# ANTI-INFLAMMATORY EFFECT OF CBD ON CULTURED MICROGLIA

by

Caroline O'Connor

Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 2, 2022

ANTI-INFLAMMATORY EFFECT OF CBD
ON CULTURED MICROGLIA

Project Approved:

Supervising Professor: Michael Chumley, Ph.D.

Department of Biology

Gary Boehm, Ph.D.

Department of Psychology

Lisa Vanderlinden, Ph.D.

Department of Anthropology

## ABSTRACT

Alzheimer's Disease (AD) is a progressive neurodegenerative disease associated with old age and marked by deficits in memory and learning skills. AD pathology is characterized by amyloid-beta (AB) accumulation, which leads to plaque formation and ultimately neuronal death. Additionally, AB activates microglial cells, which function as an immune cell in the brain. Microglial cells secrete proteins that induce inflammation, known as pro-inflammatory cytokines. The chronic activation of pro-inflammatory cytokines engenders neuroinflammation and oxidative stress, which then further exacerbate AD pathologies. This project aims to study the effectiveness of cannabidiol (CBD) as a potential treatment for AD, due to its known anti-inflammatory properties. We measured the inflammatory response of cultured BV2 immortalized murine microglial cells following lipopolysaccharide (LPS) treatment. We then included a CBD treatment to study its therapeutic capabilities in reducing inflammation. We hypothesized that treatment with CBD would decrease the pro-inflammatory cytokines TNF-alpha and IL-6 induced by LPS stimulation. We performed enzyme-linked immunosorbent assays (ELISAs) to detect and quantify the cytokine levels. The overall goal of the research is to demonstrate the capacity of CBD to minimize the immunological mechanisms that drive AD pathologies. Our research will contribute to the understanding of the link between the immune system and central nervous system in AD development. AD is the sixth leading cause of death in America, but the availability of therapies is limited. CBD represents a natural and possibly effective therapy for those suffering from Alzheimer's disease, and our research will contribute to determining its efficacy.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank Dr. Chumley for serving as the primary supervisor for this honors project and as my mentor in the research lab for the past three years. Additionally, I would like to thank Dr. Boehm and Dr. Vanderlinden for serving as my committee members. This project truly would not have been possible without the guidance and unending support of graduate students Paige Braden-Kuhle, Kelly Brice, and Chelsy Mani. Lastly, I would like to thank my fellow undergraduate students for their support of this project with hours spent in the lab and of me with their bright smiles and laughter.

# INTRODUCTION

Alzheimer's disease (AD) is a devastating progressive neurodegenerative disease without a cure. Approximately 6 million Americans are currently battling Alzheimer's disease, which is the sixth leading cause of death in America. AD is the most common cause of dementia and accounts for nearly 70% of dementia cases around the world (Liu et al., 2019). AD cases are rapidly proliferating now and will remain a major healthcare concern as the world's life expectancy continues to increase. Researchers predict that by mid-century, 14 million Americans and 100 million people worldwide will suffer from AD (Alzheimer's Association, 2019). AD is characterized by progressive decline in at least two cognitive domains, which include memory, language, personality, behavior, and functioning. These symptoms cause a loss of ability and make it difficult for those with AD to engage in the basic activities of daily life (Weller & Budson, 2018).

AD is primarily characterized by two hallmark pathologies: the deposition of amyloid beta (Aβ) plaques and neurofibrillary tangles of hyperphosphorylated tau proteins (Liu et al., 2019). Existing research has well established a link between these AD pathologies and chronic inflammation, which occurs due to activation of the innate immune system and the secretion of pro-inflammatory cytokines (Amor et al., 2014; Cunningham et al., 2009; Yirmiya et al., 2011). Preceding studies have shown that AD patients have increased levels of pro-inflammatory cytokines circling in their blood (Dursun et al., 2015; Engelhart et al., 2004). Chronic inflammation and the presence of pro-inflammatory cytokines exacerbate the proteolytic cleavage of amyloid precursor protein (APP) into amyloid beta, which aggregates and forms extracellular plaques in the brain. Subsequently, the development of Aβ plaques disrupts the synaptic communication of neurons, as well as learning and memory processes. Aβ plaques

develop first in the cerebral cortex and then progress to the hippocampus, a region that plays a crucial role in learning and memory. The hippocampus is one of the first brain regions to experience atrophy during the development of AD (LaFerla et al., 2007). Therefore, a possible beneficial therapeutic for AD patients would be able to attenuate chronic inflammation and the accumulation of amyloid beta.

Two key factors involved in the progression of AD pathologies are chronic inflammation and oxidative stress. Inflammation is a typically beneficial natural response in the body that aims to eliminate the cause of cellular damage, remove dead cells, and initiate repair mechanisms (Ahmed et al., 2017). While acute inflammation is self-limiting and can be beneficial, chronic inflammation, especially when uncontrolled, can be harmful and lead to cellular damage (Ahmed et al., 2017). Prolonged chronic inflammation and the presence of damaged tissue activates microglia and astrocytes, the two main cellular mediators of the inflammatory response (Fischer & Maier, 2015). The activation of microglia and the dysfunction of the antioxidant system can cause oxidative stress. Oxidative stress can activate pro-inflammatory cytokines and increase production of reactive oxygen species (ROS), such as peroxides and superoxides (Agostinho et al., 2010). Both inflammation and the presence of ROS can lead to the cleavage of APP and consequently the accumulation of Aβ plaques. Aβ plaque production and aggregation is also a cause of increased inflammation and oxidative stress. These two factors form a self-perpetuating cycle that ultimately causes the progression of AD pathologies. As a result, treatments that limit pro-inflammatory cytokine or ROS production are important potential therapies.

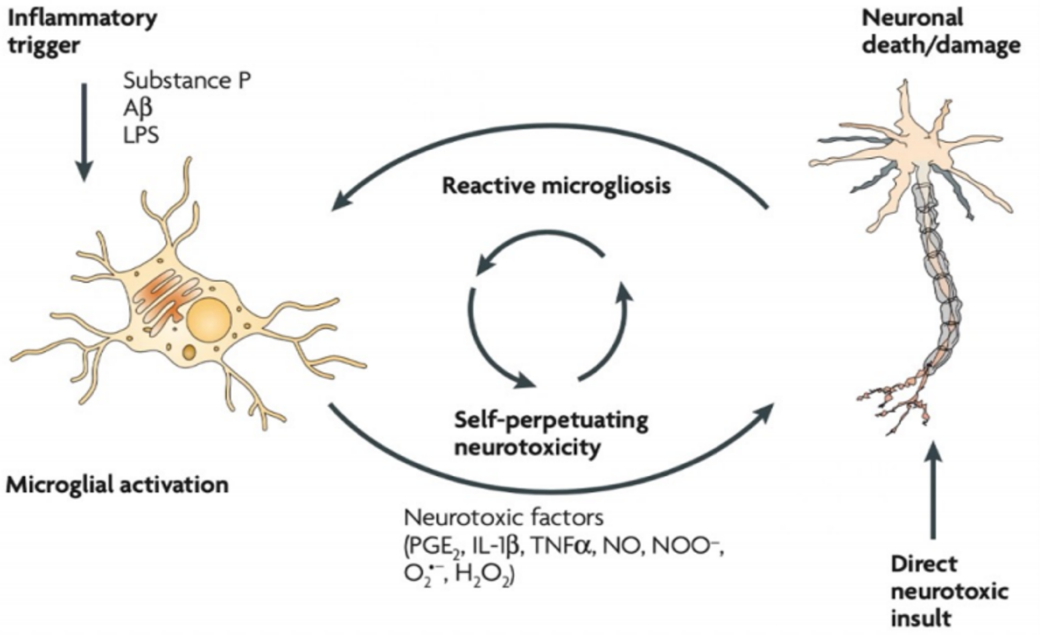


**Figure 1.** Aβ plaque aggregation and inflammation form a positive feedback loop, which exacerbates the progression of AD pathologies (Block et al., 2007).

Microglia are immune cells that act as macrophages in the central nervous system (CNS). Microglial cells most commonly populate the hippocampal region of the brain essential to learning and memory processes (Stansley et al., 2012). They are involved in the innate immune response and help maintain homeostasis (Zheng et al., 2018). Microglia become activated in areas of high inflammation and release pro-inflammatory cytokines (Dursun et al., 2015; Kahn et al., 2012; Lee et al., 2008). Factors that microglia release in response to inflammation are part of the TLR4 pathway include ROS such as nitric oxide (NO), inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokine such as tumor necrosis factor (TNF-$\alpha$), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (Lee & Kang, 2014). The most common cytokine released by microglial cells is TNF-$\alpha$. In AD, chronic inflammation resulting from Aβ plaque accumulation leads to the chronic activation of microglia and the incessant release of pro-inflammatory

cytokines, all of which contribute to the progression and development of AD pathologies (Block & Hong, 2005).

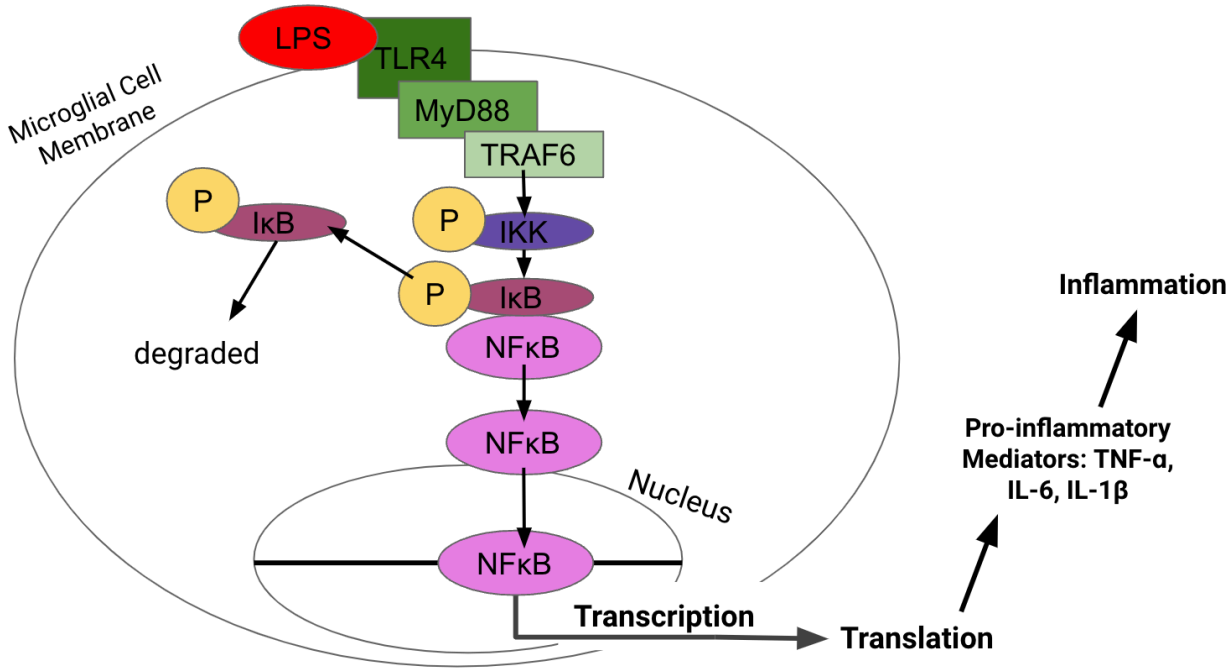


**Figure 2.** Signaling pathways involved in inflammation, simplified for the purpose of this study. LPS interacts with TLR4 and initiates a signaling cascade. Phosphorylated IkB is removed and degraded, allowing NFkB to enter the nucleus and act as a transcription factor. This results in an increase in the production of pro-inflammatory mediators, such as TNF-$\alpha$ and IL-6. (Figure based on information from Ahmed et al., 2017. Layout based on pathway from Caitlyn Vilas, TCU Departmental Honors Project 2020.)

Cannabidiol is a cannabinoid from the *Cannabis sativa* plant with both antioxidant and anti-inflammatory properties (Klein, 2005; Nagarkatti et al., 2009). Since oxidative stress and inflammation are key causes of AD, CBD may have the potential to reduce the biological, immunological, and behavioral mechanisms that lead to chronic inflammation of the CNS and cause AD pathologies. A first step in evaluating CBD's potential as an AD therapeutic is understanding the mechanism by which CBD mitigates pro-inflammatory cytokines and the production of Aβ. Previous in-vitro research has demonstrated that the use of a CBD pre-treatment decreases the amount of pro-inflammatory cytokines produced and released by microglial cells (Iuvone et al., 2004; Janefjord et al., 2013; Kozela et al., 2009). Additionally,

previous research has shown CBD's antioxidant capabilities are the result of phenolic properties (Klein, 2005). The phenolic properties have beneficial effects in neuronal cells by attenuating ROS in the brain and thereby attenuating oxidative stress (Omar et al., 2017). CBD's phenolic properties may also be remedial against the accumulation of Aβ (Kim et al., 2020). The existing literature clearly shows that CBD has the potential to attenuate the two primary pathologies of AD and serve as an effective therapeutic due to its antioxidant and anti-inflammatory capabilities.

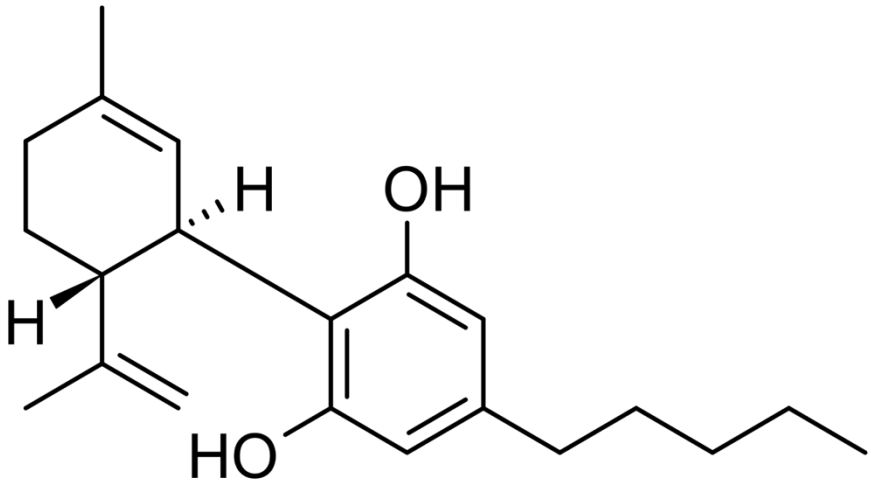


**Figure 3.** Molecular structure of cannabidiol (Mechoulam & Hanuš, 2002). Depiction created by Caroline O'Connor.

There are limited therapies currently available to AD patients, and not all patients respond to these therapies. Therefore, the aim of this research is to explore the effectiveness of cannabidiol (CBD) as a potential new therapy for AD patients. We will assess CBD therapy in vitro using BV2 microglial cells, which are an immortalized cell line of murine neonatal cells frequently utilized as a model for neurodegenerative diseases. Microglial cells are utilized because they express cannabinoid-like receptors CB1 and CB2 that are activated by CBD (Stella, 2010). BV2 cells specifically express CB2 receptors which, when activated, decrease the secretion of pro-inflammatory cytokines (Ramirez et al., 2005). The BV2 cells can be stimulated

with lipopolysaccharide (LPS), a bacterial mimetic that induces CNS inflammation. LPS is an endotoxin and an element of the cell wall in gram-negative bacteria (Lee et al., 2008). It is used often in AD research to activate the immune system and induce inflammation because cells recognize it as foreign (Chen et al., 2012). In an initial experiment BV2 cells were seeded in 6-well plates, treated with one of five concentrations of LPS and collected at one of three time intervals to evaluate the pro-inflammatory cytokines produced following LPS stimulation. In this study, BV2 microglial cells were seeded in 6-well plates, pre-treated for four hours with one of five concentrations of CBD, and then treated with one of two concentrations of LPS. To quantify the amount of TNF-$\alpha$ produced in each experiment, Enzyme Linked Immunosorbent Assays (ELISA) were completed.

# MATERIALS AND METHODS

## BV2 Cell Maintenance

BV2 microglial cells were maintained in a cell incubator at 37 degrees Celsius at 5% $CO_2$. Cells were grown in 10 cm tissue culture dishes in complete cell medium containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM - Caisson Laboratories, Smithfield, UT), 5% Pen/Strep, 5% L-Glutamine, and 5% Fetal Bovine Serum. When the cells became around 80-90% confluent, they were sub-cloned. Cells were sub-cloned by aspirating the old medium, washing with 10 mL phosphate-buffered saline (Dulbecco's PBS - Caisson Laboratories, Smithfield, UT), and adding 5 mL of fresh complete medium to the dish. A cell scraper was used to lift cells from the bottom of the dish. Cells were subcloned by transferring 10-20% of the cells to new 10 cm dishes with 10 mL of fresh complete medium and returned to the incubator.

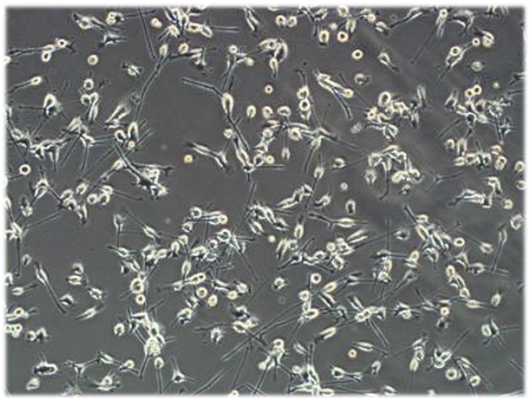

**Figure 4.** Photo of BV2 microglial cells taken under the microscope.

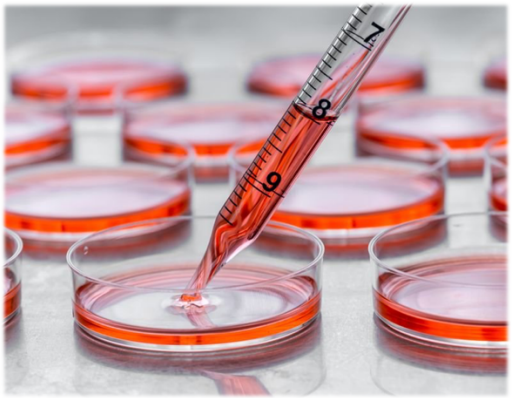

**Figure 5.** Photo of tissue culture dishes with complete medium and cells adhered to the plate.

To prepare for experiments, cells were seeded in 3.5 cm 6-well plates at 200,000 cells per well. Old medium was aspirated from a 10 cm dish, cells were washed in 10 mL of PBS, and 10 mL of fresh complete medium was added to the dish. A cell scraper was used to loosen cells from the bottom of the dish. Cells were pipetted into a 15 mL conical tube and centrifuged for 10 minutes at 1.2 RCF to spin the cells down into a pellet. 8 mL of supernatant was aspirated off

and the pellet was broken up into the remaining 2 mL. The cells were counted by adding 10 µL of the cells resuspended in solution to each side of a hemocytometer and manually counted using a microscope.

LPS and CBD Preparation

The lipopolysaccharide (LPS) used is from the Escherichia coli serotype: 055:B5 and was purchased from SigmaAldrich, St Louis, MO. LPS was dissolved in phosphate buffered saline (PBS) to create a 5 µg/mL stock solution. Aliquots of this stock solution were stored in a -20-degree Celsius freezer. Immediately prior to LPS treatment, one aliquot of stock solution was removed from the freezer and thawed. Concentrations of 0.5 µg/mL, 0.05 µg/mL, 0.005 µg/mL, and a control of 0 µM were prepared by serial dilution of the 5 µg/mL stock solution with serum free media.

CBD powder isolate (Eureka93, Eureka, MT) was dissolved in dimethyl sulfoxide (DMSO) to create a 100 mM stock solution. Aliquots of this stock solution were stored in a -20-degree Celsius freezer. Immediately prior to CBD pretreatment, one aliquot of stock solution was removed from the freezer and thawed. The stock solution was diluted with serum free media (SFM) to prepare a top concentration of 20 µM. Concentrations of 10 µM, 5 µM, 1 µM, 0.1 µM, and a control of 0 µM were prepared by serial dilution of the 20 µM concentration with serum free media.

LPS Treatment of Cells

In a preliminary experiment, cells were treated with various concentrations of lipopolysaccharide (LPS) and supernatant and lysates were collected at various time points to determine the cytokines produced in the resulting inflammatory response. After a four-hour incubation period, one concentration of LPS was added to each well and the plates were returned

to the incubator. Cell supernatants and lysates were collected after treatment times of 4, 8, and 24 hours. Supernatant was aliquoted into microfuge tubes, snap frozen in dry ice, and stored in a -20-degree Celsius freezer. For cell lysate collection, 200 µL of non-sterile PBS was added to each well and aspirated off to remove any dead cells. The plate was placed on ice and 300 µL of lysis buffer containing mammalian protein extraction reagent (M-PER; Invitrogen, Waltham, MA), protease inhibitor, and phosphatase inhibitor. A cell scraper was used to dislodge cells from the bottom of the wells. The cell lysate was aliquoted into microfuge tubes, snap frozen in dry ice, and stored in a -20-degree freezer for later analysis.

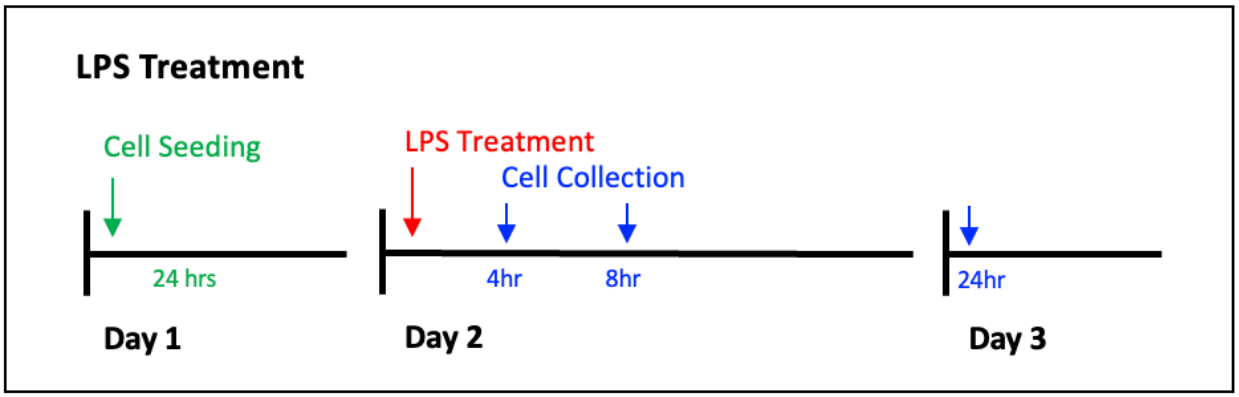


**Figure 6.** Timeline of experimental procedures for LPS treatment. Cells were seeded on day one. At the start of day 2, cells were treated with various concentrations of LPS. Cell supernatant and lysates were collected at 4, 8, and 24 hours after LPS treatment.

CBD Pretreatment of Cells

Based on the results from the LPS experiment, treatment concentrations of 0.05 µg/mL and 0.005 µg/mL and a treatment time of four hours were selected for an initial cannabidiol (CBD) experiment. On day one, cells were seeded in 6-well plates at 200,000 cells per well using the same protocol as above and placed in the incubator overnight. On day two, cells were pretreated for four hours with various concentrations of CBD and returned to the incubator. After a four-hour incubation period, the 0.05 µg/mL or the 0.005 µg/mL concentration of LPS was

added to each well and the plates were returned to the incubator. After the four-hour treatment time, cell supernatant and cell lysate were collected using the same procedure as above.

A following CBD experiment included pre-treatment CBD concentrations of 20 µM, 10 µM, 5 µM, 1 µM, 0.1 µM, and a negative control of 0 µM; LPS treatment concentrations of 0.05 µg/mL and 0.005 µg/mL; and cells were collected after treatment times of four hours or eight hours. Treatment and collection protocols were the same as above.

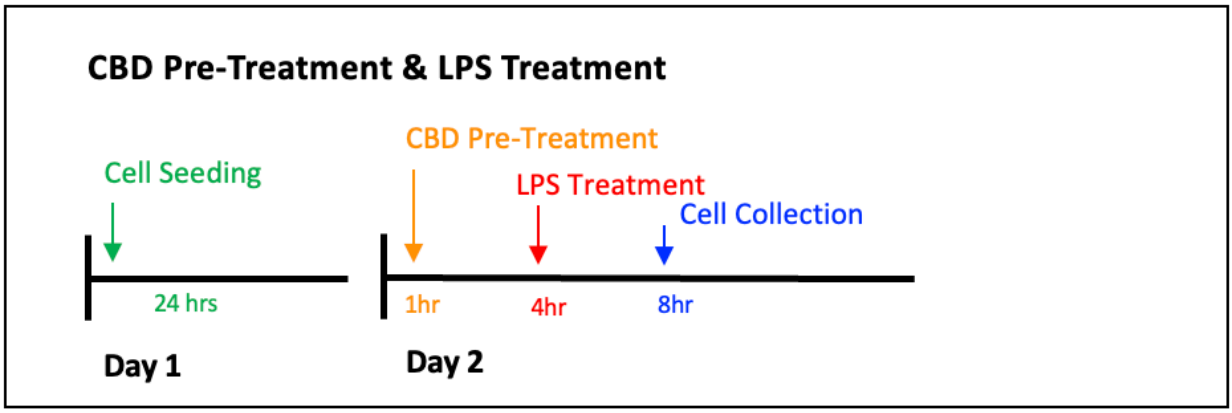


**Figure 7.** Timeline of experimental procedures for CBD pre-treatment and LPS treatment. Cells were seeded on day one. At the start of day 2, cells were treated with various concentrations of CBD and treated with LPS 4 hours later. Cell supernatant and lysates were collected after 4 or 8 hours of LPS treatment.

Enzyme Linked Immunosorbent Assays

Enzyme Linked Immunosorbent Assays (ELISAs) were performed using cell supernatants collected in the tissue culture experiments to determine the amount of TNF-α or IL-6 pro-inflammatory cytokines released by the BV2 cells. Materials included BioLegend Mouse cytokine ELISAs - ELISA MAX Deluxe Set or ELISA MAX Standard Set (BioLegend, San Diego, CA). A 96-well plate was coated with 100 µL of diluted Capture Antibody in each well, sealed, and placed in a 4-degree Celsius refrigerator overnight. The following day, the plate was washed four times using a wash buffer composed of PBS and 0.05% Tween-20. The wells were blocked using Assay Diluent A, sealed, and incubated for an hour at room temperature on a plate shaker (500 rpm with a 0.3 cm circular orbit). The plates were then washed four times and 100

µL of standard or supernatant sample was added to the appropriate wells. All samples were run neat. The plate was then sealed and incubated for two hours at room temperature on a plate shaker. The plate was washed four times and 100 µL of detection antibody was added to each well before they were sealed and incubated for one hour at room temperature on the plate shaker. The plate was washed four times and 100 µL of Avidin HRP was added to each well. Then the plate was sealed and incubated for 30 minutes at room temperature on the plate shaker. The plate was washed five times and the wash buffer was allowed to rest for one minute during each wash. 100 µL of TMB substrate solution was added to each well and the plate was incubated at room temperature in the dark for 15 minutes. Finally, 100 µL of stop solution ($2NH_2SO_4$) was added to each well. The plate was then read using a FLUOstar Omega spectrophotometer (BMG LabTech, Cary, NC) at absorbances of 450 nm and 570 nm.

# RESULTS

## LPS Induces Production of Pro-inflammatory Cytokines

Experiments were conducted to quantify the inflammatory effect of lipopolysaccharide (LPS) in BV2 microglial cells. LPS was added to wells of cells at a variety of concentrations. Cell supernatant and cell lysates were collected following the assigned treatment times. The production of pro-inflammatory cytokines, TNF-$\alpha$ and IL-6, was quantified using ELISA assays. The ELISA assays revealed a dose-dependent relationship between increasing LPS concentration and increasing cytokine production.

## TNF-$\alpha$ ELISA Results

A one-way ANOVA revealed that there was a significant main effect of LPS treatment (4 hours, μg/ml) on TNF-alpha production in BV2 cells, $F(5, 43) = 8.347$, $p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct this. Post hoc tests revealed that all LPS concentrations were significantly different from one another, with the exception of 0.05 & 0.5 μg/ml, 0.05 & 5 μg/ml, and 0.5 & 5 μg/ml, $p$s $\geq 0.362$.

Additionally, a one-way ANOVA revealed that there was a significant main effect of LPS treatment (8 hours, μg/ml) on TNF-alpha production in BV2 cells, $F(5, 45) = 19.893$, $p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct this. Post hoc tests revealed that all LPS concentrations were significantly different from one another, with the exception of 0.005 & 0.05 μg/ml and 0.5 & 5 μg/ml, $p$s $\geq 0.750$.

Furthermore, a one-way ANOVA revealed that there was a significant main effect of LPS treatment (24 hours, μg/ml) on TNF-alpha production in BV2 cells, $F(5, 42) = 25.311$, $p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct this. Post hoc tests revealed that all LPS concentrations were significantly different from one another, with the

exception of 0.005 & 0.05 µg/ml, 0.005 & 0.5 µg/ml, 0.05 & 0.5 µg/ml, and 0.05 & 5 µg/ml, *p*s ≥ 0.111. See Figure 8.

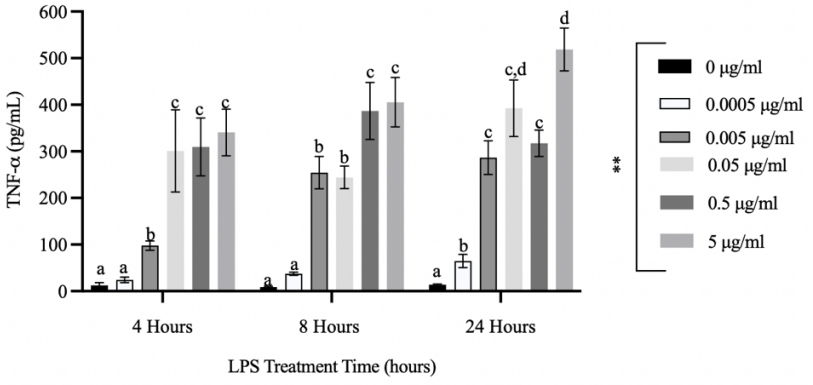


**Figure 8. LPS Treatment Induces TNF-alpha Production in BV2 Cells.** ANOVA revealed a significant main effect of LPS treatment (µg/ml) on TNF-alpha production at 4, 8, and 24 hours of LPS treatment, ($p \leq 0.001$). Different letters (a, b, c, d) represent significant differences at $p \leq 0.05$. Bars represent mean ± SEM. N's = 6 – 8.

IL-6 ELISA Results

Additional assays were conducted with cell supernatant to quantify the amount of IL-6 secreted by BV2 cells following LPS stimulation. Figure 9 shows the amount of IL-6 (pg/mL) produced in BV2 cell supernatant post LPS treatment at 4, 8, and 24 hours. This experiment was not repeated, and therefore n = 1. See Figure 9.

Collectively, these data demonstrate that TNF-$\alpha$ is produced in significant quantities beginning as early as 4 hours after LPS stimulation. Conversely, IL-6 is not produced in significant quantities until at least 8 hours after LPS stimulation. Based on these results, 0.005 µg/mL and 0.05 µg/mL of LPS were selected for use in further experiments because both showed a significant increase in cytokine production from the control and were significantly different from each other.

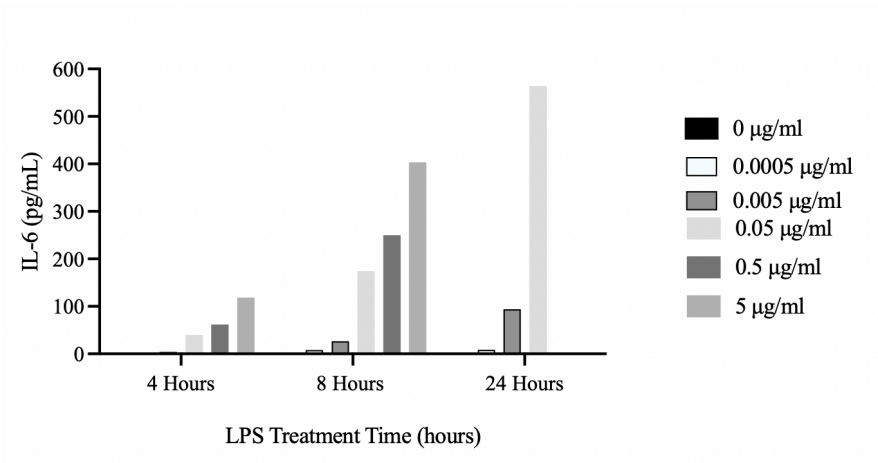


**Figure 9. LPS Treatment Induces IL-6 Production in BV2 Cells**. BV-2 cells were treated with five different concentrations of LPS and allowed to incubate for 4, 8, or 24 hours. Supernatants were collected and IL-6 production (pg/mL) determined. Due to time constraints, we were only able to complete one experiment testing for IL-6 production (N =1), thus no statistics are presented.

CBD Treatment Attenuates Inflammation in Microglial Cells

CBD experiments were conducted to quantify the anti-inflammatory effect of CBD in BV2 microglial cells stimulated with lipopolysaccharide (LPS). CBD was added to wells of cells at a variety of concentrations. LPS was added to the wells of cells at one of the two concentrations determined by the LPS experiment, as previously described. The production of pro-inflammatory cytokines TNF-$\alpha$ and IL-6 was quantified using ELISA assays. The ELISA assays revealed a dose-dependent relationship between increasing CBD concentration and decreasing cytokine production.

TNF-$\alpha$ ELISA Results Following 4 Hours of LPS Treatment

ANOVA revealed that there was a significant main effect of LPS treatment (4 hours, µg/ml) on TNF-alpha production in BV2 cells, $F(2, 49) = 20.266, p \leq 0.001$. Additionally, there was a significant main effect of CBD treatment $F(5, 49) = 27.508, p \leq 0.001$. This was qualified by a significant interaction, $F(7, 49) = 9.365, p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct this.

Figure 10A shows the amount of TNF-$\alpha$ in pg/mL produced in BV2 cell supernatant post CBD treatment for 4 hours and LPS treatment at a concentration of 0.005 μg/mL for 4 hours. Post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 μM and 5 μM ($p = 0.001$), 0 μM and 10 μM ($p \leq 0.001$) ,0 μM and 20 μM ($p \leq 0.001$), 0.1 μM and 10 μM ($p \leq 0.001$), 0.1 μM and 20 μM ($p \leq 0.001$),1 μM and 10 μM ($p \leq 0.001$), 1 μM and 20 μM ($p \leq 0.001$), 5 μM and 10 μM ($p \leq 0.001$), 5 μM and 20 μM ($p \leq 0.001$) of CBD and 0.005 μg/mL of LPS. See Figure 10A.

Figure 10B shows the amount of TNF-$\alpha$ in pg/mL produced in BV2 cell supernatant post CBD treatment for 4 hours and LPS treatment at a concentration of 0.05 μg/mL for 4 hours. Post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 μM and 5 μM ($p = 0.002$), 0 μM and 10 μM ($p \leq 0.001$), 0 μM and 20 μM ($p \leq 0.001$), 0.1 μM and 5 μM ($p = 0.036$),0.1 μM and 10 μM ($p \leq 0.001$), 0.1 μM and 20 μM ($p \leq 0.001$),1 μM and 10 μM ($p \leq 0.001$), 1 μM and 20 μM ($p \leq 0.001$), 5 μM and 10 μM ($p = 0.009$), 5 μM and 20 μM ($p = 0.002$) of CBD and 0.05 μg/mL of LPS. See Figure 10B.

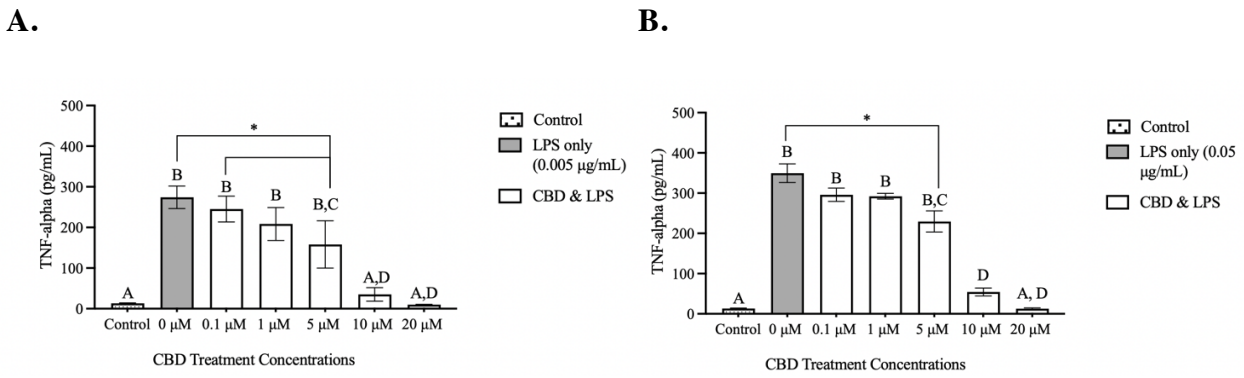


**Figure 10. CBD pre-treatment attenuates LPS-induced TNF-alpha production following 4 hours of LPS treatment (0.005 μg/mL of LPS).** A) BV-2 cells were treated with five different concentrations of CBD followed 4 hours later with by LPS (0.005 μg/mL). Supernatants were collected 4 hours after LPS treatment. ANOVA revealed a significant main effect of 4 hours of CBD treatment ($p = 0.002$) and 4 hours of LPS treatment ($p \leq 0.001$) on TNF-alpha production. **B)** BV-2 cells were treated with five different concentrations of CBD followed 4 hours later with by LPS (0.05 μg/mL). Supernatants were collected 4 hours after LPS treatment. ANOVA revealed a significant main effect of 4 hours of CBD treatment ($p \leq 0.001$) and 4 hours of LPS treatment ($p \leq 0.001$) on TNF-alpha production. Controls did not receive any treatment or drug. Different letters (A, B, C, D) represent significant differences at $p \leq 0.05$. Bars represent mean ± SEM. Ns = 2 – 6.

TNF-$\alpha$ ELISA Results Following 8 Hours of LPS Treatment

ANOVA revealed that there was a significant main effect of LPS treatment (8 hours, 0.005 μg/ml) on TNF-alpha production in BV2 cells, $F(1, 32) = 49.451, p \leq 0.001$. Additionally, there was a significant main effect of CBD treatment $F(5, 32) = 20.634, p \leq 0.001$. This was qualified by a significant interaction, $F(2, 32) = 32.216, p \leq 0.001$. The data violated homogeneity of variance, and a log transformation was performed to correct this. Post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 μM and 5 μM ($p \leq 0.001$), 0 μM and 10 μM ($p \leq 0.001$) ,0 μM and 20 μM ($p \leq 0.001$), 0.1 μM and 5 μM ($p = 0.003$), 0.1 μM and 10 μM ($p \leq 0.001$), 0.1 μM and 20 μM ($p \leq 0.001$), 1 μM and 5 μM ($p = 0.015$),1 μM and 10 μM ($p \leq 0.001$), 1 μM and 20 μM ($p \leq 0.001$), 5 μM and 10 μM ($p < 0.006$), 5 μM and 20 μM ($p \leq 0.001$), and 10 μM and 20 μM ($p = 0.022$) of CBD and LPS (8 hours, 0.005 μg/ml). See Figure 11A.

Additionally, ANOVA revealed that there was a significant main effect of CBD treatment on TNF-alpha production in BV2 cells treated with 0.05 μg/ml of LPS for 8 hours, $F(5, 19) = 8.030, p \leq 0.001$. Post hoc analyses revealed a significant difference in TNF-alpha production between cells treated with 0 μM and 10 μM ($p \leq 0.001$), 0 μM and 20 μM ($p \leq 0.001$), 0.1 μM and 10 μM ($p < 0.018$), 0.1 μM and 20 μM ($p = 0.002$),1 μM and 10 μM ($p = 0.019$), 1 μM and 20 μM ($p = 0.002$), 5 μM and 10 μM ($p < 0.013$), 5 μM and 20 μM ($p \leq 0.001$) of CBD and LPS (8 hours, 0.05 μg/ml). See Figure 11B.

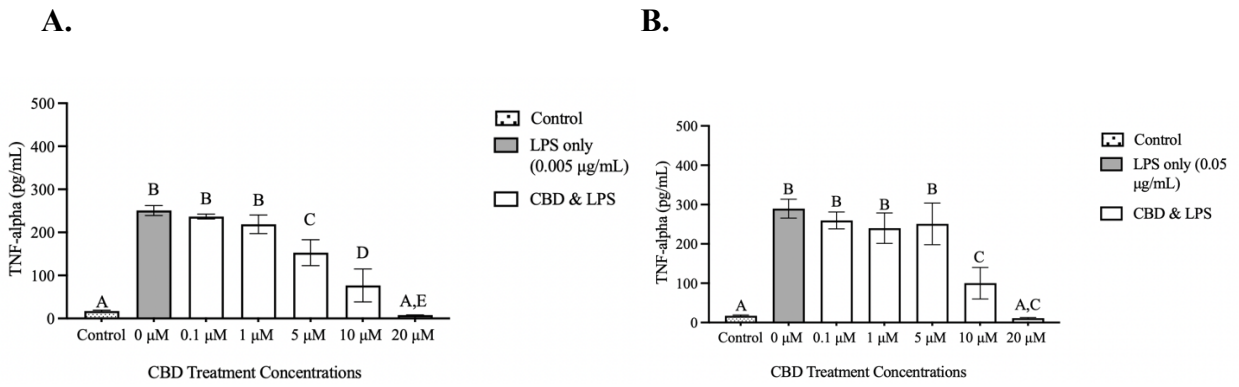


**Figure 11. CBD pre-treatment attenuates LPS-induced TNF-alpha production following 8 hours of LPS treatment (0.005 µg/mL of LPS).** A) BV-2 cells were treated with five different concentrations of CBD followed 4 hours later with by LPS (0.005 µg/mL). Supernatants were collected 8 hours after LPS treatment. ANOVA revealed a significant main effect of 4 hours of CBD treatment ($p \leq 0.001$) and 8 hours of LPS treatment ($p \leq 0.001$) on TNF-alpha production. **B)** BV-2 cells were treated with five different concentrations of CBD followed 4 hours later with by LPS (0.05 µg/mL). Supernatants were collected 8 hours after LPS treatment. ANOVA revealed a significant main effect of 4 hours of CBD treatment ($p \leq 0.001$) and 8 hours of LPS treatment ($p \leq 0.001$) on TNF-alpha production. Controls did not receive any treatment or drug. Different letters (A, B, C, D) represent significant differences at $p \leq 0.05$. Bars represent mean ± SEM. Ns = 2 – 6.

## IL-6 ELISA Results Following 8 Hours of LPS Treatment

ANOVA revealed that there was a significant main effect of CBD treatment on IL-6 production in BV2 cells, $F(5, 50) = 11.05$, $p \leq 0.001$. Additionally, there was a significant main effect of LPS treatment (8 hours, 0.05 µg/mL or 0.005 µg/mL) on IL-6 production in BV2 cells, $F(2, 50) = 7.607$, $p = 0.002$. Figure 12A shows the amount of IL-6 in pg/mL produced in BV2 cell supernatant post CBD treatment for 4 hours and LPS treatment at a concentration of 0.005 µg/mL for 8 hours. Post hoc analyses revealed a significant difference in IL-6 production between cells treated with 0 µM and 5 µM ($p = 0.002$), 0 µM and 10 µM ($p \leq 0.001$) ,0 µM and 20 µM ($p \leq 0.001$), 0.1 µM and 5 µM ($p = 0.007$), 0.1 µM and 10 µM ($p = 0.002$), 0.1 µM and 20 µM ($p = 0.002$), 1 µM and 5 µM ($p = 0.016$),1 µM and 10 µM ($p = 0.005$), and 1 µM and 20 µM ($p = 0.005$) of CBD and 0.005 µg/mL of LPS treatment for 8 hours.

Additionally, Figure 12B shows the amount of IL-6 in pg/mL produced in BV2 cell supernatant post CBD treatment for 4 hours and LPS treatment at a concentration of 0.05 µg/mL

for 8 hours. Post hoc analyses revealed a significant difference in IL-6 production between cells treated with 0 μM and 5 μM ($p = 0.007$), 0 μM and 10 μM ($p \leq 0.001$), and 0 μM and 20 μM ($p = 0.003$), 0.1 μM and 5 μM ($p = 0.038$), 0.1 μM and 10 μM ($p = 0.009$), 0.1 μM and 20 μM ($p = 0.013$),1 μM and 10 μM ($p = 0.019$), and 1 μM and 20 μM ($p = 0.027$) of CBD and LPS (8 hours,

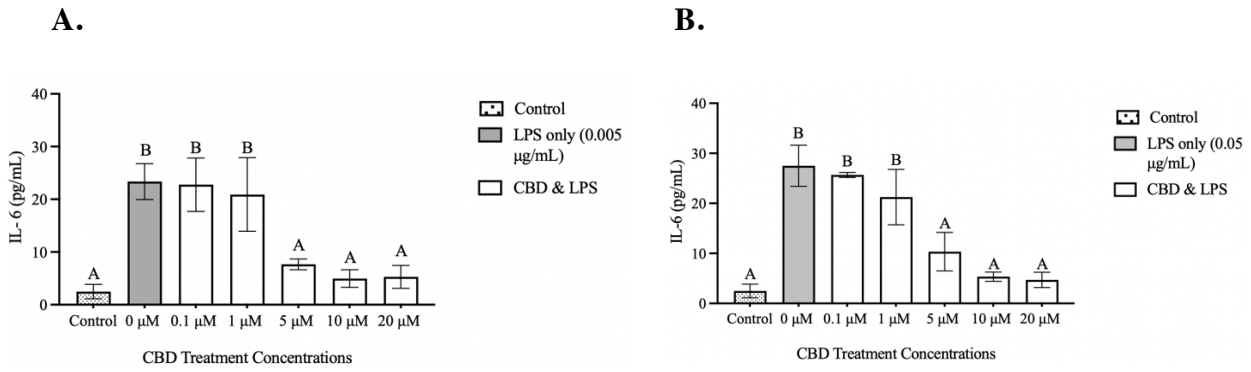


**Figure 12. CBD pre-treatment attenuates LPS-induced IL-6 production following 8 hours of LPS treatment (0.005 μg/mL of LPS).** A) BV-2 cells were treated with five different concentrations of CBD followed 4 hours later with by LPS (0.005 μg/mL). Supernatants were collected 8 hours after LPS treatment. ANOVA revealed a significant main effect of CBD treatment ($p \leq 0.01$) and 8 hours of LPS treatment ($p \leq 0.01$) on IL-6 production. **B)** BV-2 cells were treated with five different concentrations of CBD followed 4 hours later with by LPS (0.05 μg/mL). Supernatants were collected 8 hours after LPS treatment. Controls did not receive any treatment or drug. Different letters (A, B) represent significant differences at $p \leq 0.05$. Bars represent mean ± SEM. N's = 3 – 6.

# DISCUSSION

One hallmark pathology of AD is the deposition of amyloid beta (Aβ) plaques in the brain (Liu et al., 2019). Existing research has well established a link between AD pathologies and chronic inflammation, which occurs due to activation of the innate immune system and the secretion of pro-inflammatory cytokines (Amor et al., 2014; Cunningham et al., 2009; Yirmiya et al., 2011). Chronic inflammation and the presence of pro-inflammatory cytokines exacerbate the proteolytic cleavage of amyloid precursor protein (APP) into amyloid beta, which aggregates and forms extracellular plaques in the brain. Subsequently, the development of Aβ plaques disrupts the synaptic communication of neurons, as well as learning and memory processes. Aβ plaque production and aggregation is also a cause of increased inflammation and oxidative stress. In AD, chronic inflammation resulting from Aβ plaque accumulation leads to the chronic activation of microglia and the incessant release of pro-inflammatory cytokines, all of which contribute to the progression and development of AD pathologies. Cannabidiol represents a potential AD therapeutic due to its known antioxidant and anti-inflammatory properties (Klein, 2005; Nagarkatti et al., 2009). BV2 microglial cells express CB2 receptors which, when activated, minimize the secretion of pro-inflammatory cytokines (Ramirez et al., 2005).

This research sought to understand the mechanism by which CBD mitigates pro-inflammatory cytokines and the production of Aβ in order to evaluate its potential as an AD therapeutic. In the first experiments, we demonstrated that BV2 microglial cells exhibit an increased inflammatory response when stimulated with a lipopolysaccharide treatment. ELISA assays quantified the production of the proinflammatory cytokines TNF-$\alpha$ and IL-6 and showed that increasing concentrations of LPS increased the expression of the cytokines. This supports previous AD research that used LPS to activate the immune system and induce inflammation

(Chen et al., 2012). In following experiments, we included a CBD pre-treatment to determine its capability to reduce the inflammation brought on by LPS at two concentrations. The data show similar trends at both the lower and higher doses of LPS. 10 and 20 μm of CBD treatment were the most effective concentrations used to reduce TNF-α production. 5, 10, and 20 μm of CBD treatment significantly reduced IL-6 production. Based on these results, CBD may represent a natural therapeutic intervention to mitigate chronic inflammation in AD patients.

There are several potential avenues of future direction for this project. Similar experiments could be conducted with a broader range of concentrations of LPS and CBD to further examine the therapeutic properties of CBD against the inflammatory response of BV2 microglial cells following LPS activation. Western blots could be used to measure biomarkers and determine if CBD activates the Nrf2 anti-inflammatory pathway. Further ELISA assays and western blotting could be used to elucidate the effect of CBD on production of anti-inflammatory cytokines, such as IL-10. These studies would be beneficial in gaining a greater understanding of the role of inflammation and oxidative stress in the development of AD pathologies. Additionally, further research would buttress existing knowledge of the antioxidant and anti-inflammatory properties of CBD and support its potential use as a therapeutic for AD and other neurodegenerative diseases.

REFERENCES

Agostinho, P., Cunha, R. A., & Oliveira, C. (2010). Neuroinflammation, oxidative stress and the pathogenesis of Alzheimers disease. *Current Pharmaceutical Design*, 16(25), 2766–2778.https://doi.org/10.2174/138161210793176572

Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J., & Tang, X. (2017). Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1863(2), 585–597. https://doi.org/10.1016/j.bbadis.2016.11.005

Alzheimer's Association (2019). Alzheimer's disease facts and figures. (2019). *Alzheimers & Dementia*, 15(3), 321–387. doi: 10.1016/j.jalz.2019.01.010

Amor, S., Peferoen, L.A.N., Vogel, D.Y.S., Breur, M., Valk, P.V.D., Baker, D., & Noort, J.M.V. (2014). Inflammation in neurodegenerative diseases. *Immunology*, 142 (2), 151-166. doi: 10.1111/imm.12233

Block, M. L., & Hong, J.-S. (2005). Microglia and inflammation-mediated neurodegeneration: Multiple triggers with a common mechanism. *Progress in Neurobiology*, 76(2), 77–98. https://doi.org/10.1016/j.pneurobio.2005.06.004

Block, M., & Hong, J. (2007). Chronic microglial activation and progressive dopaminergic neurotoxicity. *Biochemical Society Transactions*, 35(5), 1127-1132. doi:10.1042/bst0351127

Chen, Z., Jalabi, W., Shpargel, K. B., Farabaugh, K. T., Dutta, R., Yin, X., ... Trapp, B. D. (2012). Lipopolysaccharide-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4. *Journal of Neuroscience*, 32(34), 11706–11715. https://doi.org/10.1523/jneurosci.0730-12.2012

Cunningham, C., Campion, S., Lunnon, K., Murray, C. L., Woods, J. F., Deacon, R. M., Perry, V. H. (2009). Systemic inflammation induces acute behavioral and cognitive changes and accelerates neurodegenerative disease. *Biological Psychiatry*, 65(4), 304-312. doi: 10.1016/j.biopsych.2008.07.024

Dursun, E., Gezen-Ak, D., Hanağası, H., Bilgiç, B., Lohmann, E., Ertan, S., Atasoy, İ. L., Alaylıoğlu, M., Araz, Ö. S., Önal, B., Gündüz, A., Apaydın, H., Kızıltan, G., Ulutin, T., Gürvit, H., & Yılmazer, S. (2015). The interleukin 1 alpha, interleukin 1 beta, interleukin 6 and alpha-2-macroglobulin serum levels in patients with early or late onset Alzheimer's disease, mild cognitive impairment or Parkinson's disease. *Journal of Neuroimmunology*, 283, 50–57. https://doi.org/10.1016/j.jneuroim.2015.04.014

Engelhart, M. J., Geerlings, M. I., Meijer, J., Kiliaan, A., Ruixtenberg, A., Swieten, J. C. V., Stijnen, T., Hofman, A., Witteman, J.C.M., Breteler, M. M. B. (2004). Inflammatory proteins in plasma and the risk of dementia. *Archives of Neurology*, 61(5), 668. doi: 10.1001/archneur.61.5.668

Fischer, R., & Maier, O. (2015). Interrelation of oxidative stress and inflammation in neurodegenerative disease: Role of TNF. *Oxidative Medicine and Cellular Longevity*, 2015. https://doi.org/10.1155/2015/610813

Iuvone T, Esposito G, Esposito R, Santamaria R, Di Rosa M, Izzo AA (2004) Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on beta-amyloid-induced toxicity in PC12 cells. *Journal of Neurochemistry*, 89(1):134–141

Janefjord, E., Mååg, J. L. V., Harvey, B. S., & Smid, S. D. (2014). Cannabinoid effects on β amyloid fibril and aggregate formation, neuronal and microglial-activated neurotoxicity

in vitro. *Cellular and Molecular Neurobiology*, 34(1), 31–42. doi: 10.1007/s10571-013-9984-x

Kahn, M., Kranjac, D., Alonzo, C., Haase, J., Cedillos, R., McLinden, K., Boehm, G.W., Chumley, M.J. (2012). Prolonged elevation in hippocampal Aβ and cognitive deficits following repeated endotoxin exposure in the mouse. *Behavioral Brain Research*, 229(1), 176–84. doi: 10.1016/j.bbr.2012.01.010

Kim, J., Choi, J. Y., Seo, J., & Choi, I. S. (2020). Neuroprotective effect of cannabidiol against hydrogen peroxide in hippocampal neuron culture. *Cannabis and Cannabinoid Research*. doi: 10.1089/can.2019.0102

Klein, T. W. (2005). Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nature Reviews Immunology*, 5(5), 400–411. doi: 10.1038/nri1602

Kozela, E., Pietr, M., Juknat, A., Rimmerman, N., Levy, R., & Vogel, Z. (2009). Cannabinoids Δ9-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-κB and interferon-β/STAT proinflammatory pathways in BV-2 microglial cells. *Journal of Biological Chemistry*, 285(3), 1616–1626. doi: 10.1074/jbc.m109.069294

LaFerla, F. M., Green, K. N., & Oddo, S. (2007). Intracellular amyloid-β in Alzheimer's disease. *Nature Reviews Neuroscience*, 8(7), 499–509. doi: 10.1038/nrn2168

Lee, J. W., Lee, Y. K., Yuk, D. Y., Choi, D. Y., Ban, S. B., Oh, K. W., & Hong, J. T. (2008). Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. *Journal of Neuroinflammation*, 5(37), 1–14.

Lee, S.-G., & Kang, H. (2014). Inhibition of lipopolysaccharide-stimulated neuro-inflammatory kuntze in BV-2 microglial cell mediators by tetragonia tetragonoides (Pall). *Tropical Journal of Pharmaceutical Research*, 13(12). https://doi.org/10.4314/tjpr.v13i12.8

Liu, Y., Nguyen, M., Robert, A., & Meunier, B. (2019). Metal ions in Alzheimer's disease: A key role or not? *Accounts of Chemical Research*, 52(7), 2026–2035. https://doi.org/10.1021/acs.accounts.9b00248

Mechoulam, R., & Hanuš L. (2002). Cannabidiol: An overview of some chemical and pharmacological aspects. part I: Chemical aspects. *Chemistry and Physics of Lipids*, 121(1-2), 35–43. https://doi.org/10.1016/s0009-3084(02)00144-5

Nagarkatti, P., Pandey, R., Rieder, S. A., Hegde, V. L., & Nagarkatti, M. (2009). Cannabinoids as novel anti-inflammatory drugs. *Future Medicinal Chemistry*, 1(7), 1333–1349. Doi: 10.4155/fmc.09.93

Omar, S. H., Scott, C. J., Hamlin, A. S., & Obied, H. K. (2017). The protective role of plant biophenols in mechanisms of Alzheimers disease. *The Journal of Nutritional Biochemistry*, 47, 1–20. doi: 10.1016/j.jnutbio.2017.02.01

Ramirez B.G., Blazquez C., Gomez del Pulgar T., Guzman M., de Ceballos M.L. (2005). Prevention of Alzheimer's disease pathology by cannabinoids: Neuroprotection mediated by blockade of microglial activation. *Journal of Neuroscience*, 25(8), 1904–1913. doi: 10.1523/jneurosci.4540-04.2005

Stansley, B., Post, J., & Hensley, K. (2012). A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *Journal of Neuroinflammation*, 9(1). https://doi.org/10.1186/1742-2094-9-115

Stella, N. (2010). Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia*, 58(9), 1017–1030. doi: 10.1002/glia.20983

Weller, J., & Budson, A. (2018). Current understanding of Alzheimer's disease diagnosis and treatment. *F1000Research*, 7, 1161. https://doi.org/10.12688/f1000research.14506.1

Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain, Behavior, and Immunity*, 25(2), 181–213. doi: 10.1016/j.bbi.2010.10.015

Zheng, Q., Sun, W., & Qu, M. (2018). Anti-neuro-inflammatory effects of the bioactive compound capsaicin through the NF-κB signaling pathway in LPS-stimulated BV2 microglial cells. *Pharmacognosy Magazine*, 14(58), 489. https://doi.org/10.4103/pm.pm_73_18