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The Immunomodulating Effects of Delta-9 Tetrahydrocannabinol (THC) and Cannabidiol (CBD) in the Context of Inflammation and Infection in Vitro

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ABSTRACT

The therapeutic potential of cannabinoid-based medicines has led many U.S. states and countries to authorize their clinical use. Delta-9 tetrahydrocannabinol (THC) and cannabidiol (CBD), the biologically active compounds of cannabis, possess a wide range of immune regulatory properties. Macrophages are specialized immune cells that express endocannabinoid receptors which can affect inflammatory phenotypes and phagocytosis. Increasing prevalence, and legalization of cannabis, has led to regulatory findings of various aspects of physiological, behavioral, and metabolic function; however, the effects on immunological regulation in the setting of infection is less well understood. The purpose of the current study was to test the immunoregulatory effects of various THC and CBD doses in the context of infection. Secondary, THC and CBD temporal and tissue-specific cytotoxic effects were evaluated at 2 or 6 h. Macrophages were pre-treated with THC or CBD (0, 2, 5, 10, 15, 25 μg/mL) and challenged with LPS (2 h) or live *Escherichia coli* (*E. coli*) (6 h). Extracellular bacteria were eliminated, macrophage cells lysed, and intracellular bacteria quantified. Unlike CBD, THC-induced phagocytosis was significantly decreased in a dose-dependent manner. CBD-induced phagocytosis was inversely increased at 25 μg/mL. In macrophages, THC increased cytotoxicity and CBD decreased cytotoxicity at doses 15 μg/mL and greater. These findings demonstrate the multifaceted interplay between THC and CBD that affect the immunological interaction between host and microbes. Taken together, it is necessary to understand the immunoregulatory underpinnings of Phyto cannabinoids to maximize therapeutic potential and reduce opportunistic infections.

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Introduction

Cannabis is legal in many U.S. states [1]. In Colorado the sales tax of cannabis sold in stores is 2.9%, retail cannabis sales tax is 15%, and retail cannabis excise tax 15%. [2]. Sales tax revenue does not include additional revenue from cannabis license and application fees. The total cannabis revenue in Colorado in 2022 was more than $300 million. The total revenue from the date of legalization to year end in 2022 was more than $2.3 billion [2]. Similarly, the state of Washington generated $515.2 million in cannabis sales tax and fees in 2022 [3]. In addition to revenue, the legalization of cannabis impacted the economy through job growth, and investment opportunities. From 2011 to 2016, the state of Colorado gained 238,000 jobs, which was largely attributed to the legalization of cannabis [4]. Although the legalization of cannabis has created an economic boom, research to test the therapeutic potential of cannabis is limited with varying results.

Delta-9 tetrahydrocannabinol (THC) and cannabidiol (CBD), are the biologically active compounds in cannabis. THC and CBD have a similar chemical formulation and molecular mass [5]. However, structurally THC contains a cyclic ring whereas CBD contains a hydroxyl group [5]. The variation in physiologic function is attributed to the differences in structural composition. Less is known about the physiological, immunological, and mechanism of action for CBD compared to THC. There are also multiple types of THC, where the structure is similar, yet the psychoactive effect can vary. Delta-8 and delta-9 vary because of the double bond location in the cyclic ring. Delta-8 THC contains a double bond on the 8th carbon, whereas delta-9 has a double bond on the 9th carbon [6]. Kruger. et al. reported similar effects from users of both delta-9 and delta-8; however, important physiological and immunological differences remain to be elucidated.

CBD and THC are currently used recreationally, and increasingly more prevalent for medical use. Martin-Santos et al. studied the acute effects of both THC and CBD in healthy individuals. They rated psychologic symptoms, 1, 2, and 3 hours post 10 mg of either CBD or THC was administered orally [7]. In individuals that were given THC as compared to placebo, an association with anxiety, dysphoria, positive psychotic symptoms, mental and physical sedation, and tachycardia was recorded. With individuals that were given CBD and psychological symptoms were analysed, no statistical difference was recorded compared to placebo control [7]. Conversely, Khan and Rabia et al's. analysis of CBD indicates a therapeutic potential in treating schizophrenia, social anxiety disorder, autism spectrum disorder, attention deficit hyperactivity disorder, insomnia, anxiety, bipolar disorder, post-traumatic stress disorder, and Tourette syndrome [8]. Although a positive

correlation was measured for treatments with CBD, the therapeutic mechanisms for each disorder were not evaluated [8]. Stanley et al. looked more closely at the anti-inflammatory properties of CBD especially in the vascular system. A common finding with CBD is its anti-inflammatory and antioxidant properties.

More specifically, CBD has been found to have a vasorelaxant response in blood vessels, providing protection against ischemia-reperfusion associated with diabetes, reduce infarct size, increase blood flow in animals with stroke, induce survival and death in white blood cells, and influence cell migration and platelet aggregation [9]. Baczynsky, W. O. T., and A. M. Zimmerman looked at the effect of THC and CBD on the humoral immune response post vaccination in mice. These findings indicate that THC suppressed the humoral response post primary vaccination, but had less of an effect on secondary vaccination, and CBD had no change in humoral response post vaccination [9]. Taken together, these data suggest THC has a direct effect on the humoral immune system. CBD is less well described, and more research is needed on the adaptive immune system, especially in the context of infection. It is clear THC and CBD have varying, influential effects on multiple in vivo systems such as the vascular system, psychological effects, and the immune system; however, more research is needed to better understand the cannabis-mediated inflammatory mechanisms that modulate the immune system.

Tissue macrophages and blood monocytes are critical immune cells that link the innate and adaptive immune systems. Macrophages act to initiate homeostatic, inflammatory, reparative, and protective functions for the body. Like many other important immune cells, they originate from the bone marrow as well as the fetal liver and yolk sac during development [10]. Macrophages play a major role in inflammation, anti-inflammation, cytokine release, phagocytosis, antigen presentation and wound healing. Activation of each role depends on a multitude of ligands, epigenetics, and post-transcriptional modification such as microRNA [11,12]. Dysregulation in inflammatory responses from macrophages can result in conditions like sepsis [13]. Phagocytosis is an engulfment and degradation process that is critical to fight off infections and maintain immunological homeostasis [14]. Several tissue systems including the immune system express CB1 and CB2 receptors, which THC is a partial agonist for, and CBD is thought to be an antagonist [15]. CB1 and CB2 have been specifically identified on macrophages suggesting they have an impact on macrophages function. Staiano, Rosaria I., et al discovered that activation of CB1 and CB2 on macrophages impacts their role in blood vessel and lymph angiogenesis [16]. Additionally, McCoy found that cannabis can disrupt the antigen presenting function of macrophages, thus impairing the immune system, specifically in B-cell activation [17]. The influence of CBD, CBG, and a combination of CBD and THC on cytokines has been described to decrease TNF-alpha, IL-1, and IL-6, all of which are important cytokines in inflammation often released by macrophages and other immune cells [18,19]. The connection between CB1, CB2, cytokine release, angiogenesis, and other functions of macrophages, is not fully understood. Perhaps more important is to better understand the modulating of effects CBD and THC on the processes of phagocytosis in the context of inflammation and infection. This information was previously published as a poster abstract presentation [20].

The purpose of the current study was to test the immunomodulating effects of THC and CBD *in vitro*. Secondary, the inflammatory mechanisms of THC and CBD were analysed in the context of macrophage phagocytosis function with a live bacterial infection. Fundamental pharmacological properties of THC and CBD were also tested. This study provides important evidence that could be used to improve therapeutic applications for the medical use of cannabis and supports the need for further testing of the quantity of CBD and THC use separately and in mixtures. Investigating the therapeutic potential of CBD and THC will improve a knowledge gap that moves beyond the medical field to include local, state, and national policies for the legalization of cannabis.

Materials and Methods

Reagents and Cell Culture

This study's protocol was submitted and approved by Noorda College of Osteopathic medicine (#PN023). The human A549 cells and murine RAW 264.7 cell lines were obtained from ATCC, and the cells were grown in F-12K and Dulbecco's modified Eagle medium (DMEM) complete, respectively (Gibco BRL, Grand Island, NY, USA). The mediums contained 10% fetal bovine serum (FBS), 100 U/mL penicillin, 50 mg/ml streptomycin. 2 mM L-glutamine, 10 mM HEPES, 0.1 mM non-essential amino acids, and 1.5 g/l sodium bicarbonate (Gibco BRL). Bacterial LPS from *Escherichia coli* serotype 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA). THC (1mg/mL; Millipore Sigma, T4764) was diluted in heptane at 0- 25 µg/mL, and CBD (1mg/mL; Millipore Sigma, C-045) was diluted in methanol at 0 – 25 µg/mL. Cells were seeded in 24 well plates at 1x10^5 per well; viability was 90% or greater for all experiments.

Bacterial Growth and Culture Conditions

For each study, frozen stock cultures *Escherichia coli* (ATCC, BAA-2469) was inoculated into Luria Bertani broth (LB) and incubated overnight at 37°C in an orbital shaker incubator (200 rpm) (New Brunswick C25, Edison, NJ, USA). Bacteria were diluted 1:10 and grown to late-logarithmic phase measured by optical density at OD600 absorbance in a spectrophotometer (Eppendorf Bio Photometer AG2233, Hamburg, Germany). Bacteria were collected in 1mL by centrifugation and resuspended in 1mL with pre-warmed antibiotic-free-DMEM complete at a concentration of 1 X 10^5 cfu/25µL as described previously [21]. Actual numbers of viable bacteria were determined by standard plate counts of the bacterial suspensions on LB agar plates. An MOI of 0.5:1 was established for experiments.

Cytotoxicity

To assess cytotoxicity, lactate dehydrogenase was measured using the CyQUANT LDH Cytotoxicity Assay Kit (ThermoFisher, C20301) according to the manufacturer's instructions. In brief, 2 hours after incubation and again after 6 hours of incubation, 50 µL was collected and transferred to a corresponding well on a 96 well flat bottom plate (Cat. No. C20301). Triplicate experimental replicates and three assay replicates were used.

Infection and Phagocytic Engulfment

Confluent monolayers of macrophages were pre-treated with media supplemented with THC or CBD (0, 2, 5, 10, 15, 25 µg/mL) for 2 hours. E. coli was grown overnight in sterile LB media. Prior to co-culturing conditions, the bacteria were diluted to late logarithmic growth, centrifuged, and the pellet was washed twice in fresh non-antibiotic DMEM media. Treatment was removed, and cells infected with live E. coli for 6 hours at 37˚ C, 5.5% CO2 to allow for attachment and engulfment to occur. After 6 h, extra-cellular bacteria were removed by washing cells with PBS and replacing culture media supplemented with 250 µg/ml of kanamycin for 1 h to completely kill any residual extracellular and attached bacteria. Antibiotic dose and duration were verified by culturing supernatants on LB plates overnight (data not shown). Following an additional PBS wash, intracellular bacteria were

released after cell monolayers were lysed with PBS containing 0.1% Triton X-100. Viable intracellular bacteria were quantified by plating serial dilutions of the lysate, and average CFU determined. Bacterial intracellular invasion assays were replicated independently at least twice.

Cytokines

In combination with the infection and phagocytic engulfment assay, Enzyme Linked Immunosorbent Assay (ELISA) was used to measure IFN-γ (ThermoFisher: KMC4021), IL-12 (ThermoFisher: EMIL12B), TNF-α (ThermoFisher: MTA00B), and IL-10 (ThermoFisher: BMS614) release from macrophage following incubation in either THC or CBD at varying concentrations. The IL-10 protocol was followed exactly except for an addendum in which wells were incubated with the Streptavidin-HRP for 1 hour.

Lipopolysaccharide (LPS) Challenge

Macrophages were cultured as listed above, media was disposed of, and wells were rinsed with 500 µL phosphate-buffered saline. Cells were grown in media supplemented with THC or CBD at varying concentrations in duplicate for 2 hours. Treatment was removed 10 µg of LPS (00-4976-93) was added to each well and incubated for 2 hours. Wells were then stored at -80C for ELISA testing as described previously.

Statistical Analysis

Data statistics were analysed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) and presented as standard error of the mean (SEM). A Two-way ANOVA was used for analysis of three or more groups. Significance was defined as $p \leq 0.05$.

Results

CBD Decreases Cytotoxicity in a Time-and-Tissue-Specific Manner

Toxicity is a fundamental pharmacological feature evaluated for drug therapeutic potential. The cytotoxic effects of THC and CBD were tested at various doses, against two tissue types from different species, and at different temporal perspectives. First, to establish the cytotoxic effects of the vehicle utilized for THC and CBD dilutions, methanol, and heptane at 2h or 6h were tested. There was no significant increase in cytotoxicity for methanol and heptane at 2h or 6h, compared to controls (Figure 1).

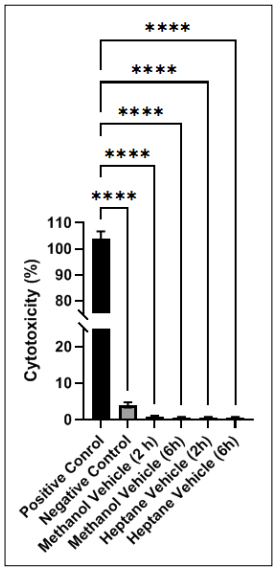

Figure 1: Control and vehicle cytotoxicity values. Methanol is the vehicle for CBD and heptane the vehicle for THC. Data represented in percent or % (ordinate) and positive control (lysis buffer), negative control (PBS), and methanol or heptane for 2 and 6 h (abscissa). Data statistics presented as standard error of the mean (SEM). Lines and asterisk (*) represent statistical comparison by one-way ANOVA with Tukey's multiple comparisons test. ****, $p \leq 0.0001$.

Second, the cytotoxic effects of THC and CBD compounds were tested against human A549 epithelial cells and murine RAW 264.7 macrophage cells after a two-hour incubation. THC at 2, 5, 10, and 15 µg/ml did not significantly increase cytotoxicity in A549 cells compared to control (0 µg/ml). Although not statistically significant, a dose-dependent trend can be observed (Panel A). Similarly, CBD at 2, 5, 10, and 15 µg/ml did not significantly increase cytotoxicity in A549 cells compared to control (Panel C). THC at the same doses did not significantly increase cytotoxicity in RAW 264.7 cells compared to control (Panel B). However, CBD decreased cytotoxicity in RAW 264.7 cells in a dose-dependent manner. CBD at 15 µg/ml significantly decreased cytotoxicity in RAW 264.7 cells compared to control and doses 2-10 µg/ml (Panel D). Still, THC and CBD did not exceed 20% cytotoxicity in RAW 264.7 cells (Figure 2).

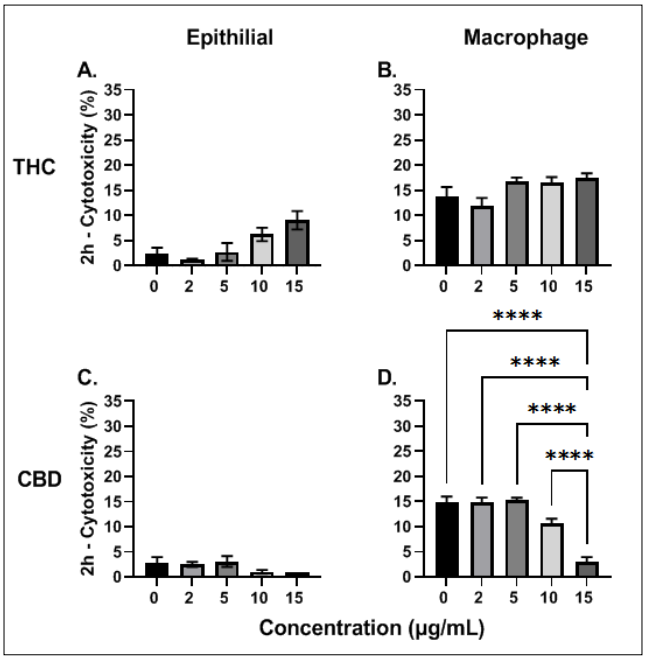

Figure 2: Epithelial cells (left) and macrophage cells (right) cytotoxicity after 2-hour incubation with various doses of THC (A and B) or CBD (C and D). Data represented in percent or % (ordinate) and concentration of THC or CBD in micrograms per millilitres or µg/mL (abscissa). Data statistics presented as standard error of the mean (SEM). Lines and asterisk (*) represent statistical comparison by two-way ANOVA. *, $p \leq 0.05$, **, $p \leq 0.01$.

Third, the cytotoxic effects of THC and CBD compounds were tested against human A549 epithelial cells and murine RAW 264.7 macrophage cells after a six-hour incubation. THC at 2, 5, 10, 15 µg/ml increased A549 cell cytotoxicity in a dose-pendent manner. THC at 15 µg/ml significantly increased cytotoxicity approximately 10-fold compared to control (Panel A). Conversely, CBD did not significantly increase A549 cell cytotoxicity compared to control (Panel C). THC and CBD significantly increased RAW 264.7 cytotoxicity at 15 µg/ml. Like the two-hour incubation, cytotoxicity did not exceed 20% at the six-hour incubation (Figure 3).

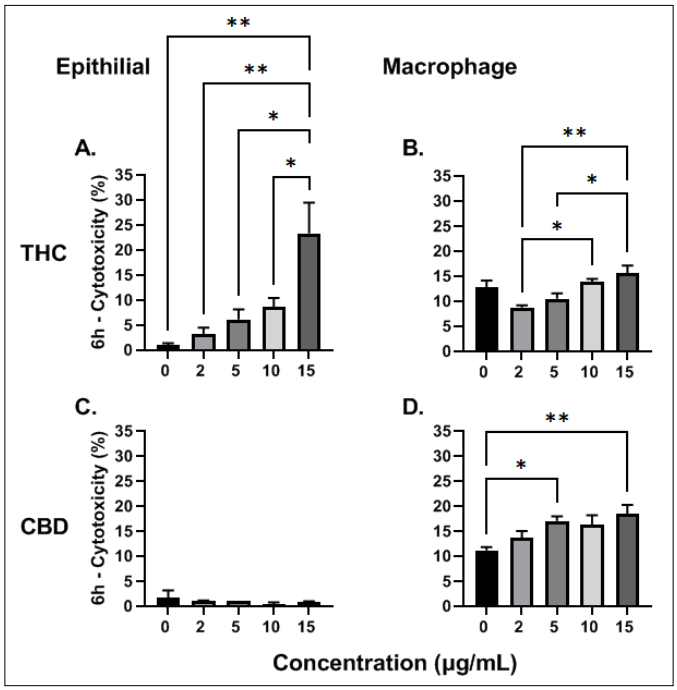

Figure 3: Epithelial cells (left) and macrophage cells (right) cytotoxicity after 6-hour incubation with various doses of THC (A and B) or CBD (C and D). Data represented in percent or % (ordinate) and concentration of THC or CBD in micrograms per millilitres or µg/mL (abscissa). Data statistics presented as standard error of the mean (SEM). Lines and asterisk (*) represent statistical comparison by two-way ANOVA. *, $p \leq 0.05$, **, $p \leq 0.01$.

To test the potential for cytoprotective features with endocannabinoid compounds and macrophages, increasing doses (25-75 µg/ml) were tested at 2 and 6 h. CBD, not THC, significantly decreased cytotoxicity in RAW 264.7 cells at 2 h and 6h. CBD at 25 µg/ml decreased cytotoxicity to approximately 1.5%, while cytotoxicity remained at 0% with increasing CBD (35-75 µg/ml) concentrations (Supplemental Figure 1).

THC, Not CBD, Decreases Macrophage Function in a Dose-Dependent Manner

Tissue and blood macrophages are a critical immune cell that bridge the innate and adaptive immune system, which cells function to attach, engulf, and eliminate foreign microbes, thus managing the potential for disease. From the cytotoxicity data, THC and CBD may have a greater effect on primary immune-mediated cells, and to a lesser extent secondary immune cells, such as epithelial or endothelial cells. RAW 264.7 macrophage cells were used as the primary cell of interest for the remainder of the study. To test the effects of THC and CBD on macrophage function, different doses of CBD and THC were used to challenge the engulfment function of RAW 264.7 macrophage infected with live bacteria. THC at 5, 15, or 25 µg/ml decreased the number of engulfed bacteria in a dose-dependent manner after 6 h, compared to the untreated control. THC at 25 µg/ml significantly decreased phagocytic engulfment approximately 3-fold compared to control. Conversely, CBD significantly decreased phagocytic engulfment at 15 µg/ml, but not at 25 µg/ml, compared to control (Figure 4).

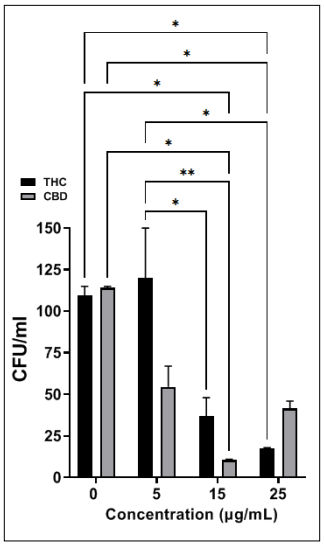

Figure 4: RAW 264.7 macrophage engulfment function after pre-treatment with various doses of THC or CBD followed by infection with live E. coli. Data represented in Colony forming units (CFU) per millilitres (ordinate) and concentration of THC or CBD in µg/mL (abscissa). Data statistics presented as standard error of the mean (SEM). Lines and asterisk (*) represent statistical comparison by two-way ANOVA. *, $p \leq 0.05$, **, $p \leq 0.01$.

CBD, Not THC, Decreases Pro-Inflammatory TNF-α After Live Infection

Excess pro-inflammatory cytokine expression can lead to cellular disfunction and immune suppression. To better understand the effects of THC and CBD on macrophage function in the context of inflammation and a live infection, pro – and – anti-inflammatory cytokine secretion was measured. THC at 25 µg/ml increased TNF-α compared to control. THC (5 µg/ml) was significantly decreased compared to CBD (5 µg/ml); however, CBD at doses 15 and 25 µg/ml were significantly decreased compared to THC at the same doses (Panel A). CBD (25 µg/ml) significantly increased IFN-γ compared to THC at the same dose. Although not statistically different, a dose-dependent decrease in IFN-γ was measured at 5 and 15 µg/ml for THC (Panel B). No statistical differences were measured for IL-12 and IL-10 with THC and CBD (Figure 5).

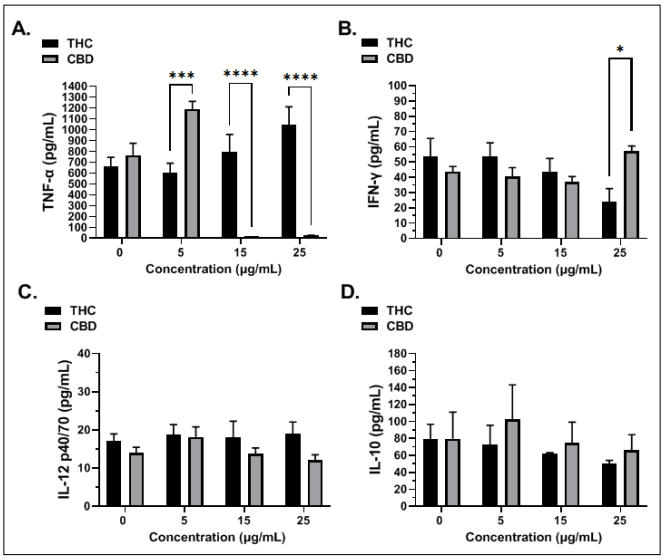

Figure 5: TNF- α (panel A), IFN-γ (panel B), IL-12 (panel C), and IL-10 (panel D) cellular protein expression after pre-treatment

(2 h) with THC or CBD at various doses, followed by infection with live E. coli for 6 h. Data represented in picograms (pg) per millilitres (ordinate) and concentration of THC or CBD in micrograms per millilitres or µg/mL (abscissa). Data statistics presented as standard error of the mean (SEM). Lines and asterisk (*) represent statistical comparison by two-way ANOVA. *, $p \leq 0.05$, ***, $p \leq 0.001$.

CBD, Not THC, Decreases Pro-Inflammatory TNF-α After LPS Challenge

LPS is a major antigenic protein associated with Gram-negative bacteria, that induces a pro-inflammatory response typically through Toll-like-Receptor 4 (TLR-4). To better understand the inflammatory and temporal effect of THC and CBD, macrophages were pretreated (2 h) with different doses of THC and CBD and challenged with LPS for an additional 2 h (Figure 6). THC (2 – 10 µg/ml) decreased TNF-α in a dose-dependent manner. THC at 15 and 25 µg/ml increased TNF-α relative to 10 µg/ml. Although not statistically significant, THC at 25 µg/ml did not return to control level at 2 h post LPS challenge (Panel A). Conversely, CBD significantly decreased TNF-α in a dose-dependent manner. However, unlike THC, CBD significantly decreased TNF-α at 15 and 25 µg/ml, compared to THC at the same doses. TNF-α remained significantly decreased when pre-treated with CBD and not THC, at doses greater than 25 µg/ml (data not shown). A pre-treatment of THC and CBD at multiple doses decreased IFN-γ at 2 h post LPS challenge (Panel B).

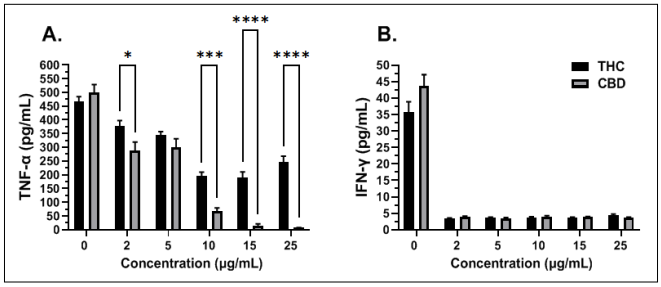

Figure 6: IFN-γ (panel A) and TNF-α (panel B) cellular protein release after pre-treatment with THC or CBD and challenged with LPS. Data represented in picograms (pg) per millilitres (ordinate) and concentration of THC or CBD in micrograms per millilitres or µg/mL (abscissa). Data statistics presented as standard error of the mean (SEM). Lines and asterisk (*) represent statistical comparison by two-way ANOVA. *, $p \leq 0.05$, ***, $p \leq 0.001$, and ****, $p \leq 0.0001$.

Discussion

CBD and THC have a broad range of pharmacological effects, some of which are therapeutic properties; however, the question of inflammatory and immune system modulation still stands. The current study tested the immunomodulating effects of THC and CBD with RAW 264.7 macrophage cells in vitro. Secondary, the inflammatory effects of THC and CBD were analysed with a live bacterial infection and LPS challenge. Fundamental pharmacological properties of THC and CBD were also tested. First, THC and CBD expressed cytotoxic effects that varied dependent on time and tissue type. At 2 hours after pre-treatment with THC, cytotoxicity was not different between A549 and RAW 264.7 cells. However, macrophages pre-treated with CBD at 15 µg/mL for 2 hours exhibited significantly less cytotoxicity compared to its THC counterpart. Of considerable note is that CBD cytotoxicity decreased at higher doses (≥15µg/mL) and remained elevated at lower doses; cytotoxicity dropped below the control expression of cytotoxicity with CBD doses greater than 25 µg/mL (Figure 2 and Supplemental Figure 1). Inverted U-shaped dose-response profiles have been reported in animal and human studies of anxiety and psychiatric disorder, but there has not been an explicit focus of research on CBD's effects on immune-mediated cells [22]. These curves were first described in rats tested in models of anxiety [23]. Similar curves were reported with zebrafish treated with 0.5 mg/kg CBD, while the behaviour of fish treated with the lowest dose (0.1 mg/kg) and the highest dose (10 mg/kg) did not differ from controls [24]. Significant adverse effects have not been reported in humans; however, from a therapeutic perspective it is critical to determine if a similar inverted U-shaped dose-response pattern is present when testing the immunological impact of CBD and THC. Moreover, in the current study, human A549 cells were derived from alveolar basal epithelial cells, whereas RAW 264.7 cells are derived from a murine ascites environment. It is plausible that pulmonary epithelial cells are more resistant to toxic chemicals and require an extended contact duration to induce a cytotoxic response [25]. Additionally, inverted U-shaped dose-response effects may provide a basis for understanding the cytotoxic effects of CBD at the extreme doses of the descending arm of the inverted U-shape curve. The implications for extreme doses of THC and CBD in mixtures or ratios is beyond the scope of the current study yet requires further investigation.

Second, after a 6-hour exposure to A549 cells, THC is approximately 10-fold more cytotoxic than CBD at 15 µg/ml (Figure 3). A similar finding was reported by Sarafian, T. A., et al's study on the cytotoxic effects of THC on A549 cells [26]. However, unlike the current study, the deleterious effects of THC were attributed to smoke-induced oxidative stress and necrotic cell death. Like tobacco smoke, cigarettes derived from cannabis inhibit Fas, which cannabis tar is a potent inhibitor of Fas-induced caspase 3 activity leading to increased levels of intracellular reactive oxygen species. These data suggest that THC may affect both the carcinogenic and immunologic consequences of smoke-derived cannabis use. In the current study, the THC compound in the absence of smoke-induced oxidative stress increased cytotoxicity in A549 cells. The implications of these data include cellular stress from different consumption patterns beyond inhalation and extend to infection susceptibility for immunocompromised consumers. Conversely, increased cytotoxicity of small doses of CBD were determined beneficial to eliminate cancer cells, specifically the reduction of head and neck tumor growth, and increased the chemotherapeutic drug effects through cytotoxicity [27]. In the current study, the longer temporal exposure with RAW 264.7 cells increased THC and CBD cytotoxicity in a dose dependent manner (Figure 3).

Macrophages are a critical immune cell that are involved in the first phases of disease progression and resolution. Indeed, the dysfunction of macrophages are among the abnormal characteristics in severe bacterial and viral pathologies [28]. Additionally, an increase in inflammatory monocyte-derived macrophages replace tissue-resident macrophages in a healthy host, whereas a marked reduction in macrophage phagocytosis activity is detected in abnormal immunocompromised hosts. In the current study, CBD at low doses (5 -15 µg/ml) decreased RAW 264.7 cell phagocytic engulfment. At high doses (25 µg/ml) CBD increased phagocytic engulfment (Figure 4). Macrophage treatment with CBD may lead to an increase in FcγRII and CD36 gene expression, thus initiating increased phagocytosis by the ligation of Fcγ receptors to IgG-opsonin on the target cell. CD36 is an important scavenger receptor for phagocytosis of bacteria such as Streptococcus pneumoniae [28]. Furthermore, CBD enhanced

microglial phagocytic function in Alzheimer's Disease patients by increasing β-amyloid-mediated phagocytosis and degradation [29]. Comparable to related studies, THC decreased phagocytic engulfment in a dose-dependent manner in the current study [30]. In addition, THC at doses of 20 μg/mL can inhibit macrophage movement [31]. The inhibition of macrophage phagocytic engulfment and dysfunction will increase the risk of infection. Moreover, an increase in macrophage polarization by cannabis-based treatment may potentially lead to an exacerbated cytokine storm identified in patients with severe pulmonary infections [32]. For instance, for now, users and healthcare personnel should avoid the use of cannabis for COVID-19 prevention treatment [28].

Cytokines are important messenger molecules used to inform the immune system of where and how to act when foreign molecules or pathogens are present [33]. To build on the previous live infection assay, cytokines were measured after pretreatment with THC or CBD, followed by infection with live E. coli. In the current study, CBD significantly decreased TNF-α, and increased IFN-γ, at 25 μg/mL. TNF-α is a primary pro-inflammatory cytokine that promotes phagocytic activity; however, prolonged, and elevated levels of TNF-α is associated with so called cytokine storm conditions that exhaust the immune response and promote macrophage dysfunction [19]. CBD treatment led to an increase in IFN-γ levels in an inverse U-shaped dose-response manner. Both IL-12 and IL-10 were not significantly different compared to control when RAW 264.7 cells were pre-treated with THC or CBD (Figure 5). However, Aswad, Marin et al. identified that at high levels of CBD anti-inflammatory and master regulator IL-10 is elevated [34]. In agreement with the current study, CBD was shown to decrease pro-inflammatory IFN-γ and TNF-α cytokine release and increase anti-inflammatory IL-4 and IL-10 cytokines, in diabetic mice with insulitis [35]. Taken together, it is plausible that the increase in phagocytic engulfment when cells were pre-treated with 25 μg/mL is correlated to the CBD-induced reduction in TNF-α observed at doses 15 and 25 μg/mL (Figure 4 and 5). To better understand the more global implication of CBD and THC on cytokine secretion and cellular activation, a more thorough and expansive cytokine study is necessary.

To better understand the inflammatory modulating effects of THC and CBD, LPS derived from E. coli was utilized to induce an inflammatory response in RAW 264.7 cells after a pre-treatment with different doses of THC or CBD. Like the live infection, CBD significantly decreased TNF-α in cells pre-treated with 15 or 25 μg/mL. THC, not CBD, produced a dose-dependent decrease in TNF-α, yet inversely increased TNF-α at 15 and 25 μg/mL. Surprisingly, both THC and CBD significantly decreased IFN-γ at all doses tested, compared to control. A significant limitation with the assay was a use of one LPS dose. In addition, the study was conducted in vitro, which provides limitations for translatability in terms of dose and effect and does not completely demonstrate biological outcomes compared to whole system studies. The dose of LPS and the duration of incubation were selected based on consistent findings under similar experimental conditions [36]. Nevertheless, pre-treatment with larger doses of CBD may improve macrophage phagocytic function by reducing elements that contribute to a cytotoxic environment.

Overall, a unique contribution of the current study is the identification of CBD doses (> 15 μg/mL) that promote an anti-inflammatory microenvironment by targeting TNF-α that maintains or improves the immunological role of RAW 264.7 macrophage cells by influencing phagocytosis and regulating beneficial cytokine secretion. Furthermore, the live infection data demonstrates the THC– and– CBD- regulation of critical immunological mechanisms (i.e., macrophage phagocytosis) and direct effect of cytokine secretion. The implications of these data extend beyond cellular dysregulation to include human infection and pathogenesis of several pulmonary and CNS diseases [37]. Moreover, these results highlight the need to expand the research on the interplay between mixtures or ratios of THC/CBD and other endocannabinoids. In addition, to better understand the biological effects associated with the increasing prevalence of commercial THC and CBD 'extreme doses', further investigation of large doses beyond the scope of the current study is required [38]. High levels of THC can have deleterious effects on neurological cellular mechanisms that result in psychosocial disorders and immunological dysfunction that exacerbates a harmful microenvironment. Lastly, the positive effects of CBD may be counteracted by extreme doses of THC and/or CBD [39]. Nevertheless, the therapeutic potential of cannabis extracts such as THC and CBD extend to anxiolytic, antidepressant, antiepileptic, anti-inflammatory, and analgesic conditions. These results support the caution against the arbitrary use of cannabis or cannabinoid compounds for recreational or therapeutic use.

Conclusions

In conclusion, these data show that the cannabinoids THC and CBD indicate both similarities and differences of the impact on immunoregulation and immune cell function in vitro. In this and other studies, the results indicate that THC has deleterious effects on cell viability and function in a dose-dependent manner; the effects were independent of murine or human origin and harmful effects had a greater impact on RAW 264.7 macrophages compared to A549 epithelial cells. Similarly, CBD has deleterious effects at lower doses, yet demonstrated beneficial effects at higher doses that support other existing inverse U-shaped dose-response results. The temporal component of the study suggests a linear correlation; however, meaningful data to better understand acute and chronic effects remain to be elucidated. These results suggest that THC and CBD can modulate the immune response through the cytokine system and other inflammatory mechanisms.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request. In addition, the files are available from Figshare database (accession number(s) 10.6084/m9.figshare.24529879)

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Supplemental Figure 1. Cytotoxicity of RAW 264.7 cells after pre-treatment with CBD at doses 25, 35, 45, 55, 65, or 75 μg/mL for 2 hours (left) and 6 hours (right). The solid black bar

represents the average with SEM from 3 experimental replicates and 3 assay replicates.

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