# Receptor-mediated effects of $\Delta^9$-THC & CBD on the inflammatory response of alveolar macrophages

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Running title: Cannabinoids affect alveolar macrophages

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**Keywords**: cannabis, cannabinoid, inflammation, macrophage, RNA-sequencing

## ABSTRACT

$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD) are cannabinoids found in *Cannabis sativa*. While research supports cannabinoids reduce inflammation, the consensus surrounding receptor(s) mediated effects has yet to be established. Here, we investigated the receptor-mediated properties of $\Delta^9$-THC and CBD on alveolar macrophages, an important pulmonary immune cell in direct contact with cannabinoids inhaled by cannabis smokers. MH-S cells, a mouse alveolar macrophage cell line, were exposed to $\Delta^9$-THC and CBD, with and without lipopolysaccharide (LPS). Outcomes included RNA-sequencing and cytokine analysis. $\Delta^9$-THC and CBD alone did not affect the basal transcriptional response of MH-S cells. In response to LPS, $\Delta^9$-THC and CBD significantly reduced the expression of numerous pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6, an effect that was dependent on $CB_2$. The anti-inflammatory effects of CBD- but not $\Delta^9$-THC- were mediated through a reduction in signaling through NF-κB and ERK1/2. These results suggest that CBD and $\Delta^9$-THC have potent immunomodulatory properties in alveolar macrophages, a cell type important in immune homeostasis in the lungs. Further investigation into the effects of cannabinoids on lung immune cells could lead to the identification of therapies that may ameliorate conditions characterized by inflammation.

## INTRODUCTION

*Cannabis sativa* L. (Cannabaceae) is a plant that produces hundreds of secondary metabolites, including cannabinoids. Cannabinoids are terpenophenolic compounds with a $C_{10}$ monoterpene subunit. Of the approximately 120 cannabinoids derived from *C. sativa*, $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBD) represent two of the most abundant. Structurally, $\Delta^9$-THC and CBD are nearly identical and share the exact same molecular formula of $C_{21}H_{30}O_2$. However, they differ in that $\Delta^9$-THC forms a cyclic ring whereas CBD forms a hydroxy group[1]. Ultimately, it is this subtle molecular discrepancy that accounts for the differences in the three-dimensional structure of the two compounds, thereby altering their affinities and interactions with the endogenous cannabinoid receptors $CB_1$ and $CB_2$. Activation of $CB_1$ and $CB_2$ results in the stimulation of mitogen-activated protein kinase (MAPK) activity and the inhibition of cyclic AMP (cAMP) production[2].

$\Delta^9$-THC is the primary psychoactive cannabinoid that produces hypoactivity, hypothermia as well as spatial and verbal memory impairment via $CB_1$ in the central nervous system[3]. Conversely, CBD is non-psychoactive and may exert anti-inflammatory effects via $CB_2$ on immune cells[1]. Within immune cells under basal conditions, $CB_2$ receptors have differential levels of expression, with the highest in B cells followed by macrophages, monocytes, natural killer (NK) cells and T cells. However, the expression of $CB_2$ is highly inducible and can increase up to 100-fold in response to tissue injury or inflammation[4]. The ability of cannabinoids to modulate the immune response has been proposed to occur in four main ways: (1) immune cell apoptosis, (2) suppressed cell proliferation, (3) pro-inflammatory cytokine/chemokine inhibition and promotion of anti-inflammatory cytokines, as well as (4) regulatory T cell ($T_{regs}$) induction[5]. Of the immune cells that may respond to cannabinoids, macrophages are a particularly important component of immunity. Macrophages are a

heterogeneous population of myeloid cells that are positioned throughout the body in a manner that facilitates the ingestion and degradation of dead cells, debris, foreign material, and the orchestration of inflammatory processes[6]. Macrophages produce cytokines and other mediators including nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukins (IL) such as IL-1β and IL-6. Macrophages typically exist in two distinct sub-sets: classically-activated macrophages (M1) or alternatively-activated macrophages (M2)[7]. M1 macrophages are pro-inflammatory and can be polarized by LPS or in conjunction with T helper type 1 (Th1) cytokines including GM-CSF or IFN-γ[8]. Once polarized, M1 macrophages produce pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNF-α. M2 macrophages are anti-inflammatory and immunoregulatory and can be polarized by T helper type 2 (Th2) cytokines including IL-4 and IL-13[8]. M2 macrophages produce anti-inflammatory cytokines such as IL-10 and TGF-β.

There also exist specialized tissue-resident macrophages that can be characterized according to their anatomical location. Notable among these are alveolar macrophages, which reside in the lung airspace and so are in direct contact with cannabinoids in people who smoke cannabis. Alveolar macrophages comprise a large majority of cells in the lung and are embryonically-derived. The main functions of alveolar macrophages are to engulf dead cells and debris, recycle surfactant, phagocytose pathogens, organize inflammatory processes, and recruit additional immune cells. Although studies have shown the effects of cannabinoids on macrophage function, including their ability to downregulate the production of acute phase proteins such as NO [9, 10], there is a scarcity of information with regards to alveolar macrophages. This includes a lack of information on the effects of cannabinoids on inflammatory signaling molecules and the mechanism through which this is accomplished. Therefore, we sought to investigate whether the inflammatory response of alveolar macrophages can be reduced by CBD and $\Delta^9$-THC.

Herein, we describe the receptor-mediated effects of two prominent cannabinoids- CBD and $\Delta^9$-THC- on alveolar macrophage function.

## RESULTS

### RNA-seq analysis identifies distinct gene signatures altered by cannabinoids during the inflammatory response

Given that cannabinoids can affect the immune response by causing alterations in cell survival[5, 11], we first determined the concentration of $\Delta^9$-THC and CBD which did not affect cell viability in MH-S cells to be 3 μM (**Supplementary figure 1**). Then, we used RNA-seq to comprehensively profile changes in gene expression by $\Delta^9$-THC and CBD in response to LPS. Based on changes in inflammatory gene expression in MH-S cells, we chose an LPS concentration of 0.1 μg $mL^{-1}$ for a duration of 24 hours (**Supplementary figure 2**). Principal component analysis (PCA) demonstrated that there was minimal intra-group variability (≤ 10%) and substantial inter-group variability (≥ 81%) in conditions containing LPS (**Supplementary figures 3 and 4**). Pearson Correlation was used to highlight the variation that was present between samples, showing correlation values of $r > 0.99$ when MH-S cells were treated with LPS (**Supplementary figure 5**). RNA-seq analysis of differentially expressed genes (DEGs) further revealed that there was no change in genes that met the threshold criteria ($log_2$fold change of 2; FDR $P < 0.05$) between MeOH and CBD, MeOH versus $\Delta^9$-THC or MeOH versus untreated (doi: 10.17632/g98tsfd8bh.1). This indicates that $\Delta^9$-THC and CBD alone do not significantly impact gene expression in MH-S cells. However, there was a total of 399 DEGs in response to LPS, of which 313 (78%) were upregulated and 86 (22%) were downregulated (**Figure 1a**). Among the inflammatory- and immune response-related genes that exhibited significant induction were *IL-1β, IL-6, CXCL2, CCL5, PTGS2, SAA3, LCN2, ACOD1, GBP3,* and *TRAF1* (**Figure 1a**). Genes that were significantly downregulated in response to LPS included *CCL24, CYTIP, ST6GAL1, KCTD12B, FCGR3, RNF150, SERPINB1A, TNS1, RASGRP3,* and *CD28* (**Figure 1a**). In contrast to the effect of LPS alone, the combination of LPS with CBD (LPS + CBD) and $\Delta^9$-THC (LPS + $\Delta^9$-THC) reduced gene expression. For LPS

159 + CBD, there was a total of 286 differentially expressed genes, of which 209 (73%) were
160 upregulated and 77 (27%) were downregulated (**Figure 1b**) and LPS with $\Delta^9$-THC, there was
161 a total of 287 differentially expressed genes, of which 212 (74%) were upregulated and 75
162 (26%) were downregulated (**Figure 1c**). As such, there was a net suppressive effect of CBD
163 and $\Delta^9$-THC on the LPS-induced gene expression profile, predominantly on the genes that were
164 increased in response to LPS.
165
166 Of the genes with increased expression, there were 173 that were common to all three treatment
167 groups (**Figure 1d**). There were also genes unique to each of the three conditions. Treatment
168 with LPS elicited the largest increase in genes (101), whereas co-treatment with either CBD or
169 $\Delta^9$-THC each had 13 uniquely upregulated genes (**Figure 1d**). The number of genes that were
170 uniquely decreased across the three conditions were 19, 11, and 10 for the LPS alone, LPS +
171 CBD, and LPS + $\Delta^9$-THC, respectively (**Figure 1e**). Therefore, despite considerable overlap
172 across the DEG profiles in response to LPS, there were significantly fewer upregulated genes
173 following treatment with either CBD or $\Delta^9$-THC, indicating a suppressive effect for CBD and
174 $\Delta^9$-THC on LPS-induced gene expression.
175
176 To further characterize the effects of CBD and $\Delta^9$-THC on the LPS-induced gene expression,
177 DEGs were classified into gene ontology (GO) biological processes (FDR 0.05) and KEGG
178 (Kyoto Encyclopedia of Genes and Genomes) pathways. Based on these analyses, DEGs are
179 mainly involved in processes such as immunity and inflammation, including the innate immune
180 response, response to cytokine stimulus and inflammatory response (GO:0006954) (**Figure 1f**).
181 Additional pathways identified included JAK-STAT, TNF signaling (mmu04668) and NF-κB
182 (**Figure 1g**). There was a reduction in GO enrichment when cells were treated with CBD and
183 $\Delta^9$-THC in conjunction with LPS **(Figure 1f**). Most notably, this reduction in GO enrichment

can be seen in biological processes such as inflammatory response and cellular response to cytokine stimulus (**Figure 1f**) and in pathways such as NF-κB (mmu04064) and JAK-STAT-signaling (mmu04630); there was no GO enrichment in the LPS + CBD group for these pathways (**Figure 1g**). Thus, in response to LPS, CBD and $\Delta^9$-THC alters the expression of genes associated with relevant biological processes and pathways in alveolar macrophages.

Because we were interested in the ability of cannabinoids to modulate inflammation in alveolar macrophages, we compared DEGs that comprised the inflammatory response (GO:0006954) and NF-κB signaling (mmu04064) pathway in response to CBD and $\Delta^9$-THC. Comprising the inflammatory response (GO:0006954) biological process were 36 genes that were differentially regulated by LPS; CBD decreased the expression of 32 of these genes relative to LPS. Genes that exhibited significant reductions (FDR-adjusted $P <$ value) by CBD included *II-1β, IL-6, SERPINE1, IL-1a* and *PTGS2* (**Table 1**). $\Delta^9$-THC decreased the expression of 35 of the 36 genes with a mean $\log_2$-fold change value of -0.613 relative to LPS alone (**Table 1**). Genes which demonstrated the most dramatic reduction by $\Delta^9$-THC included *IL-6, SERPINE1, PTGS2, IL-12β* and *CCL5* (**Table 1**). The NF-κB signaling (mmu04064) pathway had 11 genes that were differentially regulated by LPS. CBD decreased the expression of 9 of those genes with a mean $\log_2$-fold change value of -0.567 (**Table 2**). Genes with the most dramatic reduction by CBD included *IL-1β, PTGS2, CXCL2, TNF* and *TRAF1*. Similarly, $\Delta^9$-THC reduced the induction of 9 genes comprising the NF-κB signaling pathway, with a mean $\log_2$-fold change value of -0.514 (**Table 2**). *PTGS2, TNF*, *CXCL11, CD40* and *TRAF1* were most significantly down-regulated by $\Delta^9$-THC (**Table 2**). Therefore, CBD and $\Delta^9$-THC reduced the induction of genes associated with the inflammatory response and NF-κB signaling pathway in response to LPS.

**CBD and Δ$^9$-THC down-regulate inflammatory genes and proteins in alveolar macrophages**

Several of the genes identified by RNA-seq analysis to be reduced by CBD and Δ$^9$-THC following treatment with LPS were further validated through real-time qPCR. *IL-1β, IL-6* and *TNF-α* were selected based on their reduced expression by CBD and Δ$^9$-THC relative to LPS. As expected, CBD and Δ$^9$-THC alone did not affect the expression of *IL-1β* mRNA at the 6- or 24-hour timepoints (**Figure 2a** and **2b**). *IL-1β* mRNA was significantly induced by LPS at both timepoints; this induction was significantly reduced by CBD and Δ$^9$-THC at the 24-hour timepoint (**Figure 2b**). *IL-6* mRNA was similarly unaffected by treatment with CBD and Δ$^9$-THC (**Figure 2c** and **2d**). The induction of *IL-6* mRNA by LPS was significantly inhibited by both CBD and Δ$^9$-THC at the 24-hour timepoint (**Figure 2c** and **2d**). CBD and Δ$^9$-THC alone did not affect the expression levels of *TNF-α* mRNA (**Figure 2e** and **2f**). Similarly, there was a trend towards a reduction in *TNF-α* mRNA by both CBD and Δ$^9$-THC at the 24- but not 6-hour timepoint (**Figure 2e** and **2f**). Thus, CBD and Δ$^9$-THC significantly reduced the induction of genes associated with inflammation following exposure to LPS.

To next assess if the immunomodulatory effects of CBD and Δ$^9$-THC were also observed at the protein level, a multiplex assay was carried out on the cell supernatants. CBD and Δ$^9$-THC alone did not have any effect on the proteins examined (**Figure 3**). LPS significantly increased protein levels of all cytokines. There was a significant reduction in IL-1β and IL-6 protein when alveolar macrophages were pre-treated with either CBD or Δ$^9$-THC (**Figure 3a** and **3b**). For TNF-α and GM-CSF, CBD significantly reduced the induction by LPS (**Figure 3c** and **3d**, respectively). Finally, MCP-1 induction by LPS was unaffected by CBD or Δ$^9$-THC (**Figure 3e**). IFN-γ, IL-2, IL-4, IL-10 and IL-12p70 were also assessed in this assay but were below the

limit of detection (data not shown). Overall, these results demonstrate that CBD and $\Delta^9$-THC attenuate the cytokine levels produced by alveolar macrophages in response to LPS.

**CBD reduces LPS-induced activation of the NF-κB- and ERK1/2-signaling pathways**

From our RNA-seq data, we observed that while LPS caused significant enrichment in the NF-κB pathway, there was no gene enrichment with the inclusion of CBD, suggesting that CBD may be exerting its immunomodulatory properties via inhibition of this pathway. Therefore, we next addressed the role of NF-κB signaling in the anti-inflammatory properties of CBD and $\Delta^9$-THC in alveolar macrophages. **Figure 4a** illustrates that neither CBD nor $\Delta^9$-THC alone caused significant change in p65 phosphorylation, a marker of its activation[12]. In response to LPS, there was a significant increase in the phosphorylation of p65, an effect that was inhibited by CBD at the 15-minute time point (**Figure 4a**). $\Delta^9$-THC led to a slight, but non-significant, decrease in p65 activation in response to LPS. Thus, CBD inhibits p65 phosphorylation.

Next, ERK1/2 signaling was evaluated, as ERK1/2 plays a major role in the activation of several transcription factors that contribute to the inflammatory response, including NF-κB. CBD alone had no effect on the activation of ERK1/2; however, $\Delta^9$-THC led to a significant increase in ERK1/2 activation at both the 15-minute and 2-hour time points **(Figure 4b**). The was a noticeable (but not significant) induction of ERK1/2 phosphorylation by LPS that was reduced by CBD (**Figure 4b**). Overall, these data indicate that CBD and $\Delta^9$-THC have opposing effects on the activation of ERK1/2 at basal level. Modulation of ERK1/2 phosphorylation by CBD represents another signaling pathway through which CBD may be exerting its anti-inflammatory properties.

**$CB_2$ is required to mediate the anti-inflammatory properties of CBD and $\Delta^9$-THC**

Many of the pharmacological effects imparted by cannabinoids act through the endogenous cannabinoid receptors $CB_1$ and $CB_2$. As such, we next investigated the role of these receptors in mediating the anti-inflammatory properties of CBD and $\Delta^9$-THC in alveolar macrophages. To address this, we first evaluated $CB_1$- and $CB_2$- receptor expression in MH-S cells. There was no $CB_1$ protein detected in alveolar macrophages (data not shown), whereas $CB_2$ was constitutively expressed (**Figure 5a**). Therefore, we speculated that $CB_2$ may contribute to the reduction in pro-inflammatory cytokines induced by LPS. To assess this, we transiently transfected MH-S cells for a duration of 68 hours with either $CB_2$ receptor-specific siRNA ($siCB_2$) or with control siRNA (siCTRL), which reduced $CB_2$ by 50% (**Figure 5b**). Cells were pre-treated with CBD or $\Delta^9$-THC 40 hours into the transfection time for 1-hour followed by the addition of LPS for the remainder of the 68-hour transfection time. There was a decrease in *IL-1β* mRNA expression in response to CBD and $\Delta^9$-THC compared to LPS in the siCTRL-transfected cells (**Figure 5c**). However, in cells with reduced $CB_2$ expression, this reduction in response to CBD and $\Delta^9$-THC was less (**Figure 5c**). Similarly, there was no reduction of *IL-6* mRNA by CBD or $\Delta^9$-THC in the $siCB_2$-transfected cells (**Figure 5d**). It is noteworthy that there was a significant reduction in LPS-induced *IL-6* expression in MH-S cells where $CB_2$ expression was knocked-down. Finally, *TNF-α* expression was negligibly reduced by CBD and $\Delta^9$-THC (**Figure 5e**). Taken together, these data indicate that the $CB_2$ receptor plays a role in mediating the anti-inflammatory properties of CBD and $\Delta^9$-THC in alveolar macrophages.

## DISCUSSION

Countries such as Canada have recently legalized cannabis for both medicinal and recreational use. The medical potential of cannabis is largely attributed to the presence of cannabinoids, particularly CBD and $\Delta^9$-THC, and their ability to alter immune signaling including inhibition of acute-phase proteins such as NO, TNF-α, IL-1β, and IL-6[13]. Moreover, an increasing number of studies have revealed the therapeutic potential of these cannabinoids in pathologies characterized by a dysregulated immune response, including multiple sclerosis, inflammatory bowel disease and arthritis[14-16]. Despite optimism for the medical use of cannabis, a broader understanding of the effects of cannabinoids on immune function is lacking, particularly for alveolar macrophages, a highly specialized pulmonary innate immune cell that responds to environmental signals. Herein, we show that CBD and $\Delta^9$-THC exert immune regulatory effects on alveolar macrophages, including suppression of pro-inflammatory cytokine production and downregulation of signaling pathways implicated in the immune response.

In macrophages, their phenotype and polarization are dependent upon factors present within their environment[7]. Therefore, we evaluated the effects of CBD and $\Delta^9$-THC in response to LPS, a component of the cell wall of gram-negative bacteria that induces inflammation. Using RNA-seq, we identified a unique transcriptional response in LPS-induced alveolar macrophages treated with CBD or $\Delta^9$-THC. This unbiased profiling revealed the importance of CBD and $\Delta^9$-THC in the regulation of key inflammatory genes involved in innate immunity. Here, CBD and $\Delta^9$-THC repressed the expression of an important subset of inflammatory genes that were increased by LPS, including *IL-1β, IL-6,* and *TNF-α*. Excessive production of IL-1β, IL-6 and TNF-α by macrophages has been associated with disease progression and severe inflammation pathologies, including inflammatory lung disease[8]. Thus, the ability of CBD and $\Delta^9$-THC to reduce the expression of pro-inflammatory mediators is highly relevant in

pulmonary conditions wherein aberrant expression of these cytokines is at the core of their pathology. For instance, acute lung injury (ALI) is a pulmonary condition in which the generation and release of pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS) produced by activated lung macrophages leads to damage of the lung parenchyma. LPS is commonly employed to mimic ALI. As such, the ability of cannabinoids CBD and $\Delta^9$-THC to down-regulate the expression of IL-1β, IL-6, and TNF-α may have substantial utility in conditions such as ALI.

It is also interesting to note that neither CBD nor $\Delta^9$-THC elicited a transcriptional response in macrophages in our study. However, this is inconsistent with previous studies in $CD4^+$ T cells, where functional analysis of genes differentially expressed in response to $\Delta^9$-THC revealed significant enrichment in the inflammatory response pathway[17]. Furthermore, *in vitro* studies evaluating the effects of cannabinoids, including $\Delta^9$-THC, on cytokine production in B cells, $CD8^+$ T cells, NK cells, monocytes, and eosinophilic cell lines demonstrated variable results, depending on the cell line and concentration of cannabinoids used[18]. For instance, in RAW264.7 monocytes, CBD at a concentration of 21.2 μM significantly increased the levels of G-CSF, GM-CSF, IFN-γ, IL-1a, IL-6, IL-27, I-TAC, M-CSF, MCP-1, RANTES, and TNF-α[19]. Other studies have found that treatment with $\Delta^9$-THC at a concentration of 9.5 μM in LPS-activated resident peritoneal macrophages increased the production of inflammatory cytokines such as IL-1β[20]. This suggests that the differential effects of cannabinoids on cytokine induction may be attributed to varied thresholds of different cell populations; thus, the secretion of inflammatory cytokines in response to cannabinoids is variable and may depend on factors such as the cannabinoid concentration and cell type employed. Consistent with this, in certain cell culture systems, cannabinoids can display biphasic dose-response curves, which may account for the apparent discrepancy regarding cannabinoid modulation of cytokine expression. In

peripheral blood mononuclear cells, $\Delta^9$-THC exerted an inhibitory effect on the production of TNF-α, IL-6, and IL-8 at a concentration of 3 nM whereas a stimulatory effect on these cytokines was observed at a concentration of 3 μM[21]. It should also be noted that the cannabinoids employed in our study were pure analytical grade, whereas those in other studies were a commercially available e-liquid containing CBD[19], a variable that could confound interpretation of the results. Thus, the induction of pro-inflammatory markers in other studies could be attributed to other ingredients present such as chromium, copper, lead, and flavoring chemicals.

Another confounding factor in the interpretation of data between studies is the ability of cannabinoids to affect cell death pathways, which is one of the proposed mechanisms through which cannabinoids exert their immunomodulatory properties[5, 11]. In various immune cell populations, cell death has been associated with increased secretion of pro-inflammatory mediators. For instance, Zhu *et al*. demonstrated that treatment with $\Delta^9$-THC at concentrations between 15-30 μM led to apoptosis in murine macrophages through regulation of Bcl-2 and caspase activity. This increase in apoptosis was accompanied by a dose-dependent release of IL-1β as well as other inflammatory cytokines[22]. Similarly, treatment with CBD caused a dose- and time-dependent increase in apoptosis of $CD4^+$ and $CD8^+$ T cell populations[23]. Cannabinoid-induced cell-death may be of particular importance with regards to cannabis consumers, as it could be postulated to increase susceptibility to pulmonary infection. Macrophages constitute one of the primary lines of defence against foreign organisms and xenobiotics within the lungs, meaning their death may increase the frequency- and severity- of infection. This may further alter the expression of various cytokines and chemokines within the lung. To minimize the confounding effects of cannabinoid-induced cell death in our study, we ensured that cell viability in response to CBD and $\Delta^9$-THC was not affected. Therefore, our results show that

CBD and $\Delta^9$-THC can reduce the inflammatory response in alveolar macrophages without changes in cell survival.

Our unbiased approach also identified several novel genes and pathways affected by cannabinoids, including CD40 antigen which was significantly reduced in response to CBD and $\Delta^9$-THC. CD40 antigen regulates the co-stimulatory activity of APCs, induces B cells to upregulate B7 co-stimulatory proteins as well as induces DCs to increase cell surface expression of other co-stimulatory molecules such as CD54 and CD86[24]. Furthermore, ligation of CD40 antigen leads to the production of various inflammatory cytokines including IL-8, TNF-α, and macrophage inflammatory protein (MIP)[25]. Notably, CD40 co-stimulation leads to the induction of IL-12, a cytokine that was similarly reduced in response to CBD and $\Delta^9$-THC in alveolar macrophages (Table 2). IL-12 plays a key role in the polarization of Th1 immune responses. The expression of CD40 antigen can be found on a host of non-immune cells including epithelial cells, endothelial cells, fibroblasts, myofibroblasts, and more[26]. In these cell types, the CD40 system serves as an effective means of communication with immune cells, the usual outcome being amplification of immune and inflammatory processes. For instance, ligation of CD40 antigen on endothelial cells or fibroblasts leads to the production of IL-8, MCP-1, MIP-1, IL-6, and TNF-α [24, 25]. This suggests that repression of CD40 antigen in alveolar macrophages by CBD and $\Delta^9$-THC may be how cannabinoids mitigate an adaptive immune response. This notion is supported by a study by Chuchawankul and colleagues who demonstrated that treatment with $\Delta^9$-THC in peritoneal macrophages significantly impaired their ability to deliver co-stimulatory signals to a helper T cell hybridoma[27]. Additionally, pre-treatment with $\Delta^9$-THC significantly impaired the upregulation of CD40 antigen induced by anti-CD3/CD28 in mouse splenic $CD4^+$ T cells[28], and in mesenchymal stem cells, pre-treatment with CBD led to the downregulation of genes coding for antigens involved in the activation of

the immune system[29]. As such, susceptibility to bacterial infection could increase following cannabinoid exposure, given that inflammatory signaling was additionally reduced by cannabinoid exposure in the present study. Therefore, our data highlight the possibility that CBD and $\Delta^9$-THC may mitigate the immune response through suppression of co-stimulatory molecules. Another possibility to explain our results is that cannabinoids cause the internalisation of TLR4, resulting in a decrease in LPS signalling. Prolonged exposure to LPS results in the internalization of TLR4 in RAW264.7 macrophages, the effect of which requires ERK signaling[30]. Although it is not currently known, it is possible that cannabinoid-induced modulation of ERK activity directly alters TLR4 signaling, an interesting topic for future studies.

Mechanistically, the NF-κB signaling pathway is required for induction of inflammatory genes, including those encoding IL-1β, IL-6, and TNF-α[31]. Cannabinoids inhibit NF-κB activation, resulting in subsequent reductions in inflammatory mediators[10, 13]. Herein, we show that CBD reduced the induction of genes comprising the NF-κB signaling pathway. In addition, there was a significant reduction in the phosphorylation of the NF-κB p65 subunit by CBD. Interestingly, $\Delta^9$-THC did not affect either of these markers of activation, thus questioning the involvement of the NF-κB pathway in the $\Delta^9$-THC-mediated effects in alveolar macrophages. Similar to our observations, Kozela *et al.* demonstrated that CBD and $\Delta^9$-THC reduce the expression of IL-1β and IL-6 in BV-2 microglial cells, and that the effects of CBD, but not $\Delta^9$-THC, were mediated by NF-κB[13]. Although our data are suggestive of a role of the NF-κB pathway in mediating the anti-inflammatory properties of CBD, separate pathways are likely involved in the effects of $\Delta^9$-THC such as ERK1/2. ERK1/2 (p42/p44) increases pro-inflammatory signaling in monocytes and macrophages through activation of transcription factors including NF-κB and AP-1 [31]. We found that activation of ERK1/2 by LPS was significantly inhibited by CBD.

However, $\Delta^9$-THC, both alone and in combination with LPS, increased ERK1/2 activation. The differential effects of CBD and $\Delta^9$-THC on ERK1/2 activation may be the result of their reciprocal action on receptors of the endocannabinoid system, specifically the $CB_2$ receptor, where CBD act as an antagonist and $\Delta^9$-THC acts as an agonist[32]. Thus, agonism of $CB_2$ by $\Delta^9$-THC and antagonism by CBD may have resulted in the differential activation of ERK1/2 in our study. In support of this, Bouaboula *et al.* and Kobayashi *et al.* demonstrated that activation of p42/44 in response to $\Delta^9$-THC was abrogated upon addition of the $CB_2$ receptor antagonist SR144528[33, 34]. In our study, knocking-down the $CB_2$ receptor in alveolar macrophages also affects CBD- and $\Delta^9$-THC- mediated reductions in *IL-1β, IL-6,* and *TNF-α* expression. Thus, cannabinoid-mediated modulation of MAPK activity may be acting through the $CB_2$ receptor.

Our work is the first to elucidate the ability of CBD and $\Delta^9$-THC to inhibit the inflammatory response in alveolar macrophages. However, one of the limitations of our study was that we only explored the effects of these cannabinoids *in vitro* in the MH-S alveolar macrophage cell line. Another limitation in the interpretation of our findings is that while we showed reduced phagocytosis in response to CBD in combination with LPS, we did not quantify macrophage killing, an important regulatory function of phagocytosis. Another potential limitation is that we only evaluated the ability of select cannabinoids to affect functions in alveolar macrophages *in vitro*, an experimental system that does not recapitulate the complexity of human cannabis users. Smoking currently stands as the most popular method of cannabis consumption, where cells present in the lung would be in direct contact with cannabinoids as well as combustion derived constituents, of which there are thousands[35].

Despite these limitations, we are the first to report on the anti-inflammatory properties of CBD and $\Delta^9$-THC in alveolar macrophages, with the $CB_2$ receptor playing a key role in mediating

their immunomodulatory effects. We also show that the suppressive effects of CBD extend beyond its ability to inhibit pro-inflammatory cytokines in that they also reduce phagocytosis by alveolar macrophages. With continued research, we may uncover a vital link in the relationship between cannabinoids and their immunomodulatory properties in humans, leading to the identification of a molecular mechanism that could be targeted by novel cannabinoid-based therapies to ameliorate conditions characterized by acute or chronic inflammation.

## METHODS

### Cell Culture

MH-S cells were obtained from American Type Culture Collection (ATCC; Manassas, USA). Cells were cultured in RPMI 1640 media (WISENT Inc, Saint-Jean Baptiste, Canada) containing 10% FBS (WISENT Inc), gentamycin (WISENT Inc), antibiotic-antimycotic (A/A; WISENT Inc) and 2-mercaptoethanol (Millipore Sigma, Burlington, USA). Cells were cultured under 7 separate conditions; untreated, methanol (MeOH), $\Delta^9$-THC (ISO60157; Cayman Chemical, USA), CBD (ISO60156; Cayman Chemical, Ann Arbor, USA), LPS (LPS 0111: B4; Sigma-Aldrich, St. Louis, USA), LPS plus $\Delta^9$-THC and LPS plus CBD. Only purified, research grade $\Delta^9$-THC and CBD were utilized in this study. The untreated (control) condition consisted of RPMI 1640 without FBS. Methanol was used as the solvent control for $\Delta^9$-THC and CBD throughout the study. All cells were incubated in humidified chambers at 37$^o$C and exposed to 21 % $O_2$ and 5% $CO_2$.

### qRT-PCR

MH-S cells were seeded at $2x10^5$ cells/cm$^2$ in 6-well plate and upon reaching 70-80% confluency one day later, were pre-treated with either 3µM $\Delta^9$-THC or 3µM CBD for one hour followed by the addition of 0.1µg mL$^{-1}$ LPS for 6 or 24 hours. RNA was isolated using Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Saint-Laurent, Canada) in accordance with manufacturer's instructions. RNA quantification was done using a Nanodrop 1000 spectrophotometer infinite M200 pro (TECAN, Männedorf, Switzerland). Reverse transcription of RNA to cDNA was carried out using iScript Reverse Transcription Supermix (Bio-Rad Laboratories) and mRNA levels were analyzed. Primer sequences for the genes were *GAPDH* (f) GGTCCTCAGTGTAGCCCAAG, (r) AATGTGTCCGTCGTGGATCT; *TNF-α* (f) CTATGTCTCAGCCTCTTCTC, (r) GGGAACTTCTCATCCCTTT; *IL-1β* (f)

GGACATGAGCACCTTCTT, (r) CCTGTAGTGCAGTTGTCTAA and *IL-6* (f) CCAGAGTCCTTCAGAGAGATACA, (r) CCTTCTGTGACTCCAGCTTATC. Quantitative PCR (qPCR) was done by combining 1µl cDNA and 0.5µM primers with SsoFast EvaGreen (Bio-Rad Laboratories) with amplification performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Canada). Thermal cycling was initiated at 95°C for 3 minutes followed by 39 cycles of denaturation at 95°C for 10 seconds and annealing at 55°C for 5 seconds. RNA expression was analyzed using the ΔΔCt method and results presented as fold-change normalized to the housekeeping gene (*GAPDH)*.

**Cytokine Analysis**

Cells were treated as described above for 24 hours. Cell supernatants were then collected and sent to Eve Technologies Corporation (Calgary, Canada) and a Mouse Cytokine Array Proinflammatory Focused 10-plex (MDF10) Assay (IFN-γ, IL-1β, GM-CSF, IL-2, IL-4, IL-6, IL-10, IL-12p70, MCP-1 and TNF-α) was conducted.

**Western Blot**

MH-S cells were seeded at $2x10^5$ cells/$cm^2$ in 6-well plates and upon reaching 70-80% confluency, were pre-treated with 3µM of $\Delta^9$-THC or CBD for one hour followed by the addition of 0.1µg $mL^{-1}$ LPS. Total cellular protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific, Nepean, USA) containing Protease Inhibitor Cocktail (Roche, Indianapolis, USA). Following extraction, protein concentration was determined using the bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific). Protein lysate at a concentration of 15 or 20 µg was electrophoresed on 10% SDS-PAGE gels and transferred onto Immuno-blot PVDF membranes (Bio-Rad Laboratories). After the transfer, the membrane was

blocked using a blocking solution of 5% w/v non-fat dry milk in PBS/0.1% Tween-20 for one hour at room temperature. Antibodies were applied to membranes for one hour or overnight. The following is a list of the antibodies used: anti-tubulin (1:50000; ID: T6199, Sigma-Aldrich), anti-$CB_2$ Receptor (1:200; ID: 101550, Cayman Chemical, USA), anti-p-p65 (1:1000; ID: 3033, Cell Signaling Technologies, Danvers, USA), anti-p65 (1:1000; ID: 8008, Santa Cruz, Dallas, USA), anti-p-p44/42 MAPK (1:1000; ID: 4370, Cell Signaling Technologies) and anti-p44/42 MAPK (1:1000; ID: 4696, Cell Signaling Technologies). After application of the primary antibody, secondary antibodies including anti-rabbit IgG, HRP linked (1:10000; ID:7074, Cell Signaling Technologies, USA) and HRP-conjugated anti-mouse IgG (1:10000; ID: 7076, Cell Signaling Technologies, USA) were used. Membrane visualization was carried out using either Clarity western ECL substrate (Bio-Rad Laboratories) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Protein detection was performed using the ChemiDoc MP Imaging System (Bio-Rad Laboratories). Densitometric analysis was analyzed through Image Lab Software Version 5 (Bio-Rad Laboratories).

**RNA sequencing**

Total RNA was quantified using Qubit (Thermo Scientific) and RNA quality was assessed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Transcriptome libraries were generated using the KAPA RNA HyperPrep Kit with RiboErase (HMR) (Roche). Sequencing was performed on the Illumina NextSeq500, obtaining around 50M single-end reads per sample. The reads were trimmed using fastp and then aligned using the STAR aligner. From the aligned reads, HTSeq was used to get the raw read counts. If there was a known batch effect, it was accounted for using the sva R package. Then the DESeq2 R package was used to normalize the counts and run a differential expression (DE) analysis between the different conditions. The Gage and Pathview R packages were used on the most significant differentially

expressed genes (DEGs; log2 fold change > 2 and an adjusted *P*-value < 0.05) to get pathways and gene sets. Complete data tables are accessible at doi: 10.17632/g98tsfd8bh.1.

**siRNA Knockdown**

MH-S cells were seeded at $2x10^5$ cells/$cm^2$ in 6-well plate and one day later were transfected with 80 nM of siRNA targeting $CB_2$ (Santa Cruz) or non-targeting control siRNA (Santa Cruz) in accordance with manufacturer's instructions. The transfection was performed using jetPRIME Transfection Reagent (Polyplus Transfection, Illkirch-Graffenstaden, France) following manufacturer's instructions. After 24 hours, media containing transfection reagent was removed and cells washed with PBS. Fresh RPMI 1640 containing 10% FBS was then added to the cells. Twenty hours later, alveolar macrophages were pre-treated with either 3µM $\Delta^9$-THC or 3µM CBD for one hour followed by the addition of 0.1µg $mL^{-1}$ LPS for 24 hours. Confirmation of $CB_2$ knockdown was examined by western blot 68 hours after transfection.

**Statistical Analysis**

Statistical analysis was performed using two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test to assess differences between the treatment groups unless otherwise stated, using GraphPad Prism 6 (v.602; GraphPad Software Inc, San Diego, USA). Results are presented as mean ± standard error of the mean (SEM) of the fold changes compared to MeOH treated cells. Statistical significance was considered in all cases which had a *P*-value < 0.05.

## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare

## FUNDING

This work was supported by the Canadian Institutes for Health Research Project Grants (168836 and 162273) and the Natural Sciences and Engineering Research Council of Canada (NSERC). C.J.B was supported by a salary award from the Fonds de recherche du Quebec-Sante (FRQ-S). H.T. was supported by a Réseau de recherche en santé réspiratoire du Québec (RSR) Scholarship. H.T. and M.W.P were supported by a Meakins-Christie Laboratories Collaborative Research Award.

## AUTHOR CONTRIBUTIONS

Data curation and/or analysis: M.W.P., H.T., P.G., O.L., G.J.F., C.J.B.; Funding acquisition: C.J.B; Methodology: M.W.P., H.T., G.J.F.; Project administration: C.J.B.; Supervision: C.J.B.; Intellectual contributions: M.W.P., C.J.B., D.H.E., G.J.F. H.T.; Manuscript writing, review and editing: M.W.P., H.T., P.G., G.J.F., D.H.E., C.J.B.

## DATA AVAILABILITY

The RNA-seq data tables supporting the conclusions of this article are available at doi: 10.17632/g98tsfd8bh.1.

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**Table 1. CBD and $\Delta^9$-THC reduce the induction of genes comprising the inflammatory response.** List of genes comprising the inflammatory response that were differentially regulated by LPS. Each gene is presented as the $log_2$-fold change in the LPS + CBD and LPS + $\Delta^9$-THC treatment groups relative to the LPS treatment group. Genes are ranked according to significance in the LPS + CBD treatment group relative to the LPS treatment group using the -$Log_2$ *P*-value for each respective gene represented by the blue spectrum coloring.

| Gene Name | Gene Symbol | Log2[LPS] | |
|---|---|---|---|
| | | CBD | THC |
| Interleukin 1 Beta | *IL1B* | -1.327 | -1.401 |
| Interleukin 6 | *IL6* | -1.195 | -1.195 |
| Serine Peptidase Inhibitor, Clade E, Member 1 | *SERPINE1* | -1.233 | -1.082 |
| Interleukin 1 Alpha | *IL1A* | -1.090 | -1.083 |
| Prostaglandin-Endoperoxide Synthase 2 | *PTGS2* | -1.255 | -1.010 |
| Chemokine (C-X-C motif) Ligand 2 | *CXCL2* | -1.367 | -1.124 |
| Interleukin 12b | *IL12B* | -1.153 | -1.282 |
| Chemokine (C-C motif) Ligand 5 | *CCL5* | -0.973 | -1.086 |
| Solute Carrier Family 7 | *SLC7A2* | -0.719 | -0.455 |
| Tumor Necrosis Factor | *TNF* | -0.611 | -0.597 |
| Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells Inhibitor, Alpha | *NFKBIA* | -0.446 | -0.333 |
| Serum Amyloid A 3 | *SAA3* | -0.439 | -0.448 |
| Nitric Oxide Synthase 2, Inducible | *NOS2* | -0.627 | -0.580 |
| CD40 Antigen | *CD40* | -0.535 | -0.418 |
| Chemokine (C-C motif) Receptor-Like 2 | *CCRL2* | -0.570 | -0.456 |
| Chemokine (C-X-C motif) Ligand 10 | *CXCL10* | -1.007 | -1.047 |
| Chemokine (C-X-C motif) Ligand 11 | *CXCL11* | -1.264 | -1.317 |
| Adenosine A2A Receptor | *ADORA2A* | -0.977 | -0.420 |
| Hemopoietic Cell Kinase | *HCK* | -0.416 | -0.274 |
| Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells Inhibitor, Zeta | *NFKBIZ* | -0.200 | -0.208 |
| Tumor Necrosis Factor, Alpha-Induced Protein | *TNFAIP3* | -0.176 | -0.096 |
| Chemokine (C-C motif) Ligand 2 | *CCL2* | -0.885 | -0.636 |
| Chemokine (C-C motif) Ligand 7 | *CCL7* | -1.104 | -0.734 |
| Zinc Finger CCCH Type Containing 12A | *ZC3H12A* | -0.230 | -0.150 |
| Guanylate Binding Protein 5 | *GBP5* | -1.034 | -1.279 |
| T Cell-Interacting, Activating Receptor on Myeloid Cells 1 | *TARM1* | -0.641 | -0.471 |
| Formyl Peptide Receptor 2 | *FPR2* | -0.815 | -0.709 |
| Orosomucoid 1 | *ORM1* | 0.562 | 0.487 |
| Serine Peptidase Inhibitor, Clade B, Member 1a | *SERPINB1A* | -0.292 | -0.149 |
| Prostaglandin E Synthase | *PTGES* | -0.202 | -0.092 |
| Z-DNA Binding Protein 1 | *ZBP1* | -0.641 | -1.031 |
| Cytochrome b-245, Beta Polypeptide | *CYBB* | -0.551 | -0.567 |
| Tumor Necrosis Factor, Member 18 | *TNFSF18* | -0.234 | -0.133 |
| Interleukin 1 Family, Member 9 | *IL1F9* | 0.051 | -0.274 |
| Haptoglobin | *HP* | 0.059 | -0.136 |
| CD5 Antigen-Like | *CD5L* | 0.069 | -0.260 |

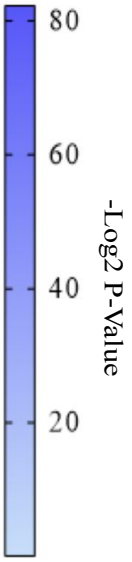
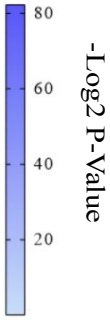


**Table 2. CBD and $\Delta^9$-THC reduce the induction of genes comprising the NF-κB signaling pathway.** List of genes comprising the NF-κB signaling pathway that were differentially regulated by LPS. Each gene is presented as the $log_2$-fold change in the LPS + CBD and LPS + $\Delta^9$-THC groups relative to LPS. Genes are ranked according to significance in the LPS + CBD group relative to LPS using the -$Log_2$ *P*-value for each respective gene represented by the blue spectrum coloring.

| Gene Name | Gene Symbol | Log2[LPS] | |
|---|---|---|---|
| | | CBD | THC |
| Interleukin 1 Beta | *IL1B* | -1.327 | -1.401 |
| Prostaglandin-Endoperoxide Synthase 2 | *PTGS2* | -1.255 | -1.010 |
| Chemokine (C-X-C motif) Ligand 2 | *CXCL2* | -1.367 | -1.124 |
| Tumor Necrosis Factor | *TNF* | -0.611 | -0.597 |
| Tumor Necrosis Factor Receptor Associated Factor 1 | *TRAF1* | -0.691 | -0.486 |
| Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells Inhibitor, Alpha | *NFKBIA* | -0.446 | -0.333 |
| CD40 Antigen | *CD40* | -0.535 | -0.418 |
| Chemokine (C-X-C motif) Ligand 11 | *CXCL11* | -1.264 | -1.317 |
| Tumor Necrosis Factor, Alpha-Induced Protein | *TNFAIP3* | -0.176 | -0.096 |
| Tumor Necrosis Factor Superfamily Member 14 | *TNFSF14* | 0.918 | 1.095 |
| Casein Kinase 2 Alpha 1 | *CSNK2A1-PS* | 0.508 | 0.023 |



## Figure Legend

**Figure 1.** Effects of CBD and $\Delta^9$-THC on the LPS-induced differential gene profile. (**a-c**) Gene expression scatter plots representing the normalized counts of all genes comprising the LPS vs. MeOH (**a**), LPS + CBD vs. MeOH (**b**) and LPS + $\Delta^9$-THC vs. MeOH (**c**). Red indicates significantly (false discovery rate [FDR]-adjusted $P < 0.05$ and $\log_2$-fold change $\geq 2$) upregulated genes in the respective treatment group whereas blue indicates significantly downregulated genes compared to MeOH. Grey indicates genes not differentially expressed. Genes highlighted represent those with the most significant induction according to adjusted *P*-value. Values are presented as the $\log_2$ value of the mean of the normalized counts of each gene (n = 3). (**d-e**) Venn diagrams displaying the number of genes that were differentially upregulated (**d**) or downregulated (**e**) across the different treatment groups as well as the number of genes that were unique or shared amongst groups. (**f**) GO term enrichment analysis for the "biological process" category of the DEGs. Biological processes are presented as the -$\log_{10}$ q-value of the DEGs for each treatment group. The GO terms are ranked by the -$\log_{10}$ q-value in the LPS treatment group. (**g**) KEGG pathways derived from the DEG profiles of the LPS, LPS + CBD, and LPS + $\Delta^9$-THC treatment conditions. Pathways are presented as the -$\log_{10}$ q-value of the DEGs for each group. The pathways are ranked and presented according to the -$\log_{10}$ q-value of LPS.

**Figure 2.** CBD and $\Delta^9$-THC reduce the mRNA of *IL-1β*, *IL-6*, and *TNF-α* mRNA in response to LPS. (**a**) There was no change in *IL-1β* mRNA in MH-S cells pre-treated with CBD or $\Delta^9$-THC followed by LPS for 6 hours. There was a significant reduction in *IL-1β* (**b**) expression by CBD and $\Delta^9$-THC with LPS for 24 hours. *IL-1β* mRNA did not change in response to CBD or $\Delta^9$-THC alone at either timepoint relative to control (n = 5). *IL-6* (**c**) mRNA levels did not change in response to cells pre-treated with CBD or $\Delta^9$-THC followed by the addition of LPS for 6 hours. Both CBD and $\Delta^9$-THC significantly reduced *IL-6* (**d**) expression with LPS for 24 hours. *IL-6* mRNA levels did not change in response to CBD or $\Delta^9$-THC alone at both 6- and 24-hour timepoints (**c-d**) relative to control (n = 5). (**e**) There was no change in *TNF-α* mRNA levels in cells pre-treated with CBD or $\Delta^9$-THC followed by the addition of LPS for 6 hours or 24 hours (**f**). *TNF-α* mRNA did not change in response to CBD or $\Delta^9$-THC alone (**e-f**). Values are presented as the mean ± SEM. Means are expressed as fold change from the control. $^{**}P < 0.01$; $^{****}P < 0.001$, as compared to LPS.

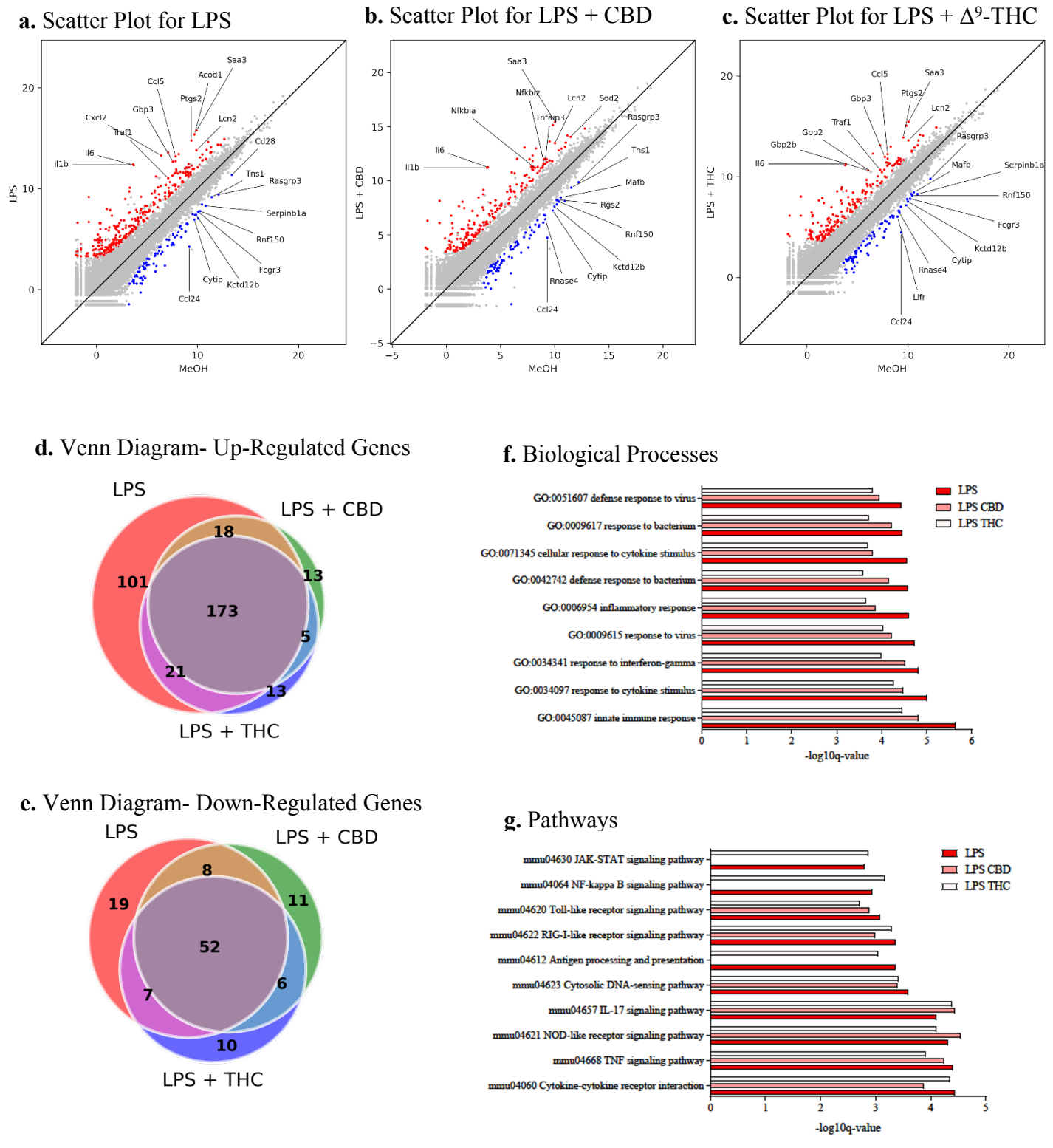
**Figure 3.** CBD and $\Delta^9$-THC reduce the level of proteins in response to LPS. Basal levels of IL-1β (**a**), IL-6 (**b**), TNF-α (**c**), GM-CSF (**d**), and MCP-1 (**e**) are unaffected by CBD or $\Delta^9$-THC in the absence of LPS. In the presence of LPS for 24 hours, CBD significantly reduced the protein level of IL-1β (**a**), IL-6 (**b**), TNF-α (**c**), and GM-CSF (**d**). There was no significant difference in MCP-1 (**e**) protein by CBD in the presence of LPS. $\Delta^9$-THC significantly reduced the protein level of IL-1β (**a**) and IL-6 (**b**) when LPS was present. However, $\Delta^9$-THC did not change the level of GM-CSF, MCP-1 or TNF-α compared to LPS alone (n = 4). Values are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ as compared to LPS alone.

**Figure 4.** Effects of CBD and $\Delta^9$-THC on activation of NF-κB and ERK1/2 pathways. (**a**) Basal p65 phosphorylation was unaffected by either CBD or $\Delta^9$-THC. LPS-induced increase in p-p65 was significantly downregulated by CBD but not $\Delta^9$-THC following 15-minute exposure. (**b**) Basal ERK1/2 phosphorylation was unaffected by CBD; however, $\Delta^9$-THC significantly increased phosphorylation at both timepoints. LPS-induced increase in

phosphorylated ERK1/2 were downregulated by CBD and unaffected by $\Delta^9$-THC. Values are presented as the mean ± SEM (depicted blots are representative of five independent experiments). Means are expressed as fold change from the control (MeOH); phosphorylated protein were normalized to total protein with all treatment groups. $^{**}P < 0.01$ and $^{****}P < 0.0001$ as compared to LPS unless otherwise stated.

**Figure 5.** Cannabinoid-mediated reduction in pro-inflammatory cytokine expression is inhibited by $CB_2$ receptor knockdown in MH-S cells. (**a**) $CB_2$ protein is constitutively expressed in MH-S cells. (**b**) Sixty-eight hours following transfection of MH-S cells with si$CB_2$ reduced the level of $CB_2$ protein to ~ 50%. Representative of 3 replicate experiments. Levels of *IL-1β* (**c**), *IL-6* (**d**), and *TNF-α* (**e**) mRNA in response to siCTRL or si$CB_2$. Cells were pre-treated with CBD or $\Delta^9$-THC 40 hours into the transfection window for 1-hour followed by the addition of LPS for the remainder of the 68-hour transfection time. Results are expressed as the mean ± SEM of 5 independent experiments. Means are expressed as fold change from MeOH. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ and $^{****}P < 0.00001$ compared to LPS.

**Figure 1**


a. Scatter Plot for LPS
LPS
MeOH
Saa3
Acod1
Ccl5
Ptgs2
Gbp3
Cxcl2
Lcn2
Traf1
Cd28
Il6
Il1b
Tns1
Rasgrp3
Serpinb1a
Rnf150
Fcgr3
Cytip
Kctd12b
Ccl24
b. Scatter Plot for LPS + CBD
LPS + CBD
MeOH
Saa3
Nfkbiz
Lcn2
Sod2
Nfkbia
Tnfaip3
Rasgrp3
Il6
Il1b
Tns1
Mafb
Rgs2
Rnf150
Kctd12b
Cytip
Rnase4
Ccl24
c. Scatter Plot for LPS + Δ9-THC
LPS + THC
MeOH
Ccl5
Saa3
Gbp3
Ptgs2
Lcn2
Traf1
Gbp2
Rasgrp3
Gbp2b
Il6
Mafb
Serpinb1a
Rnf150
Fcgr3
Kctd12b
Cytip
Rnase4
Lifr
Ccl24


d. Venn Diagram- Up-Regulated Genes
LPS
LPS + CBD
LPS + THC
101
18
13
173
5
21
13


e. Venn Diagram- Down-Regulated Genes
LPS
LPS + CBD
LPS + THC
19
8
11
52
7
6
10


f. Biological Processes
LPS
LPS CBD
LPS THC
GO:0051607 defense response to virus
GO:0009617 response to bacterium
GO:0071345 cellular response to cytokine stimulus
GO:0042742 defense response to bacterium
GO:0006954 inflammatory response
GO:0009615 response to virus
GO:0034341 response to interferon-gamma
GO:0034097 response to cytokine stimulus
GO:0045087 innate immune response
-log10q-value


g. Pathways
LPS
LPS CBD
LPS THC
mmu04630 JAK-STAT signaling pathway
mmu04064 NF-kappa B signaling pathway
mmu04620 Toll-like receptor signaling pathway
mmu04622 RIG-I-like receptor signaling pathway
mmu04612 Antigen processing and presentation
mmu04623 Cytosolic DNA-sensing pathway
mmu04657 IL-17 signaling pathway
mmu04621 NOD-like receptor signaling pathway
mmu04668 TNF signaling pathway
mmu04060 Cytokine-cytokine receptor interaction
-log10q-value

**Figure 2**

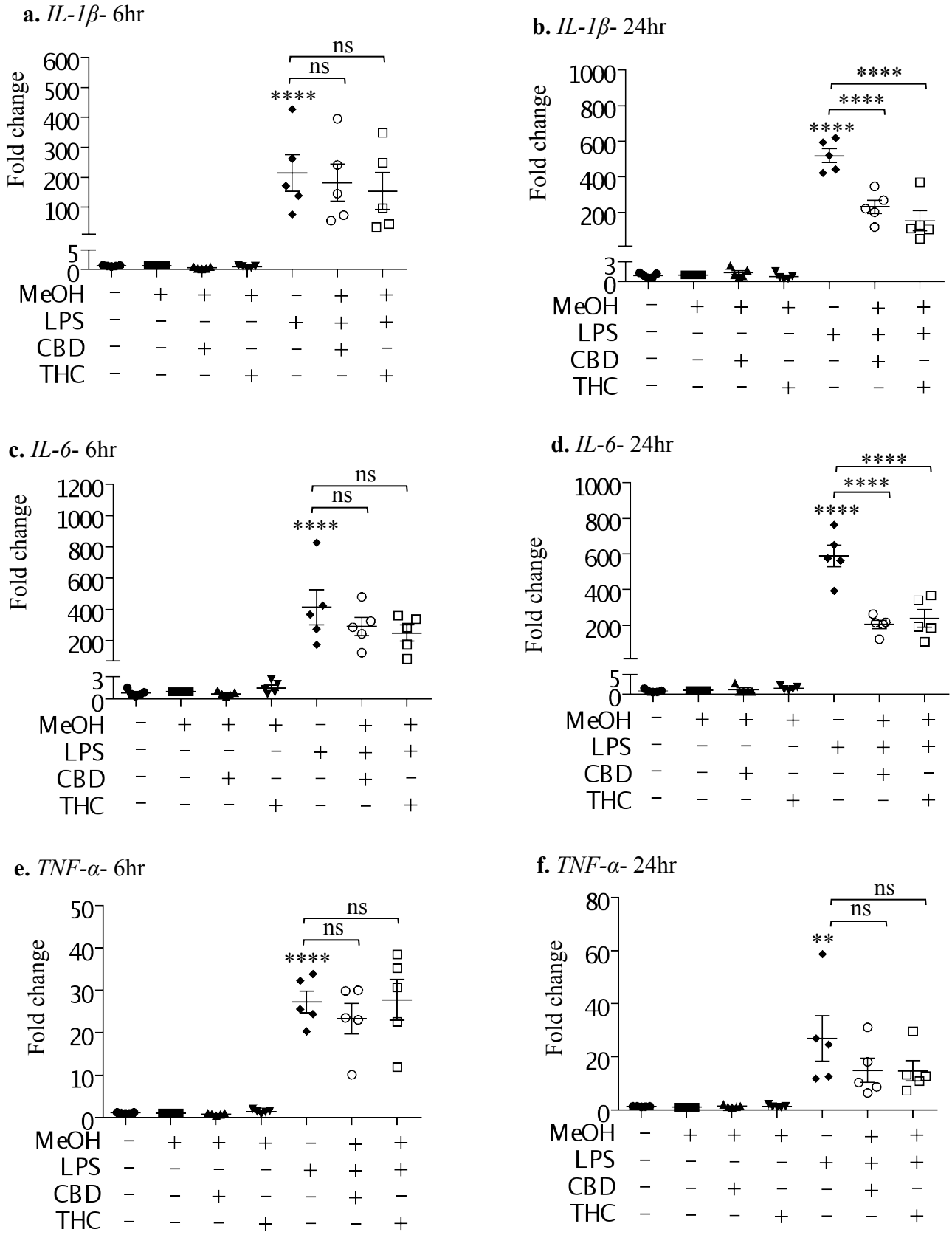

a. IL-1β- 6hr
b. IL-1β- 24hr
c. IL-6- 6hr
d. IL-6- 24hr
e. TNF-α- 6hr
f. TNF-α- 24hr
Fold change
ns
****
**
MeOH
LPS
CBD
THC

**Figure 3**

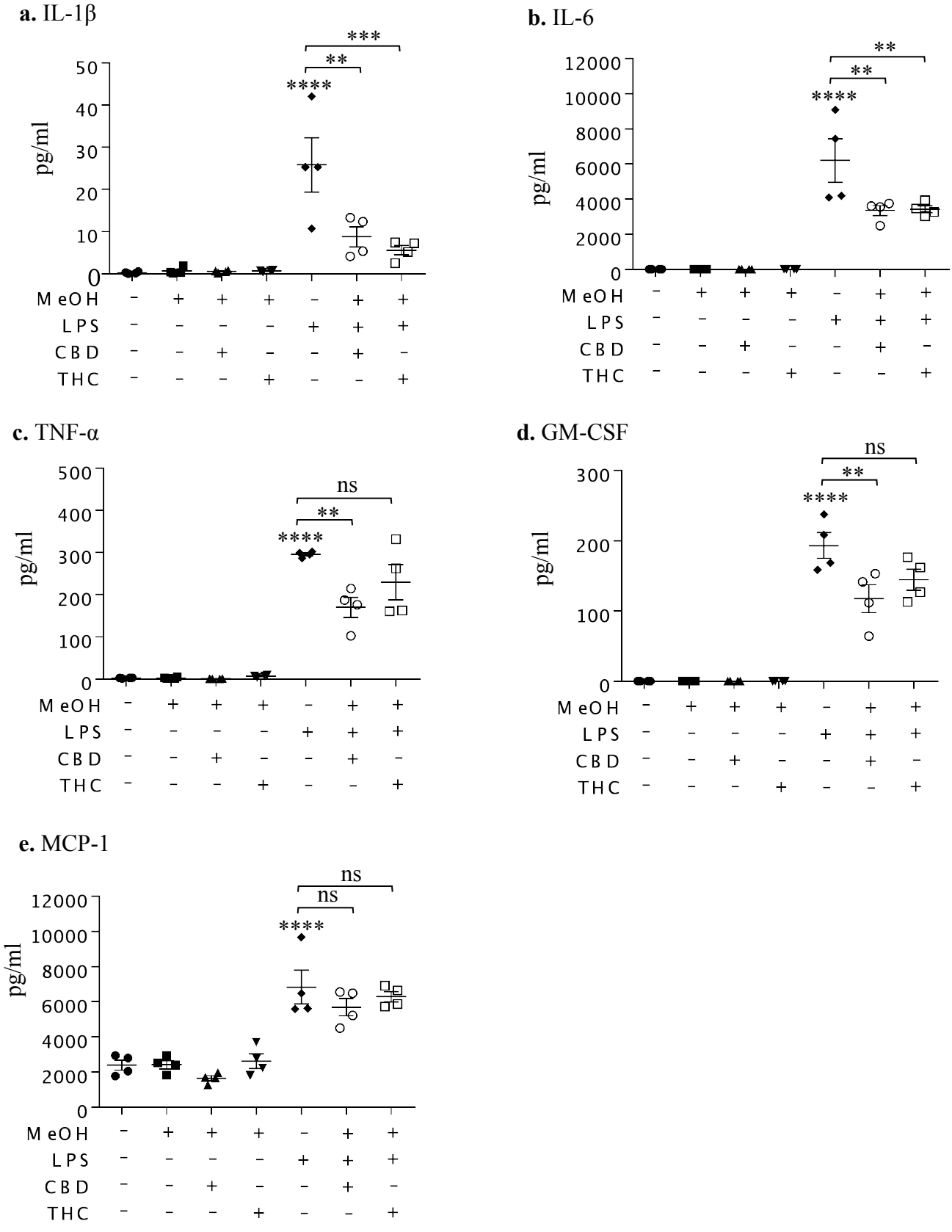

a. IL-1β
pg/ml
50
40
30
20
10
0
***
**
****
MeOH - + + + - + +
LPS - - - - + + +
CBD - - + - - + -
THC - - - + - - +
b. IL-6
pg/ml
12000
10000
8000
6000
4000
2000
0
**
**
****
MeOH - + + + - + +
LPS - - - - + + +
CBD - - + - - + -
THC - - - + - - +
c. TNF-α
pg/ml
500
400
300
200
100
0
ns
**
****
MeOH - + + + - + +
LPS - - - - + + +
CBD - - + - - + -
THC - - - + - - +
d. GM-CSF
pg/ml
300
200
100
0
ns
**
****
MeOH - + + + - + +
LPS - - - - + + +
CBD - - + - - + -
THC - - - + - - +
e. MCP-1
pg/ml
12000
10000
8000
6000
4000
2000
0
ns
ns
****
MeOH - + + + - + +
LPS - - - - + + +
CBD - - + - - + -
THC - - - + - - +

**Figure 4**

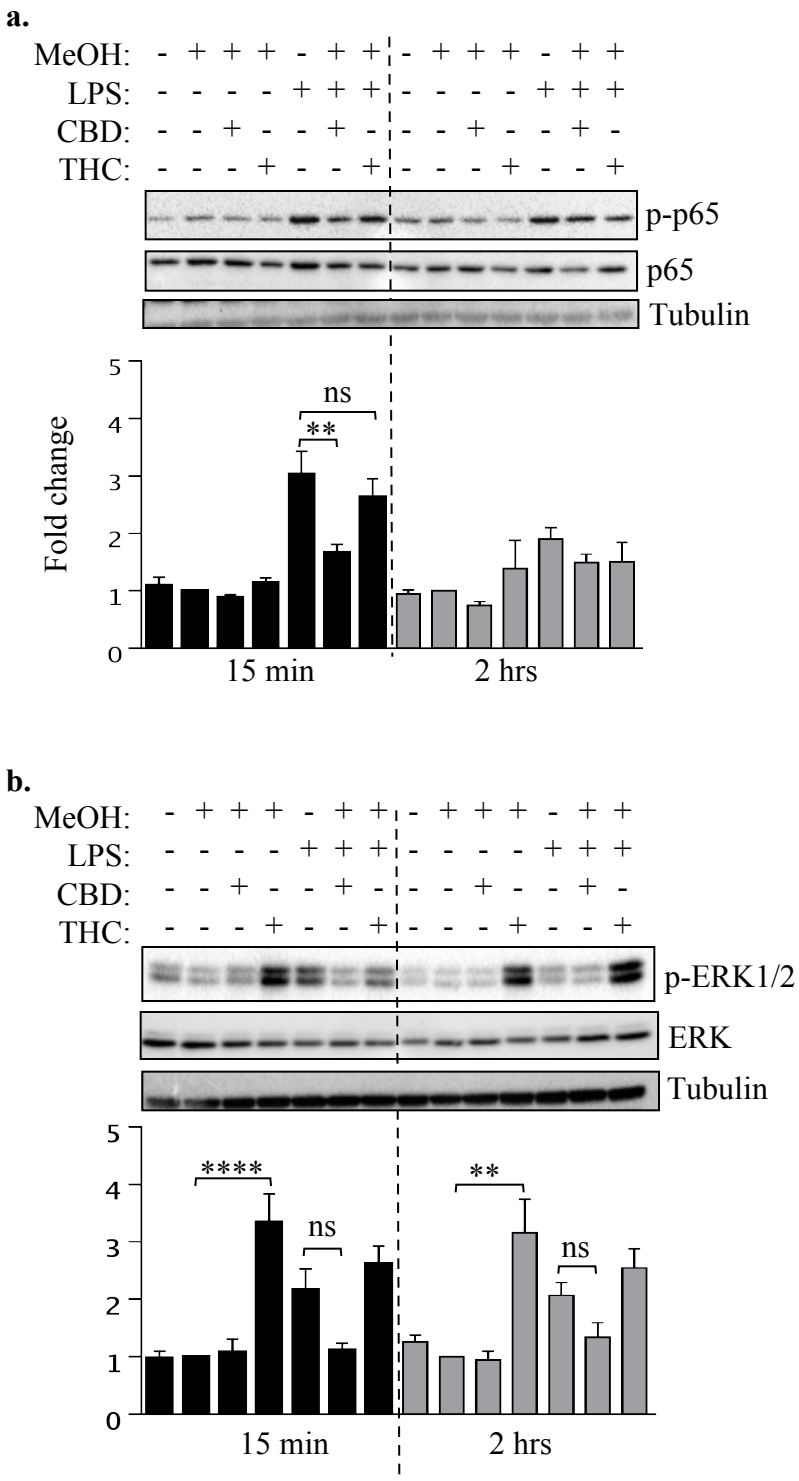
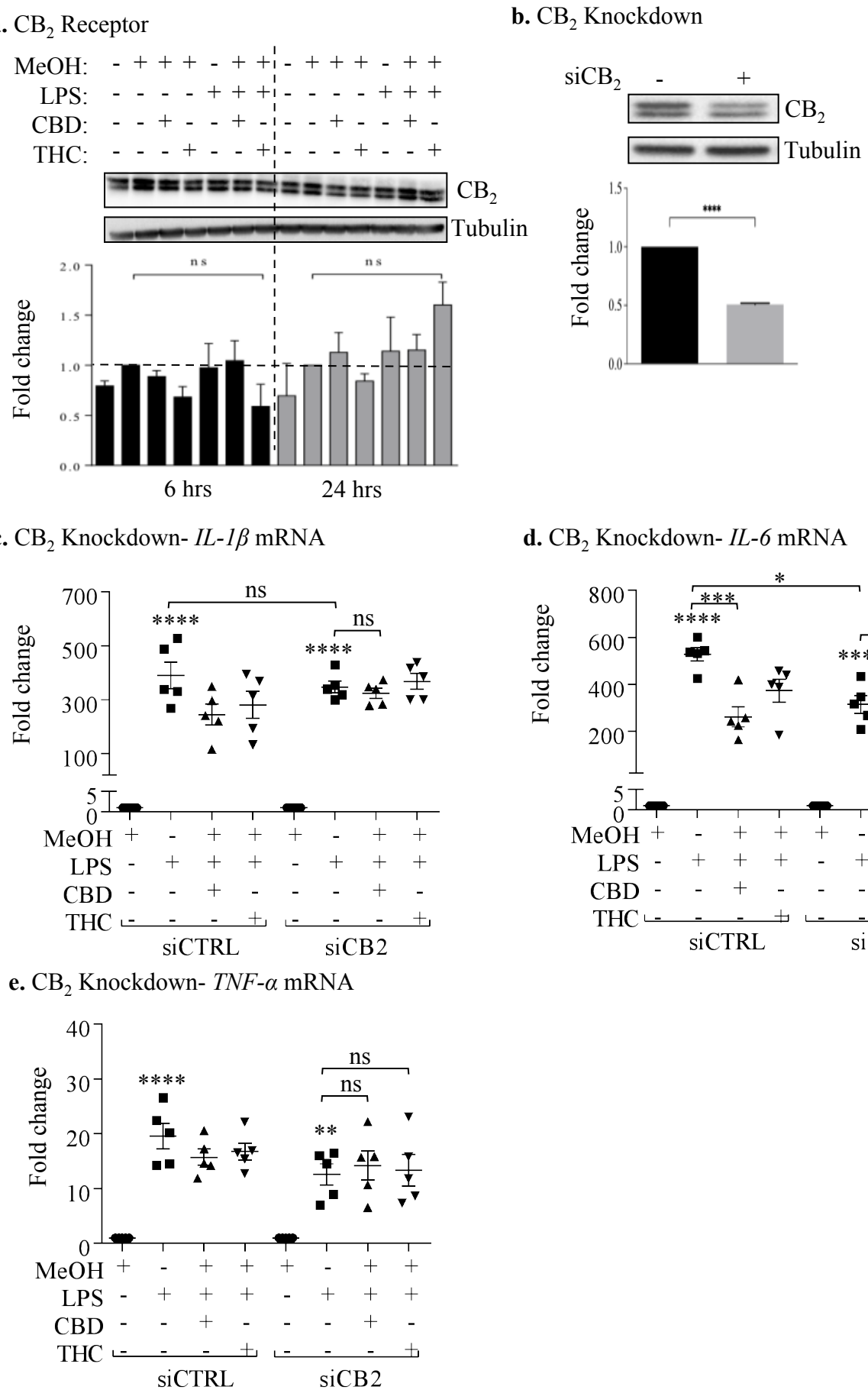

a.
MeOH:
LPS:
CBD:
THC:
p-p65
p65
Tubulin
Fold change
ns
**
15 min
2 hrs
b.
MeOH:
LPS:
CBD:
THC:
p-ERK1/2
ERK
Tubulin
****
ns
**
ns
15 min
2 hrs

**Figure 5**


a. CB2 Receptor
MeOH:
LPS:
CBD:
THC:
CB2
Tubulin
Fold change
ns
6 hrs
24 hrs
b. CB2 Knockdown
siCB2
CB2
Tubulin
Fold change
****
c. CB2 Knockdown- IL-1β mRNA
Fold change
ns
****
MeOH
LPS
CBD
THC
siCTRL
siCB2
d. CB2 Knockdown- IL-6 mRNA
Fold change
*
***
****
ns
MeOH
LPS
CBD
THC
siCTRL
siCB2
e. CB2 Knockdown- TNF-α mRNA
Fold change
****
**
ns
MeOH
LPS
CBD
THC
siCTRL
siCB2