HHS Public Access

Author manuscript

Toxicol Appl Pharmacol. Author manuscript; available in PMC 2020 November 01.

Published in final edited form as:

Toxicol Appl Pharmacol. 2019 November 01; 382: 114713. doi:10.1016/j.taap.2019.114713.

Cannabidiol differentially regulates basal and LPS-induced inflammatory responses in macrophages, lung epithelial cells, and fibroblasts

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Abstract

Introduction: Cannabidiol (CBD) containing products are available in a plethora of flavors including oral, sublingual, and inhalable forms. Immunotoxicological effects of CBD containing liquids were assessed by hypothesizing that CBD regulates oxidative stress and lipopolysaccharide (LPS) induced inflammatory responses in macrophages, epithelial cells, and fibroblasts.

Methods: Epithelial cells (BEAS-2B and NHBE), macrophages (U937), and lung fibroblast cells (HFL-1) were treated with varying CBD concentrations or exposed to CBD aerosols and reactive oxygen species (ROS), and the inflammatory mediators, were measured. Furthermore, monocytes and epithelial cells were stimulated with LPS in combination with CBD or dexamethasone to understand the anti-inflammatory effects of CBD.

Results: CBD showed differential effects on IL-8 and MCP-1, and acellular and cellular ROS levels. CBD significantly attenuated LPS-induced NF-κB activity and IL-8 and MCP-1 release from macrophages. Cytokine array data depicted a differential cytokine response due to CBD. Inflammatory mediators, IL-8, serpin E1, CXCL1, IL-6, MIF, IFN-γ, MCP-1, RANTES, and TNF-α were induced, whereas MCP-1/CCL2, CCL5, eotaxin, IL-1ra, and IL-2 were reduced. CBD and dexamethasone treatments reduced the IL-8 level induced by LPS when the cells were treated individually, but showed antagonistic effects when used in combination via MCPIP (monocytic chemotactic protein-induced protein).

Conclusion: CBD differentially regulated basal pro-inflammatory response and attenuated both LPS-induced cytokine release and NF-κB activity in monocytes, similar to dexamethasone. Thus, CBD has a differential inflammatory response and acts as an anti-inflammatory agent in pro-inflammatory conditions but acts as an antagonist with steroids, overriding the anti-inflammatory potential of steroids when used in combination.

Graphical abstract

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Authors' contributions: TM conducted most of the experiments and data analysis. TM and IR conceived/designed the experiments and prepared the manuscript.

Conflicts of interest: The authors declare no conflict of interests.

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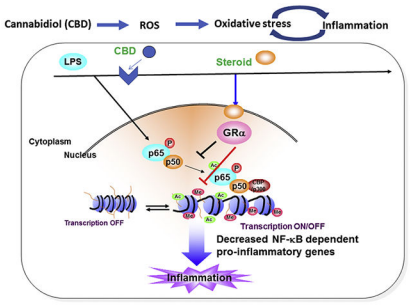

Keywords

CBD; marijuana; e-cigarette; e-liquid; vaping; lung

Introduction

Cannabidiol (CBD) is the non-psychoactive derivative of the *Cannabis sativa* (marijuana) plant. It has been widely used for medicinal benefits by all age groups, including children. CBD is self-administered for its anxiolytic, antiemetic, anti-inflammatory, and anti-cancer properties (Fraguas-Sanchez and Torres-Suarez, 2018). Numerous consumer products are available in the market containing CBD. These products include edibles, personal care products, health supplements, and e-liquids used in ENDS (electronic nicotine delivery systems). Routes of administration of CBD oil are oral, sublingual, topical, and inhalation via ENDS. The majority of CBD liquid/oil users purchase it online or at local retailers and do not require a prescription. Currently, there are no FDA regulations on these commercially available CBD containing products. In 2018, for severe forms of epilepsy, such as Dravet syndrome, the FDA approved Epidiolex, a purified form of CBD oil, as a prescribed medicine for children above two years old. It is important to assess the effects of exposure to these CBD containing products, such as vaporizable liquids used in e-cigarettes, before being available in the market.

Inhaled toxicants can generate reactive oxygen species (ROS), giving rise to an inflammatory response (Wong et al., 2016). As a result, inflammatory mediators, such as interleukin-8 (IL-8) and monocytic chemoattractant protein-1 (MCP-1), are secreted enhancing the recruitment of inflammatory cells (Turner et al., 2014). These inflammatory cells, such as alveolar macrophages and neutrophils, release more ROS, such as superoxide and hydrogen peroxides during respiratory burst (Kawabata et al., 2002; Glennon-Alty et al., 2018). These secondary reactive species act as a positive feedback loop dysregulating the oxidative homeostasis leading to inflammation as well as tissue injury. Gene expression of these inflammatory cytokines depends upon Nuclear Factor kappa beta (NF-κB) transcription factor (Ueda et al., 1994; Hildebrand et al., 2013). Therefore, we assessed the activity of NF-κB upon the treatment of CBD to understand the signaling mechanism. To further understand the mechanism of cytokine regulation, monocyte chemotactic protein-induced protein-1 (MCPIP-1) protein expression was determined in cells. MCPIP-1 has been known to dampen the inflammatory cytokine response by inhibiting NF-κB activation and toll-like receptor signaling (Li et al., 2017).

As sublingual and inhalation are the primary routes of CBD administration, in this study, we tested normal primary human bronchial cells (NHBE), bronchial epithelial cells (BEAS-2B), monocytes (U937), and human lung fibroblasts (HFL-1). It is vital to assess cellular changes and the inflammatory response upon exposure to CBD containing liquids as xenobiotics are known to cause inflammatory disorders due to chronic ROS exposure (Upham and Wagner, 2001; Pagano, 2002). We further studied the effect of CBD on LPS-induced inflammatory responses and compared the anti-inflammatory properties between dexamethasone and CBD containing liquids.

We have previously shown that e-liquids and their flavoring chemicals disrupt oxidative homeostasis in cells, thus inducing inflammation (Gerloff et al., 2017; Muthumalage et al., 2017). Many vaping products are emerging with CBD as a beneficial constituent in the e-liquid, but the validity of these claims has not been scientifically proven. Thus, we attempted to assess the CBD containing liquids and their toxicological effects on cells. We hypothesized that exposure to CBD containing e-liquids regulates inflammation and oxidative stress in monocytes/macrophages and various lung cells.

Materials and Methods

Ethics statements

We used a rigorous and unbiased approach throughout the experimental plans mentioned in this manuscript. Reproducibility has been ensured by repeating the experiments. All the key biological and chemical resources that are used in this study were validated except for the tested CBD oil/liquid containing products. Our results adhere to NIH standards of reproducibility and scientific rigor.

Procurement of CBD containing liquids

CBD oil-containing liquids, Green Roads (100 mg, 300 mg and 550 mg), and Hemplucid (1000 mg) were purchased online and from local retail stores. The milligram value following the brand name denotes the cannabidiol concentration listed on the package label. For the purpose of determining treatment doses in the in-vitro component of this study, we have assumed the CBD compound in these products to be pure. Immediately before use CBD liquids were sonicated and vortexed vigorously to attain a homogenous mixture.

Gas-chromatography and mass-spectrometry on CBD liquids

CBD containing liquids (Green Roads 100 mg and 350 mg) and the cannabidiol reference standard (CAS 13956-29-1) were prepared for analysis by diluting 100 μL of e-liquid solution into 400 μL of methylene chloride (HPLC Grade, Fisher Scientific). Samples were then vortexed at 1800 rpm for 1 minute to ensure incorporation and allowed to sit at 5 °C for 22 hours. 300 μL of supernatant was taken off of the samples and injected into the GC-MS (Hewlett Packard 5890 series [GC] equipped with a Hewlett Packard 5972 series [MS] Mass Selective Detector). Helium was used as the carrier gas at a flow of 1.0 mL min^{-1}. The initial oven temperature was set to 60 °C with an initial 4-minute hold followed by a programmed temperature ramp of 25 °C min^{-1} until a final temperature of 285 °C was reached and held for 5 minutes. The total analysis time for each sample was 18 minutes. The

instrument was equipped with a J&W Scientific Inc. column (30m x 0.250mm) with a 0.10 micron thin film of phenyl arylene polymer (Catalog # 122-5531). The samples were analyzed over the range of 50-550 m/z at 1.5 scans per second using electron-impact ionization in the positive ion mode. Using the program MS Chemstation (Hewlett Packard), data from each chromatogram obtained from the solutions were compared to known compounds using the Wiley Database incorporated with the program. GC-MS analysis and ICP-MS were used to characterize the contents of these CBD liquids.

Assessment of acellular ROS generated by CBD containing liquids

Relative levels of ROS produced by CBD containing liquids were determined using 2', 7'dichlorofluorescein diacetate (DCFH-DA) fluorescent dye (Calbiochem, CA, catalog # 287810). Hydrogen peroxide (H_2O_2) standards between 0 and 50 μM were prepared from 1 M stock. Three milliliters of the prepared DCFH solution was then added to the standards as well as samples, which were then incubated in a 37 °C water bath for 15 minutes. CBD oil-containing liquids from Green Roads (350 mg and 550 mg) were sonicated and vortexed before adding to the dye at 17.5 mg, 35.0 mg, and 52.5 mg doses (in equivalent volumes of 50 μL, 100 μL, and 150 μL) of each product respectively. Using Quantech fluorometer FM109535, the fluorescence of oxidized dichlorofluorescein (DCF) at 485 nm/535 nm was measured. Generated ROS levels at each dose were expressed as μM H_2O_2 equivalents.

Assessment of acellular ROS in aerosolized CBD containing liquids

To assess the ROS levels in aerosolized CBD containing liquids, Green Roads (100 mg CBD) was aerosolized using the Scireq in Expose (Montreal, Canada) e-cigarette system with one puff, two puffs, and three puffs per minute regimes for 10 minutes using a modified Behar II profile. Subsequently, aerosol from the CBD-liquid was bubbled through the prepared DCFH solution. The bubbled DCFH solution was then measured for ROS release using the Quantech fluorometer. Propylene glycol (PG) or vegetable glycerin (VG) was used as a control comparison group, as CBD containing liquids use PG and VG as the base humectant.

Culture and treatment of epithelial cells, monocytes, and lung fibroblasts with CBD

Human monocytic cell line (U937) from pleural tissue was procured from ATCC (Manassas, VA). Cells were cultured and grown to reach the required density in complete RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin in T75 flasks. For each experiment passages below ten with viability over 90% were seeded in 24 well plates with 500,000 cells per well with 1% FBS. After incubating the cells overnight in low serum media, the cells were then treated with CBD liquid (Green Roads, 100 mg) at varying doses, 10.6 μM, 21.2 μM, 31.8 μM, and 42.4 μM.

Normal primary human bronchial epithelial cells (NHBE) cells (CC-2540) were purchased from LONZA (Rochester, NY). Cells were cultured and grown to reach required confluency in complete BEGM media (C-3170) with suggested supplements (C-3171). In 6-well and 12-well plates, $2x10^5$ cells were grown to near confluency for CBD (Hemplucid, 1000 mg) aerosol exposure and 10.6 μM CBD (Green Roads, 100 mg) treatment respectively.

Human bronchial epithelial cells (BEAS-2B) were cultured in complete media DMEM-F12 (50:50) with 5% FBS, 1% penicillin/streptomycin, and 15 mM HEPES. Once the confluency reached approximately 90%, the cells were seeded into six-well plates with 400,000 cells per well. The complete media in these wells were replaced with 1% FBS media approximately at 80% confluency and incubated overnight prior to the CBD (Green Roads, 100 mg) liquid treatments (10.6μM, 21.2 μM, 31.8 μM, and 42.4 μM) at 90% confluency.

Human lung fibroblasts cells (HFL-1, ATCC) were cultured in Dulbecco's modified Eagle's medium and supplemented with 1% L-glutamine, 1% of penicillin/streptomycin, 1% of amino acid, and 10% fetal bovine serum (FBS) in T75 flasks. Once the confluency reached approximately 90%, the cells were seeded into six-well plates with 400,000 cells per well. The complete media in these wells were replaced with 1% FBS media at approximately 80% confluency and incubated overnight prior to the CBD liquid (Green Roads, 100 mg) treatments (10.6 μM, 21.2 μM, 31.8 μM, and 42.4 μM treatments) at 90% confluency.

Mouse RAW264.7 cells were cultured in complete media Prigrow III medium (abm, Cat. No. TM003) with 10% FBS, L-Glutamine to 2 mM, 100 μg/ml G418, and 1% Penicillin/ Streptomycin in T25 flasks incubated at 37°C and 5% carbon dioxide (CO_2). Approximately $4x10^5$ cells per well were seeded in a 24-well plate in complete medium. After serum starvation with 1% FBS, the designated wells were treated with CBD 21.2 μM (Green Roads, 100 mg) and LPS (1 μg/mL) at 90% confluency. Twenty-four hours later, the supernatants were collected for the assigned cytokine analyses. Cell pellets were washed in 1X PBS and stored in −80°C until cell lysis was performed for the described assays.

CBD and dexamethasone treatment

NHBE, BEAS-2B, and U937 at $4x10^5$ density were cultured in 12 -well, 6-well, and 24-well plates respectively. After serum starvation (except NHBE cells), designated wells were treated with dexamethasone 10 μM, 10.6 μM CBD (Green Roads 100 mg), and dexamethasone in combination with CBD. After 24 hours, the supernatants and the pellets were collected and stored at −80°C. Albeit the smaller sample size, these experiments have been repeated to increase the power and to ensure reproducibility.

Air-liquid-interface exposure to CBD

NHBE and BEAS-2B cells were seeded in six-well plates and brought to near confluency. Immediately before the exposure, culture media was removed from the plate-wells leaving only a thin layer of media. The plate was then placed in an air-tight chamber (CR1601, Enzyscreen) The chamber was then connected to Scireq inexpose system. Either propylene glycol mixed with vegetable glycerin (50:50) or CBD (Hemplucid, 1000 mg) were aerosolized using ECX Behar puffing profile (51 mL volume, 2 puffs/min, 2 L/min flow) for 30 minutes. The heating temperature of the e-liquid was 230 °C with a 0.55 Ω atomizer. After the exposure, removed media was transferred back to the respective wells and incubated for another 24-hours under optimum conditions. Conditioned media was then collected for the quantification of inflammatory mediators by ELISA or Luminex assays.

Cell ROX assay to determine cellular ROS production

In a four-chamber slide (Cell treat 229164) approximately 75,000 RAW264.7 cells were seeded in 1 mL of complete media. Chamber one was untreated and the next three chambers were treated with CBD 10.6 μM (Green Roads, 100 mg), CBD 21.2 μM (Green Roads, 100 mg), and t-butyl hydroperoxide (TBH70X) (100 μM) respectively. After 6 hours of incubation with the treatment, Cell ROX green reagent (485/520 nm) (Molecular probes, Life-technologies) was added and incubated for an additional 30 minutes. Then, the cells were rinsed with PBS three times prior to imaging with a fluorescence microscope. Each chamber image was split into three and the mean image intensity was determined using ImageJ software.

Mitochondrial ROS as MitoSOX assay

RAW264.7 macrophages were used to assess the mitochondrial superoxide production by CBD treatment. Briefly, the cells were seeded after reaching confluency in a 24-well plate ($1x10^6$) with 10% FBS. At 70% confluency, the cells were serum-starved by replacing the media with 1% FBS media overnight. Then, the designated cells were treated with LPS (1μg/mL), CBD 21.2 μM (Green Roads, 100 mg), and CBD 42.4 μM (Green Roads, 100 mg). Four hours later, the cells were prepared for flow cytometry in HSC buffer by labeling with Annexin V and MitoSOX red according to the flowCellect (Cat # FCCH100109) kit. Flow cytometry data acquisition was done on Guava millipore easycyte 8 system and the data analysis was performed using Guavasoft 3.3 software.

Cytotoxicity assessment

The cytotoxicity was determined by staining 20 μL of the respective cell sample with 20 μL of acridine orange and propidium iodide (AO/PI) dye. After gently mixing the sample, 20 μL of the total sample was pipetted to the chamber slide. Live, dead, and total cell counts and the resulted percent viability were obtained by Cellometer 2000.

Determining Interleukin-8 (IL-8) by ELISA in cell-conditioned media

Conditioned media were assayed for IL-8 using life technologies catalog #CHC1303 kit. Briefly, the supernatants and the prepared standards were added to the previously coated plate and incubated with the detection antibody for two hours. Subsequently, streptavidin-HRP was added prior to adding TMB substrate. Once the reaction was quenched with stop solution, the absorbance was measured at 450 nm with 650 nm as reference. Cytokine levels were expressed in pg/mL.

Proteome profiler cytokine arrays to screen for the inflammatory mediators

To detect an array of membrane-based cytokines and chemokines in human (BEAS-2B, HFL-1, and U937) and mouse cell (RAW264.7) supernatants, R&D systems ARY005B and ARY006 proteome profiler array kits were used respectively. Samples were pooled together to obtain 1.5 mL per treatment group or control group. Then, the prepared detection antibody cocktail was added to the samples. Subsequently, the samples were added to the membranes and incubated overnight. Membranes were then incubated with streptavidin-HRP prior to the addition of chemi reagent or detection ECL. Membranes were then exposed

using the BioRad chemi-doc. The blots were quantified using the protein array analyzer on ImageJ software.

Inflammatory mediator quantification by Luminex

To quantify inflammatory mediators released by NHBE, BEAS-2B, and U937 cells Luminex performance human XL cytokine discovery panel (FCSTM18, R&D systems) was used according to the manufacturer's instructions. Briefly, collected conditioned media subsequent to respective CBD treatments were added to the plate along with the standards. After 2-hours of incubation with antibody conjugated microparticles, biotinylated antibody cocktail was added followed by streptavidin-PE. Subsequently, data acquisition was performed by FLEXMAP3D (xPonent) instrument and the analysis by xPonent software.

NF-κB activity assessment

RAW264.7 NF-κB reporter cell line was used for the NF-κB activity assessment. Approximately $4x10^5$ cells per well were seeded on a 24-well plate. At 70% confluency, the cells were serum-deprived (1% FBS). At 90% confluency designated wells were treated with LPS (10 μg/mL), 21.2 μM of CBD (Green Roads, 100 mg), and LPS in combination with CBD. Twenty-four hours later the supernatants and the pellets were collected. According to the One Glo luciferase kit (Promega Cat#E 6110) manufacturer's guidelines, the pellets were lysed with the Glo lysis buffer (E2661) prior to the addition of the one glow reagent (E6110). Luminescence was then measured using the Cytation5 (Biotek) instrument.

Determining MCP-1 levels in cell supernatants

To determine the MCP-1 levels, Invitrogen kit 88-7399-88 was used. Manufacturer's directions were followed as described. Briefly, the conditioned media and the prepared standards were added to the previously coated plate and incubated for two hours. Then, the detection antibody was added to the well, followed by the addition of avidin-HRP. After the required incubation period, TMB substrate was added. After adding the stop solution, the absorbance was read at 450 nm and 570 nm was used as the reference. MCP-1 levels were expressed in pg/mL.

Determining changes in MCPIP-1 protein level by CBD by immunoblotting

Total protein amounts in the lysed cell samples were quantified by following the directions on the BCA assay kit (Peirce, Cat# 23225). SDS-gel-electrophoresis was conducted using 20 μg of proteins per sample along with 10 μL of kaleidoscopic protein ladder (BioRad, Cat# 161-0375). Once the am was completed the gel was transferred to a nitrocellulose membrane. Then, the membrane was blocked with BSA 1% and incubated with the primary antibody MCPIP1/ZC3H12A Rabbit Polyclonal (GeneTex, Cat# GTX110807) overnight at 4 °C. Appropriate mouse anti-rabbit secondary antibody was then used for one hour of incubation prior to imaging on the Chemdoc at the appropriate exposure. Anti-beta actin antibody, mAbcam 8226 loading control (HRP) ab20272, was used as the housekeeping protein to normalize the data. MCPIP1 levels were expressed as fold change compared to the control group.

Statistical analyses

Statistical significance between two groups was compared by Student's *t*-test and multi-group comparisons were made by one-way ANOVA or two-way ANOVA with Tukey's *post hoc* and Sidak's multiple comparison analyses. A threshold of α=0.05 was used to determine the level of significance. All values are represented as mean ± standard error of the mean (SEM) of 'n' of samples. GraphPad Prism 8 program (La Jolla, CA, USA) was used to conduct all statistical analyses.

Results

Cellular ROS generation by CBD

To assess the cellular ROS generation by CBD containing liquids RAW264.7cells were treated with 10.6 μM and 21.2 μM of the Green Roads CBD oil (100 mg) and stained with cellROX green stain. The intensity of the whole image increased with the CBD treatments compared to the untreated counterpart. CBD 21.2 μM treatment showed significantly greater image intensity (ROS intensity) compared to the 10.6 μM CBD treatment. CBD (10.6 μM) slightly increased the intensity but was not significant compared to the untreated group of cells. A positive control, TBH70X, showed the highest image intensity or ROS generation (***$p<0.001$) [Figure 1 (A–E)].

MitoROS - MitoSOX production by CBD

Mitochondrial superoxide production by CBD containing liquids was assessed by treating RAW264.7 cells with CBD, and LPS as a positive control. Representative scatter plots of the four groups, control, LPS, CBD 21.2 μM, and CBD 42.4 μM are shown in Figure 2B (I–IV). Four hours later, the CBD treatment caused a cell population to shift towards MitoSOXred+ quadrant (A- upper left quadrant) [Figure 2B (III, IV)]. This increased shift in cell population was amplified at 42.4 μM CBD showing a dose-dependently increasing trend in mitochondrial ROS (CBD 42.4 μM > 21.2 μM) [Figure 2A(I)]. There was a slight increase in mitoSOX levels in approximately 20% of cells with 21.2 μM CBD and a significant increase with 42.4 μM CBD in 30% of the cells compared to the unexposed control cells (*$p<0.05$). This increase was comparable to the superoxide levels produced by LPS treatment [Figure 2B (II, IV)]. MitoSOXred+ Annexin+ (quadrant D) and MitoSOXred− Annexin+ (quadrant C) represent very small percentages of apoptotic cell populations [Figure 2A (III and IV)]. These numbers are negligible, thus causing minimal biological relevance. The MitoSOXred-Annexin- scatter plot (quadrant B) shows the health of the cells (Figure 2A (II)). Overall, approximately 80% of the cells were viable, LPS and 42.4 μM CBD treatments slightly decreased this percentage [Figure 2A (II)]. Similar data were found in U937 monocytes (data not shown).

Acellular ROS levels in CBD

Acellular ROS generation by CBD containing solutions was determined by DCFH-DA assay. CBD liquids (350 mg and 550 mg by Green Roads) generated dose-dependently increased levels of ROS equivalent to 100 μM to 300 μM H_2O_2 compared to glycerol (Supplementary figure 1A, 1B). Glycerol showed negative values on the fuorometer, which

were assumed to be zero. Aerosolized CBD (Green Roads 100 mg) also produced significantly increased ROS levels (***p<0.01) in a dose-dependent manner proportional to the number of puffs compared to propylene glycol (Supplementary figure 1C).

Cytotoxicity caused by CBD oil containing liquids

Cytotoxicity induced by CBD containing liquids was determined by assessing the cell viability with AO/PI staining. CBD treatment did not cause significant cytotoxicity to BEAS-2B cells. U937 cell viability was significantly affected by CBD treatment (***p<0.001) at low and high concentrations. The viability of HFL-1 cells was unaffected by the CBD treatment, except for at highest concentration of 42.4 μM of CBD (*p<0.01) (Supplementary figure 2).

The release of inflammatory mediators by epithelial cells, fibroblasts, and monocytes

BEAS-2B cells: To assess the inflammatory mediators secreted by BEAS-2B cells as a result of the treatment with CBD containing liquid, proteome profiler cytokine array was performed. CBD oil significantly increased IL-8 and serpin E1 cytokines (*p<0.01) (Figure 3A).

HFL-1 cells: Treatment with CBD oil significantly elevated CXCL1, IL6, and IL8 levels compared to the untreated group (***p<0.001). Conversely, MCP-1/CCL2 levels were significantly lowered with the CBD treatment compared to the untreated group (Figure 3B).

U937 cells: With the CBD treatment IL-1ra, IL-8, IL-16, IL-32, and MIF were significantly increased (p<0.05, p**<0.01 and ***p<0.001). CCL2 and CCL5 were significantly decreased with the CBD treatment compared to the untreated counterparts (*p<0.001) (Figure 3C).

RAW264.7 cells: CBD treatment significantly elevated many cytokines, including G-CSF, GM-CSF, IFN-γ, IL-1a, IL-6, IL-27, I-TAC, M-CSF, MCP-1, RANTES, and TNF-α, compared to the untreated control group (***p<0.001). Some cytokines, including eotaxin, IL-1ra, and IL-2, were significantly decreased in the CBD treated group compared to the untreated control group (***p<0.001) (Figure 3D and Supplementary figures 3,4).

IL-8 pro-inflammatory cytokine induction by CBD in epithelial cells, monocytes, and fibroblasts.

To quantify and validate the IL-8 levels secreted by CBD treatment, an ELISA assay was performed. A dose-dependent increase in the IL-8 production in BEAS-2B cell supernatants was seen compared to the untreated control cells. A significant IL-8 response was observed with the 21.2 μM and 42.4 μM of CBD treatment (*p<0.05 and **p<0.01) (Figure 4A).

U937 monocytes produced significantly elevated levels of IL-8 as a result of CBD treatment compared to the untreated cells (**p<0.01 and ***p<0.001) (Figure 4B). Similarly, HFL-1 cells also secreted significantly high levels of IL-8 with the CBD treatment compared to the untreated control cells (*p<0.05 and **p<0.01) (Figure 4C).

Dexamethasone reduced CBD-mediated inflammation in BEAS-2B epithelial cells

CBD (10.6 μM) treated BEAS-2B cells significantly elevated IL-8 levels compared to untreated cells (**p<0.001). Dexamethasone (10 μM) alone did not induce IL-8. CBD and dexamethasone together did not induce any IL-8, suggesting that dexamethasone treatment was able to suppress the IL-8 production significantly (***p<0.001) (Figure 5A).

CBD produced anti-inflammatory effects similar to dexamethasone in U937 monocytes.

CBD-containing liquid treatment was compared to dexamethasone for its anti-inflammatory properties. As expected, LPS (1 μg/mL) stimulated U937 macrophages significantly elevated the IL-8 levels. This response was significantly decreased with the CBD (10.6 μM) treatment (*p<0.05). Dexamethasone treatment (10 μM) further reduced the IL-8 response (**p<0.01) comparatively. CBD with DEX treatment did not further cause any changes in IL-8 response (Figures 5B and 8). Other inflammatory mediators, such as MCP-1, CCL3, CCL4, CXCL2, and IL-6, also showed synergistic inhibition in inflammatory mediators when CBD used in combination with DEX in U937 cells (Figure 8).

CBD differentially regulated the inflammatory mediators induced by TNF-α in primary NHBE cells.

Primary normal bronchial cells treated with 10.6 μM CBD alone did not induce secretion of inflammatory mediators. However, CBD significantly attenuated CXCL1, G-CSF, and IL-6 levels in TNF-α stimulated cells (**p<0.01). On the other hand, CXCL2, IL-8, GM-CSF levels were significantly augmented when CBD and TNF-α were used in combination (***p<0.001) (Figure 6 A–F).

CBD and dexamethasone differentially regulated the inflammatory mediators in BEAS-2B cells.

CBD treatment significantly attenuated MCP-1, CXCL-1, IL-6, G-CSF, and GM-CSF levels induced as a response to stimulation with TNF-α. Dexamethasone significantly attenuated CXCL2, IL-8, and GM-CSF. CBD+DEX combination was able to significantly reduce almost all cytokines except IL-8 (Figure 7A–G).

CBD and dexamethasone differentially regulated the inflammatory mediators in U937 cells.

LPS-induced release of inflammatory mediators, IL-8, MCP-1, CCL3, CCL4, TNF-α, CXCL2, IL-33, IL-6, and RANTES, were significantly attenuated by CBD treatment in U937 cells. Similarly, dexamethasone attenuated the LPS-induced inflammation. However, the combined treatment of CBD with dexamethasone resulted in a similar but differential response (Figure 8 A–I).

CBD aerosols induced inflammatory cytokines in BEAS-2B epithelial cells

Inflammatory mediators, Eotaxin, MCP-1, CXCL1, CXCL2, IL-8, and IL-6 levels were significantly elevated by CBD vapor exposure compared to its vehicle PG/VG (Figure 9 A–F).

CBD attenuated MCP-1/CCL2 cytokine release in human epithelial cells, monocytes, and fibroblasts.

To confirm the MCP-1/CCL2 decrease observed in proteome profiler array, ELISA assays were performed on cell supernatants. CBD treatment significantly and dose-dependency reduced MCP-1 secretion in BEAS-2B, U937, and HFL-1 cells compared to their untreated counterparts (*p<0.05, **p<0.01, ***p<0.001) (Figure 10).

Change in NF-κB activity with CBD treatment

To understand the mechanism of MCP-1 decrease with CBD treatment, NF-κB activity was determined in LPS stimulated RAW264.7. As expected, LPS stimulation cells showed significantly increased luminescence compared to unstimulated control cells (***p<0.001). CBD alone did not change luminescence compared to the control. Cells stimulated with LPS (1 μg/mL) and treated with CBD 21.2 μM significantly decreased the relative luminescence. This suggests that CBD treatment inhibited NF-κB, which was previously activated by LPS. CBD treatment by itself did not activate NF-κB. When the LPS and CBD were combined, NF-κB activity was significantly inhibited (***p<0.001) (Figure 11).

MCP-1/CCL2 cytokine measurement by ELISA in RAW264.7 cells

In RAW264.7 cells, LPS stimulation significantly elevated the MCP-1 secretion (*p<0.05). However, this increase was significantly attenuated when these cells were also treated with CBD (*p<0.05) (Figure 12).

MCPIP1 protein level increased by CBD treatment

To determine if the MCPIP-1 protein levels had changed in the CBD treated groups, immunoblot analysis was performed. Compared to the untreated control and CBD treated groups, LPS (1 μg/mL) treated cells showed significantly downregulated MCPIP1 protein levels (*p<0.05). CBD and LPS combined treatment resulted in intermediate MCPIP1 protein expression (Figure 13 A–C).

Constituents of CBD containing liquids by GC-MS analysis

GC-MS spectra show that CBD oil (Green Roads, 100 mg) contains pure cannabidiol similar to the reference compound. However, CBD 350 mg showed other peaks exhibiting the presence of cannabidiol related compounds (Supplementary Figure 4).

Discussion

Pharmacological benefits of pure CBD have been shown in ameliorating ailments (Nagarkatti et al., 2009). We tested commonly available CBD liquids which are primarily administered sublingually and orally. Composition analysis of these liquids showed that most of these e-liquids also contain other ingredients, such as metals, trace amounts of THC, and flavoring chemicals. Elements that were present in these liquids included chromium, copper, and lead, all of which cause detrimental health effects. Peace *et al.*, have shown e-liquids to contain underestimated levels of CBD in commercially available e-liquids (Peace et al., 2016). Presence of these constituents may be a confounding factor in understanding

CBD related pharmacological and toxicological effects. Vaping CBD/THC containing liquids has recently have shown to be more harmful than smoking marijuana, with higher THC levels in the blood and long-lasting cognitive impairments (Spindle et al., 2018).

We observed that CBD containing liquids as well as aerosols generated significant amounts of acellular ROS and cellular ROS. The observed dose-dependent ROS generated by CBD-demonstrated the potential to cause oxidative stress. The continuous oxidative burden can lead to tissue injury and inflammatory disorders (Mittal et al., 2014). Exposure to CBD showed an increase in mitochondrial ROS, indicating the potential hazard of mitochondrial dysfunction. Corroborating our findings, Schultze *et al.*, showed significant alterations in mitochondrial bioenergetics in THP-1 cells at CBD IC_{50}, including maximal respiration and respiratory spare capacity by 60% (Schultze et al., 2017).

In-vitro exposure of epithelial cells, monocytes, and fibroblasts to CBD showed varying degree of toxicity depending on the cell type. Monocytes and fibroblasts were particularly prone to cell death and damage by the exposure to CBD. Several other studies have shown similar pro-apoptotic effects in cells, including human monocytes and murine microglial cells (Wu et al., 2010; Wu et al., 2012). In this study, even though CBD showed mild cytotoxicity to monocytes, however, the increased release of inflammatory mediators suggests that the upregulation of inflammatory genes had already occurred before ensuing toxicity. CBD has shown to have a potential anti-tumorigenicity. Ramer *et al.* demonstrated decreased cell viability elicited by CBD treated lung cancer cell lines, A549 and H460, as well as in primary cells from lung cancer patients (Ramer et al., 2013). Massi *et al.* described CBD as an anticancer drug for lung, glioma, leukemia, breast, thyroid as well as colon cancers by reducing cell viability, migration, invasiveness and proliferation (Massi et al., 2013).

Cells treated with CBD showed increased IL-8 response, suggesting that CBD exposure can result in a pro-inflammatory response. Elevation of IL-8 levels is a hallmark of acute inflammation, recruitment, and activation of naïve inflammatory cells. However, the cytokine array for human epithelial cells, monocytes, and fibroblasts exhibited attenuated levels of MCP-1 cytokine levels, exhibiting anti-inflammatory characteristics in response to CBD treatment. Generally, chemokines, such as MCP-1 are secreted in response to other pro-inflammatory cytokines and inflammatory disorders, such as arthritis, asthma, and inflammatory bowel disease, (Deshmane et al., 2009; Yadav et al., 2010). One of the primary mechanisms of MCP-1 expression is dependent on the NF-κB pathway (Donadelli et al., 2000; Hayden and Ghosh, 2012). In order to understand the mechanism of MCP-1 down-regulation, we used NF-κB reporter cell line RAW264.7. Interestingly, while CBD alone did not cause alterations in NF-κB activity, endotoxin (LPS) induced NF-κB activity was significantly inhibited by the CBD treatment. This is consistent with the observed attenuation of LPS stimulated MCP-1 levels by CBD treatment. Similarly, in primary normal bronchial epithelial cells treated with CBD and TNF-α, we observed significantly elevated levels of pro-inflammatory mediators, such as IL-8 and chemoattractants (CXCL1 and CXCL2), and a highly significant suppression in IL-6 suggesting concomitant regulation in pro- and anti-inflammatory processes during acute inflammation. Wang *et al.*, demonstrated reduced hepatic inflammation due to alcohol by CBD treatment (Wang et al., 2017). In a

multiple sclerosis mouse model, Mecha *et al.*, showed the potential of CBD to reduce transmigration of leukocytes and reduction of CCL2 (MCP-1) and CCL5 (Mecha et al., 2013). In contrast, Karmaus *et al.*, found significantly increased LPS-induced inflammation in C57BL6 mice (Karmaus et al., 2013). These studies bolster our observations, wherein CBD reduced existing/ongoing inflammation and its immunomodulatory effects. Our data imply that exposure to CBD containing liquids elicits a pro-inflammatory response. However, in the presence of ongoing inflammation, CBD may be able to suppress that inflammation by inhibiting NF-κB activity. This was further evidenced in U937 cells stimulated with LPS where CBD attenuated the IL-8 response, exhibiting its anti-inflammatory characteristics in contrast to the induced IL-8 response with CBD liquid treatment without LPS stimulation. Our data has shown that CBD may have similar anti-inflammatory properties to other steroids, such as dexamethasone, to alleviate an existing inflammation.

In U937 cells, CBD attenuated IL-8 response elicited by LPS, but when used in combination with another compound, i.e., dexamethasone, there was a reduced or antagonistic effect. This phenomenon was observed in a broader-scale in BEAS-2B cells. When separately treated with CBD or dexamethasone, MCP-1, CXCL1, CXCL2, IL-6, IL-8, and G-CSF, significantly reduced compared to the LPS stimulated group. However, when used together, this effect was lessened. This suggests that there may be a competitive antagonistic interaction between the two compounds as both interact with their receptors. As glucocorticoids can transrepress NF-κB and AP-1 transcription factors, there may be a complex pharmacological interaction between CBD and dexamethasone anti-inflammatory pathway reducing or overriding the steroid effects (Adcock and Caramori, 2001; Mogensen et al., 2008). Conversely, another possible inference may be the dual receptor signal transduction mechanism, i.e., CBD via CB2 receptor and dexamethasone via glucocorticoid receptors. In our future work, understanding the CB2 and glucocorticoid receptor signaling pathways, molecules, and their interactions, such as protein kinase A(PKA), extracellular signal-regulated kinases (ERK1/2), histone deacetylase 2 (HDAC2), CREB-binding protein (CBP), and P300/CBP activating factor (PCAF), are of interest.

It has been shown that MCP-1 inducing protein (MCPIP-1) can regulate inflammation by inhibiting NF-κB activity via its deubiquitinase activity (Kolattukudy and Niu, 2012). We found increased MCPIP protein levels in the samples stimulated with LPS treated in conjunction with CBD compared to the LPS only group. This trend was observed repeatedly in our treatments. Consistent with our observation, Garg *et al.*, observed negative regulation of IL-17 mediated pulmonary inflammation by MCPIP in mice (Garg et al., 2015). This suggests that the immunosuppression observed may be due to NF-κB inhibition by MCPIP expression.

Our proteome profiler array for RAW264.7 showed differential cytokine levels. Treatment with CBD liquid alone showed there was an overall pro-inflammatory cytokine repertoire with increased levels of TNF-α, IL-6, MCP-1, and GM-CSF. However, in the LPS stimulated and CBD treated group, cytokine response was multifaceted. For example, we observed that in the LPS stimulated CBD treated cells, there was a significantly decreased IFN-γ response but an increased complement activating component C5/C5a secretion.

Marijuana smokers have exhibited symptoms, including shortness of breath, pharyngitis, asthma, and decreased lung function (Owen et al., 2014). Rotolo et al., have recently demonstrated the correlation between cannabis smoking and the incidence of lung diseases and the presence of cannabinoids in bronchoalveolar lavage (Rotolo et al., 2018). Abdallah et al., have demonstrated that acute exposure to marijuana vaping does not provide beneficial effects in airway function (Abdallah et al., 2018). Such recent findings have highlighted the respiratory effects of vaping and smoking marijuana (CBD/THC), albeit, the pro- and anti-inflammatory effects are not known (Tashkin, 2015; Ribeiro and Ind, 2018; Tashkin, 2018).

As discussed previously, the potential anti-inflammatory effects of CBD may be beneficial to alleviate an existing inflammatory condition. Conversely, this may also be harmful if the immune system is fighting an evading pathogen, such as bacterial and viral infections. Thus, it may be inferred that CBD has both pro- and anti-inflammatory mechanisms of action.

To add to the complexity, the route of administration of CBD containing liquid plays an important role. Oral administration of CBD oil may be less harmful as CBD is mainly metabolized by CYP3A4 and CYP2C19 enzymes, which are expressed more in the liver (Jiang et al., 2011; Ujvary and Hanus, 2016). Administering CBD containing e-liquids may cause harm due to substantial differences in pulmonary expression and intestinal-hepato expression of these enzymes. Lungs, in general, have lower expression of these enzymes, thus the inability to metabolize and eliminate the constituents in CBD containing e-liquids. The accuracy of the dose of the actual exposure of the target organ is a challenge when considering aerosolization as a route of administration for therapeutic purposes (Lefever et al., 2017). The increase in cytokine levels in BEAS-2B cells exposed to CBD aerosols is likely due to thermodynamically unstable free radical formation. These data suggest that administration of CBD via inhalation can induce inflammation-mediated by oxidative stress.

Our elemental analysis has shown that CBD containing liquids contain other contaminants, such as metals, including small amounts of lead (Pb) and chromium (Cr), similar to heavy metals that have been found in other e-cigarette aerosols (Olmedo et al., 2018). Bioaccumulation of these heavy metals can pose severe neural and cardiovascular toxicity. Other ingredients in CBD containing liquids, such as propylene glycol, vegetable glycerin, and flavoring chemicals, can cause respiratory irritation, mucociliary damage, and lung tissue fibrosis (Kaur et al., 2018).

One of the challenges conducting this study was the batch to batch variation in the formulation of CBD containing liquids by the manufacturers. Our data showed that CBD differentially regulates LPS-induced inflammatory responses in macrophages, lung epithelial cells, and fibroblasts. This was due to the ability of CBD to induce inflammatory responses *per se*, whereas LPS-induced inflammatory responses were attenuated by CBD in various cellular types.

In conclusion, CBD differentially regulated the inflammatory response and attenuated both LPS-induced cytokines (IL-8 and MCP-1) in lung cells and NF-κB activity in monocytes, similar to dexamethasone. Thus, CBD may induce a pro-inflammatory response but also may act as an anti-inflammatory agent under existing inflammatory conditions, acting as an

antagonist with steroids. As a pharmacological benefit, liquids containing CBD may have some therapeutic benefits in reducing an existing inflammation. However, exposure to CBD containing liquids results in a multifaceted toxicological response. This study provides insights into regulating CBD containing products and their constituents for consumption, in particular, the usage in e-cig devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the toxicology training program grant T32-ES007026, and National Institute of Health NIH 2R01HL085613, HL137738, HL135613, and the WNY Center for Research on Flavored Tobacco Products (CRoFT) under cooperative agreement U54CA228110. Melanie Prinz and Thomas Lamb helped with the acellular ROS assays. Alan Friedman, Ph.D. at the University at Buffalo, performed GC-MS analysis of the CBD containing liquids. Thanks to Tom Scrimale and Matthew Rand of the Element Analysis Facility of the Environmental Health Science Center (NIEHS P30 ES001247). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Food and Drug Administration (FDA).

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Research Highlights

- Cannabidiol (CBD) containing products are available in a plethora of flavors and forms.
- CBD showed differential pro- and anti-inflammatory effects by ROS levels.
- CBD significantly attenuated LPS-induced NF-κB activity and IL-8 and MCP-1.
- CBD and dexamethasone reduced the IL-8 level induced by LPS via MCPIP.
- CBD has a differential inflammatory response and acts as an antagonist with steroids.

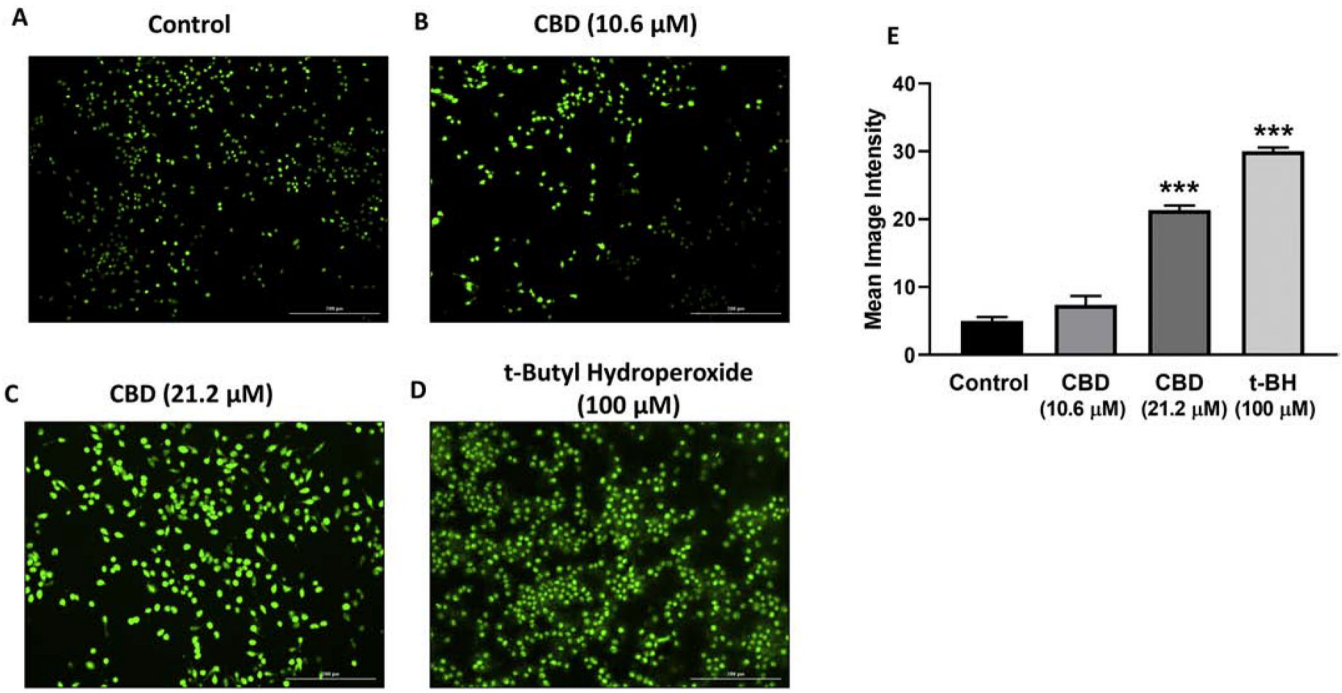

Figure 1: Cell ROX generated by CBD in murine macrophages using RAW264.7.

RAW 264.7 cells treated with 10.6 μM and 21.2 μM CBD (Green Roads, 100 mg) and a positive control tert-butyl peroxidase (TBH70X) (100 μM). Six hours post-treatment, the cells were stained with Cell ROX green. Representative images of (A) untreated control, (B) CBD 10.6 μM, (C) CBD 21.2 μM, (D) TBH70X (100 μM), and (E) quantified mean image intensity plot. ***$p<0.001$ vs. control or denoted group by one-way ANOVA, n=3 per treatment.

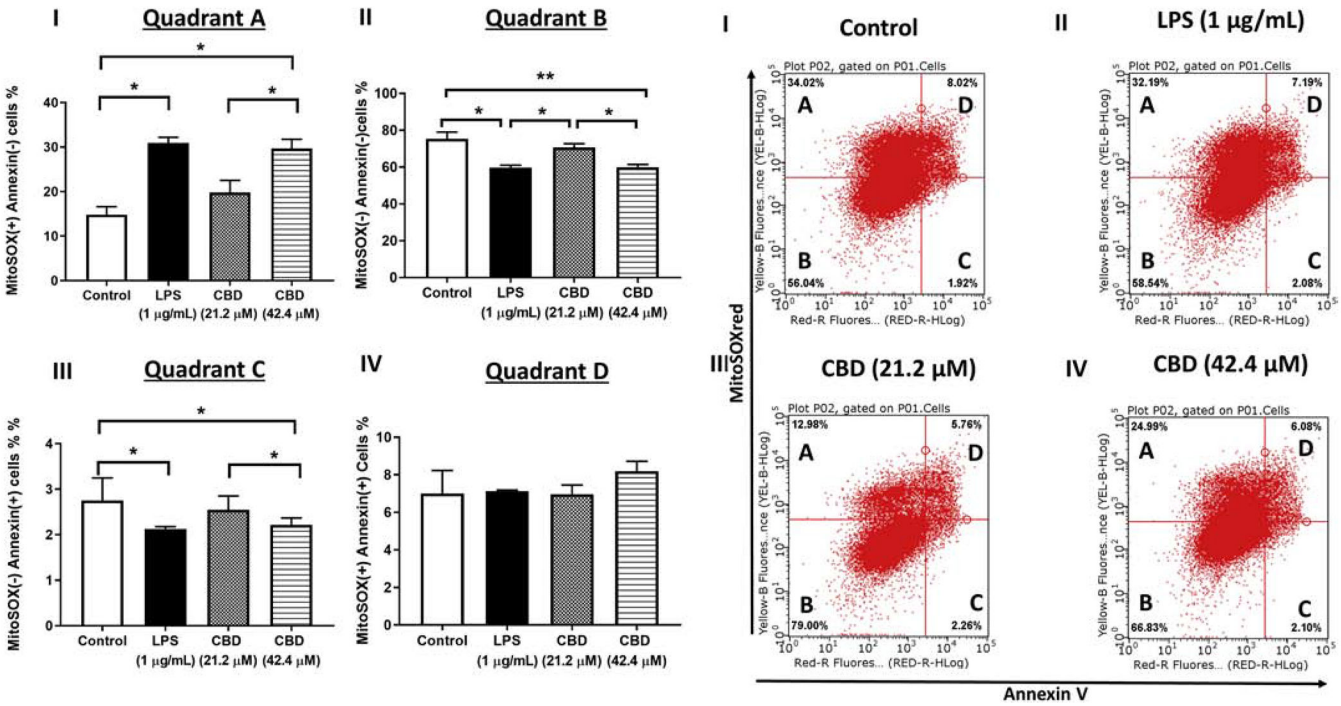

Figure 2.

A. Mitochondrial ROS generation by CBD after 4 hours in RAW264.7 murine macrophages. RAW264.7 cells ($1x10^6$) cultured in a 24 well plate with 10% FBS. After serum deprivation, the designated wells were treated with 42.4 µM CBD (Green Roads, 100 mg). Cells were collected after 4 hours. Subsequently, they were stained and assayed for ROS (MitoSOX) by Guava FlowCellect kit. (I) quadrant A- Percent MitoSOX high and annexin low populations. (II) Quadrant B- Percent MitoSOX low and Annexin low populations. (III) Quadrant C- Percent MitoSOX low and Annexin high populations. (IV) Quadrant D- Percent MitoSOX high and Annexin high populations. *p<0.05, p**<0.01 vs. respective group by one-way ANOVA, n=2-4/treatment by one-way ANOVA.

B. Mitochondrial ROS generation by CBD after 4 hours in RAW264.7 murine macrophages, representative scatter plots.

RAW264.7 cells ($1x10^6$) cultured in a 24 well plate with 10% FBS. After serum deprivation, the designated wells were treated with LPS (1 µg/mL), 21.2 µM CBD (Green Roads, 100 mg), and 42.4 µM CBD (Green Roads, 100 mg). Cells were collected after 4 hours. Subsequently, they were stained and assayed for MitoSOX by Guava FlowCellect. Guavasoft 3.3 software quadrant method was used for the scatter plot data analysis. Representative scatter plots with quadrant analysis for (I) control group, (II) LPS treated group, (III) CBD (21.2 µM) treated group, and (IV) CBD (42.4 µM) treated group.

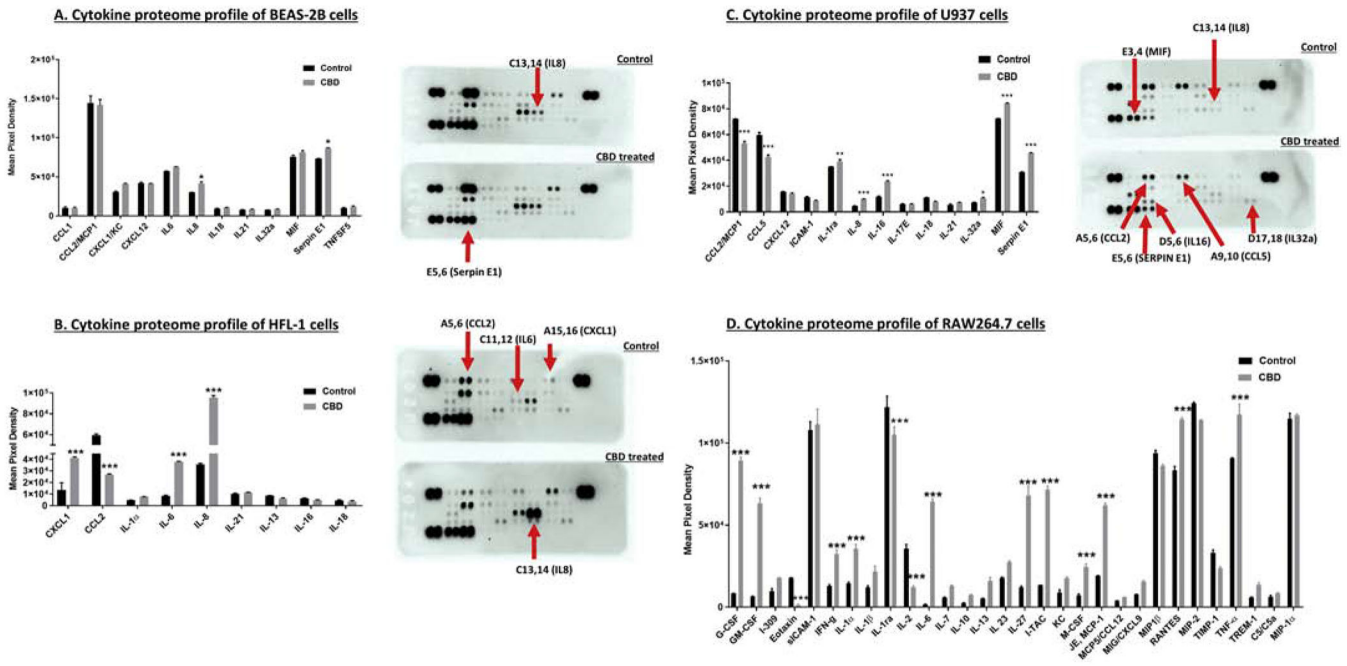

Figure 3.

A. CBD oil treatment induces a pro-inflammatory response in human bronchial epithelial, BEAS-2B cells. BEAS-2B cells were treated with 10.6 μM CBD Green Roads, 100 mg), and the conditioned media from three exposed samples were pooled and assayed for inflammatory mediators by proteome profiler. Mean Pixel Densities were determined by ImageJ software. Two-way ANOVA with Sidak's multiple comparison, *p<.05 vs. control, **p<0.01 vs. control, and ***p<0.001 vs. control, n=3 per group.

B. CBD treatment-induced pro-inflammatory response in human lung fibroblasts, HFL-1 cells. HFL-1 cells were treated with 10.6 μM CBD (Green Roads 100 mg) and the conditioned media from three exposed samples were pooled and assayed for inflammatory mediators by proteome profiler. Mean Pixel Densities were determined by ImageJ software. Two-way ANOVA with Sidak's multiple comparison, *p<.05 vs. control, **p<0.01 vs. control, and ***p<0.001 vs. control, n=3 per group.

C. CBD treatment-induced pro-inflammatory response in human monocytes, U937 cells. Monocytes were treated with 10.6 μM CBD (Green Roads 100 mg), and the conditioned media from four exposed samples were pooled and assayed for inflammatory mediators by proteome profiler. Mean Pixel Densities were determined by ImageJ software. Two-way ANOVA with Sidak's multiple comparison, *p<.05 vs. control, **p<0.01 vs. control, and ***p<0.001 vs. control, n=4 per group.

D. CBD treatment-induced pro-inflammatory response in RAW264.7 murine macrophages. Mouse monocytes were treated with 21.2 μM CBD (Green Roads, 100 mg), and the conditioned media from six exposed samples were pooled and assayed for inflammatory mediators by proteome profiler. Mean Pixel Densities were determined by ImageJ software. Two-way ANOVA with Sidak's multiple comparison, *p<.05 vs. control, **p<0.01 vs. control, and ***p<0.001 vs. control, n=6 per group.

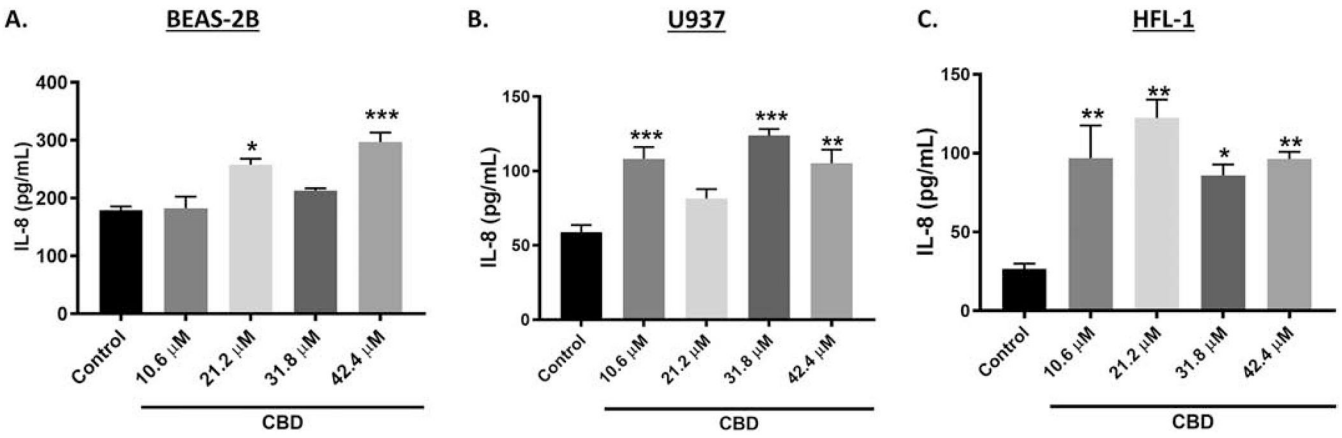

Figure 4. CBD induced dose-dependent IL-8 response in various lung cell types.

Three cell types, (A) BEAS-2B, (B) U937, and (C) HFL-1, were treated with Green Roads 100 mg CBD at 10.6 μM, 21.2 μM, 31.8 μM, and 42.4 μM respectively. TNF-α (10 ng/mL), LPS (1 μg/mL), and TGF-β (5 ng/mL) were used as positive control treatments along with an untreated control group. IL-8 was measured twenty-four hours post-treatment in conditioned media by ELISA. One-way ANOVA *p<.05 vs. control, **p<0.01 vs. control, and ***p<0.001 vs. control, n=3-4 per group.

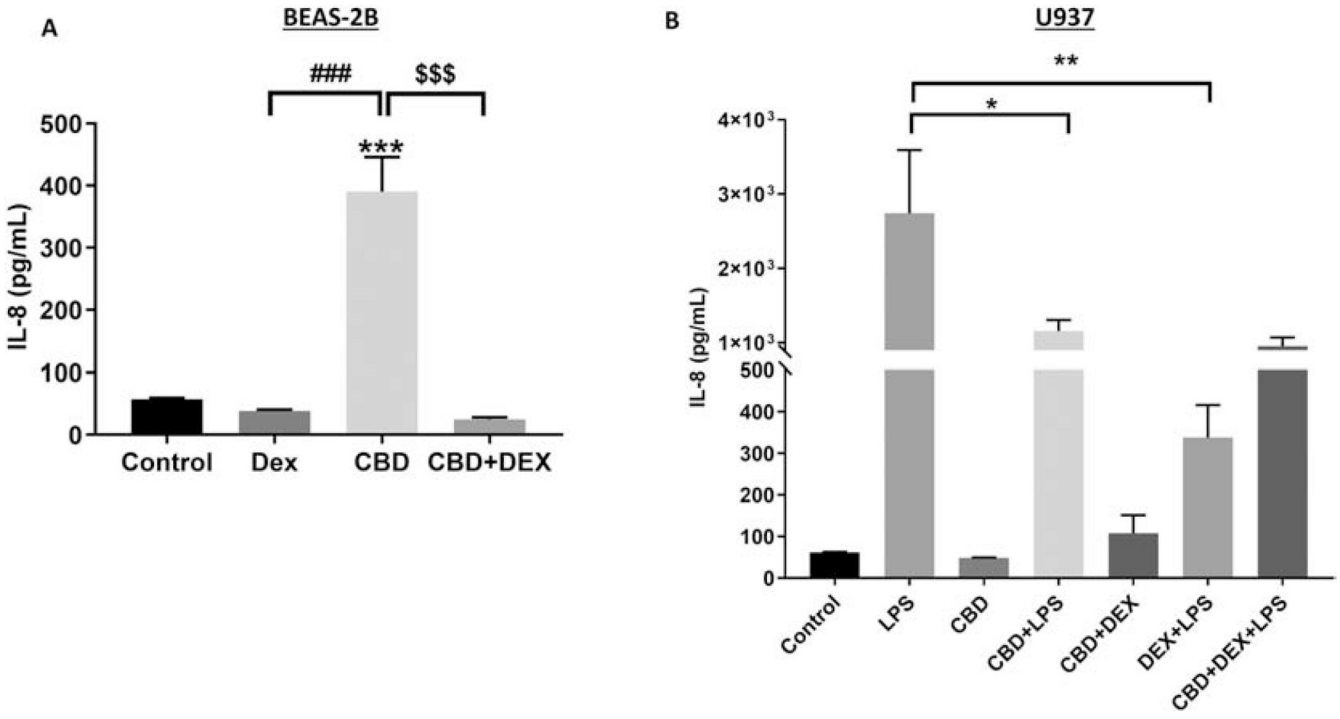

Figure 5.

A. CBD induced IL-8 cytokine response attenuated by dexamethasone in human bronchial epithelial cells (BEAS-2B). BEAS-2B cells ($4x10^5$ cells per well in six-well plates) at 1% FBS were pretreated with 10 μM dexamethasone for two hours. Subsequently, they were treated with 10.6 μM CBD (Green Roads, 100 mg). Approximately 24 hours later conditioned media was collected, and the IL-8 levels were measured in conditioned media. ***p<0.001 Vs. Control one-way ANOVA, n=3-4 per group.

B. CBD attenuated inflammatory response induced by LPS in human monocytic cells (U937). U937 cells were treated with 10.6 μM CBD (Green Roads, 100 mg), LPS (1 μg/mL), CBD/LPS in conjunction with dexamethasone (10 μM). IL-8 was measured twenty-four hours post-treatment in conditioned media by ELISA. One-way ANOVA *p<.05 vs. control and **p<0.01 vs. control, n=2-4 per group.

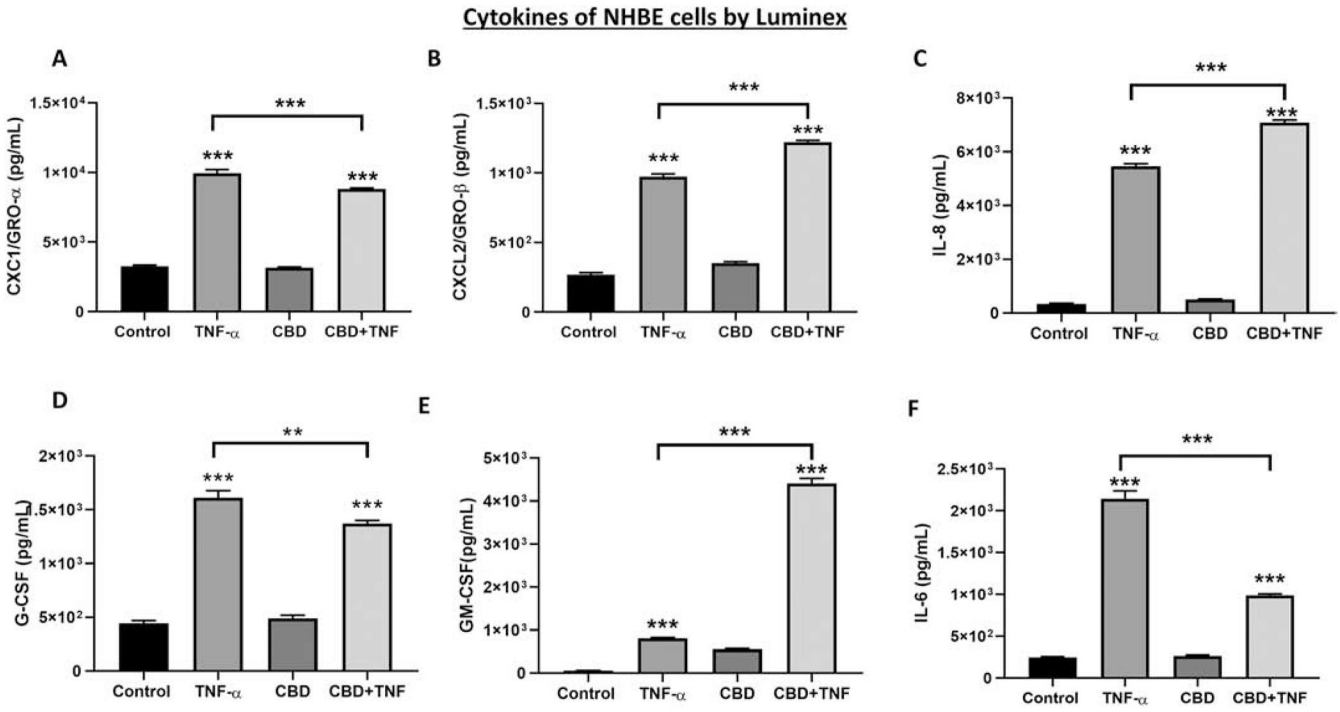

Figure 6. Differential inflammatory mediator response to CBD by normal bronchial epithelial cells, NHBE.

NHBE cells grown in complete media were treated with 10.6 μM CBD (Green Roads, 100 mg), TNF-α (10 ng/mL), and CBD+TNF. Twenty-four hours later, conditioned media was collected and inflammatory the mediators were analyzed by Luminex. The changes CXCL1, CXCL2, IL-8, G-CSF, GM-CSF, and IL-6 levels are shown. One-way ANOVA ***$p<0.001$ vs. control and denoted groups, n=3/group.

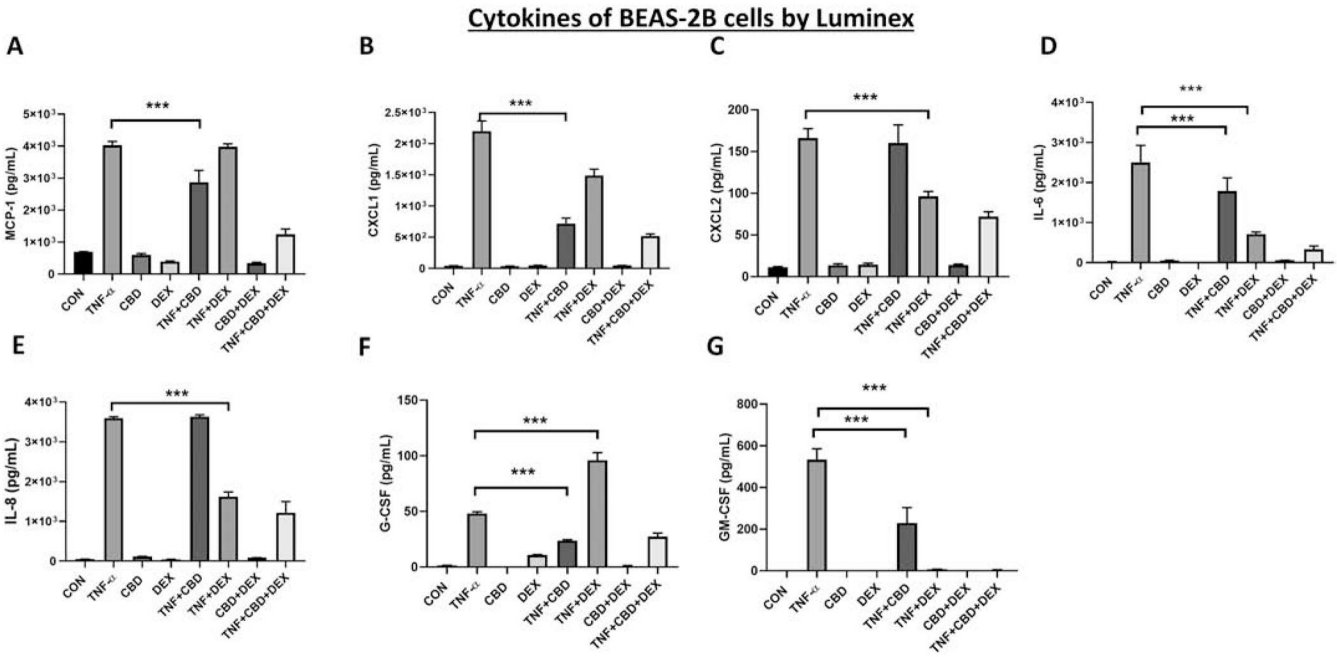

Figure 7. CBD and Dexamethasone differentially regulated inflammatory responses induced by TNF-α in human bronchial epithelial cells (BEAS-2B).

BEAS-2B cells were treated with 10.6 μM CBD (Green Roads 100 mg), TNF-α (10 ng/mL), and CBD+TNF. Twenty-four hours later, conditioned media was collected and analyzed by Luminex. MCP-1, CXCL1, CXCL2, IL-6, IL-8, G-CSF, and GM-CSF levels are shown. One-way ANOVA ***$p<0.001$ vs. control and denoted groups.

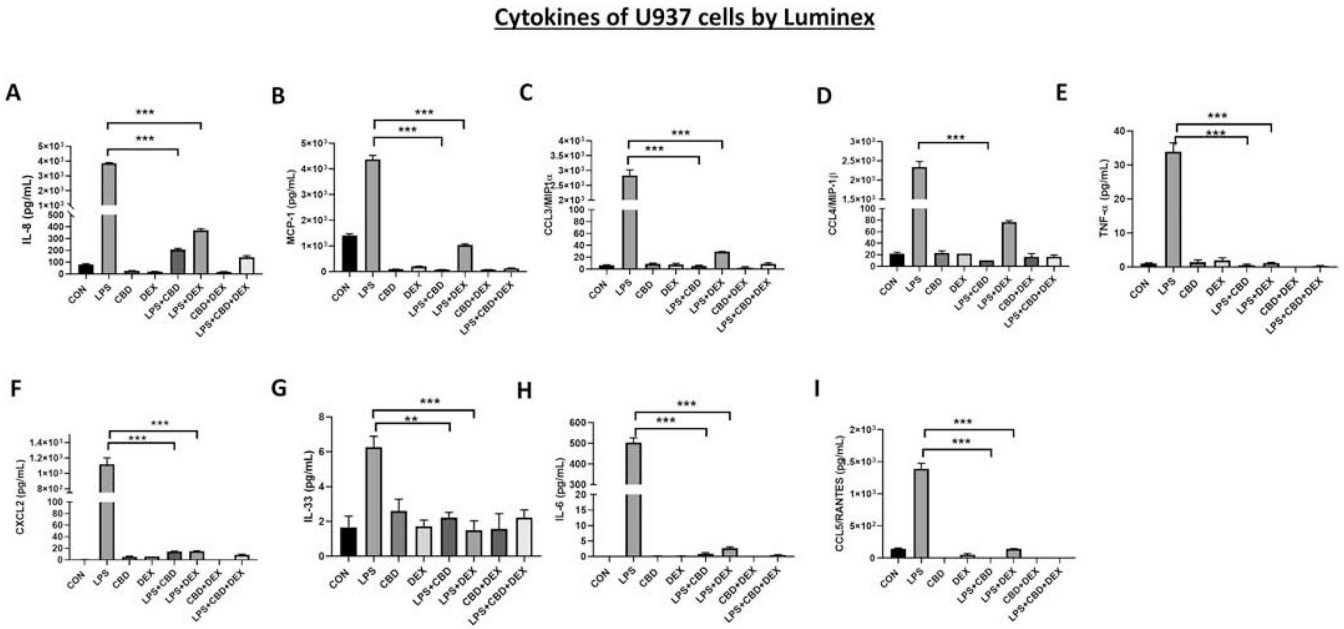

Figure 8. CBD and Dexamethasone differentially regulated inflammatory responses induced in human U937 monocytes.

U937 cells were treated with 10.6 μM CBD (Green Roads, 100 mg), LPS (1 μg/mL), Dexamethasone (10μM), combination treatments. Twenty-four hours later, conditioned media was collected and analyzed by Luminex. IL-8, MCP-1, CCL3, CCL4, TNF- α, CXCL2, IL-33, IL-6, and CCL5 are shown. One-way ANOVA **p<0.01 and ***p<0.001 vs. denoted groups.

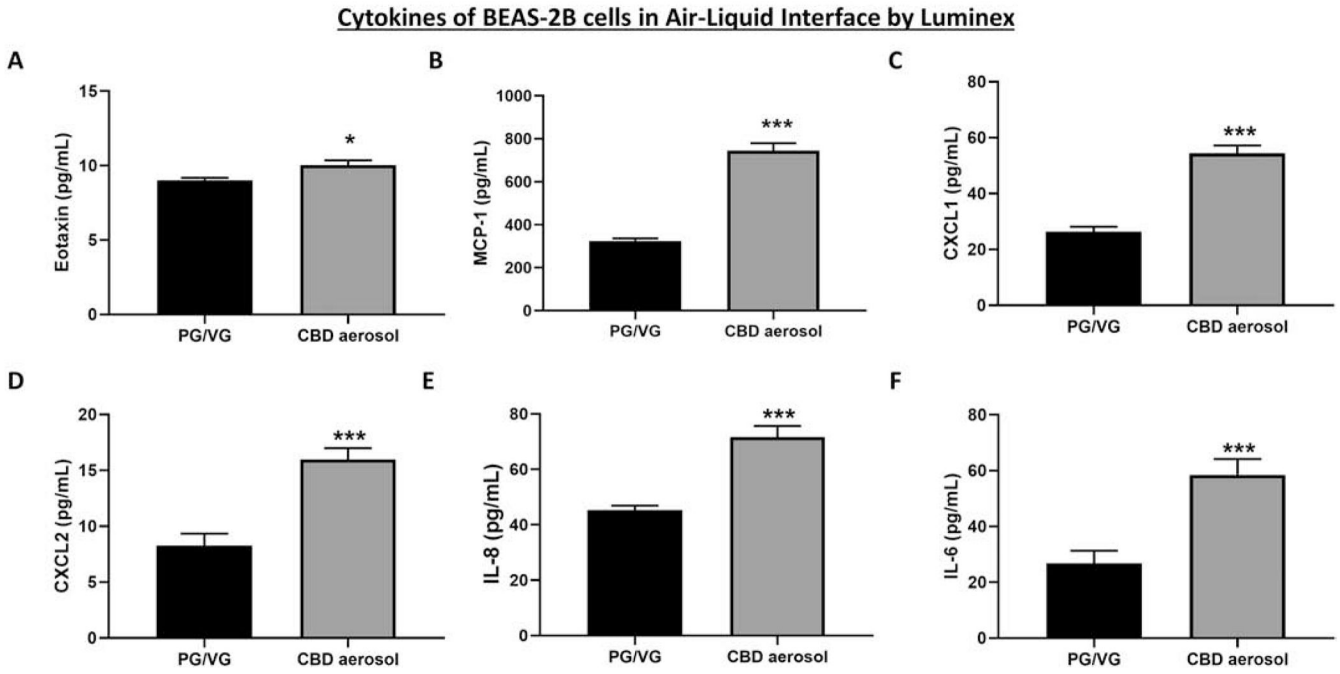

Figure 9. Induction of inflammatory response by CBD vapors in human bronchial epithelial cells (BEAS-2B).

BEAS-2B cells were exposed to 60 puffs of propylene glycol and vegetable glycerin mixture and CBD by Hemplucid separately during a 30 minute period. Twenty-four hours later, conditioned media was collected and the inflammatory mediators were analyzed by Luminex. Eotaxin, MCP-1, CXCL1, CXCL2, IL-8, and IL-6 are shown. Student's *t-test* *p<0.05, **p<0.01, and ***p<0.001 vs. PG/VG, n=6/group.

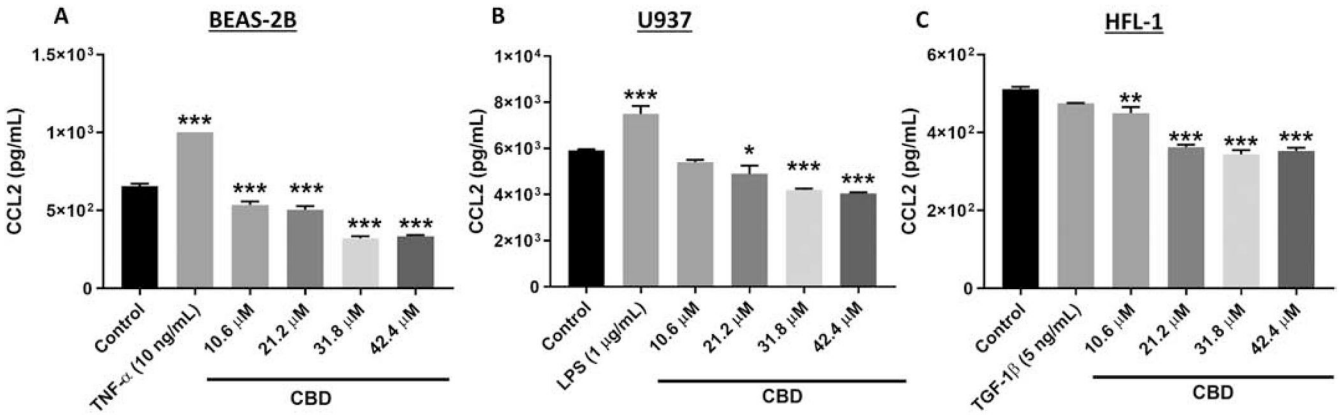

Figure 10. CBD oil attenuated CCL2 (MCP-1) response in epithelial cells, macrophages, and fibroblasts.

Three cell types, (A) BEAS-2B, (B) U937, and (C) HFL-1, were treated with CBD oil (Green Roads, 100 mg) at final well concentrations 10.6 μM, 21.2 μM, 31.8 μM, 42.4 μM respectively. TNF-α (10 ng/mL), LPS (1 μg/mL), and TGF-β (5 ng/mL) were used as positive control treatments along with an untreated control group. CCL2 was measured twenty-four hours post-treatment in conditioned media by ELISA. One-way ANOVA *p<.05 vs. control, **p<0.01 vs. control, and ***p<0.001 vs. control, n=3-4 per group.

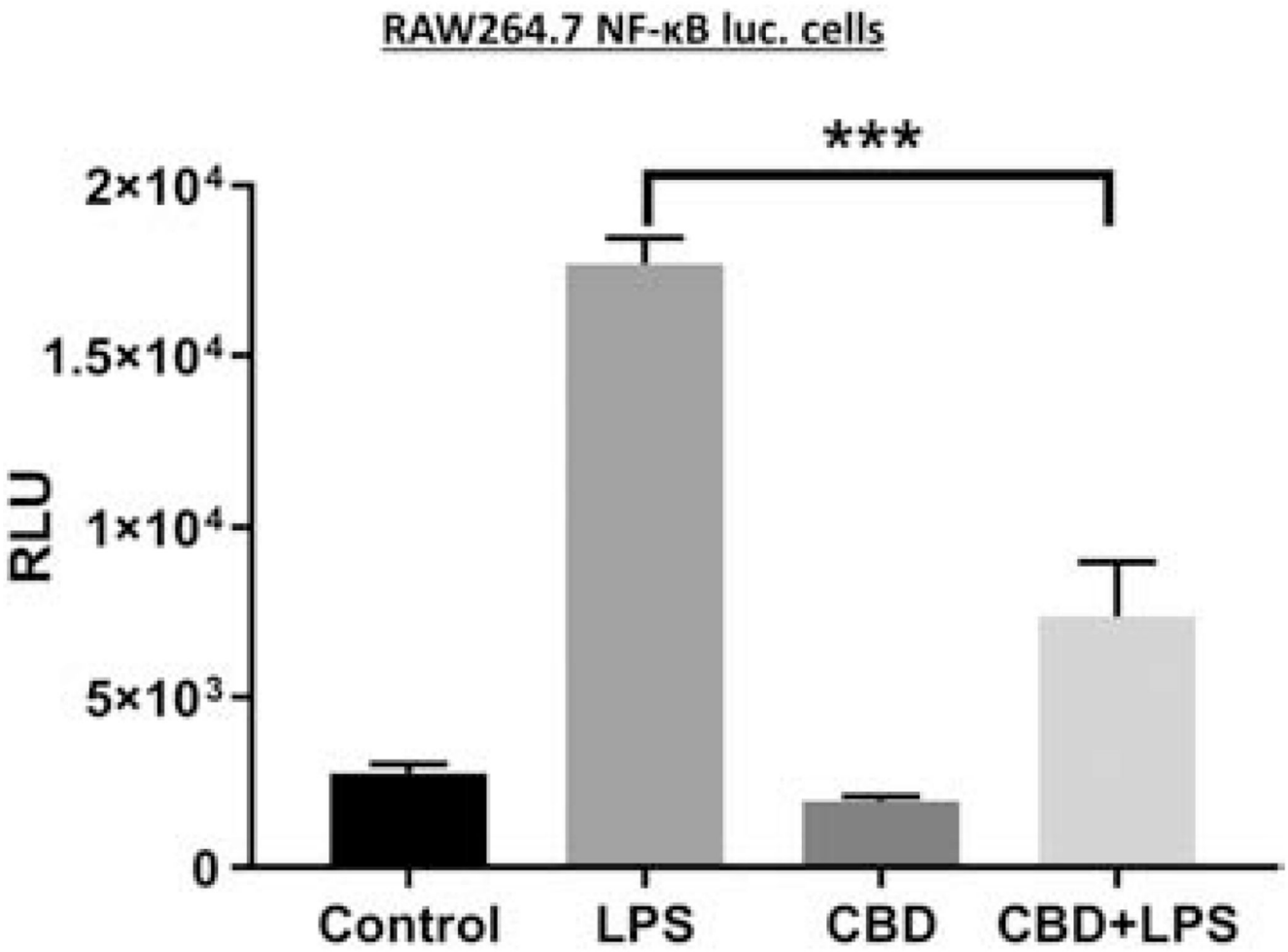

Figure 11. CBD inhibited NF-κB activity in RAW264.7 in mouse monocytes.

RAW264.7 cells were seeded in a 24 well plate with $4x10^5$ per well in 1% FBS. Designated wells were treated with LPS (1 μg/mL) and 21.2 μM CBD, and CBD with LPS. Cells were collected and lysed 24 hours, subsequently the luminescence was measured. One-way ANOVA with Tukey's multiple comparison ***$p<0.001$ CBD+LPS vs. LPS, n=3.

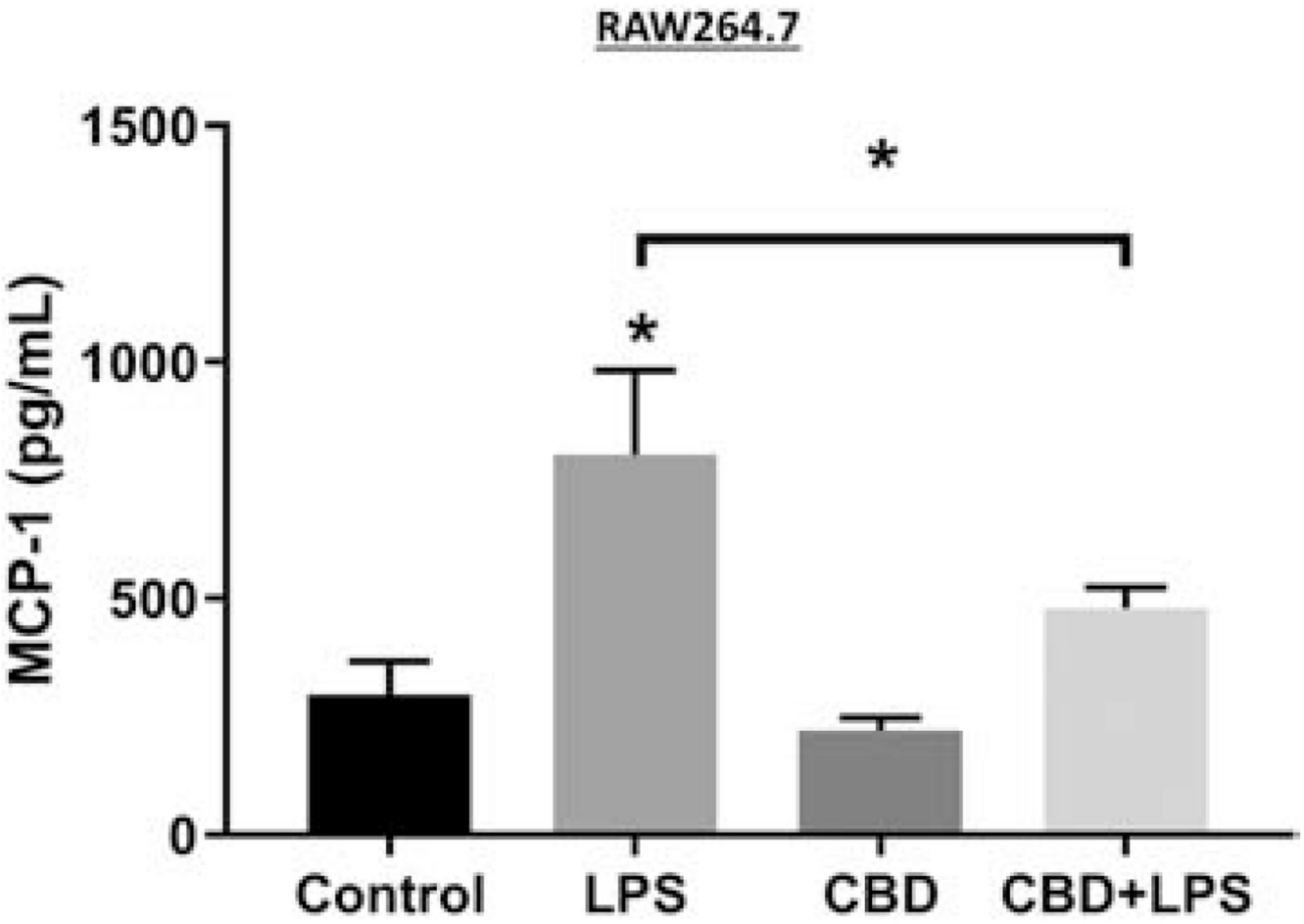

Figure 12. CBD-induced MCP-1 in mouse monocytes.

RAW264.7 cells were seeded in a 24 well plate with $4x10^5$ per well in 1% FBS. Designated wells were treated with LPS (1 μg/mL) and 21.2 μM CBD. Cells were collected 24 hours after the treatment. Subsequently, MCP-1 levels were measured in the cell supernatants. One-way ANOVA with Tukey's multiple comparison, *$p<0.05$ vs control and CBD+LPS, n=3.

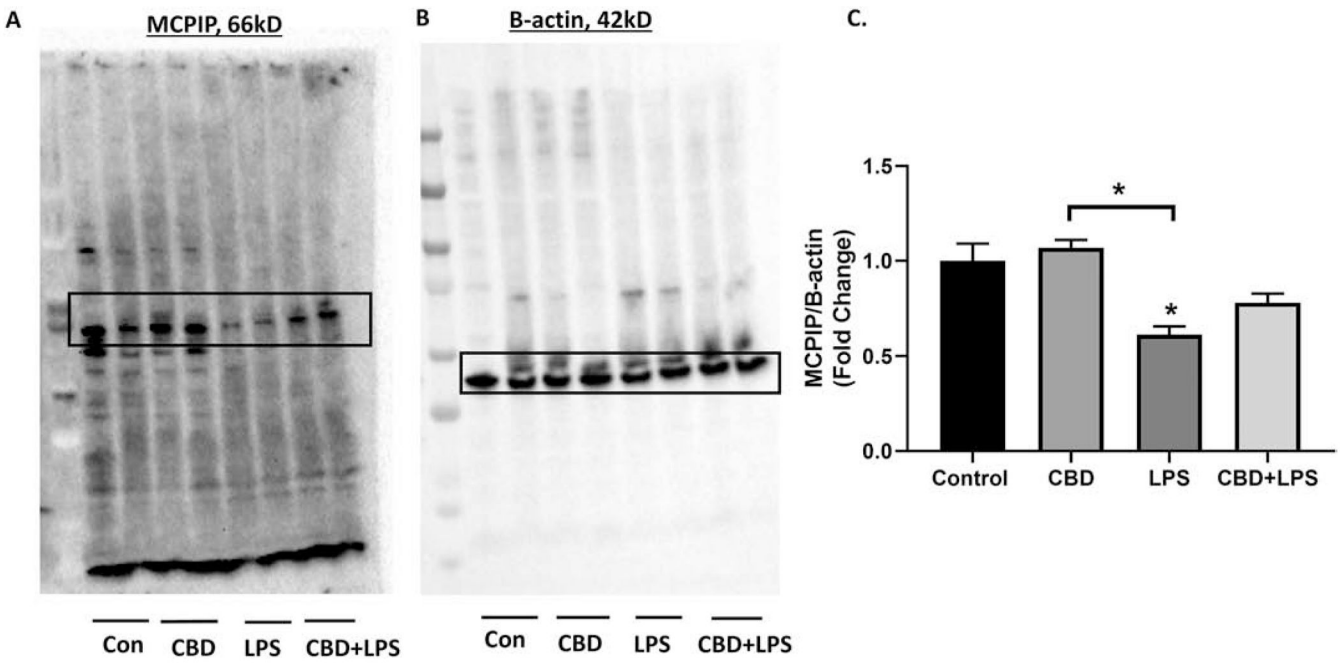

Figure 13. Immunoblot analysis of MCPIP1 protein in murine macrophages (RAW264.7) treated with CBD, LPS, and CBD+LPS.

RAW264.7 cells were treated with 21.2 μM of CBD, stimulated with LPS (1μg/mL), and CBD combined with LPS. Twenty-four hours later the cells were lysed, protein levels were quantified using BCA assay. 20 μg protein of each sample was run on SDS-PAGE and then transferred to a membrane. These were probed with MCPIP-1 (ZC3H12A Rabbit Polyclonal GeneTex) antibody. (B) For normalization, probed with the loading control, β –actin (mAbcam 8226), and the densitometry analysis was performed using ImageLab software. *$p<0.05$ vs. control and denoted group, student's t-test.