Microarray and Pathway Analysis Reveal Distinct Mechanisms Underlying Cannabinoid-Mediated Modulation of LPS-Induced Activation of BV-2 Microglial Cells

Ana Juknat1, Maciej Pietr2†, Ewa Kozela1,2, Neta Rimmerman1,2, Rivka Levy2, Fuying Gao3, Giovanni Coppola3, Daniel Geschwind3, Zvi Vogel1*

1 The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Sackler Faculty of Medicine, Physiology and Pharmacology Department, Tel Aviv University, Tel Aviv, Israel, 2 Neurobiology Department, Weizmann Institute of Science, Rehovot, Israel, 3 Program in Neurogenetics, Department of Neurology, University of California Los Angeles, Los Angeles, California, United States of America

Abstract
Cannabinoids are known to exert immunosuppressive activities. However, the mechanisms which contribute to these effects are unknown. Using lipopolysaccharide (LPS) to activate BV-2 microglial cells, we examined how Δ2-tetrahydrocannabinol (THC), the major psychoactive component of marijuana, and cannabidiol (CBD) the non-psychoactive component, modulate the inflammatory response. Microarray analysis of genome-wide mRNA levels was performed using Illumina platform and the resulting expression patterns analyzed using the Ingenuity Pathway Analysis to identify functional subsets of genes, and the Ingenuity System Database to denote the gene networks regulated by CBD and THC. From the 5338 transcripts that were differentially expressed across treatments, 400 transcripts were found to be upregulated by LPS, 502 by CBD+LPS and 424 by THC+LPS, while 145 were downregulated by LPS, 297 by CBD+LPS and 149 by THC+LPS, by 2-fold or more (p<0.005). Results clearly link the effects of CBD and THC to inflammatory signaling pathways and identify new cannabinoid targets in the MAPK pathway (Dusp1, Dusp8, Dusp2), cell cycle related (Cdkn2b, Gadd45a) as well as JAK/STAT regulatory molecules (Socs3, Cish, Stat1). The impact of CBD on LPS-stimulated gene expression was greater than that of THC. We attribute this difference to the fact that CBD highly upregulated several genes encoding negative regulators of both NFκB and AP-1 transcriptional activities, such as Trib3 and Dusp1 known to be modulated through Nrf2 activation. The CBD-specific expression profile reflected changes associated with oxidative stress and glutathione depletion via Trib3 and expression of ATF4 target genes. Furthermore, the CBD affected genes were shown to be controlled by nuclear factors usually involved in regulation of stress response and inflammation, mainly via Nrf2/Hmox1 axis and the Nrf2/ATF4-Trib3 pathway. These observations indicate that CBD, and less so THC, induce a cellular stress response and that this response underlies their high immunosuppressant activities.

Introduction

Cannabis sativa extracts (marijuana and hashish) have been used for centuries both as therapeutic agents and recreational drugs, primarily due to their ability to regulate neurobehavioral processes including memory, mood and appetite [1,2]. The spectra of possible therapeutic uses of marijuana and its active constituents, the cannabinoids, range from handling nausea, vomiting and cachexia (in cancer chemotherapy and AIDS patients) to treatment of chronic pain, glaucoma, epileptic seizures, Parkinsonian tremor as well as multiple sclerosis [1,2,6]. Amongst the 60 different cannabinoids identified from Cannabis preparations, Δ2-tetrahydrocannabinol (THC), the major psychoactive Cannabis constituent, and the non-psychoactive cannabidiol (CBD) are the most abundant and important.

Many of the beneficial effects of cannabinoids have been attributed to their potent immunosuppressive and anti-inflammatory properties [7–14]. Additionally, cannabinoids are known to possess pro-apoptotic, neuroprotective and anti-tumor properties [15–17]. To date, two cannabinoid receptors have been characterized: the CB1 and the CB2 receptors. The CB1 receptor is highly expressed in neural cells and mediates the psychoactive and addictive activities of cannabinoids, while the CB2 receptor is abundantly present in the periphery including the immune system and is involved in cannabinoid immunomodulation [18–20]. THC binds to both of these receptors with similar efficiency [21] and has

Citation: Juknat A, Pietr M, Kozela E, Rimmerman N, Levy R, et al. (2013) Microarray and Pathway Analysis Reveal Distinct Mechanisms Underlying Cannabinoid-Mediated Modulation of LPS-Induced Activation of BV-2 Microglial Cells. PLoS ONE 8(4): e61462. doi:10.1371/journal.pone.0061462

Received November 14, 2012; Accepted March 10, 2013; Published April 24, 2013

Copyright: © 2013 Juknat et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation and by the Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases. AJ is supported by the Israeli Ministry for Absorption in Science. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interest exist.

* E-mail: zvi.vogel@weizmann.ac.il
† Deceased.
been reported to have effects on both the nervous and the immune systems [7,11,18,22]. In the last years, attention has been turned to several other phytocannabinoids, most notably to CBD. CBD displays a diversity of actions, including anticonvulsive, sedative, hypnoic, antipsychotic, anti-inflammatory and neuroprotective properties [11–14,23–20]. CBD, unlike THC, is not an efficient ligand of either CB1 or CB2 receptors and therefore, is devoid of the unwanted psychotropic effects (mediated via CB2) characteristic of marijuana or THC. Thus, the effects of CBD are probably mediated through other receptors/targets, as described below and elsewhere [23,29,30].

Phytocannabinoids were reported to affect various populations of immune cells [7,11–14,31]. Both THC and CBD have been shown to decrease cytokine production in human immune cell lines [32] and to suppress T cell proliferation and their effector functions [12,33,34]. In response to stimulation with the bacterial endotoxin lipopolysaccharide (LPS), both monocytes and microglial cultures treated with either THC or CBD produce lower levels of cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and IL-6 [11,35]. However, the molecular mechanisms involved in these cannabinoid-mediated effects are not yet fully characterized. Eljaschewitsch et al. [36] showed that activation of CB1 and/or CB2 receptors in the murine microglial BV-2 cell line leads to a reduced induction of mitogen-activated protein kinase phosphatase-1 (MKP-1) and that this event switches off MAPK signal transduction which was activated by LPS stimulation. Altered adenosine signaling (through inhibition of its uptake) has been reported as a potential non-cannabinoid receptor mechanism by which CBD, but less so THC, may decrease inflammation [37]. Other studies identified the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) as a novel intracellular target, which mediates the cannabinoid-associated immunosuppression in a manner that is independent of the known cannabinoid receptors CB1 and CB2 [38,39]. Other targets including the G-protein-coupled receptors GPR55 and GPR18 as well as the transient receptor potential (TRP) channels were also suggested [19,20,30,40].

Microglial cells are the resident macrophage-like cells of the CNS. They are highly ramified cells and their processes are very dynamic under non-pathological conditions, actively scanning their environment. These cells have important roles in brain’s innate immunity and neuronal homeostasis as well as in neuroinflammatory pathologies [41]. Microglia can be activated by infection, injury or by endogenously released neurotoxic factors and their activation is associated with the production and secretion of a variety of compounds such as cytokines, reactive oxygen species (ROS), reactive nitrogen species, matrix metalloproteinases and prostaglandins. Although microglial activation is considered a protective mechanism involved in the clearance of pathogen infection and in regulating tissue repair and recovery, excessive or chronic activation can lead to harmful effects [42]. Interestingly, the mechanisms that give rise to either the protective or the damaging microglial phenotypes are not fully elucidated. Enhancing the microglial-mediated innate immunity in the CNS and/or preventing the harmful effects associated with their chronic activation may offer new therapeutic approaches for the treatment of brain injury and neurodegenerative diseases [43].

One of the most potent stimuli for microglia activation is the bacterial endotoxin LPS, that mimics infection by Gram-negative bacteria. LPS activates intracellular signaling pathways in a complex way leading to secretion of cytokines and to overexpression of several markers of the immune response. Previous studies reported that LPS stimulation induces gene expression of TNFα, IL-1β, IL-6, iNOS and COX-2 as well as the production of NO and PGE2 in primary and BV-2 microglial cell cultures [11,44,45]. We reported that CBD reduces the activity of the NF-κB pathway and upregulates the activation of STAT3 transcription factor in LPS-stimulated BV-2 cells, and that both CBD and THC decrease the activation of the LPS-induced STAT1 transcription factor, a key player in IFNβ-dependent pro-inflammatory processes [11]. Moreover, performing comparative microarray analysis of genome-wide mRNA levels in the BV-2 cells, we reported that CBD, but less so THC, shows a specific gene expression profile associated with oxidative stress and glutathione depletion involving the G2CN2/cF2α/p8/ATF4/Chop-Trib3 pathway [13]. Furthermore, the CBD-stimulated genes were shown to be controlled by nuclear factors known to be involved in suppression of stress response and inflammation, mainly via the (EpRE/ARE)-Nrf2/Atf4 system and the Nrf2/Hmox axis. We reported that CBD, but less so THC, affects the expression of genes involved in zinc homeostasis, suggesting that the regulation of zinc levels could have an important role through which CBD may exert its antioxidant and anti-inflammatory effects [14].

Although the inhibitory functions of cannabinoids on LPS-activated NF-κB and IFN-β/STAT proinflammatory pathways and on the secretion of selected cytokines in BV-2 microglial cells has been studied [11], a genome-wide search for cannabinoid molecular targets in LPS-activated BV-2 cells has not yet been performed. We have, therefore, performed gene array studies and comparative gene profiling analysis of BV-2 cells treated with LPS, CBD+LPS or THC+LPS. This approach allowed us to analyze the changes induced by CBD and THC on gene expression patterns in LPS-treated BV-2 cells and to explore the genome-wide interaction network affected by these treatments. In this regard, a structured network knowledge-based approach to analyze genome-wide transcriptional responses in the context of known functional interactions among proteins, small molecules and phenotypes has been established [46]. We applied this analysis to show the interactions and signaling networks elicited by the cannabinoids in LPS-stimulated BV-2 cells. Our results show that CBD is a potent modulator of microglial activation. Identification of the CBD- and THC-regulated genes and related networks provides a molecular basis for understanding the effects of these cannabinoids on LPS-activated microglia.

Materials and Methods

Materials

LPS from *Escherichia coli* (serotype 055:B5) was purchased from Sigma (St. Louis, MO, USA). THC and CBD were obtained from the National Institute on Drug Abuse (NIDA; Baltimore, MD, USA). Stock solutions of cannabinoids were prepared in ethanol and diluted into culture medium before experiments. Final ethanol concentration in the medium did not exceed 0.1%.

Microglial cell culture

The immortalized murine BV-2 microglial cell line was kindly provided by Prof. E.J. Choi from the Korea University (Seoul, Korea). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) containing 4.5 g/L glucose, supplemented with 5% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Biological Industries Ltd., Kibbutz Beit Haemek, Israel), under a humidified 5% CO2 atmosphere at 37 °C. Cells were pretreated for 2 h with either THC or CBD (both at 10 μM) followed by addition of LPS (100 ng/ml) for another 4 h.
Total RNA extraction

Extraction, quantification and quality of extracted RNA were performed and analyzed as described previously [13].

Microarray transcript analysis

Comparative microarray analysis was performed using the Illumina MouseRef-8 BeadChip platform, as described previously [13]. Four replicates of each experiment were carried out, resulting in four independent microarrays for each individual treatment and controls (24 total arrays). Gene products that were affected by 2-fold or more were further analyzed as described in the Result section. Differentially expressed genes were classified according to their gene ontology (GO; http://www.geneontology.org/; [47]), using DAVID Bioinformatics online tools (Ddatabase for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/; [48]). Cellular pathway association was analyzed according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) and pathway maps according to BioCarta (http://www.biocarta.com/genes/index.asp).

Ingenuity pathway analysis

Pathway and global functional analyses were performed using Ingenuity Pathway Analysis 6.0 (IPA; Ingenuity® Systems, www.ingenuity.com). A data set containing gene identifiers and corresponding expression values was uploaded into the application, and each gene identifier was mapped using the Ingenuity Pathways Knowledge Base (IPKB). The IPKB analyses identify the biological functions as well as the pathways from the IPA library that are most significant to the data set. Genes from the data sets associated with biological functions or with a canonical pathway in the IPKB, that met the P value cutoff of 0.005 were used to build the interactome as described below. Fisher’s exact test was used to calculate a p-value determining the probability that each biological function and/or canonical pathway assigned to this data set was not due to chance alone.

Interactome/network analysis of differentially expressed genes

The accession number and the ratio (CBD+LPS versus LPS and THC+LPS versus LPS) for each gene whose expression was changed by at least 2-fold by one of the treatments (focused genes), were uploaded into the IPA system. This IPA application was used to query databases for interactions between sets of differentially expressed genes and all other genes stored in the knowledge base to generate a set of interactive networks taking into consideration canonical pathways, relevant biological interactions as well as cellular and disease processes. The IPA system computes a score for each network according to the fit of the set of the supplied “focus genes”. The p value scores for such a set indicate the likelihood of focused genes to belong to a network versus those obtained by chance.

Microarray data validation by quantitative real time PCR

Many of the gene products that were found to be affected using the microarray gene analysis were validated by quantitative real time reverse transcription polymerase chain reaction (qPCR). Primer sets for qPCR were designed using Primer Quest, an online tool provided by Integrated DNA Technologies (http://eu.idtdna.com/Scitools/Applications/Primerquest/) (Table S1). Wherever possible, designs with at least one of the primer sequences located on an intron-exon boundary were chosen, thus avoiding co-amplification of minor contaminating amounts of genomic DNA that could be present in the RNA samples. All primers were analyzed using the nucleotide program BLAST to ensure primer specificity for the gene of interest (http://blast.ncbi.nlm.nih.gov/Blast.cgi). cDNA was generated by using the QuantiTect Reverse Transcription kit containing “genomic DNA wipe out” (to eliminate contamination with genomic DNA), according to the manufacturer’s instructions (QiaGen, AG, Basel, Switzerland). qPCR was carried out as detailed by Jukat et al., [13] using the Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia). Expression levels of genes of interest were normalized to the reference genes, B2m-microglobulin (B2m), whose expression was found not to be affected by the various treatments, and are expressed as fold change using the calculation method described previously [13]. The qPCR experiments were repeated 3 to 4 times using different mRNA batches from independent experiments and reactions were performed in duplicates for each cDNA sample.

Statistics

qPCR data were plotted as the mean±SEM of 3–4 independent experiments. Statistical significance was assessed using a one-way or two-way ANOVA, followed by Bonferroni post hoc multiple comparison test as implemented with the Statistics Toolbox Software MATLAB, Version 6.1 (R2007b), MathWorks (http://www.mathworks.com/help/toolbox/stats/statstoolbox.html). A probability value <0.05 was defined as statistically significant.

Results

Effect of cannabinoids on LPS-stimulated gene expression in BV-2 microglial cells

BV-2 microglial cells were pretreated for 2 h with either THC or CBD (both at 10 μM) followed by the addition of LPS (100 ng/ml) to the incubation medium for another 4 h. The control treatment with cannabinoids alone lasted for 6 h and with LPS alone for 4 h. The choice of these time points for transcriptional profiling was guided by our previous studies [11,13] as well as by other reports [44,45] which investigated the general temporal pattern of microglial activation by LPS. Moreover, according to our previous results, neither THC nor CBD treatments (both at 10 μM) significantly affected the viability of the BV-2 microglial cells during this 6 h period [11].

The RNA prepared from these samples (in sextuplicates) was analyzed for changes in transcriptional levels using the MouseRef-8 v1.1 Expression BeadChip Illumina Arrays. Each of these arrays has >24,000 mouse targets based on the NCBI mouse Reference Sequence Database, including 16,287 constitutive exons/islands based on the splice variants in the mouse transcriptome (Molecular Signature Database; MouseDB3) and NCBI LocusLink databases. The results of the analyses of the arrays showed that 32% of the transcripts were consistently “present” in the BV-2 RNA samples across all arrays. Moreover, clustering based on inter-array Pearson correlation coefficient indicated no batch effects. Microarray analysis based on a threshold of p<0.005, revealed that a total of 22% (5338 out of 24,000 transcripts) of the Illumina gene set was differentially regulated across treatments. Of these, 1319 gene probe sets were upregulated and 1829 transcripts were downregulated by the LPS treatment (Figure 1A); and from these numbers of genes, 400 transcripts were found to be upregulated and 145 downregulated by LPS by 2-fold or more. When the fold change was set on ≥3-fold (p<0.005), we found that 226 gene products were upregulated and 33 were downregulated by LPS (Figure 1A). The vast majority of the LPS-affected transcripts (89%) or 1181 gene probe sets of the upregulated genes, p<0.005 and 90% or 1641 gene probes of the downregulated transcripts,
When LPS was applied in the presence of THC, 1216 gene probe sets (22.0%) were upregulated and 1630 transcripts (30.7%) were downregulated; and from these, 424 transcripts were found to be upregulated and 149 downregulated by 2-fold or more (Figure 1A). From the 1216 upregulated genes, 157 transcripts were exclusively responsive to THC+LPS treatment alone and from the 1630 downregulated transcripts, 205 gene probes were affected only when THC and LPS were together (Figure 1B).

When the fold change was set on ≥3-fold, we found that 230 gene products were upregulated by CBD+LPS and 236 transcripts by THC+LPS whereas 51 gene products were downregulated by CBD+LPS and 31 transcripts by THC+LPS (Figure 1A).

Our results also reveal that from the 5338 transcripts found to be differentially regulated by the various treatments (p ≤ 0.005), 680 gene probe sets were found to be upregulated by CBD alone and 58 gene products by THC alone. CBD had also a much larger effect compared to THC on the number of downregulated genes, 524 gene products were downregulated by CBD and 36 by THC (Figure 1A).

The groups of LPS-upregulated and downregulated genes that showed a change in expression of ≥2-fold (p≤0.005) in either direction were subjected to gene ontology (GO) analysis allowing functional annotation using the DAVID Bioinformatics Resources and the KEGG pathways (Figure S1). All major Biological Processes, Cellular Components and Molecular Functions within GO for the LPS-upregulated transcripts, were for the most part, genes associated with the immune and defense responses as well as apoptosis and cell death. KEGG database analysis included genes related to Toll-like receptor pathways and antigen processing as well as to the MAPK pathways. IPA global functional analysis of the LPS-upregulated and downregulated genes (≥2-fold; p≤ 0.005) confirmed the DAVID representation of genes within IPA-specific GO categories (Figure S2). IPA global functional analysis of the LPS-upregulated transcripts show gene products mainly implicated in cell-to-cell signaling, cellular movements, growth and proliferation, as well as cell death, immune response and signaling, including genes involved in the NF-kB pathway and in the activation of the liver X receptor/retinoic X receptor (LXR/RXR). The largest subsets of downregulated transcripts included genes known to be involved in the regulation of macromolecules and nucleotide metabolism, differentiation and development as well as regulation of gene expression and transcription (Figures S1 and S2).

A list of 183 LPS-upregulated genes (stimulated by 2-fold or more; p≤0.005) are presented in Table S2. This list includes LPS-upregulated genes that were (in most cases) significantly affected by CBD+LPS or by THC+LPS. They appear in the Table according to their categories and specific annotations based on their GO and IPA. The highly upregulated transcripts were related to inflammation, host defense and adaptive response. Inflammatory cytokines and chemokines together with their cognate receptors comprised a large group of more than 20 genes. Interferon related transcripts formed a significant population comprised of 12 genes. Based on GO and relevant literature references, genes were also classified into other functional categories including metabolic enzymes, membrane transport and secretion, kinases, phosphatases as well as transmembrane G-protein coupled receptors. The remainder of the LPS-upregulated genes was grouped according to their involvement in several other cellular processes such as apoptosis, proliferation and cell cycle progression, transcriptional and translational control as well as stress response. Additionally, we have identified a cluster of genes associated with regulation of extracellular matrix that are known to be involved in molecular recognition between cells, cell

Figure 1. Gene array analysis of the mRNA of BV-2 cells following treatment with LPS and cannabinoids. mRNA was prepared from BV-2 cells treated with LPS and cannabinoids and subjected to array analysis as described in Methods. (A) Number of differentially expressed genes that were either significantly upregulated (red) or downregulated (green) across the different treatment conditions versus control untreated cells (p ≤ 0.005). The different shades of color represent the indicated fold of change (>1, =2 and ≥3). (B) Venn diagrams representing the numbers of BV-2 microglial transcripts that were either upregulated or downregulated at a significance of p≤0.005 following treatments with LPS, CBD and THC, or with a combination of LPS and one of the cannabinoids. doi:10.1371/journal.pone.0061462.g001
adhesion and migration. Genes implicated in cytoskeleton remodeling and control of cell motility and morphogenesis form a separate category.

The gene transcripts upregulated by LPS to the highest extent consist of interleukin 1B (Il1b; 153-fold), colony stimulating factor 3 (Csf3; 118-fold), chemokine (C-X-C motif) ligand 2 (Cxcl2; also known as MIP-2a; 109-fold), chemokine (C-X-C motif) ligand 10 (Cxcl10; 44-fold), serum amyloid A3 (Saa3; 38-fold), chemokine (C-C motif) receptor-like 2 (Cxcr2; 33-fold) and CD69 antigen (Cd69; 50-fold); \( \text{Table S2}. \)

Analysis of expression levels for the various transcripts affected by LPS showed that a much higher number of LPS-induced genes were significantly affected by pretreatment with CBD as compared to THC. From the 183 LPS-upregulated genes (by 2-fold or more; \( p \leq 0.005 \)), 73 transcripts were inhibited by 30% or more, and 15 genes were repressed by more than 60% by CBD. These later genes are shown in \( \text{Table 1} \) and contain Il-1b (reduced by 81%), dual specificity phosphatase 2 (Dusp2; by 78%), chemokine (C-C motif) ligand 12 (Cxcl12; by 77%), chemokine (C-C motif) ligand 9 (Cxcl9; by 76%), endothelin 1 (Edn1; by 71%), interferon beta 1 (Ifnb1; by 70%); chemokine (C-C motif) ligand 7 (Ccl7; by 70%); Cd69 by 69%, formyl peptide receptor, related sequence 2 (Fpr-rs2; by 69%); Il-1a (by 60%), interleukin 4 induced 1 (Il4i1; by 63%); interleukin 27 (Il27; by 62%), paired immunoglobulin-like type 2 receptor alpha (Pilra; by 62%), matrix metalloproteinase 13 (Mmp13; by 53%); and chemokine (C-C motif) ligand 2 (Cxcl2; by 52%).

When CBD was applied without LPS stimulation, it did not have a significant effect on these genes, except for Ccl7, Ccl2 and Cd69 which were downregulated by CBD by 71.4%, 71.4% and 50%, respectively. Careful evaluation of the list of genes reveals that the inhibitory effect of CBD on LPS-upregulated genes was concentrated mainly within gene categories related to inflammation. These included cytokines and chemokines, interferon regulated components of the immune response as well as molecules involved in host defense and adaptive immune response. The inhibitory effect of CBD on LPS-upregulated genes was less strong on other gene products such as those for regulatory molecules, kinases, phosphatases, metabolic enzymes, G protein-coupled receptors and molecules related to regulation of transcription.

THC inhibited the expression of only some of the LPS-upregulated genes and in most cases its effect was smaller than that of CBD: Dusp2 was reduced by 45%; Ifnb1, by 38%; Pilra, by 38%; Il-1b, by 28%; Cxcl12, by 25%; Fpr-rs2, by 24% and Il-1a, by 21%; (\( \text{Table 1} \)). The expression of genes downregulated exclusively by THC by more than 30% included only haptoglobin (Hp; reduced by 45%; \( \text{Table S2} \)). Analyzing the relationship (using DAVID Bioinformatics, KEGG pathways and BioCarta pathway maps) between the list of genes affected by LPS and those downregulated when CBD or THC were added, we found that the downregulated genes are related to a number of inflammatory

### Table 1. Repressive effect of CBD and THC on LPS-upregulated genes.

| Gene Name (Abbreviations and Accession) | Fold Change (versus untreated) | Effect on LPS |
|----------------------------------------|-------------------------------|--------------|
| **Proinflammatory cytokines**          |                               |              |
| Il1b (interleukin 1 beta; NM_008361)  | 135.3                         | –81% –28%    |
| Il1a (interleukin 1 alpha; NM_0010554)| 22.9                          | –68% –21%    |
| Il27 (interleukin 27; NM_145636)      | 7.7                           | –62% –12%    |
| **Anti-inflammatory cytokines**        |                               |              |
| Il4i1 (interleukin 4 induced 1; NM_010215.3)| 10.0                         | –63% –19%    |
| **Proinflammatory chemokines**         |                               |              |
| Ccl12 (chemokine (C-C motif) ligand 12; NM_011331)| 9.7                          | –77% –25%    |
| Ccl2 (chemokine (C-C motif) ligand 7; NM_013654)| 8.6                          | –70% –9%     |
| Ccl9 (chemokine (C-C motif) ligand 9; NM_011338.2)| 4.9                          | –76% –3%     |
| Ccl2 (MCP-1) (chemokine (C-C motif) ligand 2; NM_011333.3)| 4.5                          | –52% 0%      |
| **Interferon regulated**               |                               |              |
| Ifnb1* (interferon beta 1; NM_010510.1)| 11.1                          | –70% –38%    |
| **Host defense and adaptive response** |                               |              |
| Cd69** (CD69 antigen; NM_001033122)   | 29.9                          | –69% –8%     |
| Edn1** (endothelin 1; NM_010104.2)    | 11.8                          | –71% –5%     |
| Pilra** (paired immunoglobulin-like type 2 receptor alpha; NM_153510.3)| 6.3                          | –62% –38%    |
| **Phosphatases**                      |                               |              |
| Dusp2 (dual specificity phosphatase 2; NM_010090)| 3.1                          | –78% –45%    |
| **Motility and morphogenesis**         |                               |              |
| Fpr-rs2** (formyl peptide receptor, related sequence 2; NM_008039.2)| 11.7                          | –69% –24%    |
| **Adhesion and migration**            |                               |              |
| Mmp13* (matrix metalloproteinase 13; NM_008607.1)| 17.4                          | –53% 9%      |

*genes related to proinflammatory signaling.
**genes related to inflammatory responses.

\( \text{doi:10.1371/journal.pone.0061462.t001} \)
pathways including Toll-like receptor, Jak/STAT, IL-10 or IFNβ signaling pathways.

Interestingly, we found a significant number of LPS-upregulated genes whose expression was increased by CBD pretreatment. CBD enhanced the expression of 165 genes from the list of the ≥2-fold LPS-upregulated genes [Table S2]. The expression of 28 of these genes was enhanced by more than 50%. These genes were present mainly within the categories of regulation of transcription, cell cycle and proliferation, apoptosis, membrane transport and secretion, phosphatases, cell adhesion as well as of host defense and adaptive responses. Among them, we would like to mention Table 2, 15 genes whose expression was largely enhanced by CBD. These included aquaporin 9 (Aqp9; upregulated by 877%), growth differentiation factor 15 (Gdf15; also known as macrophage-inhibitory cytokine 1, MIC-1; by 592%), lipoctin 2 (Lcn2; by 421%), Cdkn2b/p15 (by 314%), prostaglandin 1 receptor (Pigir; by 207%), solute carrier family 7 member 11 (Slc7a11; by 157%), sequesstage 1/p62 (Sgst1/p62; by 117%), jun dimerization protein 2 (Jund2; by 117%); villin 2 (Vil2; by 110%), dual specific phosphatase 8 (Dusp8; by 107%), nuclear protein 1/p8 (Nup1/p8; by 107%), mucolipin 2 (Mcoln2/TRPML2; by 104%), Dusp1 (by 103%). C-type (calcium dependent, carbohydrate recognition domain) lectin (Clec9p; by 59%) and homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (Herpud1; by 57%). It is important to note that synergistic-like effect between CBD and LPS was observed regarding the expression of Aqp9, Gdf15, Lcn2, Cdkn2b, Pigir, Slc7a11 and Clec9p.

In contrast to CBD, a smaller number of LPS-upregulated genes showed increased expression as a result of treatment with THC+LPS (Table 2). These included Gdf15 (enhanced by 92%), Aqp9 (by 55%), interleukin 15 (Il15; by 47%), Herpud1 (by 42%), Cdkn2b (by 41%); Sgst1/p62 (by 40%); Jund2 (by 38%) and Clec9p (by 30%). From these transcripts, only two gene products, Gdf15 and Clec9p, were induced synergistically by the combination of THC+LPS. As for the remainder of THC sensitive genes, in only two cases we have seen enhancement of the LPS-upregulated mRNA expression levels by THC and not by CBD. These included guanylate nucleotide binding protein 5 (Gnb5; 36%) and myxovirus resistance 1 (Mx1; 33%; Table S2).

As for the 145 transcripts that were downregulated by ≥2-fold (i.e., by 50% or more; p≤0.005) after LPS treatment, Table S3 shows 82 gene products that were affected by CBD+LPS or by THC+LPS. The largest subsets of LPS-downregulated transcripts include genes known to be involved in cell cycle and proliferation, as well as in regulation of gene expression and transcription. Among the 33 genes that were downregulated by LPS treatment by ≥3-fold (i.e., by 73% or more; p≤0.005), the most affected genes included Epstein-Barr virus induced gene 2 (G2/p150, 90%); interleukin 15 (Il15; by 89%); Ikbkb6; by 87.5%, lymphoblastic leukemia (Lyl1; by 81.5%), pleckstrin homology, sec7 and coiled-coil domains, binding protein (Pscdbp; by 80.8%); beta galactosidase alpha 2, 6 sialyltransferase 1 (Ngal1; by 78.7%); sestrin 1 (Sesn1; by 77.3%); ubiquitin specific protease 2, transcript variant 2 (Usp2; by 76.7%); CD28 antigen (Cd28; by 75.6%) and c-mer proto-oncogene tyrosine kinase (Merkt; by 75%); (Table S3).

From the 82 LPS-downregulated genes presented in Table S3, the CBD pretreatment counteracted the reduction in mRNA levels of 16 genes (by 30% or more) and 2 were affected by more than 60%. These genes almost exclusively belong to the functional categories of cell adhesion and migration, motility and morphogenesis, regulation of transcription as well as of membrane transport and secretion. These genes [Table S3] include neuropehin 1 (Nph1; whose reduction was completely abolished), neural precursor cell expressed developmentally down-regulated gene 9 (Nedd9; reversed by 69%), basic helix-loop-helix domain containing class B2 (Bhlhb2; by 56%), unc-5 homolog B (Unc5b; by 33%); Cd28 (by 49%); SRF-box containing gene 4 (Sre4; by 42%) and potassium channel, subfamily K, member 13 (Kcnk13; by 42%). Treatment with THC resulted in a much weaker attenuation of the LPS-induced downregulation of gene expression and only in the case of Nph1 exceeded 30%. DAVID Bioinformatics Resources and KEGG pathway analyses identify Nph1 and Unc5b as genes related to axon guidance and Nedd9 as a gene related to adherens junction dynamics.

In several cases, we have observed that treatment with CBD further potentiated the LPS-downregulation of some genes. These genes were spotted across almost all identified gene categories; however they were particularly concentrated within those related to cell cycle and proliferation, stress response, metabolic processes and phosphatases. Thus, CBD significantly enhanced the LPS-downregulation of a number of important genes including cyclin D1 (Cnd1; by additional 153%), Pdkb [by (by 111%); myocyte enhancer factor 2c (Mef2c; by 95%); Ccl2 (by 93%); paladin (Pald; by 88%); Ebi2/Gpr185 (by 82%); Klf6 (by 72%) and RAS guanyl releasing protein 3 (Rasgrp3; by 66%), heat shock 70 kDa protein 5 binding protein 1 (Hspbp1, by 64%); zinc finger protein 36, CSH-type-like 1 (Zfp36l1; by 61%); carbohydrate sulfotransferase 10 (Chst10; by 59%) and SH3-binding kinase (Sbk; by 51%); (Table 4). As for THC, from the 75 genes that were downregulated when both THC and LPS were present, only two transcripts were markedly downregulated due to the THC treatment. These included Pscdbp reduced by 40% and Chst10 by 30% compared with the levels obtained with LPS alone.

Validation of microarray results

A large number of genes that were identified by microarray analysis as differentially regulated were subjected to validation by qPCR using B2m as a reference gene. Many of them were selected to be validated according to the distinct effects of cannabinoids on the LPS affected genes. The qPCR assays were repeated several times using at least 3 mRNA preparations from independent experiments. The results are expressed as fold change relative to control levels. We found that in almost all cases, there has been a very good agreement between the microarray and the qPCR data in terms of direction of change as well as of its magnitude (Table 5). For example, the qPCR data show that LPS upregulates the expression of the inflammatory chemokines Ccl2, Ccl12 and Cxcl10, the transporters and channels Agp9, Mob2 (TRPML2) and Slc7a11, the kinase inhibitor Cdkn1a, the phosphatases Dusp1 and Dusp2, the regulatory molecule Sgst1/p62, the G protein-coupled receptor Pigir and the receptor Tb2. The qPCR data also show that LPS downregulates the expression of the G protein-coupled receptors Ebi2/Gpr185, Cn2 and Gpr55, and the transcription factors Pparg1 and Pparg2. In addition, in agreement with the microarray data, the stress response genes Trib3 and Hmx1 are upregulated by CBD+LPS but not by LPS alone or by THC+LPS.

Network analysis and signaling pathways – Functional associations between the effects of cannabinoids and LPS

The differential mRNA expression patterns as revealed by the arrays of BV-2 cells treated with LPS alone or in combination with either CBD or THC, were analyzed using the Ingenuity System Database, software that includes the Ingenuity Knowledge Base (IKB) and the Global Molecular Network (GMN). These databases integrate published findings on biologically meaningful genetic or molecular gene/gene product interactions and identify functionally related gene networks.
### Table 2. Enhanced effect of CBD and THC on LPS-upregulated genes.

| Gene Name | Description | Accession | Fold Change (versus untreated) | Effect on LPS |
|-----------|-------------|-----------|--------------------------------|---------------|
| **Anti-inflammatory cytokines** | | | LPS | CBD+LPS | THC | CBD | THC |
| Gdf15 | growth differentiation factor 15 | NM_011819.2 | 8.1 | 55.7 | 15.5 | 8.2 | 2.4 | 592% | 92% |
| **Regulatory molecules** | | | | | | | | | |
| Sqstm1/p62 | sequestosome 1 | NM_011018 | 2.3 | 5.1 | 3.3 | 3.2 | 1.2 | 117% | 40% |
| **Membrane transport and secretion** | | | | | | | | | |
| Slc7a11 | solute carrier family 7 (cationic amino acid transporter, y+ system), member 11 | NM_011990.1 | 8.0 | 20.5 | 10.1 | 7.1 | 1.7 | 157% | 26% |
| Aqp9 | aquaporin 9 | NM_022026.2 | 3.1 | 30.3 | 4.8 | 14.3 | 2.0 | 877% | 55% |
| Mcoln2 (TRPML2) | mucolipin 2 | NM_026566.4 | 2.8 | 5.7 | 3.6 | 1.8 | 1.3 | 104% | 28% |
| **Phosphatases** | | | | | | | | | |
| Dusp1 (MKP-1) | dual specificity phosphatase 1 | NM_013642 | 2.5 | 5.1 | 2.9 | 2.8 | 1.1 | 103% | 16% |
| Dusp8 | dual specificity phosphatase 8 | NM_008748 | 2.5 | 5.1 | 2.5 | 1.6 | 1.0 | 107% | 0% |
| **G protein-coupled receptors** | | | | | | | | | |
| Ptgir | prostaglandin I receptor | NM_008967.1 | 3.8 | 11.7 | 4.3 | 5.4 | 1.3 | 207% | 13% |
| **Apoptosis** | | | | | | | | | |
| Lcn2 | lipocalin 2 | NM_008491.1 | 13.3 | 69.1 | 10.9 | 2.5 | 2.5 | 421% | –18% |
| **Regulation of transcription** | | | | | | | | | |
| Nupr1 | nuclear protein 1 | NM_019738.1 | 4.3 | 8.8 | 5.4 | 7.9 | 2.0 | 107% | 27% |
| Jundm2 | Jun dimerization protein 2 | NM_030887.2 | 3.0 | 6.5 | 4.1 | 3.0 | 1.5 | 117% | 38% |
| **Cell cycle and proliferation** | | | | | | | | | |
| Cdkn2b | cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) | NM_007670.2 | 2.2 | 9.1 | 3.1 | 2.9 | 1.1 | 314% | 41% |
| **Stress response** | | | | | | | | | |
| Herpud1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 | NM_022331.1 | 3.2 | 4.4 | 4.6 | 3.3 | 2.3 | 37% | 42% |
| **Adhesion and migration** | | | | | | | | | |
| Clec9* | C-type (calcium dependent, carbohydrate recognition domain) lectin | NM_019948.2 | 20.3 | 32.3 | 26.5 | 2.3 | 1.7 | 59% | 30% |
| Vil2/Ezrin | villin 2 | NM_009510.2 | 2.6 | 5.4 | 2.9 | 1.6 | 1.0 | 110% | 12% |

*gene involved in proinflammatory signaling. doi:10.1371/journal.pone.0061462.t002
The differential gene expression values (e.g., CBD+LPS versus LPS treatment or THC+LPS versus LPS) were entered into IPA to determine the most highly regulated networks of gene interactions and to highlight the biological processes that are relevant to each of the treatments. The IPA computation gives a score for each network according to the fit of the set of supplied affected genes.

### Table 3. Opposing effects of CBD and THC on LPS-downregulated genes.

| Gene Name | Description | Accession | Fold Change (versus untreated) | Effect on LPS |
|-----------|-------------|-----------|-------------------------------|--------------|
| **Adhesion and migration** | | | | |
| Cd28* | CD28 antigen | NM_007642.2 | −4.1 | −2.1 | −3.3 | 1.3 | −1.2 | −49% | −20% |
| Nrp1 | neuropilin 1 | NM_008737.1 | −3.4 | 0.0 | −2.2 | 1.6 | −1.1 | −100% | −33% |
| Unc5b | unc-5 homolog B (C. elegans) | NM_029770.1 | −2.9 | 1.4 | −2.4 | 1.7 | −1.4 | −53% | −15% |
| **Motility and morphogenesis** | | | | |
| Nedd9 | neural precursor cell expressed, developmentally down-regulated gene 9 | NM_017464.2 | −2.8 | −0.9 | −2.3 | −1.0 | −1.4 | −69% | −20% |
| **Membrane transport and secretion** | | | | |
| Kcnk13 | potassium channel, subfamily K, member 13 | NM_146037.1 | −2.2 | −1.3 | −1.6 | −0.9 | −0.9 | −42% | −27% |
| **Regulation of transcription** | | | | |
| Sox4 | SRY-box containing gene 4 | NM_009238.1 | −3.1 | −1.8 | −2.8 | 2.1 | 0.9 | −42% | −10% |
| Bhlhb2 | basic helix-loop-helix domain containing, class B2 | NM_011498.4 | −2.9 | 1.4 | −2.4 | 1.7 | −1.4 | −53% | −15% |

*gene related to inflammatory responses.
doi:10.1371/journal.pone.0061462.t003

### Table 4. Potentiated effect of CBD and THC on LPS-downregulated genes.

| Gene Name | Description | Accession | Fold Change (versus untreated) | Effect on LPS |
|-----------|-------------|-----------|-------------------------------|--------------|
| **Stress response** | | | | |
| Sesn1 | sestrin 1 | NM_001013370.2 | −4.4 | −6.3 | −4.2 | −1.7 | −1.1 | 43% | −3% |
| Hisp5bp1 | heat shock 70 kDa protein 5 binding protein 1 | NM_133804.1 | −3.6 | −5.8 | −4.1 | −1.0 | −1.5 | 64% | 14% |
| **Proinflammatory chemokines** | | | | |
| Cxcl14 | chemokine (C-X-C motif) ligand 14 | NM_019568.2 | −1.1 | −2.1 | −1.2 | −2.6 | −1.4 | 93% | 11% |
| **Metabolic enzymes** | | | | |
| Chst10 | carbohydrate sulfotransferase 10 | NM_145142.1 | −3.2 | −5.0 | −4.3 | −2.2 | −1.8 | 59% | 38% |
| **Cell cycle and proliferation** | | | | |
| Klhl6 | kelch-like 6 (Drosophila) | NM_183390.1 | −8.0 | −13.7 | −9.1 | −1.9 | −1.4 | 72% | 13% |
| Rasgrp3 | RAS guanyl releasing protein 3 | NM_207246.2 | −2.7 | −4.5 | −2.4 | −1.4 | −1.1 | 66% | −13% |
| Ccnd1 | cyclin D1 | NM_007631.2 | −2.4 | −6.0 | −2.8 | −2.9 | −1.2 | 155% | 20% |
| Zfp36l1 | zinc finger protein 36, C3H type-like 1 | NM_007564.2 | −2.0 | −3.2 | −2.2 | −1.1 | −1.0 | 61% | 9% |
| **Motility and morphogenesis** | | | | |
| Pscdbp* | pleckstrin homology, Sec7 and coiled-coil domains, binding protein | NM_139200.4 | −5.2 | −11.0 | −7.3 | −2.1 | −1.7 | 111% | 40% |
| **Phosphatases** | | | | |
| Pold | paladin | NM_013753.1 | −3.0 | −5.7 | −3.3 | −2.0 | −1.2 | 88% | 9% |
| **Kinases** | | | | |
| Sbk | SH3-binding kinase | NM_145587.1 | −2.2 | −3.2 | −2.3 | −1.2 | −1.2 | 51% | 8% |
| **G protein-coupled receptors** | | | | |
| Ebi2 | Epstein-Barr virus induced gene 2/GprPR183 | NM_183031.2 | −9.1 | −16.6 | −10.6 | −2.4 | −1.3 | 82% | 16% |
| **Regulation of transcription** | | | | |
| Met2c | myocyte enhancer factor 2C | NM_025282.1 | −2.1 | −4.1 | −2.3 | −2.1 | −1.2 | 95% | 11% |

*gene related to inflammatory responses.
doi:10.1371/journal.pone.0061462.t004
that are also contained in the GMN (focus genes: network-eligible molecules). The scores indicate the likelihood that “focus genes” belong to a network versus those that are obtained by chance. A score of >2 indicates a ≥99% confidence that a “focus gene” network was not generated by chance. According to the degree of interconnectedness among the molecules, a higher or lower IPA network score is assigned. Only networks with a score of 15 or higher were selected for further analysis. Networks describing the relationships between a subset of genes and their neighboring genes are presented in Figures 2, 3, 4 for CB and Figures 5, 6, 7 for THC. Focus genes are denoted by red symbols for THC and green symbols. The genes downstream of NFκB and the genes involved in cellular and molecular functions of cell death, protein synthesis and cancer [49,50].

**Table 5. qCR validation.**

| Gene Name                 | Fold Change |
|---------------------------|-------------|
| Inflammatory chemokines   |             |
| LPS                       | CBD+LPS     | THC+LPS     | CBD         | THC         |
| Ccl2                      | 5.2±1.5     | 2.8±0.3     | 5.7±1.3     | 0.5±0.08    | 1.2±0.1     |
| Ccl12                     | 10.7±2.5    | 3.8±0.4     | 5.8±1.2     | 0.8±0.2     | 0.9±0.1     |
| Cxcl10                    | 52.7±3.2    | 41.2±5.1    | 45.1±2.8    | 1.8±0.3     | 0.7±0.1     |
| Membrane transport and secretion |          |             |             |             |
| Apo9                      | 2.5±0.3     | 34.7±7.1    | 6.8±1.6     | 14.6±3.8    | 2.5±0.5     |
| Mcoln2 (TRPML2)           | 3.9±0.3     | 5.3±0.1     | 4.0±0.6     | 1.5±0.8     | 1.6±0.5     |
| Scl7a11                   | 6.5±0.3     | 19.8±0.1    | 15.3±0.1    | 9.2±0.6     | 1.4±0.2     |
| Cell cycle and proliferation |            |             |             |             |
| Cdkn1a/tp21               | 7.3±2.1     | 12.6±2.2    | 6.2±0.7     | 3.2±0.6     | 1.0±0.1     |
| Phosphatases              |             |             |             |             |
| Dusp1                     | 2.4±0.2     | 9.1±1.8     | 2.2±0.4     | 3.2±1.5     | 0.8±0.1     |
| Dusp2                     | 2.5±0.2     | 1.1±0.5     | 2.2±0.2     | 0.8±0.2     | 0.9±0.2     |
| Regulatory molecules      |             |             |             |             |
| Sqtmi/p62                 | 3.0±0.8     | 5.9±1.8     | 3.2±0.8     | 4.8±1.7     | 1.7±0.1     |
| G protein-coupled receptors |             |             |             |             |
| Ebi2/Gpr183               | 0.3±0.1     | 0.06±0.02   | 0.08±0.01   | 0.24±0.01   | 1.06±0.19   |
| Ptq2                      | 5.3±0.7     | 13.4±0.8    | 7.7±0.6     | 11.9±0.6    | 1.6±0.2     |
| GPRSS5                    | 0.19±0.04   | 0.15±0.03   | 0.29±0.07   | 0.83±0.06   | 1.0±0.2     |
| Ctn2                      | 0.1±0.01    | 0.1±0.02    | 0.15±0.02   | 0.9±0.1     | 1.05±0.15   |
| Host defense and adaptive response |       |             |             |             |
| Tnf2                      | 4.1±0.9     | 2.6±0.3     | 3.2±0.6     | 1.1±0.1     | 1.1±0.2     |
| Stress response           |             |             |             |             |
| Trib 3                    | 0.6±0.1     | 15.2±0.4    | 1.6±0.2     | 15.7±0.3    | 3.6±0.9     |
| Hmox1                     | 0.84±0.04   | 3.6±0.9     | 0.9±0.2     | 3.9±1.2     | 2.1±0.2     |
| Transcription factors     |             |             |             |             |
| Pparg1                    | 0.14±0.01   | 0.27±0.06   | 0.12±0.03   | 1.4±0.3     | 1.27±0.01   |
| Pparg2                    | 0.15±0.02   | 0.06±0.01   | 0.35±0.06   | 1.6±0.2     | 1.0±0.1     |

doi:10.1371/journal.pone.0061462.t005

Network 2 (Figure 3) with a score of 32 contains 27 genes. This network shows Fos, a member of the AP-1 transcription factor family, as a hub of connectivity, as it appears to play a central role possessing 15 edges (via direct connections). Two other transcripts belonging to the AP-1 superfamily, FosL2/Fra-2 and FosL1/Fra-1 (a gene whose product is known to decrease AP-1-dependent transcription) are also present in the Fos interactome. FosL2/Fra-2 was found to be downregulated while FosL1/Fra-1 is upregulated. A series of canonical pathways including those involved in glutathione and xenobiotic metabolism (Mgst2, Gstm5, Gstm6,
Network 2 is in correlation with a set of cellular and molecular functions which include amino acid-, vitamin- and mineral-transporter family, known to belong together with cytochrome P450 enzymes, NAD(P) H:quinone oxido-reductase 1 (Nqo1) and aldehyde dehydrogenase 3 to a group of genes encoding phase 1 and phase 2 xenobiotic metabolizing enzymes [51]. Altogether, Network 2 is in correlation with a set of cellular and molecular functions which include amino acid-, vitamin- and mineral-metabolism and transport as well as cell signaling.

Network 3 (Figure 4) with a score of 28, includes 25 genes. This network presents p38MAPK in a central position and overlaps with canonical pathways for calcium signaling (Nfat5, Itp1, Pka, ATP2A2, Ank2, Ogt, Me2b), FGF pathway (Fig21, ERK1/2), B cell signaling (Me2c, Itp1, ERK1/2, Sh3h2, Creb) and p38MAPK signaling (Dusp8, Me2c, Creb, Nfat3, Erro). The IPA interactome analysis has linked the CBD-stimulated expression of Dusp8 (via the negative modulation of p38MAPK) to the attenuation of LPS-mediated induction of Nfat5, a member of the inflammatory transcription family. In addition, 3 members of the complement component 1q (C1q, C1gh and C1po) that recognize antibody-bound pathogens and trigger the complement classical pathway, were found to be present in the p38MAPK interactome. This network seems to be related to functions of cell signaling, tissue morphology and neurological diseases.

The levels of changes in gene expression induced by combined treatment of LPS and THC (versus that induced by LPS alone) are smaller than those observed for the combined treatment of LPS with CBD (versus LPS alone). These LPS+THC affected genes encompassed three networks of specific molecular interactions. Network 4 (Figure 5) with a score of 32 contains 16 differentially regulated focus molecules and presents the transcription factors AP-1, Akt and NFkB in its central positions. The gene products within the displayed network were found to be part of a number of canonical pathways. For example, the genes X-box binding protein 1 (Xbp1) and Tgfb2/3 are related to the glucocorticoid receptor signaling. The progressive multifocal leukoencephalopathy (Pml) is related to retinoic acid receptor (RAR) activation and PPAR signaling, and the neuraminidase 3 (Nas3) to ceramide pathway and to p38MAPK. The gene Tmem1 is involved in immune response and Vgla and Tlbs1 are related to the angiogenesis pathway. Altogether, this network shows that THC affects several biological processes including immune and lymphatic system development and function as well as tissue morphology.

THC also led to changes in the LPS effects on several other groups of genes. Network 5 received a score of 23 and is populated with 12 genes, with retinoic acid as a transcription regulator positioned at its center (Figure 6). A number of canonical pathways related to axonal guidance (Sema3a, Rock2, Dpp32/Comp2, Dpp35/Cran, Sph3a/Atl1, Foxd3), synaptic plasticity (Rast1), cell adhesion (Adam2), actin cytoskeleton (Rock2, Foxd3) as well as gene products involved in the activation of the nuclear receptor family of transcription factors LXR/RXR (Esl, Raf113a1), were found to participate in this network. Cell to cell interaction as well as tissue development are the major biological processes related to this network.

Finally, Network 6 with a score of 16 and 9 genes shows Tp53, Cdh414 and beta-estradiol at the central nodes of the interactome (Figure 7). This network overlaps with the aryl hydrocarbon and cannabinoid anti-inflammatory networks.
Figure 3. Ingenuity Interactome analysis of the effect of CBD on LPS-affected gene expression. Network 2: The FOS interactome – Interaction between the downregulated AP1 (a modulator of inflammatory mediated cellular functions) and the upregulated FosL1 (a negative regulator of AP1) shows the connection of this network to CBD-anti-inflammatory responses. Details are as indicated in Figure 2.

doi:10.1371/journal.pone.0061462.g003

Figure 4. Ingenuity Interactome analysis of the effect of CBD on LPS-affected gene expression. Network 3: p38MAPK interactome. Details are as indicated in Figure 2.

doi:10.1371/journal.pone.0061462.g004
glucocorticoid receptor signaling. Phosphatidylinositol 3-kinase (PI3K)/Akt (Tp53/Tp53, Cdkn1A, Ccnd1), p53 (Tp53/Tp53, Prcc, Cdkn1A, Gdf15, Ccnd1, Smc3), beta estradiol (Foxn3, Gjb2, Hexim1, Gria4, Calco1) as well as JAK/Stat (Cdkn1A, Klf7) signaling are affected. This network is correlated with functions of cell cycle control, reproductive malfunctions and cancer.

Figure 5. Ingenuity Interactome analysis of the effect of THC on LPS-affected gene expression. Network 4: The AP1-Akt-NFκB interactome Network analysis of the genes whose expression was affected by LPS+THC vs LPS was performed using the Ingenuity software. Details are as indicated in Figure 2.

doi:10.1371/journal.pone.0061462.g005

Figure 6. Ingenuity Interactome analysis of the effect of THC on LPS-affected gene expression. Network 5: The retinoic acid interactome Details are as indicated in Figure 2.

doi:10.1371/journal.pone.0061462.g006
agreement with this result, Malfai et al., [53] reported that in the murine collagen-induced arthritis (CIA) CBD treatment blocks the progression of the disease.

CD69 antigen is another mediator found to be involved in the CIA model of rheumatoid arthritis. This antigen is highly expressed in the leukocyte infiltrates of various chronic inflammatory diseases. In this regard, CIA disease severity is increased by antibody-induced blockade of the transforming growth factor β (TGF-β) in wild type but not in CDB69-/- mice, suggesting that CD69 is a negative modulator of autoimmune response and inflammatory reaction [54]. Our current results show that CBD downregulates the mRNA expression of the LPS-upregulated Cd69 antigen and of Mmp13 suggesting a possible mechanism for the therapeutic activity of CBD in the murine CIA model.

The expression of several genes in the inflammatory cytokine functional category was found to be enhanced by CBD (and to a lower extent by THC). These include the LPS-upregulated expression of Gdf15/MIC-1 and Il-15. From these genes, Gdf15 mRNA expression is upregulated (8.2-fold) in surveillant/resting BV-2 cells by CBD (and 2.4-fold by THC) but synergistically upregulated by CBD+LPS (55.7-fold). Gdf15/MIC-1 is a member of the TGF-β superfamily that plays key roles in the regulation of cellular responses to stress signals, inflammation and tissue repair. Gdf15 mRNA expression was found to be upregulated in activated macrophages by secreted proinflammatory cytokines (including TNFα, IL-1β and IL-6, as well as IL-2 and the macrophage colony-stimulating factor) suggesting that Gdf15 may act through an autocrine loop as an inhibitory factor in the late phases of inflammation by suppressing inflammation through the inhibition of macrophage activation [55].

Other genes highly upregulated by CBD are Lcn2 and Il-15. Lcn2 has been implicated in many cellular processes, such as cell death/survival, cell migration/invasion, cell differentiation, inflammation and iron sequestration, including a role in the acute phase response [56] and references therein). With regard to Arg9, we have previously shown that CBD upregulates Arg9 mRNA in BV-2 cells [14-fold; [13]]. Moreover, CBD synergistically increases (by 877%) the LPS-upregulated Arg9 (3.1-fold). Arg9 participates in the transport of small solutes (such as glycerol, lactate and urea) and takes part in osmotic swelling induced by apoptotic stimuli [57].

Two major kinase-mediated signaling pathways are activated following TLR4 stimulation by LPS. These include the MAPKs and IκB kinase complexes, which lead to activation of AP-1 and NF-κB transcription factors, respectively. Here we show that LPS-upregulated expression of Dusp1 (MKP-1) was significantly upregulated by CBD (5.1-fold) but not by THC. An increase in the expression of Dusp1 was also observed following incubation of resting BV-2 cells with CBD, but not with THC. A similar CBD-mediated regulation of mRNA level was observed for Dusp8, another negative regulator of MAPKs. The IPA interactome analysis has linked this CBD-stimulated expression of Dusp8, via negative modulation of p38 MAPK, to the attenuation of LPS-mediated induction of NFAT5, a member of the inflammatory transcription complex. On the other hand, the LPS-stimulated expression of Dusp2 (Pac-1), a key positive regulator of the inflammatory MAPKs signaling [58], was significantly repressed by CBD as well as by THC. Moreover, CBD but not THC decreased basal levels of Dusp2/Pac-1 mRNA in resting BV-2 cells.

IPA and interactome analysis indicate that pretreatment with CBD, but less so with THC, result in attenuation of LPS-stimulated activation of NF-κB and its dependent gene transcription pathways. Indeed, our previous data show that CBD
This effect of CBD in decreasing NF-κB signaling activity is in line with the CBD-induced diminished transcription of NF-κB-dependent genes, e.g., IL-1β and IL-6 [11]. We and others previously reported that BV-2 cells express CB₁, CB₂, GPR55, GPR18 and TRPV2, G protein-coupled receptors and a channel that are known to interact with cannabinoids [20,30,31,59–61]. Using gene array analysis [13,31], we have observed that the relative levels of CB₁, CB₂, GPR18 and TRPV2 as well as of the fatty acid amide hydrolase (FAAH, known to cleave the endocannabinoid anandamide) gene transcripts were not significantly affected by the cannabinoid treatments and their levels did not exceed the 2-fold induction or 50% reduction by either CBD or THC treatment. On the other hand, we show here that LPS markedly downregulates CB₂ and GPR55 and that this downregulation is not affected by either CBD or THC pretreatment. This result is in agreement with our previous report showing that LPS markedly downregulates CB₂ and GPR55 mRNAs in BV-2 microglial cells and in microglial primary cultures [59].

Network analysis and signaling pathways

A relationship between CBD-mediated oxidative stress response and glutathione depletion was previously reported ([13] and references therein). More recently, we showed that CBD-specific gene expression profile in BV-2 cells displays changes normally occurring under either nutrient limiting conditions or proteasome inhibition, and that are attributed to activation of GCN2/eIF2α/p70S6K/CHOP-Trib3 pathway leading to autophagy as well as to apoptotic cell death [13]. The Trib3 gene product seems to be of high importance to the CBD effect due to its ability to serve as a master regulator of an array of pathways including AP-1 [62], ER stress [63], Akt/PKB [64] and NF-κB [65]. Trib3 expression is significantly upregulated by CBD (16-fold) as well as by THC (3.4-fold) [13] and as observed here, remains upregulated after LPS treatment (CBD + LPS, 14-fold; THC + LPS, 2.3-fold). According to these gene array studies and the qPCR results, LPS by itself does not significantly affect the expression of Trib3 mRNA. IPA interactome analysis of the microarrays data reveals an interaction between the CBD-upregulated Trib3 and the NF-κB transcription factor pathway (as shown in Figure 2). This interaction seems to be responsible for the attenuation by CBD of the transcription of many proinflammatory genes. There are several indications suggesting interaction between these two pathways. First, a direct interaction between p65/RelA and Trib3 protein which induces inhibition of PKA dependent p65 phosphorylation, was described [65]. Second, Trib3 protein can negatively regulate the serine-threonine kinase Akt/PKB [64], a downstream effector of PI3K that has been implicated in the potentiation of NF-κB-induced transcription of proinflammatory mediators [66]. This negative regulation of Akt activity by the highly induced Trib3 gene product could point to the mechanism for the CBD-mediated regulation of LPS-stimulated gene expression. Indeed, the effect of CBD treatment on a number of LPS-stimulated genes as reported here is reminiscent of the effects described for the PI3K inhibitor (LY294002) and for the NFκB inhibitor (BAY 11-7082) in the murine macrophage cell line RAW264.7 activated with LPS [67]. Both PI3K and NFκB signaling pathways exert important roles in gene expression in response to LPS, but they are not overlapping. Specifically, treatment with CBD repressed a number of typical proinflammatory genes (e.g., C/EBPβ, Hix2, Il-1β, Il-1α and Cox2/Ptgs2, which are under the control of both PI3K and NFκB pathways).

Finally, Trib3 was documented to interfere with the inflammatory MAPK signaling via direct interaction with MEK-1 and MKK7 leading to attenuation of AP-1 mediated transcriptional activity in cancer HeLa cells [62]. AP-1 is a transcription factor involved in the regulation of inflammation-mediated cellular functions and has been shown to be inhibited by Nrf2-activating agents [68]. Indeed, our IPA network analysis indicates that the observed decrease in mRNA levels for a number of genes is probably related to a reduction in AP-1 dependent transcription. Additionally, according to these IPA results, this repression is reinforced by combined treatments of CBD and LPS as observed by the induction of FoxJ1 gene product, another negative regulator of AP-1 (see Figure 3).

Trib3 has been shown to downregulate PPARγ transcription and serve as a potent negative regulator of adipocyte differentiation [69] and PPARγ is a molecular target for CBD that could be involved in mediating transcriptional effects in BV-2 microglial cells. Indeed, CBD has been shown to bind to PPARγ in vitro as well as to activate its transcriptional activity in 3T3L1 fibroblast and in HEK293 transfected cells [70]. In addition, Necela et al., [71] described a regulatory feedback loop in which PPARγ represses NF-κB-mediated inflammatory signaling in unstimulated macrophages. Moreover, they show that upon activation of TLR4 in LPS-stimulated macrophages, NF-κB drives down PPARγ expression. These results are in agreement with our results showing that LPS highly downregulates the expression of Pparg1 and Pparg2 (Table 5) in BV-2 cells.

The profiles of CBD-induced gene expression with either resting or LPS-activated BV-2 cells, show that CBD stimulates the transcription of several anabolic genes encoding amino acid biosynthetic enzymes, amino acid transporters and aminoacyl-tRNA synthetases known to be activated by ATF4, a basic leucine zipper transcription factor, that is increased when cultured cells are deprived of amino acids or subjected to endoplasmic reticulum (ER) stress [13] and references therein). The divergent types of stress (nutrient deficiency, ER stress, hypoxia and oxidative stress) converge on a single event—phosphorylation of the translation initiation factor eIF2α, resulting in a general translational pause followed by selective increase in ATF4 mRNA translation and subsequent stimulation of expression of ATF4 target genes [72]. Many of the CBD-affected transcripts (e.g., Herpud1, Hmx1, Nqo1, Gels1, Gstm1, Gstm6, upregulated by CBD and by CBD+LPS treatments) are indeed classified as Nrf2-mediated oxidative stress response genes, including enzymes involved in the biosynthesis of glutathione [13]. Thus, the observed CBD-mediated induction of ATF4-dependent anabolic genes may serve to replenish the amino acids reduced during the elevated turnover of GSH (cysteine, glutamate and glycine). The mechanism underlying CBD action presumably engages generation of ROS which in turn depletes intracellular GSH. Perturbations in redox tone and GSH levels activate the “phase 2 response”, a mechanism used by cells to mitigate oxidative stress [73]. As we have previously shown, many of the “phase 2” gene products are significantly upregulated by CBD [13].

Our present results show that CBD, and less so THC, have immunosuppressive and protective activities that are reminiscent of other clinically applied drugs such as glucocorticoids (GCs; [74]), rexinoids and synthetic triterpenoids [75]. GCs are immunomodulatory agents known to act as suppressive and protective mediators against inflammation. GCs are known to clear antigens by stimulating cell trafficking as well as scavenger systems and matrix metalloproteinases while they stop cellular...
Cannabinoid Anti-Inflammatory Networks

Supporting Information

Figure S1  Functional and pathway annotations according to DAVID Bioinformatics Resources of affected gene products following LPS treatment. Gene Ontology (GO) analysis was performed separately for upregulated (red) and downregulated (green) genes, to identify functional categories and cluster of genes significantly affected by LPS treatment. The GO terms were arbitrarily chosen at various levels within the ontology to avoid redundant allocation. Categories shown are significantly represented at p≤0.05. The bottom pie charts represent the cellular pathways according to KEGG database.

(TIF)

Figure S2  Distribution of differentially expressed genes by global functional annotation and pathway association according to IPA analysis. IPA global functional and pathway analysis was used to examine the enriched functional classes of upregulated (warm colors) and downregulated (cold colors) gene transcripts among the LPS-stimulated genes. The y axis shows the top 8 most representative high-level functions and canonical pathways associated with genes regulated in LPS-treated BV-2 cells. The x axis displays the mean p-value for each associated high-level function and canonical pathway in a -log scale. Increasing value of −log (significance) indicates increased confidence for each category. The vertical gray line in each plot indicates p = 0.05.

(TIF)

Table S1  Primer sequences for qPCR. a Amplicon length in base pairs; b Genbank accession number of cDNA and corresponding gene, available at http://www.ncbi.nlm.nih.gov/; c FW, forward primer; RV, reverse primer

(DOC)

Table S2  Effect of CBD and THC on LPS-upregulated genes. Some genes were validated by qPCR, using β2-microglobulin mRNA as a reference gene.

(XLS)

Table S3  Effect of CBD and THC on LPS-downregulated genes. Some genes were validated by qPCR, using β2-microglobulin mRNA as a reference gene.

(XLS)

Acknowledgments

This work is dedicated to the memory of Maciej Pietr.

Author Contributions

Conceived and designed the experiments: AJ MP ZV. Performed the experiments: AJ MP RL GC. Analyzed the data: AJ MP EK NR FG ZV. Performed the experiments: AJ MP RL GC. Analyzed the data: AJ MP EK NR FG ZV. Contributed reagents/materials/analysis tools: FG GC DG. Wrote the paper: AJ EK NR ZV.

References

1. Kogan NM, Mechoulam R (2007) Cannabinoids in health and disease. Dialogues Clin Neurosci 9: 413–430.
2. Hill AJ, Williams CM, Whalley BJ, Stephens GJ (2012) Phyto cannabinoids as novel therapeutic agents in CNS disorders. Pharmacology & Therapeutics 133: 79–97.
3. Pertwee RG (2002) Cannabinoids and multiple sclerosis. Pharmacol Ther 95: 165–174.
4. Guzman M (2003) Cannabinoids: potential anticancer agents. Nat Rev Cancer 3: 745–755.
5. Di Marzo V, De Petrocellis L (2006) Plant, synthetic and endogenous cannabinoids in medicine. Ann Rev Med 57: 553–574.
6. Goveran A, Noonan J, Campbell VA (2011) The multiplicity of action of cannabinoids: Implications for treating neurodegeneration. CNS Neuroscience & Therapeutics 17: 637–644.
7. Cabral GA, Staab A (2005) Effects on the immune system. Handb Exp Pharmacol 168: 385–423.
8. Klein TW, Cabral GA (2006) Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. J Neuroimmunol Pharmaco 1: 50–64.

Cannabinoid Anti-Inflammatory Networks

immune responses by inhibiting antigen presentation and T cell activation [74]. Synthetic oleanane triterpenoids (SO) were shown to be highly effective (much more than the natural triterpenoids) in many in vivo models in the prevention and treatment of cancer and other diseases with an inflammatory component. Molecular targets of SO include KEAP1 (the inhibitor of the transcription factor Nrf2), PPARγ, IkB kinase, TGF-β signaling and STAT signaling [75]. SO are among the most potent known inducers of the phase 2 response both in vivo and in vitro and affect the expression of several key cell cycle proteins (e.g., cyclin D1, Cdkn1a/p21 and caveolin 1). In some cancer cells, SO signal through PPARγ to inhibit proliferation. The rexinoids bind almost exclusively to the RXRs and are involved in regulation of development, cell proliferation, differentiation and apoptosis [76]. Because RXRs heterodimerize with other receptors (such as RARs, vitamin D receptor, thyroid hormone receptor, PPARx, PPARγ and LXRα), rexinoids modulate the actions of many steroid-like molecules that control metabolism and cellular energetics. In view of these results, triterpenoids and rexinoids are defined as multifunctional drugs. Their targets are either regulatory proteins that control the activity of transcription factors (triterpenoids) or transcription factors themselves (rexinoids). These complex modulatory activities exerted by GCs, rexinoids and SO display a panorama of effects that closely resembles the complex actions of CBD.

Conclusions

We found that CBD induces robust responses related to oxidative stress and inflammation and that CBD, and to a lesser extent THC, have immunosuppressive and protective activities. The anti-inflammatory effects are mediated by mainly downregulating the expression of proinflammatory genes but also by upregulating anti-inflammatory mediators. The impact of pre-treatment with CBD on LPS-stimulated gene expression was much greater than that of THC.

We show here, that CBD highly upregulates genes encoding negative regulators of NF-κB and AP-1 transcriptional activities (e.g., Trib3, Dusp1). CBD response to oxidative stress and inflammation is controlled by Nrf2 and ATF4 transcription factors and seem to involve the Nrf2/Hmox1 and the Nrf2/ATF4 systems. The central role of phase 2-response genes and the ATF4-dependent Trib3 induction offer an explanatory mechanism by which CBD (but less so THC) lowers the LPS-induced activation of microglial cells and leads to attenuation of transcription of many proinflammatory genes.

CBD and THC effects are also found to be linked to new targets, notably the MAPK pathway (Dusp1/Mkp-1, Dusp8 and Dusp2/Pac-1), the JAK/STAT regulatory molecules (Socs3, Cish, Stat1) and the cell cycle related genes (Cdkn2b/p15, Gadd45a, Cdk1a/p21).

All together, our results show that CBD and to a lesser extent THC, are potent modulators of microglial activation and affect several signaling pathways and related networks including induction of cellular stress responses that underlies their high immunosuppressant activity.

References

1. Kogan NM, Mechoulam R (2007) Cannabinoids in health and disease. Dialogues Clin Neurosci 9: 413–430.
2. Hill AJ, Williams CM, Whalley BJ, Stephens GJ (2012) Phyto cannabinoids as novel therapeutic agents in CNS disorders. Pharmacology & Therapeutics 133: 79–97.
3. Pertwee RG (2002) Cannabinoids and multiple sclerosis. Pharmacol Ther 95: 165–174.
4. Guzman M (2003) Cannabinoids: potential anticancer agents. Nat Rev Cancer 3: 745–755.
5. Di Marzo V, De Petrocellis L (2006) Plant, synthetic and endogenous cannabinoids in medicine. Ann Rev Med 57: 553–574.
6. Goveran A, Noonan J, Campbell VA (2011) The multiplicity of action of cannabinoids: Implications for treating neurodegeneration. CNS Neuroscience & Therapeutics 17: 637–644.
7. Cabral GA, Staab A (2005) Effects on the immune system. Handb Exp Pharmacol 168: 385–423.
8. Klein TW, Cabral GA (2006) Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. J Neuroimmunol Pharmaco 1: 50–64.
10. Rieder SA, Chauhan A, Singh U, Nagarkatti M, Nagarkatti P (2010) Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression. Immunology 131: 586–603.

11. Kozea E, Pietr M, Jakub A, Rimmerman N, Levy R, et al. (2010) Cannabinoids Δ9-tetrahydrocannabinol and cannabidiol differentially inhibit the LPS-activated NF-κB and IFNβ/STAT1 proinflammatory pathways in BV-2 reactive microglia. J Biol Chem 285: 1616–1625.

12. Kozea E, Lev N, Kahanovsky E, Eilam R, Rimmerman N, et al. (2011) Cannabidiol inhibits pathogenic T-cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in mice. Brit J Pharmacol 163: 1507–1519.

13. Juknat A, Pietr M, Kozea E, Rimmerman N, Levy R, et al. (2012) Differential transcriptional profiles mediated by exposure to the cannabinoids cannabidiol and Δ9-tetrahydrocannabinol in BV-2 reactive microglia. Brit J Pharmacol 165: 512–2308.

14. Velasco G, Sánchez C, Guzmán M (2012) Towards the use of cannabinoids as antimutagen agents. Nature Rev Cancer 12: 436–444.

15. Wu HY, Goldin R, Meca M, Wang CC, Huang CH, et al. (2012) Cannabidiol inhibits pathogenic T-cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in mice. Brit J Pharmacol 163: 1507–1519.

16. Mechoulam R, Peters M, Murillo-Rodriguez E, Hanus LO (2007) Cannabidiol - the non-psychoactive plant cannabinoid. Series: The Receptors, Vol. 24, pp. 143–171.

17. Ricaux N, Caritis N, Dourish C, Francois JR, Remião FM, Baker HV, et al. (2005) A network-based analysis of systemic inflammation in humans. Nature 437: 1032–1037.

18. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000) Gene ontology: tool for the unification of biology. Nat Genet 25: 25–29.

19. Sullivan SE (2007) Cannabinoids go nuclear: evidence for activation of the NFκB pathway by cannabinoids. Brit J Pharmacol 152: 576–580.

20. Esposito G, Scuderi C, Valenzia M, Togni GA, Latina V, et al. (2011) Cannabidiol reduces LPS- and IL-1β-induced microglial activation and promotes hippocampal neurogenesis through PPARγ involvement. PLOS One 6: e26868.

21. De Petrocellis L, Di Marzo V (2010) Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids and synthetic cannabinomimetics: focus on G-protein coupled receptors and transient receptor potential channels. J Neuropharmacol 5: 103–121.

22. Graber MB, Streit WJ (2010) Microglia: biology and pathology. Acta Neuropathol 119: 89–105.

23. Mechoulam R, Smolikova M, Hanus L, Farkas R, Motro M, et al. (2012) The non-psychoactive cannabinoid constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. PNAS 97: 9561–9566.

24. McHugh D, Hu SS, Rimmerman N, Juknat A, Vogel Z, et al. (2010) N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR12, the putative cannabinoid receptor. J Clin Invest 121: 787–793.

25. Sullivan SE (2007) Cannabinoids go nuclear: evidence for activation of the NFκB pathway by cannabinoids. Brit J Pharmacol 152: 576–580.

26. Sullivan SE (2007) Cannabinoids go nuclear: evidence for activation of the NFκB pathway by cannabinoids. Brit J Pharmacol 152: 576–580.

27. Sullivan SE (2007) Cannabinoids go nuclear: evidence for activation of the NFκB pathway by cannabinoids. Brit J Pharmacol 152: 576–580.

28. Sullivan SE (2007) Cannabinoids go nuclear: evidence for activation of the NFκB pathway by cannabinoids. Brit J Pharmacol 152: 576–580.
68. Kim J, Cha Y-N, Surh Y-J (2010) A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. Mutat Res 690: 12–23.

69. Takahashi Y, Ohoka N, Hayashi H, Sato R (2008) Trb3 suppresses adipocyte differentiation by negatively regulating PPARγ transcriptional activity. J Lipid Res 49: 880–892.

70. Sullivan SE, Sun Y, Bennett AJ, Randall MD, Kendall DA (2009) Time-dependent vascular actions of cannabidiol in the rat aorta. Eur J Pharmacol 612: 61–68.

71. O'Sullivan SE, Sun Y, Bennett AJ, Randall MD, Kendall DA (2009) Time-dependent vascular actions of cannabidiol in the rat aorta. Eur J Pharmacol 612: 61–68.

72. Nguyen T, Sherratt PJ, Nsoi P, Yang CS, Pickett CB (2005) Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. J Biol Chem 280: 32485–32492.

73. Talalay P, Dinkova-Kostova AT, Holczclaw WD (2003) Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Adv Enzyme Regul 43: 121–134.

74. Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, et al. (2002) Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. Faseb J 16: 61–71.

75. Laby KT, Yore MM, Sporn MB (2007) Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. Nat Rev Cancer 7: 357–369.

76. Tanaka T, De Luca LM (2009) Therapeutic potential of rexinoids in cancer prevention and treatment. Cancer Res 69: 4945–4947.