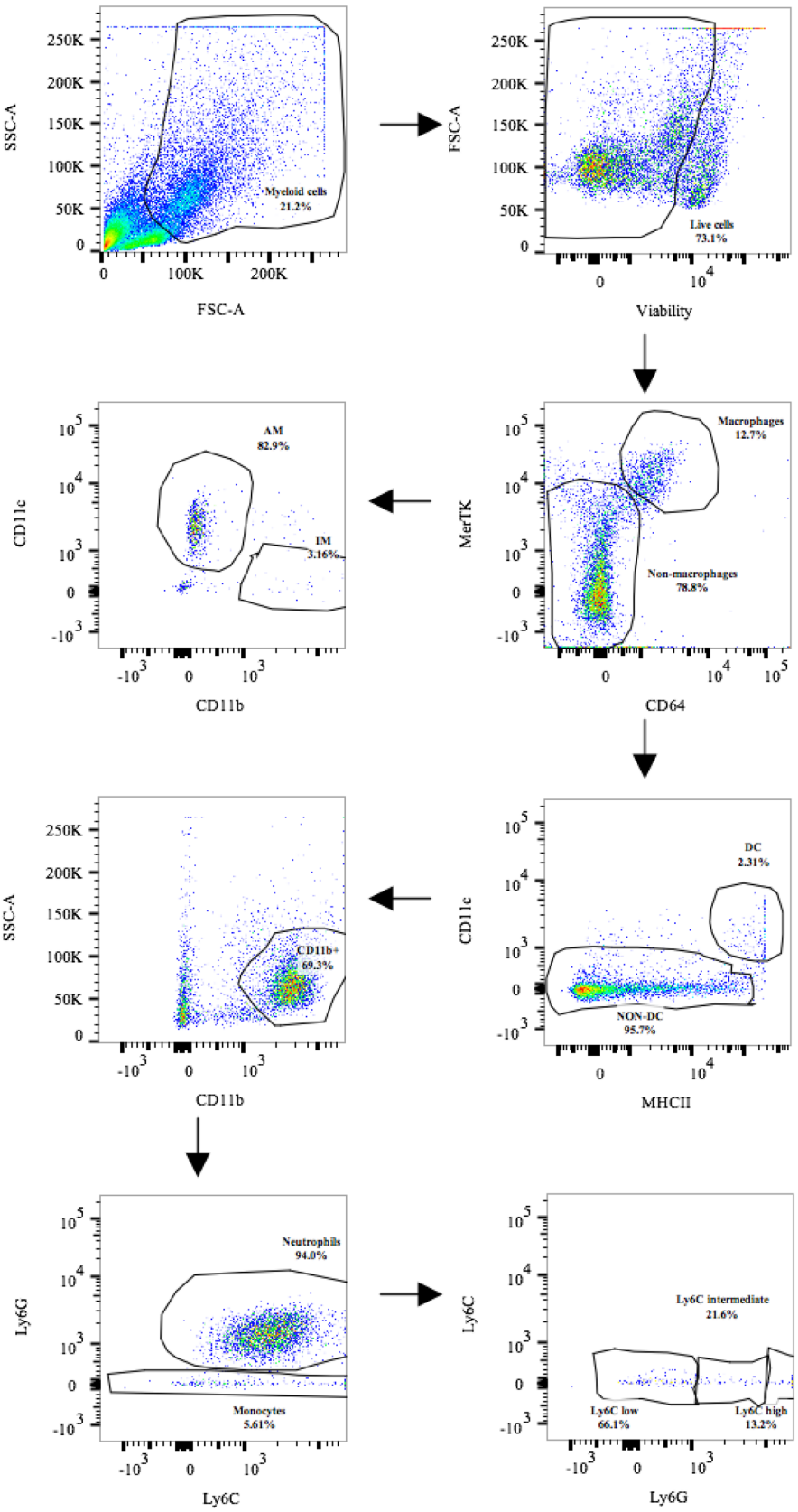

Fig. 2 Gating strategy utilized for flow cytometry of myeloid cells in lung tissue of mice. Total live cells were selected using viability dye after removing debris. Total myeloid cells were then plotted as MerTK versus CD64. The $MerTK^{+}CD64^{+}$ (macrophage) gate was plotted as CD11c versus CD11b to identify alveolar macrophages (AMs) and the interstitial macrophages (IMs). $MerTK^{-}CD64^{-}$ macrophage-deficient gate was plotted with CD11c and MHCII to identify dendritic cells (DCs). $CD11c^{-}MHCII^{-}$ Non-DCs gate was plotted as side scatter (SSC) versus CD11b to identify $CD11b^{+}$ population. $CD11b^{+}$ population were plotted as Ly6G versus Ly6C to identify $Ly6G^{+}$ neutrophils and three types of Ly6C monocytes ($Ly6C^{low}$, $Ly6C^{intermediate}$, $Ly6C^{high}$). MHC, major histocompatibility complex. Representative plots shown are from an air control mouse

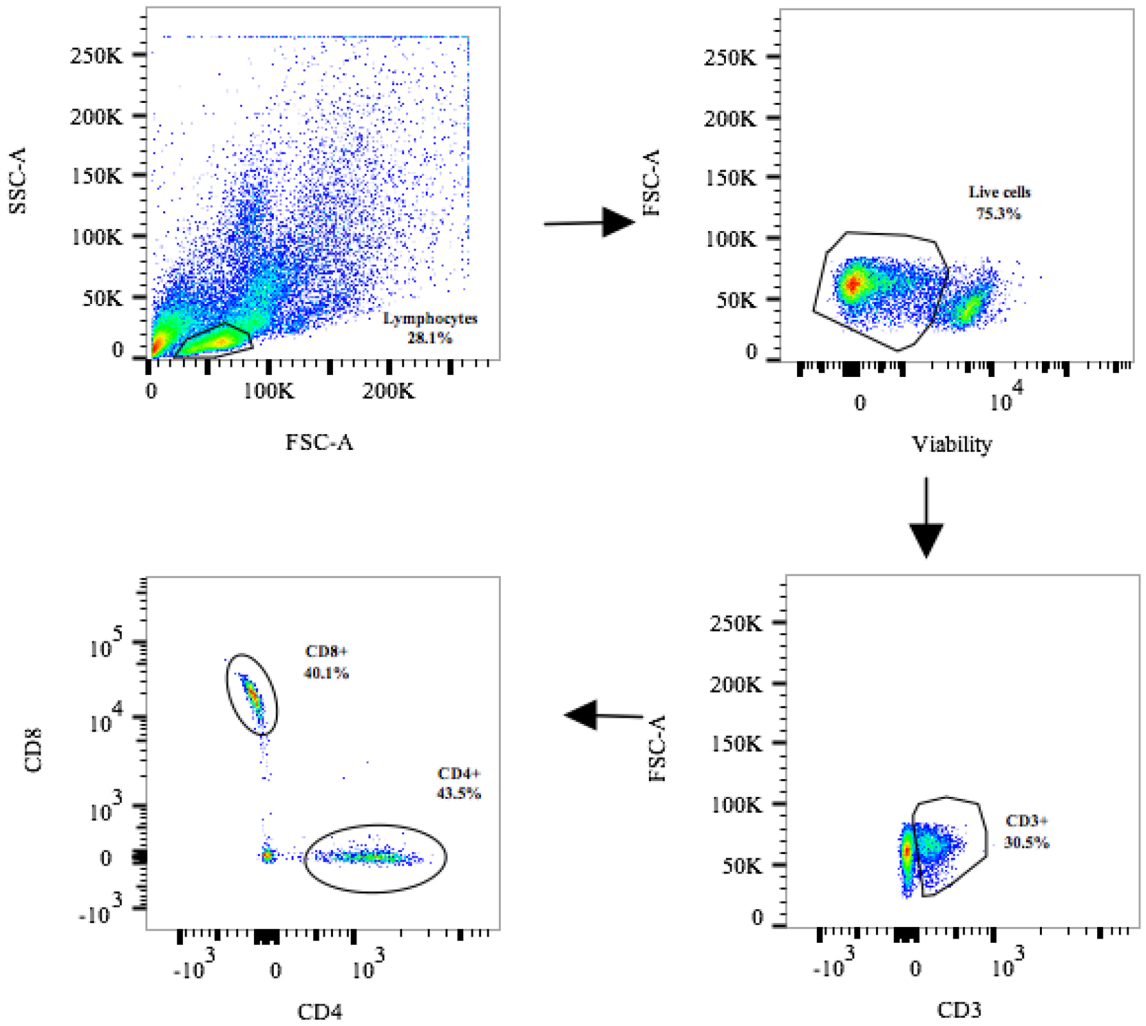

Fig. 3 Gating strategy for identifying lymphoid cells by flow cytometry in lung tissue of mice. Total live cells were selected using viability dye after removing debris. Total lymphocytes were then plotted as FSC versus CD3. Then, the CD3 population was plotted with CD4 and CD8 to identify the $CD4^+$ cells and $CD8^+$ T cells, respectively. Representative plots shown are from an air control mouse

THC-dominant and CBD-dominant varieties. There was no significance change in neutrophils (Fig. 6E) or monocytes (Fig. 6F) in the BAL in response to cannabis smoke. There was also no change in BAL lymphocyte subsets from acute cannabis smoke exposure (Fig. 7). Overall, these results demonstrate that acute cannabis smoke exposure changes the lung immune landscape.

Differential effect of cannabis smoke exposure on immune mediators in the BAL

To better understand whether differential changes in cytokine levels could potentially account for immune cell recruitment to the lungs and airways, we performed a 44-plex cytokine array on the BAL fluid. The cytokines included in this array were eotaxin, erythropoietin, 6Ckine, fractalkine, G-CSF, GM-CSF, IFNB1, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17, IL-20, IP-10, KC, LIF, LIX, MCP-1, MCP-5, M-CSF, MDC, MIG, MIP-1α, MIP-1β, MIP-2, MIP-3α, MIP-3B, RANTES, TARC, TIMP-1, TNFα and VEGF-A. Cytokine profiling in the BAL of mice following acute cannabis smoke exposure revealed that there were significant changes in 14 out of the 44 cytokines analyzed (raw data are in Supplemental Table 1). Overall, these changes were more pronounced when mice were exposed to S-CBD compared to I-THC dominant varieties. We then organized these cytokines based on their main mode of action, including those associated with macrophage biology (Fig. 8A). Here, inhalation of smoke from the S-CBD variety significantly increase the levels of monocyte chemotactic protein 5 (MCP-5; CCL12), thymus- and activation-regulated chemokine (TARC; CCL17) and macrophage-derived chemokine (MDC; CCL22); there was no significant change

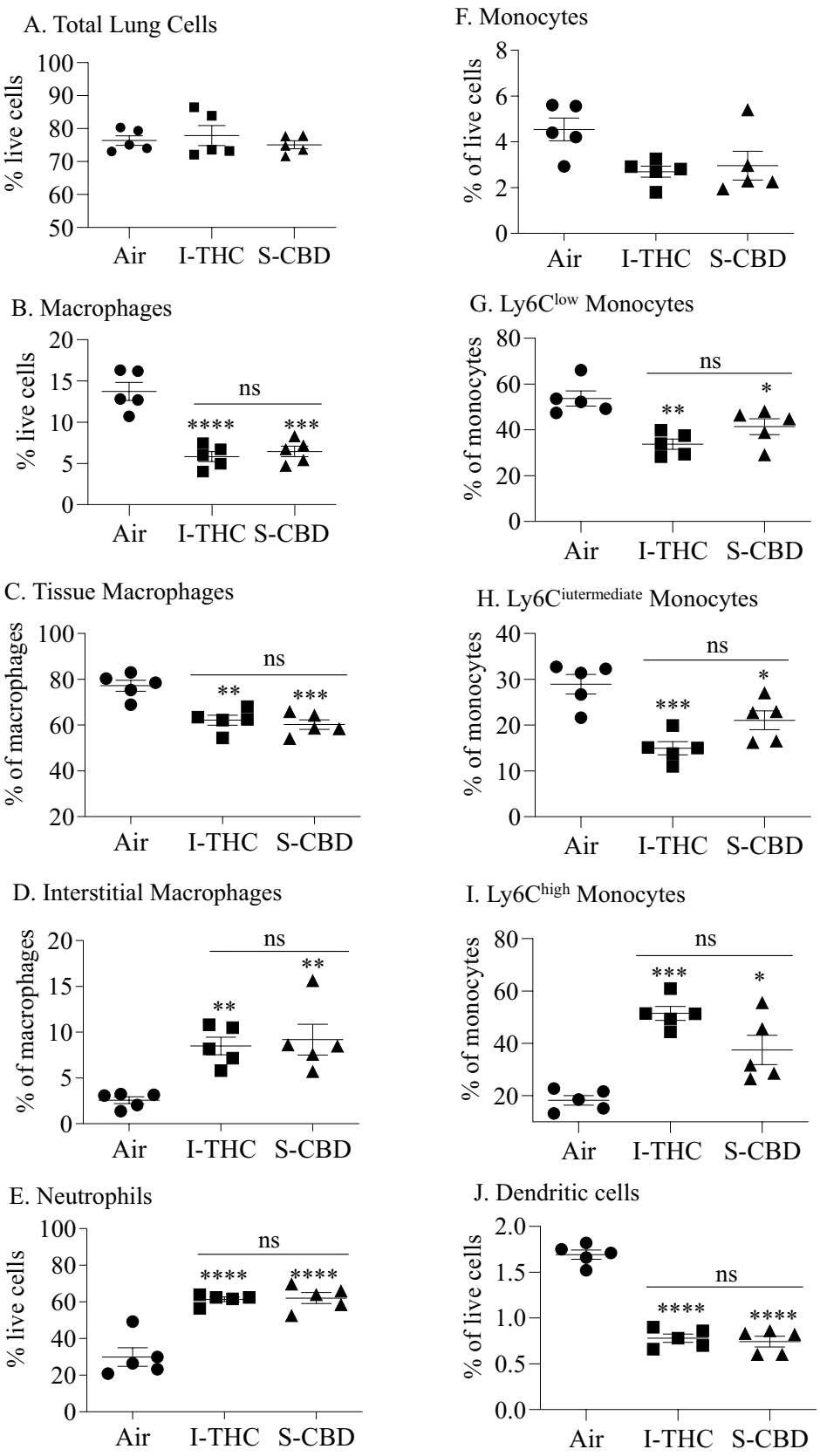

Fig. 4 Cannabis smoke exposure modifies the percentage of innate immune cell populations in the lungs. **A** Total lung cells—there was no difference in the percentage of total live cells in mice exposed to cannabis vs mice exposed to room air. **B** Macrophages—there was a significant decrease in macrophage percentages in mice exposed to smoke from the I-THC and S-CBD varieties compared to air group (****$p < 0.0001$ and ***$p = 0.0001$, respectively). **C** Tissue macrophages were decreased in mice exposed to smoke from the I-THC (**$p = 0.0015$) and S-CBD (***$p = 0.0005$) varieties compared to air group. **D** Interstitial macrophages were significantly higher in mice exposed to smoke from the I-THC (**$p = 0.0097$) and S-CBD (**$p = 0.0045$) varieties compared to air group. **E** Neutrophils—there was a significant increase in neutrophil percentages in mice exposed to smoke from the I-THC and S-CBD varieties compared to air group (****$p < 0.0001$). **F** Monocytes—the percentage of monocytes was unchanged in mice exposed to cannabis smoke compared to air group. **G** Ly6C^{low} monocytes were decreased in mice exposed to I-THC and S-CBD varieties compared to air group (**$p = 0.0017$ and *$p = 0.0432$). **H** Ly6C^{intermediate} monocytes were significantly less in mice exposed to smoke from the I-THC (***$p = 0.0007$) and S-CBD (*$p = 0.0381$) varieties compared to air group. **I** Ly6C^{high} monocytes were higher in mice exposed to I-THC and S-CBD varieties compared to air group (***$p = 0.0001$ and *$p = 0.0100$). **J** Dendritic cells were decreased in mice exposed to smoke from the THC-dominant and CBD-dominant varieties compared to air group (****$p < 0.0001$). Data represent mean ± SEM; $n = 5$ per group

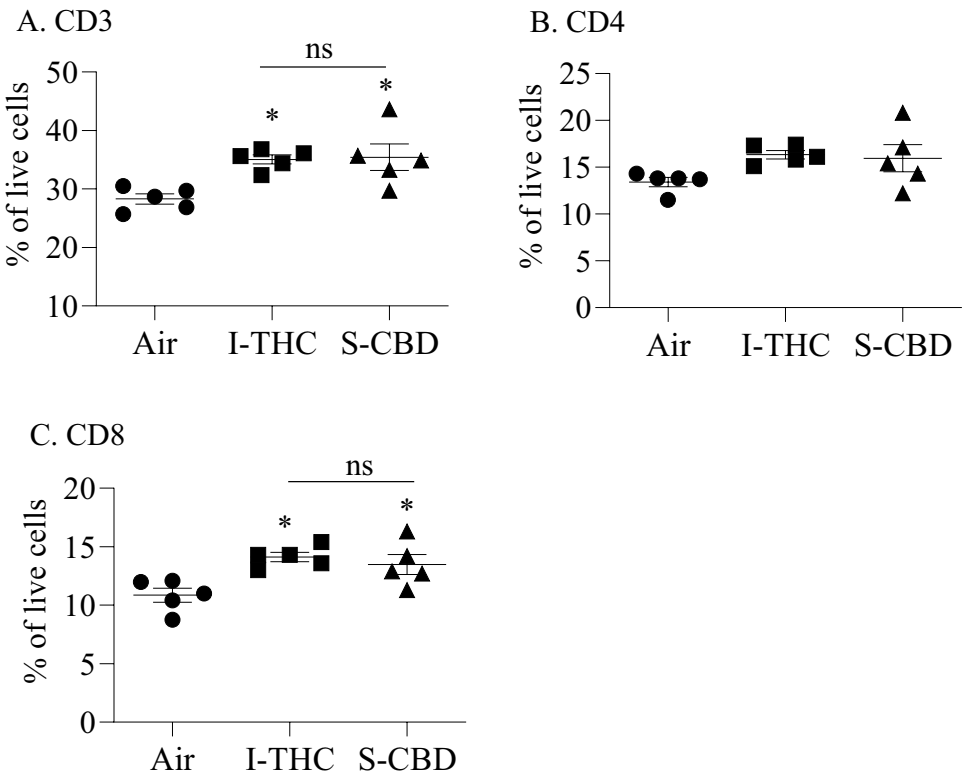

Fig. 5 Acute cannabis exposure alters the percentage of lymphoid cells in the lungs. **A** CD3—the percentage of $CD3^+$ cells were significantly increased in mice exposed to smoke from both the I-THC (*$p = 0.0220$) and S-CBD (*$p = 0.0157$) varieties. **B** CD4—there was no difference in $CD4^+$ T cells percentages in mice exposed to cannabis smoke. **C** CD8—$CD8^+$ T cells were significantly increased in the lungs in response to inhalation of cannabis smoke using the THC-dominant strain (*$p = 0.0111$) and CBD-dominant strain compared to the air-exposed mice (*$p = 0.0410$). Data represent mean ± SEM; $n = 5$ per group

in the levels of these mediators from inhalation of the smoke derived from the I-THC variety. There was also a significant increase in the levels of eotaxin (CCL11) (Fig. 8B) in mice exposed to smoke only from the CBD-dominant variety. Antiviral cytokines such as CXCL10 (IP-10) and CCL5 (RANTES) were differentially regulated by S-CBD, where there was a significant increase in CXCL10 but a decrease in CCL5 (Fig. 8C); neither CXCL10 nor CCL5 were significantly changed by I-THC. There was also an overall decrease in BAL levels of select interleukins (IL) (Fig. 8D); this includes pro-inflammatory cytokines such as IL-1α (Botelho et al. 2011), cytokines that promote survival and maturation of immune cells (IL-3, IL-7), Th2 cytokines that are involved in IgE production (IL-13) (Van der Pouw Kraan et al. 1998), IFN production (IL-11) (Miyawaki et al. 2019) and, finally, IL-20, an interleukin that enhances innate immune function (Rutz et al. 2014). S-CBD significantly reduced BAL levels of these interleukins, with I-THC causing suppression only for IL-11 and IL-20 (Fig. 8D). Finally, two growth factors that were differentially regulated by S-CBD were vascular endothelial growth factor (VEGF) and granulocyte-stimulating factor (G-CSF), with S-CBD reducing levels of VEGF, but increasing BAL levels of G-CSF (Fig. 8E). Overall, these results show that the levels of immune mediators in the BAL is differentially modulated by acute exposure to cannabis smoke, with more alterations occurring because of smoke generated from the combustion of a high CBD variety.

Acute cannabis smoke exposure modulates the levels of neutrophils in the spleen

One advantage of our preclinical exposure system is that cannabis smoke is delivered directly to the respiratory system, allowing us to better ascertain whether inhalation can also cause changes in organs systems distal to the lungs; this model therefore avoids confounding factors related to whole-body exposures. Thus, we next characterized the effects of cannabis smoke inhalation on the immune cell composition in spleen using this acute (3-day) model. Flow cytometric analysis of cells from the spleen revealed that the percentage of total live cells in the spleen was not changed in cannabis smoke-exposed animals (Fig. 9A). Analysis of the myeloid cell population in spleen showed that the percentage of neutrophils was increased only in mice exposed to I-THC compared to air group (Fig. 9B). There was also no difference in the total percentage of spleen monocytes (Fig. 9C), monocyte subsets (Fig. 9D–F) or DCs (Fig. 9G). Finally, flow cytometry analysis revealed that there was also no significant change in splenic proportion of lymphocyte subsets (Fig. 10). Overall, our results demonstrate that exposing mice to cannabis smoke for 3 days alters the pulmonary immune landscape with minimal effect on distal organs such as the spleen.

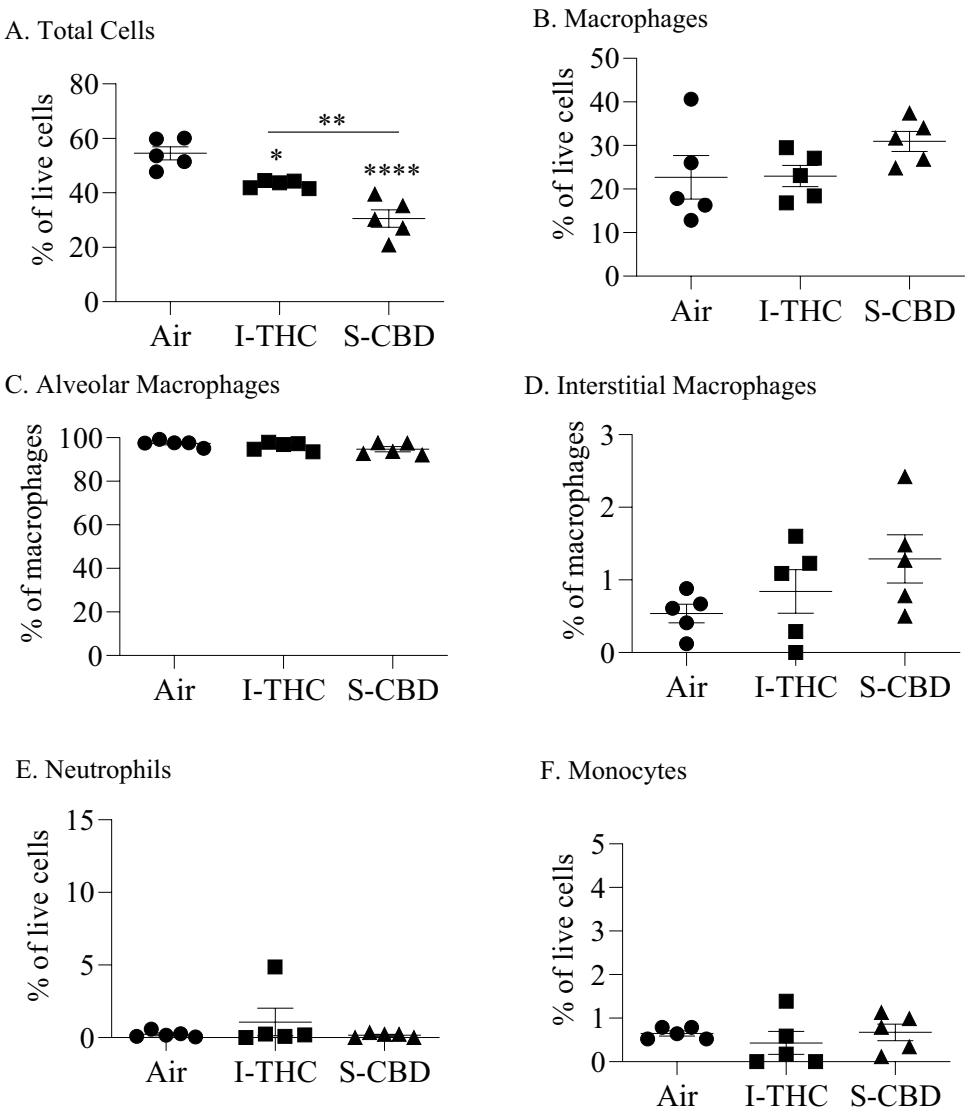

Fig. 6 Acute cannabis smoke exposure does not modulate cellularity in the bronchoalveolar lavage (BAL). **A** Total cells—the percentage of total live cells was significantly decreased in mice exposed to smoke from both the I-THC (*$p=0.0153$) and S-CBD (****$p=<0.0001$) varieties. This decrease was also significant in S-CBD exposed mice compared to mice exposed to I-THC (**$p=0.0077$). **B** Macrophages—the percentage of macrophages was not changed in mice exposed to cannabis smoke from both varieties compared to air group. There was also no difference in the percentage of **C** AMs, **D** IMs, **E** neutrophils or **F** monocytes in mice exposed to cannabis compared to mice exposed to air. Data represent mean ± SEM; $n=5$ per group

Discussion

Despite legalization in many parts of the world, our knowledge of the effects of cannabis smoking on the respiratory system remains limited. Clinical studies suggest that cannabis smoking increases the risk of symptoms associated with chronic bronchitis, including inflammation and injury in the airways. However, gaps and inconsistencies in the literature exist, in part because comparison between studies is complicated by numerous factors including historical issues with legality that prevented scientific advancement, differences in species (human versus mouse) and the array of cannabis products available for study. To better address gaps in knowledge regarding cannabis use and pulmonary health, we utilized a mouse model of cannabis smoke exposure using a nose-only inhalation exposure system for 3 days to compare two varieties of cannabis that differed predominately in the THC:CBD ratio; we then assessed the effects of exposure on lung immune and inflammatory responses. Overall, we show that acute exposure to cannabis smoke leads to significant alteration in the immunological profile of respiratory system, with the THC–CBD ratio affecting this response.

One of the key findings in our study was alterations in the macrophage populations in the lungs. Different macrophage subtypes occur in the healthy lung including alveolar macrophages and IMs. Alveolar macrophages are long-lived, embryonically derived cells that self-renew to main their population (Gangwar et al. 2020). Alveolar macrophages include tissue-resident alveolar macrophages (TR-AMs) but also monocyte-derived alveolar macrophages (Mo-AMs) (Hou et al. 2021). When injury occurs, blood monocytes are recruited to the alveolar lumen and develop into Mo-AMs, and can cause tissue damage by releasing numerous cytokines (Hou et al. 2021). Conversely, IMs are thought to have a mixed origin, being initially derived from yolk sac precursors, and later replaced by circulating monocytes.

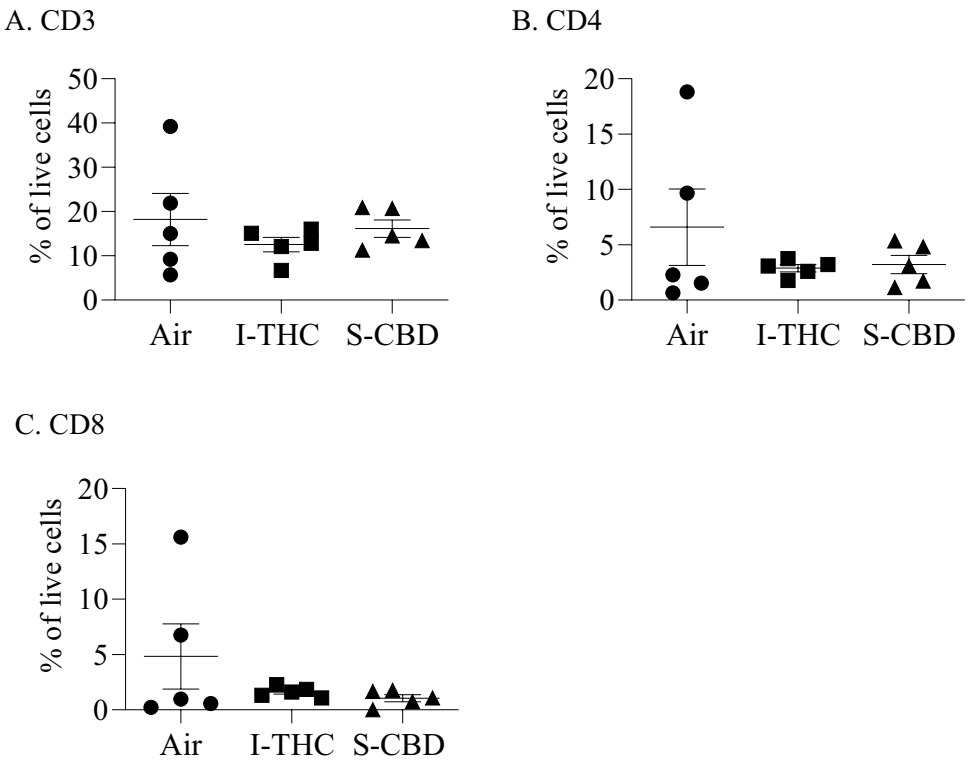

Fig. 7 Acute cannabis smoke exposure does not impact lymphocytes in the BAL. **A** CD3—there was no difference in the percentage of $CD3^+$ T cells in mice exposed to cannabis smoke. The percentage of **B** $CD4^+$ T cells and **C** $CD8^+$ T cells was also unchanged in mice exposed to cannabis versus mice exposed to room air. Data represent mean ± SEM; $n = 5$ per group

There are three subsets of IMs (IM1, IM2 and IM3), constituting 4% of lung monocyte/macrophages at steady-state (Sabatel et al. 2017), and are largely immunosuppressive/tolerogenic due to their constitutive production of IL-10 (Kawano et al. 2016; Schyns et al. 2019). In this study, we showed that there is a significant decrease in TR-AMs in lungs of cannabis smoke-exposed mice and an increase in IMs. Our observations that cannabis smoke affects macrophage subpopulations is partially in line with those seen in models of tobacco smoke, where exposure of mice to tobacco smoke increased $CD11b^+$ macrophages subpopulations, including both Mo-AM as well as IM subpopulations (Cass et al. 2021). Interestingly, we did not find the Mo-AM population ($MerTK^+$, $CD64^+$, $CD11b^+$, $CD11c^+$), which could be due to the relatively short time frame of exposure in our study (i.e., 3 days).

The $CD11b^+$ population of macrophages have been reported to be derived from monocytes (Gibbings et al. 2017; Joshi et al. 2020). Murine monocytes are divided into three different populations based on surface expression of Ly6C: $Ly6C^{high}$, $Ly6C^{intermediate}$, and $Ly6C^{low}$ monocytes (Yang et al. 2021). $Ly6C^{high}$ and $Ly6C^{intermediate}$ monocytes are considered the counterpart of human classical/inflammatory monocytes, while $Ly6C^{low}$ monocytes are represented by human non-classical/patrolling monocytes (Yang et al. 2021). $Ly6C^{high}$ monocytes are the main recruited monocytes during inflammation and perform pro-inflammatory functions (Auffray et al. 2007; Epelman et al. 2014). When infection or injury occurs, they enter the alveolar cavity and develop into Mo-AMs (Hou et al. 2021). Like $Ly6C^{high}$ monocytes, $Ly6C^{intermediate}$ monocytes are also recruited to sites of inflammation but can differentiate into DC (Sprangers et al. 2016; Yang et al. 2021). Conversely, $Ly6C^{low}$ monocytes, also known as patrolling monocytes, constantly survey the endothelium as part of the innate local surveillance vasculature and are involved with early responses to inflammation and tissue repair via their anti-inflammatory properties (Kratofil et al. 2017). Interestingly, Schyns et al., showed that that $Ly6C^{low}$ patrolling monocytes can transition into extravasating monocytes to give rise to one of the IM populations (Schyns et al. 2019). We observed in lungs that the level of total monocytes was unchanged, but that there was a significant increase in $Ly6C^{high}$ monocytes. It is therefore plausible that the inflammatory $Ly6C^{high}$ monocytes were recruited to the lungs but either failed to differentiate into Mo-AMs or disappeared due to apoptosis. However, we showed a decrease in lung $Ly6C^{low}$ monocytes as well as pulmonary DCs from cannabis smoke inhalation. DCs are antigen-presenting cells that connect the innate and adaptive immune systems. DCs recognize surrounding antigens, then process and present the antigens to T cells, which starts the adaptive immune responses (Wculek et al. 2020). DCs express cannabinoid receptors, and cannabinoids were predicted to play an essential role in regulating DC biology (Svensson et al. 2010). Importantly, cannabinoids have been implicated in apoptosis of DCs, suggesting that the decrease

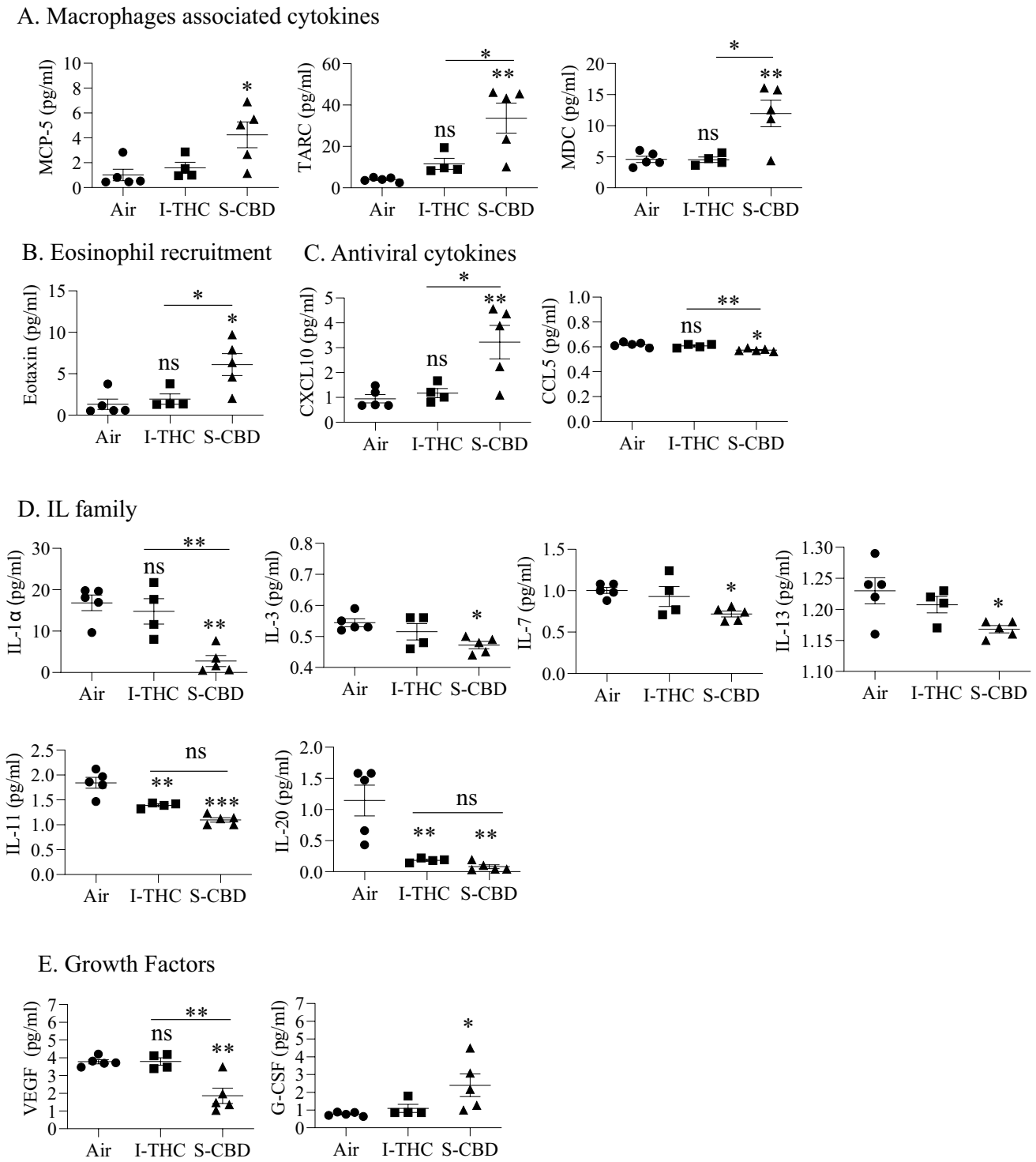

Fig. 8 Differential impact of acute cannabis smoke exposure on immune mediators in the BAL. Only immune mediators with significant differences are shown. Macrophages-associated cytokines (**A**) such as MCP-5, TARC, and MDC were significantly higher only in S-CBD smoke-exposed mice compared to air group (*$p=0.0253$, **$p=0.0022$, and **$p=0.0073$ respectively). **B** The level of eosinophil recruiting cytokine eotaxin was elevated in mice exposed to S-CBD compared to controls (*$p=0.0115$). **C** Cytokines involved in the antiviral response were differentially regulated by S-CBD cannabis smoke, including CXCL10, which was increased (**$p=0.0093$), and CCL5, which was decreased (**$p=0.0029$). **D** ILs were decreased in S-CBD cannabis smoke-exposed mice, including IL-1α (**$p=0.0012$), IL-3 (*$p=0.0263$), IL-7 (*$p=0.0282$), IL-13 (*$p=0.0347$), IL-11 (****$p<0.0001$) and IL-20 (**$p=0.0012$); IL-11 and IL-20 were also decreased in mice exposed to I-THC (**$p=0.0044$, **$p=0.0041$, respectively) compared to the air group. **E** Growth factors exhibited differential regulation in response to S-CBD, with VEGF being significantly decreased, whereas G-CSF was significantly higher compared to control mice (*$p=0.0466$). Data represent mean ± SEM; $n=5$ per group

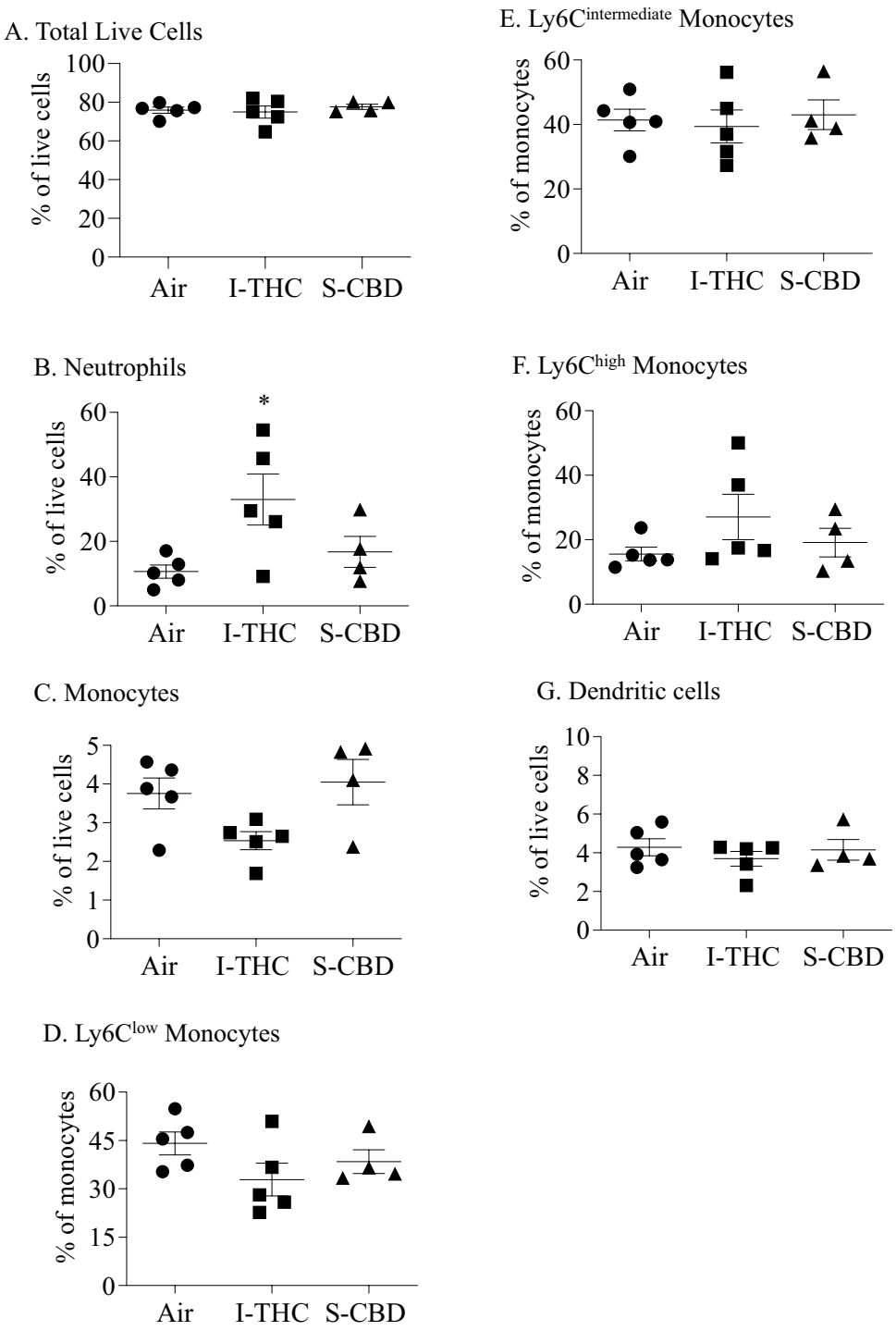

Fig. 9 Acute cannabis smoke exposure does not affect the levels of innate immune cell populations in the spleen. **A** Total live cells—the percentage of total live cells was unaffected in mice exposed to cannabis smoke. **B** Neutrophils—the percentage of neutrophils was increased only in mice exposed to I-THC (*$p = 0.0421$). **C** Monocytes—there was no difference in monocytes percentages and subtypes of monocytes including **D** Ly6C^{low} monocytes, **E** Ly6C^{intermediate} monocytes and **F** Ly6C^{high} monocytes. **G** Dendritic cells were not affected in the spleen of mice exposed to cannabis smoke compared to mice exposed to air. Data represent mean ± SEM; $n = 4–5$ per group

in cells in lungs could be due to apoptosis from cannabinoids present in both the I-THC and S-CBD smoke. Future studies would be needed to evaluate the mechanism behind the decrease in specific immune cell subsets caused by inhalation of cannabis smoke.

There was induction of an acute pulmonary neutrophilic response, with there being an increase in the number of neutrophils in the lungs due to inhalation of smoke from both varieties of cannabis. Neutrophils are short-lived cells involved in microbial killing during infection. Inhalation of particulates is associated with neutrophil accumulation in the lungs (Salvi et al. 1999). Neutrophils are a main source of reactive oxygen species (ROS), inflammatory cytokines, lipid mediators and antibacterial peptides (Hiemstra et al. 1998). The recruitment and activation of neutrophils can lead to lung tissue damage via production of large amounts

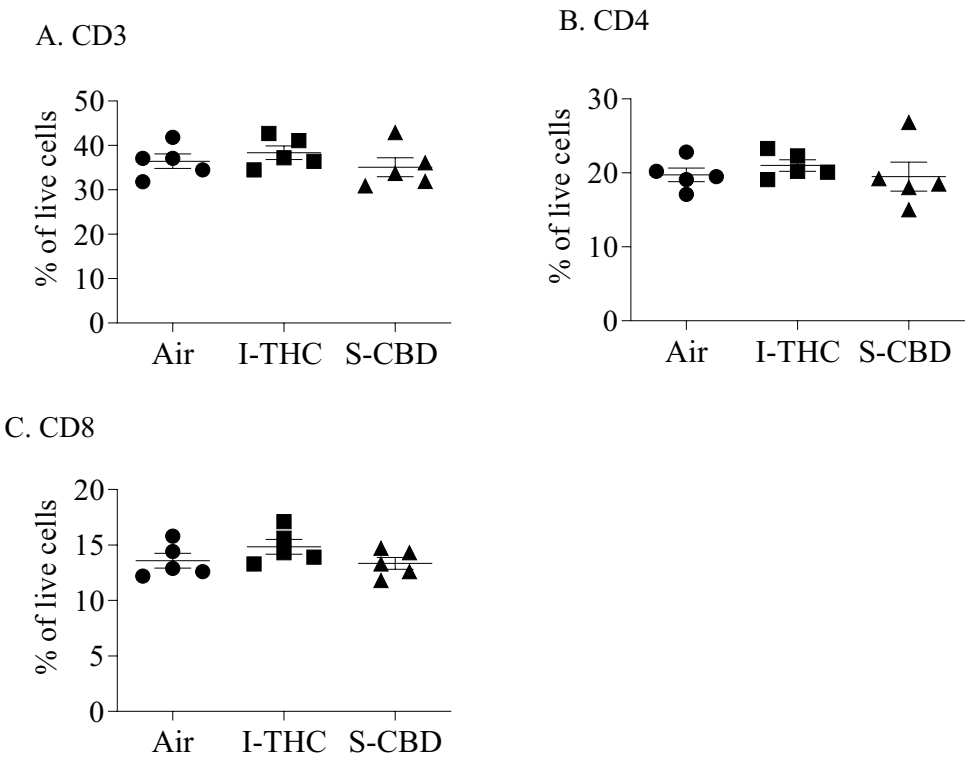

Fig. 10 Cannabis smoke exposure does not change the levels of lymphocytes in the spleen. The percentage of **A** $CD3^+$, **B** $CD4^+$ and **C** $CD8^+$ T cells were unchanged in mice exposed to cannabis smoke vs mice exposed to room air. Data represent mean ± SEM; $n = 5$ per group

of oxygen radicals and the release of granule-associated enzymes such as serine proteases and matrix metalloproteinases (MMP) (Rossi 2003). In this study, the increase in neutrophils caused by cannabis smoke could be postulated to cause lung tissue damage in chronic users. It is noteworthy that there was no difference in lung neutrophils caused by cannabis smoke between I-THC and S-CBD, suggesting that the neutrophilic response is an effect of other components of the smoke (i.e., particulates).

Another finding of interest is the differential change in the proportion of T cells. T cells are a major subset of immune cells that mediate adaptive immunity. T cells express antigen-specific cell-surface receptors for recognition of different pathogens and are a significant source of various cytokines. Two main subsets of T cells are well known by the surface molecule CD4 and CD8 (Berger 2000). $CD4^+$ T cells are called "helper" cells because they do not neutralize infections but rather trigger the body's response to infections (Luckheeram et al. 2012). $CD8^+$ T cells are known as cytotoxic T lymphocytes, which play a vital role in host immune defense via killing infected or damaged cells (Qiu et al. 2017). Although cannabinoids can induce apoptosis in T cells (Rieder et al. 2010), we show an increase in $CD8^+$ T cells, but no change in $CD4^+$ T cells. Additional studies are needed to better understand how inhalation of cannabis smoke alters the proportion of T cell subsets in the lungs.

Despite there being no difference in immunological composition between the I-THC and S-CBD varieties, there were considerable differences in lung cytokine levels. Thus, it could be surmised that the alterations in BAL cytokines may affect the function of resident and recruited immune cells even though there are no differential changes in immune cell populations. It is known that cannabinoids can modulate the production of cytokines as one of their anti-inflammatory mechanisms of action. Interestingly, our findings revealed that the changes in BAL cytokines were more pronounced when mice were exposed to S-CBD compared to I-THC variety, an effect that could be due to the ability of CBD to act as an inverse agonist on CB2 receptors (Pertwee 2008). We noted a general suppressive effect of S-CBD smoke on members of the interleukin family of cytokines, many of which are produced by activated immune cells. This includes IL-3 that is synthesized almost entirely by T cells in response to antigen (Pixley and Stanley 2010), and IL-7, which is important for T cell development and survival and is synthesized by stromal cells in the bone marrow and lymph nodes (ElKassar and Gress 2010). It is also interesting that S-CBD significantly reduced BAL levels of IL-11. IL-11 is produced by structural cells, including fibroblasts, epithelial cells and endothelial cells in response to numerous stimuli including infection (Srinivasan et al. 2017). IL-11 is involved in hematopoiesis but is also anti-fibrotic and can protect cells from injury (Cook and Schafer 2020; Yuzhalin

and Kutikhin 2015). Thus, smoke from the S-CBD variety may exert inhibitory actions on the function of numerous cells in the lungs.

It remains unclear whether these immunological changes from acute cannabis smoke exposure, therefore, alter pathological outcomes, including susceptibility to bacterial and viral infection (Khoury et al. 2022). This could be relevant given our data showing differential regulation of antiviral cytokines, particularly those implicated in early innate immune signaling. There is evidence that cannabinoids regulate immunity and pathology during respiratory infection (Turcotte et al. 2016), including that THC treatment of mice infected with influenza yielded lower influenza-specific $CD4^+$ and $CD8^+$ T cells and higher viral load (Buchweitz et al. 2007). This is also relevant in light of the current COVID-19 pandemic, with studies showing that cannabis consumption is associated with lower COVID-19 severity (Shover et al. 2022) and that there are antiviral effects of cannabis extracts containing CBD including control over virus entry and cytokine release (Santos et al. 2022; van Breemen et al. 2022). Recently, Fantauzzi et al. reported that CXCL10, IFNγ and CCL5 were unchanged in mice exposed to cannabis smoke containing THC (Fantauzzi et al. 2021), a finding consistent with that seen in our study. In contrast, exposure to smoke from the S-CBD variety increased CXCL10 and decreased CCL5. This not only suggests that modulation of the pulmonary immune response differed based on the type of cannabis being utilized, but also that strains with higher CBD may exert more potent antiviral responses. However, we cannot rule out that there are additional chemical differences between the two varieties of cannabis used in this study. It should be noted that CXCL10 attracts Th1 cells (leukocytes, T cells, eosinophils, and monocytes) such that the binding of CXCL10 to CXCR3 allows immune cell migration to the site of infection (Oliviero et al. 2021). CCL5 is produced by $CD4^+$ lymphocytes, epithelial cells, fibroblasts and platelets (Appay and Rowland-Jones 2001) and helps to control virus infection (Culley et al. 2006). Thus, it could be that alterations in key antiviral cytokines is one reason for the previously documented effects of cannabis on viral infection that may have implications for COVID-19.

We also saw differential alterations in select growth factors, including VEGF, a growth factor highly expressed in the lung epithelium and implicated in angiogenesis. VEGF was decreased by S-CBD in our study, a finding that may be related to its ability to control the recruitment of inflammatory cells (Jeong et al. 2021). Conversely, G-CSF, a growth factor that stimulates the bone marrow to produce neutrophils (Roberts 2005), was increased by smoke from the S-CBD variety. Because both the I-THC and S-CBD varieties increased neutrophils but only the S-CBD variety increased G-CSF, it is unlikely that this cytokine was contributing to neutrophil recruitment to the lungs. We also observed a general suppressive effect for S-CBD on interleukins, including for IL-3 and IL-7, cytokines that promote survival and maturation of immune cells, and IL-20, an interleukin that enhances innate immune function. This is in line with reports with the ability of cannabis/cannabinoids to suppress inflammatory cytokine production (Eisenstein and Meissler 2015). Interestingly, IL-10, a potent anti-inflammatory cytokine, was unchanged in cannabis smoked mice even though the percentage of anti-inflammatory IM increased. Overall, these results suggest that cannabis smoke has an overall immunosuppressive effect on cytokine production in the lungs.

In summary, we characterized a preclinical model of acute cannabis smoke exposure and used this to evaluate the effect of two varieties of cannabis with different cannabinoid profiles with which to perform comprehensive lung immune profiling. Not only can utilization of this model accelerate our understanding of how the pulmonary immune landscape has been impacted by inhalation of cannabis smoking, but these results highlight that the pulmonary immune system is differentially impacted by cannabis varieties that vary in their THC:CBD ratio. This enhances our understanding of how cannabis smoke can impact not only immune cell populations, but also whether this has the potential to lead to adverse lung health outcomes in people who smoke cannabis on a regular basis.

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Data availability Data not provided in the online supplement are available upon reasonable request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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