Investigating Receptor-Mediated Effects of Select Cannabinoids on the Innate Inflammatory Response

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LIST OF ABBREVIATIONS

Abbreviation	*Meaning*
2-AG	2-Arachidonylglycerol
A/A	Antibiotic-Antimycotic
A2A	Adenosine A2A receptor
AEA	Anandamide
ALI	Acute lung injury
AM	Alveolar macrophage
ANOVA	Analysis of variance
AP	Activator protein
APC	Antigen-presenting cell
BCA	Bicinchoninic acid
BM	Bone-marrow
BMDM	Bone-marrow-derived macrophage
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CBC	Cannabichromene
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBE	Cannabielsoin
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBL	Cannabicyclol
CBN	Cannabinol
CBND	Cannabinodiol
CBR	Cannabinoid receptor
CBT	Cannabitriol
CCL	Chemokine ligand
CCR	Chemokine receptor
CCRL	Chemokine receptor ligand
CCS	Canadian Cannabis Survey
CD	Cluster of differentiation
CIA	Collagen-induced arthritis
CNS	Central nervous system
COX	Cyclooxygenase
CPZ	Capsazepine
CRE	Cyclic AMP-response element
CSF	Colony-stimulating factor
CXCL	C-X-C motif chemokine ligand
DAG	Diacylglycerol
DC	Dendritic cell
DE	Differential expression
DEG	Differentially expressed gene

DMSO	Dimethyl sulfoxide
DNBS	2,4,6-Dinitrobenzene
ECS	Endocannabinoid system
ENT	Equilibrative nucleoside transporter
ERK1/2	Extracellular signal-regulated kinase ½
FAAH	Fatty acid amide hydrolase
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDR	False-discovery rate
GABA	Gamma-aminobutyric acid
G_i	G-inhibitory alpha subunit
GO	Gene ontology
GPCR	G-protein coupled receptor
$G_{\beta\gamma}$	G beta-gamma dimer
HLA	Human-leukocyte antigen
IBD	Inflammatory bowel disease
IC	Immune complex
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IL-R	Interleukin receptor
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
JAK	Janus kinase
JNK	c-Jun N terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
K_i	Inhibitory constant
LPS	Lipopolysaccharide
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MAPK	Mitogen-activated protein kinase
MCP	Macrophage chemoattractant protein
MEK	MAP/ERK kinase
MeOH	Methanol
MEP	Methylerythritol phosphate
MGS	Mononuclear phagocyte system
MIP	Macrophage inflammatory protein
MKK	MAP kinase kinase
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase
MMPR	Marijuana for Medical Purposes Regulations
MS	Multiple sclerosis
MTT	Tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
nM	Nanomolar
NO	Nitric oxide
NOD	Non-obese model of diabetes
OPC	Oligodendrocyte precursor cell
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PTGS	Prostaglandin-endoperoxide synthase
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated upon activation, normal T cell expressed and secreted
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
siCB2	Small-interfering CB2
siCTRL	Small-interfering Control
siRNA	Small-interfering RNA
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
TAK1	TGF beta activated kinase
TAM	Tumor-associated macrophage
Th1	T-helper type 1
Th2	T-helper type 2
TLR	Toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus-induced demyelinating disease
TNB	Trinitrobenzene
TNF-α	Tumor necrosis factor alpha
T_{reg}	Regulatory T cell
TRP	Transient receptor potential channel
TRPV1	Vanilloid receptor 1
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
Δ^9-THC	Delta-9-tetrahydrocannabinol
Δ^9-THCA	Delta-9-tetrahydrocannabinolic acid
μM	Micromolar

ABSTRACT

There are more than 120 compounds called cannabinoids produced by the plant *Cannabis sativa*. Cannabinoids represent a class of terpenophenolic compounds which interact with the endogenous cannabinoid system (ECS) to exert a diverse assortment of physiological effects from modulation of psychoactivity to the immune response. Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are two of the most abundant cannabinoids found in *C. sativa*. Their ability to impart diverse physiological effects is the result of their interaction with receptors of the ECS that are dispersed throughout the central nervous system (CNS) as well as immune cell surfaces. The two primary receptors of the ECS are the cannabinoid receptor 1 (CB_1) and the cannabinoid receptor 2 (CB_2). CB_1 is predominantly expressed within the central and peripheral nervous tissues and is known to mediate the psychoactive effects of Δ^9-THC. CB_2 is a highly inducible receptor expressed on immune cells with potential roles in inflammatory and oxidative stress processes. While preliminary research has demonstrated the ability of cannabinoids to reduce inflammatory signaling, the consensus surrounding receptor(s) mediated effects has yet to be conclusively demonstrated. Here, we investigated the receptor-mediated properties of Δ^9-THC and CBD on alveolar macrophages, an important pulmonary immune cell that would be in direct contact with cannabinoids inhaled by cannabis smokers. Based on the current state-of-knowledge regarding cannabinoids, we hypothesize that Δ^9-THC and CBD may reduce the innate inflammatory response in alveolar macrophages and promote an anti-inflammatory phenotype. The aims of this study are: (1) to assess the effects of Δ^9-THC and CBD on the inflammatory response of alveolar macrophages; (2) investigate the receptor-mediated effects of these cannabinoids on the inflammatory response; and (3) characterize alveolar macrophage polarization and functional response to these cannabinoids. Through the use of RNA-sequencing and qPCR, we found that

both Δ^9-THC and CBD alone did not affect the basal transcriptional response of MH-S cells, a murine alveolar cell line. Conversely, during lipopolysaccharide (LPS)-induced inflammation, both Δ^9-THC and CBD significantly reduced levels of numerous pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α*),* interleukin (IL) 1 beta*,* and IL-6. This reduction in pro-inflammatory cytokines was accompanied by a shift in macrophage polarization away from an M1 phenotype but not towards an M2 phenotype. Additionally, CBD reduced alveolar macrophage phagocytosis in response to LPS. Further investigation revealed that the anti-inflammatory effects of CBD- but not Δ^9-THC- were mediated through a reduction in signaling through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and extracellular signal-regulated kinase 1/2 (ERK1/2). MH-S cells do not express CB_1 but have constitutive expression of CB_2. To understand the potential role of CB_2, AMs were transfected with small-interfering RNA (siRNA) for CB_2 ($siCB_2$) or control siRNA (siCTRL). The reductions in *TNF-α, IL-1β,* and *IL-6* by CBD and Δ^9-THC in the siCTRL-transfected cells were not present in the $siCB_2$-transfected cells. Thus, CB_2 knockdown abrogates the reductions in pro-inflammatory markers by CBD and Δ^9-THC. This study is the first to investigate the immunomodulatory properties of CBD and Δ^9-THC in alveolar macrophages, which could help provide the basis for new targeted cannabinoid-based therapies in conditions characterized by inflammation.

RESUMÉ

La plante *Cannabis sativa* contient plus de 120 composés qui sont collectivement surnommés cannabinoïdes. Ces cannabinoïdes représentent une classe de composés terpénophénoliques qui interagissent avec le système des cannabinoïdes endogène et exercent divers effets psychologiques médiés allant de la psycoactivité à la réponse immunitaire. Le Δ^9-tétrahydrocannabinol (Δ^9-THC) et le cannabidiol (CBD) sont les composés que l'on retrouve le plus abondamment dans le *Cannabis sativa*. Leur habileté à causer divers effets physiologiques est le résultat de leur interaction avec les récepteurs du système des cannabinoïdes endogène, qui sont dispersés à travers le système nerveux central ainsi qu'à la surface des cellules immunitaires. Les deux récepteurs primaires du système des cannabinoïdes endogène sont communément appelés récepteur cannabinoïde 1 (CB_1) et récepteur cannabinoïde 2 (CB_2). Le CB_1 est principalement exprimé au sein du système nerveux central et périphérique et est reconnu pour médier les effets du Δ^9-THC. Le CB_2 est un récepteur très inductible qui est exprimé sur les cellules immunitaires avec des rôles potentiels dans les procédés inflammatoires et stress oxydatif. Bien que les recherches préliminaires aient démontré l'habileté des cannabinoïdes à réduire la réponse inflammatoire, le consensus concernant les effets médiés par le récepteur(s) n'as été démontré de façon concluante. Dans notre étude, nous avons observé les propriétés du Δ^9-THC et du CBD à la suite de leur liaison avec leur récepteur sur les macrophages alvéolaires, une cellule pulmonaire importante qui serait en contact direct avec les cannabinoïdes lors de l'inhalation du cannabis par les fumeurs. En nous basant sur les données les plus récentes concernant les cannabinoïdes, nous formulons l'hypothèse que le Δ^9-THC et CBD peuvent réduire la réaction inflammatoire des macrophages alvéolaire et promouvoir un phénotype anti-inflammatoire. Cette recherche a pour but : (1) évaluer les effets du Δ^9-THC et du CBD sur la réaction inflammatoire des macrophages

alvéolaire; (2) investiguer la réponse immunitaire suite a la liaison de cannabinoïde à leur récepteur; (3) caractériser la polarité cellulaire et la fonction des macrophages alvéolaires à la suite d'une exposition aux cannabinoïdes. A l'aide du séquençage de l'ARN et du qPCR, nous avons démontré que le Δ^9-THC et le CBD seul n'ont pas eu d'impact sur l'activité transcriptionnelle basale des cellules MH-S, une ligne cellulaire alvéolaire murine. A l'opposé, lors de l'inflammation induite par lipopolysaccharide (LPS), le Δ^9-THC et le CBD réduit considérablement le taux de cytokines pro-inflammatoires dont TNF-α, IL-1β, et IL-6. Cette réduction de cytokines pro-inflammatoires est accompagnée par un changement de polarité des macrophages, s'éloignant du phénotype M1 sans toutefois s'approcher du phénotype M2. De plus, le CBD a eu un effet négatif sur la phagocytose des macrophages alvéolaire. De plus amples investigations ont révélés que les effets anti-inflammatoires du CBD, mais non du Δ^9-THC, s'expriment par une réduction des signaux par le NF-κB et le ERK1/2. Les cellules MH-S n'expriment pas de CB_1 mais ont une expression constitutive de CB_2. Afin de mieux comprendre le rôle des CB_2, les cellules MH-S ont été transfectées avec le CB2 siARN (siCB2) ou avec le contrôle siARN (siCTRL). Le Δ^9-THC et le CBD ont réduit les taux de TNF-α, IL-1β, et IL-6 dans les cellules transfectées avec siCTRL, mais cette réduction n'a pas été observée dans les cellules transfectées avec siCB2. Ainsi, l'abattre du CB_2 abroge les réductions des marqueurs pro-inflammatoires par le Δ^9-THC et le CBD. Cette étude est la première à s'intéresser aux propriétés immunomodulatrices du Δ^9-THC et du CBD dans les macrophages alvéolaires. Ce travail pourrait donc constituer la base de nouvelles thérapies ciblées à base de cannabinoïdes dans le but de traiter les maladies caractérisées par l'inflammation.

CHAPTER 1 INTRODUCTION

1.1 Historical Advents and Medicinal Applications of *Cannabis Sativa*

1.1.1 Cannabis Sativa Prior to the Christian Era

The cultivation of cannabis can be traced back as far as 12,000 years, placing the plant among humanity's oldest cultivated crops[1]. The exact origin of the plant is believed to be in Central Asia, more specifically in the regions of Mongolia and southern Siberia. Dating back to 4800 BCE, the earliest cultural evidence of cannabis use can be derived from the Yangshao, a Neolithic culture in China that appeared along the Yellow River Valley[1]. During this time up and until 3000 B.C.E., their economy was primarily driven by cannabis with archaeological and historical findings indicating the plant was cultivated for fibers[1]. Those cannabis stem-derived fibers would subsequently be used to manufacture strings, ropes, textiles, and even paper, some of which have been discovered in the tomb of Emperor Wu (104-87 B.C.) of the Han dynasty[1]. The Chinese additionally consumed cannabis as a form of fruit that was small, smooth, and had a hard shell. With the Christian Era beginning and the Han dynasty ending, cannabis was no longer considered an important food in China. However, the seeds are still used to this day as cooking oil in regions of Nepal[2].

The first recorded use of cannabis as a medicinal drug occurred in 2737 B.C. by the Chinese emperor Shen Nung, and was reported in the world's oldest pharmacopoeia, the *pen-ts'ao ching*[2]. While the first reference of cannabis as a psychoactive drug appeared in this text, it also documented its effectiveness in the treatment of pain associated with rheumatism, intestinal disorders, gout, malaria as well as others. The ancient Chinese took advantage of every part of the cannabis plant: the root would typically be used for medicines; the stem for textiles, rope, and paper; the leaves and flowers for intoxication and medicine; and the seeds for food and oil[2]. This

demonstrated the wide range of use of cannabis sp. in ancient China. The first medical journals in China were made of hemp and eventually came to replace papyrus, clay tablets and expensive silks as the primary source of paper[1]. During the beginning of the Christian Era, Hua T'o, the founder of Chinese surgery, would administer a portion of the cannabis plant, in addition to wine, as an anesthetic during surgical operations[1]. The portion of the plant used primarily for medicinal purposes were the seeds, which are deficient in the primary active constituent of cannabis- delta-9-tetrahydrocannabinol (Δ^9-THC)[3].

Aside from the *pen-ts'ao ching*, few other ancient Chinese texts describe cannabis as a hallucinogen, which is perhaps the result of shamanism, a common religion in central Asia that declined in popularity during the Han dynasty. The use of cannabis in shamanism was incredibly popular and based on the principles that it brought on a long-lived trance-like state, promoted deep and profound experiences with others, relieved anxiety and fear, and put the dead to rest as a funerary herb[2]. Although shamanism became restricted in China, it was widely disseminated in regions of Central and Western Asia, as well as India, leading to an increase in cannabis use for both recreational and medicinal purposes in those regions[4]. Moreover, many coastal farmers from China, between 2000 B.C. and 1000 B.C., took cannabis to countries including Korea and India. Such broad use in these countries may be attributed to the association between cannabis and religion that was maintained during these times. Many religious practices assigned sacred virtues to cannabis, including the *Atharva Veda*, a collection of sacred texts that highlighted cannabis as one of five sacred plants that "donated joy, freedom, and happiness,". As such, the use of cannabis in many religious rituals became common practice[2].

The psychoactive properties of cannabis were well known in India prior to the Christian era, likely due to three different preparations that were commonly used. The first preparation was called *Bhang*, which solely consisted of the dry leaves, with the flowers of the plant being removed. *Bhang* was the most common preparation in India when the region was controlled by Aryans, a group that spoke an archaic Indo-European language. The Aryan religion was documented in the four Vedas, which are large bodies of religious texts originating in India, where they worshipped the spirits of plants and animals, with cannabis and hemp being primary constituents in rituals, clothing, and medicine[2]. However, *Bhang* was the weakest of the three preparations, followed by *Ganja*, a preparation consisting of the female flowers. The strongest of all three was *Charas*, which was made exclusively from the resin that covers these flowers[2]. Regardless of the preparation, active cannabinoids were present as cannabis contains solitary resin glands found on the flowers, leaves, and trichome stalks which contain large amounts of active cannabinoids that are liberated upon consumption[5]. The timeline for the medical and religious uses of cannabis in India can be dated around 1000 B.C. with the plant having many uses including as an analgesic, anti-inflammatory, anti-biotic, diuretic, *etc*.,[6].

Other regions and cultures that have documented evidence of cannabis use prior to the Christian era were in Tibet, where Tantric Buddhism was commonly practiced, and cannabis considered sacred[2]. The medical uses of cannabis were very common as a result of Hindi medical practices that were derived by the Tibetans. Botany played a key component in their pharmacopeia; cannabis was very plentiful in that region and was commonly used in meditation[2]. Another region with historical and archaeological evidence of cannabis use prior to the Christian era was in Europe. Evidence suggests that the plant came alongside Scythian invaders that had originated in Central Asia and settled near the Mediterranean[7]. According to Herodotus, Scythians in the year 450 B.C.

inhaled the vapors produced from burning cannabis seeds during a funeral ceremony for ritualistic and euphoric purposes. This was discovered by archaeologists that found charred cannabis seeds in Scythian tombs[7]. Similarly, archaeologists unearthed thirteen female cannabis plants from an ancient tomb in northern China that were found lying diagonally across a man believed to be a shaman[8].

1.1.2 Beginning of Christian Era to 18th Century

While there is little evidence of cannabis use in Greek and Roman culture prior to the Christian era, once this era began there are some references to the use of cannabis seed-oil as a means to treat earaches and to deter insect infections[6,7]. During that time, the medical use of cannabis remained very popular in India and had spread to regions in the Middle East and Africa. Mentions of the medical uses of cannabis can be found in the compendiums of well-known physicians such as Avicenna as well as in Muslim texts that describe its use as a diuretic and in treating conditions of the digestive system and the ear[9]. One specific document refers to the son of the caliph's chamberlain who was cured of his epilepsy from treatment with cannabis resin[7]. The use of cannabis in Africa has been documented since the 15th century and is believed to originate from Arab traders that had a connection with India[10]. Uses of cannabis in Africa tended to differ from those of other cultures and regions among which its uses include snakebite, childbirth, malaria, asthma and dysentery[10]. By the 16th century, cannabis had made its way to South America, likely as a result of slaves arriving from Angola, as most references to cannabis in these regions have their origin in the Angolan language[11]. Religious rituals praising African deities such as the 'Catimbó' were common practice at the time and often included the use of cannabis for its magical properties and ability to treat pains relating to tooth ache and menstrual cramps[11]. During the 16th century in Europe, the use of cannabis was restricted to its cultivation for fibers, with very few

references to the medicinal properties of the plant[7]. The manufacturing of paper from cannabis was initially introduced by the Chinese in 1534 BCE and was then adapted by Muslims in 1150 initially in Spain and eventually in Italy[7]. The cultivation of cannabis in these regions has been documented in texts as far back as the 18th century, with references to the distinctions between male and female cannabis plants and the various practical uses of their fibers[7].

1.1.3 Medicinal Applications of Cannabis in the 19th & 20th Centuries

During the 19th century, the use of cannabis in western medicine was investigated by the Irish physician William B. O'Shaughnessy and French psychiatrist Jacques-Joseph Moreau. O'Shaughnessy was first introduced to cannabis while working as an assistant surgeon in India and researching possible therapies for diseases such as cholera[12]. From his time there, O'Shaughnessy studied the available literature on cannabis including the various preparation methods and toxicities in animals. From this, he later tested the effects of these preparations in patients with various pathologies. In 1839, he published his work '*On the preparations of the Indian hemp, or gunjah (Cannabis Indica), their effects on the animal system in health, and their utility in the treatment of tetanus 6 and other convulsive disorders*' where he described the various effects of cannabis in animals and humans[13]. In animals such as fish, dogs, and cats, he observed the intoxicating effects of the drug. However, when administered in large animals including horses, cows, and goats, even at a wide range of doses, only minimal effects were observed. While he did note the inability of cannabis to treat conditions like rheumatism or cholera, cannabis was capable of calming and pain relief effects in patients. Even though cannabis did not cure many of these ailments, it was noted that in conditions characterized by muscle spasms such as tetanus and rabies, cannabis was able to ease muscle spasticity[13]. Ultimately, O'Shaughnessy concluded that while cannabis may not be capable of curing many of these conditions, it remains beneficial as part of

their treatment. Upon his return to England, O'Shaughnessy produced two more written works entitled '*The Bengal Dispensatory*' in 1842 and '*The Bengal Pharmacopoeia*' in 1844[14,15], both of which have been described as the most comprehensive assessments of the properties of cannabis and have led to increased interest in the medicinal properties of the plant in the western world.

Jacques-Joseph Moreau was first introduced to cannabis while working as an assistant physician at the Charenton Asylum near Paris[16]. At the time, it was common practice to accompany psychiatric patients on long trips to distant countries. It was during this time that Moreau observed the common use of hashish (cannabis resin) in the Middle East. Upon his return to France in 1840, Moreau began conducting systematic experiments using different cannabis preparations, initially on himself and then subsequently on his students. In 1845 he published '*Du Hachisch et de l'Alienation Mentale: Études Psychologiques*' wherein he describes the acute effects of cannabis exposure[17]. In contrast to O'Shaughnessy- who researched the therapeutic properties of the plant- Moreau investigated the psychoactive effects of cannabis on mental abilities to understand the genesis of mental illness. Ultimately, it was the contributions of both O'Shaughnessy and Moreau that had the greatest impact on the use of cannabis in Western medicine because during this time, therapeutic interventions for disease such as rabies, cholera, and tetanus were scarce. As such, the medical use of cannabis had spread from England and France to all over Europe and eventually America.

As the use of cannabis in Western medicine gained attention, the first cannabis monograph was introduced into the 3rd edition of the American Herbal Pharmacopoeia in 1851, classifying cannabis as a botanical medicine[18]. This monograph marked a compendium of scientific data, and included standards for plant identity, purity, quality as well as botanical properties. Most

importantly, the monograph provided a foundation for healthcare professionals to integrate the use of cannabis-based therapies into their practices based on scientific understanding, components and biological effects. This led to 100 scientific articles being published in the latter half of the 19th century in Europe and America on the therapeutic effects of cannabis[19]. One of those articles was written by Wood and colleagues in 1899, who isolated the first cannabinoid in pure form from the exuded resin of Indian hemp[20]. From the constancy of the compound found in preparations of 'Charas' it was believed to be a chemical of the formula $C_{18}H_{24}O_2$. The compound they isolated was later determined to be cannabinol (CBN) but was initially (wrongly) assumed to be the main active component of cannabis responsible for its psychoactive effects[20].

1.1.4 Commercial Cannabis-Based Medications

The peak of cannabis use in Western medicine occurred in the late 19th and early 20th century. During this time various cannabis laboratories had begun selling cannabis extracts or tinctures that were made by soaking the dried flowers of the female cannabis plant in ethanol, leading to the cannabinoids being dissolving[9]. Other forms of extraction would use the water-based plant products such as chlorophyll or involve drying the cannabis leaves, leading to decarboxylation, which increases the active cannabinoids in the extract[9]. Companies that were making cannabis extracts included Merck (Germany), Burroughs-Wellcome (England), Bristol-Meyers Squibb (United-States), Parke-Davis (United States) and Eli Lilly (United States)[9]. However, the medical use of cannabis tapered off in the early decades of the 20th century for a multitude of reasons, but most notably due to the difficulty of obtaining replicable effects as a result of the variability in the plant samples. At the time, the primary active component of cannabis had not been isolated and the drug was most commonly administered through tincture or extracts whose efficacy was largely dependent on origin, age and mode of preparation[9]. Additionally, cannabis was rivaled by the

development of novel medications used to treat the same conditions. For instance, vaccines had been developed for diseases such as tetanus, analgesics such as aspirin for pain management, hypodermic needles for administration of morphine and for its sedative properties, and substances such as chloral hydrate, paraldehyde, and barbiturates were much more effective[9]. Moreover, the production of these cannabis extracts and tinctures had come to a halt due to the Marihuana Tax Act of 1937. This stated that anyone using the plant had to register and pay taxes depending on the use of the plant[21]. For medicinal purposes, the tax was $1 for every ounce and for any other use it was $100 per ounce. However, non-payment of this tax would result in a $2000 fine and/or 5 years imprisonment[21]. Ultimately, this law made it very difficult to make use of the plant due to the excess of required paperwork and the risk of a severe punishment. Eventually cannabis was removed from the American Herbal Pharmacopoeia in the 12th edition released in 1942[18].

1.1.5 Key Advents in Cannabis Research

In 1963, a second cannabinoid was isolated by Mechoulam and Shvo that turned out to be cannabidiol (CBD)[22]. The following year, the primary active constituent of cannabis, Δ^9-THC, was isolated by Gaoni and Mechoulam[23]. At the same time, a meteoric increase in cannabis consumption throughout the western world was occurring, especially in the younger age-range of the population. In 1967, the percentage of young adults who had used cannabis more than once was 5%; by 1971 usage increased to 44%, and by 1980 was 68%[24,25]. A combination of both increased consumption by the general public and the isolation of key cannabinoids led to a significant increase in scientific interest for cannabis. Publications during this time period primarily followed the isolation of select cannabinoids and evaluation of their interactions. However, from the mid 1970's until the 1990s cannabis research saw a decline in the number of publications. It was not until the identification of the specific binding sites for THC in the brain

were discovered by Devane and colleagues that cannabis research was renewed[26]. This was followed by the cloning of CB_1 by Matsuda and colleagues in 1990, leading to what is termed the 'cannabinoid receptor system' due to the binding affinity of THC to this receptor as a partial agonist[27]. Shortly after, CB_2 was discovered by Munro and colleagues in 1993[28]. It was ultimately these findings that led to the discovery of the endocannabinoid system (ECS) by Devane and colleagues who extracted an ethanolamine of arachidonic acid (AEA) that bound to these receptors[29]. The endocannabinoid agonist was named anandamide, which is a Sanskrit word meaning 'bliss'. It was only 3 years later that the second endocannabinoid was discovered by Mechoulam and colleagues which was termed 2-arachidonylglycerol (2-AG)[30]. From that point forward, publications about cannabis have been growing, attesting to the immense interest in the plant and its potential applications, and most notably the therapeutic properties associated with the primary cannabinoids. Current studies are investigating THC for its role as an anti-emetic, stimulant of appetite, analgesia, and ability to treat symptoms associated with multiple sclerosis (MS)[31]. As for CBD, studies are investigating its potential therapeutic benefit in epilepsy, insomnia, anxiety, inflammation, and many more[32,33].

1.1.6 Medicinal & Recreational Legislation on Cannabis

While the therapeutic benefits of cannabis are promising, it has taken considerable time for many countries to begin recognizing them. In Canada, cannabis was added to the Confidential Restricted List in 1923 under the *'Narcotics Drug Act Amendment Bill'* in order to prohibit the improper use of opium and other drugs. While there were many proponents that contributed to the criminalization of cannabis, one of the primary drivers in Canada was the country's involvement in international conferences such as the League of Nations where the topic was discussed. As such, it was not until July of 2001 that the legislation on access to cannabis for medicinal purposes was

established by Health Canada[34]. Under this legislation, physicians could prescribe cannabis at their liberty if the patient can be classified in one of two categories. Category 1 describes a patient undergoing end-of-life care or has symptoms relating to pain from MS, spinal cord injury/disease, cachexia, anorexia, weight loss, nausea, arthritis, or seizures from epilepsy. Category 2 describes patients who have debilitating symptoms other than those mentioned in Category 1[34]. In April of 2014, the Medical Marijuana Access Program was replaced by the Marijuana for Medical Purposes Regulations (MMPR) by Health Canada wherein legal medical cannabis production was authorized to licensed producers by Health Canada. Patients wishing to fulfill a medical cannabis prescription had to receive a medical document from a healthcare worker and needed to obtain authorization to possess from Health Canada. Individuals would then register with a licensed producer of their choice[34]. On April 13th of 2017, a bill to legalize cannabis by July 1st of 2018 was introduced to parliament that would allow individuals older than 18 years of age to possess cannabis in quantities less than 30 grams. On June 19th of 2018 the senate passed the bill and the then Prime Minister Justin Trudeau announced legalization that would begin October 17th, 2018, making Canada the second nation (behind Uruguay) to federally legalize cannabis for medicinal and recreational purposes (Figure 1.1)[34]. Currently there are in excess of 147 million people worldwide (2.5% of the world population) who consume cannabis, ranking it among the most widely cultivated and consumed drug considered to be illicit internationally[35].

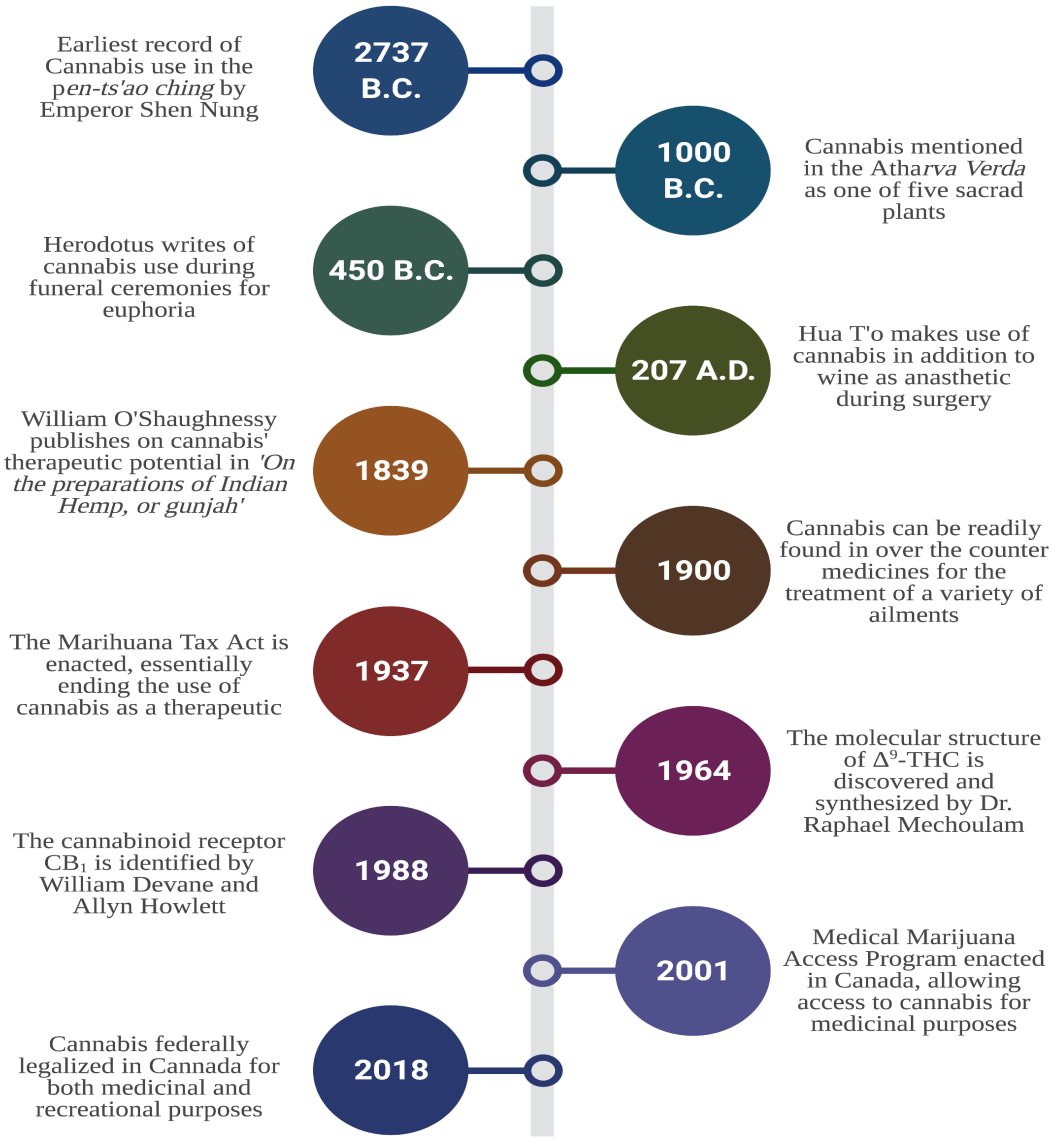


Figure 1.1. Timeline of cultural and medical milestones in cannabis. Summary of events beginning with the first recorded use of cannabis in 2737 B.C. up until the federal legalization of cannabis in Canada for both medicinal and recreational use.

1.1.7 Cannabis Use in Canada

Based on statistics conducted in the 2020 Canadian Cannabis Survey (CCS), there has been an increase in the percentage of Canadians who consume cannabis[140]. Twenty seven percent of Canadians have reported using cannabis within the past 12 months, an increase from 25% in the

2019 CCS, of which, people between the ages of 16 to 24 years reported cannabis use at a percentage nearly double that of people 25 years and older; males recorded a higher percentage of cannabis use compared to females. Of note however, is the frequency in which cannabis is consumed, with 47% having reported cannabis use at least 1-2 days per week, and 18% reporting daily cannabis use. Smoking remains the most popular method of consumption (79%) followed by eating it in food (52%), vaporizing using a vape pen or e-cigarette (24%), and vaporizing using a dry herb vaporizer (12%). Despite prominent cannabis use in young adolescents, many of the diverse physiological effects of cannabis have yet to be ascertained.

1.2 Cannabinoids and the Endogenous Cannabinoid System (ECS)

1.2.1 Cannabis Taxonomy

Cannabis sativa L. (Cannabaceae) is a plant that can be found in a variety of different habitats and altitudes, ranging from sea level all the way up to the foothills of the Himalayas[36]. The number of species within the genus *Cannabis* has long been debated. Previously, cannabis was characterized based on its polytypic nature that was further subdivided into three separate species: *C. sativa, C. indica,* and *C. ruderalis*[37]. Conversely, many taxonomists only recognize the *C. sativa* and *C. indica* species of the *Cannabis* genus[38]. Regardless of these interpretations, *Cannabis* is widely recognized as constituting a single, highly diverse species called *C. sativa* L. with *C. sativa, C. indica,* and *C. ruderalis* being acknowledged as varieties of the species[39]. The *sativa* and *indica* varieties are far more widespread than *ruderalis*, which is considered a hardier variety characterized by sparse growth and minimal drug content[40]. When comparing the *sativa* and *indica* varieties, these can often be distinguished by their physical features. *Sativa* plants are typically taller, with heights ranging from 2.5 to 3.5m, whereas *indica* plants are generally shorter (1.8m),

bushier, broader and have darker green leaves[39,40]. Other methods of differentiating varieties is through the cannabinoid profile of the plant, with *sativa* being primarily Δ^9-THC dominant and *indica* being CBD dominant[41]. *C. sativa* L. is typically dioecious in nature, *i.e.,* male and female flowers developing on separate plants. At the vegetative state, it is very difficult to differentiate male and female plants morphologically, as sexual dimorphism typically does not occur until the onset of flowering, at which point sex can be determined. Moreover, male plants typically contain lower amounts of Δ^9-THC compared to female plants[42].

1.2.2 Cultivation

C. sativa can be grown annually under both indoor and outdoor conditions, each with respective advantages and disadvantages. Seeding of cannabis crops outdoors typically occurs at the beginning of April and goes until November depending on the variety. While outdoor crops tend to yield more biomass than indoor crops, growth is limited by the solar photoperiod which regulates flowering and maturation of the plants. This means that outdoor conditions are limited to one crop per year whereas indoor conditions, which permit regulation of the photoperiod, can yield three to four crops per year[43]. Moreover, due to the difficulty of regulating crop conditions, outdoor crops can have altered chemical profiles as a result of cross-fertilization. While vegetative propagation through cuttings can be used to maintain uniformity in the chemical profile, indoor cultivation achieves this in a much more efficient manner. Indoor cultivation allows for complete control of the plant cycle through light level, photoperiod, humidity, temperature, CO_2 concentration and air circulation; this means that the quality and quantity of biomass can be managed[44]. This is particularly important under circumstances where the production of *C. sativa* requires a specific chemical profile for pharmaceutical use. Another key component in the cultivation of *C. sativa*, regardless of indoor or outdoor conditions, is to ensure the chemical profile

characterized by optimal Δ^9-THC content during the harvesting, processing, and storage periods. The content of Δ^9-THC increases with plant age and peaks at the budding stage wherein that level is maintained for one to two weeks with an ensuing decrease at the onset of senescence[43]. During the processing stage, it is crucial the material be well-dried and sealed to prohibit the degradation of cannabinoids. Δ^9-THC for instance is very sensitive to oxygen and UV light, which oxidizes and converts it to CBN[43]. This process can even occur at room temperature. Therefore, the preferred conditions for long-term storage are low-temperature and the absence of light.

1.2.3 Cannabis Chemotaxonomy

C. sativa is chemically-complex due to its production of hundreds of secondary metabolites. In order to characterize the phenotype and cannabinoid profile of *C. sativa*, qualitative and quantitative analysis can be employed. Ultimately, it is the concentration of Δ^9-THC in the dried inflorescence that determines the psychoactivity of the plant. However, the three primary strains of *C. sativa* are dependent not only on Δ^9-THC content, but also on the quantity of CBD[45]. *C. sativa* can be classified into three separate strains according to the ratio of Δ^9-THC/CBD content. Strain I, or the drug-type, is based on strong presence of Δ^9-THC (1-20%) and a Δ^9-THC/CBD ratio greater than 1. Strain II, or the intermediary-type, is characterized primarily by the presence of CBD but also with Δ^9-THC present (0.3–1.0%), meaning a Δ^9-THC/CBD ratio roughly equivalent to 1. The third and final strain, commonly known as fiber-type or hemp, is based upon a low content of Δ^9-THC (<0.2-0.3%) with a Δ^9-THC/CBD ratio less than 1[46]. While the cannabinoid profiles in the various regions of the plant at different growth stages are subject to environmental factors, the ratio of Δ^9-THC and CBD in most strains are genetically controlled.

1.2.4 Cannabinoid Biosynthesis and Constituents

Cannabinoids belong to a class of secondary metabolites called terpenophenolic compounds that are chemically-related to terpenes with a ring structure derived from a C_{10} monoterpene subunit. The biosynthesis of cannabinoids follows the plastidial methylerythritol phosphate (MEP) pathway which begins with the coupling of olivetolic acid with geranyl phosphate through prenylase, leading to the formation of cannabigerolic acid (CBGA)[47]. CBGA is the precursor for many cannabinoids. CBGA becomes oxidized by different flavin adenine dinucleotide (FAD)-dependent oxidases, particularly Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) synthase and cannabidiolic acid (CBDA) synthase, which form Δ^9-tetrahydrocannabinolic acid and cannabidiolic acid, respectively (Figure 1.2)[48]. Δ^9-THCA and CBDA undergo a decarboxylation reaction during heating, forming Δ^9-THC and CBD[48]. In recent decades, the total number of natural compounds identified or isolated from *C. sativa* has greatly increased. In 1980, there were 423 compounds isolated from *C. sativa*. As of 2015, this number has increased to 565[43,49]. Of those isolated compounds, only 120 constitute the definition of a cannabinoid, which exhibits the typical C_{21} terpenophenolic skeleton. Classification of these cannabinoids follows 11 general types based upon their structure: Δ^9-THC, Δ^8-THC, cannabigerol (CBG), cannabichromene (CBC), CBD, cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), CBN, cannabitriol (CBT) and miscellaneous types[43].

1.2.5 Δ^9-THC & CBD

Of the approximately 120 cannabinoids derived from *C. sativa*, Δ^9-THC and CBD represent two of the most extensively-studied. The literature on these compounds has shown that while they display similar effects in many physiological domains including inflammation and immunomodulation, they conversely can impart diverse effects such as psychotropic mediation and memory impairment. Structurally, Δ^9-THC and CBD are near identical and share the exact

same molecular formula of $C_{21}H_{30}O_2$. However, they differ in that Δ^9-THC forms a cyclic ring whereas CBD forms a hydroxy group (Figure 1.2)[50]. This leads to Δ^9-THC existing in a planar conformation whereas CBD adopts a conformation in which the two rings are at a right angle to each other. Ultimately, it is this subtle molecular discrepancy that accounts for the differences in the three-dimensional structure of the compounds, thereby altering their affinities and interactions with endogenous cannabinoid receptors (CBRs). This results in CBD being unable to bind to or activate the CB_1 receptor, unlike Δ^9-THC[50]. Δ^9-THC is the primary psychoactive compound in *C. sativa* that dose-dependently produces hypoactivity, hypothermia as well as spatial and verbal memory impairment. Conversely, CBD is non-psychoactive and is incapable of regulating locomotor activity, body temperature, or memory[51]. CBD has also been implicated as a potential anti-convulsant, anti-inflammatory and anti-tumorigenic therapy.

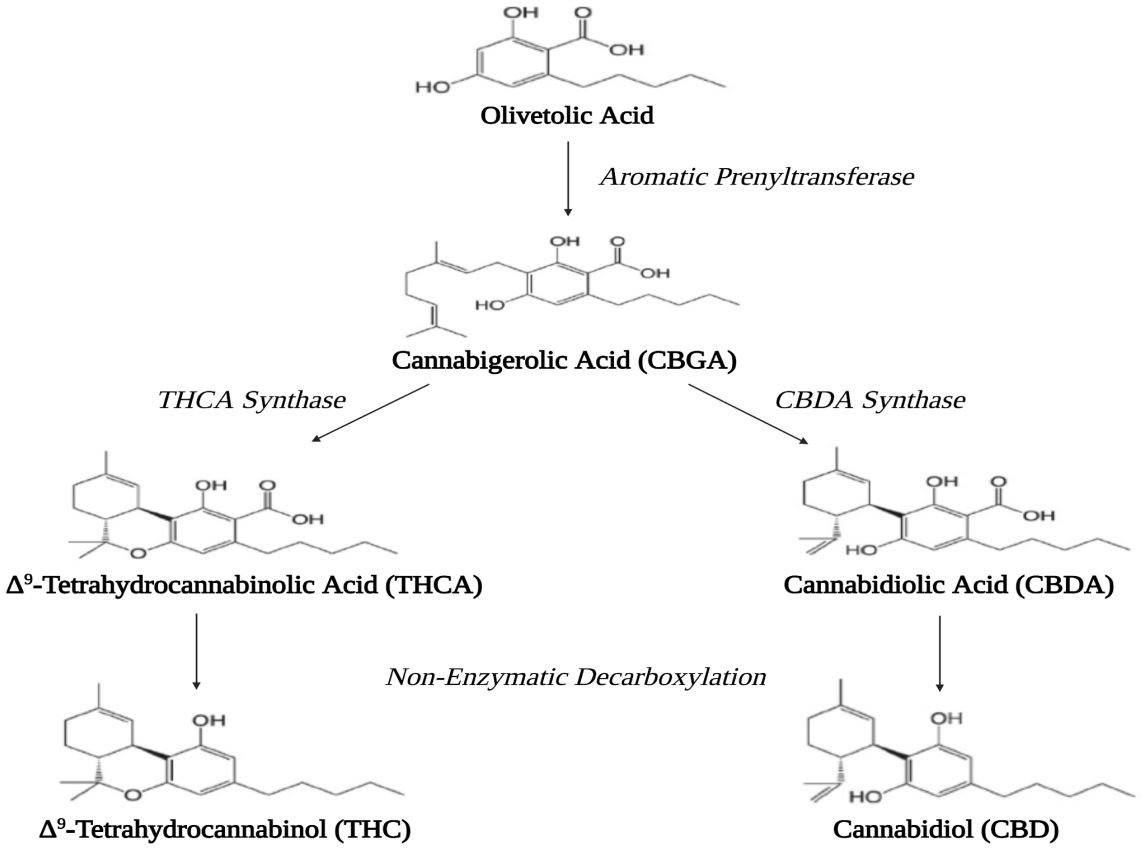


Figure 1.2. Biosynthesis of cannabinoids and structural differences between Δ^9-THC and CBD. Conversion of olivetolic acid to cannabigerolic acid (CBGA) occurs through the use of aromatic prenyltransferase. CBGA acts as the point of differentiation from which cannabinoid-specific FAD-oxidases (THCA synthase & CBDA synthase) convert CBGA to precursor cannabinoid acids. Subsequent decarboxylation of cannabinoid acids results in active cannabinoids. Δ^9-THC forms a cyclic ring whereas CBD has a hydroxy group resulting three-dimensional structural differences. Adapted from McCormick *et al*[142].

1.2.6 Cannabinoid Receptors 1 & 2

The majority of the pharmacological effects of Δ^9-THC are mediated by CB_1 and CB_2[52]. CB_1 and CB_2 share 44% amino acid homology and are G protein coupled receptors (GPCRs), meaning that they have seven transmembrane domains. CB_1 and CB_2 are coupled to the G-inhibitory (G_i) alpha subunit such that binding of an agonist to the GPCR results in a conformational change that is transmitted to this particular subunit which forms a heterotrimeric G protein complex. Then, the

G_i subunit dissociates from the G beta-gamma ($G_{\beta\gamma}$) dimer and the receptor. This allows the G_i and $G_{\beta\gamma}$ subunits to interact with intracellular proteins to propagate the signal transduction cascade, while the freed CB_1 or CB_2 is able to rebind another heterotrimeric G protein to form a new complex. Upon release, the $G_{\beta\gamma}$ subunit inhibits voltage-dependent calcium channels and activates inward rectifying potassium channels. Simultaneously, the G_i subunit inhibits adenylate cyclase which stimulates the phosphorylation and early activation of extracellular signal-regulated kinase ½ (ERK1/2). ERK1/2 activity is additionally promoted by the activation of β-arrestin-1, another downstream product of CB_1 and CB_2 activation[52]. Ultimately, activation of CB_1 and CB_2 receptors results in the stimulation of mitogen-activated protein kinase (MAPK) activity and the inhibition of cyclic AMP (cAMP) production. Δ^9-THC acts as an agonist of both CB_1 and CB_2 receptors, displaying inhibitory constant (K_i) values in the nanomolar (nM) range. CBD on the other hand acts as an antagonist of CB_1 and CB_2 receptors with K_i values in the micromolar (μM) range. Cannabinoid mediated activation of CB_1 and CB_2 results in the promotion of similar downstream signaling pathways; however, the roles these receptors play in cellular physiology including synaptic function, gene transcription, and cell motility can be quite diverse. Another key difference is that CB_1 is expressed heterogeneously throughout the central nervous system (CNS) with expression being densest in areas of cognition and short-term memory (cerebral cortex and hippocampus) in addition to motor function and movement (basal ganglia and cerebellum). CB_1 receptors are expressed to a lesser degree in peripheral tissues but does include the liver, thyroid, uterus, and bones[53]. CB_1 mediates the psychotropic effects of Δ^9-THC and has additionally been shown to regulate pain, stress responses, energy regulation, as well as lipogenesis. Within the brain, CB_1 receptors can be found at the terminals of central and peripheral neurons to inhibit the action of a number of excitatory and inhibitory neurotransmitter systems including dopaminergic,

gamma-aminobutyric acid (GABA), acetylcholine and more[52]. Conversely, the expression of CB_2 within the CNS is very minimal and is significantly less than that of CB_1. It has however, been characterized under certain pathological conditions including nerve injury or inflammation. Ultimately, the expression of CB_2 receptors is predominantly limited to immune cells in addition to certain organ systems such as the respiratory system. Within populations of immune cells, CB_2 receptors follows decreasing levels of expression in the following order (highest to lowest): B cells, macrophages, monocytes, natural killer (NK) cells, and T cells. The expression of CB_2 is highly inducible and can increase up to 100-fold in response to tissue injury or inflammation. Additionally, the activation of CB_2 is devoid of any undesired psychotropic effects[54]. As such, agonists targeting CB_2 have been proposed as potential therapies for the treatment or management of conditions characterized by acute and chronic inflammatory pain[54].

1.2.7 Other GPCRs

The interaction of cannabinoids with endogenous receptors is not limited to CB_1 and CB_2, as other GPCRs with no endogenous ligands are proposed to bind cannabinoids. Among the orphan GPCRs, five have been linked with the ECS, including GPR5, GPR18, GPR55, GPR92 and GPR119, many of which have limited sequence homology with CB_1 and CB_2[55]. Additionally, the pharmacology of these orphan receptors displays significant overlap with CB_1 and CB_2, particularly for GPR18 and GPR55. GPR55 is activated by Δ^9-THC, CBD, certain synthetic cannabinoids, and the endocannabinoids AEA and 2-AG[55]. GPR55 differs from CB_1 and CB_2 in that it is coupled to the $G_{12/13}\alpha$ subunit rather than the $G_{i\alpha}$ subunit and increases levels of intracellular calcium upon activation[56]. Compared to CB_1 and CB_2, GPR55 has limited sequence homology of 14% and 15% respectively[57]. However, cannabinoids such as Δ^9-THC have weak GPR55 activity while CBD may be an antagonist of the receptor. GPR18 is expressed within

immune tissues such as the spleen, thymus, peripheral blood leukocytes and lymph nodes[58]. Δ^9-THC, CBD and anandamide act as partial agonists of GPR18[59]. Of the previously mentioned GPCRs, GPR119 has the most limited homology with CB_1 and CB_2 receptors; however endogenous ligands that have demonstrated activity at these receptors also have activity for GPR119[60].

1.2.8 Other Receptors

While many of the pharmacological effects of cannabinoids have been attributed to the CB_1 and CB_2, there is a growing body of literature demonstrating the ability of cannabinoids to interact with other receptors, including the adenosine receptors (A2A). There are four known A2A receptors that comprise a class of purinergic GPCRs named A1, A2A, A2B, and A3[132]. Their primary ligand is the purine nucleoside adenosine, which functions in mitigating excessive cellular damage and inflammation during periods of acute stress[133]. The protective effects of adenosine are mediated by A2A receptors that can be found on virtually all immune cells[133]. This is accomplished through an induction of cyclic-AMP leading to subsequent activation of protein kinase A. This cascade leads to inhibition of T cell differentiation, downregulation of neutrophil superoxide production, as well as an inhibition of pro-inflammatory cytokine production[132]. Adenosine signaling is negatively regulated by equilibrative nucleoside transporters (ENTs) which function in the reuptake of adenosine[132]. Cannabinoids, including CBD, have been shown to act as competitive ENTs resulting in subsequent anti-inflammatory and immunosuppressive effects through an inhibition of adenosine uptake, thereby leading to enhanced adenosine signaling[93]. As such, further investigation into the interaction between cannabinoids and A2A receptors is currently being conducted.

Another class of receptor that has garnered significant attention as it relates to the pharmacological effects of cannabinoids is the vanilloid receptor 1 (TRPV1). TRPV1 is a homotetrameric membrane protein belonging to the transient receptor potential channel (TRP) family of which there are six known channels. This receptor is found predominantly within afferent sensory neurons and is involved in processes including regulation of body temperature, nociception, and detection of noxious environmental stimuli[92]. TRPV1 may be activated by a number of exogenous and endogenous stimuli however, the best characterized activators include temperatures greater than 43°C, low pH, capsaicin, and allyl isothiocyanate[131]. TRPV1 is responsible for the detection of stimuli which in turn promote the influx of calcium as a second messenger for the induction of pro-inflammatory cytokines and chemokines. Similar to the CB receptors, the activation of TRPV1 may lead to downstream signaling such as ERK1/2 and other MAP kinase cascades which activate nuclear factor-κB (NF-κB), resulting in the induction of pro-inflammatory cytokines[92]. Cannabinoids such as CBD can bind TRPV1 receptors[131]. It has been proposed that the interaction between cannabinoids and TRPV1 leads to the desensitization of the receptor which subsequently promotes an analgesic phenotype; however, this has yet to be demonstrated[131]. As such, the role of TRPV1 receptors in mediating the pharmacological effects of cannabinoids is currently of particular interest.

1.2.9 Endocannabinoid System

The discovery of CBRs prompted the search for endogenous ligands that bind these receptors. AEA and 2-AG are the primary endogenous ligands for CB_1 and CB_2[61]. The precursor molecules of AEA and 2-AG are present within lipid membranes, where upon activation, follow one or two enzymatic steps that liberate the endocannabinoids into the extracellular space[61]. This process, in addition to endocannabinoid degradation, occurs in a very timely manner. In contrast to

neurotransmitters, endocannabinoids are not stored in vesicles or cells but rather are synthesized on demand from lipid precursors within the cytoplasmic membrane in response to increases in intracellular calcium[61]. In the case of AEA biosynthesis, precursor phosphatidylcholine and phosphatidylethanolamine lipids are converted to N-arachidonoyl phosphatidylethanolamine and subsequently to AEA via the actions of N-arachidonoyl ethanolamine-phospholipase[65]. The biosynthesis of 2-AG is contingent on the conversion of 2-arachidonate containing phosphoinositides to diacylglycerols (DAGs) which are then converted to 2-AG through DAG lipase[65]. Endocannabinoids are removed from the site of action through cellular uptake processes such as simple diffusion, membrane associated binding proteins, or by a transmembrane carrier protein and are metabolized by fatty acid amide hydrolase (FAAH)[65]. Despite both AEA and 2-AG demonstrating a shared ability to be released on demand, they make use of analogous but distinct receptor-dependent pathways. They additionally share similarities in chemical structure such as their arachidonic acid skeleton characterized by a 20-carbon chain and four cis-double bonds; however, their intrinsic efficacies within the ECS vary[62]. 2-AG is a high-efficacy agonist of the CB_1 and CB_2 receptors with K_i values of 472 nM and 1400 nM, whereas AEA is a very low-efficacy agonist for CB_1 and a low-efficacy agonist for CB_2 receptors with K_i values of 5810 nM and 1930 nM, respectively[62,63]. In certain pathological conditions such as cancer, cardiovascular disorders, MS, and excitotoxicity, the tissue concentration of endocannabinoids, CBR density, as well as the efficiency with which the CBRs couple to endocannabinoids is increased, which results in a reduction in symptoms[64]. As such, there is a growing body of literature to support a role of the ECS in physiological processes such as lipogenesis, nociception, inflammation, and immune modulation. In cell types such as macrophages and leukocytes, treatment with inflammatory stimuli such as LPS increases secretion of AEA and 2-AG in addition to a decrease in the

concentration of FAAH[65]. Endocannabinoids are well described for their ability to negatively regulate the activity of adenylyl cyclase and MAPKs[66]. Likewise, both CB_1 and CB_2 receptors have similar properties in modulating MAPK and adenylyl cyclase activity which both play a significant role in lymphocyte regulation[67]. For instance, cAMP signaling cascades have a positive role on immune cell function, indicating that CBR stimulation antagonizes the acute response during immune cell activation[68]. This is supported in studies demonstrating that AEA inhibits macrophage-mediated killing of tumor necrosis factor-sensitive fibroblasts in addition to reducing the expression of LPS-induced pro-inflammatory mediators such as nitric oxide[69]. 2-AG has a similar ability to suppress pro-inflammatory cytokines, including IL-6 in macrophages, an effect mediated by CB_2[70]. It is now evident that endocannabinoid stimulation of the CBRs within the immune system modulates processes such as cyclic AMP and MAP kinase activity, which both play key roles in immune homeostasis. Despite the function of CBRs within the immune system, their underlying mechanisms are yet to be fully defined.

1.2.10 Evidence of Cannabinoid Immunomodulation in Disease Models

Given the body of literature supporting a role of the ECS in modulating the immune response, the therapeutic potential of cannabinoids including Δ^9-THC and CBD have been investigated under a variety of disease models. At the core, many of these pathologies can be characterized by acute or chronic inflammation that further contributes to the severity of the disease. As such, greater understanding of the mechanisms governing the immunomodulatory properties of cannabinoids can have profound implications with regards to novel cannabinoid-based therapies.

1.2.10.1 Effects of Cannabinoids on Cytokines & Chemokines

Bouts of inflammation, acute or chronic, are regularly characterized by excess cytokines and chemokines at the site of action. The immunomodulatory properties of cannabinoids are founded in their ability to mitigate or reduce the number of cytokines and chemokines in models of disease characterized by inflammation. In a model of acute ocular inflammation, CBD inhibited LPS-induced release of TNF-α due to inhibition of p38 MAPK phosphorylation[82]. Similarly, treatment with either CBD or Δ^9-THC prevented microglial activation, macrophage infiltration, and inhibited serum and retinal release of TNF-α in LPS-treated rats. CBD additionally decreased the production and release of pro-inflammatory cytokines IL-1β, IL-6, and interferon (IFN) β in microglial cells treated with LPS while also reducing the activity of the pro-inflammatory transcription factor NF-κB and increasing the activation of the signal transducer and activator of transcription (STAT) 3 transcription factor[83]. Collectively, these studies support the ability of cannabinoids such as CBD and Δ^9-THC to exert anti-inflammatory properties, an effect that is characterized by a reduction in pro-inflammatory cytokine production.

Conversely, the current body of literature surrounding the mechanism by which cannabinoids exert their anti-inflammatory properties has yet to see consistent findings with regards to receptor mediation. One study that investigated this phenomenon evaluated the effects of CBD in a mouse model of Alzheimer's disease-related neuroinflammation[81]. Using this model, mice were inoculated with human amyloid-beta peptide and treated with or without CBD. Here, CBD dose-dependently reduced the protein expression of both inducible nitric oxide synthase (iNOS) and IL-1β and similarly decreased nitric oxide (NO) production. While the inhibitory effects of CBD on inflammatory markers were believed to be CB_2 mediated, no direct evidence was presented. In a similar model of neuroinflammation using hypoxic-ischemic mice, CBD significantly reduced IL-6, TNF-α, cyclooxygenase (COX)-2, and iNOS levels. This effect however, was mitigated upon

application of the A2A antagonist SCH58261[93]. Further evidence in support of A2A receptors mediating the reductions in pro-inflammatory cytokines by cannabinoids can be seen in a study whereby CBD significantly reduced the levels of TNF-α found in the serum of LPS-treated mice[94]. However, this reduction was markedly reduced in the presence of the A2A receptor antagonist ZM241385 and was abolished in mice not expressing the A2A receptor ($A2A^{-/-}$). Similarly, in a model of liver inflammation using concanavalin A, treatment with CBD significantly reduced the levels of pro-inflammatory cytokines IL-2, TNF-α, IFN-c, IL-6, IL-12p40, IL-17, macrophage chemoattractant protein (MCP) 1, and chemokine ligand (CCL) 11[92]. However, the use of vanilloid receptor 1 knockout ($TRPV1^{-/-}$) mice showed that the immunosuppression imparted by CBD in concanavalin A-treated mice was dependent on TRPV1. Despite consistent findings with regards to the ability of cannabinoids to suppress the production of pro-inflammatory cytokines across different models of inflammation, the receptor-mediated effects have yet to be validated.

1.2.10.2 Effects of Cannabinoids on Edema and Hyperalgesia

In addition to the anti-inflammatory effects of cannabinoids on cytokine production, they have notable effects on edema and hyperalgesia, two additional features of inflammation. Following the acute onset of inflammation induced by intraplantar injection of carrageenan, CBD was administered orally once a day for 3 days in rats, and prostaglandin E_2 (PGE_2), COX, NO, and other oxygen-derived free radical activity was assayed in the paw tissue; these increased following carrageenan paw injections[84]. CBD decreased PGE_2 plasma levels, COX activity, as well as oxygen-derived free radical and NO production. However, co-treatment with the TRPV1 antagonist capsazepine (CPZ) reversed the anti-hyperalgesia effects of CBD. In a similar model of carrageenan-induced edema and hyperalgesia, Δ^9-THC given through oral gavage resulted in reduced paw withdrawal latency and paw thickness. This was shown to be contingent on the CB_1

receptor, as administration of SR141716 (CB_1 antagonist) abolished the anti-hyperalgesia effects[165]. Studies have also investigated the therapeutic potential of CBD in a 2,4,6-dinitrobenzene (DNBS)-induced murine model of colitis. CBD significantly reduced colitis-associated edema, hyperemia, as well as macroscopic damage associated with DNBS administration[120]. Thus, there is evidence of a protective effect of CBD in pathologies characterized by excess inflammation and its associated clinical symptoms including edema and hyperalgesia.

1.2.10.3 Cannabinoids and Arthritis

In models of collagen-induced arthritis (CIA), the expression of pro-inflammatory cytokines such as TNF-α and IL-1β within the arthritic joints are significantly increased and are a major contributor of clinical symptoms[85]. As such, CBD has been investigated as a potential therapeutic agent. In a mouse model of CIA, oral CBD significantly diminished production of collagen II, IFN-y, as well as TNF-α from synovial cells[85]. Similarly, CBD dose-dependently suppressed lymphocyte proliferation as well as the production of reactive oxygen species (ROS). These data suggest that CBD improves clinical outcomes and is associated with protection of the joints and inhibition on the progression of CIA. This led to the conclusion that the combined anti-inflammatory and immunosuppressive actions of CBD yield potent anti-arthritic effects. In the same model of CIA in mice, Δ^9-THC reduced arthritis through a prevention of inflammatory cell infiltration, synovium hyperplasia, and cartilage damage[166]. It additionally inhibited the expression of inflammatory and catabolic genes within the knee joints, an effect that was shown to be contingent on the CB_1 receptor. *In vitro* culture of human synovial fibroblasts from patients with rheumatoid arthritis treated with CBD revealed increased intracellular calcium levels, reduced cell viability, and led to reductions in IL-6, IL-8 and matrix metalloproteinase (MMP) 8 production. Additionally, the suppressive effect of CBD appeared to be enhanced by TNF-α pre-treatment

meaning CBD may preferentially target pro-inflammatory (activated) synovial fibroblasts, suggesting potential anti-arthritic activity[128].

1.2.10.4 Effects of Cannabinoids on Demyelinating Pathologies Including Multiple Sclerosis

Oligodendrocytes play a key role in maintaining the myelin sheath surrounding neurons. In pathologies characterized by demyelination, the efficiency in which nerve impulses are transmitted is significantly reduced. CBD may protect against damage to oligodendrocyte precursor cells (OPCs)[86]. In OPCs treated with CBD, there is protection against oxidative stress through decreased production of ROS. Another facet through which CBD is protective is by attenuating OPC apoptosis via a decrease in caspase-3 induction. In an animal model of MS, Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV) is characterized by inflammation[87]. In this model, CBD reduced leukocyte infiltration in the brains of TMEV-infected animals and also significantly reduced microglial activation in the cerebral cortex. The levels of pro-inflammatory cytokines TNF-α and IL-1β were also reduced by CBD. Another study using the TMEV model further investigated the role of the CB_2 receptor through the use of the receptor-specific agonist JWH-015. Administration of JWH-015 in mice subjected to TMEV reduced microglial activation, abrogated MHC II antigen expression, and decreased the number of $CD4^+$ T cells infiltrating the spinal cord. The recovery of motor function and reduction in inflammation by JWH-015 were also observed in accordance with extensive remyelination[121]. Further evidence to support the role of the CB_2 receptor in ameliorating disease characterized by demyelination was demonstrated in a study by Docagne and colleagues[122]. In a model of TMEV, the CB_2 receptor-specific agonist HU-210 prevented axonal damage, reduced excitotoxicity, and increased motor activity. However, these effects were mitigated upon application of the CB_2 receptor antagonist SR144528. Finally, in patients with MS, the novel CB_2-selective agonist COR167 dose-

dependently reduced the proliferation of both peripheral blood mononuclear cells (PBMCs) and myelin basic protein-reactive T cells. This was due because of an incomplete shift in phenotype from T-helper type 1 (Th1) to T-helper type 2 (Th2) with reductions in IL-4, IL-5, and Th17-related cytokines[127]. Similarly, the immunosuppressive effects of CBD by inhibition of adenosine reuptake and regulation of A2A receptors have additionally been evaluated in a TMEV model of MS[87]. Treatment with CBD inhibited the production of vascular cell adhesion molecule (VCAM) 1, a mediator of lymphocyte adhesion and leukocyte infiltration, that is typically increased in inflammation. Furthermore, CBD also reduced CCL2 and CCL5 chemokine expression as well as TNF-α and IL-1β. However, these effects were A2A-dependent, as they were not observed following administration of ZM241385. Taken together, these studies highlight the therapeutic potential of cannabinoids and their receptors in demyelinating pathologies through modulation of inflammatory mediators, enhancement of remyelination, and stimulation of OPC survival and maturation.

1.2.10.5 Cannabinoids and Inflammatory Bowel Disease (IBD)

Many of the protective functions of cannabinoids have been attributed to their antioxidant functions. For example, such antioxidant activity can markedly inhibit colon injury as well as iNOS induction and IL-1β production in a murine model of colitis induced by intracolonic administration of trinitrobenzene (TNB)[88]. Another study that made use of the TNB model investigated the therapeutic potential of Δ^9-THC and observed marked decreases in myeloperoxidase activity, neutrophil infiltration, as well as the frequency in which motility disturbances occurred[167]. Moreover, in a mouse model of croton oil-induced hypermotility, CBD reduced hypermotility[89]. It was suggested that this in part, was the result of CBD indirectly inhibiting FAAH. The protective effects likely involve the CB_2 receptor. Studies also made use of

the TNB model to evaluate the role of the CB_2 receptor through the use of receptor-specific agonists JWH-133 and AM1241. Both agonists significantly reduced macroscopic damage scoring, myeloperoxidase activity, and colonic shortening. However, these effects were mitigated upon the application of the CB_2 receptor antagonist AM630[90]. Additionally, JWH-133 and AM12421 were ineffective when applied in CB_2 receptor knockout mice. Other studies have made use of IL-10 deficient mice (IL-$10^{-/-}$) in studying IBD due to altered lymphocyte and myeloid profiles, elevated serum amyloid A, altered responses to autoimmune or inflammatory stimuli, and spontaneous development of chronic enterocolitis. One study that made use of the IL-$10^{-/-}$ model showed that JWH-133 reduced the percentage of $CD4^+$ T cells, neutrophils, mast cells, NK cells, and activated T cells within the colon and that these effects were reversed following administration of AM630[123].

The route of administration of CBD may be important in protecting the gut. In the TNB model of colitis, CBD, when given via rectal and intraperitoneal routes markedly reduced the extent of colitis whereas oral administration was ineffective[88]. Finally, in *ex vivo* cultured human colonic biopsies from patients with ulcerative colitis, treatment with CBD reduced protein levels of TNF-α, iNOS, and the astroglial signaling neurotrophin S100B[130]. This was the result of CBD targeting reactive enteric gliosis which counteracted the inflammatory environment present in patients with ulcerative colitis. Collectively these studies demonstrate that cannabinoids acting through the CB_2 receptor reduce inflammation and pathology associated with IBD.

1.2.10.6 Effects of Cannabinoids on Inflammatory Lung Disease

Acute lung injury (ALI) can be characterized by a disruption of the vascular endothelium and the alveolar epithelium that subsequently leads to the loss of alveolar-capillary membrane integrity,

excessive neutrophil transmigration, and the release of pro-inflammatory mediators[91]. As such the impact of endocannabinoids, cannabinoids and even synthetic CBR agonists have been investigated as a means to better understand their therapeutic potential. In a model of ALI, prophylactic treatment with CBD reduces inflammation[91]. In LPS-induced ALI, for example, CBD was given at a concentration of 20 mg/kg or 80 mg/kg and pulmonary mechanics and inflammatory markers were measured. CBD decreased total lung resistance and elastance, neutrophil migration into the lungs, and myeloperoxidase activity in the lung tissue. It also reduced the concentration and production of pro-inflammatory cytokines TNF-α and IL-6 as well as chemokines MCP-1 and macrophage inflammatory protein (MIP) 2. Δ^9-THC similarly has a therapeutic effect in an LPS-induced model of ALI in mice whereby intranasal administration significantly reduced TNF-α levels as well as the number of infiltrating neutrophils[163]. Additionally, in an endotoxin B-induced model of lung inflammation in mice, Δ^9-THC given through intraperitoneal injection decreased mortality, vascular leakage, leukocyte infiltration, as well as the concentration of pro-inflammatory cytokines[164]. As such, these studies demonstrate the positive impact that cannabinoids impart on lung damage and inflammation while highlighting novel strategies in ALI therapy development.

1.2.10.7 Cannabinoids and Diabetes

Type 1 diabetes (T1D) can be characterized by excess invading immune cells within the pancreas leading to inflammation and eventual death of insulin-producing beta cells. In a non-obese model of diabetes (NOD), CBD lowered the incidence of diabetes. Prophylactic treatment with CBD resulted in a later onset of T1D in addition to significantly reducing leukocyte activation within the pancreas[124]. Similar studies revealed that CBD pre-treatment significantly reduced insulitis as well as the incidence of T1D in NOD mice[125]. Additionally, administration of CBD to NOD mice

in a latent stage of T1D significantly ameliorated features of the disease including reductions in TNF-α and IFN-y within the plasma as well as an increase in the synthesis of Th2-associated cytokines IL-4 and IL-10[125,126]. In a separate mouse model of diabetes that made use of streptozotocin, a compound that damages pancreatic β cells leading to hyperinsulinemia and hyperglycemia, Δ^9-THC given orally transiently attenuated streptozotocin-induced elevation in serum glucose and loss of pancreatic insulin[168]. Additionally, Δ^9-THC was able to reduce the mRNA expression of IFN-y, TNF-α, and IL-12. Collectively, these studies demonstrate the potential of cannabinoids to modify the inflammatory process and delay the appearance of hyperglycemia-related complications typically observed in patients with diabetes.

1.2.11 Considerations in Cannabinoid-Based Therapies

Cannabinoids possess the ability to modulate immune function and thus may be a therapeutic target for pathologies characterized by acute or chronic inflammation. However, the mechanistic processes that mediate the immunomodulatory properties of major cannabinoids are inconclusive. In neuropathic conditions characterized by pain, inflammation and degeneration, CB_1 is a potential molecular target for therapeutic intervention, particularly given the density of its expression within neural tissues. The caveat to this is that activation of CB_1 also has psychotropic effects, dependence and cognitive impairment[71]. As such, much attention has been given to CB_2 largely due to its expression in cells and tissues of the immune system and because its activation is devoid of psychotropic effects. In many models of inflammation, agonists of the CB_2 receptor have demonstrated dose-dependent reductions in inflammation. This includes inhibition of the mixed lymphocyte reaction (MLR), reductions in the concentrations of pro-inflammatory cytokines such as TNF-α, IL-6, nitric oxide synthase (iNOS), as well as increases in the concentration of anti-inflammatory cytokines such as IL-10[72]. The majority of the immunomodulatory effects of Δ^9-

THC have been attributed to the activation of the CB_2 receptor; however, the activation of CB_2 by Δ^9-THC also results in the activation of CB_1 leading to unwanted psychotropic effects. As such, therapies specific to CB_2 including the synthetic ligand WIN 55212-2 and CBD analog HU-320 are under investigation[72]. Although the immunomodulatory properties of CBD are described, its specific receptor-mediated effects have yet to be elucidated.

1.2.12 Cannabinoids & Immune Cell Function

Despite numerous studies exploring immunomodulation by cannabinoids using both *in vitro* and *in vivo* systems in the context of disease, there still remain many questions surrounding their underlying mechanism of action. It is well known that the interaction of cannabinoids with CBRs causes inhibition of adenylate cyclase activity and subsequent prevention of forskolin-stimulated cAMP activation. This ultimately leads to decreased activity of protein kinase A as well as binding of transcription factors to cAMP-response element (CRE) consensus sequences[72]. Cannabinoid modulation of the immune response during inflammation has been demonstrated in many disease models, as previously mentioned. Results from these studies indicate that cannabinoids exert their immunomodulatory properties in four different ways: 1) induction of immune cell apoptosis, 2) suppression of cell proliferation, 3) inhibition of pro-inflammatory cytokine/chemokine production and increase in anti-inflammatory cytokines, and 4) induction of regulatory T cells (T_{regs})[73]. While discussion on the second and third pathways mentioned above have been central themes to many of these studies, the importance of the effects of cannabinoids on individual immune cell populations cannot be overstated. As such, there is a growing body of literature describing the effects of cannabinoids on cells comprising both the innate- and adaptive-immune systems.

1.2.13 Cannabinoids & Adaptive Immunity

In the context of disease, cannabinoids have demonstrated a potent ability to modulate the cytokine and chemokine profiles at the site of action. However, the individual cell types that secrete and respond to these cell-signaling molecules have additionally been characterized in response to cannabinoids. Δ^9-THC for example inhibits proliferation of human lymphocytes in culture and leads to apoptosis of murine macrophages and T cells through the regulation of Bcl-2 and caspase activity[74]. The effects of Δ^9-THC are significantly greater in naïve rather than activated lymphocytes, which may be the result of decreased CB_2 expression in activated cells[75]. This was further investigated in a study that made use of the CB_2 agonist JWH-015[143]. In a dose-dependent manner, JWH-015 not only inhibited proliferation, but also induced apoptosis in naïve- and activated-splenocytes and -thymocytes. In a separate study, apoptosis was similarly observed in $CD4^+$ and $CD8^+$ T cell populations in response to CBD at concentrations in the micromolar range in a time- and dose-dependent manner[144]. In concanavalin A-treated mice, Δ^9-THC increased levels of apoptosis in activated T cells while simultaneously increasing the number of T_{regs}. It has been proposed that the T_{regs} not only inhibited the induction of cytokines induced by concanavalin A but also may be resistant to apoptosis in response to Δ^9-THC and suppress the activation of T cells that escape apoptosis. Similarly, Δ^9-THC and CB_2 agonists can also inhibit the differentiation of monocytes into antigen-presenting dendritic cells (DCs) which results in an inability of DCs to stimulate T cell proliferation or mature into functional effector/memory T cells[77].

B cells express high levels of CB_2, and a number of studies have investigated the potential for immunomodulation of B cells, the antibody-producing cells of the adaptive immune system, in response to cannabinoids. Several reports have shown that cannabinoids have different effects on B cells including altered proliferation as well as reduced antibody production[145]. One study

demonstrated that in response to Δ^9-THC or the synthetic cannabinoid analog WIN 55212-2, there is a dose-dependent increase in B cell proliferation[146]. Conversely, following an increase in B cell proliferation by LPS, cannabinoids can inhibit this effect[147]. In human B lymphoblastoid cells, Δ^9-THC and CBD have a protective effect in response to serum-deprived cell death[148]. This effect was independent of CB_1 and CB_2. In the same cells, treatment with Δ^9-THC resulted in a protective effect against oxidative stress and cell death induced by retinod anhydroretinol[148]. Endocannabinoids, including 2-AG, may stimulate the migration of splenocytes in a CB_2-dependent manner in addition to inducing B cell differentiation[149]. While this may suggest a positive role for cannabinoids in mobilizing B cells during immune responses, it is not clear whether these effects are the result of direct interaction with B cells or indirectly through T cells or other cells of the innate immune system.

1.2.14 Cannabinoids & Innate Immunity

The innate immune system comprises several different cell types which play critical roles in the maintenance and regulation of acute phase proteins as well as defense against foreign pathogens. In addition to acting as the first line of defense, the innate immune system functions in collaboration with adaptive immunity in order to mount a full immune response. Given the immunomodulatory properties of cannabinoids, their interaction with the various cells of the innate immune system have been investigated.

NK cells function in host defense against infectious pathogens and limit the degree to which infection spreads through the termination of infected cells. Very few studies have investigated the effect of cannabinoids on NK cells however, it has been shown that in response to Δ^9-THC or CBD, there is an inhibition of the constitutive expression of IL-8, MIP-1α, MIP-1β, regulated upon

activation, normal T cell expressed and secreted (RANTES), TNF-α, GM-colony stimulating factor (CSF), and IFN-y[150]. Similarly, *in vitro* studies have shown that Δ^9-THC can suppress NK cell function including cytolytic activity in rats, mice, as well as humans[151,152,153]. Extending upon this finding was a study that showed Δ^9-THC administered subcutaneously in mice could inhibit NK cell cytolytic activity, an effect that could be reversed by antagonists targeting either CB_1 or CB_2[154]. As such, there is evidence in support of a suppressive effect of cannabinoids on NK cell function.

Neutrophils play important roles in early anti-microbial responses. Despite being one of the first cells shown to express CB_2, there are considerably few studies that have described neutrophils in response to cannabinoids. It has, however, been shown that, cannabinoids induce the release of lysosomal enzymes from neutrophils in addition to modulating their response to chemokines[155]. Cannabinoids have additionally been shown to inhibit superoxide formation by neutrophils in a manner that was independent of CB_1 or CB_2[156]. In summary, the current body of literature describing the interaction between cannabinoids and neutrophils is rather sparse and warrants further investigation.

Mast cells are bone marrow-derived cells commonly found within connective- and mucosal-tissues that play a predominant role in mediating inflammatory reactions. Currently, the expression of CBRs on mast cells has not seen consistent findings. One study in human mast cells was unable to detect the CB_1 or CB_2 but did however demonstrate an ability of mast cells to transport and release large quantities of AEA[157]. Conversely, two mast cell lines do express CB_1 and CB_2 at both the mRNA and protein level[158]. Additionally, the application of selective CB_1/CB_2 agonists resulted in the activation of ERK1/2. Another study was able to measure levels of CB_2 mRNA in rat

peritoneal mast cells wherein administration of Δ^9-THC dose-dependently released histamine irrespective of the CBRs[159]. Similarly, treatment with AEA, WIN 55212-2, or HU-210 induced secretion of histamine in rat mast cells, an effect that was once again independent of the CBRs[160]. In summary, there is considerably more to research to be done with regards to the role of cannabinoids in mast cell immunology.

DCs represent potent and specialized antigen-presenting cells (APCs) of the immune system. Their roles encompass both the initiation of immune responses and the development of T cell responses. Both CB_1 and CB_2 are expressed in human DCs in addition to anandamide, 2-AG, and FAAH, suggesting they have a fully functioning ECS[161]. The immunosuppressive effects of cannabinoids on DCs have been demonstrated whereby *in vivo* administration of Δ^9-THC decreased amounts of splenic DCs as well as reduced expression of MHC II[162]. *In vitro* studies have demonstrated that Δ^9-THC and AEA induce apoptosis in murine bone marrow-derived DCs through an activation of caspases 2, 8, and 9. This effect was dependent on the engagement of the cannabinoids with both CB_1 and CB_2[162]. Collectively, these studies suggest that DCs may be important peripheral targets for cannabinoids.

Macrophages are important mediators in both innate and adaptive immunity with roles that include phagocytosis of infectious agents, presentation of antigenic peptides, and secretion of acute phase proteins including NO, TNF-α, IL-1β, and IL-6. In mouse peritoneal macrophages, treatment with Δ^9-THC significantly downregulates NO production as well as TNF-α maturation and secretion[134]. In RAW264.7 macrophages, reduction in NO production was a consequence of Δ^9-THC inhibiting the activation of NF-κB[135]. Δ^9-THC can also impair the phagocytic activity of the P388D1 cell line.[136]. Subsequent studies have also revealed that in response to Δ^9-THC, macrophages display

a differential response in the ability to process antigens necessary for presentation to $CD4^+$ T lymphocytes[137]. There is also an impaired ability to produce Th1 cytokines as well as a downregulation in the expression of co-stimulatory molecules and MHC II. These effects involve both CB_1 and CB_2. Collectively, these studies indicate a negative overall effect of Δ^9-THC on antigen presentation, which may impair the immune response to pathogens.

Although studies have shown effects for Δ^9-THC on macrophage function, evidence for CBD is sparser. One study that made use of the human monocytic cell line U-937 investigated the ability of CBD to affect pro-inflammatory cytokines with and without LPS[138]. This study revealed differential effects of CBD on IL-8, MCP-1, and cellular ROS levels. Following induction by LPS, CBD attenuated NF-κB activity, as well as IL-8 and MCP-1 production. However, at the basal level CBD induced the production of IL-8, C-X-C motif chemokine ligand (CXCL) 1, Serpin E1, IL-6, IFN-y, MCP-1, RANTES, and TNF-α, indicating that the effects of CBD may depend on the activation state of the cells. In microglial cells, CBD potently inhibited the production of TNF-α and IL-1β following stimulation with LPS[139]. It was subsequently observed CBD resulted in an intrinsic free-radical scavenging capacity, meaning that CBD was exerting its anti-inflammatory properties through an intrinsic antioxidant effect. In summary, the current body of literature characterizing macrophages in response to CBD is limited and has yet to demonstrate consistent findings with regards to its effects on innate immune modulation.

1.3 Macrophages

1.3.1 Macrophage Ontogeny

Macrophages were initially classified as part of the mononuclear phagocyte system (MGS) by van Furth and Cohn in 1968[95]. The MGS holds that tissue-resident macrophages in adulthood are

dependent on replenishment by bone marrow (BM)-derived blood monocytes. However, it has now been established that the long-term persistence of adult macrophage populations can be maintained through self-renewal[95]. The currently-accepted paradigm states that the majority of tissue-resident macrophages are established prenatally from the yolk sac and can self-maintain locally- independent of any hematopoietic input[96]. However, macrophages in adulthood also develop from tissue-infiltrating monocytes which are often associated with pathological, homeostatic and inflammatory reactions and typically display limited lifespans[97]. Both embryonic and adult-derived cell populations can co-exist in certain tissues, with their relative abundances reflecting the nature and history of a particular tissue. Monocytes make up between 4% and 10% of nucleated cells in the peripheral circulation and exhibit a half-life of roughly 20 hours[98]. Monocytes are highly plastic and dynamic in their abilities to compliment classic tissue-resident mononuclear phagocytes on demand. Monocytes typically function as short-lived effector cells within tissues in physiological processes such as angiogenesis and arteriogenesis[98]. Embryonic tissue-resident macrophages play a pivotal role in tissue remodeling, whereas adult-derived macrophages from bone-marrow assist in host defense[99]. Both cell types co-exist in tissues[96].

Specialized tissue-resident macrophages can be divided based on their anatomical location as well as their functional phenotype. Examples include microglia in the CNS, osteoclasts in the bone, alveolar macrophages in the lung and histocytes in the spleen[96]. These tissue-specific macrophages engulf dead cells, debris, foreign antigens, organize inflammatory processes and recruit additional macrophages as needed. Moreover, tissue-specific macrophages can comprise a significant proportion of cell-types found within a tissue. For instance, under non-inflamed conditions within the lung, the composition of bronchoalveolar lavage fluid is greater than 85% alveolar macrophages[193]. When distinguishing between monocyte and tissue-resident macrophages, surface

markers are employed. Human monocytes express high levels of cluster of differentiation (CD)14 and low levels of CD16 whereas macrophages possess the opposite profile with low levels of CD14 and high levels of CD16[100]. Other commonly employed markers include the chemokine receptor CCR5 and CD71 which are highly expressed in macrophages but not in monocytes[100]. Moreover, bone marrow-derived monocytes require the chemokine receptor type 2 (CCR2) for their exit from the bone marrow. Thus, CCR2 dependence of a given tissue-resident macrophage population can serve as an indicator of its monocyte origin and can be used to distinguish fetal- and adult-derived populations[100].

1.3.2 Macrophage Function

Macrophages are a heterogeneous population of myeloid cells that are positioned throughout the body in a manner that facilitates the ingestion and degradation of dead cells, debris, foreign material and the orchestration of inflammatory processes[101]. Macrophages were the first cell type identified as part of the innate immune system by Élie Metchnikoff in 1884, who coined the term "macrophage" meaning "macro = big and phage = eater"[102]. Macrophages are small populations of leukocytes that can be defined by their location, phenotype, morphology and gene expression profile. Macrophages are predominantly known for their phagocytotic ability in removing dying cells or cellular debris[101]. In cases of chronic inflammation characterized by large quantities of neutrophils at the site of infection, macrophages ingest these cells upon release of apoptotic or senescent signals[103]. This function is primarily carried out by tissue-resident macrophages rather than circulating monocytes in locations such as the lungs, liver, bone and spleen[101]. Phagocytosis follows the ingestion of a pathogen by a macrophage that then gives rise to an internal compartment in the macrophage called a phagosome, followed by the fusion of the phagosome with a lysosome where enzymes and peroxides digest the pathogen[104].

Another critical function of macrophages is their role as APCs. The capture, endocytosis, and presentation of self or foreign antigen on the cell surface is a key process that initiates and regulates the immune response. Macrophages are distributed throughout peripheral tissues and are constantly monitoring for invading pathogens. Upon presentation of a pathogen, macrophages will engulf it and subsequently process the antigens and present the peptide fragments on their surface. These cell surface fragments are bound to human leukocyte antigens (HLA-DP, HLA-DM, HLA-DO, HLA-DQ, and HLA-DR) corresponding to MHC class II[105]. Following antigen presentation, macrophages migrate towards helper T cell populations in peripheral lymphoid organs to prime and stimulate them, leading to an adaptive immune response. The ability of macrophages to mount an immune response is due in part to their high expression of co-stimulatory molecules and antigen presenting molecules such as CD80, CD86 and MHC class I and II molecules on their surface[106]. Finally, macrophages play an essential role in orchestrating inflammatory reactions due to their polarization and plasticity. This response, characterized by the release of a distinct set of cytokines and chemokines, is dependent upon micro-environmental stimuli which determine the pro-inflammatory, anti-inflammatory or intermediary response of the macrophages.

1.3.3 Macrophage Polarization & Plasticity

Macrophages typically exist in two distinct sub-sets: classically-activated macrophages (M1) or alternatively-activated macrophages (M2)[107]. M1 macrophages are pro-inflammatory and can be polarized by LPS or in conjunction with Th1 cytokines including GM-CSF or IFN-y[108]. Once polarized, M1 macrophages produce pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNF-α. M2 macrophages are anti-inflammatory and immunoregulatory and can be polarized by

Th2 cytokines including IL-4 and IL-13[108]. Once polarized, M2 macrophages produce anti-inflammatory cytokines such as IL-10 and TGF-β.

M1 and M2 macrophages have very different functions and responses, most notably their abilities to mitigate infection by pathogens or by repairing inflammatory-associated tissue damage, respectively[109]. Macrophages are not only subject to the effects of cytokines and chemokines but are also potent producers. It can be discerned based on the various phenotypes of macrophages that their cytokine and chemokines profiles differ accordingly. In the case of M1 macrophages, polarization results in many notable changes in the surrounding micro-environment. In addition to the release of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, M1 macrophages promote cytotoxic adaptive immunity through the up-regulation of MHC class II molecules and co-stimulatory molecules CD40, CD80, and CD86, resulting in the activation of APCs[99]. Moreover, M1 macrophages express Th1 and Th17-polarizing cytokines IL-12, IL-23, IL-17, as well as Th1 recruiting chemokines CXCL9, CXCL10, and CXCL11[110]. Conversely, as M2 polarized macrophages facilitate the resolution of inflammation, their cytokine profile can be defined by IL-10, TGF-β, and IL-1Ra. M2 macrophages additionally express high levels of endocytic receptors such as CD163, Stabilin-1, and c-type lectin receptors CD206, CD301, dectin-1, and CD209[111]. M2 macrophages also recruit Th2 cytokines, Tregs, eosinophils, and basophils through the release of CCL17, CCL18, CCL22, and CCL24[110,111]. Ultimately, it is the balance between M1 and M2 polarization that governs the fate of a tissue undergoing inflammation or injury. As such, if a tissue becomes infected or severely inflamed, macrophages will initially display an M1 phenotype characterized by the release of TNF-α, IL-1β, IL-12, and IL-23[107]. This occurs through the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, with subsequent generation of ROS[112]. While ROS production is a consequence of the robust anti-

microbial and anti-tumoricidal activity of M1 macrophages, ROS can induce tissue damage, prevent tissue regeneration as well as promote wound healing. Depending on the duration of the infection, a chronic M1 response can result in tissue damage. M2 macrophages polarized through the Th2 cytokines IL-4 and IL-13 occurs through STAT6 and the IL-4 receptor (IL-R) alpha [112]. Alternatively, cytokines such as IL-10 can govern M2 polarization by activating STAT3 via the IL-10R alpha. M2 macrophages secrete high amounts of IL-10 and TGF-β to suppress inflammation, contribute to tissue repair and remodeling, vasculogenesis, and retain homeostasis[107]. Moreover, M2 polarized macrophages maintain phagocytotic capability and can scavenge debris as well as apoptotic cells[108].

The ability of macrophages to rapidly and effectively modify their phenotype highlights their plasticity, a plasticity largely determined by the cytokine milieu[107]. M2 macrophages can be further subdivided into M2a, M2b, M2c, or M2d (Figure 1.3)[113]. The M2a phenotype can be induced by IL-4 or IL-13 and produce high levels of CD206, decoy receptor IL-1RII and IL-1R antagonist which function to promote tissue remodeling. The M2b phenotype can be induced by stimulation with immune complexes (ICs), toll-like receptor (TLR) agonists, or IL-1 receptor ligands. The M2b subset most accurately reflects the intermediate phase between M1 and M2 with the release of both pro- and anti-inflammatory cytokines including IL-10, IL-1β, IL-6 and TNF-α which function in promoting a Th2 response as well as tissue remodeling. M2c macrophages can be polarized by glucocorticoids in addition to IL-10 and exhibit anti-inflammatory properties against cells undergoing apoptosis via the release of IL-10 and TGF-β. Finally, macrophages with a M2d phenotype are induced by TLR agonists through the adenosine receptor; this leads to a reduction in the secretion of pro-inflammatory cytokines and an increase in the secretion of anti-inflammatory cytokines (characterized as IL-10^{high} and IL-12^{low}). Additionally, vascular

endothelial growth factor (VEGF) is secreted by M2d macrophages, contributing to the proangiogenic properties of tumor-associated macrophages (TAMs)[113,114]. Ultimately, the ability of macrophages to 're-polarize' or 're-program' upon exposure to various signals in the micro-environment is evidence for their high functional plasticity and polarization.

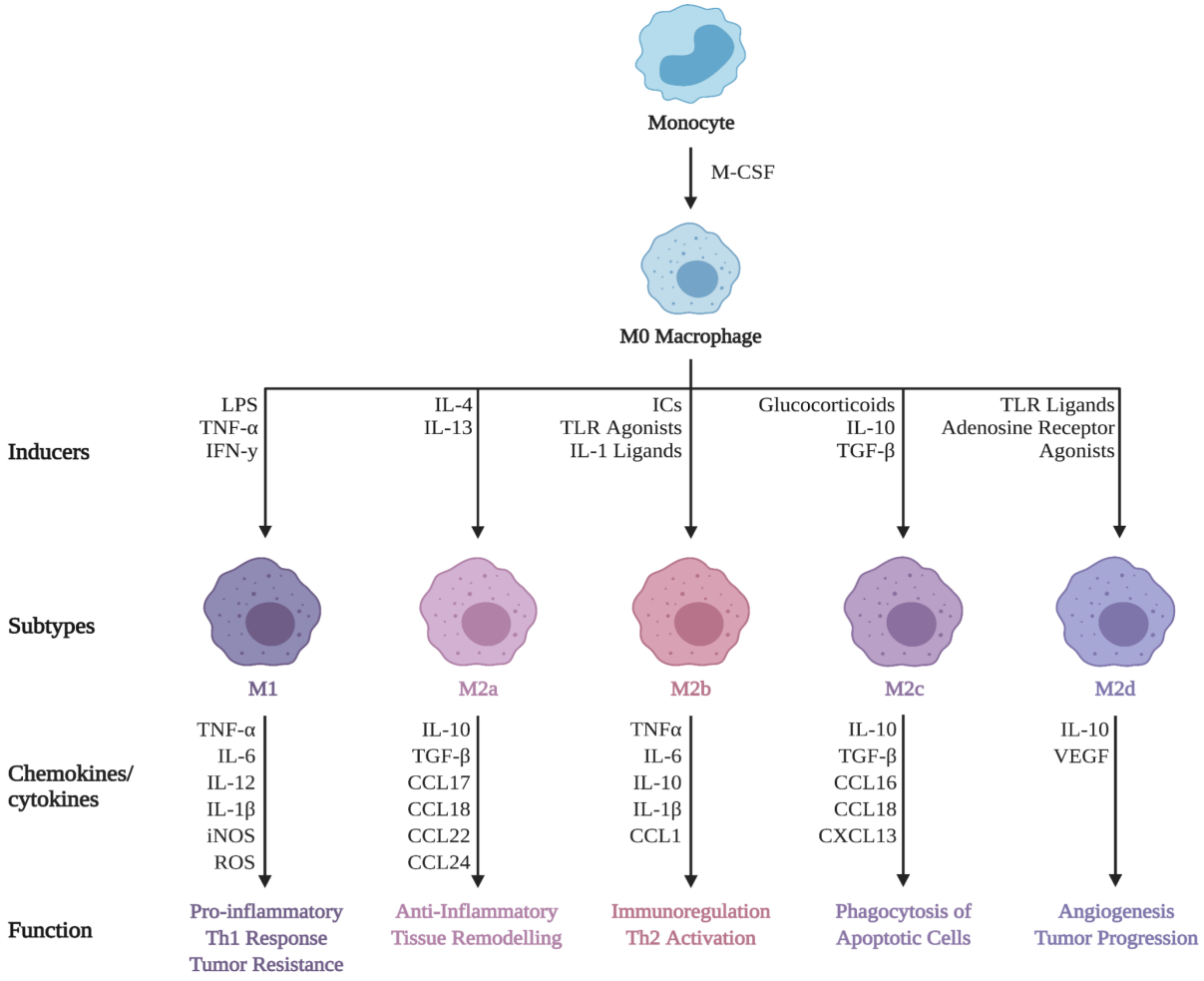


Figure 1.3. Differing biological and physiological features of M1 and M2 macrophage phenotypes. Monocytes stimulated by macrophage-colony stimulating factor (M-CSF) differentiate into M0 macrophages. M0 macrophages subjected to certain stimuli promote a phenotype of either M1, M2a, M2b, M2c, or M2d. Each phenotype has characteristic cytokine/chemokine secretion profiles with respective cellular and molecular functions. Adapted from Shapouri-Moghaddam *et al*[141].

1.3.4 Macrophages & Cannabis

Owing to their importance in the immune response, the modification of macrophages in relation to cannabis exposure has been studied. In habitual smokers of cannabis, the number of alveolar macrophages is significantly increased when compared to non-smokers[115]. The increased numbers of alveolar macrophages in cannabis smokers is also seen in tobacco smokers[115]. The mechanism by which this occurs has yet to be determined but has been hypothesized to be the result of tissue-infiltrating monocytes migrating in response to the inhaled smoke. Phenotypically, these macrophages are significantly enlarged and contain large amounts of inclusion bodies and particulates consistent with tar[116]. Macrophages recovered from cannabis smokers also contain measurable levels of Δ^9-THC and Δ^9-THC metabolites[117]. In addition to increased numbers, exposure to cannabis smoke also results in functional impairment in macrophages. When challenged with a common respiratory pathogen such as *Staphylococcus aureus* (*S. aureus*), alveolar macrophages from cannabis smokers are deficient in both bacterial phagocytosis and killing[118]. The reduced phagocytotic ability of alveolar macrophages is supported by their altered metabolic profiles characterized by reduced oxygen consumption and formation of superoxide. Consistent with these findings is the inability of the alveolar macrophages from cannabis smokers to produce NOS, TNF-α, GM-CSF, and IL-6 when compared to non-smokers as well as tobacco smokers[118]. However, incubation of these cells with GM-CSF or IFN-y restores NOS production, suggesting that cannabis exposure causes a decrease in cytokine priming that weakens host defense. As such, there is clear evidence to support an immune-suppressive effect of cannabis smoke that impairs anti-microbial defenses.

Year in and year out, the consumption of cannabis continues to increase with smoking remaining the primary method among users[140]. Recurrently, it has been demonstrated that smoking of cannabis imparts deleterious effects on the lung marked by inflammation and diminished host

defense[115,118]. Alveolar macrophages (AMs) are one of the predominant cell types found within the lung are an in direct contact with large quantities of cannabinoids in persons who smoke cannabis. AMs play a critical role in orchestrating inflammatory processes as well as serving as one of the primary lines against foreign pathogens. However, the interaction between cannabinoids and AMs has yet to be investigated in the context of their effects on inflammatory mediators and cellular function. As such, research into the immunomodulatory properties of cannabinoids on AMs warrants attention.

CHAPTER 2 HYPOTHESIS AND AIMS

Hypothesis:

The innate inflammatory response by alveolar macrophages can be reduced by CBD and Δ^9-THC to promote an anti-inflammatory phenotype.

Aims:

1. Assess the effects of CBD and Δ^9-THC on the inflammatory response of alveolar macrophages.
2. Investigate the receptor-mediated effects of these cannabinoids on the inflammatory response.
3. Characterize alveolar macrophage polarization and functionality in response to these cannabinoids.

CHAPTER 3 MATERIALS AND METHODS

3.1 Cell Culture

The MH-S AM cell line was obtained from ATCC (Manassas, USA). This cell line was established following transformation of cells obtained by bronchoalveolar lavage from Balb/cJ mice with simian virus 40[194]. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (WISENT Inc, Canada) containing 10% fetal bovine serum (FBS; WISENT Inc), gentamycin (WISENT Inc), Antibiotic-Antimycotic (A/A; WISENT Inc), and 2-mercaptoethanol (OmniPur, USA). All experiments were carried out with cell passage number being between 18 and 24. Bone-marrow-derived macrophages (BMDMs) were isolated from wild-type C57BL/6 mice. Femur and tibia bones were isolated from sacrificed mice and epiphyses were cut off. Bones were then washed using a 10 ml syringe connected to a 25 G needle containing RPMI. The marrow was then aspirated using a 10 ml syringe connected to an 18 G needle until the bone marrow aggregates were broken. The final cell suspension was then centrifuged at 300g for 7 minutes. Cells were then maintained in RPMI 1640 containing 10% FBS, 30% L929, A/A, non-essential amino acids (WISENT Inc), essential amino acids (WISENT Inc,), sodium pyruvate (WISENT Inc,), HEPES (Sigma-Aldrich, USA), and sodium hydroxide (NaOH; Sigma-Aldrich). BMDMs were then grown for 7 days with fresh media being added on day 3.

3.2 Cell Treatments

AMs were cultured under 7 separate conditions; untreated, methanol (MeOH), THC (Cayman Chemical, USA) alone, CBD (Cayman Chemical) alone, LPS (0111:B4; Sigma-Aldrich) alone, LPS plus THC, and LPS plus CBD. The untreated (control) condition consisted of RPMI 1640 that did not contain FBS. For the methanol, THC alone, and CBD alone conditions, AMs were cultured with serum-free RPMI 1640 containing equivalent concentrations of each respective drug. The

LPS alone, LPS plus THC, and LPS plus CBD conditions followed pre-treatment with either THC or CBD and subsequent addition of LPS directly to the plate. The 24-hour incubation window was selected according to 1) CBD and THC induced cytotoxicity on MH-S cells and 2) the induction of inflammatory markers by LPS. All cells were incubated in humidified chambers at 37^{o}C and exposed to 21 % O_2 and 5% CO_2.

3.3 Tetrazolium Bromide Assay (MTT Assay)

AMs were plated at 1x10^4 cells/cm^2 in a sterile, flat-bottom 96-well plate in RPMI 1640 containing 10% FBS. One-day later AMs were treated with varying concentrations of methanol, THC, or CBD and incubated for 24 hours. Then, a 5mg/ml solution of MTT (Sigma-Aldrich M-2128) in phosphate buffered saline (PBS; Gibco, USA) was prepared and 10 µl was applied to each well. AMs were then incubated for 4 hours, the point at which a visible purple precipitate forms, and the plate was then centrifuged at 800 RPM for 5 minutes. Media was then removed and 200µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. The plate was then read using an iMark Microplate Reader (Bio-Rad Laboratories, Canada) using Microplate Manager Software Version 6 (Bio-Rad Laboratories, Canada) at 510 nm.

3.4 Annexin V & Propidium Iodine Staining

Following AM collection, cell suspensions were stained using APC Annexin V Apoptosis Kit with PI (BioLegend). Cells were initially washed twice using Cell Staining Buffer (BioLegend, USA) and resuspended in Annexin V Binding Buffer (BioLegend). In 100 µL of cell suspension, 5µL of APC Annexin V (BioLegend) and 3µL of propidium iodine (PI; BioLegend) were mixed. Cells were then incubated in the dark at room temperature (25°C) for 15 minutes followed by the addition of 400 µL of Annexin V Binding Buffer. Data was then acquired using a fluorescence-

activated cell sorting (FACS) Canto (BD Biosciences, Canada) flow cytometer and analyzed using FlowJo version 9 software (BD Biosciences, Canada).

3.5 Quantitative RT-PCR (qRT-PCR)

AMs were seeded at $2x10^5$ cells/cm^2 in 6-well plate and upon reaching 70-80% confluency one day later, were pre-treated with either 3μM THC or 3μM CBD for one hour followed by the addition of 0.1μg/ml LPS for 6 or 24 hours. RNA was isolated using Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Canada) in accordance with manufacturer's instructions. RNA quantification was done using a Nanodrop 1000 spectrophotometer infinite M200 pro (TECAN, Switzerland). Reverse transcription of RNA to cDNA was carried out using iScript Reverse Transcription Supermix (Bio-Rad Laboratories) and mRNA levels of *CB2R, GAPDH, TNFα, IL-1B,* and *IL-6* were analyzed using gene specific primers (Table 1). Quantitative PCR (qPCR) was done by combining 1μl cDNA and 0.5μM primers with SsoFast EvaGreen (Bio-Rad Laboratories) with amplification performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Canada). Thermal cycling was initiated at 95ºC for 3 minutes followed by 39 cycles of denaturation at 95ºC for 10 seconds and annealing at 55ºC for 5 seconds. Genomic RNA expression was analyzed using the ΔΔCt method and results presented as fold-change normalized to the housekeeping gene (*GAPDH)*.

Table 1: Primer sequences used for qRT-PCR analysis

Gene	**Forward Primer Sequence**	**Reverse Primer Sequence**
CNR2	GTTACCCGCCTACCTACAAAG	GAGCGGCAGGTAAGAAATCA
GAPDH	GGTCCTCAGTGTAGCCCAAG	AATGTGTCCGTCGTGGATCT
TNFα	CTATGTCTCAGCCTCTTCTC	GGGAACTTCTCATCCCTTT

IL-1β	GGACATGAGCACCTTCTT	CCTGTAGTGCAGTTGTCTAA
IL-6	CCAGAGTCCTTCAGAGAGATACA	CCTTCTGTGACTCCAGCTTATC

3.6 Cytokine & Chemokine Protein Analysis

AMs were treated as described above for 24 hours. Cell supernatants were then collected and sent to Eve Technologies Corporation (Alberta, Canada) and a Mouse Cytokine Array Proinflammatory Focused 10-plex (MDF10) Assay was carried out. The markers evaluated in the assay included IFN-γ, IL-1β, GM-CSF, IL-2, IL-4, IL-6, IL-10, IL-12p70, MCP-1, and TNF-α. A standard curve was generated using fluorescent intensity of the bead population for a specific marker. The observed concentration of the marker in the samples was generated within this standard curve. This was done using cubic spline as a regression applied against standard curve fluorescent intensity values to calculate the corresponding observed concentration of each marker.

3.7 Western Blot

AMs were seeded at $2x10^5$ cells/cm^2 in 6-well plates and upon reaching 70-80% confluency one day later were pre-treated with either 3μM THC or 3μM CBD for one hour followed by the addition of 0.1μg/ml LPS for 0.25, 2, 6, or 24 hours. Total cellular protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific, USA) in addition to Protease Inhibitor Cocktail (Roche, USA). Following extraction, protein concentration was determined using the bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific). Protein lysate at a concentration of 15 or 20 μg was electrophoresed on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels and transferred onto Immuno-blot PVDF membranes (Bio-Rad Laboratories). After the transfer, the membrane was blocked using a blocking solution of 5% w/v

non-fat dry milk in 1X PBS/0.1% Tween-20 for one hour at room temperature. Antibodies were applied to membranes for one hour or overnight. The following is a list of the antibodies used: anti-tubulin (1:50000; Sigma-Aldrich), anti-CB1 Receptor (1:1000; Cell Signaling Technologies, USA), anti-CB2 Receptor (1:200; Cayman Chemical, USA), anti-p-NF-κB p65 (1:1000; Cell Signaling Technologies), anti-NF-kB p65 (1:1000; Santa Cruz, USA), anti-p-p44/42 MAPK (1:1000; Cell Signaling Technologies), and anti-p44/42 MAPK (1:1000; Cell Signaling Technologies). After the application of the primary antibody, secondary antibodies including anti-rabbit IgG, HRP linked (1:10000; Cell Signaling Technologies, USA) and HRP-conjugated anti-mouse IgG (1:10000; Cell Signaling Technologies, USA) were used. Membrane visualization was carried out using either Clarity western ECL substrate (Bio-Rad Laboratories) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Protein band detection was then performed using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Canada). Densitometric analysis was analyzed through Image Lab Software Version 5 (Bio-Rad Laboratories, Canada) and protein expression was normalized to tubulin or one of the two respective total proteins and presented as fold change compared to the methanol condition.

3.8 Measurement of Phagocytic Ability

AMs were treated as described above. Twenty-four hours later, IgG coated beads (0.1μM) conjugated to phycoerythrin (PE; Cayman Chemical, USA) were diluted in serum-free RPMI 1640 at a dilution of 1:200 and applied to the wells. AMs were then incubated with the beads for 4 hours. Media was then removed, and cells were washed 3 times with PBS followed by the addition of 200μl of Accutase (Innovative Cell Technologies, USA) Cell Detachment Solution. Cell exclusion was then done by quenching with 50μl of trypan blue (0.4%; Gibco, USA). Data was then acquired

using a FACS Fortessa (BD Biosciences, Canada) flow cytometer and analyzed using FlowJo version 9 software (BD Biosciences, Canada).

3.9 RNA sequencing & Analysis

Total RNA was quantified using Qubit (Thermo Scientific) and RNA quality was assessed with the 2100 Bioanalyzer (Agilent Technologies). Transcriptome libraries were generated using the KAPA RNA HyperPrep Kit with RiboErase (Roche). Sequencing was performed on the Illumina NextSeq500, obtaining around 50M single-end reads per sample. The reads are trimmed using fastp and then aligned using the STAR aligner. From the aligned reads, HTSeq is used to get the raw read counts. If there is a known batch effect, it is accounted for using the sva R package. Then the DESeq2 R package was used to normalize the counts and run a differential expression (DE) analysis between the different conditions. The Gage and Pathview R packages are used on the most significant differentially expressed genes (DEG) (typically a log2 fold change > 2 and an adjusted p-value < 0.05) to get pathways and gene sets associated with those genes. Finally, the regionReport R package was applied on the results from DESeq2 to create an HTML report and summary.

3.10 CB2-siRNA Knockdown

AMs were seeded at $2x10^5$ cells/cm^2 in 6-well plate and one day later were transfected with 80 nM of siRNA targeting CB2 (Santa Cruz, USA) or non-targeting control siRNA (Santa Cruz, USA) in accordance with manufacturer's instructions. The transfection was performed using jetPRIME Transfection Reagent (Polyplus Transfection, USA) following manufacturer's instructions. After 24 hours, media containing transfection reagent was removed and cells washed with PBS. Fresh RPMI 1640 containing 10% FBS was then added to the cells. Twenty hours later, AMs were pre-

treated with either 3μM THC or 3μM CBD for one hour followed by the addition of 0.1μg/ml LPS for 24 hours. Confirmation of CB2 knockdown was examined by western blot 68 hours after transfection.

3.11 Statistical Analysis

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

CHAPTER 4 CONTRIBUTION OF CO-AUTHORS

This thesis is founded on original work and has been prepared as a manuscript for the purpose of peer-review and submission.

- Dr. David H. Eidelman assisted in experimental design and project management.
- Dr. Carolyn J. Baglole provided intellectual support and guidance as well as experimental design, project management, and thesis editing.
- Dr. Hussein Traboulsi provided insight regarding experimental design and conduct.
- Dr. Gregory Fonseca assisted with the framework and analysis of the RNA-sequencing data.
- Patrick Greiss assisted with RNA-sequencing analysis as well as experiments relating to primary BMDMs.

CHAPTER 5 RESULTS

5.1 Optimization of experimental conditions

Δ^9-THC and CBD may modulate the inflammatory response in AMs, a cell type integral to the inflammatory process through their production of cytokines and chemokines. It is not known if impaired ability of AMs to produce many of these cell-signaling molecules in habitual cannabis consumers is due to Δ^9-THC and CBD. In order to address whether Δ^9-THC or CBD affect macrophage function, we first determined the concentration of Δ^9-THC and CBD that did not affect cell viability. This was initially assessed through the use of MTT, a colorimetric cell viability assay whereby MH-S cells were treated with either Δ^9-THC or CBD at concentrations ranging from 1-5 μM for 24 hours. Methanol was used as negative control as it is the solvent for Δ^9-THC and CBD. At concentrations between 1-4 μM, both Δ^9-THC and CBD did not have any significant effect on cell viability (Figure 5.1A and 5.1B, respectively). However, 5 μM of both Δ^9-THC and CBD significantly reduced cell viability (Figure 5.1A and 5.1B). As a complimentary technique, we also used flow cytometry with PI to detect live and dead cells. At concentrations between 1-4 μM, Δ^9-THC and CBD did not qualitatively increase the number of PI-positive (PI^+) AMs (Figure 5.1C and 5.1D, respectively). Consistent with the findings of the MTT assay, both Δ^9-THC and CBD at 5 μM increased the percentage of PI^+ cells (Figure 5.1C and 5.1D, respectively). Quantification confirmed that there was a significant increase in PI^+ cells at the 5 μM concentration for both Δ^9-THC and CBD when compared to the methanol control (Figure 5.1E and 5.1F, respectively). Based on these findings, we selected 3 μM as the concentration for both Δ^9-THC and CBD used in all subsequent experiments.

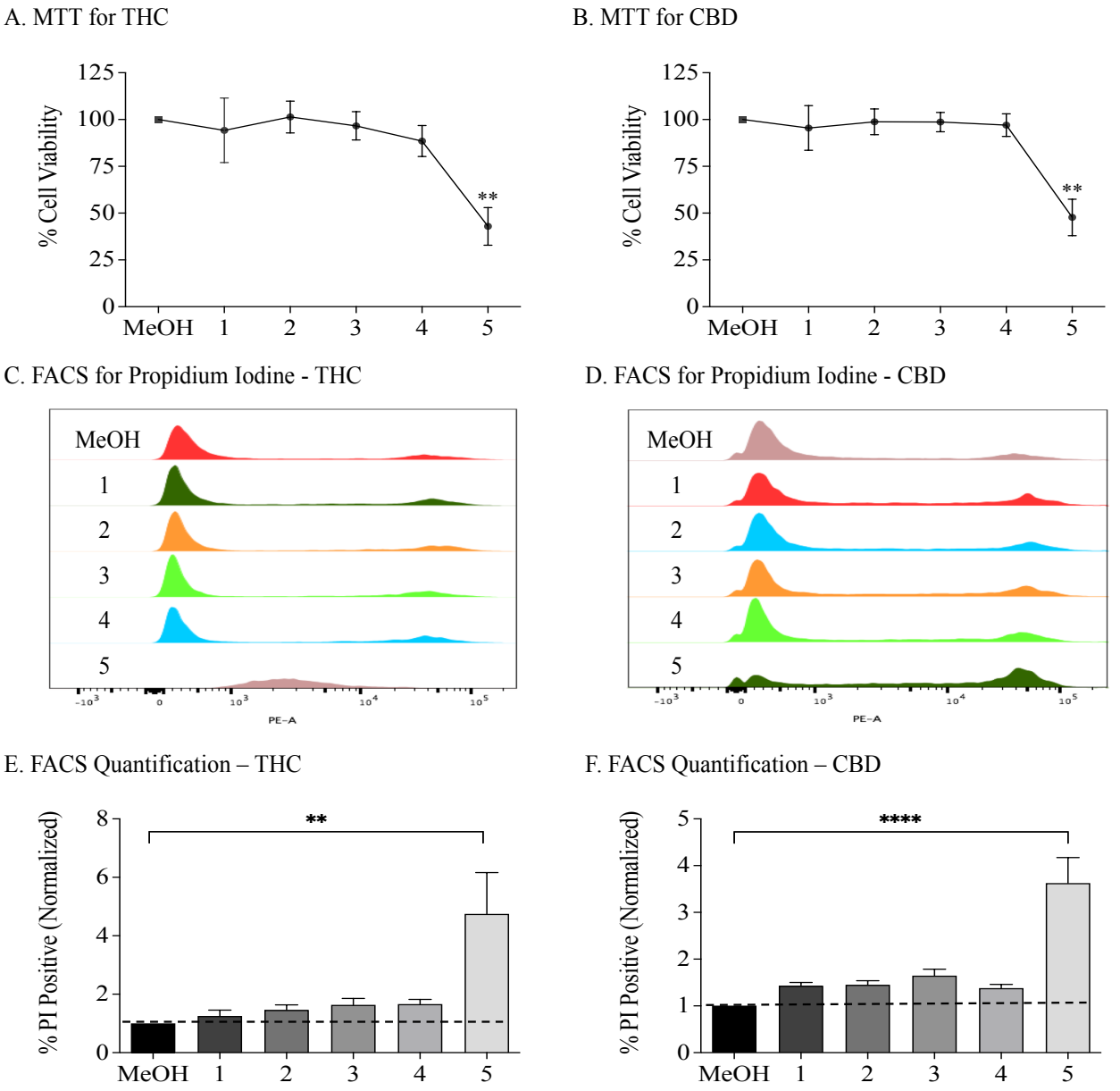
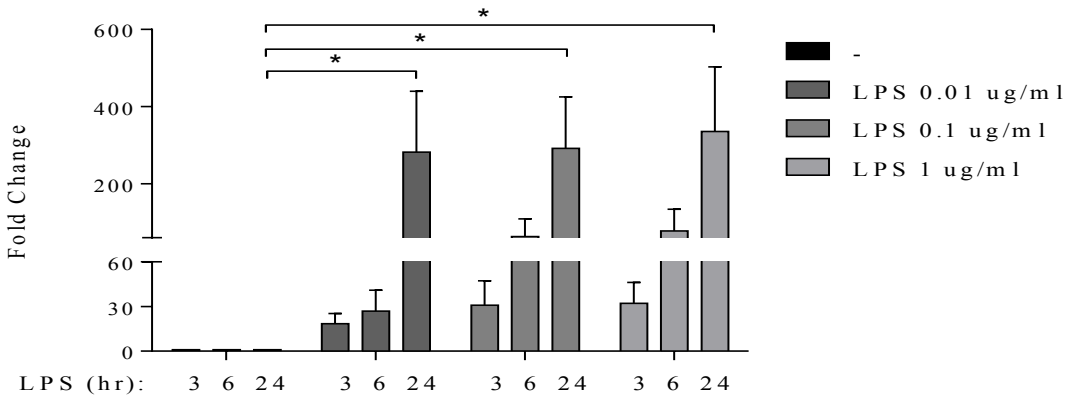


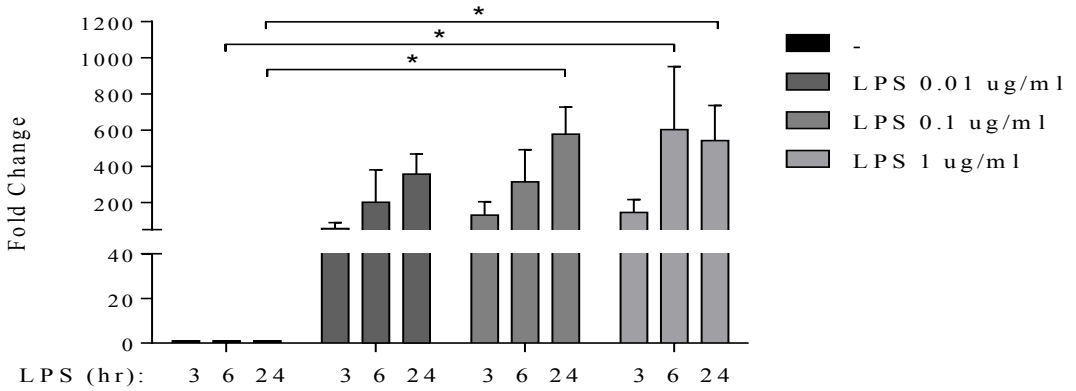
Figure 5.1. There is a decrease in cell viability in response to CBD and Δ^9-THC. Cell viability is significantly decreased by Δ^9-THC (A) and CBD (B) at a concentration of 5 µM after 24 hours. At concentrations of 4 µM or less, there was no significant effect of Δ^9-THC or CBD on cell viability (n=5). In response to Δ^9-THC (C) or CBD (D) at a concentration of 5 µM for 24 hours, there is an observable increase in the number of PI$^+$ cells compared to methanol control. Δ^9-THC (C) and CBD (D) at a concentration of 4 µM or less did not qualitatively increase the number of PI$^+$ cells. (E) There is a significant increase in the number of PI$^+$ cells in response to Δ^9-THC at a concentration of 5 µM compared to methanol control. (F) CBD at a concentration of 5 µM significantly increased the quantifiable number of PI$^+$ cells compared to methanol control (n=4). Values are presented as the mean ± SEM; **p<0.01, ****p<0.0001 compared to control (methanol). FACS: fluorescence-activated cell sorting.

Next, based on its well-defined mechanism of action through the TLR4, we evaluated the ability of LPS to increase inflammatory gene expression in a dose- and time-dependent manner. To assess the inflammatory response, we analyzed the expression of *IL-1β*, *IL-6*, and *TNF-α* by qPCR. At all concentrations, LPS significantly induced the transcription of *IL-1β* for the 24-hour time point (Figure 5.2A). However, for *IL*-6, only the 0.1 and 1 μg/ml concentrations of LPS significantly increased mRNA levels after 24 hours (Figure 5.2B). For *TNF-α*, there were comparable levels of induction between the 0.1 and 1 μg/ml concentrations at the 24-hour time point; however, only the 1 μg/ml concentration was statistically significant (Figure 5.2C). Therefore, LPS at a concentration of 0.1 μg/ml and for a duration of 24 hours was utilized for the remainder of experiments.

A. *IL-1β* mRNA



B. *IL-6* mRNA



C. *TNF-α* mRNA

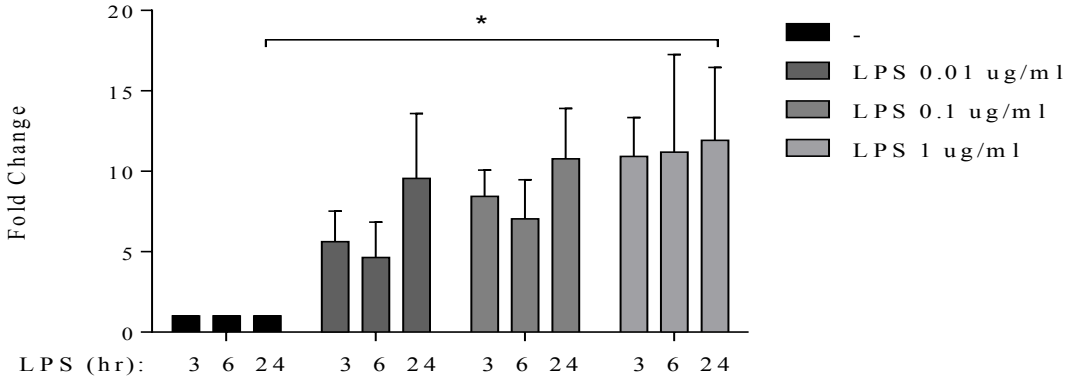


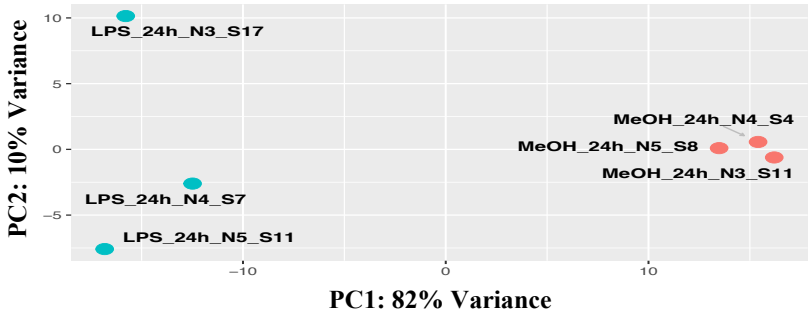
Figure 5.2. There is an increase in the mRNA expression of *IL-1β*, *IL-6*, and *TNF-α* in response to LPS. (A) Levels of *IL-1β* mRNA are significantly increased in response to LPS (0.01, 0.1 and 1, and μg/ml) at 24 hours; however, at 3- and 6-hours there was no significant increase. (B) The expression of *IL-6* mRNA is significantly increased by LPS (0.1 and 1 μg/ml) at 24; hours however at a concentration of 0.01 μg/ml, there was no significant increase. In response to LPS (1 μg/ml), the expression of *IL-6* mRNA is significantly increased at the 6-hour but not the 3-hour timepoint. LPS (0.01 and 0.1 μg/ml) did not significantly affect IL-6 mRNA at 3- and 6-hours. (C)

The expression of *TNF-α* is significantly increased in response to LPS at a concentration of 1 μg/ml for 24 hours; however, there is no significant effect at 3- or 6-hours. At concentrations of 0.01 and 0.1 μg/ml, LPS did not significantly increase *TNF-α* mRNA expression at 3-, 6-, or 24-hours (n=4). Values are presented as the mean ± SEM. Means are expressed as fold change from the control (serum-free media). *$p<0.05$ compared to control (6-, 24-h serum-free media) unless otherwise stated.

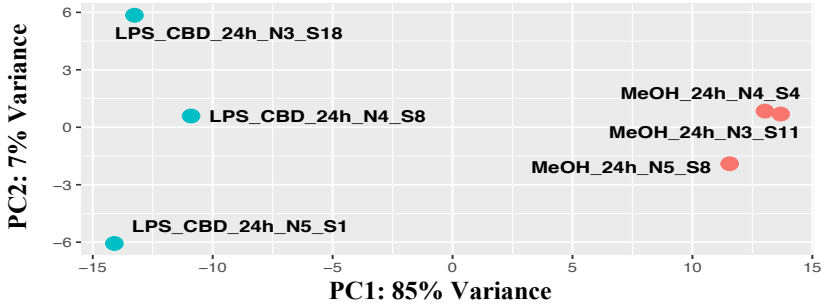
5.2 RNA-seq analysis identifies distinct gene signatures altered by cannabinoids during the inflammatory response

To determine the impact of Δ^9-THC and CBD in response to LPS, RNA-seq analysis was conducted. For these experiments, there were seven experimental conditions: untreated (media only), methanol, CBD, Δ^9-THC, LPS, LPS + CBD and LPS + Δ^9-THC for 24 hours. Principal component analysis (PCA) and Pearson correlation revealed that of the six comparisons, only treatment conditions in which LPS was present (LPS alone, LPS + CBD, and LPS + Δ^9-THC) clustered successfully and revealed differentially expressed gene (DEG) profiles (Figure 5.3 and 5.4). PCA demonstrated minimal intra-group variability ($\leq$ 10%) and substantial inter-group variability ($\geq$ 81%) (treatment condition vs. methanol) in conditions containing LPS (Figure 5.3). Pearson correlation analysis (Figure 5.4) highlighted the variation that was present between samples showing correlation values of $r > 0.99$. The other three treatment conditions (media alone, Δ^9-THC alone, and CBD alone) did not effectively cluster, indicating that their gene expression profiles relative to the methanol control did not differ significantly (Figure 5.5 and 5.6).

A. Principal Component Analysis for LPS vs. MeOH



B. Principal Component Analysis for LPS + CBD vs. MeOH



C. Principal Component Analysis for LPS + THC vs. MeOH

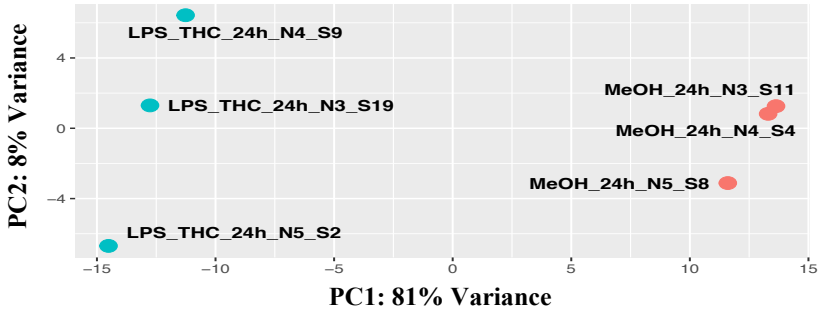
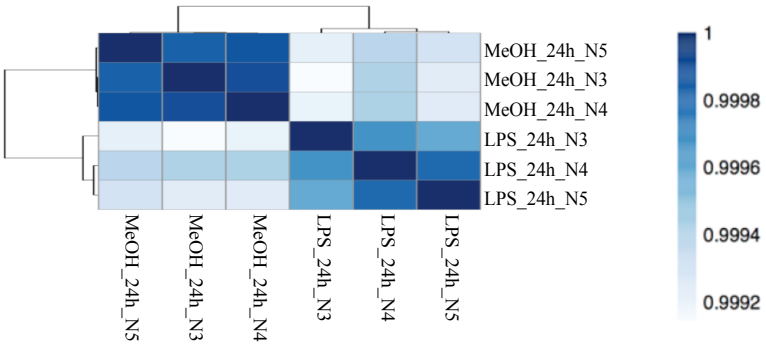
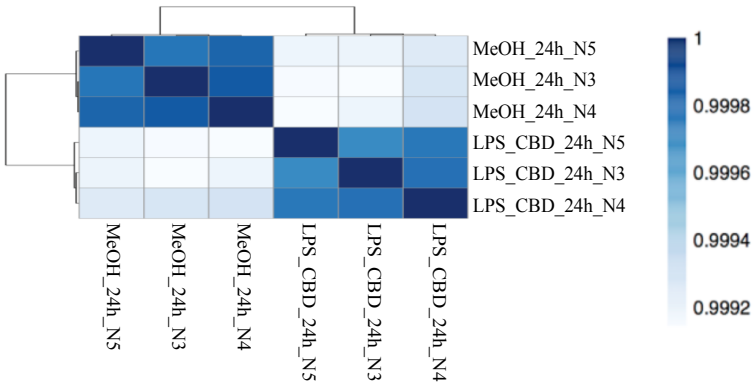


Figure 5.3. PCA of RNA-seq comparisons reveals successful clustering in conditions containing LPS. (A) PCA comparing LPS (n=3) to the methanol control (n=3) in which there was a PC1 variance of 82% and PC2 variance of 10%. (B) PCA analysis comparing LPS + CBD treatment group (n=3) to the methanol control with a PC1 variance of 85% and a PC2 variance of 7%. (C) PCA analysis comparing LPS + Δ^9-THC treatment group (n=3) to the methanol control with a PC1 variance of 81% and a PC2 variance of 8%.

A. Pearson Correlation for LPS vs. MeOH



B. Pearson Correlation for LPS + CBD vs. MeOH



C. Pearson Correlation for LPS + THC vs. MeOH

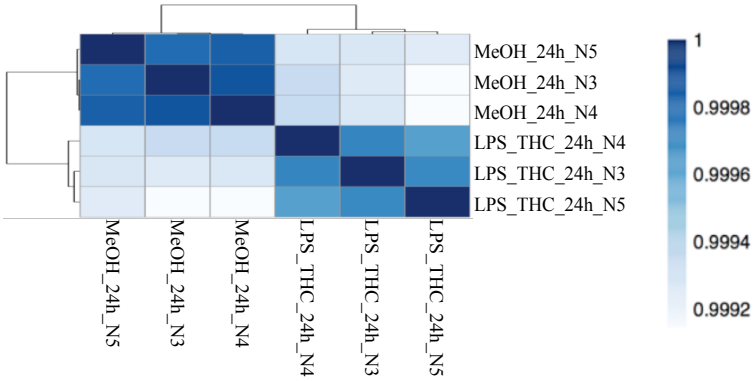
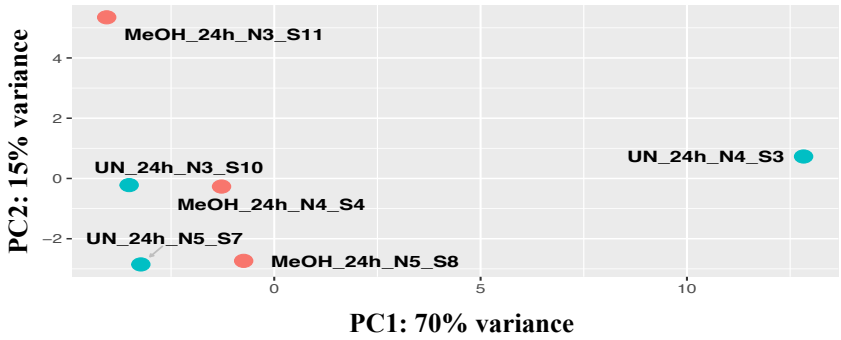
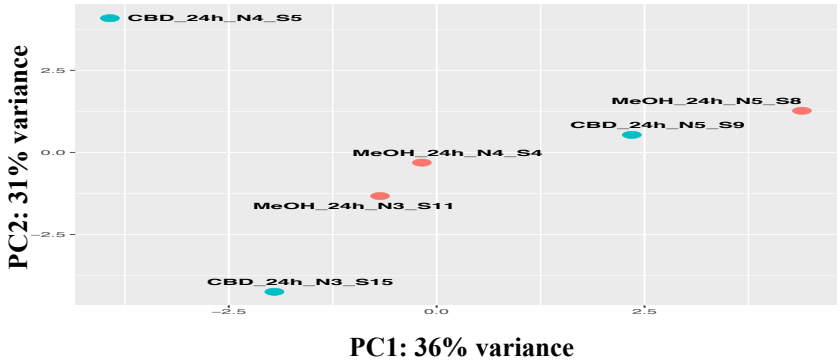


Figure 5.4. Pearson correlation confirms successful clustering in conditions containing LPS. Pearson correlation plot visualizing the correlation (*r*) values between LPS and methanol control (A), LPS + CBD treatment group and methanol control (B), and LPS + Δ^9-THC treatment group and methanol control (C).

A. Principal Component Analysis for Media vs. MeOH



B. Principal Component Analysis for CBD vs. MeOH



C. Principal Component Analysis for THC vs. MeOH

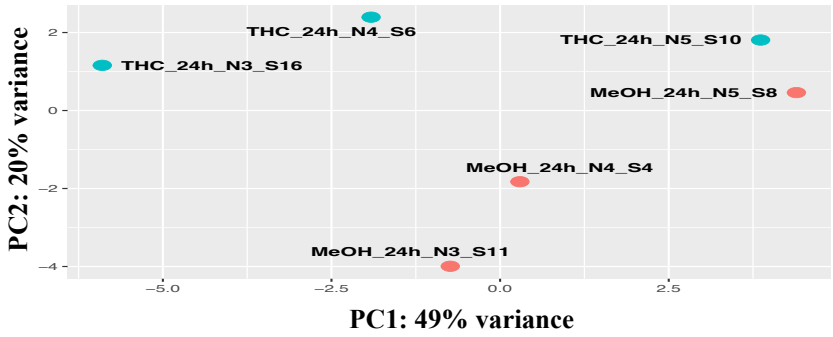
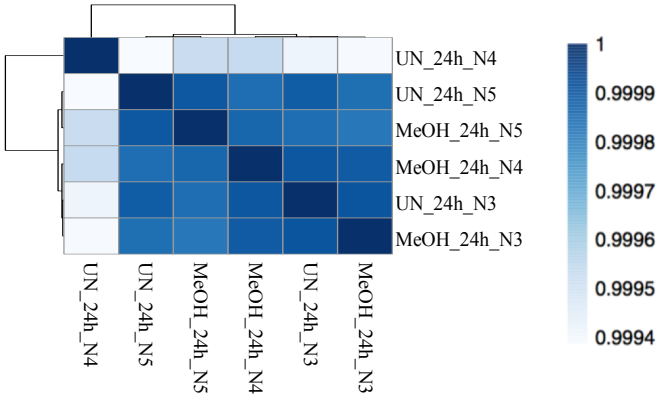
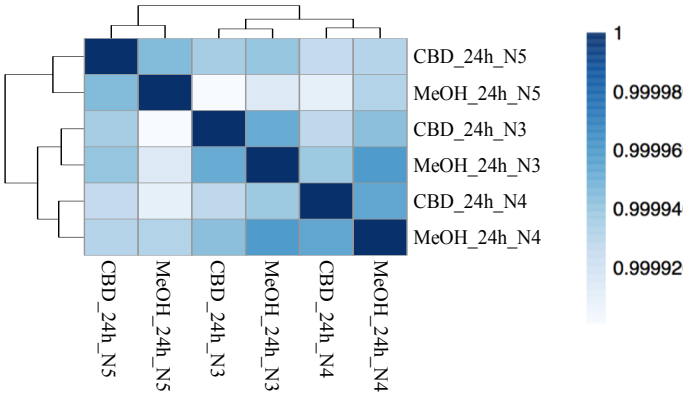


Figure 5.5. PCA of RNA-seq comparisons reveals a lack of successful clustering in conditions without LPS. (A) PCA comparing the media only (un) treatment group (n=3) to the methanol control (n=3) in which there was a PC1 variance of 70% and PC2 variance of 15%. (B) PCA analysis comparing the CBD alone treatment group (n=3) to the methanol control with a PC1 variance of 36% and a PC2 variance of 31%. (C) PCA analysis comparing the Δ^9-THC alone treatment group (n=3) to the methanol control with a PC1 variance of 49% and a PC2 variance of 20%.

A. Pearson Correlation for Media vs. MeOH



B. Pearson Correlation for CBD vs. MeOH



C. Pearson Correlation for THC vs. MeOH

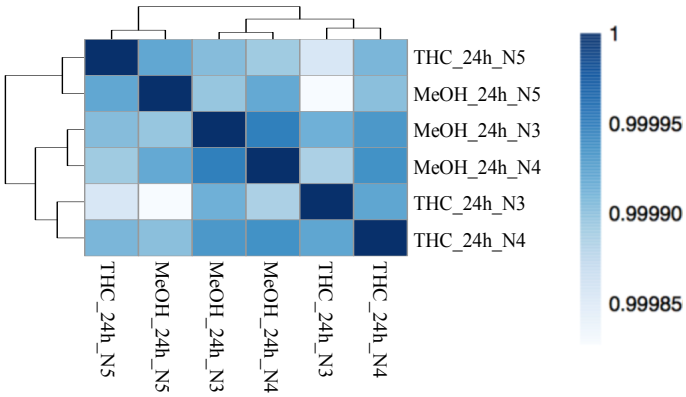


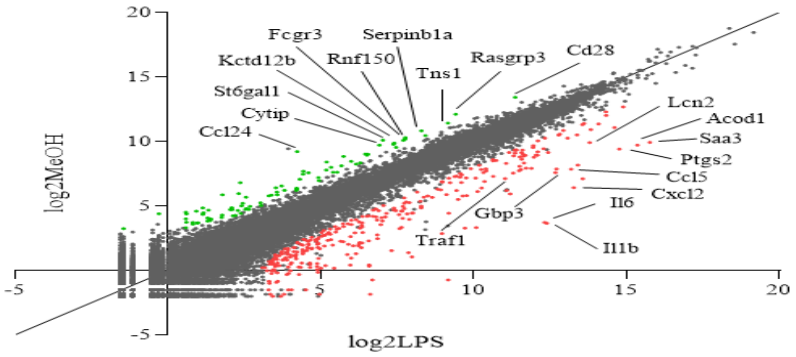
Figure 5.6. Pearson correlation confirms lack of successful clustering in conditions without LPS. Pearson correlation plot visualizing the correlation (*r*) values between the media only (un) treatment group and methanol control (A), CBD alone and methanol control (B), and Δ^9-THC and methanol control (C).

Next, we evaluated DEG profiles. There was no change in genes that met the threshold criteria (log_2fold change of 2; FDR $p < 0.05$) between methanol and CBD (Appendix Table 1), methanol versus Δ^9-THC (Appendix Table 2) or methanol versus untreated (Appendix Table 3). This indicates that Δ^9-THC and CBD do not significantly impact gene transcription in MH-S cells. However, there was a total of 399 genes differentially expressed in response to LPS, of which 313 (78%) were upregulated and 86 (22%) were downregulated (Figure 5.7). Among the inflammatory- and immune response-related genes that exhibited significant induction were *IL-1β, IL-6, CXCL2, CCL5,* prostaglandin-endoperoxide synthase *(PTGS) 2, SAA3, LCN2, ACOD1, GBP3,* and *TRAF1* (Figure 5.7A). Genes that were significantly downregulated in response to LPS included *CCL24, CYTIP, ST6GAL1, KCTD12B, FCGR3, RNF150, SERPINB1A, TNS1, RASGRP3,* and *CD28* (Figure 5.7A). In contrast to the effect of LPS alone, the combination with CBD (LPS + CBD) and Δ^9-THC (LPS + Δ^9-THC) reduced gene expression. In the LPS + CBD group, there was a total of 286 differentially expressed genes, of which 209 (73%) were upregulated and 77 (27%) were downregulated (Figure 5.7B). With LPS + Δ^9-THC, there was a total of 287 differentially expressed genes, of which 212 (74%) were upregulated and 75 (26%) were downregulated (Figure 5.7C). As such, a net suppressive effect of CBD and Δ^9-THC on the LPS-induced gene expression profile was observed, predominantly on the genes that were increased in response to LPS.

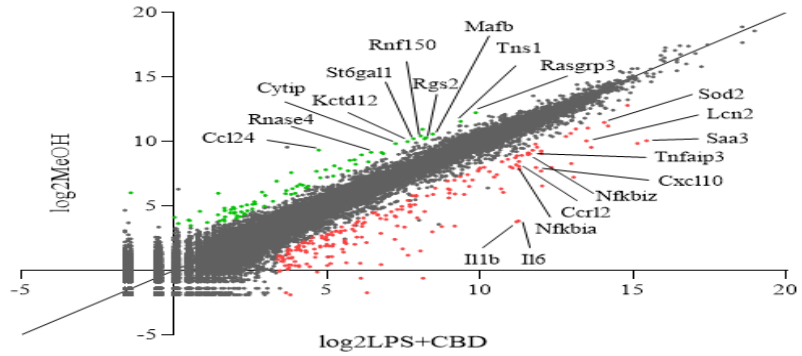
To further classify differences in the LPS-induced gene expression caused by CBD and Δ^9-THC, we compared their DEG profiles. Of the genes that had an increased differential expression, there were 173 genes that were common to all three treatment groups (Figure 5.8A). Despite significant overlap in genes that were increased, there were several genes that were unique to each of the three

conditions. Treatment with LPS elicited the largest increase in genes (101), whereas co-treatment with either CBD or Δ^9-THC each had 13 uniquely upregulated genes (Figure 5.8A). Notable LPS-induced inflammation- and immunity-related genes that were uniquely increased included *CCL3,* janus kinase *(JAK) 2, IL1F6, TNFRSF1B, GBP9, CXCL3, CXCL1,* and *CCL22.* Of the genes that had decreased differential expression, 52 were shared across the three treatment groups (Figure 5.8B). The number of genes that were uniquely decreased across the three conditions were 19, 11, and 10 for the LPS alone, LPS + CBD, and LPS + Δ^9-THC, respectively (Figure 5.8B) (Appendix Table 4-6). Therefore, despite considerable overlap across the DEG profiles in response to LPS, there were significantly fewer upregulated genes following treatment with either CBD or Δ^9-THC, thus indicating a suppressive effect of CBD and Δ^9-THC on LPS-induced gene expression.

A. Gene Expression Scatter Plot for LPS vs. MeOH



B. Gene Expression Scatter Plot for LPS + CBD vs. MeOH



C. Gene Expression Scatter Plot for LPS + THC vs. MeOH

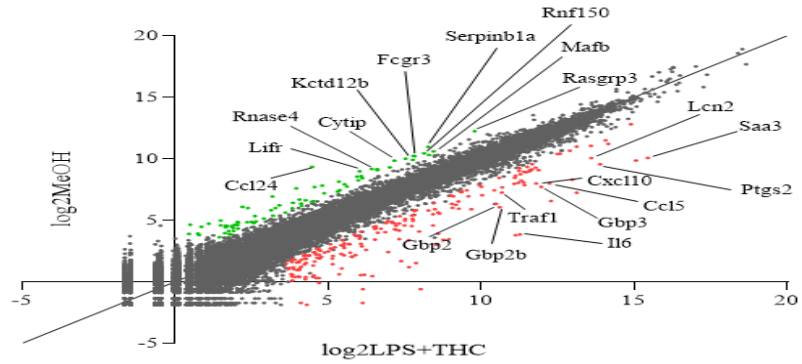
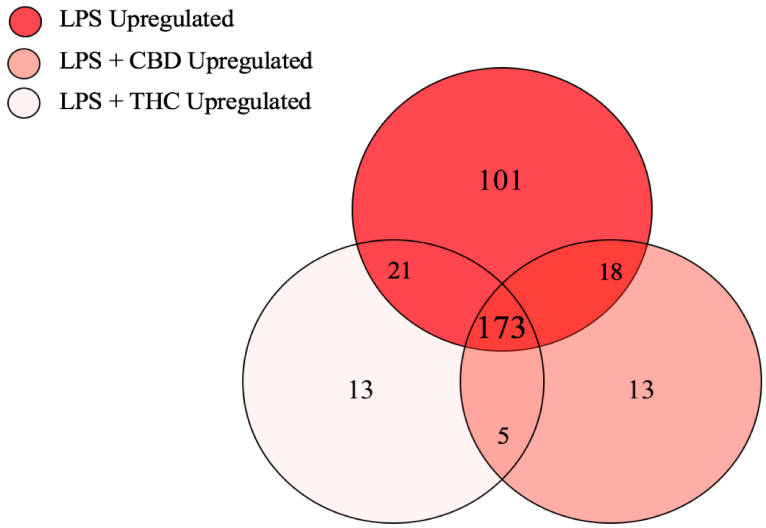


Figure 5.7. Effects of CBD and Δ^9-THC on the LPS-induced differential gene profile. Gene expression scatter plots representing the normalized counts of all genes comprising the LPS vs. MeOH comparison (A), LPS + CBD vs. MeOH (B), and LPS + Δ^9-THC vs. MeOH (C). Red indicates significantly (false discovery rate [FDR]-adjusted $P<0.05$ and $\log_2$-fold change ≥ 2) upregulated genes in the respective treatment group whereas green indicates significantly downregulated genes compared to the methanol treatment group. Grey indicates genes not differentially expressed. Genes highlighted represent those with the most significant induction according to adjusted p-value. Values are presented as the $\log_2$ value of the mean of the normalized counts of each gene (n=3).

A. Venn Diagram of Differentially Up-Regulated Genes



B. Venn Diagram of Differentially Down-Regulated Genes

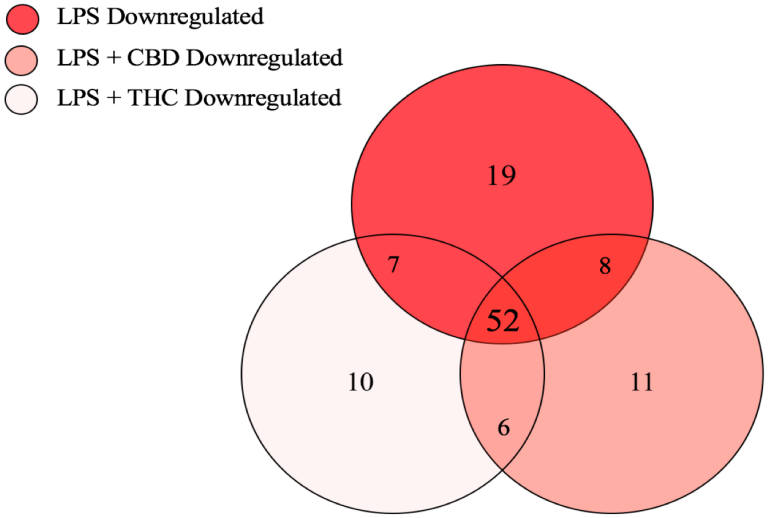


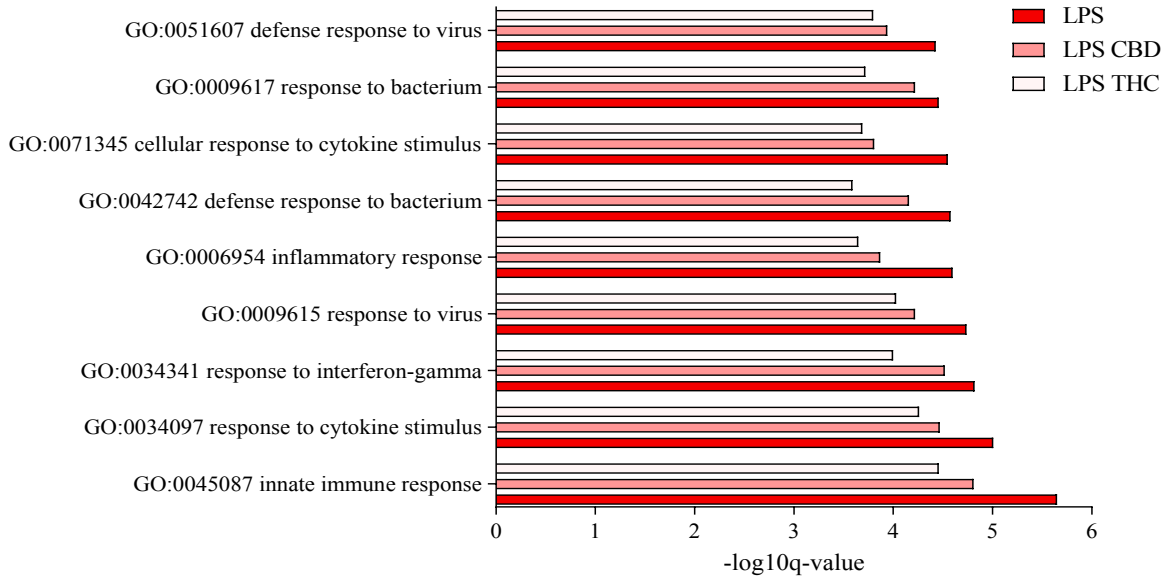
Figure 5.8. Overlapping and unique DEGs in response to LPS, LPS + CBD, and LPS + Δ^9-THC. Venn diagrams displaying the number of genes that were differentially upregulated (A) or downregulated (B) across the different treatment groups as well as the number of genes that were unique or shared amongst groups.

5.3 Functional & pathway analyses following CBD or Δ^9-THC pre-treatment in LPS-induced MH-S cells

To further characterize the effects of CBD and Δ^9-THC on the LPS-induced transcriptome, the DEGs were classified into gene ontology (GO) biological processes (FDR 0.05) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. Based on these analyses, DEGs are mainly involved in processes such as immunity and inflammation, including the innate immune response (GO:0045087), response to cytokine stimulus (GO:0034097), response to interferon-gamma (GO:0034341), and inflammatory response (GO:0006954) (Figure 5.9A). Additional pathways identified included JAK-STAT signaling (mmu04630), TNF signaling (mmu04668) and NF-κB (mmu04064) (Figure 5.9B).

To compare the GO biological processes and pathways according to treatment condition, GO enrichment analysis was conducted to determine the significance with which a particular process or pathway is associated with the group of DEGs. For each biological process that was identified, there was a reduction in GO enrichment in the LPS + CBD and LPS + Δ^9-THC treatment groups when compared to LPS (Figure 5.9A). Most notably this reduction in GO enrichment can be seen in biological processes such as inflammatory response (GO:0006954) and cellular response to cytokine stimulus (GO:0071345) (Figure 5.9A) and in pathways such as NF-κB (mmu04064), antigen-processing and presentation (mmu04612) and JAK-STAT-signaling (mmu04630); there was no GO enrichment in the LPS + CBD treatment group (Figure 5.9B) for these pathways. This indicates that when in a state of LPS-induced inflammation, CBD and Δ^9-THC may alter genes associated with relevant biological processes and pathways.

A. Biological Processes Regulated by DEGs



B. Pathways Regulated by DEGs

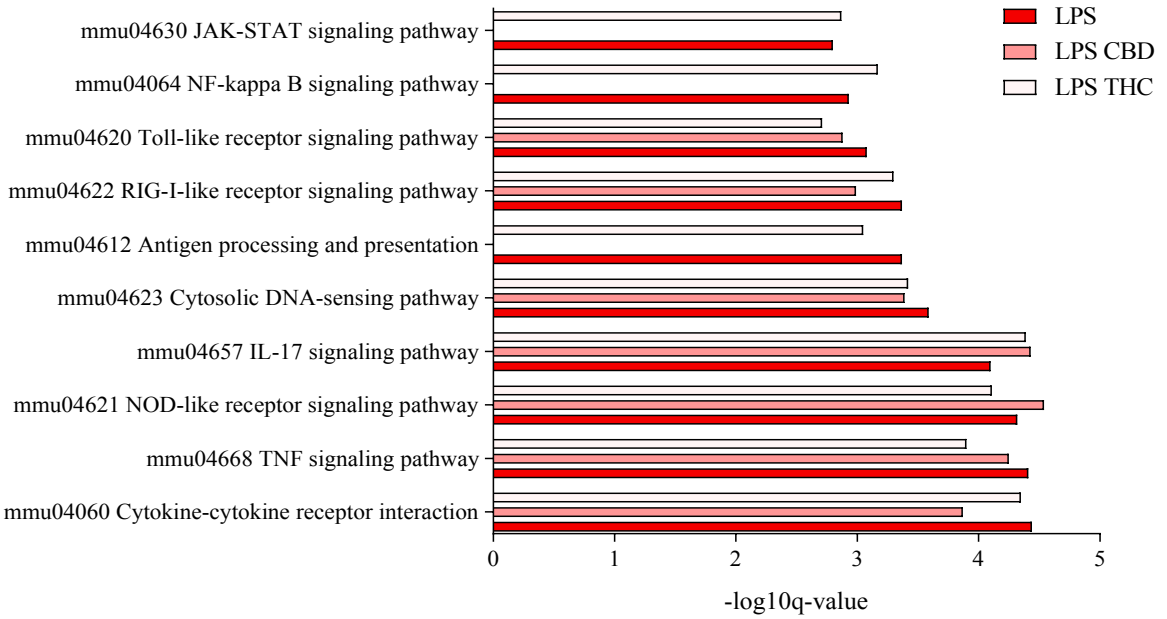


Figure 5.9. Functional annotation and biological pathways of DEGs in the LPS, LPS + CBD, and LPS + Δ^9-THC treatment groups. (A) GO term enrichment analysis for the "biological process" category of the DEGs. Biological processes are presented as the -$\log_{10}$ q-value of the DEGs for each treatment group. The GO terms are ranked by the -$\log_{10}$ q-value in the LPS treatment group. (B) KEGG pathways derived from the DEG profiles of the LPS, LPS + CBD, and LPS + Δ^9-THC treatment conditions. Pathways are presented as the -$\log_{10}$ q-value of the DEGs for each treatment group. The pathways are ranked and presented according to the -$\log_{10}$ q-value of the LPS treatment group.

To further quantify differences in the DEG profiles caused by CBD or Δ^9-THC, we used these RNA-seq data to compare the level of induction of the genes that were differentially regulated. Because we were specifically interested in the ability of cannabinoids to modulate inflammation in AMs, DEGs that comprised the inflammatory response (GO:0006954) as well as NF-κB signaling (mmu04064) pathway were further evaluated. Comprising the inflammatory response (GO:0006954) biological process were 36 genes that were differentially regulated by LPS. CBD decreased the expression of 32 of those genes with a mean $\log_2$-fold change value of -0.652 relative to LPS (Table 2). Genes that exhibited the most significant reduction (FDR-adjusted p-value) by CBD included *IL-1β, IL-6, SERPINE1, IL1A* and *PTGS2* (Table 2). Δ^9-THC decreased the expression of 35 of the 36 genes with a mean $\log_2$-fold change value of -0.613 relative to LPS alone (Table 2). Genes which demonstrated the most dramatic reduction by Δ^9-THC included *IL-6, SERPINE1, PTGS2, IL-12β* and *CCL5* (Table 2).

The NF-κB signaling (mmu04064) pathway had 11 genes that were differentially regulated by LPS. CBD decreased the expression of 9 of those genes with a mean $\log_2$-fold change value of -0.567 (Table 3). Genes which had the most dramatic reduction by CBD included *IL-1β, PTGS2, CXCL2, TNF* and *TRAF1*. Similarly, Δ^9-THC reduced the induction of 9 genes comprising the NF-κB signaling pathway, with a mean $\log_2$-fold change value of -0.514 (Table 3). *PTGS2, TNF, CXCL11, CD40* and *TRAF1* were most significantly down-regulated by Δ^9-THC (Table 3). Therefore, both CBD and Δ^9-THC reduced the induction of genes associated with the inflammatory response and NF-κB signaling pathway in response to LPS.

Table 2. CBD and Δ^9-THC reduce the induction of genes comprising the inflammatory response. List of genes comprising the inflammatory response GO term that were differentially regulated by LPS. Each gene is presented as the log_2-fold change in the LPS + CBD and LPS + Δ^9-THC treatment groups relative to the LPS treatment group. Genes are ranked according to significance in the LPS + CBD treatment group relative to the LPS treatment group.

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

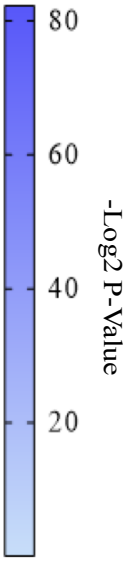
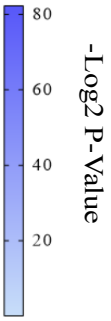


Table 3. CBD and Δ^9-THC reduce the induction of genes comprising the NF-κB signaling pathway. List of genes comprising the KEGG NF-κB signaling pathway that were differentially regulated by LPS. Each gene is presented as the log_2-fold change in the LPS + CBD and LPS + Δ^9-THC treatment groups relative to the LPS treatment group. Genes are ranked according to significance in the LPS + CBD treatment group relative to LPS.

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



5.4 CBD and Δ^9-THC reduce genes associated with M1 macrophage polarization without promoting genes associated with M2 polarization

Macrophages can exist as classically-activated macrophages (M1) that are pro-inflammatory, or alternatively-activated macrophages (M2) that are anti-inflammatory[107]. Owing to their plasticity in this polarization, as well as the decrease in inflammatory gene expression, we next investigated the effects of CBD and Δ^9-THC on the level of induction of genes comprising M1 and M2 macrophage phenotypes using RNA-seq data. Twenty-seven genes comprising the M1 phenotype were selected for evaluation according to literature demonstrating their distinct expression in M1 macrophages[169,170]. Of the 27 genes evaluated, CBD and Δ^9-THC decreased the expression of 26 (Table 4). The mean $\log_2$-fold change value of those genes in response to CBD was -0.542 with *SAA3, NOS2, CCRL2, ISG15* and *CXCL10* demonstrating the most significant reductions (p-value). The mean $\log_2$-fold change decrease of those M1 genes in response to Δ^9-THC was -0.543 with *CXCL10, SAA3, NOS2, ITGAL* and *XAF1* being most significantly reduced (Table 4). Twenty-one genes comprising the M2 phenotype were selected for evaluation according to literature demonstrating their distinct expression in M2 macrophages[169,170]. Of the genes that were evaluated, CBD increased the expression of 12 genes and decreased the expression for 9 genes. The mean $\log_2$-fold change value of the M2 genes in response to CBD was 0.014 (Table 5). Δ^9-THC increased the expression of 13 genes and decreased the expression of 8 with their mean $\log_2$-fold change value being 0.021 (Table 5). Therefore, our results show that both CBD and Δ^9-THC shift macrophage polarization away from an M1 phenotype but not towards an M2 phenotype. Overall, these RNA-seq data provide insight into the transcriptional changes exerted by CBD and Δ^9-THC in MH-S cells under inflammatory conditions.

Table 4. CBD and Δ^9-THC reduce the induction of genes associated with an M1 macrophage phenotype. List of genes associated with an M1 macrophage phenotype that were differentially regulated by LPS. Each gene is presented as the log_2-fold change in the LPS + CBD and LPS + Δ^9-THC treatment groups relative to the LPS treatment group. Genes are ranked according to significance in the LPS + CBD treatment group relative to the LPS treatment group.

Gene Name	Gene Symbol	Log2[LPS]	
		CBD	THC
Serum Amyloid A 3	*SAA3*	-0.439	-0.449
Nitric Oxide Synthase 2, Inducible	*NOS2*	-0.627	-0.580
Chemokine (C-C motif) Receptor-Like 2	*CCRL2*	-0.570	-0.457
Interferon-Stimulated Gene 15	*ISG15*	-1.030	-0.814
Chemokine (C-X-C motif) Ligand 10	*CXCL10*	-1.007	-1.049
2'-5'-Oligoadenylate Synthase-Like Protein 1	*OASL1*	-0.434	-0.376
G Protein-Coupled Receptor 18	*GPR18*	0.809	-0.741
Toll-Like Receptor 2	*TLR2*	-0.275	-0.322
Integrin Subunit Alpha L	*ITGAL*	-0.326	-0.411
Membrane-Spanning 4-Domains, Subfamily A, Member 4c	*MS4A4C*	-1.622	-1.640
Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells Inhibitor, Zeta	*NFKBIZ*	-0.200	-0.208
Complement Factor B	*CFB*	-0.583	-0.426
Guanylate Binding Protein 6	*GBP6*	-0.469	-0.447
Histocompatibility 2, Q Region Locus 6	*H2-Q6*	-0.283	-0.171
CD38 Antigen	*CD38*	-1.402	-0.335
HECT Containing E3 Ubiquitin Protein Ligase 6	*HERC6*	-0.276	-0.333
Schlafen 4	*SLFN4*	-0.431	-0.389
Formyl Peptide Receptor 2	*FPR2*	-0.816	-0.709
Radical S-Adenosyl Methionine Domain Containing 2	*RSAD2*	-0.551	-0.597
DExD/H-Box Helicase 60	*DDX60*	-0.467	-0.604
XIAP-Associated Factor 1	*XAF1*	-0.396	-0.573
Interleukin 1 Receptor Associated Kinase 3	*IRAK3*	-0.163	-0.296
Formyl Peptide Receptor 1	*FPR1*	-0.825	-0.274
Schlafen 1	*SLFN1*	-0.798	-1.863
Z-DNA Binding Protein 1	*ZBP1*	-0.642	-1.031
Cytochrome b-245, Beta Polypeptide	*CYBB*	-0.552	-0.567
MX Dynamin Like GTPase 1	*MX1*	-0.252	-0.475

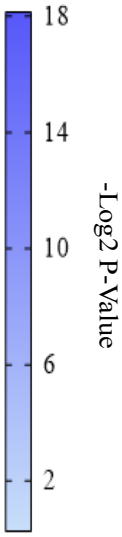
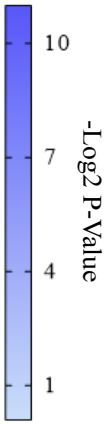


Table 5. CBD and Δ^9-THC do not affect the induction of genes associated with an M2 macrophage phenotype. List of genes associated with an M2 macrophage polarization phenotype that were differentially regulated by LPS. Each gene is presented as the $\log_2$-fold change in the LPS + CBD and LPS + Δ^9-THC treatment groups relative to the LPS treatment group. Genes are ranked according to significance in the LPS + CBD treatment group relative to the LPS treatment group.

Gene Name	Gene Symbol	Log2[LPS]	
		CBD	THC
7-Dehydrocholesterol Reductase	*DHCR7*	0.661	0.189
Matrix Metalloproteinase-12	*MMP12*	-0.323	-0.143
Matrix Metalloproteinase-9	*MMP9*	-0.287	-0.095
Olfactomedin 1	*OLFM1*	0.211	-0.027
Transmembrane Protein 158	*TMEM158*	0.212	0.241
ATPase H+ Transporting V0 Subunit D2	*ATP6V0D2*	0.449	0.635
MYC Proto-Oncogene, bHLH Transcription Factor	*MYC*	0.156	0.364
C-Type Lectin Domain Containing 7A	*CLEC7A*	0.225	0.074
Tetratricopeptide Ankyrin Repeat and Coiled-Coil Containing 2	*TANC2*	-0.090	-0.176
Purinergic Receptor P2Y1	*P2RY1*	-0.238	0.170
Interleukin 6 Cytokine Family Signal Transducer	*IL6ST*	0.067	0.008
Fibronectin Leucine Rich Transmembrane Protein 2	*FLRT2*	0.117	0.049
C-Type Lectin Domain Containing 10A	*CLEC10A*	-0.919	-0.233
Arginase 1	*ARG1*	0.193	0.192
Prostaglandin-Endoperoxide Synthase 1	*PTGS1*	-0.028	0.016
Megakaryocyte-Associated Tyrosine Kinase	*MATK*	-0.101	0.188
Von Willebrand Factor	*VWF*	0.039	0.185
Early Growth Response 2	*EGR2*	-0.153	-0.887
Archaelysin Family Metallopeptidase 1	*AMZ1*	0.043	-0.239
BCAR3 Adaptor Protein, NSP Family Member	*BCAR3*	-0.023	-0.127
Mannose Receptor C-Type 1	*MRC1*	0.082	0.050



5.5 CBD and Δ^9-THC down-regulated inflammatory genes and proteins in alveolar macrophages

Several of the genes identified by RNA-seq analysis to be reduced by CBD and Δ^9-THC following treatment with LPS were further assessed through real-time qPCR. *IL-1β, IL-6* and *TNF-α* were selected based on their reduced expression by CBD and Δ^9-THC relative to LPS. As expected, CBD and Δ^9-THC alone did not affect the expression of *IL-1β* mRNA at the 6- or 24-hour timepoints (Figure 5.10A, B). *IL-1β* mRNA was significantly induced by LPS at both the 6- and 24-hour timepoints; pre-treatment with either CBD or Δ^9-THC significantly reduced the expression at the 24-hour timepoint (Figure 5.10A, B). *IL-6* mRNA was similarly unaffected by treatment with CBD and Δ^9-THC at both timepoints (Figure 5.10C, D). The induction of *IL-6* mRNA by LPS was significantly inhibited by both CBD and Δ^9-THC at the 24-hour timepoint alone (Figure 5.10C, D). CBD and Δ^9-THC alone did not affect the expression levels of *TNF-α* mRNA (Figure 5.10E, F). Similarly, there was a trend towards a reduction in *TNF-α* mRNA by both CBD and Δ^9-THC at the 24- but not 6-hour timepoint (Figure 5.10E, F). Therefore, both CBD and Δ^9-THC significantly reduced the induction of genes associated with inflammation following incubation with LPS.

To next assess if the immunomodulatory effects of CBD and Δ^9-THC was also observed at the protein level, a multiplex assay was carried out on the cell supernatants. CBD and Δ^9-THC alone did not have any effect on the proteins examined (Figure 5.11). There was a significant reduction in IL-1β and IL-6 when AMs were pre-treated with either CBD or Δ^9-THC (Figure 5.11A, C). Of the other cytokines assessed, only CBD significantly reduced their expression (Figure 5.11B-E). Treatment with Δ^9-THC led to a non-significant reduction of GM-CSF and TNF-α (Figure 5.11B

and E). Finally, MCP-1 induction by LPS was unaffected by CBD or Δ^9-THC (Figure 5.11D). IFN-γ, IL-2, IL-4, IL-10 and IL-12p70 were also assessed but were below the limit of detection (data not shown). Overall, these results demonstrate that CBD and Δ^9-THC attenuate the inflammatory response in AMs.

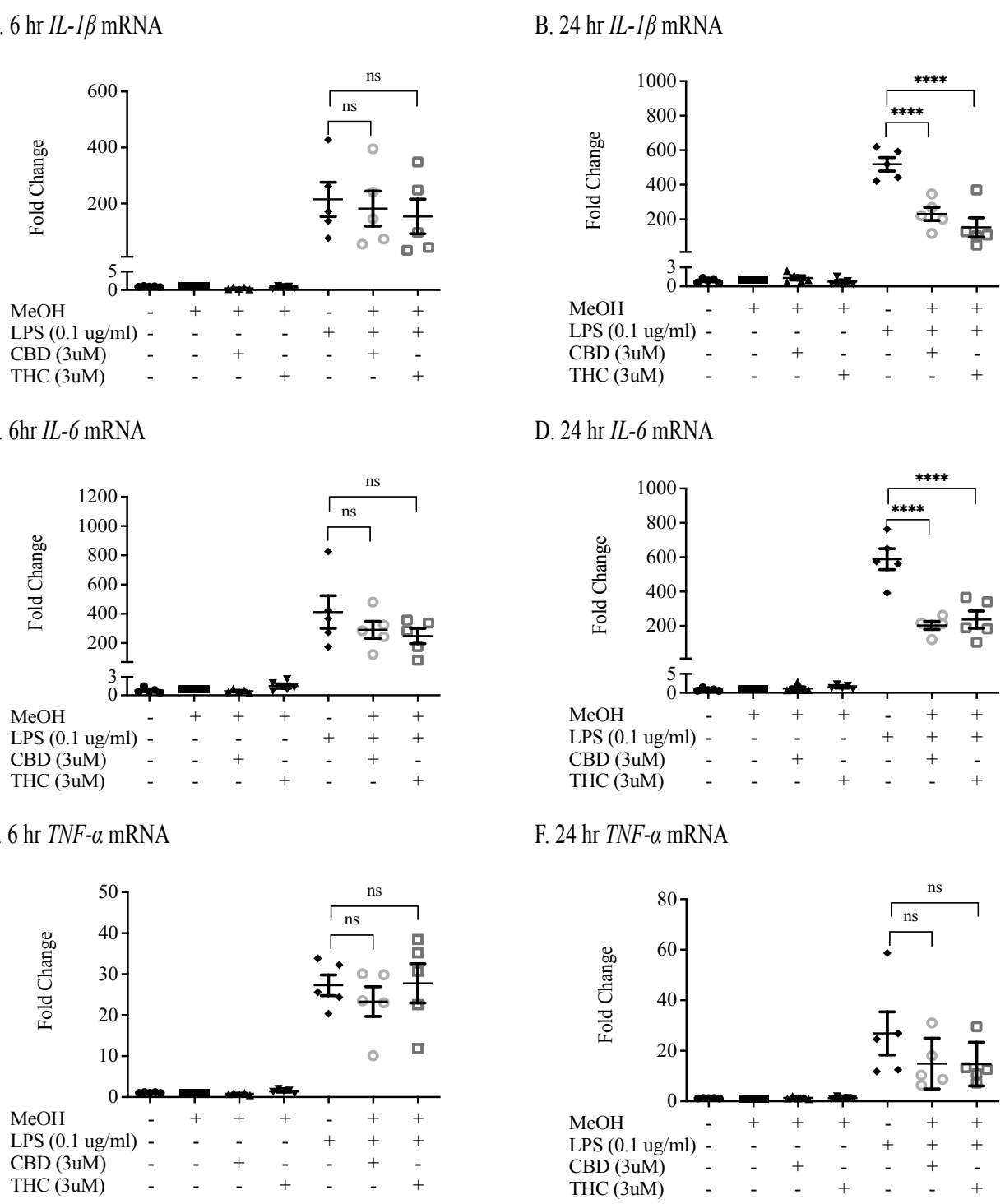


Figure 5.10. CBD and Δ^9-THC reduce the mRNA of *IL-1β*, *IL-6*, and *TNF-α* in response to LPS in a time-dependent manner. (A) There was no change in *IL-1β* mRNA levels in MH-S cells pre-treated with CBD or Δ^9-THC followed by the addition of LPS for 6 hours. There was a significant reduction in *IL-1β* (B) expression by CBD and Δ^9-THC with LPS for 24 hours. *IL-1β* mRNA did not change in response to CBD or Δ^9-THC alone at either timepoint (A-B) relative to

methanol control (n=5). *IL-6* (C) mRNA levels did not change in response to cells pre-treated with CBD or Δ^9-THC followed by the addition of LPS for 6 hours. Both CBD and Δ^9-THC significantly reduced *IL-6* (D) expression with LPS for 24 hours. *IL-6* mRNA levels did not change in response to CBD or Δ^9-THC alone at both 6- and 24-hour timepoints (C-D) relative to methanol control (n=5). (E) There was no change in *TNF-α* mRNA levels in cells pre-treated with CBD or Δ^9-THC followed by the addition of LPS for 6 hours or 24 hours (F). *TNF-α* mRNA did not change in response to CBD or Δ^9-THC alone (E-F) relative to methanol control (n=5). Values are presented as the mean ± SEM. Means are expressed as fold change from the control (methanol). ns; not statistically significant, ****$p<0.0001$, as compared to control (LPS alone).

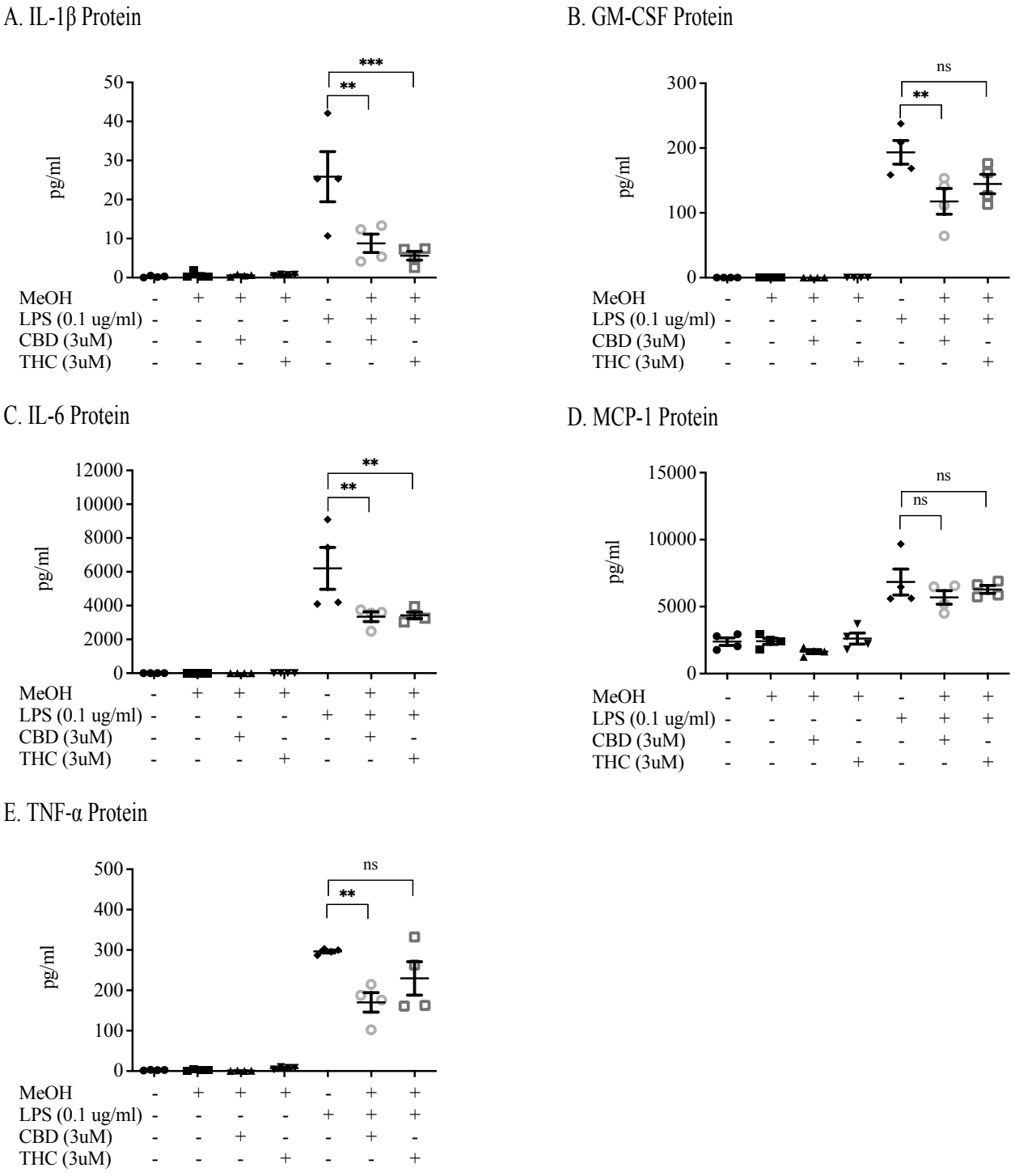


Figure 5.11. CBD and Δ^9-THC reduce the level of proteins associated with inflammation in response to LPS. Quantification of inflammatory proteins was evaluated through Multiplex Assay in cells treated with CBD or Δ^9-THC with or without LPS for 24 hours. Basal levels of IL-1β (A), GM-CSF (B), IL-6 (C), MCP-1 (D), and TNF-α (E) are unaffected in response to CBD or Δ^9-THC in the absence of LPS. In the presence of LPS, CBD significantly reduced the protein level of IL-

1β (A), GM-CSF (B), IL-6 (C), and TNF-α (E). There was no significant difference in MCP-1 (D) protein by CBD in the presence of LPS. Δ^9-THC significantly reduced the protein level of IL-1β (A) and IL-6 (C) when LPS was present. However, Δ^9-THC did not change the level of GM-CSF (B), MCP-1 (D), and TNF-α (E) protein when compared to the LPS control (n=4). Values are presented as the mean ± SEM. ns; not statistically significant, **$p<0.01$, ***$p<0.001$, as compared to control (LPS alone).

5.6 CBD reduces alveolar macrophage phagocytosis in response to LPS

The function of AMs centers in part on their ability to phagocytose dead cells, debris, and foreign material; in habitual consumers of cannabis, this ability may be impaired. However, there is no information on whether THC or CBD affect AM phagocytosis, a question we next addressed. To assess this, cells were treated with CBD or Δ^9-THC in the presence or absence of LPS, followed by the addition of IgG-coated beads. Phagocytosis of the beads was subsequently assessed by flow cytometry. MH-S cells treated with CBD or Δ^9-THC alone had no significant effect on bead uptake (Figure 5.12A, C). However, CBD significantly reduced the uptake of the beads in the presence of LPS (Figure 5.12B, D). There was also a non-significant decrease in bead uptake when cells were treated with Δ^9-THC and LPS (Figure 5.12B, D). These data indicate that CBD exerts a suppressive effect on the phagocytic ability of AMs.

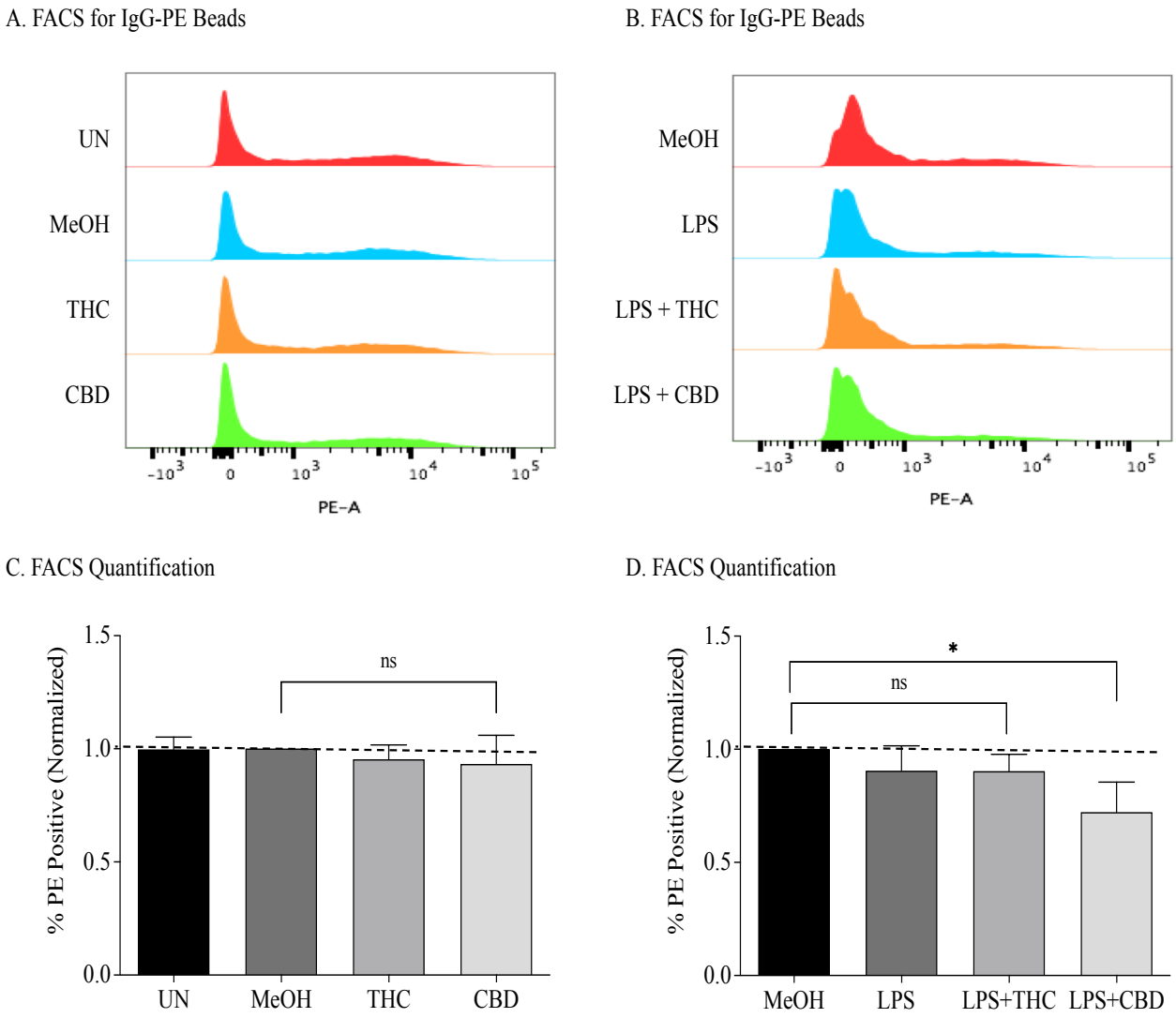


Figure 5.12. CBD reduces alveolar macrophage phagocytosis. Data shown are phycoerythrin (PE) positive cells in response to CBD or Δ^9-THC in the absence (A) or presence (B) of LPS for 24 hours. Normalized quantification of the number of PE positive cells in response to CBD or Δ^9-THC relative to methanol in the absence (C) or presence (D) of LPS. (C) There is no change in the number of PE positive cells in response to CBD or Δ^9-THC. (D) In the presence of LPS, CBD significantly decreases the number of PE positive cells compared to methanol control. There is no significant decrease in the number of PE positive cells in response to Δ^9-THC in the presence of LPS (n=4). Values are presented as the mean ± SEM. ns; not statistically significant, *$p<0.05$, normalized to control (MeOH). FACS: fluorescence-activated cell sorting.

5.7 The anti-inflammatory properties of CBD and Δ^9-THC in bone-marrow-derived macrophages

To extend our findings on the immunomodulatory properties of CBD and Δ^9-THC in AMs, we next utilized primary BMDMs from wild-type C57BL/6 mice. We first optimized the concentration of CBD and Δ^9-THC in BMDMs via MTT. Here, there was a dose-dependent decrease in viability in BMDMs treated with Δ^9-THC (Figure 5.13A). Δ^9-THC at a concentration of 1 μM did not lead to a significant decrease in cell viability (Figure 5.13A). Similarly, BMDMs treated with CBD 1 or 2 μM did not lead to a significant decrease in cell viability (Figure 5.13B). Based on this we selected 1 μM as the concentration for both CBD and Δ^9-THC in the experiments utilizing BMDMs.

To ensure an adequate inflammatory response in BMDMs, cells were treated with LPS at 1, 0.1, or 0.01 μg/ml for24 hours. LPS at concentrations of 0.1 and 1 μg/ml significantly increased *IL-1β* (Figure 5.13C), *IL-6* (Figure 5.13D), and *TNF-α* mRNA induction (Figure 5.13E). LPS at a concentration of 0.01 μg/ml did not significantly increase the expression of pro-inflammatory genes (Figure 5.13C-E). As such, LPS at a concentration of 0.1 μg/ml and for 24 hours was utilized in the following experiments.

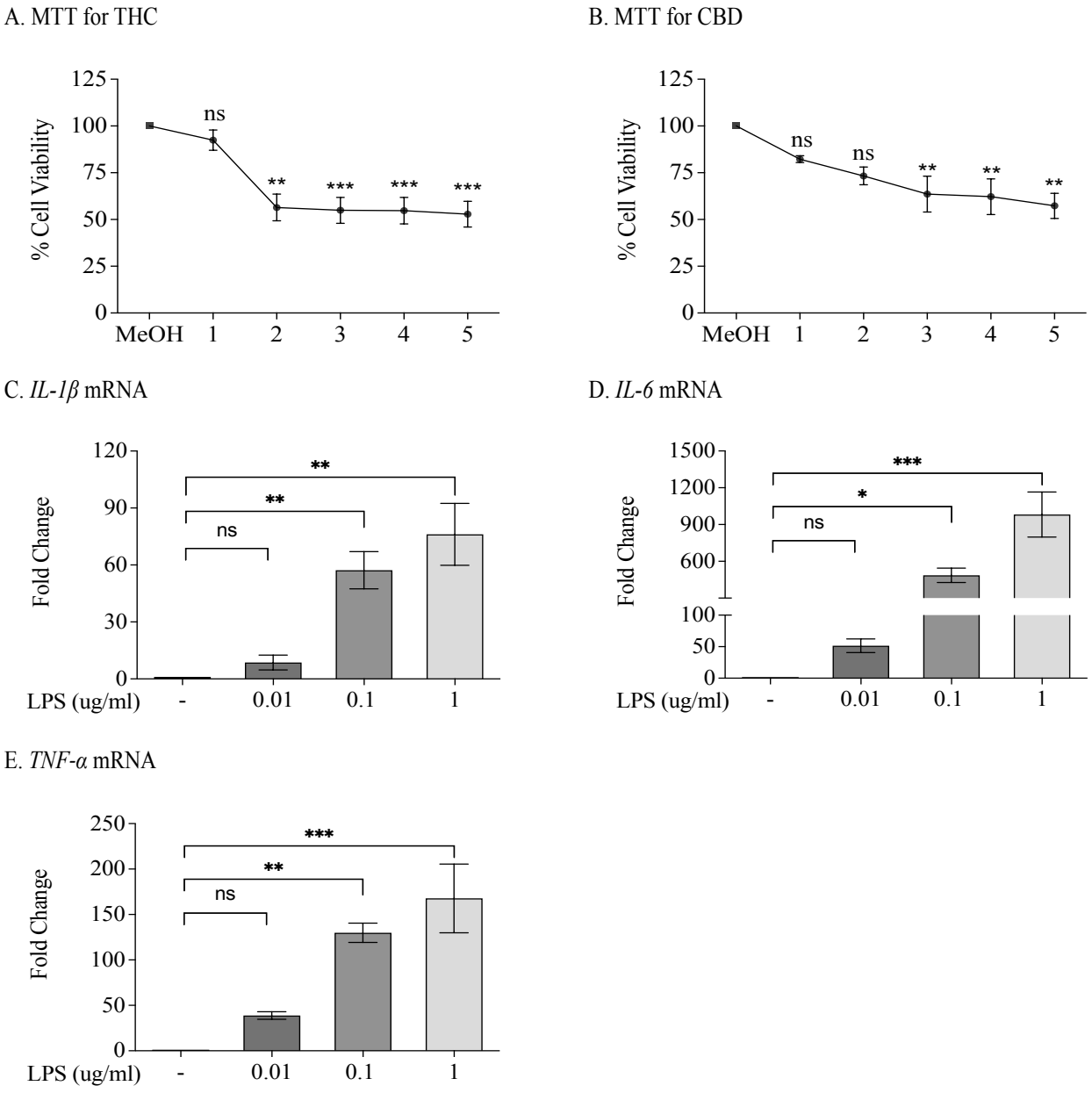
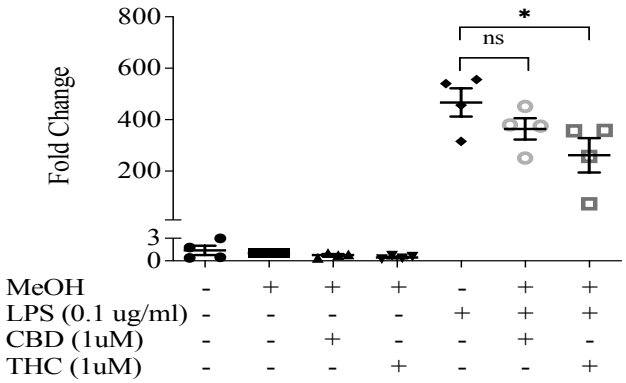


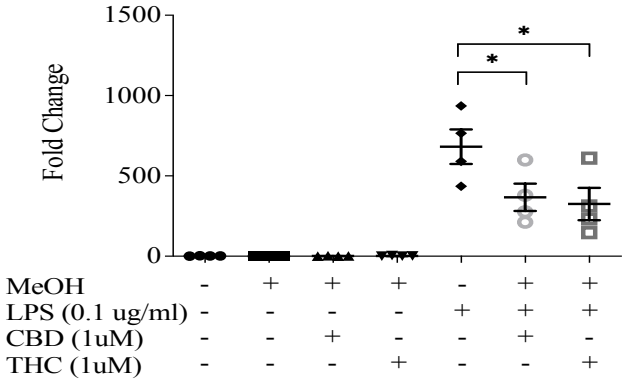
Figure 5.13. Optimization of experimental conditions in BMDMs. (A-B) MTT colorimetric cell viability assay evaluating Δ^9-THC (A) and CBD (B) at concentrations ranging from 1-5 μM. Both Δ^9-THC (A) and CBD (B) at concentrations of 3 μM or greater significantly decreased cell viability. At concentrations of 1 μM there was no significant effect on cell viability by Δ^9-THC (A) or CBD (B) (n=4). (C-E) Induction of *IL-1β*, *IL-6*, and *TNF-α* mRNA in response to LPS at concentrations ranging from 0.01-1 μg/ml for 24 hours. Levels of *IL-1β* (C), *IL-6* (D), and *TNF-α* (E) are significantly increased in response to LPS at a concentration of 1 and 0.1 μg/ml. However, 0.01 μg/ml LPS, there was no significant increase in all these genes (C-E) (n=3). Values are presented as the mean ± SEM. Means are expressed as fold change from the control (serum-free media). ns; not statistically significant, *p<0.05, **p<0.01, ***p<0.001, compared to control.

To next assess the immunomodulatory properties of CBD and Δ^9-THC, BMDMs were pre-treated with either CBD or Δ^9-THC for 1 hour followed by the addition of LPS for 24 hours. The expression of *IL-1β, IL-6,* and*, TNF-α* were subsequently assessed by qPCR. Neither CBD or Δ^9-THC alone had any effect on the induction of *IL-1β, IL-6,* or*, TNF-α* mRNA (Figure 5.14). Following the induction of *IL-1β* mRNA by LPS, there was a non-significant decrease in expression by CBD (Figure 5.14A). However, Δ^9-THC significantly reduced *IL-1β* mRNA when compared to LPS (Figure 5.14A). The expression of *IL-6* following induction by LPS was significantly decreased by both CBD and Δ^9-THC (Figure 5.14B). *TNF-α* was significantly induced by LPS; however, neither CBD or Δ^9-THC significantly modulated its expression (Figure 5.14C). Therefore, CBD and Δ^9-THC have differential effects on the expression of pro-inflammatory cytokines in primary BMDMs in response to LPS.

A. *IL-1β* mRNA



B. *IL-6* mRNA



C. *TNF-α* mRNA

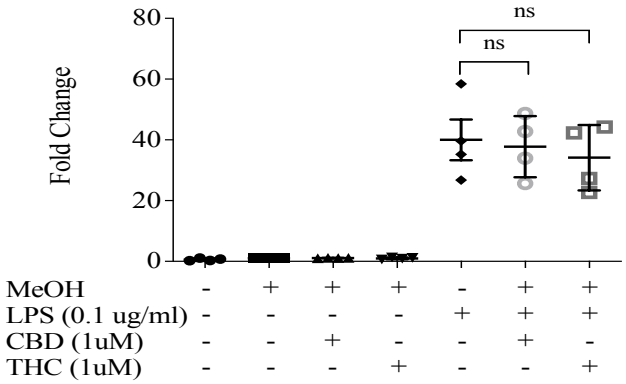


Figure 5.14. CBD and Δ^9-THC exert immunomodulatory properties in BMDMs. (A) Following significant induction of *IL-1β* mRNA by LPS, Δ^9-THC significantly reduced its expression. (B) *IL-6* mRNA was significantly reduced by both CBD and Δ^9-THC following treatment with LPS. (C) The induction of *TNF-α* mRNA by LPS was not affected by treatment with either CBD or Δ^9-THC (n=4). Values are presented as the mean ± SEM. Means are expressed as fold change from the control (methanol). ns; not statistically significant, *p<0.05, as compared to control (LPS alone).

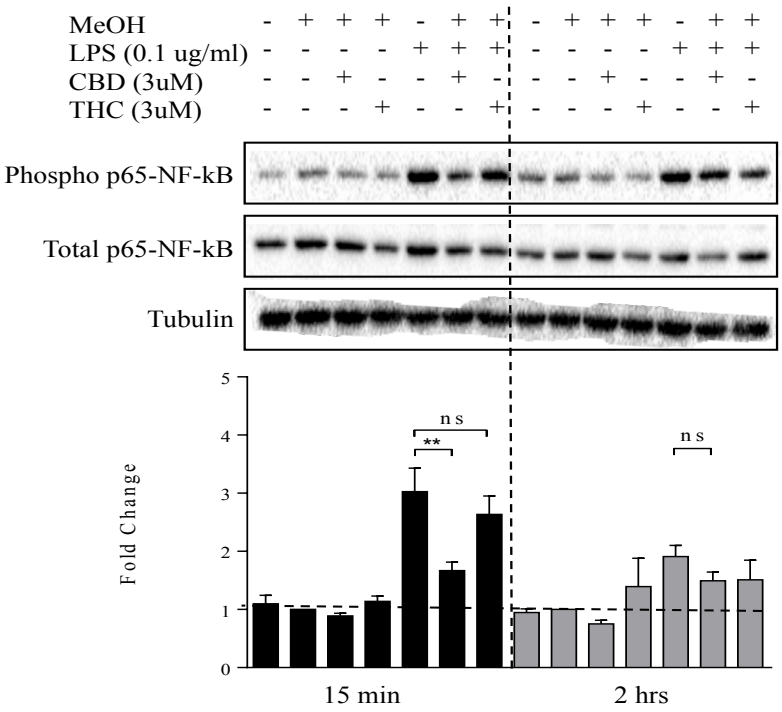
5.8 CBD reduces LPS-induced activation of the NF-κB- and ERK1/2-signaling pathways

Given that CBD and Δ^9-THC significantly reduced markers associated with the inflammatory response, our next question focused on the mechanism by which this was occurring. The NF-κB pathway plays a major role in mediating the induction of pro-inflammatory genes. From our RNA-seq data, we observed that while LPS caused significant enrichment in this pathway, there was no gene enrichment for the LPS + CBD treatment group (see Figure 5.9B), suggesting that CBD may be exerting its immunomodulatory properties through an inhibition of this pathway. Therefore, we next addressed the role of NF-κB signaling in the anti-inflammatory properties of CBD and Δ^9-THC in AMs. First, we analyzed activation of the p65 subunit. Figure 5.15A illustrates that neither CBD or Δ^9-THC alone caused significant change in p65 phosphorylation, a marker of its activation[171]. In response to LPS, there was a significant increase in the phosphorylation of p65, an effect that was inhibited by CBD at the 15-minute time point (Figure 5.15A). Δ^9-THC, in combination with LPS, led to a slight but non-significant decrease in p65 activation (Figure 5.15A). At the 2-hour time point, both CBD and Δ^9-THC partially attenuated the phosphorylation of p65 by LPS (Figure 5.15A). In summary, CBD, but not Δ^9-THC, inhibits p65 phosphorylation.

Next, the MAPK ERK1/2 was evaluated. ERK1/2 signaling plays a major role in the activation of several transcription factors that contribute to the inflammatory response, including NF-κB. To assess ERK1/2 activity, we measured changes in phosphorylation, a marker of its activation. CBD alone had no effect on the activation of ERK1/2; however, treatment with Δ^9-THC alone led to a significant increase in ERK1/2 activation at both the 15-minute and 2-hour time points (Figure 5.15B). The significant induction of ERK1/2 phosphorylation by LPS was reduced by CBD to near basal levels (Figure 5.15B). Δ^9-THC, in combination with LPS, also resulted in a slight but non-

significant increase in ERK1/2 phosphorylation when compared to LPS at both time points (Figure 5.15B). Overall, these data indicate that CBD and Δ^9-THC have opposing effects on the activation of ERK1/2 at basal level as well as in combination with LPS. Additionally, the modulation of ERK1/2 phosphorylation by CBD represents another signaling pathway through which CBD may be exerting its anti-inflammatory properties. Δ^9-THC does not appear to modulate ERK1/2 activity.

A. Phospho p65-NF-kB Protein



B. Phospho ERK1/2 Protein

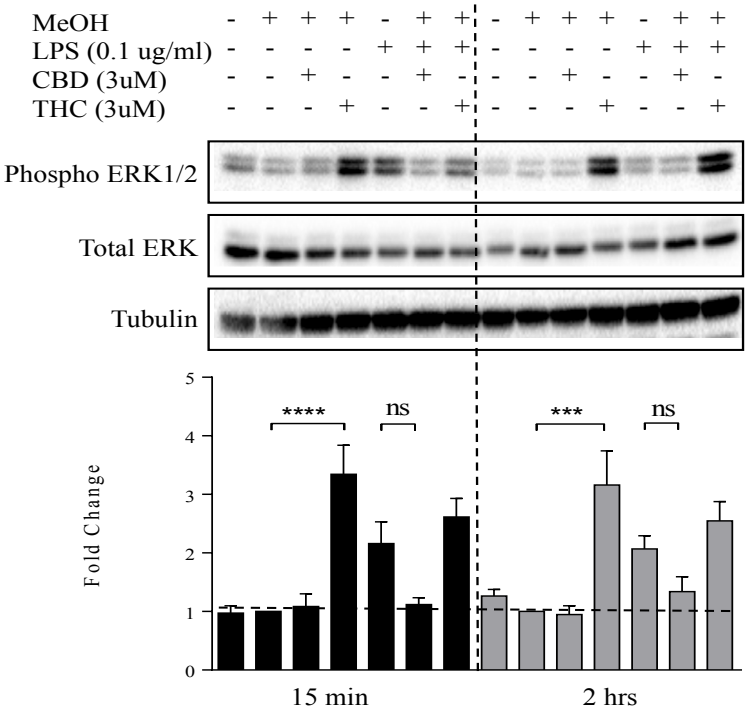


Figure 5.15. Effects of CBD and Δ^9-THC on basal & LPS-induced activation of NF-κB and ERK1/2. MH-S cells were pre-treated with CBD or Δ^9-THC for one hour followed by the addition of LPS for 15 minutes or 2 hours. Cellular protein was collected for western blot analysis of

phosphorylated p65 (A) and ERK1/2 (B) proteins. Basal p65 phosphorylation was unaffected by either CBD or Δ^9-THC at both time points as compared to control methanol (A). LPS-induced increase in phosphorylated p65 (A) was significantly downregulated by CBD but not Δ^9-THC following 15-minute exposure. There was a non-significant increase in phosphorylated p65 following 2-hour exposure in response to LPS (A). Basal ERK1/2 phosphorylation was unaffected by CBD; however, Δ^9-THC significantly increased phosphorylation at both timepoints as compared to methanol control (B). LPS-induced increase in protein levels of phosphorylated ERK1//2 were downregulated non-significantly by CBD and were unaffected by Δ^9-THC at both timepoints. Values are presented as the mean ± SEM (depicted blots are representative of five independent experiments). Means are expressed as fold change from the control (MeOH). ns; not statistically significant, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ as compared to control LPS for each treatment unless otherwise stated.

5.9 The CB_2 receptor is required to mediate the anti-inflammatory properties of CBD and Δ^9-THC

Many of the pharmacological effects imparted by cannabinoids have been reported to act through the endogenous cannabinoid receptors CB_1 and CB_2. As such, we next investigated the role of these receptors in mediating the anti-inflammatory properties of CBD and Δ^9-THC in AMs. To address this, we first evaluated CB_1- and CB_2- receptor expression. We assessed the expression of the CB_1 receptor in MH-S cells by western blot, using mouse brain as positive control. However, we were unable to detect CB_1 receptor protein in these AMs (Figure 5.16A). However, CB_2 protein was readily detectable in AMs, and that the level of expression was not significantly altered by the treatments utilized in this study (Figure 5.16B).

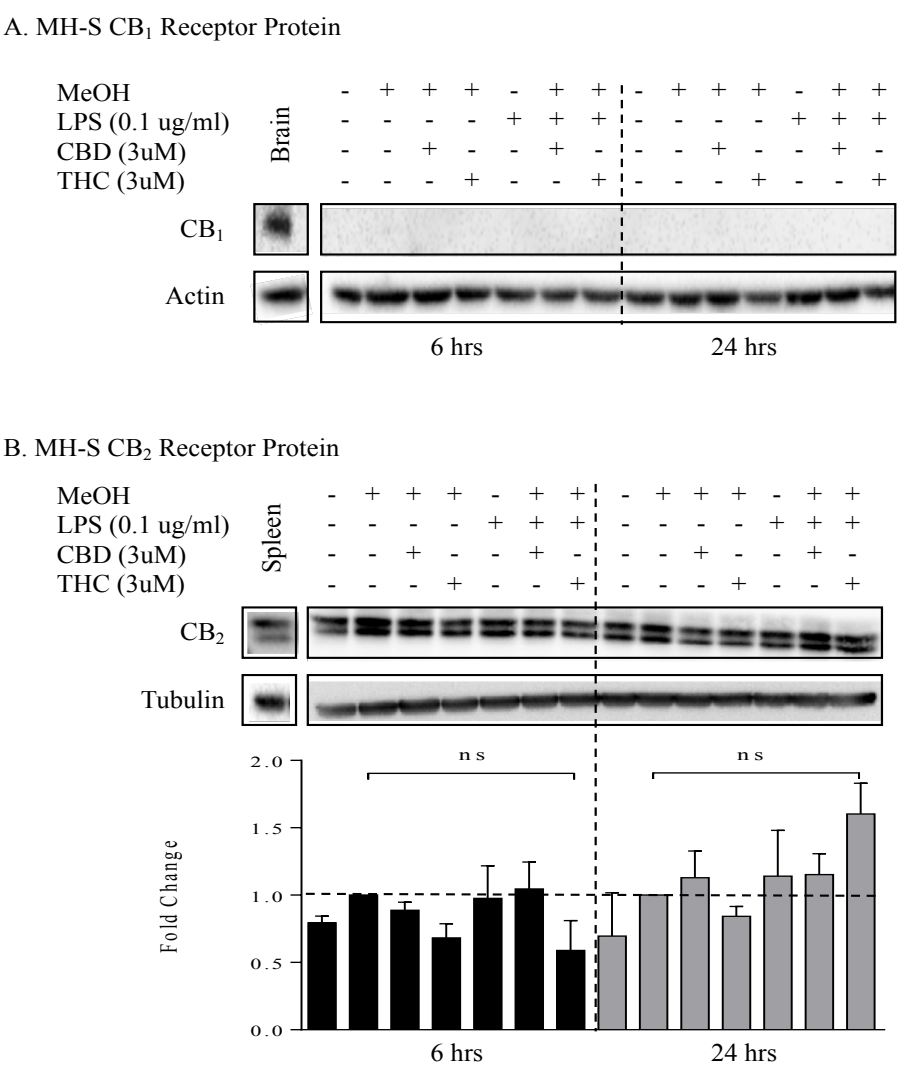


Figure 5.16. CB receptor expression in MH-S cells. (A) Total CB_1 protein is not expressed in MH-S cells under basal-, LPS-, or cannabinoid-induced conditions for both 6- and 24-hour timepoints (n=3). (B) Total CB_2 protein is constitutively expressed in MH-S cells (n=3). Values are presented as the mean ± SEM. Means are expressed as fold change from the control (MeOH). ns; not statistically significant,

Therefore, we speculated that the CB_2 receptor may contribute to the reduction in pro-inflammatory cytokines induced by LPS. To assess the role of the CB_2 receptor, we transiently transfected AMs with either CB_2 receptor-specific siRNA ($siCB_2$) or with control siRNA (siCTRL). We confirmed that the level of the CB_2 receptor was significantly reduced by 50% in the $siCB_2$-transfected cells comparing to siCTRL-transfected cells by western blot (Figure 5.17A). siCTRL and $siCB_2$-transfected cells were then pre-treated with CBD or Δ^9-THC for 1-hour, followed by the addition of LPS for an additional 24 hours. RNA was then collected and the expression of pro-inflammatory cytokines *IL-1β*, *IL-6*, and *TNF-α* was assessed by qPCR. There was a slight but non-significant decrease in *IL-1β* expression in response to CBD and Δ^9-THC relative to the LPS treatment group in the siCTRL-transfected cells (Figure 5.17B). However, in the $siCB_2$-transfected cells there was no reduction in *IL-1β* expression in response to CBD and Δ^9-THC (Figure 5.17B). Similarly, the expression of *IL-6* in siCTRL-transfected cells was reduced by both CBD and Δ^9-THC relative to LPS, although only for CBD did this reach statistical significance (Figure 5.17C). There was no reduction of *IL-6* mRNA by either CBD or Δ^9-THC in the $siCB_2$-transfected cells (Figure 5.17C). A similar finding was observed upon evaluation of *TNF-α* expression whereby the non-significant reduction in expression by CBD and Δ^9-THC in the siCTRL-transfected cells, was not observed in the $siCB_2$-transfected cells (Figure 5.17D). Taken together, these data indicate that the CB_2 receptor is playing a role in mediating the anti-inflammatory properties of both CBD and Δ^9-THC in AMs.

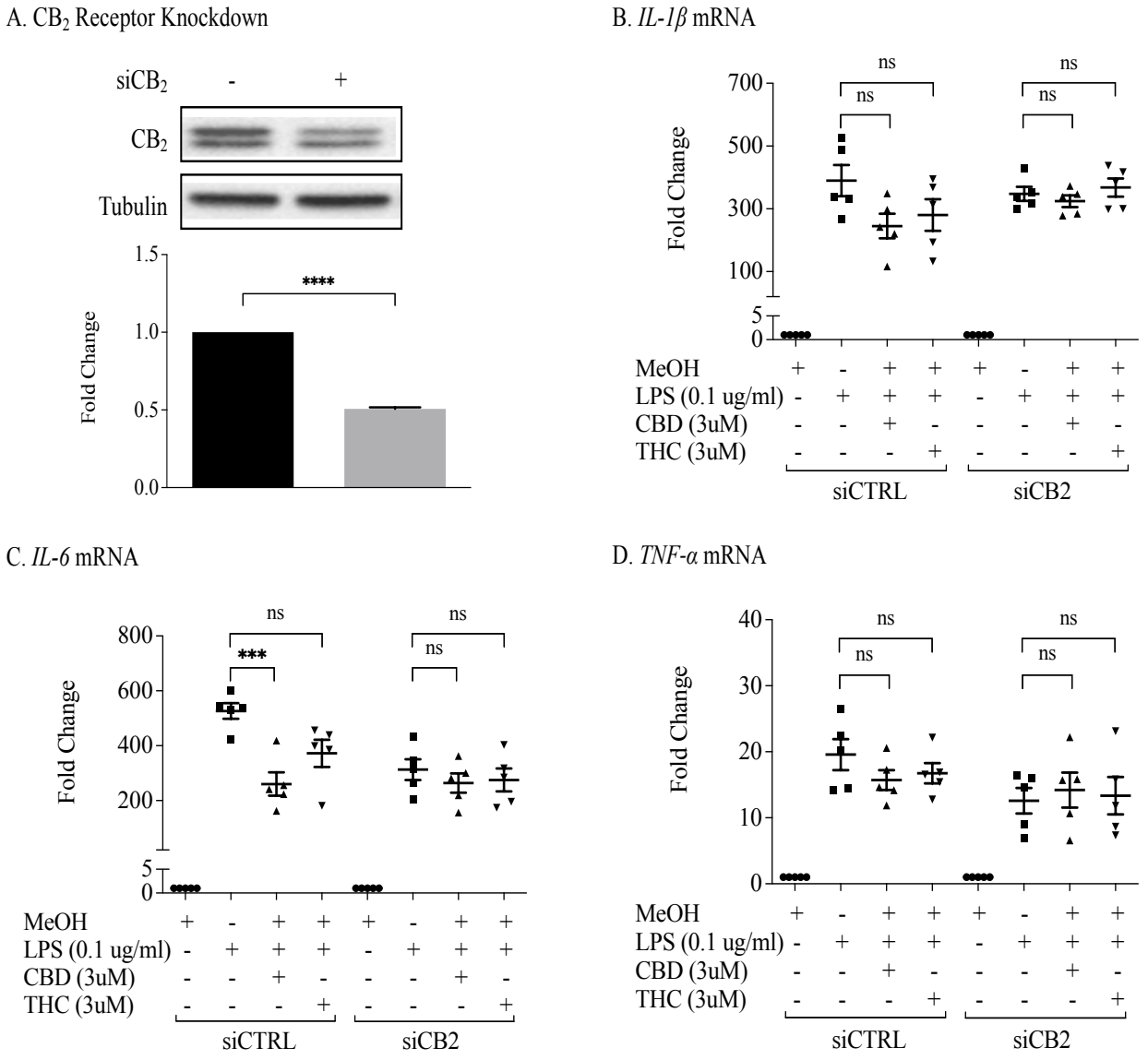


Figure 5.17. Cannabinoid-mediated reduction in pro-inflammatory cytokines is inhibited in response to CB_2 receptor knockdown in MH-S. (A) $siCB_2$- western blot- densitometry: transfection of MH-S cells with $siCB_2$ reduced the level of CB_2 protein to ~ 50%. Levels of *IL-1β* (B), *IL-6* (C), and *TNF-α* (D) mRNA in response to siCTRL or $siCB_2$- transfected cells pre-treated with either CBD or Δ^9-THC followed by the addition of LPS. Results are expressed as the mean ± SEM of 5 independent experiments. Means are expressed as fold change from the control (MeOH). ns; not statistically significant, ***p<0.001, ****p<0.0001 as compared to control LPS for each treatment.

In conclusion, our study has demonstrated five novel findings:

1. Using RNA-seq, we demonstrated that both CBD & Δ^9-THC exert anti-inflammatory properties in AMs characterized by reductions in pro-inflammatory cytokines in response to LPS.
2. CBD and Δ^9-THC partially shift AM polarization away from an M1 phenotype.
3. The suppressive effects of CBD extend beyond its ability to inhibit pro-inflammatory cytokines by also reducing phagocytosis, a key feature of macrophage function.
4. CBD inhibits activation of the NF-κB and ERK1/2 signaling pathways.
5. The CB_2 receptor is constitutively expressed in AMs and mediates some of the anti-inflammatory properties of both CBD and Δ^9-THC.

CHAPTER 6 DISCUSSION

As it currently stands, *C. sativa* ranks as the most widely cultivated and consumed drug considered to be illicit internationally[35]. Even with a lack of scientific knowledge surrounding its physiological effects, countries such as Canada have recently legalized cannabis for both medicinal and recreational use[34]. One of the primary unknowns related to the effects of cannabis consumption is that of its signature compounds called cannabinoids. Despite their isolation from *C. sativa* in the early 1960's, CBD and Δ^9-THC have not been extensively studied[22,23]. In recent decades, advances in molecular biology have led to the discovery of several interactions between these cannabinoids and endogenous signaling proteins which has shed light on the physiological effects imparted by CBD and Δ^9-THC. For instance, both CBD and Δ^9-THC can inhibit the induction of pro-inflammatory acute-phase proteins such as NO, TNF-α, IL-1β, and IL-6[82,83]. As such, an increasing number of studies have revealed the therapeutic potential of these cannabinoids in pathologies characterized by a dysregulated immune response including MS, IBD, arthritis, and many more[85,86,88]. Although the mechanisms have yet to be elucidated, these findings have greatly broadened our view on the ability of select cannabinoids to serve as novel regulators in disease progression. Overall, our data show powerful immune regulatory effects of CBD and Δ^9-THC on AMs.

Among the physiological functions exerted by CBD and Δ^9-THC, their ability to modulate the pulmonary inflammatory response has significant implications with regards to the health of cannabis consumers. Smoking currently stands as the most popular method of cannabis consumption wherein cells present in the lung are in direct contact with cannabinoids[140]. However, the smoke generated from burning cannabis delivers not only cannabinoids, but an array of other

plant and combustion derived constituents. The volatile and particulate phase components of cannabis smoke contain carbon monoxide, aldehydes, acrolein, phenols, and carcinogenic polycyclic aromatic hydrocarbons[49]. Many of these compounds have pro-apoptotic properties in addition to being potent inducers of the inflammatory response. This confounds the interpretation of direct effects of cannabinoids themselves on lung cell populations likely to come into contact with cannabinoids. Moreover, the current body of literature describing the interaction between cannabinoids and pulmonary cells is rather sparse including their potential effects on AMs. AMs constitute one of the primary cell types present in the lungs which orchestrate immune processes related to both the promotion and resolution of inflammation[101]. In the lungs of both cannabis- and cigarette-smokers there is a 3-fold increase in the number of alveolar macrophages present[115]. Functional impairments in AM function are also observed in those collected from cannabis users. When co-cultured with *S. aureus*, clear deficits in both bacterial phagocytosis and killing were observed in AMs from cannabis smokers but not from tobacco smokers[115]. The functional differences between cells from cannabis- and tobacco-smokers suggests a unique biological consequence from the cannabinoids inhaled during cannabis smoking. In the present study we observed a net suppressive effect on the ability of AMs to carry out phagocytosis when treated with a combination of CBD and LPS (Figure 5.12). In support of our findings, a report by Hassan and colleagues revealed that in BV-2 microglial cells treated with CBD in combination with LPS, there was a 43% reduction in phagocytosis relative to the control[173]. Taken together, this study and our data indicate that CBD inhibits the function of MH-S alveolar macrophages, as demonstrated by reductions in phagocytosis.

AMs are derived from yoke sac precursors of fetal monocytes, which populate the alveoli shortly after birth and persist over the lifespan via self-renewing embryo-derived populations- independent of a bone-marrow contribution[95,96]. However, in response to inflammatory insults, tissue-infiltrating monocytes derived from the bone-marrow are recruited to the lung and differentiate into macrophages[97]. Tissue-infiltrating monocytes act as short-lived effector cells; however, they are highly plastic and dynamic in their abilities to compliment classic tissue-resident macrophages[97]. Due to their remarkable plasticity, AMs are highly specialized in reacting to environmental signals, leading to rapid and reversible changes in their inflammatory phenotype. In this study, we hypothesized that CBD and Δ^9-THC would exhibit differential effects on the inflammatory response of macrophages. Our rationale was the fact that the phenotype of macrophages is contingent upon stimuli present with their milieu, and that the anti-inflammatory properties of cannabinoids are predominantly described in the context of an inflammatory state. For instance, macrophages polarized to an M1 phenotype via tissue injury, or another inflammatory stimulus, leads to increased expression of cytokines and chemokines such as TNF-α, IL-1β, and IL-6[107]. The increased expression of these mediators is a significant contributor in pathological processes where CBD and Δ^9-THC have demonstrated therapeutic value. Because both tissue-resident- and BMDMs are present in the lungs, we evaluated the ability of CBD- and Δ^9-THC-alone to influence the expression of pro-inflammatory mediators in these relevant cell-types. Under the experimental conditions utilized in this study, neither CBD or Δ^9-THC elicited a transcriptional response in either MH-S or BMDs. This suggests that on their own, neither CBD or Δ^9-THC are influencing aspects related to macrophage function, including polarization or cytokine expression. This result is inconsistent with previous studies in $CD4^+$ T cells, where functional analysis of genes differentially expressed in response to Δ^9-THC revealed significant

enrichment in the inflammatory response pathway[172]. In addition, *in vitro* studies evaluating the effects of cannabinoids, including Δ^9-THC, on cytokine production in B cells, $CD8^+$ T cells, NK cells, monocytes, and eosinophilic cell lines demonstrated variable results, depending on the cell line and concentration of cannabinoids used[150]. For instance, in RAW264.7 monocytes, CBD significantly increased the levels of G-CSF, GM-CSF, IFN-γ, IL-1a, IL-6, IL-27, I-TAC, M-CSF, MCP-1, RANTES, and TNF-α[138]. This suggests that the differential effects of cannabinoids on cytokine induction may be attributed to varied thresholds of different cell populations. It should be noted that the cannabinoids employed in our study were pure analytical grade, whereas those in the previously mentioned study were commercially available e-liquids containing CBD[138]. Thus, the induction of pro-inflammatory markers in that study may be attributed to other ingredients present such as chromium, copper, lead, and flavoring chemicals, as stated in their compositional analysis. Altogether, our results suggest that CBD and Δ^9-THC alone do not influence the transcriptional profile of alveolar- and bone-marrow-derived- macrophages.

Another confounding factor in the interpretation of data between studies is the ability of cannabinoids to affect cell death pathways. This is in fact one of the proposed mechanisms through which cannabinoids exert their immunomodulatory properties[73]. Moreover, in various immune cell populations, cell death has been associated with increased secretion of pro-inflammatory mediators. For instance, Zhu *et al*. demonstrated that treatment with Δ^9-THC at concentrations between 15-30 μM led to apoptosis in murine macrophages through regulation of Bcl-2 and caspase activity[74]. The resultant increase in apoptosis was accompanied by a dose-dependent release of IL-1β as well as other inflammatory cytokines. Similarly, treatment with CBD in the micromolar range caused a dose- and time-dependent increase in apoptosis of $CD4^+$ and $CD8^+$ T

cell populations[144]. Cannabinoid-induced cell-death may be of particular importance with regards to cannabis consumers, as it could be postulated to increase susceptibility to pulmonary infection, specifically as it pertains to lung macrophages. Macrophages constitute one of the primary lines of defence against foreign organisms and xenobiotics within the lungs, meaning their death as a result of high cannabinoid concentration may increase the frequency- and severity- of infection. This may additionally alter the expression and subsequent signaling of various cytokines and chemokines within the lung. To minimize the confounding effects of cannabinoid-induced cell death in our studies, we first ensured that cell viability in response to CBD and Δ^9-THC was not adversely affected, and thus chose a concentration that had no significant effect on cell death. Therefore, choosing a concentration that does not alter survival may underlie discrepancies between our results versus those in the literature with regards to the lack of induction of inflammatory genes by CBD and Δ^9-THC alone.

The therapeutic potential of CBD and Δ^9-THC has predominantly been described within the context of inflammation. In cell types such as macrophages, their phenotype and polarization are dependent upon factors present within their environment[107]. Therefore, we evaluated the effects of CBD and Δ^9-THC in response to LPS, a component of the cell wall of gram-negative bacteria that is a powerful inducer of inflammation. Using RNA-seq, we identified a unique transcriptional response in LPS-induced alveolar macrophages treated with CBD or Δ^9-THC. This unbiased profiling revealed the importance of CBD and Δ^9-THC in the regulation of key inflammatory genes involved in the establishment of innate immunity. Importantly, our RNA-seq data revealed that CBD and Δ^9-THC repressed the enrichment of an important subset of inflammatory genes that were increased by LPS; this included reduced expression of *IL-1β, IL-6,* and *TNF-α* mRNA.

However, this effect occurred in a time-dependent manner, as reduced expression of these cytokines by CBD and Δ^9-THC was only observed at the 24-hour timepoint but not the 6-hour timepoint (Figure 5.10). We speculate that the inability of CBD and Δ^9-THC to reduce the expression of these pro-inflammatory markers at 6 hours may reflect an incomplete transition in macrophage polarization. In support of this, we observed an increase in the induction of M1 macrophage polarization markers by LPS at the 24- versus 6-hour incubation window. This finding is in accordance with the current body of literature in which CBD and Δ^9-THC repress the induction of inflammatory cytokines when subjected to an inflammatory stimulus[82,83]. It is interesting to note that in opposition to other investigations, treatment with Δ^9-THC in LPS-activated macrophages can increase the production of inflammatory cytokines such as IL-1β[174]. Cannabinoids display biphasic dose-response curves for cytokine secretion in some cell-culture systems, which may account for the apparent discrepancy regarding cannabinoid modulation of cytokine expression. Excessive production of IL-1β, IL-6, and TNF-α by macrophages has been associated with disease progression and severe inflammation pathologies, including IBD, multiple sclerosis, and inflammatory lung disease[108]. IL-1β and IL-6 are potent inducers of the acute phase inflammatory response through their ability to induce B cell proliferation as well as co-stimulate APC's and T-cells at the site of action[105]. TNF-α similarly is a pro-inflammatory cytokine that mediates its effects through activation of the NF- κB/MAPK pathway. In turn, this pathway promotes the transcription of several pro-inflammatory cytokines and chemokines, including TNF-α, leading to a positive feedback loop[175]. The ability of CBD and Δ^9-THC to reduce the expression of pro-inflammatory mediators is highly relevant in pulmonary conditions wherein aberrant expression of these cytokines is at the core of their pathology. For instance, ALI is a common pulmonary condition in which the generation and release of pro-inflammatory cytokines,

chemokines, and ROS produced by activated lung macrophages leads to damage of the lung parenchyma. Similar to the model used in our study, LPS is commonly employed as a means to mimic the resultant molecular environment and phenotype of ALI. As such, the ability of cannabinoids CBD and Δ^9-THC to down-regulate the expression of IL-1β, IL-6, and TNF-α may have substantial utility in conditions such as ALI defined by a dysregulated inflammatory response.

Co-stimulatory activity is a primary facet through which cells of the innate immune system communicate with those of the adaptive immune system. Macrophages serve as a primary cell-type that express cell-surface antigens to co-stimulate T cells to fully activate the immune response[176]. One of the unique genes significantly reduced in response to treatment with CBD and Δ^9-THC was CD40 antigen (Table 2). CD40 antigen regulates the co-stimulatory activity of APCs, induces B cells to upregulate B7 co-stimulatory proteins as well as induces DCs to increase cell surface expression of other co-stimulatory molecules such as CD54 and CD86[176]. Furthermore, ligation of CD40 antigen leads to the production of various inflammatory cytokines including IL-8, TNF-α, and MIP[177]. Notably, CD40 co-stimulation leads to the induction of IL-12, a cytokine that was similarly reduced in response to CBD and Δ^9-THC (Table 2). IL-12 plays a key role in the polarization of Th1 immune responses. The expression of CD40 antigen can be found on a host of non-immune cells including epithelial cells, endothelial cells, fibroblasts, myofibroblasts, and more[178]. In these cell-types, the CD40 system serves as an effective means of communication with immune cells, the usual outcome being amplification of immune and inflammatory processes. For instance, ligation of CD40 antigen on endothelial cells or fibroblasts leads to the production of IL-8, MCP-1, MIP-1, IL-6, and TNF-α[176,177]. This suggests that repression of CD40 antigen in

alveolar macrophages by CBD and Δ^9-THC may be how cannabinoids can mitigate an adaptive immune response. Such as notion is supported by a study by Chuchawankul and colleagues who demonstrated that treatment with Δ^9-THC in peritoneal macrophages significantly impaired their ability to deliver co-stimulatory signals to a helper T cell hybridoma[179]. Additionally, pre-treatment with Δ^9-THC significantly impaired the upregulation of CD40 antigen induced by anti-CD3/CD28 in mouse splenic $CD4^+$ T cells[180], and in mesenchymal stem cells, pre-treatment with CBD led to the downregulation of genes coding antigens involved in the activation of the immune system[181]. In the same study, other co-stimulatory molecules including CD109, CD151, CD46, CD59, CD68, CD81, CD82, and CD99, were similarly downregulated. Therefore, our data highlight the possibility that CBD and Δ^9-THC may mitigate the immune response through suppression of co-stimulatory molecules.

Another intriguing finding of our work was the effects of CBD and Δ^9-THC on the polarization of AMs. Macrophages typically exist in two distinct sub-sets: classically-activated macrophages (M1) or alternatively-activated macrophages (M2)[107]. M1 macrophages are pro-inflammatory and can be polarized by LPS whereas M2 macrophages are anti-inflammatory and can be polarized by Th2 cytokines including IL-4 and IL-13[108]. That is, M1 macrophages play a pro-inflammatory role in the early stages of inflammation, and M2 macrophages promote tissue repair in the late stages of inflammation[182]. Given the suppressive effects of CBD and Δ^9-THC on the LPS-induced inflammatory response, we postulated that this would reflect a transition in AM polarization away from an M1 phenotype and towards M2. While we did indeed observe a transition away from an M1 phenotype, there was not a resultant promotion of M2 by either CBD or Δ^9-THC. This is evidenced by a reduced transcription of genes comprising an M1 phenotype such as *SAA3, NOS2,*

and *CCRL2* by both CBD and Δ^9-THC (Table 4). M2 gene transcription including *ARG1, PTGS1,* and *EGR2* was unaffected by CBD or Δ^9-THC (Table 5). We speculate that this may be the result of the relatively short incubation time of 1 day, as the transition for macrophage polarization and expression of M1 markers peaks between 1-3 days following injury or stimulation. The expression of M2 markers, conversely, occurs following a reduction in the expression of M1 markers, typically at 3-5 days post- injury or stimulation[182]. In accordance with this, treatment with LPS resulted in a gradual increase in the level of M1 markers through 24 hours (Figure 5.2). As such, our data suggest that CBD and Δ^9-THC may be influencing macrophage polarization by promoting a transition away from an M1 phenotype. However, their ability to promote polarization towards an M2 phenotype warrants further investigation consisting of an extended incubation window in addition to phenotyping of cell-surface markers by flow cytometry.

The spectrum of subtypes in which macrophages can polarize is often accompanied by distinct profiles of cytokines and chemokines that they both secrete and are sensitive to. As such, each state of polarization has specific molecular mechanisms and cell-signaling pathways that are implicated. As previously noted, both CBD and Δ^9-THC promote a transition in AM polarization away from an M1 phenotype with associated reductions in the pro-inflammatory cytokines that they secrete. The NF-κB signaling pathway is a central mediator of M1 macrophage polarization and is required for induction of inflammatory genes, including those encoding *IL-1β, IL-6,* and *TNF-α*[175]. The NF-κB p65-p50 protein complex is present in the cytoplasm through association with the inhibitor protein, IκB, which masks the nuclear localization signal within the p65 subunit. The activation of NF-κB by LPS depends on the rapid phosphorylation of IκB by upstream interleukin-1 receptor-associated kinase (IRAK) 1. The p65 subunit then undergoes

phosphorylation, followed by translocation to the nucleus where it promotes the transcription of target pro-inflammatory genes by binding to a specific DNA element[175]. Cannabinoids inhibit NF-κB activation, resulting in subsequent reductions in inflammatory mediators[83,135]. Herein, we provide several lines of evidence that CBD decreases the activity of NF-κB in AMs. First, CBD reduced the induction of genes comprising the NF-κB signaling pathway (Table 3). Second, there was a significant reduction in the phosphorylation of the NF-κB p65 subunit by CBD (Figure 5.15). Interestingly, treatment with Δ^9-THC did not significantly affect either of these markers of activation, thus questioning the involvement of the NF-κB pathway in the Δ^9-THC-mediated effects in AMs (Figure 6.1). Similar to our observations, Kozela *et al.* demonstrated the ability of CBD and Δ^9-THC to reduce the expression of IL-1β and IL-6 in BV-2 microglial cells[83]. The effects of CBD, but not Δ^9-THC, were mediated by NF-κB. The expression of IL-1β and IL-6 are tightly regulated, and their promoter regions possess binding sites for specific inducible transcription factors[175]. Although our data are suggestive of a role of the NF-κB pathway in mediating the anti-inflammatory properties of CBD, separate pathways are likely involved in the effects of Δ^9-THC.

Although NF-κB is a primary regulator of pro-inflammatory cytokines such as IL-1β and IL-6, it is not the only one. CBD and Δ^9-THC can inhibit the production and release of IFNβ, whose expression activates a wave of genes including CXCL10, CCL5, and CCL2[83]. Similarly, our RNA-seq data demonstrated a reduction in CXCL10, CCL5, and CCL2 by both CBD and Δ^9-THC following LPS stimulation (Table 2). The two primary mediators of IFNβ signaling are STAT1 and STAT3, which are elevated following treatment with LPS[183]. Activation of the STAT pathways involves the formation of STAT1 and STAT3 homo- or heterodimers within the

cytoplasm followed by translocation to the nucleus and binding to respective promoter sites[190]. STAT1 and STAT3 dimers bind selectively to similar- although not identical elements- leading to the activation of varied downstream genes that likely account for their diverse effects. In the case of STAT1 homodimers, these exert pro-inflammatory effects through binding to IFN sequence elements, inducing the expression of chemokines such as CCL2 and CXCL10. STAT3 conversely, exerts anti-inflammatory effects through synthesis of anti-inflammatory interleukins such as IL-10[190]. Previous reports have demonstrated STAT3-mediated reductions in inflammatory processes through reductions in IL-6, TNF-α, and IL-12 in LPS-treated macrophages and neutrophils[191,192]. In the present study, STAT pathways were not directly evaluated. Downstream mediators of STAT1 including *IL-12, CSF2, CSF3,* and *JAK2* were, however, decreased in response to both CBD and Δ^9-THC. STAT3 target genes such as *MYC, LIFR,* and *BCL-3* were increased in response to CBD, and to a lesser degree Δ^9-THC (Appendix Table 7 & 8). These findings are consistent with another study that demonstrated reduced activation of STAT1 in response to CBD and Δ^9-THC. Similarly, CBD- -but not Δ^9-THC- strengthened the activation of the anti-inflammatory STAT3[83]. As such, the anti-inflammatory effects of CBD and Δ^9-THC may be due to alterations in STAT signaling.

We also postulated that the MAPK protein ERK1/2 may be involved in the immunomodulatory properties of CBD and Δ^9-THC, as ERK1/2 (p42/p44) increases pro-inflammatory signaling in monocytes and macrophages through activation of transcription factors including NF-κB and activator protein (AP) 1[175]. We found that, although exposure of AMs to CBD did not change the phosphorylation of ERK1/2, there was a significant increase in phosphorylation in response to Δ^9-THC (Figure 5.15). Moreover, activation of ERK1/2 by LPS was significantly inhibited following

administration by CBD. However, treatment with Δ^9-THC in combination with LPS further increased ERK1/2 activation (Figure 5.15). The differential effects of CBD and Δ^9-THC on ERK1/2 activation may be the result of their reciprocal action on receptors of the endocannabinoid system, specifically the CB_2 receptor which is expressed in macrophages (Figure 6.1). Upon ligand activation, the G_i alpha subunit of the CB_2 receptor forms a heterotrimeric G protein complex. Subsequent dissociation of the G_i subunit from the $G_{\beta\gamma}$ dimer facilitates downstream signal transduction cascades including the phosphorylation of ERK1/2[52]. CBD and Δ^9-THC can have differential effects on the CB_2 receptor, wherein CBD acts as an antagonist and Δ^9-THC acts as an agonist[61]. Therefore, we speculated that agonism of the CB_2 receptor by Δ^9-THC and antagonism by CBD may have resulted in the differential activation of ERK1/2 in our study. In support of this, Bouaboula *et al.* and Kobayashi *et al.* demonstrated activation of p42/44 in response to Δ^9-THC was abrogated upon addition of the CB_2 receptor antagonist SR144528[184,185]. Similarly, in human prostate epithelial PC-3 cells, activation of p42/44 by Δ^9-THC is mediated by the phosphoinositide 3-kinase (PI3K)/PKB pathway, resulting in Raf-1 translocation to the membrane[186]. SR144528 inhibited this effect, further suggesting a role of the CB_2 receptor. WIN 55212-2, a synthetic cannabinoid with affinity for the CB_2 receptor, can also inhibit ERK1/2 activation in a concentration-dependent manner in activated mouse splenocytes; this decreased the production of pro-inflammatory cytokines such as IL-2[187], indicating that cannabinoid-mediated modulation of MAPK activity may be acting through the CB_2 receptor.

CB_1 is heterogeneously expressed throughout the CNS whereas the expression of the CB_2 receptor is predominantly in immune cells[53]. Indeed, there was no detectable expression of CB_1 whereas there was constitutive expression of CB_2 in MH-S AMs (Figure 5.16). Because CB_2 receptors were

expressed in MH-S cells, these seem to be primary candidates for cannabinoid immunomodulation. Indeed, knocking-down the CB_2 receptor in AMs significantly attenuates CBD- and Δ^9-THC-mediated reductions in the pro-inflammatory cytokines *IL-1β, IL-6,* and *TNF-α* (Figure 5.17). This finding is in partial accordance with what has been shown in the literature. First, one study demonstrated that the CB_2 receptor antagonist SR144528 reversed CBD modulation of pro-inflammatory cytokines in mouse peritoneal macrophages *in vitro* and that CBD decreased the chemotaxis of macrophages in a CB_2-dependent manner[188]. However, there is a dichotomy in the literature with regards to this finding. In BV-2 microglial cells, both CBD and Δ^9-THC reduced LPS-induced expression of IL-1β and IL-6 in both the presence and absence of SR144528[83]. The reasons for these different results are not clear. One possibility is that expression of the CB_2 receptor is cell- and tissue-type specific. Additionally, the CB_2 receptor is highly inducible and can increase in response to tissue injury or inflammation. However, CB_2 induction may be specific to the stimulus applied as well as the type of cell in question[54]. This is supported in reports detailing a lack of basal CB_2 expression in microglial cells, a specialized population of macrophages found within the CNS, in addition to very low expression in response to inflammatory stimuli[189]. Interestingly, CB_2 receptor expression was not altered in response to treatment with LPS in MH-S alveolar macrophages (Figure 5.16). Varied expression of the CB_2 receptor across populations of macrophages may suggest that its expression is cell and/or tissue-specific. This is supported in reports detailing high levels of CB_2 expression in immune tissues such as the spleen, in contrast to minimal CB_2 expression within the CNS[54]. Thus, the expression of CB_2 in certain cell types may be inadequate to mediate the immunomodulatory properties of cannabinoids, thus suggesting cannabinoid-receptor independent mechanisms of action. In agreement with this, novel targets such as the adenosine A2A- and TRPV1-receptors have been identified as a means by which

cannabinoids can exert their immunomodulatory properties. In a model of neuroinflammation, treatment with the A2A receptor antagonist SCH58261 abrogated the CBD-mediated reductions in IL-6, TNF-α, and COX-2[93]. Similarly, in concanavalin-A treated hepatic cells, cannabinoid-mediated reductions in IL-2, TNF-α, IFN-c, IL-6, IL-12p40, IL-17, MCP-1, and CCL11 were the result of the TRPV1 receptor[92]. Altogether, these results indicate that the immunomodulatory effects of cannabinoids can be both CB receptor- and non-CB receptor-mediated even in the same system.

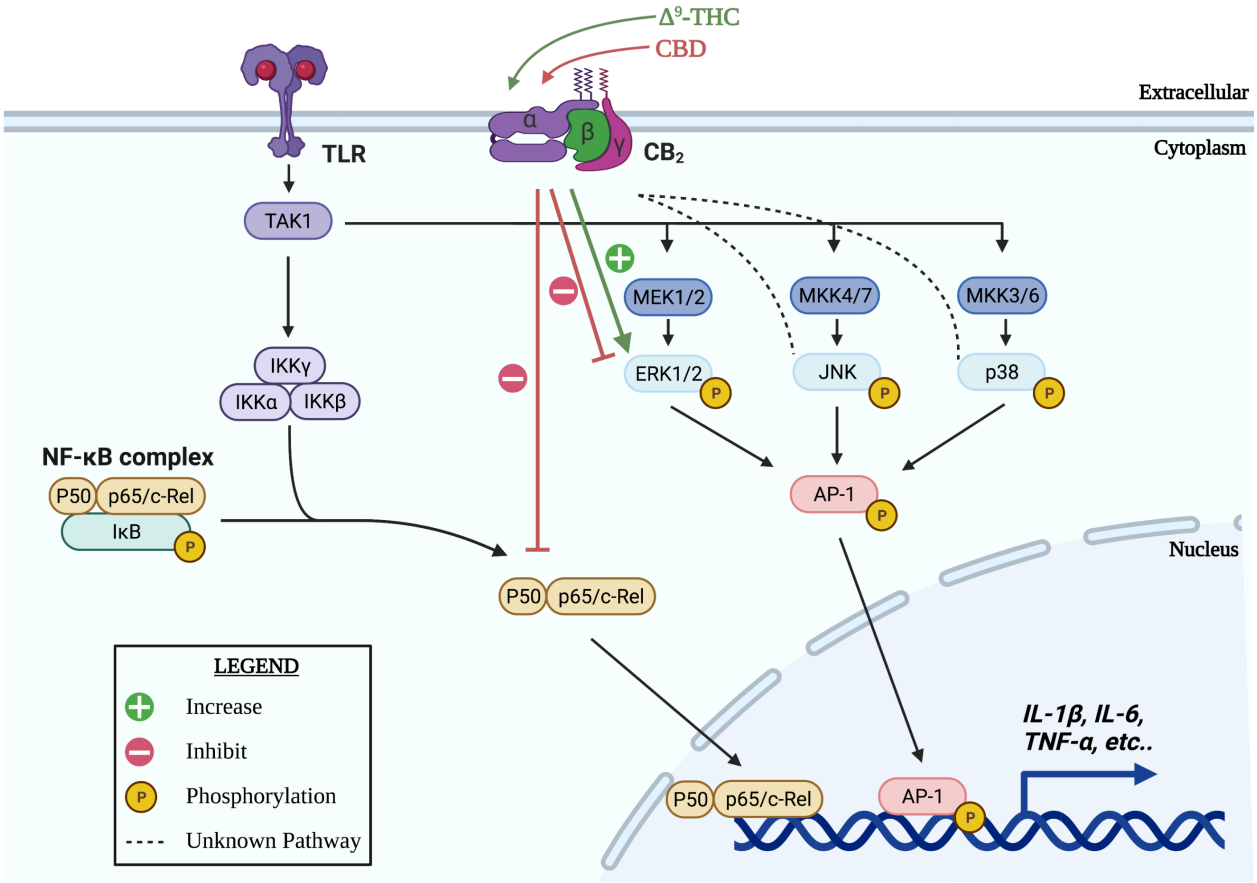


Figure 6.1. A schematic representation of the pathways modulated in response to CBD or Δ^9-THC in alveolar macrophages. In LPS-treated alveolar macrophages, there was an inhibitory effect on the induction of inflammatory cytokines including *IL-1β, IL-6,* and *TNF-α,* in response to CBD and Δ^9-THC. The effects were mediated through the CB_2 receptor. However, CBD and Δ^9-THC had differential effects on downstream pathways involving NF-κB and ERK1/2. Furthermore, the role of separate pathways in mediating the anti-inflammatory effects including the MAP kinase JNK and p38 are not known. TLR: toll-like receptor; CB_2: cannabinoid receptor

2; TAK1: TGF beta activated kinase 1; IKKγ: IκB kinase-γ; IKKα: IκB kinase-α; IKKβ: IκB kinase-β; MEK1/2: MAP/ERK kinase 1/2; MKK4/7: MAPK kinase 4/7; MKK3/6: MAPK kinase 3/6; ERK1/2: extracellular-regulated kinase 1/2; JNK: c-Jun N-terminal kinase; p38: p38 MAPK; AP-1: activator protein 1.

Our work is the first to elucidate the ability of CBD and Δ^9-THC to inhibit the inflammatory response in AMs (Figure 6.1). However, one of the limitations of our study was that we only explored the effects of these cannabinoids *in vitro* in the MH-S AM cell line and primary BMDMs, both of which were mouse-derived. Similar to freshly isolated AMs, the MH-S cell line exhibits typical macrophage morphology, is Fc receptor-positive, capable of phagocytosis, secretes IL-1 in response to LPS, and can suppress the *in vitro* plaque forming cell response. However, the MH-S cell line does differ from human AMs in that MH-S cells are much more homogeneous than would be found within a typical human lung[194]. It would be of interest to confirm our results in AMs isolated from the human lung. Despite this, our study strongly supports the notion that CBD and Δ^9-THC exert anti-inflammatory effects. Additionally, we show that both of these cannabinoids may influence macrophage polarization away from an M1 phenotype but not towards M2. This finding is limited by our evaluation of transcriptional markers without validation at the protein level. Future experiments should consider measuring cell-surface markers through flow cytometry to gain a better understanding of macrophage phenotype in response to cannabinoids. Additionally, extending the window of incubation beyond 24 hours would allow for a greater quantification of M2 markers that may be increased by CBD and Δ^9-THC. Another limitation in the interpretation of our findings is that while we showed reduced phagocytosis in response to CBD in combination with LPS, we did not quantify macrophage killing, an important regulatory function of phagocytosis. It would be of interest to utilize a bacterial challenge with *S. aureus* to quantify bacterial killing in addition to phagocytosis. Measuring oxygen consumption and superoxide

formation in response to cannabinoids would further provide a more comprehensive evaluation of the function of AMs. We also showed that while CBD and Δ^9-THC both suppress the expression of several inflammatory mediators, the mechanism by which this is accomplished follows separate pathways. CBD acted through ERK1/2 and NF-κB whereas Δ^9-THC had no effect on NF-κB. Future experiments could evaluate the effects of CBD and Δ^9-THC on other MAPKs such as JNK and p38 or other pathways including the STAT family of transcription factors; these have been previously described to play key roles in inflammatory processes[175]. While our data suggest that the CB_2 receptor mediates some of the anti-inflammatory properties of CBD and Δ^9-THC, it would of further interest to confirm if this is through ERK1/2 and NF-κB. Furthermore, our approach could have been expanded through the use of pharmacological inhibitors targeting the CB_2 receptor such as AM630. Knockdown approaches as well as pharmacological inhibitors have previously been described to have off-target effects in various models[83,93]; therefore, an approach making use of both techniques could further validated our findings.

Despite these limitations, our study strongly supports the notion that not only are CBD and Δ^9-THC anti-inflammatory, but their effects are at least, in part, mediated by the CB_2 receptor. Furthermore, given the experimental evidence supporting a role for non-cannabinoid receptors in mediating the immunomodulatory properties of cannabinoids, an important experiment would be to assess their roles within the same system. Thus, an experimental approach making use of receptor specific antagonists for A2A and TRPV1 would be extremely beneficial in delineating the underlying mechanism(s) of cannabinoid immunomodulation. It would be interesting to investigate these effects using different cell types and tissues systems where the density of receptor

expression may vary such as in nervous tissues and immune cell populations where differential expression of cannabinoid receptors has been previously described.

In conclusion, we are the first to report on the anti-inflammatory properties of CBD and Δ^9-THC in AMs, with the CB_2 receptor playing a key role in mediating the immunomodulatory effects of these cannabinoids. We also show that the suppressive effects of CBD extend beyond its ability to inhibit pro-inflammatory cytokines in that they also reduce phagocytosis by AMs. Further investigation is needed into the pathways and receptors underlying the suppressive effects of CBD and Δ^9-THC on the inflammatory response. With continued research, we may uncover a vital link in the relationship between cannabinoids and their immunomodulatory properties in humans, leading to the identification of a molecular mechanism that could be targeted by novel cannabinoid-based therapies to ameliorate conditions characterized by acute or chronic inflammation.

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