The Effects of Cannabidiol on Interleukin-2 Production and Viability of Human T Lymphocytes

Dipannita Purkayastha

A Thesis

in the

Department

of

Health, Kinesiology & Applied Physiology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science (Exercise Science)

Concordia University

Montreal, Quebec, Canada

July 2023

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared

By: Dipannita Purkayatha

Entitled: The Effects of Cannabidiol on Interleukin-2 Production and Viability of Human T Lymphocytes

and submitted in partial fulfillment of the requirements for the degree of

Master of Science (Exercise Science)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

Dr. Veronque Pepin — Chair

Dr. Robert Kilgore — Examiner

Dr. Andres Bergdhal — Examiner

Dr. Peter Darlington — Thesis Supervisor(s)

_____ Thesis Supervisor(s)

Approved by _____

Dr. Maryse Fortin, Graduate Program Director — Chair of Department or Graduate Program Director

Dr. Pascale Sicotte — Dean of Faculty of Arts & Science

ABSTRACT

The Effects of Cannabidiol on Interleukin-2 Production and Viability of Human T Lymphocytes

Dipannita Purkayastha

The legalization of cannabis in Canada resulted in an increased consumption of cannabis and cannabis-related products by the Canadian population. Cannabidiol (CBD) is one of the active chemicals within the cannabis plant. It is of particular interest because it is thought to suppress T cells, which are an integral part of the adaptive immune system. Suppressing T cells is not the desired effect for most individuals consuming cannabis. One of the problems with commercially available CBD is a lack of information on safe or effective amounts. More research needs to be conducted to determine if CBD at various doses causes health problems or can be therapeutic in patients with autoimmunity. The objectives were: (1) to determine the most effective way to deliver CBD in vitro by testing different solvents, (2) to investigate whether CBD alters the amount of the IL-2 cytokine produced by T cells, and (3) to examine the effects of CBD dose on T cell death. The findings demonstrated (1) glycerol was determined to be a better solvent compared to DMSO, (2) CBD causes cell death and decreased Il-2 production in T cells and PBMCs at higher doses.

The relevance of these findings is to better understand the interplay between CBD and the immune system by elucidating what a safe and effective dose of CBD might be for suppressing cytokine production in T cells. The results of thesis also pave the ground for future studies on the mechanism of action of CBD on T cells.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Peter Darlington for all the support, guidance, and supervision throughout my undergraduate and graduate studies. I would also like to thank my family, my brother and my sister and most importantly my parents. Thank you for crossing the Atlantic Ocean to give me a better life; I am forever in your debt. I would also like to thank my peers: Fadi Touma, Zahra Motaghi Moghadam, Maria Xavier for all the help, support and friendship throughout these last few years. I would like to thank all the undergraduate students who have contributed their time to my project, notably: Lou Meziere, who brought with her the last little bit of luck I needed to complete my data collection. I would be remiss if I did not thank my colleagues at L'Espoir: Lea, Alexa, Joelle and Eric. Thank you for all the memories and laughter, I will cherish them always. A huge thank you to my friends, Iqra, Nehaam and Judy; thank you for your wisdom and patience.

Finally, I would like to thank my best friend, for being at my side when I needed it and when I did not; most importantly when I did not know I needed it. It has made all the difference.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AP-1 - Activator protein 1
APC – Antigen Presenting Cell
CA^{2+} - Calcium
CBD – Cannabidiol
CD – Cluster of Differentiation
CN - Calcineurin
CTL – Cytotoxic T cell lymphocytes
DAG -Diacylglycerol
DNA - Deoxyribonucleic acid
DMSO – Dimethyl Sulfoxide
GTP – Guanosine- 5'- triphosphate
IL-2 – Interleukin-2
NF-kB - Nuclear factor kappa light chain enhancer of activated B cells
NFAT – Nuclear factor of activated T cells
NK – Natural Killer
MAPK - Mitogen-activated protein kinase
MHC – II – Major Histocompatibility Complex Class II
MEK - Methyl Ethyl Ketones
PBMC - Peripheral blood mononuclear cell
PKC – Protein Kinase C
PMA - Phorbol myristate acetate
T Cell – Thymus Cells
THC - Tetrahydrocannabinol
TCR – Thymus Cell Receptor
PKB – Protein Kinase B pathway
PIP2 - Phosphatidylinositol 4,5-bisphosphate
PLC - Phosphorylation of phospholipase C
RNA - Ribonucleic Acid

THEORETICAL CONTEXT

1 Introduction

1.1 Innate and Adaptive Immune System

The innate immune system is the first line of defense against infections. The cells responsible for innate immunity are rapidly activated when they interact with potentially dangerous microbes (Hato & Dagher, 2015). Innate responses are not adaptive to specific pathogens, although innate immune cells can recognize conserved features of pathogens such as lipopolysaccharide on certain bacteria. The innate immune system relies on proteins and phagocytic cells that are able to recognize harmful foreign pathogens (Chaffey, 2003). The innate immune system consists of macrophages, dendritic cells, natural killer cells and special innate subsets of T cells and B cells that have reduced receptor diversity (Hato & Dagher, 2015). The idea of reduced receptor diversity means that the innate immune system has a smaller repertoire of receptors as compared to the adaptive immune system. However, the innate immune receptors are very specific for microbial determinants, they can react quickly upon exposure to a microbe. The main objective of an innate immune response to pathogens to is slow down the spread of pathogens in the body, allowing the adaptive immune system to begin fighting the infection.

The adaptive immune system response is capable of recognizing an almost limitless range of microbial determinants. There are two types of adaptive immune responses: humoral immune response and cell mediated immune response. Activated B cells and antibodies are responsible for humoral immune response and T cells are responsible for cell-mediated immune response.

Helper T cells are normally required for initiating the humoral response by activating the B cells (Medzhitov & Janeway, 1998). Antibodies produced by B cells destroy extracellular microorganisms, slow the spread of infections, and provide immunological memory. The T cell receptor and antibodies have a randomized re-arrangement of their DNA in the binding regions, which creates an almost limitless capability to recognize new pathogen determinants (Daniel *et al*, 2022).

1.2 The role of T cells in adaptive immunity

T lymphocytes (T cells) circulate throughout the circulatory and lymphatic systems in active search of antigen presenting cells (APC), including in secondary lymphoid tissues such as lymph nodes and spleen. APCs express surface protein complexes known as Class II Major Histocompatibility Complex (MHC-II), which present antigen fragments to circulating T cells. T cell receptors (TCR) on the T cell surface recognize specific antigens presented by MHC-II and will initiate T cell activation upon detecting a recognized antigen. Since each T cell has unique TCRs programmed to recognize one specific antigen, eventually one will fit like a lock and key when binding to a MHC-II complex and initiate clonal expansion. The activation of T cells causes various downstream signaling pathways signal 1 and signal 2. Signal 1 occurs when the TCR/CD3 complex binds to MHC-II and signal 2 occurs when CD 28 binds with B7, a family of surface proteins including CD80 and CD86 (Li *et al*, 1999). Successful T cell activation requires completion of signal 1 and signal 2. Once a T cell is activated, it initiates a signal transduction cascade, causing transcription factors NF-κB and NFAT to enter the nucleus and activate genes (Paul & Schaefer,

2013). T cells are an essential part of the adaptive immune system, they are responsible for initiating a prolonged inflammation and coordinating the attack against the pathogen. After clearing the pathogen, T cells become memory cells and help fight a second infection should it occur. Unfortunately, in individuals with autoimmune disease, the T cells act against self tissue and create chronic inflammation and tissue damage (Scholler *et al*, 2020). Thus, researchers are constantly exploring more effective and new ways to suppress the immune system.

1.3 Cytokines

Cytokines are small hormone-like proteins produced by immune cells. They are responsible for cell signaling which results in an altered immune response when a pathogen is present (Ross & Cantrell, 2018). Furthermore, cytokines stimulate their targets cells to release additional cytokines (Guo *et a*l, 2012). Interleukin-2 (Il-2) is predominantly produced by T (Yiemwattana *et al*, 2012). IL-2 is a pleiotropic cytokine that is able to promote inflammatory reactions in the adaptive immune system (De Rham, 2007). IL-2 signaling pathways are responsible for controlling differentiation and homeostasis of pro and anti-inflammatory T cells (Ross & Cantrell, 2018). Immature T cells are turned into regulatory T cells because IL-2 aids in T cell differentiation. After the body encounters an infection, naïve antigen specific effector T cells proliferate and differentiate to effector T cells. IL-2 interacts with cytotoxic T cell lymphocytes (CTL) to promote the release of these pro-inflammatory cytokines. IL-2 influences CTLs to produce more pro-inflammatory cytokines by stimulating the cytolytic effector molecule granzyme B and perforin (Ross and Cantrell, 2018). IL-2 controls the size of CTLs by determining

the amount of amino acid uptake and protein synthesis. IL-2 sends signals within its signal transduction network that has the potential to determine the transcriptional and metabolic functions of several different types of T cells (Ross and Cantrell, 2018). In summary, IL-2 is a cytokine with growth-factor properties and is often used as a measure of T cell activation in cell culture experiments.

1.4 T Cell Activation

The first step in the activation process occurs when the TCR on the T cell binds to the antigen that is presented by MHC-II (Smith-Garvin *et al*, 2009). MHC-II is located on the surface of the APC membrane and is responsible for detecting foreign antigens (Wieczorek *et al*, 2017). The second step of the T cell activation requires the binding of CD28 to CD86 or CD80 molecules on the APC (Smith-Garvin *et al*, 2009). During an adaptive immune response, the APC will process a microbe and present bits of the antigens in MHC-II in order for T cells to scan over the shape of the MHC/antigen complex.

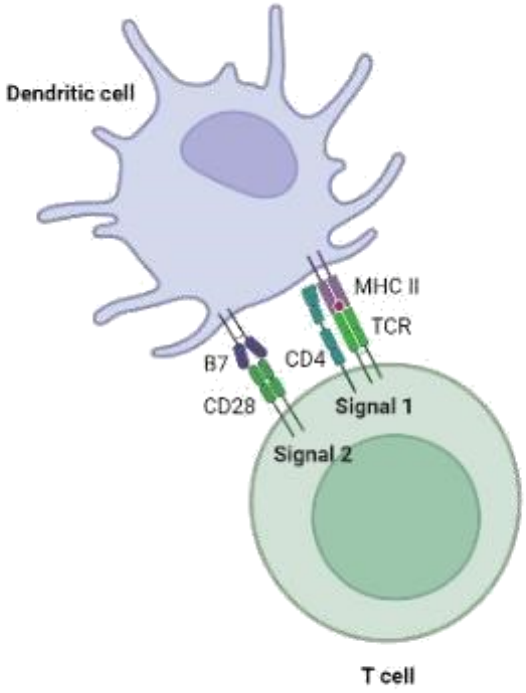

Figure 1.1: Schematic diagram demonstrating signal 1 and signal 2 during T cell activation. Signal 1 occurs when the TCR on the T cell binds to the antigen that is held by the MHC. Signal 2 occurs when CD28 binds to B7 on the APC. Created with Biorender by Dipannita Purkayastha.

For example, phorbol myristate acetate (PMA) is a small organic molecule that enters the cell membrane and cytoplasm and activates protein kinase C (PKC) (Paul Held, 2018). In doing so, PMA simulates the signaling pathway of the co-stimulatory receptor responsible for supplying signal 2. PKC is an important enzyme that regulates two stages of the cell cycle (Black & Black, 2013). PKCs affect G0/G1and G2 by modulating molecules responsible for cell cycle regulation (Black, 2000). To stimulate the TCR, antibodies that recognize CD3 are often used in cell culture experiments. The TCR forms a complex with the CD3 protein; the CD4 receptor is a co receptor on the TCR. CD3 communicates with ZAP 70, a cytoplasmic protein tyrosine kinase that plays an essential role in T cell responses (Wang *et al*, 2010). Once the T cell is activated, ZAP 70 leads to an increase in phosphorylation of phospholipase C (PLC) (Williams *et al*, 1999). Phosphatidylinositol 4,5-bisphosphate (PIP2) is a lipid that is essential for cellular metabolism and cell signaling pathways. PLC cleaves PIP2, which produces diacylglycerol (DAG) and inositol trisphosphate (IP3). PIP2 also acts as a messenger and is important for signaling pathways, IP3 is a signaling molecule that activates calcium signaling (Steelman *et al*, 2015). The release of IP3 from the membrane causes receptors on the endoplasmic reticulum to activate the release of intracellular calcium stores and activates the PKC pathway (Steelman *et al*, 2015). The increased intracellular calcium leads to an induction of calcineurin (CN). CN is a calcium is calmodulin dependent protein phosphatase (Kraner & Norris, 2018). CN promotes Nuclear Factor of Activated T cell (NFAT) pathway dephosphorylation (Macián & López-Rodríguez, 2001). NFAT is an activator for the transcription of IL-2 in T cells (Boss et al, 1996).

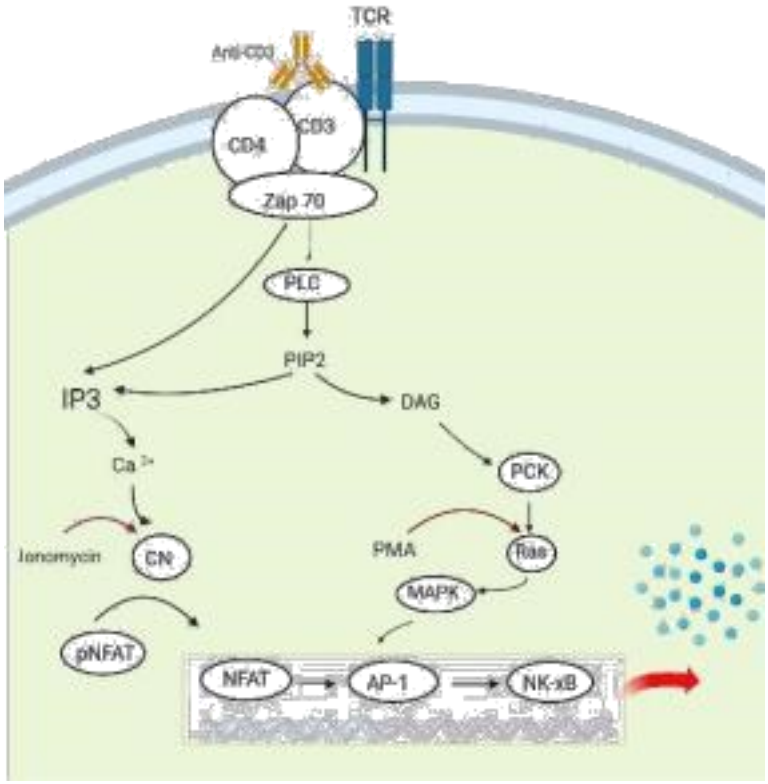

Figure 1.2: Schematic depiction of Il-2 production in Jurkat T cells. Anti CD3 attaches to the CD3 receptor on the TCR, which causes Zap70 to communicate IP3. Zap 70 also causes increased phosphorylation of PLC. PLC cleaves PIP2 which produces DAG and IP3. Two different pathways form: one starting with IP3 and the second with DAG. The release of IP3 causes the release of intracellular calcium stores and activates CN. Ionomycin further triggers calcium release, which also activates CN. CN causes NFAT dephosphorylation. The NFAT pathway takes place in the nucleus of the cell. NFAT promotes AP-1. AP-1 activates NF- κB, which is a transcription factor that regulates inflammatory responses. In the second pathway DAG causes the release of PCK which activates Ras. PMA enters and mimics the action of DAG. Ras then induces MAPK. MAPK proteins activates AP-1 which regulates immune responses such as Il-2 release. Created using Biorender by Dipannita Purkayastha.

The NFAT pathway occurs within the nucleus of the cell, where it promotes Activator Protein 1 (AP-1). AP-1 is a group of transcription factors that bind to DNA enhancer sequences for gene expression, cellular differentiation, proliferation and apoptosis (Frigo *et al*, 2004; Macián & López-Rodríguez, 2001). AP-1 then activates Nuclear Factor kappa light chain enhancer of activated B cells (NF-κB). NF-κB is a transcription factor that regulates cellular behavior such as inflammatory responses (Lai *et al*, 1995). PLC cleaves PIP2, which produces DAG and IP3 (Steelman *et al*, 2015). These downstream effects can be directly stimulated by PMA, which enters and directly modulates PKC by mimicking the action of DAG (Huang, K. P., 1989). PKC activates Ras, which is a small Guanosine-5'-triphosphate (GTP) binding protein. It is an essential component of the signal transduction pathways. Activation of cells causes Ras to activate the protein kinase pathway (MAPK). (Margolis & Skolnik, 1994). Ras activates RAF kinase; which phosphorylates and activates Methyl Ethyl Ketones (MEK). MEK are responsible for phosphorylating and activation MAPK (Guo *et al*, 2020). MAPK proteins activate AP-1, which causes increased secretion of Il-2 in Jurkat T cells (Gopalakrishnan *et al*, 2006). Ionomycin is an ionophore, which in combination with PMA produces a low-level signal for IL-2 secretion. However, when PMA is combined with another stimulator, both molecules stimulate IL-2 signal cascade concurrently resulting in a significant increase in IL-2 production (Paul Held, 2018).

1.5.1 Experimentation on Jurkat T Cells

To facilitate research advancements, immunologists have used a cell line called Jurkat T cells. Most of the knowledge regarding how the TCR works, and how IL-2 is regulated, was discovered

in this cell line (Abraham & Weiss, 2004). The cell line was derived from a leukemia patient who had the last name Jurkat ; the Jurkat E6.1 clone was selected because it produced high levels of IL-2 when the TCR was activated (Meng *et al*, 2021). Using the Jurkat E6.1 cells, immunologists performed saturation mutagenesis and identified many of the key signalling molecules in the TCR pathway (Abraham & Weiss, 2004). Saturation mutagenesis is a technique in which one or several positions in the protein sequences are changed to a more beneficial position and then randomized (Steffens & William, 2007). Jurkat T cells can be easily transfected and grow continuously in culture, making them an ideal cell to study TCR-dependent IL-2 production in human T cells. Despite the many advantages of using this cell line, Jurkat T cells are leukemic cells that constantly proliferate and thus do not accurately represent healthy human T cells. More specifically, Jurkat T cells lack a tumor-suppressor enzyme called PTEN which normally suppresses T cell growth (Shan *et al*, 2000). Despite this proliferation defect, Jurkat T cells are still the gold standard for studying TCR signalling and regulation of IL-2.

1.5.2 Experimentation on primary human lymphocytes

To account for the limitations of Jurkat T cells, experiments in this project were replicated on primary human PBMC samples. These samples are obtained from the venous blood of human participants, which is why the word 'peripheral blood' is used in the acronym. Mononuclear cell refers a heterogenous mixture of immune cells including macrophages, dendritic cells, B cells, monocytes, NK cells, and T cells. The procedures used to purify the PBMC excludes the polymorpho-nuclear immune cells such as neutrophils, mast cells, basophils, and eosinophils,

and also excludes platelets and erythrocytes, all of which are very toxic if included in cell culture experiment. Those elements are toxic in cell culture because they tend to quickly degrade and release enzymes and a plethora of inflammatory mediators. By removing the poly-morpho nuclear cells, the mononuclear lymphocytes survive in culture and can be studied exclusively. Human PBMCs are very different between people which presents an opportunity to study diversity, but also makes it necessary to repeat the experiments on several participants to know if the findings are representative. The percentage of T Cells also varies in PBMC samples depending on the participant. Despite these limitations, mononuclear lymphocytes from venous blood represent one of the best ways to study the human immune system that is feasible in an *in vitro* research setting.

1.6 Cannabinoids history, chemistry, and *in vitro* experimentation

Cannabis is one of the most consumed psychoactive compounds in the world (Sharma, Murphy, & Bharath, 2012). There was a 7 % increased in cannabis consumption amongst Canadians during the pandemic (Scott *et al*, 2023). Cannabis has many chemical components; the two main ones are cannabidiol (CBD) and tetrahydrocannabinol (THC). THC is responsible for inducing psychoactive triggers as well as causing paranoia, nausea, and euphoria (Hollister, 1971). CBD, however, is less psychoactive and has anti-inflammatory properties and is often used as an analgesic (Sunda & Arowolo, 2020).

One major issue with CBD is the lack of information and knowledge regarding the safe amounts to consume. When purchasing CBD or CBD products, there is no recommended usage on the labels. It is not known whether CBD can have a harmful effect to the immune system if consumed at a certain dose. In the literature, experiments using different types of CBD were reported to further understand the effects of CBD on cytokine production, cell death, and proliferation. For example, PBMC samples were incubated with various concentrations of CBD suspended in ethanol and cytokines were measured after 24 hours. The results showed that CBD at a concentration of 4 μg/mL (12.8 μM) significantly reduced production of IFN-γ and TNF-α. Moreover, CBD at a concentration of 2 μg/mL (6.4 μM) significantly reduced IL-10 production (Turner *et al*, 2021). CBD reduced the production of IL-2 in immune cells by suppressing NFAT in immune cells at concentrations of 10 and 20 μM (Kaplan *et al*, 2008). CBD at 10 μM causes apoptosis in a manner driven by reactive oxygen species and caspases. Exposing Jurkat T cells to a CBD solution of 10 μM reduces cell viability by apoptosis caused by reactive oxygen species damaging the DNA, RNA and mitochondria (Kalenderoglow *et al*, 2017). Moreover, CBD deactivated the Protein Kinase B/Akt (PKB) pathway. The PKB pathway is a signal pathway which is responsible for the survival and growth of cells in response to extracellular messages. When the PKB pathway is activated, it mediates downstream signaling which controls cell survival, proliferation, growth, and angiogenesis (Kalenderoglow *et al*, 2017).

Cell death occurring after exposure to CBD has been documented (Shrivastava *et al.* 2011) A proposed mechanism by Olivas-Aguirre et al. (2019), states that CBD causes cellular death in immune cells by allowing a high amount of of calcium ions Ca^{2+} in the mitochondria. CBD's lipophilic nature allows it to pass through the plasma membrane easily. CBD causes the

voltage gated ion channels to open which results in increased calcium in the mitochondria. The increased amount of Ca^{2+} in the mitochondria results in an overload (Olivas-Aguirre et al., 2019). The formation of mPTP allows cytochrome C to be released from the mitochondria. In the cytoplasm, cytochrome C results an apoptosome formation, caspase activation and activates the cells intrinsic apoptosis mechanisms. The mPTP triggers release of Ca^{2+} from the endoplasmic reticulum which causes a toxic amount of Ca^{2+} to be present in the mitochondria, increased oxidative stress, and lowered ATP production (Olivas-Aguirre *et al*, 2019). This proposed mechanism sheds light on the exact pathway utilized.

1.7 Solvents utilized in *In Vitro* Experiment with Cannabidiol

CBD is a hydrophobic chemical that requires a solvent to dissolve (Mangal *et al*, 2021). Drug-delivery devices such as CBD-patches, use propylene glycerol and glycerol to solubilize and allow skin penetration of CBD (Casiraghi *et al*, 2020). Transdermal patches of CBD used to treat skin inflammation and conditions such as psoriasis, dermatitis, and acne contain glycerol as a solvent and propylene glycol as a skin penetrant (Casiraghi *et al*, 2020). Experiments with CBD typically use ethanol and DMSO (dimethyl sulfoxide) as organic solvents which can all be toxic at vehicle concentrations over 1% and even change gene expression at levels below 0.1% volume by volume of cell culture media (Galveo *et al*, 2014). Similarly, previous research in the Darlington laboratory on the suitability of methanol as an alternative drug vehicle for CBD found that methanol itself had modulatory effects on the amounts of cytokines being produced by T cells at low doses and decreased cell viability at high doses (Norhan Mehrez, MA thesis, unpublished data).

Follow up experiments were conducted by vaporizing methanol and replacing it with DMSO, which was found to be less toxic at the concentrations used but nevertheless significantly altered the cytokines produced by the cells (Norhan Mehrez, MA thesis, unpublished data). A survey of literature in this regard revealed that DMSO interacts with ion channels, which is concerning since it may lead to confounding effects. CBD is also thought to interact with ion channels which may explain its biological effects (Rivers-Auty & Ashton, 2013). Thus, it would be desirable to avoid the use of methanol or DMSO in future experiments.

Propylene glycol and glycerol are both gel-like substances which are considered safe for consumption (Fowles et al, 2013). The table below lists publications conducted with CBD and different vehicle controls. The authors found various effect at different concentrations of CBD. Kaplan et al. (2008), conducted similar experimentation to the one proposed here. Kaplan et al. (2008) obtained CBD and THC from the National Institute of Drug Abuse; it is not known if they CBD obtained was already in a solvent. Kaplan et al, utilized ethanol at 1% as a vehicle control, they activated Jurkat T cells with either PMA and ionomycin or anti-CD3 and anti-CD28 (Kaplan et al, 2008). The results demonstrated that CBD at doses of 5, 10 and 15 µM suppressed IL-2 production. The studies below conducted *in vitro* experimentation with CBD with solvents. All of the studies used vehicle controls; however, the vehicle controls utilized were ethanol and DMSO, which as previously discussed is problematic as a solvent. I proposed that glycerol would be a non-toxic candidate as a solvent for CBD for the purposes of *in vitro* experiments with T cells. Glycerol was chosen as it is not toxic to the cells; CBD has been administered through dermal patches utilizing glycerol as a solvent.

Table 1.1: Drug vehicles used for CBD in selected studies with corresponding findings.

Reference	*Vehicle*	*Dosage*	*Findings*
Milan *et al.*, 2020	Ethanol	10- 100 µM	CBD inhibited the proliferation of Epidermal Growth Factor Receptor in cancer cells.
Kaplan & Kaminski, 2008	Ethanol	0.1- 20 µM	CBD suppressed IL-2 production in Jurkat T cells at 15 and 20 µM.
Turner *et al.*, 2021	DMSO	1.59-3.18 µM	CBD at 3.18 µM reduced IFN-γ and TNF-α in PBMCs.
Shangguan *et al.*, 2021	DMSO	0-160µM	CBD at 40 µM reduces Hepatocellular carcinoma cell growth *in vitro*
Sun *et al.*, 2017	DMSO	1 -10 µM	CBD enhances glucose metabolism in hippocampal neurons at 5 µM
Shrivastava *et al.* 2011	Ethanol	1 -10 µM	CBD at concentrations of 5 & 10 µM resulted in apoptotic cell death in breast cancer cells.
Cocetta *et al.*, 2021	Ethanol	0.14-12.7 µM	CBD at 1.27 µM decreased paracellular permeability inflammation in intestinal inflammatory diseases.
Kalenderoglow *et al.*, 2017	DMSO	0.01- 10 µM	CBD reduced cell size and cell viability at 10 µM in Jurkat T cells

1.8 CBD used in Advanced Cancer Patients

Individuals who experience pain have limited treatment options apart from addictive painkillers such as opiates. Clinical trials have started exploring the effects of CBD on autoimmune diseases and pain, however, there are very few *in-vivo* studies done on the effects of CBD on the immune system on healthy people. In the context of cancer treatment, it would not be desirable to inhibit T cells, in fact, many new therapies rely on boosting T cells in cancer patients. If CBD inhibits T cells, it would not be beneficial to the cancer patient in theory, even if it has analgesic effects. Patients with cancer experience pain symptoms that require prescription medication; CBD has been utilized to alleviate pain symptoms in palliative care for individuals diagnosed with cancer. There is currently an ongoing clinical trial, a double-blind randomized study that is investigating the pain reducing effects of 600 mg of CBD compared to placebo (CBD for chronic radicular pain on chronic opioid therapy, 2022*).* Therefore, it is important to better understand the effects of different doses of CBD on the immune system by performing *in-vitro* experimentation first. Moreover, clinical trials utilizing CBD in individuals who experience pain are measuring CBD's efficacy as analgesic. This phase-I clinical is done on individuals who are experiencing pain; it does not provide information regarding the effects on immune cells for healthy participants who use CBD recreationally.

2. Specific Aims

The specific aims of my masters research thesis were:

Specific Aim 1: To determine whether DMSO interacts with CBD to affect the production of IL-2 in Jurkat T cells.

Specific Aim 2: To observe if CBD reduces IL-2 production in Jurkat T cells and PBMCs when CBD in dissolved glycerol.

Specific Aim 3: To investigate if CBD causes cell death in a dose-dependent manner in Jurkat T cells and PBMCs when CBD is dissolved in glycerol.

3. Methods

3.1 Participants

The protocols for the human portion of this research study were approved by Concordia University Research Ethics committee. Exclusion criteria for the participants included the following: being below the age of 18, if they had any prior history of immune disease, had received a flu shot in the previous 3 months, had any severe medical conditions (diabetes, heart disease, etc), and/or were frightened of needles. Participants signed an informed consent form prior to participating. After the blood draw, the participants were observed for 15 minutes to ensure they have no adverse side effects. A total of 6 to 8 vials of blood was obtained; each participant was assigned a specific code to maintain anonymity. A total of 3 participants were recruited.

3.2 Cell Culture Medium and culture

The cell culture medium utilized for incubating cells was RPMI 1640 (Wisent Bioproducts). A total of 60 ml was removed from the RPMI bottle, and 5ml of penicillin/streptomycin and 5ml of Glutamax (Thermal Fisher Scientific), and 50 ml fetal bovine serum (FBS) (Wisent, Inc) were added. The cells, both Jurkat cells and PBMC, were cultured at 37°C with 5% CO_2 in an incubator.

The reasons for adding these reagents was as follows. RPMI has vitamines, minerals and buffers, the antibiotics prevent bacterial contaminations, glutamax is the main source of nitrogen for protein growth, FBS contains hormones growth factors and glucose, and the CO_2 maintains the bicarbonate buffer in the media at neutral pH.

3.3 PBMC Isolation

Blood was obtained from antecubital vein of participants by venipuncture by a qualified professional. The blood was then transferred from the blood collection vials to 50mL conical tubes and diluted with equal volume of phosphate buffer saline solution (PBS). A total of 15 ml of blood was added to 15 ml of PBS in a 50 ml centrifuge tube. Next, 10 to 15 ml of Ficoll Hypaque density gradient was added to the same centrifuge tube. It was centrifuged for 30 minutes at 398 RCF at room temperature (21°C). After centrifugation, the blood separated into different layers including the PBMC layer which contains lymphocytes. It was extracted with a transfer pipette and transferred to a 50mL centrifuge tube. PBS was then added to this new centrifuge tube until a total amount of 50 ml was reached. It was centrifuged again at 177 G force for 15 minutes at room temperature. The centrifugation resulted in a cell pellet at the bottom of the

centrifuge tube. The supernatant was decanted, and the pellet was separated by scraping the tube on a rack and 50 ml of PBS was added and the cells were centrifuged at 177 G force for 12 minutes at room temperature. The supernatant was then discarded, and the cell pellet was resuspended. Media was added, and the mixture was transferred into a flask in the incubator until it was needed for the experiment. PBMC's were counted and the appropriate calculations were used to obtain the desired number of cells per well. Approximately 0.2 x 10^{6} cells were added per well in the 96 cell culture plate for the experiment. PBMCs were activated by using anti-CD3 and anti-CD28 each at 1 µg/mL.

3.4 Jurkat T Cell Activation

Jurkat T cells were incubated in a RPMI 1640 solution which consists of 5% FBS, 1% l-glutamine, and 1% penicillin streptomycin. The cells were kept in the incubator at 37°C and 5% CO_2, by passing the cells every 2 or 3 days. The day the experimentation occurred the T cells were counted using the trypan blue method to ensure that they were under 1.0 x 10^{6} per ml during cell culture. In brief, 12.5 µl of the Jurkat T cells and 12.5 µl of the trypan blue solution was put in an microcentrifuge tube, mixed and placed on a hemocytometer under the microscope. The cells were then counted to ensure that the cells were less than 1.0 x 10^{6} per ml to ensure there were enough cells for the experiment. Anti CD3 (eBioscience) and PMA (Sigma-Aldrich-Millipore) was used to activate the Jurkat T cells. The final concentration of Anti-CD3 was 0.1 µg/ml, the final concentration of PMA was 20 ng/ml.

3.5 CBD Cell Culture

The cell culture plate included five concentrations of CBD in order to create a dose - response graph. CBD (Sigma-Aldrich-Millipore C-045-1ML) came as an analytical standard at 1.0 mg/ml in 1mL methanol. It was not possible to obtain CBD powder without a solvent, due to federal restrictions on controlled substances, which CBD falls under. The methanol was evaporated off the CBD solution using a vacuum concentration procedure, then it was resuspended in DMSO. The molar mass of CBD is 314.47 g/mol, and the stock solution of CBD is 3180 µM. From the stock, various concentrations of CBD were made including 1.25 µM, 2.5 µM, 5 µM, 10 µM and 20 µM.

To make 100 L of 200 M working solution of CBD: C1 = 3180 M, V2 = 100 L, C2 = 200 M; therefore, V1 = C2V2/C1 = (200 M*100 L)/3180 M = 6.289 M. Therefore, 93. 71 L of media and 6.289 L of stock solution was put in a microcentrifuge tube; this was labeled as 200 M of the CBD solution. A serial dilution was then performed to obtain the 2 fold dilutions.

T cells and PBMCs were incubated in media with the various CBD for 48 hours at concentrations of: 1.25 µM, 2.5 µM, 5 µM, 10 µM and 20 µM. A timeline of 48 hours was chosen based on literature review where this was the optimal time to measure IL-2 (Kim *et al.*, 2021, Zhang *et al.*, 2019, Kalenderoglow *et al.*, 2017).

3.6 Measurement of cytokines by ELISA

Enzyme Linked Immunosorbent Assay (ELISA) was performed on T cells and PBMC's utilizing the IL-2 kit according to manufacturer's protocol (BD biosciences). The capture antibody

added to the plate 1 to 3 days before performing the experiment. A total of 24 uL of the antibody was be added to 6 ml of coating buffer (prepared in house) for a concentration of 1:250; 50 µL of this solution was added to a 96 well ELISA plate. The day of the experiment, the capture antibody solution was removed with ELISA wash. ELISA wash buffer was prepared in house, by adding 2.5 ml of tween to 1 liter of 1XPBS. The plate was then washed three times with ELISA wash. To prevent background binding, 200 µL of blocking buffer (Thermo Fischer Scientific) was added for one hour while the standards and samples were prepared. The frozen supernatants were thawed and centrifuged for 12 minutes at 177 G force (1200 rpm) at room temperature. The samples were then be transferred to a V-well plate (referred to as the dilution plate). The samples were diluted with blocking buffer according to the experiment plan. The blocking buffer was aspirated, and the ELISA plate was washed 3 times. Afterward, 50 µL of diluted sample and standard was added to the ELISA plate based on the plate layout. The plate was then left to incubate for a total of 2 hours. The ELISA plate was washed five times and 50 µL of detection antibody solution and Sav-HRP (prepared in house by diluting the detector antibody with blocking buffer for a total dilution of 1:250) was added to the ELISA plate. The ELISA plate was left to incubate for an hour. The plate was washed 7 times and 100 uL of detection substrate solution (Thermo Fisher Scientific) was pipetted into the ELISA plate. It was be left in the dark for approximately 10 to 20 minutes until the wells became dark blue. To stop the reaction, 50 uL of 2N sulfuric acid was added. Results were analyzed with a Biotek plate reader using Gen5 Microplate Reader software. Absorbency was measured at 450 nm and background absorbency, measured at 570 nm, was subtracted. The difference in absorbency between 450 nm and 570 nm was calculated for each well of the ELISA plate.

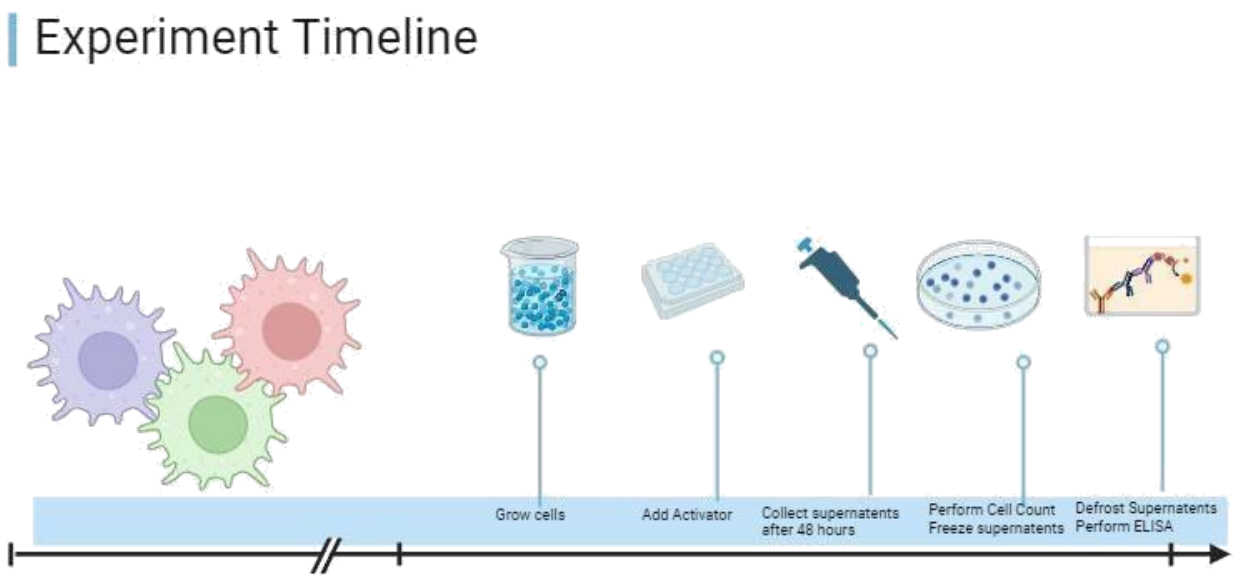

Figure 3.1: Experiment Timeline. Cells were grown until they reach half a million/mL. The cells were then be put into a 96 well plate and the activator, drugs, and the vehicle controls were added. After 48 hours, supernatants were collected and frozen and a cell count was performed. Supernatants were then defrosted and ELISAs were performed. Created with Biorender by Dipannita Purkayastha.

3.7 Cell Viability

Cell viability was obtained by utilizing the countess machine which is an automatic device that provides a cell count and cell viability by clearly dividing cell boundaries and distinguishing between debris and dead cells, which provides a more accurate count compared to the hemocytometers (Rigor *et al*, 2018). The countess machines measures size, circularity, and darkness to provide an accurate measure of cell death. In brief, 12 µL of Jurkat T cells or PBMCs from each condition was mixed with 12 µL of trypan blue solution in a cell culture plate. 10 µL of the cell and trypan solution was added to the reusable slide and then the slide was entered into the Countess machine. This was repeated twice for each concentration.

The Countess has automated software that counts live and dead cells based on size, sphericity, and trypan blue exclusion. If a cell excludes trypan it is considered to be alive, if it includes trypan that means the cell membrane is compromised and the cell is dead.

3.8 Data Analysis

ELISA data obtained from Gen5 Microplate Reader software were processed in Microsoft Excel. Absorbency data were provided by the Gen5 software for each of the wells of the ELISA plate. A standard curve was generated using the absorbency values associated with the standards of known cytokine concentration. This curve was used to interpolate the cytokine concentrations in each of the remaining wells on the plate.

Statistical analysis and figure generation were both done using GraphPad PRISM software. For data that involved multiple conditions, a one-way ANOVA was used to determine if there were significant differences between conditions. Tukey's post-hoc test was used to determine which conditions differed significantly. A significance level of 0.05 was used for the statistical analyses performed.

4. Results

4.1 Objective 1

Specific Aim 1: To determine whether DMSO interacts with CBD to affect the production of IL-2 in Jurkat T cells. To test the effects of CBD and its solvent DMSO on human T cells, a cell line

called Jurkat T cells were tested in cell culture experiments. To induce IL-2 production, which is a common measure of T cell activation, the cells were activated with anti-CD3 and PMA which stimulate signal one and signal two, respectively. Without activation, Jurkat T cells did not produce IL-2, in contrast, Jurkat T cells activated with anti-CD3 and PMA produced significantly higher amounts of IL-2 following 48 hours of incubation (Figure 4.1.1). These results confirm that the activation condition was performing as expected, and it served as a quality control measure for all experiments performed. If an experiment had IL-2 in the non-activated condition, or the activation condition failed to produce IL-2, the results were not included in the analysis.

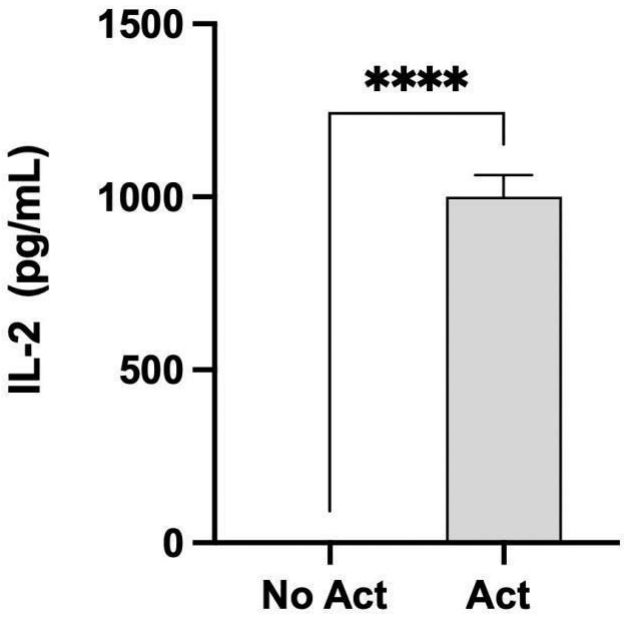

Figure 4.1.1: Jurkat cells produce IL-2 following activation with anti-CD3 and PMA. Cells were incubated for 48 hours without activation, or with activation. The cell culture supernatants were assayed for IL-2 using ELISA. Error bars represent SD. (****$p<0.0001$). The data is representative of successful activation of Jurkat T cells prior to experiments with different doses of CBD.

After establishing the activation protocol, the Jurkat T cells were activated in the presence or absence of CBD dissolved in DMSO, as compared to just DMSO at matching dilution. The dose response of CBD was chosen based on a literature review of values measured in human blood samples (Pigliasco *et al*, 2020). I observed IL-2 production in the activated condition, with no significant change in the activated plus CBD condition. DMSO did not significantly change the IL-2, in some data points it substantially increased the SD as seen in three replicate experiments (Figure 4.1.2). The importance of using DMSO was to control for the vehicle that CBD was dissolved in. The expectation was that DMSO would not alter the activation-induced IL-2. While statistically DMSO did not affect the results, its increased SD especially seen in Figure 4.1.2 B) and C) was problematic in that it prevented the interpretation of the CBD effect on T cells.

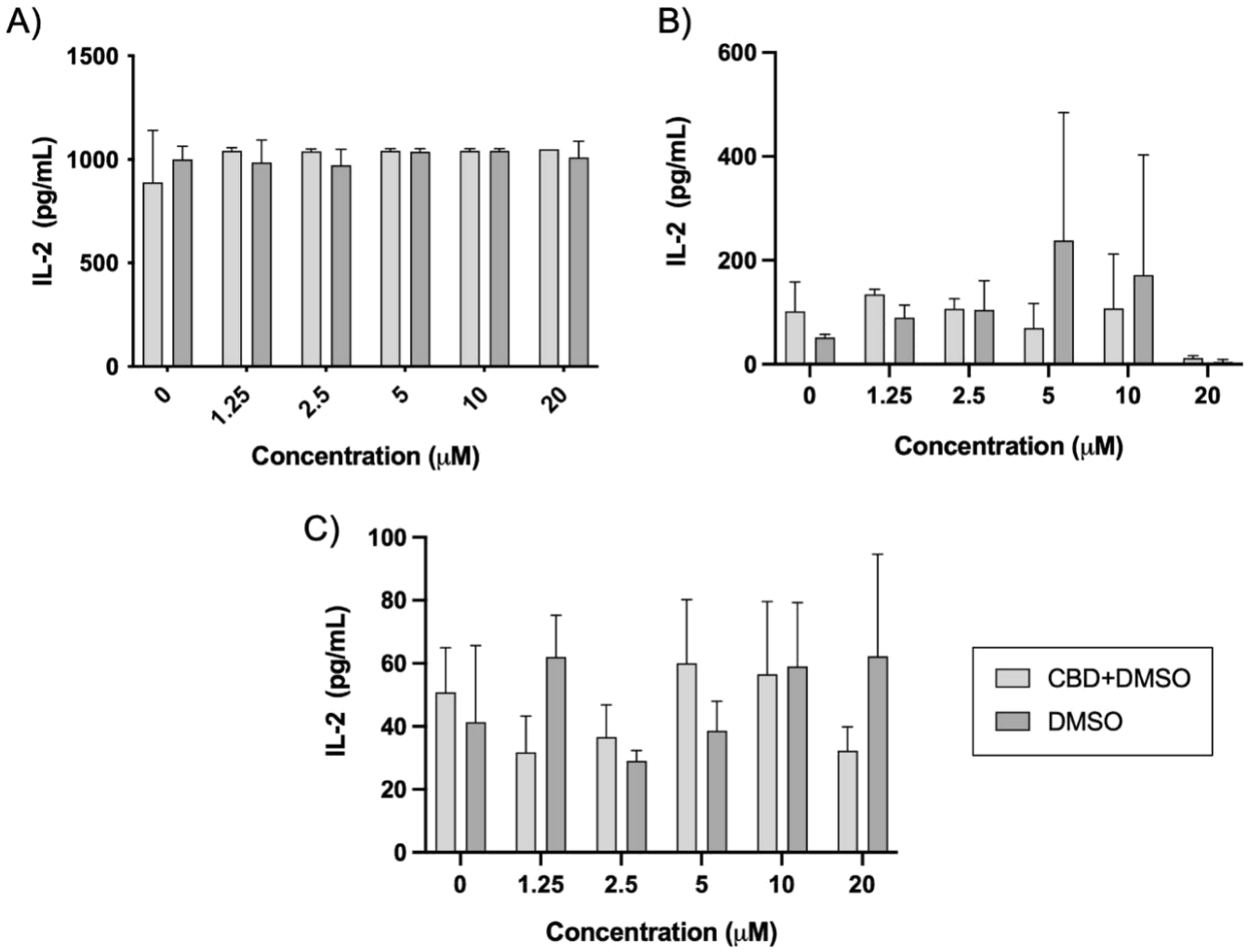

Figure 4.1.2: IL-2 production by activated Jurkat T cells incubated for 48 hours with CBD dissolved in DMSO, or DMSO as a control group. IL-2 was measured by ELISA in the cell culture supernatants. Three replicate experiments are shown in this figure (A-C). A negative control condition with non-activated Jurkat T cells (not shown in figure) showed negligeable production of IL-2. Error bars represent SD.

4.2: Objective 2

Specific Aim 2: To observe if CBD reduces IL-2 production in Jurkat T cells and PBMCs when CBD in dissolved glycerol. Considering the statistical issues with using DMSO as a solvent, the next experiments were conducted with CBD that was dissolved in glycerol as a solvent. In the three replicate experiments shown in Figure 4.2.1, CBD suppressed the production of IL-2 by activated Jurkat T cells at 5, 10 and 20 µM as compared to the glycerol vehicle control (Figure 4.2.1, A-C). The lower concentrations of CBD, which were 1.25 and 2.5 µM, increased the production of IL-2 by Jurkat T cells as compared to the VC, although this was not observed in the experiment in Figure 4.2.1 C) for CBD at a concentration of 2.5 µM. The data supports the use of glycerol for a solvent since the SD appeared to be less of an issue as compared to the DMSO experiments. The results indicate that CBD suppresses IL-2 at higher doses, but increases IL-2 when used at lower doses.

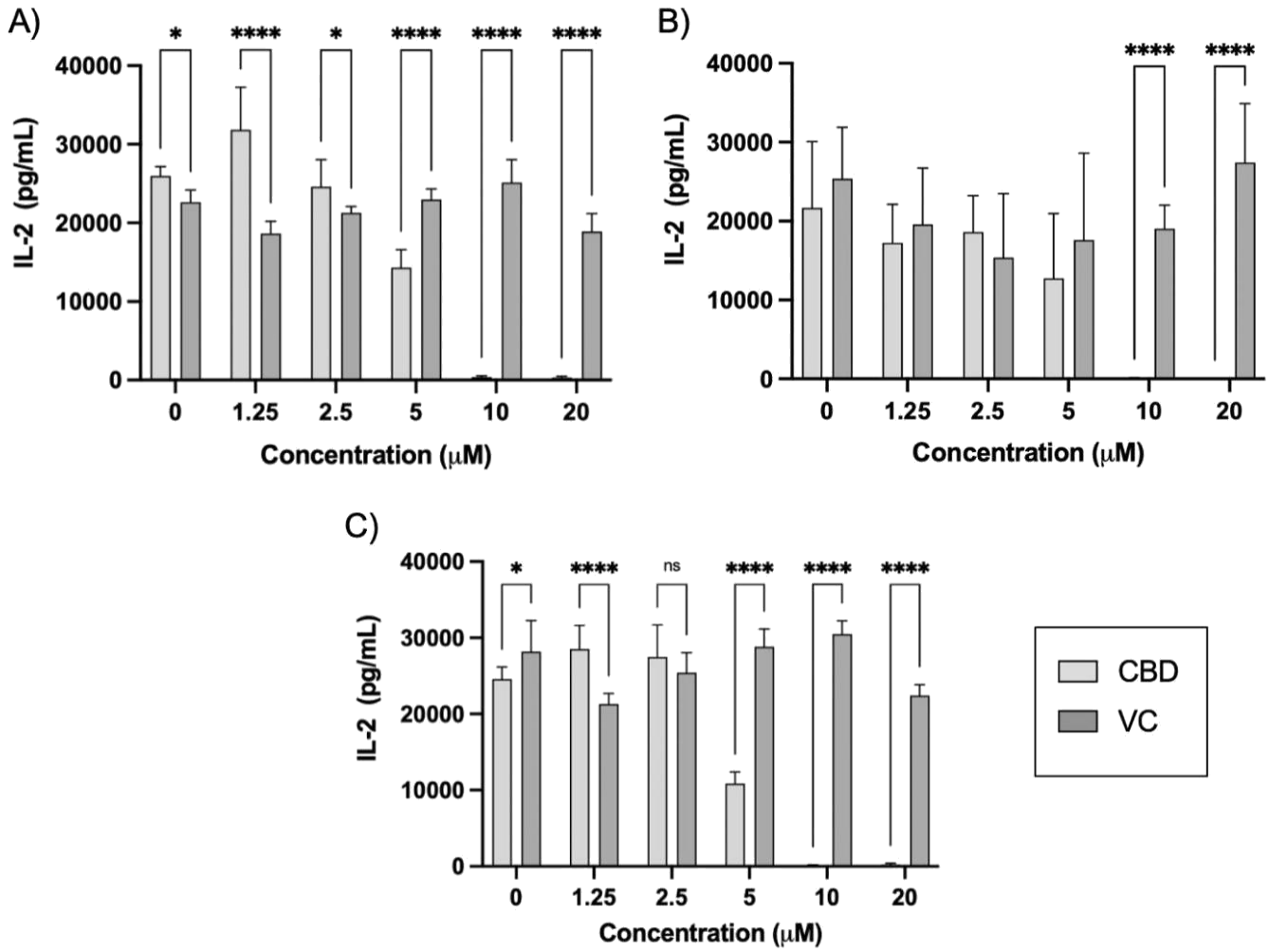

Figure 4.2.1: IL-2 production by Jurkat T cells incubated for 48 hours in CBD or glycerol vehicle control (VC). Vehicle control contain the amount of drug vehicle present in each concentration of CBD. Three replicate experiments are shown (A-C). A negative control condition with non-activated Jurkat T cells (not shown in figure) was included and showed no production of IL-2. Error bars represent standard deviation. (*p<0.05, ****p<0.0001)

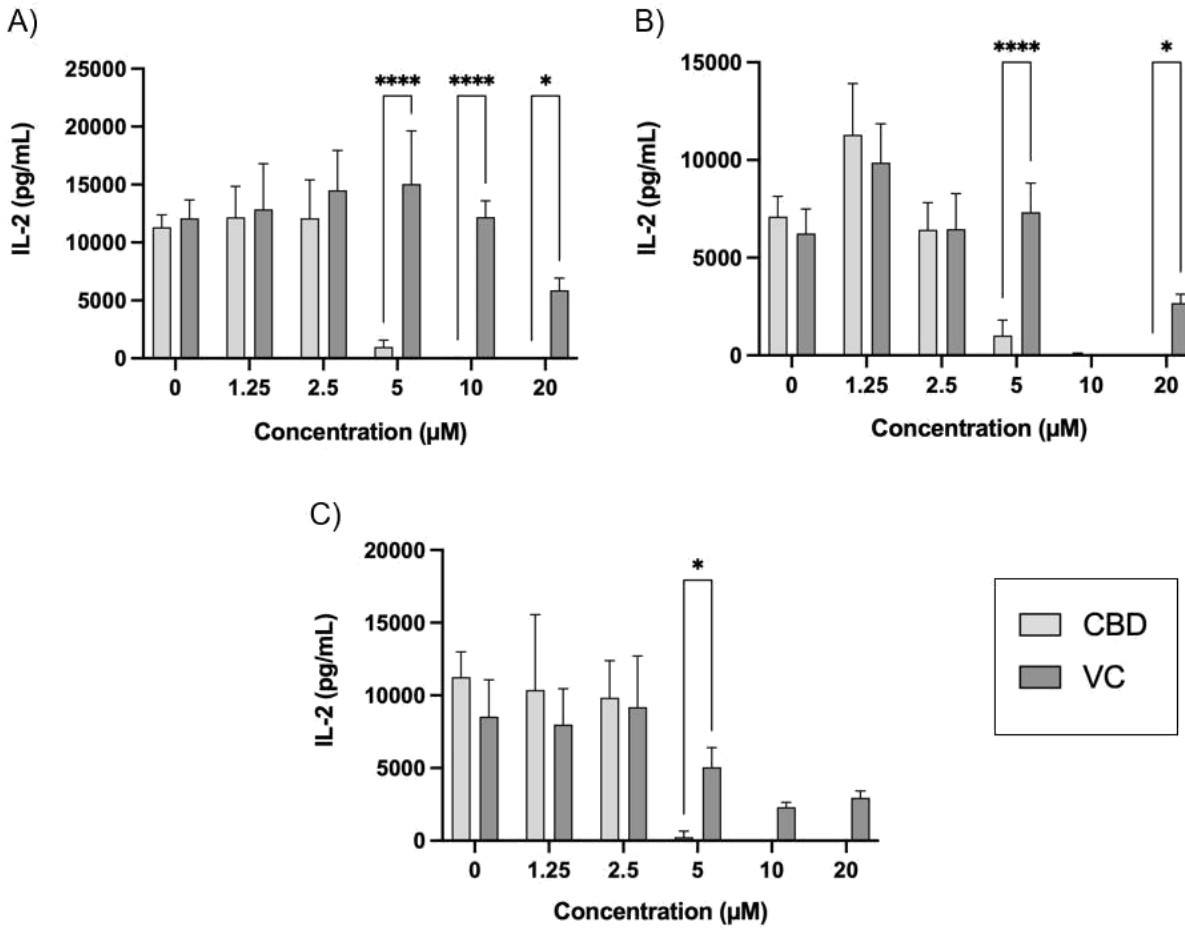

Figure 4.2.2: IL-2 production by PBMCs incubated for 48 hours in CBD or glycerol vehicle control (VC). Figure A refers to LCR 197, figure B refers to LCR 203 and figure C refers to LCR 207. Vehicle controls contain the amount of drug vehicle present in each concentration of CBD. Three replicate experiments are shown. Error bars represent standard deviation. (*p<0.05, ****p<0.0001)

4.3: Objective 3

Once the IL-2 cytokine is reduced by CBD at high doses, it was possible that the T cells were undergoing the process of cellular death. This necessitated a careful measure of cell death in the experiments. CBD increased the amount of cell death in the Jurkat T cells in a dose-dependent manner compared with vehicle controls for concentrations of 5, 10, and 20 µM (Figure 4.3.1 A-C). At the lower CBD concentrations of 1.25 and 2.5 µM, cell death was increased in two of the three experiments (Figure 4.3.1 B, C). The amount of cell death appeared to be greater with the higer concentrations of the drug as compared to the respective vehicle control. To determine if CBD would induce cell death in primary lymphocytes, the same measures were performed on PBMC exposed to CBD dissolved in glycerol as compared to glycerol as a vehicle control. CBD increased the amount of cell death in the PBMCs in a dose-dependent manner compared with vehicle controls for concentrations of 5, 10, and 20 µM (Figure 4.3.2 A-C). At the lower CBD concentrations of 1.25 and 2.5 µM, cell death was increased in PBMC in two of the three experiments (Figure 4.3.2 A, B). Higher doses of CBD were associated with more observed cell death. The amount of cell death for each concentration of CBD varied between individual participants. For example, CBD caused approximately 50 percent increase in of cell death in the participant shown in Figure 4.3.2(C), at the highest concentration, 20 µM.

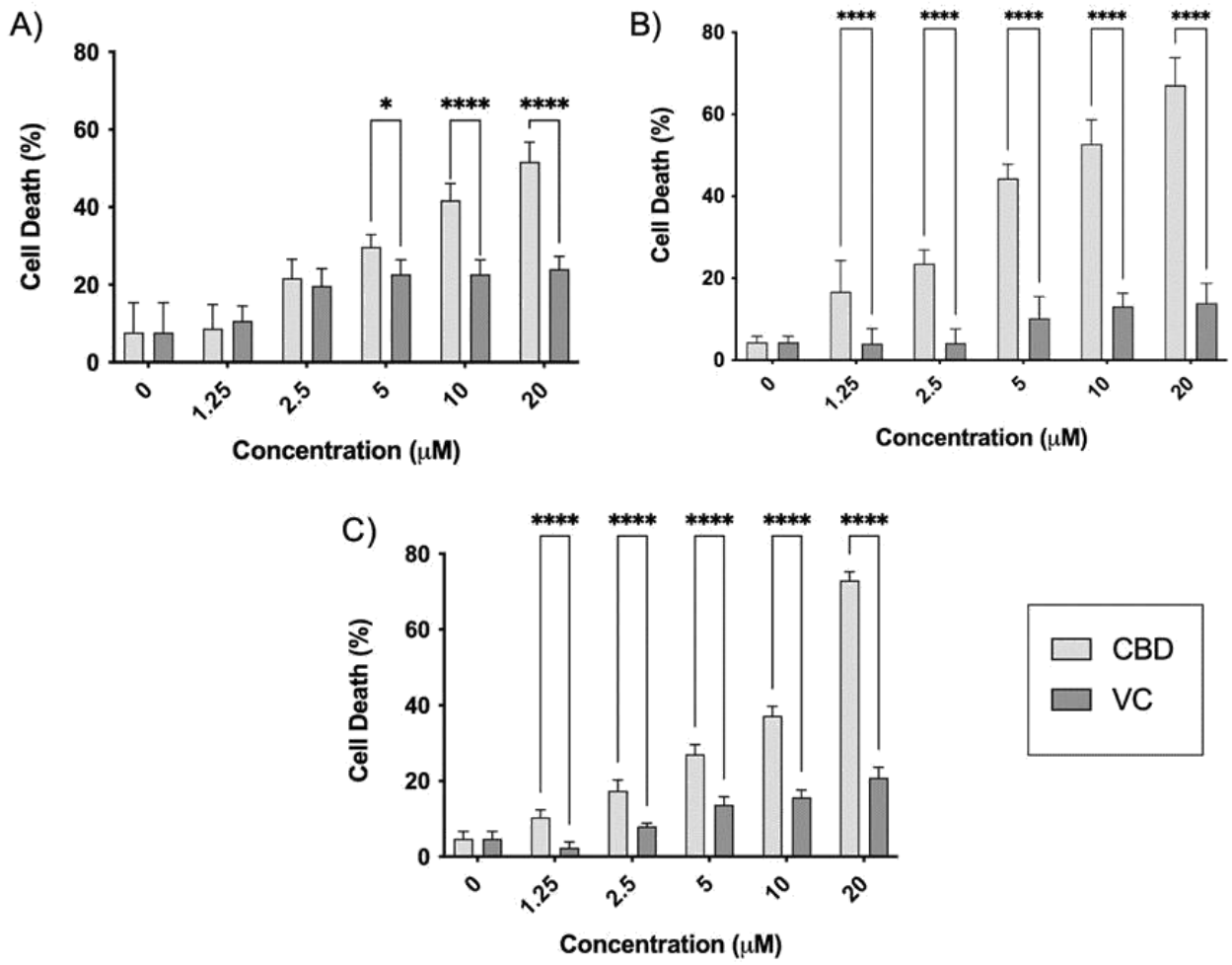

Figure 4.3.1: Cell death in Jurkat T cells incubated for 48 hours in CBD or glycerol vehicle (VC). CBD increases cell death in Jurkat T cells in a dose-dependent manner. Three replicate experiments are shown in this figure. Error bars represent SD. (*p<0.05, ****p<0.0001)

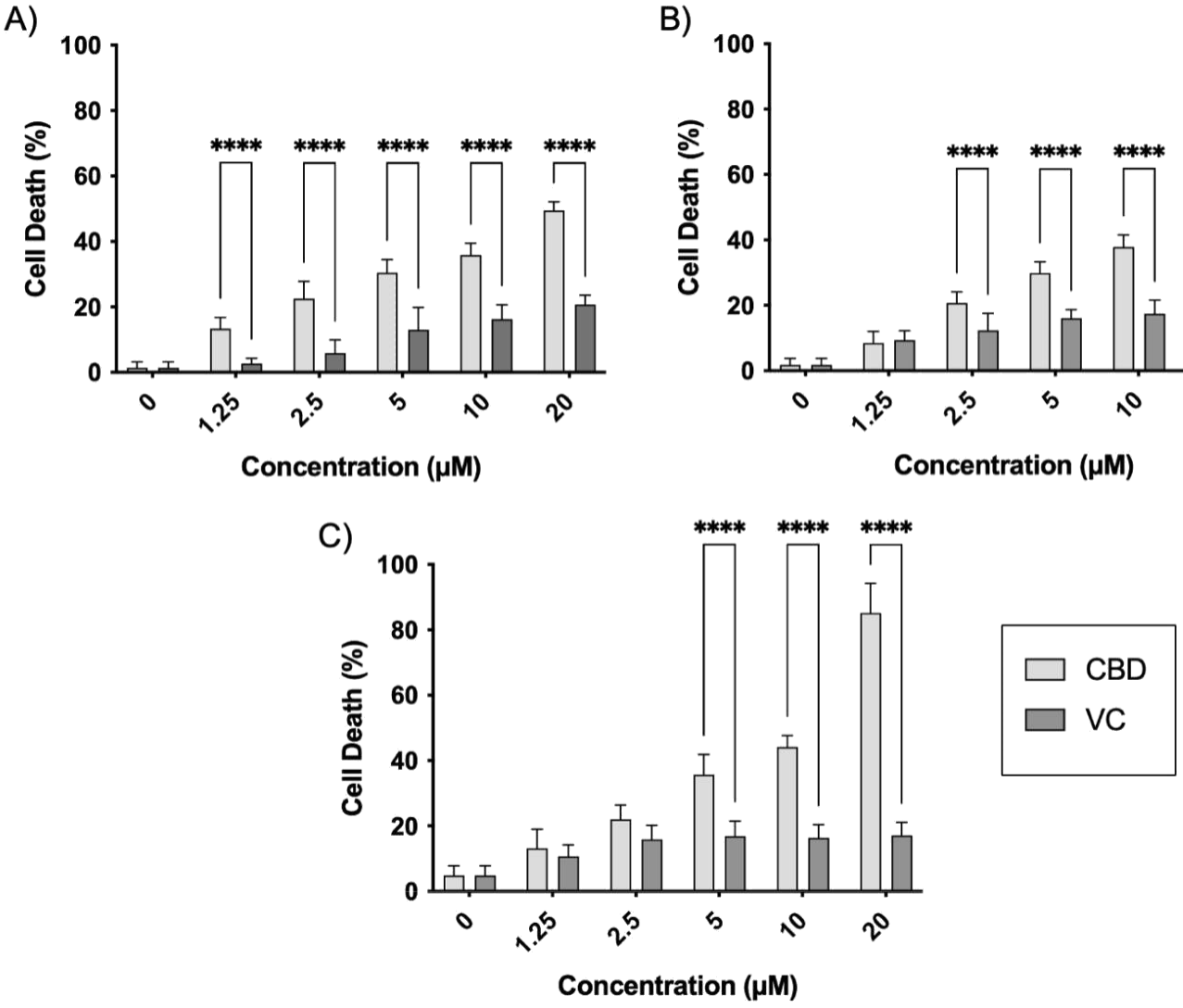

Figure 4.3.2: Cell death in PBMCs incubated for 48 hours in CBD or glycerol vehicle (VC). CBD increases cell death in Jurkat T cells in a dose-dependent manner. Three replicate experiments are shown in this figure, each one using PMBCs isolated from a different participant. Error bars represent SD. (****p<0.0001)

5. Discussion

Cannabis is one of the most consumed psychoactive compounds in the world (Sharma et al., 2012). Canadians have increased their consumption of cannabis during the COVID-19 pandemic (Scott *et al*, 2023). However, CBD has been suggested to have immunosuppressant effects, with such effects being of potential interest for individuals with autoimmune disease who consume CBD (Giorgi *et al*, 2021). CBD is currently used by individuals for analgesic effects (e.g., to alleviate pain from advanced cancer) and to help with depression and anxiety (Wieckiewicz *et al*, 2022). However, the CBD is often sold without any dosage recommendations. Canadians are increasing their consumption of CBD without fully knowing the physiological effects it has, including the effects it has on their immune system.

In order to learn more about the safety and efficacy of CBD as a drug, the effects of CBD on IL-2 production by immune cells were examined in both a human T cell line and primary T cells from healthy participants. To simulate an inflammatory response *in vitro*, a T cell activator (anti-CD3) and a co-stimulatory compound (PMA) were added, which induced the production of large amounts of IL-2 indicative of T cell activation. To determine if CBD could suppress this inflammatory reponse, various doses were added along with the volume-matched vehicle control. The results demonstrated that DMSO was not a suitable vehicle control for CBD as it altered IL-2 production and introduced large variances in the replicate conditions. Timm et al. (2013) warned against utilizing DMSO as a vehicle control as it confounds results by interacting with ion channels in the cells. It should also be noted that CBD is lipophilic and can also potentially interact with ion channels (Mangal et al, 2021). The results in this project showed that DMSO had inconsistent effects each time it was used as a drug vehicle. Figure 4.1.2A shows there are no statistically significant

difference in the amount of IL-2 production between CBD suspended in DMSO compared to DMSO alone at any concentration. Notably, the SD in IL-2 production was higher in the DMSO group for concentrations of 5 and 10 µM. At a concentration of 20 µM, there was very little IL-2 production in both CBD and DMSO conditions. CBD and DMSO had similar values of IL-2 production in the activated group and at concentrations of 1.25, 2.5 and 10 µM. This data indicate that DMSO may be either interacting directly with the cells or modifying the way CBD interacts with the cells. In the second replicate of this experiment (Figure 4.1.2B), there was no difference found in the IL-2 production in T cells incubated for 48 hours in CBD suspended in DMSO and DMSO alone in different concentrations. The values for IL-2 production in the CBD condition and DMSO-only condition were similar for concentrations of 1.25, 2.5, 5, 10 and 20 µM. As noted in the previous graph; perhaps these values provide support that DMSO interacts with the cells, confounding CBD's effects on the T cells and rendering it unsuitable as a drug vehicle for *in vitro* experimentation.

In the final replicate of this experiment (Figure 4.1.2C), there was no statistical significance in IL-2 production in the two groups (T cells incubated for 48 hours in CBD suspended in DMSO and DMSO alone) in different concentrations. After conducting three replicates of the same experiment with varying results; it was decided that DMSO could no longer be utilized as a solvent for CBD due to its inconsistent effects on IL-2 production. Though DMSO continues to be used commonly as a solvent in cell culture experiments, especially as a cryopreservation chemical when freezing cells, there has not been much conclusive findings as to how it interacts with cells or the mechanisms by which it does this. One possible explanation is that DMSO has been shown to inhibit acetylcholinesterase (AChE), which would make it

susceptible to interacting with cholinergic signaling pathways in T cells (Kumar & Darreh-Shori, 2017; Cox et al., 2020). Cholinergic signaling pathways have been shown to modulate the production of cytokines by T cells, including IL-2 (Mashimo et al., 2017). Further experiments would be needed, however, to draw any definitive conclusions. In this project, instead of exploring the immunomodulatory effects of DMSO further, I decided to dissolve CBD in a more natural solvent called vegetable glycerin (also known as glycerol). This compound was chosen as a prospective drug vehicle because it has been used as a solvent for CBD in commercial preparations, such as in transdermal patches and electronic cigarettes, and has not been demonstrated to interact with cells in existing literature (Peace et al., 2016).

In light of this, the experiments in this project were repeated utilizing glycerol as a drug vehicle to dissolve CBD. All three graphs in Figure 4.2.1 show that at 10 and 20 µM CBD reduced IL-2 production in Jurkat T cells. The CBD effect was statistically greater than the volume-matched vehicle control (glycerol) indicating that the glycerol does not interact with the cells and was thus a more effective vehicle than DMSO. The same experiment was replicated three times with PBMCs. There appears to be more variation among the PBMC experiments compared to the T cell experiments; this may be due to variation amongst individuals. In Figures 4.2.2, there were negligeable amounts of IL-2 production at a concentration of 20 µM for both CBD suspended in glycerol and glycerol alone. Moreover, CBD decreased IL-2 production at 5, 10 and 20 µM. Though glycerol also decreased IL-2 production at the same concentrations, the effect of glycerol alone was less than that of CBD. The fact that this effect was observed in the PBMC experiments only seems to indicate that glycerol interacts with some of the immune cells present in the PBMC samples used, though to a lesser extent than DMSO. CBD caused a substantial increase in

cell death in both Jurkat T cells and PBMCs at 10 and 20 µM (Figures 4.3.1 and 4.3.2). Although glycerol elevated cell death at the higher amounts, CBD caused significantly more cell death suggesting that this may reflect an effect of CBD and not the vehicle alone. As such, one novel finding of this project was the suitability of glycerol as a drug vehicle, especially given its relatively low vehicle effects compared with DMSO.

These findings indicate that CBD may be effective in suppressing T cells, however, it may also kill T cells at higher doses. The two effects may be related, that is, killing the T cells might be responsible for inhibiting the IL-2 production. Individuals consuming CBD recreationally might not be aware that it can have an immunosuppressant effect. The immunosuppressant effect observed in this project may be relevant to those living with immunodeficiencies or to healthy individuals looking to take care of their immune health, as high levels of CBD consumption might compromise immune activity. Conversely, those living with autoimmune disorders or leukemia may be interested in these same immunosuppressant effects. Therefore, in addition to the findings presented in this project, further research should continue to examine how CBD suppresses the immune system, with a focus on using drug vehicles without the potential to interact with the drug or the cells.

There are several limitations to this study. The first limitation is not being able to determine the amount of methanol that remained in CBD after removal of methanol (used as a solvent for the original product) by centrivaping. This would have required the use of quantitative mass spectrometry. There could also have residual remains of methanol in the CBD which is problematic due to the fact that methanol is toxic to the cells (Nguyen *et al*, 2020). The solvent limitation could be solved by obtaining federal permission to procure CBD powder direct from

the company which could be directly suspended in glycerol. Methanol standards are the only form of CBD legally available for research with the permits we were accorded. The second limitation is that flow cytometry was not used for cell counting. Flow cytometry would have provided a more accurate representation of the live/dead count. Although flow cytometry was not used, the Countess machine was used which does automated counting of hundreds of cells per image, which offers an improvement in accuracy and reliability compared with traditional hemocytometer counting methods. Another limitation was the participant sample size, since only three individuals were recruited for the primary healthy human part of the study. PBMC data (Figure 4.3.2) shows that there is individual variation in IL-2 response. Therefore, a larger sample size may have shown data that better represents the general population. A further limitation was that using human participants introduces confounding variables such as variations in recreational drug use, variations in medication regimens, as well as other biological differences between individuals. In this project, recent recreational drug use was given as an exclusion criterion to minimize the confounding effects of this variable. However, a greater sample size would have been a more ideal way of attenuating the effects of individual differences.

6. Future Directions

Part of the reason that I optimized the cell culture conditions was to study the mechanisms of action of CBD on the T cells. Now that the vehicle, dosing, and cell death parameters have been established in the cell line and primary human cells, future *in-vitro* studies with glycerol as a solvent need to be performed. Western blotting techniques can be used to understand the

effects of CBD has on immune cells' cell signaling pathways that are important for TCR and co-stimulation. To verify the CBD preparation, mass spectrometry should be done to verify that methanol was completely removed before beginning in-vitro experimentation. Furthermore, flow cytometry should be used to gather more detailed cell counting results that can identify T cells. Finally, PBMC's from a larger sample size should be collected to better understand patterns and account for individual variation. Once a safe range of CBD in the immune cells has been determined in *in-vitro* studies*, in-vivo* clinical trials can further establish whether CBD is immunosuppressant for healthy participants. For example, participants would be asked to consume CBD or placebo, and their blood samples would be drawn at various time points and analyzed for T cell responses. Furthermore, observational studies should be performed to determine if there is a significant decrease in symptoms in patients with autoimmune diseases. Considering the potential cell death that occurs with CBD at higher doses, it stands to reason that CBD should be obtained by prescription only and in consultation with a doctor. The current situation of unregulated CBD consumption by the general public, despite its strict research regulations, would appear to put people who consume CBD at a risk of unknown proportions.

7. REFERENCES

Abraham, R. T., & Weiss, A. (2004). Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nature reviews immunology*, *4*(4), 301-308.

Black, A. R., & Black, J. D. (2013). Protein kinase C signaling and cell cycle regulation. *Frontiers in immunology*, *3*, 423.

Black, J. D. (2000). Protein kinase C-mediated regulation of the cell cycle. *Frontiers in Bioscience-Landmark*, *5*(3), 406-423.

Boss, V., Talpade, D. J., & Murphy, T. J. (1996). Induction of NFAT-mediated transcription by Gq-coupled receptors in lymphoid and non-lymphoid cells. *Journal of Biological Chemistry*, *271*(18), 10429-10432.

Casiraghi, A., Musazzi, U. M., Centin, G., Franzè, S., & Minghetti, P. (2020). Topical administration of cannabidiol: Influence of vehicle-related aspects on skin permeation process. *Pharmaceuticals*, *13*(11), 337.

CBD for chronic radicular pain on chronic opioid therapy (COT) - full text view. ClinicalTrials.gov. (2022.). https://classic.clinicaltrials.gov/ct2/show/NCT04760613

Chaffey, N. (2003). Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. Molecular biology of the cell. 4th edition.

Cocetta, V., Governa, P., Borgonetti, V., Tinazzi, M., Peron, G., Catanzaro, D., & Montopoli, M. (2021). Cannabidiol isolated from Cannabis sativa L. protects intestinal barrier from in vitro inflammation and oxidative stress. *Frontiers in pharmacology*, *12*, 64121

Cox, M. A., Bassi, C., Saunders, M. E., Nechanitzky, R., Morgado-Palacin, I., Zheng, C., & Mak, T. W. (2020). Beyond neurotransmission: acetylcholine in immunity and inflammation. Journal of internal medicine, 287(2), 120–133. https://doi.org/10.1111/joim.13006

Daniel, B., Yost, K. E., Hsiung, S., Sandor, K., Xia, Y., Qi, Y., ... & Satpathy, A. T. (2022). Divergent clonal differentiation trajectories of T cell exhaustion. *Nature Immunology*, 1-14.

De Rham, C., Ferrari-Lacraz, S., Jendly, S., Schneiter, G., Dayer, J. M., & Villard, J. (2007). The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors. *Arthritis research & therapy*, *9*(6), 1-15.

Fowles, J. R., Banton, M. I., & Pottenger, L. H. (2013). A toxicological review of the propylene glycols. *Critical reviews in toxicology*, *43*(4), 363-390.

Frigo, D. E., Tang, Y., Beckman, B. S., Scandurro, A. B., Alam, J., Burow, M. E., & McLachlan, J. A. (2004). Mechanism of AP-1-mediated gene expression by select organochlorines through the p38 MAPK pathway. *Carcinogenesis*, *25*(2), 249-261.

Galvao, J., Davis, B., Tilley, M., Normando, E., Duchen, M. R., & Cordeiro, M. F. (2014). Unexpected low-dose toxicity of the universal solvent DMSO. *The FASEB Journal*, *28*(3), 1317-1330.

Giorgi V, Marotto D, Batticciotto A, Atzeni F, Bongiovanni S, Sarzi-Puttini P. Cannabis and Autoimmunity: Possible Mechanisms of Action. Immunotargets Ther. 2021 Jul 21;10:261-271. doi: 10.2147/ITT.S267905. PMID: 34322454; PMCID: PMC8313508.

Gopalakrishnan, A., Xu, C. J., Nair, S. S., Chen, C., Hebbar, V., & Kong, A. N. T. (2006). Modulation of activator protein-1 (AP-1) and MAPK pathway by flavonoids in human prostate cancer PC3 cells. *Archives of pharmacal research*, *29*(8), 633-644.

Guo, L., Junttila, I. S., & Paul, W. E. (2012). Cytokine-induced cytokine production by conventional and innate lymphoid cells. *Trends in immunology*, *33*(12), 598-606.

Guo, Y. J., Pan, W. W., Liu, S. B., Shen, Z. F., Xu, Y., & Hu, L. L. (2020). ERK/MAPK signalling pathway and tumorigenesis. *Experimental and therapeutic medicine*, *19*(3), 1997-2007.

Hato, T., & Dagher, P. C. (2015). How the innate immune system senses trouble and causes trouble. *Clinical Journal of the American Society of Nephrology*, *10*(8), 1459-1469.

Hollister, L. E. (1971). Hunger and appetite after single doses of marihuana, alcohol, and dextroamphetamine. *Clinical Pharmacology & Therapeutics*, *12*(1), 44-49.

Huang, K. P. (1989). The mechanism of protein kinase C activation. *Trends in neurosciences*, *12*(11), 425-432.

Kalenderoglou, N., Macpherson, T., & Wright, K. L. (2017). Cannabidiol Reduces Leukemic Cell Size - But Is It Important?. *Frontiers in pharmacology*, *8*, 144. https://doi.org/10.3389/fphar.2017.00144

Kaplan, B. L., Springs, A. E., & Kaminski, N. E. (2008). The profile of immune modulation by cannabidiol (CBD) involves deregulation of nuclear factor of activated T cells (NFAT). *Biochemical pharmacology*, *76*(6), 726-737.

Kim, J., Choi, H., Kang, E. K., Ji, G. Y., Kim, Y., & Choi, I. S. (2021). In Vitro Studies on Therapeutic Effects of Cannabidiol in Neural Cells: Neurons, Glia, and Neural Stem Cells. *Molecules*, *26*(19), 6077.

Kraner, S. D., & Norris, C. M. (2018). Astrocyte activation and the Calcineurin/NFAT pathway in cerebrovascular disease. *Frontiers in aging neuroscience*, *10*, 287.

Kumar, A., & Darreh-Shori, T. (2017). DMSO: A Mixed-Competitive Inhibitor of Human Acetylcholinesterase. ACS chemical neuroscience, 8(12), 2618–2625. https://doi.org/10.1021/acschemneuro.7b00344

Lai, J. H., Horvath, G., Subleski, J., Bruder, J., Ghosh, P., & Tan, T. H. (1995). RelA is a potent transcriptional activator of the CD28 response element within the interleukin 2 promoter. *Molecular and cellular biology*, *15*(8), 4260–4271. https://doi.org/10.1128/mcb.15.8.4260

Li, Y., Li, X. C., Zheng, X. X., Wells, A. D., Turka, L. A., & Strom, T. B. (1999). Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nature medicine*, *5*(11), 1298-1302.

Macián, F., López-Rodríguez, C., & Rao, A. (2001). Partners in transcription: NFAT and AP-1. *Oncogene*, *20*(19), 2476-2489

Mangal, N., Erridge, S., Habib, N., Sadanandam, A., Reebye, V., & Sodergren, M. H. (2021). Cannabinoids in the landscape of cancer. *Journal of Cancer Research and Clinical Oncology*, *147*(9), 2507–2534.

Margolis, B., & Skolnik, E. Y. (1994). Activation of Ras by receptor tyrosine kinases. *Journal of the American Society of Nephrology*, *5*(6), 1288-1299.

Mashimo, M., Iwasaki, Y., Inoue, S., Saito, S., Kawashima, K., & Fujii, T. (2017). Acetylcholine released from T cells regulates intracellular Ca2+, IL-2 secretion and T cell proliferation through nicotinic acetylcholine receptor. Life sciences, 172, 13–18. https://doi.org/10.1016/j.lfs.2016.12.015

Medzhitov, R., & Janeway Jr, C. A. (1998, October). Innate immune recognition and control of adaptive immune responses. In *Seminars in immunology* (Vol. 10, No. 5, pp. 351-353). Academic Press.

Meng, J., Su, R., Wang, L., Yuan, B., & Li, L. (2021). Inhibitory effect and mechanism of action (MOA) of hirsutine on the proliferation of T-cell leukemia Jurkat clone E6-1 cells. *PeerJ*, *9*, e10692.

Milian, L., Mata, M., Alcacer, J., Oliver, M., Sancho-Tello, M., Martín de Llano, J. J., ... & Carda, C. (2020). Cannabinoid receptor expression in non-small cell lung cancer. Effectiveness of tetrahydrocannabinol and cannabidiol inhibiting cell proliferation and epithelial-mesenchymal transition in vitro. *PLoS One*, *15*(2), e0228909.

Nguyen, S. T., Nguyen, H. T. L., & Truong, K. D. (2020). Comparative cytotoxic effects of methanol, ethanol and DMSO on human cancer cell lines. *Biomedical Research and Therapy*, *7*(7), 3855-3859.

Olivas-Aguirre, M., Torres-López, L., Valle-Reyes, J. S., Hernández-Cruz, A., Pottosin, I., & Dobrovinskaya, O. (2019). Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia. *Cell death & disease*, *10*(10), 1-19.

Paul, S., & Schaefer, B. C. (2013). A new look at T cell receptor signaling to nuclear factor-κB. *Trends in immunology*, *34*(6), 269-281.

Peace, M. R., Butler, K. E., Wolf, C. E., Poklis, J. L., & Poklis, A. (2016). Evaluation of Two Commercially Available Cannabidiol Formulations for Use in Electronic Cigarettes. Frontiers in pharmacology, 7, 279. https://doi.org/10.3389/fphar.2016.00279

Pigliasco, F., Barco, S., Dubois, S., Marchese, F., Striano, P., Lomonaco, T., ... & Cangemi, G. (2020). Cannabidiol determination on peripheral capillary blood using a microsampling method and ultra-high-performance liquid chromatography tandem mass spectrometry with on-line sample preparation. *Molecules*, *25*(16), 3608.

Rivers-Auty, J., & Ashton, J. (2013). Vehicles for Lipophilic Drugs: Implications for Experimental Design, Neuroprotection, and Drug Discovery. *Current Neurovascular Research*, *10(4),* 356-360

Ross, S. H., & Cantrell, D. A. (2018). Signaling and Function of Interleukin-2 in T Lymphocytes. *Annual review of immunology*, *36*, 411–433

Scott, C., Hall, S., Zhou, J., & Lehmann, C. (2023). Cannabinoids and the Endocannabinoid System in Early SARS-CoV-2 Infection and Long COVID-19—A Scoping Review. *Journal of Clinical Medicine*, *13*(1), 227.

Shangguan, F., Zhou, H., Ma, N., Wu, S., Huang, H., Jin, G., & Lan, L. (2021). A novel mechanism of cannabidiol in suppressing hepatocellular carcinoma by inducing GSDME dependent pyroptosis. *Frontiers in cell and developmental biology*, 1785.

Shan, X., Czar, M. J., Bunnell, S. C., Liu, P., Liu, Y., Schwartzberg, P. L., & Wange, R. L. (2000). Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Molecular and cellular biology*, *20*(18), 6945-6957.

Sharma, P., Murthy, P., & Bharath, M. S. (2012). Chemistry, metabolism, and toxicology of cannabis: clinical implications. *Iranian journal of psychiatry*, *7*(4), 149-156.

Sholler, D. J., Schoene, L., & Spindle, T. R. (2020). Therapeutic efficacy of cannabidiol (CBD): a review of the evidence from clinical trials and human laboratory studies. *Current addiction reports*, *7*(3), 405-412.

Shrivastava, A., Kuzontkoski, P. M., Groopman, J. E., & Prasad, A. (2011). Cannabidiol Induces Programmed Cell Death in Breast Cancer Cells by Coordinating the Cross-talk between Apoptosis and AutophagyCBD Induces Programmed Cell Death in Breast Cancer Cells. *Molecular cancer therapeutics*, *10*(7), 1161-1172.

Smith-Garvin, J. E., Koretzky, G. A., & Jordan, M. S. (2009). T cell activation. *Annual review of immunology*, *27*, 591-619.

Sourisseau, M., Sol-Foulon, N., Porrot, F., Blanchet, F., & Schwartz, O. (2007). Inefficient human immunodeficiency virus replication in mobile lymphocytes. *Journal of virology*, *81*(2), 1000-1012.

Steelman, Z. A., Tolstykh, G. P., Estlack, L. E., Roth, C. C., & Ibey, B. L. (2015, March). The role of PIP2 and the IP3/DAG pathway in intracellular calcium release and cell survival during nanosecond electric pulse exposures. In *Energy-based Treatment of Tissue and Assessment VIII* (Vol. 9326, p. 932611). International Society for Optics and Photonics.

Steffens, D. L., & Williams, J. G. (2007). Efficient site-directed saturation mutagenesis using degenerate oligonucleotides. *Journal of biomolecular techniques: JBT*, *18*(3), 147.

Sun, S., Hu, F., Wu, J., & Zhang, S. (2017). Cannabidiol attenuates OGD/R-induced damage by enhancing mitochondrial bioenergetics and modulating glucose metabolism via pentose-phosphate pathway in hippocampal neurons. *Redox biology*, *11*, 577-585

Sunda, F., & Arowolo, A. (2020). A molecular basis for the anti-inflammatory and anti-fibrosis properties of cannabidiol. *The FASEB Journal*, *34*(11), 14083-14092.

Timm, M., Saaby, L., Moesby, L., & Hansen, E. W. (2013). Considerations regarding use of solvents in in vitro cell based assays. *Cytotechnology*, *65*(5), 887-894.

Turner, S., Barker, V. D., & Adams, A. A. (2021). Effects of cannabidiol on the in vitro lymphocyte pro-inflammatory cytokine production of senior horses. *Journal of Equine Veterinary Science*, 103668.

Wang, H., Kadlecek, T. A., Au-Yeung, B. B., Goodfellow, H. E. S., Hsu, L. Y., Freedman, T. S., & Weiss, A. (2010). ZAP-70: an essential kinase in T-cell signaling. *Cold Spring Harbor perspectives in biology*, *2*(5), a002279.

Wieckiewicz, G., Stokłosa, I., Stokłosa, M., Gorczyca, P., & Pudlo, R. (2022). Cannabidiol (CBD) in the self-treatment of depression-exploratory study and a new phenomenon of concern for psychiatrists. *Frontiers in Psychiatry*, *13*, 461.

Wieczorek, M., Abualrous, E. T., Sticht, J., Álvaro-Benito, M., Stolzenberg, S., Noé, F., & Freund, C. (2017). Major histocompatibility complex (MHC) class I and MHC class II proteins: conformational plasticity in antigen presentation. *Frontiers in immunology*, *8*, 292.

Williams, B. L., Irvin, B. J., Sutor, S. L., Chini, C. C., Yacyshyn, E., Bubeck Wardenburg, J., Dalton, M., Chan, A. C., & Abraham, R. T. (1999). Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-gamma1 and Ras activation. *The EMBO journal*, *18*(7), 1832–1844.

Yiemwattana, I., Ngoenkam, J., Paensuwan, P., Kriangkrai, R., Chuenjitkuntaworn, B., & Pongcharoen, S. (2012). Essential role of the adaptor protein Nck1 in Jurkat T cell activation and function. *Clinical & Experimental Immunology*, *167*(1), 99-107

Zappacosta, B., Persichilli, S., De Sole, P., Mordente, A., & Giardina, B. (1999). Effect of smoking one cigarette on antioxidant metabolites in the saliva of healthy smokers. *Archives of oral biology*, *44*(6), 485-488.

Zhang, X., Qin, Y., Pan, Z., Li, M., Liu, X., Chen, X., ... & Li, D. (2019). Cannabidiol induces cell cycle arrest and cell apoptosis in human gastric cancer SGC-7901 cells. *Biomolecules*, *9*(8), 302.