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Are Natural Killer Cells Novel Mediators of The Effects of Cannabidiol on Mental Health, Sleep Quantity, Sleep Quality, And Immune Function?

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ARE NATURAL KILLER CELLS NOVEL MEDIATORS OF THE EFFECTS OF CANNABIDIOL ON MENTAL HEALTH, SLEEP QUANTITY, SLEEP QUALITY, AND IMMUNE FUNCTION?

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ABSTRACT

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Cannabidiol (CBD) has increased in popularity since the United States Farm Bill legalized hemp production in 2018 and is now projected to become a $24.4 billion dollar industry by 2025. Since its legalization, CBD is thought to be an antiepileptic, anxiolytic, and antipsychotic, as well as an agent to improve mental health, quality of life, aspects of sleep and immune function. However, many of these claims lack scientific evidence. The purpose of this randomized, double blind, placebo-controlled trial was to determine the effects of an 8-week CBD intervention on measures of mental health, sleep quantity, sleep quality, and natural killer cell (NKC) quantity and function. Researchers hypothesized that following an 8-week CBD intervention, participants consuming CBD will experience improvements in measures of mental health, increased sleep quantity and quality, and will have an increased percentage of NKC in peripheral blood, as well as decreases in NKC function determined by a measure of K562 cell viability. Physically active men and women (18-45y) were randomly assigned to placebo (CN, n=12) or CBD (CB, n=15) groups. Participants consumed a capsulized control; coconut derived medium chain triglycerides (MCT; 225mg/day) or CBD (50mg/day with 175mg of MCT) daily for 8 weeks. Before and after the intervention period, participants completed measures of mental health, sleep analysis (FITBIT), body size, body composition (BodPod), a peripheral blood draw, anaerobic fitness (Wingate), aerobic fitness (VO2max), 1 repetition strength testing (1RM).

Peripheral blood mononuclear cells (PBMC) were extracted from peripheral blood to determine immune cell population percentages, and natural killer cell (NKC) cytotoxicity. Immune population percentage measures involved, staining the cells with anti-CD3 and anti-CD56 for the determination of NKC (CD3-/CD56+), T cells (CD3+/CD56-) and natural killer T cells (NKT; CD3+/CD56+). NKC cytotoxicity analysis involved co-incubated of PBMC with K562 leukemia cells for 4 hours at ratios of 1:1, 5:1, 10:1, and 20:1 effector: target cell (E:T). K562 cell viability was determined using median fluorescence intensity using a flow cytometer. Data are presented as mean ± standard deviation with significance set at α=0.05. An independent-samples T-test was ran on all outcome measures at the pre intervention time point to ensure homogeneity between groups, and a 2 (group) x 2 (time) analysis of variance (ANOVA) was used to identify any interactions or main effects that occurred throughout the intervention. At the pre intervention time points, there were no significant differences between groups with respect to participant anthropometrics or exercise performance measures (age: 26.1 ± 6.2y; height: 169 ± 8.8cm; weight: 71.6 ± 12.8kg; lean body mass: 55.8 ± 11.3kg; body fat: 21.8 ± 8.3%; Peak Power: 647.8 ± 169.9W; Mean Power: 459.1 ± 121.1W; Anaerobic Fatigue: 56.9 ±7.2%; VO_2Peak: 45.0 ± 7.6ml/kg/min; 1RM Squat: 88.2 ± 31.4kg; 1RM Bench:61.43 ± 28.44kg); however, VO_2Peak significantly decreased after 8-weeks regardless of intervention group (p=0.038; Pre: 45.05 ± 7.61 Post: 43.75 ± 7.34ml/kg/min). There were no significant differences pre to post intervention between groups in measures of mental health (QOL; Pre: 9.49 ± 1.82 Post:19.81 ± 1.42; PFS; Pre: 3.22 ± 1.58 Post:2.74 ± 1.58; BDI; Pre: 4.56 ± 3.83 Post: 4.44 ± 3.72; GAD-7; Pre: 6.3 ± 6.0 Post: 4.9 ± 4.3), sleep quantity (TST; Pre: 386.75 ± 67.69 Post: 388.92 ± 57.89min) or sleep quality (WE; Pre: 26.44 ± 7.53 Post: 26.16 ± 7.70; SE; Pre: 88.41 ± 1.46 Post: 88.02 ± 1.93%). Furthermore, the fraction of NKC within $1x10^6$ peripheral blood mononuclear cells remained

unchanged (Pre: 8.44 ± 5.34% Post: 8.79 ± 4.12%), following the intervention period. Finally, there were no differences in K562 cell viability assessed through mean and median fluorescence intensity of Calcein-AM, following an 8-week CBD intervention. Eight weeks of CBD (50mg/day) did not alter measures of mental health, sleep quantity or quality, NKC percentage in peripheral blood or NKC function. This suggests that CBD may not alter mental health, sleep quantity, sleep quality, NKC percentage in peripheral blood or NKC cytolytic function.

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INTRODUCTION

Inflammation acts to elicit healing following varying types of damage. This healing process is triggered through a network of chemical signals aimed at recruiting varying cell types, such as leukocytes, to the damaged area to begin the repair process (Murphy & Weaver, 2016). These repair mechanisms can be triggered through a variety of stimuli such as viral, parasitic, or bacterial infections as well as more acute instances of muscle damage from exercise and injury (Aoi et al., 2004).

The immune system is divided into two parts, the innate and the adaptive immune systems. The innate immune system is more commonly associated with acute responses and often observed in viral and/or an inflammatory response. Adaptive immunity is involved in immunological memory and long-term immunity (Murphy & Weaver, 2016). During innate immune responses, varying types of immune cells are recruited to the damaged site and release additional signaling factors which recruit additional immune cells to begin the repair process. During this period of cell infiltration, redness, swelling, heat, and pain can occur at the injury site. The first immune cells recruited to the inflamed site under normal repair conditions will signal for apoptosis and eventually be phagocytosed by macrophages once their function is complete. These macrophages will then phenotypically change to aid in the cessation of the inflammatory process and release anti-inflammatory cytokines, including transforming growth factor-beta (TGF-ß) and interleukin-10 (IL-10) (Medzhitov & Janeway, 2000). If the inflammatory stimulus is not removed or the initially recruited cells are not phagocytized, the

inflammatory stimulus will remain, resulting in a state of chronic inflammation or autoimmunity, in which additional lymphocytes, such as T-lymphocytes, may be further activated. Acutely, this inflammatory process is an extremely beneficial mechanism which allows the body to repair and return to homeostasis. However, if the inflammatory stimulus remains, and chronic inflammation arises, additional health complications can occur.

Chronic inflammation is associated with negative health conditions such as obesity (Monteiro & Azevedo, 2010), metabolic syndrome (Monteiro & Azevedo, 2010), cancer (Coussens & Werb, 2002), cardiovascular disease (Gleeson, 2007), diabetes (Monteiro & Azevedo, 2010), and other chronic diseases. Acute inflammation is associated with increases in tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). Interleukin-6 has a multitude of functions in the human body and is involved in the activation and stimulation of diverse lymphocytes, neural survival, and the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Czarkowska-Paczek et al., 2005). More importantly, IL-6 as well as TNF-α stimulate acute phase responses, including release C-reactive protein (CRP), in the liver (Czarkowska-Paczek et al., 2005). Acute increases in IL-6 and TNF-α are associated with increases in the time spent in non-rapid eye movement (NREM) sleep, and more specifically slow wave sleep (SWS), which has restorative properties (Jewett & Krueger, 2012; Simpson & Dinges, 2007; Ware et al., 2010). An acute, transient increase in NREM sleep may be considered a beneficial outcome when SWS is increased, but in more advanced stages of inflammation, such as chronic bouts of inflammation, the sleep promoting effects of these inflammatory markers are reduced and are associated with increased wakefulness and perturbations in sleep architecture (Krueger & Takahashi, 1997; Pollmächer et al., n.d.; Spath-Schwalbe et al., 1998; Takahashi et al., 1981). The effects of acute and chronic inflammation follow a similar pattern and have

beneficial as well as negative effects on mental health status such as depression (Dantzer et al., 2008; Kivimäki et al., 2014; Raison & Miller, 2013), anxiety (Anderson et al., 2012), fatigue (Valentine et al., 2011), and quality of life (Nowakowski, 2014). Overall, acute, transient bouts of inflammation are associated with similar patterns of improvement in sleep and mental health status, while chronic inflammation is linked to the development of negative outcomes related to mental health, obesity, metabolic syndrome, and other diseases such as cancer (Anderson et al., 2012; Coussens & Werb, 2002; Dantzer et al., 2008; Gleeson, 2007; Kivimäki et al., 2014; Monteiro & Azevedo, 2010; Nowakowski, 2014; Raison & Miller, 2013; Valentine et al., 2011).

Chronic inflammation poses negative health risks. In fact, in both disease and healthy states, chronic inflammation can be a strong predictor of disability and mortality (Beavers et al., 2010). Despite this relationship, very few long-term, sustainable interventions decrease chronic inflammation (Beavers et al., 2010). Statins and angiotensin converting enzyme inhibitors (ACE) interventions reduce inflammatory profiles through the reduction of CRP, but these interventions have not been approved for long-term reduction of inflammation (Beavers et al., 2010). Other dietary and physical activity-related interventions may decrease chronic inflammation and further optimize the immune response (Beavers et al., 2010). An acute exercise bout significantly and transiently increases the biomarkers of inflammation. When regular exercise is completed over a longer time frame, the chronic, pulsatile inflammatory stimulus of an exercise bout acts to downregulate many of the inflammatory biomarkers including CRP (Abramson & Vaccarino, 2002), thereby decreasing chronic inflammation and disease risk. Additional benefits of chronic exercise are evident in a variety of immune cell populations, including natural killer cells (NKC). Natural killer cells are a subset of lymphocytes which act in both innate and adaptive immune responses (Cerwenka & Lanier, 2016; Mackinnon, 1989). These cells are most well-known for

their cytotoxic actions on both tumor and virally infected cells. The cytotoxic ability and population number of NKC transiently increases following acute exercise and increases by as much as 55% after chronic exercise training (Nieman et al., 1990). This is a notable finding given that an increase in NKC population and cytotoxic ability is beneficial to overall health and acts in the prevention of illnesses and invading pathogens as well as in the prevention of tumor growth.

Although many other interventions aimed at controlling chronic inflammation and enhancing NKC number and activity have been explored, relatively few programs, including exercise, have been administered without significant barriers. Cannabidiol (CBD), a non-psychoactive hemp derivative, is an attractive therapeutic target. Cannabidiol, is most supported by the scientific community as an antiepileptic (Stockings et al., 2018), anxiolytic (Zuardi et al., 2006), and antipsychotic (McGuire et al., 2018). Cannabidiol may also improve aspects of sleep (Babson et al., 2017), quality of life (McGuire et al., 2018), and mental health including depression, anxiety, fatigue, as well as overall physical health (Burstein, 2015a), but more research is needed to fully elucidate the clinical usefulness of CBD in humans.

Cannabidiol is also associated with anti-inflammatory and neuroprotective actions in the human body (Burstein, 2015a), which may have valuable applications in both healthy and diseased populations. Murine models demonstrate that CBD administration at varying doses increases NKC quantity, but have failed to adequately address their function (B. Ignatowska-Jankowska et al., 2009; Kishimoto et al., 2005). Additionally, CBD administration in a murine model is associated with significant reductions in IL-6 and TNF-α and is mediated through the cannabinoid 2 receptor (CB2R). Cannabidiol works through cannabinoid receptor pathways, specifically as an exogenous agonist binding to CB2R located in the periphery of the human

body. Immune cells contain CB2R at varying levels of expression, with NKC, B- lymphocytes, and monocytes possessing the highest expression of CB2R on the cell surface (Graham et al., 2010). Cannabidiol increases the number of NKC in adult male Wistar rats following 14 days of CBD (2.5mg/kg or 5mg/kg) (B. Ignatowska-Jankowska et al., 2009) and CBD has more recently been linked to improvements in stimulated NKC cytotoxic activity (Haustein et al., 2014).

In summary, inflammation is beneficial to the healing process and returning the body to homeostasis, but in excessive or chronic situations, inflammation can be detrimental and even deadly. Chronic inflammation can also lead to decrements in sleep quality and architecture, as well as increase depression, anxiety, fatigue, and decrease quality of life, and health status.

Purpose

The overall purpose of this investigation was to determine the effects of an 8-week CBD intervention on measures mental health, sleep quantity, and sleep quality. In addition, this study explored the potential CBD mechanism of action with a focus on NKC number and function. The specific aims for this study were:

Aims

A1 Determine whether measures of mental health are altered after 8 weeks of CBD intervention.

A2 Determine whether sleep quantity and sleep quality are altered after 8 weeks of CBD intervention.

A3 Determine whether NKC quantity and function are altered after 8 weeks of CBD intervention.

Hypothesis

H1 Following an 8-week CBD intervention, participant measures of mental health will be increased when compared to control. Participants consuming CBD will experience a decrease in measures of fatigue, depression, and anxiety defined by lower scores on the Piper Fatigue Scale, Beck Depression Inventory, and General Anxiety Disorders-7 scale, respectively. Furthermore, participants consuming

CBD will have an increased measure of quality of life defined as a higher score on the Ferrans and Powers Quality of Life Index.

H2 Following an 8-week CBD intervention, participants consuming CBD will have increased sleep quantity determined through an increase in total sleep time (TST) when compared to controls. Furthermore, participants consuming CBD will have increased sleep quality determined through a decrease in number of wake episodes (WE), and an increase in sleep efficiency (SE).

H3 Following an 8-week CBD intervention, participant NKC quantity will increase when compared to control. More specifically, participant NKC quantity in peripheral blood will increase when compared to control after an 8-week CBD intervention. Participants consuming CBD will also have an increased percentage of NKC in peripheral blood as determined through an increase in the percentage of cells expressing CD56 while lacking CD3.Additionally, participants consuming CBD will have increased K562 cell viability determined through an increased median fluorescence intensity in the BL1 laser wavelength (Calcein-AM).

CHAPTER II

REVIEW OF LITERATURE

Cannabidiol

While the cannabinoid delta 9-tetrahydrocannabinol (THC) has been scrutinized for the past 40 years due to its label as a schedule one drug, interest in a non-psychotropic cannabinoid, CBD has been exponentially growing since the United States Farm Bill legalized hemp production in 2018. In fact, the CBD industry is expected to generate more than $24.4 billion dollars in annual revenue by 2025 (Group, 2019). Cannabidiol, unlike THC, lacks the psychotropic effects and is not associated with feelings of euphoria or a “high” (Millar et al., 2018). However, CBD affects the central nervous system (CNS) and may be considered psychoactive, but not psychotropic. In other words, CBD can act upon the CNS, but the compound fails to cause euphoric effects (Nichols & Kaplan, 2020). The lack of CBD-induced psychotropic effects is linked to the chemical structure of CBD. Both CBD and THC have some structural overlap; however, THC has a relatively planar confirmation whereas CBD forms a right angle due to the position of its two terpene rings (Burstein, 2015a). The conformation of CBD inhibits its ability to bind to the cannabinoid 1 receptor (CB1R), thereby preventing psychotropic activity. Consequently, CBD is an attractive target for pharmacological uses and other treatments although relatively little is known about CBD pharmacokinetics. Other CBD-related literature is not standardized and makes determining agreed upon actions very difficult.

The potency of CBD is highly dependent on factors such as biological sex, whether the user is in a fed or fasted state, as well as the route of administration (Millar et al., 2018). For

example, a previous investigation administered oral soft gels containing 10 mg THC and 5.4mg CBD to both male and female participants in a fed or fasted state. In the fed state, CBD reached a max concentration (C_{max}) of 1.13ng/ml versus 0.93ng/ml in a fasted state (Nadulski et al., 2005). This study also highlights differences in the way each sex responded to CBD. More specifically, C_{max} of CBD and the time to reach maximal CBD concentrations (T_{max}) were higher in females than males in all CBD supplementation groups (Nadulski et al., 2005). In addition to alterations in the C_{max} of CBD in fed and fasted states as well as potential sex-related differences, the route of CBD administration is linked to significant alterations in C_{max} and T_{max}. Intravenous administration of CBD is the fastest and most potent route of administration. For example, a 20mg dose of CBD administered intravenously results in a C_{max} of 686ng/ml 3 minutes following CBD administration and decreases to a CBD concentration of 48ng/ml after 1h. The pulmonary route of ingestion has a similar speed in reaching C_{max} albeit, the Cmax value is often much lower when compared to intravenous administration (Hložek et al., 2017; Ohlsson et al., 1986). Most CBD users are not consuming CBD intravenously, and are taking CBD as oral-mucosal drops, sprays, oral ingestion through pills, or pulmonary routes such as smoking or inhalation (Millar et al., 2018). When combined with differences in recommended CBD dosages, all of these methods produce vastly different plasma CBD concentrations. For example, oral-mucosal spray at a dose of 10-20mg resulted in Cmax range between 2.5-3.3ng/ml between 1.6- 4.2h following CBD administration (Atsmon et al., 2018; Karschner et al., 2011). When the same dose (10mg) is ingested orally, maximal CBD concentrations averaged 2.47ng/ml at the 1.27h post ingestion time point (Guy & Robson, 2014). Together, these results suggest that oral ingestion and oral-mucosal ingestion of CBD have similar C_{max} and T_{max}. However, these circulating concentrations of CBD are significantly different from those obtained after

intravenous administration or pulmonary inhalation (Millar et al., 2018). This variation in C_{max} and T_{max} may be due to the low bioavailability of orally administered CBD. In fact, the oral bioavailability of CBD is relatively low, approximately 13-19% (Mechoulam & Hanuš, 2002) and is due to the excretion of high concentrations of CBD metabolites by the kidneys through the first pass effect of drug metabolism (Huestis, 2007). However, this bioavailability obstacle can be overcome through the use of lipid CBD carriers, administration in the fed vs a fasted state, and optimizing the dosage used during oral administration (Millar et al., 2018).

Cannabidiol can act as an anxiolytic, antipsychotic, anti-inflammatory, and antiemetic agent (Mechoulam & Hanuš, 2002). In fact, CBD may invoke anxiolytic effects which were originally thought to be solely caused by THC (Zuardi et al., 1982). The anxiolytic effects of CBD are demonstrated in a study where 8 healthy college-aged men and women were provided with CBD, THC, CBD+THC, and placebo. Following administration of placebo, no anxiety symptoms were measured (Zuardi et al., 1982). Following the administration of THC alone, there was a significant increase in anxiety scores from baseline ($p<0.05$), which coincides with previous investigations (Karniol et al., 1974; Malit et al., 1975; Zuardi et al., 1982). When CBD+THC were administered together at a dose of 1mg/kg CBD:0.5mg/kg THC, the addition of CBD diminished the feelings of anxiety from THC alone (Karniol et al., 1974; Zuardi et al., 1982). Further evidence of CBD acting as an anxiolytic compound was shown again by the same investigators when CBD (300mg) was administered in comparison to the pharmaceutical anxiolytic drugs Diazepam (10mg), Ipsapirone (5mg), and placebo. Following administration of the 4 treatments, participants, who were healthy, college-aged men and women, underwent a simulated public speaking test and all treatments, when compared to the placebo group, resulted

in significantly lower anxiety scores asassessed through visual analogue mood scale (VAMS; CBD, p=0.017; Diazepam, p=0.042; Ipsapirone, p= 0.037) (Zuardi, Cosme, et al., 1993).

The anxiolytic actions of CBD were initially thought to act through CB1R and CB2R; however, CBD has a low affinity for these receptors. In fact, at low concentrations, CBD can antagonize these receptors resulting in contradictory actions when compared to the mood-altering actions brought upon by THC. It is important to note that not all of the physiological effects of THC are blocked or altered by co-administration of THC and CBD. For example, tachycardia induced by THC administration alone was unaltered following co-administration of CBD (Zuardi et al., 1982). Cannabidiol administration alone reduces psychosis; however, its ability to reduce the psychotic episodes brought upon by THC must further be elucidated (Zuardi, 2008). First demonstrated in rats, doses of CBD (15-480mg/kg) reduce the stereotyped actions of psychosis (sniffing and biting) induced by apomorphine and these findings are similar in effectiveness to the clinical anti-psychotic drug haloperidol (Zuardi et al., 1991). In humans, ketamine (0.26mg/kg) induced both positive and negative effects of schizophrenia. However, following CBD (600mg) administration, participant Clinician Administered Dissociative States Scale (CADSS) scores decreased, demonstrating the anti-psychotic effects of CBD (Bosi et al., 2003). These results coincide with previous literature, demonstrating the effects of CBD administration in reducing psychotic episodes and side effects in humans and animal models (Zuardi et al., 1991, 1995).

Cannabidiol has anti-inflammatory properties as well (Zuardi, 2008). An *in vitro* investigation where human PBMC were extracted from peripheral blood and incubated with either THC or CBD, found that lower doses of CBD resulted in PBMC secretion of the inflammatory cytokine IL-1. In the same study, higher doses of CBD completely suppressed the

secretion of IL-1 (B. Watzl et al., 1991). Furthermore, the same investigation administered supraphysiological CBD at 5.0 ug/mlwhich completely suppressed TNF and interferon (INF) secretion (B. Watzl et al., 1991). Cannabidiol administration also reduces prostaglandin E2, and nitric oxide production through both inhibitory and downregulatory actions on cyclooxygenase (Burstein, 2015a); however, there were no alterations in nuclear factor-kappa B (NF-kB) concentrations which provides some clarity as to the cellular mechanisms related to CBD action. Further discussion on CBD and the inflammatory response will be discussed later in this chapter.

The initial discovery of the antiemetic effects of the cannabinoid system was with Dronabinol and Nabilone, which are Food and Drug Administration (FDA) approved versions of THC. Unlike THC, CBD does not elicit antiemetic effects through the CB1R but instead works as a ligand for the serotonin receptor antagonist 5-HT_{1A}. Upon CBD binding to the 5-HT_{1A} receptor, serotonin release is reduced, thereby reducing the potential to trigger emesis (Mersiades et al., 2018). In fact, some cannabinoid compounds are approved by the FDA and are used to prevent vomiting, a common side effect of chemotherapy. To summarize, CBD has anxiolytic, antipsychotic, anti-inflammatory, and antiemetic actions. However, the molecular pathways associated with these outcomes remain unexplored.

Cannabidiol activates a number of different molecular pathways. For example, CBD acts as an antagonist of G protein-coupled receptor 55 (GPR55), and an agonist in the transient receptor protein of vanilloid types 1 and 2 (TRPV1; TRPV2) (Iffland & Grotenhermen, 2017; Izzo et al., 2009). G protein-coupled receptor 55 is an orphan G-protein coupled receptor and belongs to the rhodopsin-like G-protein receptor family. Similar to many of the receptors involved in cannabinoid signaling, GPR55 possesses diverse biological functions based on its location. G protein-coupled receptor 55 is upstream to the molecular signaling pathways

including extracellular-signal-regulated kinase (ERK), p38 mitogen activated protein kinase (MAPK) and induces calcium (Ca^{2+}) release, which can lead to stimulation of some or all of the transcription factors NF-kB, nuclear factor of activated T-cells (NFAT), and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (Pellati et al., 2018). Cannabidiol acts as an antagonist with the GRP55 and has a higher affinity towards this receptor when compared to CB1R and CB2R (Kapur et al., 2009; Ryberg et al., 2007). When CBD is used to treat breast cancer cells in culture, the compound induces cell death through endoplasmic reticulum stress as well as inhibition of protein kinase B (AKT) and mechanistic target of rapamycin (mTOR) (Shrivastava et al., 2011). The results of CBD-induced cancer cell death will be further discussed later in this chapter. The GPR55 is not the only non-cannabinoid receptor responsive to CBD. Transient receptor protein of vanilloid types 1 and 2, which play a role in thermoregulation, temperature perception, and cellular proliferation, also bind to CBD (Pellati et al., 2018). The TRP receptor family promotes Ca^{2+} entry into the cell and is expressed at high concentrations in the CNS (Nilius et al., 2008). When activated by CBD, both TRPV1 and TRPV2 induce increases in intracellular Ca^{2+} concentrations (Ligresti et al., 2006). In a similar fashion to the GPR55, TRPV1 and TRPV2 play a role in cancer progression; however, their role has yet to be fully explained. This research effort is further confounded by the wide variation of TRPV1 and TRPV2 expression in each cancer cell type.

Endocannabinoid System

Cannabis sativa, an ancient plant discovered 2700 years ago, has been used for its medicinal and recreational qualities throughout history (Russo et al., 2008). It has been used for products ranging from textiles to medication; however, its phytochemical and psychotropic components have led to a renewed interest in the plant in recent decades. Approximately 140

different compounds including phytocannabinoids and terpenoids are found within *Cannabis sativa.* These compounds directly modulate various aspects of human physiology (Freeman et al., 2019; Izzo et al., 2009). Delta 9-tetrahydrocannabinol is the most widely investigated phytocannabinoid found within *Cannabis sativa*. In addition to THC, CBD, is well-known for its non-psychotropic medicinal properties and is used as a treatment for psychosis, anxiety, and movement disorders (Izzo et al., 2009). Both THC and CBD are also exogenous ligands of a more recently discovered physiological system known as the endocannabinoid system.

The endocannabinoid system encompasses more than the ability to bind exogenous phytocannabinoids, such as THC and CBD. The endocannabinoid system includes many endogenous ligands, including lipid derivates of arachidonic acid produced from various metabolic pathways (Kilaru & Chapma, 2020). The endocannabinoid system was first discovered when researchers were in search of the receptor responsible for psychotropic effects of THC. Initially discovered in slices of rat brain, CB1R was discovered through the activation of these novel receptors by a synthetic cannabinoid which inhibited adenylate cyclase (AC) and its downstream secondary messenger cAMP (Devane et al., 1988). The CB1R were further characterized through a translated sequence of cDNA from slices of rat cerebral cortex that produced a novel 473-amino acid (472 in humans) long G-protein coupled receptor (GPCR) which was later found to activate the G_i pathway (Matsuda et al., 1990). Upon initial discovery of CB1R in the neurons of the CNS and dorsal root ganglion (DRG), it was believed that the CB1R was only found in neural tissues (Anand et al., 2009). However, more recent research suggests that this belief is oversimplified and that the CB1R is found in other, non-neural tissues (Tokanovic et al., 2007). More specifically, an alternative splice variant consisting of 33 amino acids at the N-terminusof the CB1R is present in rat pancreas, muscle, liver, and adipose tissue

as well as the prostate gland (Kilaru & Chapma, 2020; Tokanovic et al., 2007). Following the discovery of CB1R, a second 360 amino acid GPCR was discovered. This receptor, CB2R, was originally discovered in the human promyelocytic cell line (HL60) cDNA library and has 44% homology with the CB1R. The affinity of CBD towards the discovered CB2R is similar to the affinity that THC has to CB1R (Munro et al., 1993).

Following the discovery of the CB1R and CB2R, it was hypothesized and later discovered that rats and humans produce endogenous ligands of these specific receptors (Devane et al., 1992; Evans et al., 1992). The first endogenous ligand discovered was arachidonoylethanolamide and was later named anandamide (AEA), which is a derivate of arachidonic acid originally isolated from porcine brain (Devane et al., 1992). Anandamide is catalyzed by N-acylphosphatidylethanolamine-specific phospholipase d-like hydrolase (NAPE-PLD) and degraded into arachidonic acid by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Cristino et al., 2020; Okamoto et al., 2004). Following its discovery, AEA is considered a potent agonist to CB1R and produces downstream effects similar to THC. These effects include inhibition of AC (Vogel et al., 1993), working through the G_i pathway (Howlett et al., 1986). Anandamide is also associated with inhibiting the N-type Ca^{2+} channels (Mackie et al., 1993; Mackie & Hille, 1992), opening rectifying K^+ channels (Mackie et al., 1995), and stimulating MAPK, c-Jun N-terminal kinase (JNK), and activator protein-1 transcription factor (AP-1) (Sugiura et al., 2002). Additionally, 2-arachadonylglycerol (2-AG), an analogue of arachidonic acid connected to a glycerol backbone, is an agonist of the CB1R with higher affinity towards this receptor than AEA (Mechoulam et al., 1995; Sugiura et al., 2002). Biosynthesis of 2-AG is catalyzed by diacylglycerol lipase α and β catalase (DAGLα and DAGL, respectively), and 2-AG is degraded by hydrolysis which is catalyzed by monoacylglycerol lipase (MAGL) (Bisogno et

al., 2003; Cristino et al., 2020; Dinh et al., 2002). Though the affinity of AEA and 2-AG differ, their physiological effects are relatively similar to one another when bound to the CB1R. However, the effects elicited by 2-AG on CB1R are more dramatic when compared to AEA. Consequently, some have suggested that 2-AG was the original endogenous ligand for CB1R (Sugiura et al., 1999). Furthermore, 2-AG has similar agonistic activity to CB2R, a receptor most commonly found in cells of hematopoietic origin.

In a similar fashion to the CB1R, CB2R were originally believed to be solely located in the periphery and in nerve endings with no presence in the CNS (Kilaru & Chapma, 2020). However, it was later discovered that the CB2R has 2 variants, the CB2Ra and CB2Rb, with the former expressed predominantly in the testes and lower brain regions associated with reward, and the latter expressed in the cells of hematopoietic origin (Liu et al., 2009). Cannabinoid 2 Receptor-b is activated by the same agonist of CB1R, which is 2-AG, at similar affinities; however, the effects differ between cell types (Derocq et al., 2000). Two-arachidonylglycerol is a potent ligand of CB2R and stimulates Ca^{2+} release in the HL-60 cell line. The release of Ca^{2+} is lost when HL-60 cells were pretreated with a CB2R antagonist (Sugiura et al., 2000). The release of Ca^{2+} through 2-AG works through the CB2R and not the CB1R, this is demonstrated when Ca^{2+} release still occurs when HL-60 cells were pretreated with a CB1R antagonist (Sugiura et al., 2000). Furthermore, 2-AG stimulation on HL-60 cells upregulates 9 different genes involved in cell differentiation, cytokine stimulation, and transcription, most of which work downstream of NF-kB (Derocq et al., 2000). It should be noted that although AEA, 2-AG, THC, and CBD interact with the CB1R and CB2R, the wide array of physiological outcomes evoked some degree of confusion due to the lack consistent ligand binding outcomes. This confusion led to the discovery of these ligands and their derivatives as they are interacting with additional

cannabinoid and orphan receptors involved in the endocannabinoid system such as the TRPV1, proliferator-activated receptor-α (PPARα), proliferator-activated receptor-γ (PPARγ), and the GPR55 (Cristino et al., 2020; Kilaru & Chapma, 2020).

Anandamide and other endocannabinoids have similar binding properties to the TRPV1 channel, which is also located in the CNS and periphery (Di Marzo, 2010; Hakimizadeh et al., 2012). Activation of the TRPV1 channel causes long-term depression (LTD) in neurons of various regions of the brain, which, in turn, regulates the strength of the neuronal synapse (Di Marzo, 2010). In addition to activation of the TRPV1 channel by AEA resulting in LTD, further TRPV1 activation results in alterations in nociception, thermoregulation, itching, and bladder function (Hakimizadeh et al., 2012; Moran et al., 2011). Along with TRPV1, the endocannabinoids act as a ligands for the nuclear receptor transcription factor superfamily PPAR, both α and γ (Cristino et al., 2020; Kilaru & Chapma, 2020).Administration of endocannabinoids in CB1R and CB2R knockout mouse models still resulted in the onset of the expected physiological responses, which suggests the involvement of different receptors (Howlett et al., 2002; Y. Sun et al., 2006). Specific activation of PPAR α and γ stimulate anti-inflammatory responses in various tissues. Endocannabinoid administration to PPARα knockout mice and rats produces significant alterations related to feeding behavior and increased lipolysis (Guzmán et al., 2004). Additionally, a reduction in inflammation occurred following administration of endocannabinoids in PPARα knockout mice (LoVerme et al., 2005). In addition to PPAR stimulation leading to anti-inflammatory responses, THC works with PPARγ in a concentration dependent manner leading to stimulation of adipocyte differentiation as well as vasorelaxation (O'Sullivan et al., 2005; Y. Sun et al., 2006). Therefore, it is clear that CB1R and CB2R, which were once believed to be the only receptors involved with the

endocannabinoid system, are now part of a growing list of potential targets for the endocannabinoids.

In summary, the longtime use of *Cannabis sativa* as a medicinal and recreational plant has mental and physical effects. Further exploration into the actions of THC and CBD paved the way to the discovery of the endogenous ligands AEA and 2-AG as well as their quasi-location specific receptors CB1R and CB2R. Furthermore, it is becoming clear that the endocannabinoid system is not limited to just the ligands and receptors discussed above as a plethora of new ligands and receptors are discovered every year.

Immunology

The human immune system is generally divided into two main sections, the innate immune system and the adaptive immune system. Both of these systems rely on leukocytes and lymphocytes that arise from precursor cells in the bone marrow as well as tissue specific cells that stem from the yolk sacks of fetal liver during development (Murphy & Weaver, 2016; Yanes et al., 2017). Cells of the innate immune system include leukocytes as well as various lymphocytes, whereas the cells of the adaptive immune system are predominantly of lymphocyte origin. The innate immune system is considered the first line of defense. In fact, the innate immune response occurs within seconds tominutes of antigen or immunogenic exposure (Medzhitov & Janeway, 2000; Murphy & Weaver, 2016). Leukocytes such as neutrophils, basophils eosinophils, dendritic cells and monocytes are some of the main cell types involved in the innate immune response. Additionally, some innate cells can be derived from lymphoid origin, such as B-1 cells, gamma delta-T cells ($\gamma\delta$-T cells), innate lymphoid cells 1-3 (ILC1-3), and NKC (Artis & Spits, 2015; Murphy & Weaver, 2016). The adaptive immune system, on the other hand, takes much longer to take action and requires days to weeks to become fully

activated following immunogenic exposure. This difference in activation time course between the innate and adaptive immune system is due to the antigen specific response involved in the adaptive immune system, compared to the non-specific antigen response of the innate immune system. Cells of the adaptive immune system are predominantly in the form of lymphocytes, including B-2 cells, and alpha beta T-cells (αβ T-Cell), and derived from common lymphoid progenitor cells (Murphy & Weaver, 2016; K. Suzuki et al., 2010). While various innate and adaptive immune cells can be separated into their respective systems, there is a lot of redundancy and an argument can be made for many innate cells to serve in the adaptive response and vice versa. Due to the complexity of the immune system, this document will provide more details related innate immunity and address NKC and their roles in the human body.

The innate immune system is associated with a non-specific immune response that occurs within seconds to minutes of antigen or pathogen exposure (Medzhitov & Janeway, 2000; Murphy & Weaver, 2016). The first line of defense in the innate immune system are the physical barriers of the human body. These barriers include mucosal linings of the gastrointestinal tract, respiratory tract, and other physical barriers. These barriers contain layers of epithelial tissue that are comprised of layers of live and dead cells as well as cellular and soluble defensive factors, including mucins, defensins, cathelicidins, histatins, lectins and commensal microbes (Goto & Kiyono, 2012; Murphy & Weaver, 2016). If these epithelial barriers are not penetrated, no immune response will occur. The complement system (C') is a mechanism of the innate immune system that contains proteins and innate antibodies that line the epithelial barriers of the human body. The complement system contains 30 different proteins that are produced by hepatocytes and macrophages in addition to natural antibodies produced by B-1 cells of the innate immune system (Murphy & Weaver, 2016; Sarma & Ward, 2011). Upon antigen or pathogen activation

of the C', the foreign compound is targeted for removal or destruction. These functions include neutralization which is the binding of complement proteins and antibodies to an antigen or pathogen to prevent further binding in the human body; agglutination which is the binding of a pathogen or antigen molecule by C' proteins and antibodies to form large complexes leading to the precipitation of the pathogen-protein complexes; pore formation, the binding of C' proteins leading the creation of pores in the pathogen membrane causing cellular disruption and death; and opsonization which is the binding of C' proteins and antibodies to mark and or signal to phagocytes to increase phagocytosis resulting in the removal of the pathogen. An additional innate immune response involving various cell types, in combination with C' activation, aids in the removal or degradation of an antigen (Murphy & Weaver, 2016; Sarma & Ward, 2011).

A major cell type involved in the innate immune response are the NKC. Natural killer cells are derived from common lymphoid progenitor cells, which are similar to T-cells and B-cells (Murphy & Weaver, 2016; J. C. Sun & Lanier, 2015). However, unlike T and B-cells, NKC lack the T-cell receptor (TCR), and B-cell receptor (BCR) which are vital in the recognition of specific antigen to activate the adaptive immune response (Murphy & Weaver, 2016). Unlike CD8-T cells, NKC do not need prior antigen exposure to elicit cytotoxicity effects (Abel et al., 2018). Even though NKC are categorized as cells of the innate immune system, NKC also share many properties of adaptive immune cells including immune cell education, selection during development, and receptor-antigen specificity, as well as the ability of NKC to undergo clonal expansion and to generate long lived memory cells (J. C. Sun & Lanier, 2015). Natural killer cells function by releasing cytokines, chemokines, and interferons, which aid in coordinating responses from other cells of the immune system. These actions provide aid in the downstream removal of an immunogenic substrates or virally infected cells. Additionally, NKC can produce a

cytotoxic killing effect similar to CD8-T cells. Natural killer cells can induce cytotoxicity in other cells by causing them to undergo apoptosis through the release of their cytotoxic granules containing perforin and granzyme (Abel et al., 2018; Murphy & Weaver, 2016; Trinchieri, 1989). Natural killer cells induce cytotoxic killing using 3 mechanisms. In one mechanism, NKC are activated via surface receptors that are germline coded, pattern recognition receptors (PRR; unlike CD8 T cells), which will act to release their cytotoxic granules (Murphy & Weaver, 2016). The second mechanism involves activation of NKC TNF-related apoptosis inducing ligand (TRAIL) receptors (Murphy & Weaver, 2016). Natural killer cells express TRAIL on their surface, which will interact with death receptor 4 and 5 (DR4; DR5) (Murphy & Weaver, 2016). Death receptor 4 and 5 are expressed by many other cell types (Özören & El-Deiry, 2003). Recognition of a target cell through TRAIL-DR4-5 binding causes downstream signaling to proenzyme caspase 8 which leads to apoptosis of a cell. The third mechanism is known as antibody dependent cellular cytotoxicity (ADCC). In this situation, NKCcytotoxic abilities will activate when an antibody-coated pathogen binds with their surface fragment crystallizable region (Fc) receptors (Murphy & Weaver, 2016).

Natural killer cells can utilize any or all of their 3 cytotoxic mechanisms. This action usually involves NKC stimulation by various cytokines. Interleukin-12 (IL-12), which is released by macrophages and dendritic cells during pathogen stimulation, can further activate NKC effector functioning (Murphy & Weaver, 2016; Vivier et al., 2008). Natural killer cells can also respond to type 1 interferons (INF-α/INF-β), which are released during a viral infection (Murphy & Weaver, 2016). This NKC mobilization can act to suppress the viral infection until the adaptive immune system can begin its effector functioning. In addition to IL-12 enhancement of NKC cytotoxic actions, macrophages will release Interleukin-18 (IL-18) that binds to NKC and

causes the release of interferon-gamma (INF-γ) acting upon macrophages and enhancing their capacity to kill pathogens (Murphy & Weaver, 2016). Interferon-gamma can also act on dendritic cell as well as CD4-T cells, which will differentiate into pro-inflammatory T helper 1 cells (TH1), which will produce additional INF-γ, thereby propagating the type 1 immune response. Natural killer cells also produce TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF), and chemokines such as CCL3-5 which act to recruit additional macrophages to the site of the immune response (Murphy & Weaver, 2016).

Natural killer cells are triggered to induce their effector functioning through a unique pathway of activation. Unlike most cell types, which become active upon the binding of one or two ligands to specific receptors, NKC work through the interplay between both activating and inhibitory receptors (Murphy & Weaver, 2016; C. Watzl & Long, 2010). Various cells of the body express dysregulated self-signals or stress- induced self-signals. Consequently, these cellular proteins are upregulated due to damage, infection, or other imbalances. Upon stimulation of the NKC activating receptors, the potential for NKC to perform their cytotoxic functions and release of INF-γ to induce an immune response will increase. However, NKC also need downregulation of their inhibitory receptors to perform their effector function. Natural killer cells express inhibitory receptors that will bind with self-cells which are represented by expression of major histocompatibility complex I (MHCI) on their cell surface. Major histocompatibility complex 1 molecules are used to present antigen to the adaptive immune cells and signal a problem. However, MHCI are recognized by NKC as self-cell markers resulting in inhibition of their effector functioning (Murphy & Weaver, 2016; C. Watzl & Long, 2010). Major histocompatibility complex 1 molecules are expressed on all cell types and are a marker of self to prevent autoimmunity. However, some pathogens will downregulate the expression of

MHCI to evade adaptive immune responses. This lack of MHCI expression in infected cells can still be recognized by NKC, signaling that the infected cell is missing self, ultimately resulting in the reduced NKC inhibitory functions (C. Watzl & Long, 2010). The interplay between activating and inhibitory receptor stimulation in NKC is the primary method of initiating downstream effector functions. There are 3 types of receptors on NKC and all have the potential to be activating or inhibitory (Murphy & Weaver, 2016; Pegram et al., 2011). The most common of these receptors are the killer cell immunoglobulin-like receptors (KIR) which have 2 isoforms, KIR-2D and KIR-3D which contain 2 to 3 immunoglobulin-like domains. Killer cell immunoglobulin-like receptor types are either inhibitory or stimulatory. Inhibitory KIR contain a long cytoplasmic tail with an immunoreceptor tyrosine-based inhibition motif (ITIM). When the inhibitory KIRs are activated, the tyrosine on the ITIM will become phosphorylated and interact with Src-homology region 2-containing protein tyrosine phosphatase-1 and 2 (SHP-1;SHP-2) (Murphy & Weaver, 2016; Pegram et al., 2011). These complexes will then localize near the cell membrane and remove phosphatases from other phosphorylated tyrosine, which will inhibit the activation signaling of other receptors. Conversely, stimulatory KIRs have two short cytoplasmic tails and interact with the protein DAP12, which contains an immunoreceptor tyrosine-based activation motif (ITAM) (Murphy & Weaver, 2016; Pegram et al., 2011).

When a ligand binds to DAP12, the ITAM becomes phosphorylated resulting in downstream signaling in the NKC, eventually leading to effector functioning (Murphy & Weaver, 2016; Pegram et al., 2011). The second receptor type in NKC are killer cell lectin like receptors (KLR). Similar to KIRs, KLRs also have inhibitory and stimulatory types of receptors which act in a similar downstream fashion as the KIR. This receptor is actually a heterodimer of 2 different C-type lectin receptors, NKG2 and CD94. The NKG2 receptor has multiple isoforms

which will function as either stimulatory or inhibitory to NKC (Murphy & Weaver, 2016; Pegram et al., 2011). Finally, the third receptor type present on the surface of NKC are natural cytotoxicity receptors (NCR) which include NKp30, NKp44, and NKp46. All receptors are members of the natural cytotoxicity KIR or KLR receptor families which will bind to signals from infected cells, malignantly transformed cells, or cells that have undergone physical or chemical damage (Murphy & Weaver, 2016; Pegram et al., 2011). The NCRs are activating receptors for NKCs, and each type of receptor can be activated by various ligands and work through downstream signaling. For instance, NKp30 and NKp46 signal downstream through the CD3 chain, and NKp44 signaling involves activating the ITAM on DAP12, which is similar to the KIRs (Murphy & Weaver, 2016; Pegram et al., 2011). Finally, a special NCR receptor is the homodimer of NKG2D. The NCR NKG2D binds to several MHCI molecules, MHCI polypeptide-related sequence A and B (MIC-A;MIC-B), and retinoic acid early transcript (RAET1), and are induced by various cellular stressors (Murphy & Weaver, 2016). These molecules have similar structures to the alpha1 and alpha2 domains in the MHCI structure. They are expressed during various forms of cellular stress and are recognized by the NKG2D homodimer. Once recognized, the NKG2D homodimer will signal though DAP10 to activate the PI-3K pathway and directly induce cytotoxicity in cells (Murphy & Weaver, 2016). Once the interplay between the inhibitory and stimulatory receptors of NKC reaches a threshold to inhibit or stimulate its function, NKC will proceed with either leaving the cell unharmed or initiate functions to cause cell death.

Natural killer cells can use their inhibitory receptors to bind to MHC molecules on the surface of cells. Once bound, NKC recognize the cell as self and inhibit the killing of the cell. Many virally infected cells will downregulate, degrade, or inhibit MHC molecule presentation

which will reduce the inhibitory signal on NKC and may potentially induce a larger activation signal. However, some viruses are destroyed even if they express MHC molecules through activating signals on the NKC. Specialized MHCI molecules act as ligands for activating and inhibition of NKC. As discussed above, MHC class I polypeptide-related sequence A and B (MIC-A and B, respectively) are classes of the MHCIb genes and are under different regulation when compared to MHCI. These proteins are recognized by the NKG2D receptor which is expressed on NKC and can signal for the activation of the NKC to kill MIC expressing targets. The NKG2D is categorized as an NKC activation receptor working through DAP10 and activation of phosphoinositide 3-kinase (PIk3) (Murphy & Weaver, 2016). In addition to MIC proteins, NKC are also stimulated for activation through the recognition of RAET1 with the NKG2D receptor. Cellular stress such as heat shock will upregulate MIC proteins and malignant transformation of cells will upregulate the RAET1 gene (Murphy & Weaver, 2016; Pegram et al., 2011). Human leukocyte antigen-E (HLA-E) complexes bind to the inhibitory receptor NKG2A:CD94 which is expressed on NKC. The HLA-E is an MHC Ib molecule which is activated on CD4 T cells and helps avoid NKC detection and destruction (Murphy & Weaver, 2016). KIRs can bind to MHC class Ia molecules such as HLA-a, b, and c. Killer cell immunoglobulin-like receptors on NKC binding to HLA-a, b, and c is recognized as a self-signal and inhibits NKC killing function. Major histocompatibility complex Ib molecules HLA-F and HLA-G can also inhibit NKC killing because even though HLA-F and HLA-G are class MHCIb proteins, they are not expressed in a cellular stress environment and are only expressed in a few locations in the body. For example, human leukocyte antigen-G is expressed on fetus derived placental cells that migrate into the uterine wall. Upon recognition by NKC, HLA-G will prevent NKC mediated killing through the activation of NKC inhibitory receptors (Murphy & Weaver,

2016; Pegram et al., 2011). Natural killer cell binding to HLA-G prevents killing of the placental cells and is recognized by an additional inhibitory receptor known as the leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) (Murphy & Weaver, 2016). Studies exploring new NKC receptor types and functions are currently underway.

Sleep

At the age of 70 years, most adults will have slept for approximately 20 to 25 years, but recently this number has been declining (Scheving, 1959). Although it is not clear as to how much sleep the average person should be getting in part due to intra-individual variability, sleep is an essential part of mammalian life. In mammals, the sleep cycle can be divided into 2 stages, rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep. During the aging process,our quality of sleep becomes diminished and older individuals often spend different amounts of time in different stages of sleep creating an age-dependent continuum. Neonates spend the greatest amount of time in REM sleep, and seniors spend the greatest amount of time lying awake or in a light sleep stage (Bliwise, 1993; Carskadon & Dement, 2011).

Researchers have attempted to understand sleep using the new technology of their time such as the electroencephalogram (EEG), electromyography (EMG), and electrooculography (EOG) (Carskadon & Dement, 2011). These devices are able to record eye movement, muscle activity, and brain activity, respectively, and record these actions on a revolving drum. These devices identified 5 separate sleep stages (A-E), with each stage coinciding with a different pattern marked on the EEG (Leeder et al., 2012). Sleep occurs in progression starting in the A state, moving down into the B state, and eventually down to the final E state, with the onset of sleep occurring somewhere between the later stages of A and the beginning of B states (Leeder et al., 2012). These sleep stages eventually progressed into the two primary stages of REM and

NREM, which are universally accepted. Interestingly, the NREM phase is further separated into stages 1-4, with stages 3 and 4 commonly known as slow wave sleep (SWS). Using EEG recordings, NREM sleep stages can be classified as follows: 1) Wakefulness which is characterized by low amplitude fast oscillations between 20-60hz and the presence of muscular tone, 2) NREM stage 1 which is characterized by sharp vertical waves, low voltage 3-7hz, a transitional sleep stage with the person still able to respond to questions, 3) NREM stage 2, which is characterized by slow oscillation with distinctive sleep spindles of less than 1hz, and the person in initial onset of sleep, 4) NREM stages 3 and 4 (SWS) which are characterized by high voltage, low frequency, deep sleep that is considered to be physically restorative, and 5) REM sleep which is characterized by low amplitude waves with fast oscillations, little to no muscle tone, and clusters of rapid eye movements and light sleep that signals the end of a sleep cycle (Leeder et al., 2012; Steiger, 2007). In human adults, REM sleep occurs in cycles for approximately 90 minutes, and begins approximately 70 minutes following the onset of sleep (Driver & Taylor, 2000). Between the cycles of REM, humans will enter NREM sleep, cycling between stages 1-4 (Driver & Taylor, 2000; Horne, 2000). Rapid eye-movement sleep was first reported by Aserinsky and Kleitman (Aserinsky & Kleitman, 1953) during the observation of infant sleep. To further explore this phenomenon, investigators examined the sleep patterns in men while attached to the EEG and reported that grown men also showed signs of eye movement following caseation of gross body movements. Investigators reported that eye movements were shown to occur in clusters approximately 90-120 minutes following the onset of sleep, and these periods of eye movements could last up to 50 minutes (Aserinsky & Kleitman, 1953). The brain's regulation of this sleep cycle has been reported elsewhere and is beyond the scope of this review.

Sleep and Exercise

The American Sleep Disorders Association has recommended exercise as a beneficial, non-pharmaceutical option to improve sleep quality. To determine whether sleep is improved or worsened following exercise, researchers use 3 markers of sleep. The first marker is sleep onset latency (SOL). Sleep onset latency is the time it takes for participants to reach NREM stage 2 and fall asleep from the time participants initially lie in bed. The second marker used to quantify sleep quality is time spent in slow wave sleep. Finally, the number of awakenings during a sleep period can be used to quantify the quality of sleep (Steiger, 2007). Using these sleep markers to quantify sleep quality, previous research shows that adolescents and the physically active population, have faster SOL times in addition to greater amounts of time spent in slow wave sleep (Driver & Taylor, 2000; Horne, 2000; Horne & Porter, 1975). It is believed that when the body is in slow wave sleep, it undergoes a restorative process (Dworak et al., 2008; Griffin & Trinder, 1978; Taylor, 2001). Previous observations found that during periods of sleep, peak cell division and protein synthesis rates occur in a multitude of tissue types (Adam & Oswald, 1983). Although most of the increases in cell division and protein synthesis rates were observed initially in animal models, bone growth and skin cell replication are known to increase in humans during sleep (Adam & Oswald, 1983). Additionally, the belief that sleep is a restorative process comes from the peak secretion rates of growth hormone (GH), following the initial onset of sleep. Growth hormone has been previously shown to increase protein synthesis, RNA synthesis, and amino acid uptake, all of which are initial steps in the restorative process (Adam & Oswald, 1983).

Given that slow wave sleep is considered the restorative aspect of sleep, daytime exercise has been hypothesized to increase the amount of time spent in slow wave sleep. This relationship

is likely due to the transient damaging processes of exercise. Multiple researchers have reported a positive correlation between acute daytime exercise and the amount of slow wave sleep the following night (Kubitz et al., 1996). Conversely, a few experiments have reported no relationship between daytime exercise and slow wave sleep the following night (Driver & Taylor, 2000). Limiting factors such as fitness status, exercise type and intensity, sex, and exercise time of day alter sleep quality and can explain the dissimilar findings reported in the research.

Cannabidiol and Sleep

It is well established that THC increases sleep quantity in humans likely due to its effects in the CNS and activation of the CB1R (Feinberg et al., 1975, 1976; Murillo-Rodríguez et al., 2006). However, the literature exploring the effects of CBD on sleep quantity and quality remains contradictory. Cannabidiol lacks the ability to bind to the CB1R predominately expressed in the CNS, yet in both rat and human models CBD is shown to modulate sleep. Male Wistar rats increase wakefulness, decrease REM sleep, and activate neurons in the dorsal raphe nucleus of the hypothalamus following intracerebroventricular (icv) administration of 10µg/5µl CBD (Murillo-Rodríguez et al., 2006). These results indicate that CBD induces alertness and decreases sleep quality in rats as determined through decreases in REM. Similar results are shown in humans at varying doses of CBD. A decrease in stage 3 SWS was shown in humans following oral administration of 5mg CBD, with further decreases in sleep quality though increased wakefulness following 15mg of oral CBD administration (Nicholson et al., 2004). The same effects of decreased SWS were shown in rats following CBD injections of 20mg/kg (Monti, 1977); however, following a higher dose of CBD at 40mg/kg in the same population, SWS significantly increased with a decrease in wakefulness ($p<0.05$) (Monti, 1977). Finally,

improvements in sleep following CBD administration were shown in humans following 160mg of oral CBD with increases in sleep time when compared to a vehicle control (Karniol et al., 1974). Overall, while some literature proposes that CBD has a negative influence on sleep quantity and quality, some have shown that CBD can elicit improvements. There is a small body of literature assessing CBD on its ability to manage sleep disorders, and thus far, there is insufficient evidence to support its use as a clinical sleep therapy (Suraev et al., 2020). Therefore, based on the current literature assessing the effects of CBD and modulation of sleep quantity and quality, there is inclination that responses may be dose dependent. More literature is needed to determine the sleep related effects of CBD.

Cannabidiol and Immunology

Cannabidiol modulates inflammation and immune function in both animal and human models (Burstein, 2015a); however, the pathways involved in these actions vary. While cannabidiol has little to no affinity towards the CB1R that is predominantly expressed in the CNS and neural tissue (Burstein, 2015a), CBD functions in CNS tissues. Cannabidiol acts to prevent cognitive impairments and hippocampal neurodegeneration, increase brain-derived neurotrophic factor, and promote neurogenesis in mice following bilateral common carotid artery occlusion (Mori et al., 2017). Conversely, the effects of CBD on CB2R are less defined. It is still unclear as to whether or not CBD can bind directly to the CB2R or whether CBD works through allosteric regulation (Martínez-Pinilla et al., 2017). For example, when 2 concentrations of CBD (2.5 and 5mg/kg) were administered i.p. to male Wistar rats for 14 days, there were significant body weight losses, which were reversed following administration of AM630, a potent CB2R blocker (B. Ignatowska-Jankowska et al., 2011). Furthermore, when a CB2R agonist (JWH133) is used, there is a downregulation of cAMP and activation of the MAPK pathways (Laprairie et

al., 2015; Martínez-Pinilla et al., 2017). Yet, CBD is also an inverse agonist to the CB2R in Chinese hamster ovary cells (CHO) (Thomas et al., 2007). Despite all of these various findings, the most popular belief is that CBD acts as a negative allosteric modulator of cannabinoid receptors based on its ability to reduce the effects of 2-AG and THC on the CB1R (Martínez-Pinilla et al., 2017).

Cannabidiol plays a role in immune function; however, due to the extensive number of immune cells and their varying receptor expressions, all the cellular pathways involved remain somewhat unexplored. Cannabidiol administration in a hypoxic–ischemic brain injury model reduced IL-6, TNF-a, cyclooxygenase-2 (COX2), and inducible nitric oxide synthase (iNOS) production, which suggests that CBD has anti-inflammatory potential (Castillo et al., 2010). Interestingly, these changes in IL-6, TNF-a, COX2, and iNOS were lost following the use of CB2R selective antagonists (Castillo et al., 2010). Additionally, 25mg/kg of CBD induces immunomodulatory effects through the TRPV1 receptor in a C57BL/6 mouse model. Briefly, mice were treated with concanavalin A to induce acute hepatitis, which led to cytokine induction in the liver and subsequent liver damage. In a dose-dependent manner, CBD reduced hepatitis and liver damage through the suppression of inflammatory biomarker production and a reduction in T-cell proliferation through the infiltration of myeloid-derived suppressor cells (MDSCs) (Hegde et al., 2011). Furthermore, CBD failed to induce MDSC infiltration and changes in inflammatory biomarkers upon treatment with a TRPV1 receptor antagonist (Hegde et al., 2011). These results suggest that CBD may invoke immunomodulatory effects through activation of the TRPV1 receptor. Additional immunomodulatory effects of CBD may also work through the heterodimerization of CB2R and serotonin $5HT_{1A}$ receptors. For example, in a model of hypoxic-ischemic brain injury in newborn piglets, when a vehicle, CBD at 1mg/kg, CBD with a

CB2R antagonist, and CBD with a serotonin $5HT_{1A}$ antagonist were administered i.p., recovery of both brain activity and EEG patterns occurred following hypoxic-ischemia in the CBD only group. When receptor antagonists were incorporated into this model, the protective effects of CBD were absent (Pazos et al., 2013). Finally, the last receptor pathway CBD works through to stimulate immunomodulatory effects is the orphan G-protein coupled receptor GPR55. The GPR55 is stimulated through induction of plant-derived cannabinoids such as CBD and has a wide distribution across cell types in the human body, including cells of the gastrointestinal tract, spleen, and bone (Chiurchiù et al., 2015). The stimulation of GPR55 by CBD and other exogenous and endogenous cannabinoids leads to the suggestion that GPR55 may be the third type of cannabinoid receptor (type 3; CB3R). However, controversy related to low amino acid sequence similarities of the GPR55 when compared to CB1R and CB2R make it unclear as to whether GPR55 is a novel cannabinoid receptor. G-protein coupled receptor 55 is most associated with prominent immunomodulatory roles shown to affect inflammatory pain (Staton et al., 2008), and osteoclast function in bone (Chiurchiù et al., 2015; Whyte et al., 2009). Orphan G-protein coupled receptor 55 agonist O-1602 administration on PBMCs significantly increases IL-12 and TNF-a production in monocytes, along with concomitant upregulation of granzyme B and CD107a, a marker of degranulation in NKC cytotoxicity. These effects are antagonized upon treatment with CBD (Chiurchiù et al., 2015). The information provided above demonstrates that CBD provides immunomodulatory actions in animal and human models, although the mechanism of action remains to be fully elucidated.

Cannabidiol and Natural Killer Cells

Cannabidiol acts to mediate immunomodulatory effects in the human body (Borrelli et al., 2009; Jan et al., 2003; Kozela et al., 2011; Malfait et al., 2000; Weiss et al., 2008; Zgair et

al., 2017). Immune cells express receptors involved in CBD signaling at varying concentrations across most immune cell types. Natural killer cells express high concentrations of the CB2R and GPR55 on the cell surface. Additionally, these stimulated NKC may interact with monocytes by cooperating through the serotonin $5HT_{1A}$ receptor. Additionally, NKC and monocytes express the highest concentrations of CB2R on the cell surface when compared to other PBMC populations (Graham et al., 2010). Cannabidiol, when administered i.p. at 5mg/kg to adult Wistar rats, reduced the total number of total leukocytes in peripheral blood as well as reduced the total number of T-cells, B-cells, T-helper cells, and cytotoxic T-cells (B. Ignatowska-Jankowska et al., 2009). However, this CBD-linked immunosuppressive effect was not observed in total number of NKC or Natural Killer T-cell (NKT) measures. Furthermore, in the same study, a lower, 2.5mg/kg dose, of CBD increased the total number of both NKC and NKT (B. Ignatowska-Jankowska et al., 2009), suggesting a potential dose-response relationship.

Further investigation by the same group found a similar reduction in total and relative lymphocyte cell populations in the spleen with no change in NKC populations (B. M. Ignatowska-Jankowska et al., 2009). These results indicate that the administration of CBD can cause immunomodulatory effects on PBMC populations, but due to the lack of affinity for CBD to CB2R, CBD may be interacting through a different receptor. In fact, flow cytometry analysis revealed high concentrations of GPR55 on monocytes and NKC (Chiurchiù et al., 2015). Following treatment of NKC with GRP55 agonist, increases in IL-12 and TNF-α were found, with further increases in granzyme B, CD107a, and INF-γ in NKC. However, these results were negated upon administration of CBD, acting as a GPR55 receptor antagonist (Chiurchiù et al., 2015).

The serotonin $5HT_{1A}$ receptor is the final CBD linked receptor observed in NKC. Following extraction of PBMC, monocytes suppress NKC cytotoxicity in a monocyte-NKC contact dependent manner. Upon stimulation with serotonin, the downregulation of NKC cytotoxicity occurred, which is believed to be due to monocyte-NKC cellular contact (Hellstrand & Hermodsson, 1993). In summary, CBD interacts with the serotonin $5HT_{1A}$ receptor in an agonistic manner. Consequently, CBD administration may increase or decrease NKC cellular cytotoxicity through its antagonist modulations of the GPR55 and serotonin $5HT_{1a}$ receptor.

Conclusions

The current body of literature has focused on the physiological and psychological effect of CBD in both animal and human models. Given that the endocannabinoid system was only discovered within the past 40 years, it is not surprising that there are large variations and discrepancies related to our understanding of the true mechanisms of this system. It is suggested that CBD can act as an anxiolytic, antipsychotic, anti-inflammatory, and antiemetic agent (Mechoulam & Hanuš, 2002), in addition to a sleep modifier (Babson et al., 2017). As a modulator of the immune system, CBD and its receptors are highly expressed throughout the immune system (Atalay et al., 2020; Dhital et al., 2017; B. M. Ignatowska-Jankowska et al., 2009; Nichols & Kaplan, 2020); however, due to the abundance of cell types, the overall effects of CBD on the immune system remain unclear. Additionally, the current body of literature on CBD and its role in sleep modulation contradict itself and show that varying doses may elicit varying responses in different aspects of the sleep cycle (Babson et al., 2017; Monti, 1977; Murillo-Rodríguez et al., 2006; Nicholson et al., 2004). This body of literature is still in its infancy.

Since 2018, there has been a boom in the sale and use of CBD as well as a drastic increase in the messaging related to the proposed benefits of CBD. Many of these proposed benefits are inflated, under researched, and ultimately unclear. At the present time, there is limited research on the effects of CBD on the quantity and function of NKC, with no literature investigating its effects in the human model. Moreover, the discrepancies related to the effects of CBD on sleep quantity and quality make it unclear as to whether CBD is beneficial or detrimental to sleep architecture. Yet, more people are beginning to use CBD every day. Therefore, it is necessary to expand the current body of literature on CBD and its effects on human physiology.

CHAPTER III

METHODOLOGY

Participants

Physically active males and females (N=27; CB=15, CN=12) between the ages of 18-50 years of age were recruited through flyers and word of mouth at the University of Northern Colorado and nearby communities to participate in this study. All participants were required to be in good health and had been completing 150 minutes of moderate to vigorous exercise per week for the 3 months prior to visit 1. Exclusion criteria included the presence of chronic disease conditions, such as cardiovascular disease, cancer or diabetes; consistent use of anti-inflammatory medications or medications that act through the liver metabolism throughout the duration of this investigation; sleep aid or sleep medication use; use of THC or CBD in the 6 weeks prior to visit 1; have a BMI above 29.9kg/m^2 classifying them as obese; diagnosed with or vaccinated for COVID-19 within the 2 weeks prior to visit 1; or any foreseen changes in their regular exercise regimen within the study intervention period.

Study Overview

In this double-blind investigation, participants underwent 8 total visits separated by an 8-week intervention period (Figure 1). During the first pre-intervention visit participants were asked to review and sign the informed consent form approved by the University of Northern Colorado Institutional Review Board. At this time, participants were allowed to ask any questions. Upon signing, participants were given a copy of the informed consent form for their records. Following completion of the informed consent, participants were asked to complete a

medical health history questionnaire and the physical activity readiness questionnaire (PARQ) to ensure all inclusion criteria were met and the participant was healthy enough for exercise. Upon completion of the informed consent, medical health history, and PARQ, participants underwent an 8h fasted blood draw, anthropometric measurements, body composition analysis (BODPOD), and questionnaires assessing mental health, sleep quality, and illness. Following thecompletion of visit 1, participants were given a sleep tracker (Fitbit, San Francisco, CA) to wear for 7 days before the 8-week intervention. Sleep quantity and quality analysis was completed again during the second to last week (week 7) of the intervention period. Following the first 4 visits, participants were randomly assigned to 1 of 2 groups: CBD (CB; 50mg/day) or calorie-matched placebo (CN).

Each week of the 8-week intervention, participants were given a 7-day pill box containing either one liquid gel containing 50mg of purified, hemp-derived CBD (Six Degrees Wellness, Boulder, CO), or a 250mg medium-chain triglyceride (MCT) capsule (Nutiva, Point Richmond, CA) as the calorie matched placebo. Participants were instructed to consume one pill per day following their last meal, 1-1.5h before bed. Investigators met bi-weekly with the participants to refill the supplement, and to report any adverse side effects or changes in their physical activity routine. At the final week of the 8-week intervention, participants were given a sleep tracker to wear for the final 7 days of the intervention. Upon completion of the 8-week intervention, participants completed the post-intervention testing (Visits 5-8) which consisted of the same measures as pre-intervention.

Blood Sampling

Two separate blood samples; Pre-intervention (week 0), and post-intervention (week 8) were collected from all participants for assessment of NKC quantity and function. All blood

samples were collected with the participant 8h fasted between 0600-1200h. Prior to the pre-intervention blood collection, participants were asked to complete a 24h diet log which they were asked to replicate 24h prior to the post-intervention blood collection. Participants were required to arrive 24h fasted from exercise, caffeine and alcohol to aid in the control of immune cell population distribution and function. During each blood draw, which was performed by a trained phlebotomist, participants donated approximately 10ml of blood through venipuncture of the antecubital vein of the forearm. Blood was collected using Becton, Dickinson and Company Saftey-Lok butterfly needles (Franklin Lakes, NJ) collected into vacuum sealed Becton, Dickinson and Company EDTA vials (Franklin Lakes, NJ) EDTA vials were immediately used to assess NKC quantity and NKC functional analysis.

Body Weight and Height Assessment

Participants were instructed to remove their shoes, socks, and any additional clothing other than the participant's base layer prior to height and weight assessment. Height and weight were obtained using a stadiometer SECA 220 (Chino, California, USA) and the Detecto standing digital scale (Webb City, Missouri, USA), respectively.

Air Displacement Plethysmography

Body composition, lean body mass (LBM) and body fat percentage (BF%) was evaluated using air displacement plethysmography with a BODPOD (COSMED USA Inc., Concord, CA). Participants were instructed to remove their shoes, socks, jewelry, and all additional clothing other than a base layer. Participants were then given a swim cap to wear, and body composition analysis was performed via manufacturer's guidelines (Vescovi et al., 2001).

Supplement or Placebo Description

Upon completion of all baseline and physiological characteristic measurements 1 week prior to supplementation, participants were randomly assigned 1 of 2 intervention groups (CB or CN). Participants and researchers were blinded to the intervention groups. All participants were given pill boxes containing 14 days of supplementation (50mg CBD or calorie matched placebo), that were re-filled and re-administered bi-weekly throughout the duration of the 8-week intervention period to ensure supplement adherence. Participants were instructed to consume their assigned pill once per day after their last meal within 1-1.5h of going to bed. The dosage of 50mg/d was selected to ensure participant safety. This was the first investigation at the University of Northern Colorado to administer CBD to human participants. Therefore, to limit adverse side effects and ensure participant safety, a lower dose of CBD was selected. Following completion of data collection and interpretation, researchers were then unblinded to supplementation groups.

Depression

At weeks 0 and 8, participants were asked to complete the Beck's Depression Inventory (BDI, Appendix D) for assessment of their state of depression. The BDI is a 21-question depression assessment tool in which participants respond with 0, 1, 2, or 3. The sum of all 21 questions is totaled and depression is determined as follows; 1-10 "these ups and downs are considered normal", 11-16 "Mild mood disturbance", 17-20 "Borderline clinical depression", 21-30 "Moderate depression", 30-40 "Severe depression", >40 "Extreme depression."

Anxiety

At weeks 0 and 8, participants were asked to complete the General Anxiety Disorder, 7 question screening tool (GAD-7; Appendix E) (Spitzer et al., 2006). The GAD-7 consists of 7

questions assessing participants anxiety, with participants answering the 7 questions on a 0-3 scale (0-“not at all”; 1-“several days”; 2-“more than half the days”; 3-“nearly every day”). Example questions of the GAD-7 include “over the past 2 weeks, how often have you been bothered by the following problems?” “Feeling nervous, anxious, or on edge?” “Worrying too much about different things?” And “Trouble relaxing?”

Fatigue

At weeks 0 and 8, participants were asked to complete the Piper Fatigue Scale (PFS; Appendix G) (Kelly et al., 2003). The PFS is a 27-question fatigue screening tool using a Likert based system (1-10) with 1 indicating little to no fatigue and 10 indicating maximal fatigue symptoms. Sample questions of the PFS are as follows; “To what degree would you describe the fatigue which you are experiencing as being?” and, “How would you describe the degree of intensity or severity of the fatigue which you are experiencing now?”

Quality of Life Assessment

At weeks 0 and 8, participants will be asked to complete the Ferrans and Powers Quality of Life Index (QOL; Appendix F) (Hagell et al., 2010). The QOL is a 2-part questionnaire, totaling 66 questions. Part 1 asks how satisfied the participant is in various portions of their life including but not limited to; “Your health”, “Your family’s health”, and “The emotional support you get from your family?” Available responses range between 1-6 with 1 indicating “Very dissatisfied” and 6 indicating “Very satisfied.” Part 2 of the QOL asks the participant how important various portions of their life is to them. Questions in part 2 are identical to the questions in part 1, but the available responses range between 1-6 with 1 indicating “Very unimportant” and 6 indicating “Very important.” For assessment of the QOL index, 3.5 was subtracted from the raw scores of the first 33 questions assessing how satisfied they are with

various aspects of their life. Next, the altered values were multiplied by their corresponding scores of the second 33 questions assessing how important various aspects of their life were to them. Finally, all 33 modified answers were summed and divided by total number of responses followed by the addition of 15. This process created a range of values between 0-30 with higher values indicating higher QOL and was suggested for optimal analysis by the creators of the survey (Ferrans & Powers, 1985).

Leeds Sleep Evaluation Questionnaire

At week 0 and 8, participants were asked to complete the *Leeds Sleep Evaluation Questionnaire* (LSEQ; Appendix H)(Tarrasch et al., 2003). The LSEQ is a 10-question visual analog scale (VAS) questionnaire divided into 4 sections assessing "getting to sleep" (GTS), "quality of sleep" (QOS), "awake following sleep" (AFS), and "behavior following wakening" (BFW). Participants were instructed to mark a vertical "tick mark" along a 100mm horizontal line closer or further away from their answer. For example, "How do you feel when you wake up?" participants placed a vertical tick mark closer to "Tired" or "Alert." The location of the tick mark was then measured and quantified as a quantitative millimeter value out of 100. Each question assesses a different aspect of perceived sleep quality with answers to the left indicating lower perceived sleep quality and answers to the right indicating higher perceived sleep quality. Therefore, all answers are measured from 0-100 with higher scores indicating greater perceived sleep quality.

Wrist Actigraphy Sleep Assessment

During the week prior to the start of the intervention (week 0) and the final week of supplementation (week 7), participants were given the FITBIT Inspire HR (Fitbit, San Francisco CA) to assess sleep quantity and quality to wear on their wrists. Participants were instructed to

wear the Fitbit Inspire HR band at all hours of the day and night for 1 full week for baseline and post treatment analysis. The face of the Fitbit was covered with black electrical tape so that the participant could not receive immediate feedback about his/her/their sleep performance. Previous investigations have shown that when wrist actigraphy is directly compared to polysomnography (PSG) measuring overall sensitivity and accuracy of sleep variables, wrist actigraphy scored >80% similarity to PSG measurements (Marino et al., 2013). Therefore, we felt confident in the accuracy of this sleep data collection technique in addition to its low barriers for use, entry, and analysis when compared to PSG.

Cell Culture

The K562 cell line (ATCC, Manassas, VA) originally derived from a 53-year-old female patient with chronic myeloid leukemia in blast crisis (Lozzio & Lozzio, 1977), was used to evaluated NKC function.Two days prior to NKC functional assays, a vial of K562 cells suspended in liquid nitrogen for long-term storage was thawed by gentle agitation in a 37°C water bath with the cap held above the water line to prevent contamination. Once thawed, the vial was removed from the water bath and sprayed with 70% ethanol. Once out of the water bath, all operations were carried out under aseptic conditions. The thawed vial of K562 cells was transferred from the cryovial into a 15 ml Falcon tube (Corning, Tweksbury MA) containing 9.0ml of pre-warmed complete cell culture medium (RPMI-1640; 10% FBS; 1% Pen-Strep). Cells were then spun down at 125xg for 10 minutes using a centrifuge (Eppendorf 5810R; Hamburg Germany). After a pellet of K562 cells was formed, the cells were resuspended in complete cell culture medium at a concentration of 5.0×10^5cell/ml and dispensed into a $75cm^2$ culture flask with a vented cap (Fisher Scientific, Waltham, MA). Cells were then be incubated

at 37°C in a cell incubator at 5% CO_2 atmosphere for 2 days prior to use to ensure cell health and viability.

Peripheral Blood Mononuclear Cell Extraction

Fresh blood was used for the extraction of PBMC to ensure high viability of the cell sample. Ficoll-Paque density gradient media (15ml) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was added to a 50ml conical tube. In a separate 15ml conical tube, 7-10ml of fresh whole blood was mixed with DPBS in a 1:1 ratio and inverted 4 times to mix. The blood: DPBS mixture was then carefully and slowly layered on top of the Ficoll-Paque density gradient media, without mixing the liquids. Once layered, the 50ml conical tube containing fresh blood and Ficoll-Paque density gradient media was spun down at 400xg for 40 min with the centrifuge brake off. Once spun, the plasma layer of the solution was removed, and the buffy coat was extracted and placed in a conical tube. The removed buffy coat was then mixed with DPBS at 3x volume of the buffy coat (1ml buffy coat: 3ml DPBS) to wash cells. Cells were then spun down at 400xg for 5 minutes. The wash step was repeated 3 times then counted using a Countess 3 Automated Cell Counter (ThermoFisher Scientific; Waltham, MA, USA) and cells were then resuspended at a concentration of 1.0×10^6.

Natural Killer Cell Quantity and Functional Analysis

Flasks of K562 cells were removed from the cell incubator and transferred into a conical tube. Cells were counted using a Countess 3 Automated Cell Counter (Invitrogen™, Waltham, MA) and spun down at 400xg for 10 min and resuspended in complete cell media at a concentration of 1.0×10^6cell/ml. A sample of 700ul of K562 cells (7.0×10^5 total cells) were placed in a 2.0ml Eppendorf tube (Eppendorf AG, Hamburg, Germany), and incubated with 2µl

of Alexa-Fluro 647-conjugated anti-CD-71 (Biolegend, San Diego, CA) and 2µl of Calcein-AM and placed on ice in the dark for 20min. Following the incubation, K562 cells were spun down using a microcentrifuge (Eppendorf AG, Hamburg, Germany) at 400xg for 8min and resuspended in complete cell culture media. Cells were then cultured alone (K562 only; PBMC only) at 1.0 x 10^5 and co-cultured at an effector: target (E:T) cell ratio of 1:1 (1.0 x 10^5), 5:1, 10:1, and 20:1. The 6 cell cultures were then be incubated at 37°C in a cell incubator at 5% CO_2 atmosphere for 4h. Following the 4h incubation, cells were removed, and spun down at 400xg for 8min. Cells were then resuspended in 1ml of DPBS and incubated with 2µl of efluro450-conjugated anti-CD3 (Invitrogen, Carlsbad, CA) and 2µl of PerCP-efluro 610-conjugated anti-CD56 (Invitrogen, Carlsbad, CA) on ice in the dark for 20min. Following incubation, cell were again spun down as described above, resuspended in DPBS, and analyzed for NKC quantity and NKC function using the Attune™ NxT Flow Cytometer (Thermo Fisher Scientific,Carlsbad, CA). Analysis of acquired raw data was preformed using FlowJo software (version 10, TreeStar, Ashland, OR).

Cell cultures containing only PBMC populations were used for percentage of cell populations in PBMCs. Gating strategy to determine cell populations in PBMCs began by doublets and debris were gated out using a singlets gate with forward scatter area on the X-axis and forward scatter height with a log scale on the Y-axis. Next singlet cell populations were gated on a histogram gate for CD3+/-. Daughter plots of CD3+/- gates remained as a histogram and were further gated for CD56+/-. Cell populations were then recognized as CD3+CD56+ (NKT), CD3+CD56- (T cell), and CD3-CD56+ (NKC).

Natural killer cell cytotoxicity was assessed using the mean and median fluorescence intensity of K562 human leukemia cells positive for the cell-permeant dye calcein-AM which

fluoresces in the blue laser 1 (BL1) channel. Calcein-AM is a nonfluorescent cell-permeant dye capable of freely passing into a cell's cytosol. If the cell is living, calcein-AM is converted to a green-fluorescent calcein by intracellular esterase preforming acetoxymethyl ester hydrolysis. If a cell stained with calcein-AM expresses a positive event in the BL1 channel, it is considered to be a live cell; however, once cells containing green-fluorescent calcein undergo cellular death, calcein no longer exists in the cell and is considered a dead cell. For cytotoxicity analysis, both mean and median fluorescence intensities of calcein-AM were used due to the differences in robustness between measures. Fluorescent intensity is measured on a logarithmic scale, indicating that the more that data is skewed from non-normal data points or outliers, the more exaggerated mean fluorescence intensity will become, resulting in data which is less representative of the population. Median fluorescence intensity will result in a value that is the mid-point of the data that we are observing, making it less susceptible to change in the presence of skewed data or outliers.

Furthermore, K562 leukemia cells were cultured alone as a control for cell viability. Therefore, data was processed using raw median fluorescence intensity in addition to data being normalized to the control K562 only culture. Median fluorescence intensity of 1:1, 5:1, 10:1, and 20:1 ratios were divided by the K562 only control median fluorescence intensity then multiplied by 100, to normalize data to the control cell viability of K562 only and expressed as percent cell viability as previously shown (Carreño et al., 2021).

Supplement or Placebo Description

Upon completion of all pre-intervention measurements 1 week prior to the intervention period, participants were randomly assigned to 1 of 2 intervention groups (CB or CN). Participants and researchers were blinded to the intervention groups. All participants were given

weekly, individual pill boxes containing 14 days of each compound in a pill form (50mg CBD or calorie-matched placebo, containing 250ul of coconut derived medium chain triglycerides without the CBD), that were re-filled and re-administered bi-weekly throughout the duration of the 8-week intervention period to ensure adherence. CBD product was provided by 6° wellness (©2020 6º Wellness). All products were hemp-derived, within legal limits and have had its purity verified. Participants were instructed to consume their assigned pill once per day after their last meal, within 1-1.5h prior to going to bed. Following completion of data collection and interpretation, researchers were unblinded to supplementation groups.

Statistical Analyses

To avoid Type I error and achieve a desired level of 0.95 power with an $\alpha = 0.05$, an a priori analysis (G*Power, Dusseldorf, Germany) showed a total sample size of 8 is needed. Means and standard deviations of NKC cytotoxicity of K562 cells when assessed using PBMC treated with THC, compared to cells treated with DMSO control were used for computational analyses (Specter et al., 1989). Cannabidiol cell culture population numbers were not used for power analyses due to the lack of current literature evaluating the effect of CBD on NKC cytotoxicity. Additionally, human subject numbers were not used for the power calculation due to the lack of current literature evaluating the effect of CBD on NKC cytotoxicity. To maximize the potential to detect group differences, a total sample size of 24 (CBD; n=12, PLA; n=12,) was decided. However, participant dropout was found to be more prevalent in CN group compared CB, resulting in a slightly skewed group distribution. Therefore, additional participants were included in CB analysis (CB: n=15; CN: n=12).

All data were evaluated for normality and homogeneity of variance prior to analysis. Significance was set at $p<0.05$ and all statistical analyses were performed using SPSS 25 (IBM,

Corp., Chicago, IL) and GraphPad Prism (GraphPad Software, San Diego, CA). An independent-samples t-test was used to determine whether there are group differences at the pre intervention time point. A 2 (group) x 2 (time) analysis of variance (ANOVA) was used to determine if there was a significant treatment effect on all study outcomes following 8-weeks of CBD supplementation. Additionally, one and two-way analysis of covariance (ANCOVA) tests were used to determine if there are significant treatment effects on measures of NKC quantity and quality with sleep quantity and quality being used as covariates. One and two-way ANCOVA tests were additionally used to determine if there are significant treatment effects on measures mental health with sleep quantity and quality used as covariates.

Upon first assessment of NKC cytotoxicity data with a boxplot, there were 4 outliers >3 SD outside of the mean at both pre and post intervention time points. However, these outliers were still within an appropriate range for the NKC cytotoxicity assessment based on data still being normally distributed at both pre and post intervention, as assessed by Shapiro-Wilk's test ($p>0.05$). Additionally, there was homogeneity of variances as assessed by Levene's test for equality (0.483), therefore the complete data set was used for statistical analysis.

CHAPTER IV

RESULTS

Participant Characteristics

A total of 27 participants (12 males and 15 females) completed all pre and post measures. Dosing of 50mg/d of CBD equated to a milligram per kilogram body weight dose of 1.42 ± 0.30 and 1.45 ± 0.19mg/kg for CB and CN respectively, with a total participant dose of 1.43 ± 0.26mg/kg. When all groups were combined, the average age of the study participants was 26.1 ± 6.2y and there were no differences between CB and CN groups. Participants were an average 24.8 ± 3.2kg/m^2 BMI and 21.8 ± 8.3% body fat and there were no differences at the pre intervention time point and there were no group or time main effects or group x time interactions. Mean participant body size and lean mass was 169.2 ± 8.9cm, 71.58 ± 12.82kg, 55.79 ± 11.32kg with respect to height, weight, and lean body mass, respectively when both groups were combined at the pre intervention time point. There were no differences at the pre intervention time point and there were no group or time main effects or group x time interactions. Resting heart rate was not different between groups and there were no group or time main effects or group x time interactions (Table 1).

Table 1

Participant Characteristics

	CB-Pre	*CB-Post*	*CN-Pre*	*CN-Post*
Age (yr)	24 ± 5		28 ± 6	
Sex (M/F)	6 / 9		6 / 6	
Height (cm)	168.4 ± 9.6		170.3 ± 8.1	
BMI (kg/m)^2	24.8 ± 3.5	24.9 ± 3.6	25.0 ± 2.9	25.1 ± 3.0
Weight (kg)	70.9 ± 15.2	71.1 ± 15.4	72.5 ± 9.7	72.8 ± 10.0
Lean Body Mass (kg)	55.20 ± 12.86	55.44 ± 13.11	56.53 ± 9.56	56.51 ± 10.21
Body Fat (%)	22.0 ± 8.1	22.1 ± 7.8	21.7 ± 9.0	22.2 ± 9.7
RHR (bpm)	70 ± 6	67 ± 8	68 ± 10	67 ± 8

Note. Values presented are mean ± SD. *Abbreviations: yr* year, *M/F* male/female, *BMI* Body Mass Index, *m/kg^2* meters per kilogram squared, *cm* centimeters, *kg* kilograms, *%* percent, *RHR* resting heart rate, *bpm* beats per minute, *CB-Pre* Cannabidiol Pre Intervention, *CB-Post* Cannabidiol Post Intervention, *CN-Pre* Control Pre Intervention, *CN-Post* Control Post Intervention.

Exercise Performance

When CB and CN were combined at the pre intervention time point, participants averaged 640.61 ± 169.02W peak power, 8.83 ± 1.33W relative mean power, 455.50 ± 122.01W mean power, and 56.68 ± 7.26% anerobic fatigue. There were no significant differences among

groups. Additionally, there were no group or time main effects or group x time interactions with respect to these Wingate test outcomes. Cardiorespiratory fitness averaged 44.38 ± 6.91ml/kg/min at the pre intervention time point when CB and CN were combined and there was no significant difference between groups. Although there was no significant group main effect or group x time interaction, there was a significant main effect of time for VO_2 peak. Pre intervention values were 3.0% higher when compared to post intervention values (p=0.038; Pre: 44.38 ± 6.91ml/kg/min; Post: 43.09 ± 6.63ml/kg/min) (Table 2). When grouped together, all participants in the study did not differ with respect to measures of body strength. More specifically, overall average 1RM for back squat and bench press was 88.2 ± 31.4kg and 61.4 ± 28.4kg, respectively at the pre intervention time point and there were no group or time main effects or group by time interactions.

Table 2

Exercise Performance Measures

	CB-Pre	*CB-Post*	*CN-Pre*	*CN-Post*
Peak Power (W)	636.28 ± 192.36	642.68 ± 192.61	662.29 ± 144.11	590.71 ± 239.55
Relative Peak Power (W)	8.80 ± 1.41	8.94 ± 1.70	9.19 ± 1.64	8.72 ± 2.12
Mean Power (W)	462.18 ± 132.41	457.75 ± 140.41	455.23 ± 110.95	448.98 ± 121.09
Anaerobic Fatigue (%)	55.13 ± 8.46	56.71 ± 6.43	59.09 ± 4.69	55.29 ± 8.21
VO_2 (ml/kg/min)	45.54 ± 7.32	43.83 ± 6.78*	44.43 ± 8.23	43.64 ± 8.30*
Back Squat (1RM, kg)	90.6 ± 33.7	90.5 ± 30.0	87.7 ± 28.9	90.9 ± 30.3
Bench Press (1RM, kg)	61.2 ± 28.6	62.9 ± 29.1	64.4 ± 29.2	65.2 ± 31.9

Note. Values presented are mean ± SD. *W* Watt, *%* percent, *ml/kg/min* milliliters per kilogram per minute, *1RM* 1-Repition Max, *CB-Pre* Cannabidiol Pre Intervention, *CB-Post* Cannabidiol Post Intervention, *CN-Pre* Control Pre Intervention, *CN-Post* Control Post Intervention.

*significant time effect (p=0.038).

Mental Health Measures

Participant mental health measures were assessed using the BDI, GAD-7, PFS, and QOL. When groups were combined at the pre intervention time point, participants average scores were 4.56 ± 3.82 for the BDI, 6.3 ± 6 for the GAD-7, 3.21 ± 1.58 for the PFS, and 19.50 ± 1.82 for the QOL. There were no main effects of group or time or group x time interactions with respect to any of these mental health measures (Table 3).

While not statistically significant, pre-intervention GAD-7 scores were 7.9 ± 6.6 in CB group and 4.3 ± 4.7 in CN group. Post intervention GAD-7 measures revealed that CB group experienced a mean 2.1-point reduction in anxiety scores over the course of the study, which is 26.5% of the mean score at the pre intervention. The CN group experienced a mean 0.6-point decrease, which is 14% of the mean score at the pre intervention.

Table 3

Mental Health Questionnaire Outcomes

	CB-Pre	*CB-Post*	*CN-Pre*	*CN-Post*
Beck's Depression Inventory	5.3 ± 4.2	5.6 ± 3.6	3.6 ± 3.1	3.0 ± 3.5
General Anxiety Disorder-7	7.9 ± 6.6	5.8 ± 4.2	4.3 ± 4.7	3.7 ± 4.3
Piper Fatigue Scale	3.7 ± 1.6	3.1 ± 1.7	2.6 ± 1.3	2.3 ± 1.4
Quality of Life Index	19.0 ± 1.9	19.4 ± 1.3	20.1 ± 1.5	20.4 ± 1.4

Note. Values presented are mean ± SD. *CB-Pre* Cannabidiol Pre Intervention, *CB-Post* Cannabidiol Post Intervention, *CN-Pre* Control Pre Intervention, *CN-Post* Control Post Intervention.

Quantitative and Perceived Sleep

When CB and CN groups were combined at the pre intervention time point, participants averaged 387 ± 68min asleep, 52 ± 11min awake, 26 ± 8 wake episodes, 88.4 ± 1.5% sleep efficiency, and 439 ± 77min time in bed and there were no differences between the groups. Additionally, there were no group or time main effects or group x time interactions with respect to any of these FitBit measures.

Participant perception of sleep, as evaluated with the LSEQ, was not different between CB and CN at the pre intervention time point. There was no significant main group effect or group x time interaction with respect to the LSEQ or subscales of GTS, QOS, and BFW; however, there was a significant main effect of time for the total mean score of the LSEQ where pre intervention values were 13.4 percent lower when compared to post intervention values ($p=0.023$; pre:47.3 ± 12.2; post: 54.6 ± 8.7; Figure 1.) and the QOS subscale where pre intervention values were 26.8 percent lower compared to post intervention values ($p=0.018$; Pre: 39.8 ± 21.9; Post: 54.4 ± 18.5; Figure 1.). To further investigate this significant effect of time, a paired-samples t-test was ran to determine whether CB, CN, or both groups had significant change over time. Results of the paired-samples t-test indicate LSEQ for CB was significantly increased pre to post intervention ($p=0.006$) with no change pre to post in the CN group ($p=0.687$). Furthermore, the same analysis was ran for the QOS subscale, and found the CB group significantly increased pre to post intervention ($p=0.027$) when compared to CN ($p=0.327$) There was a significant group x time interaction for the AFS subscale where CB experienced a 48.44% increase over time compared to a 1.5% decrease in CN ($p=0.05$, Post: CB 61.7 ± 17.3 CN 43.2 ± 20.0; Table 4.).

Table 4

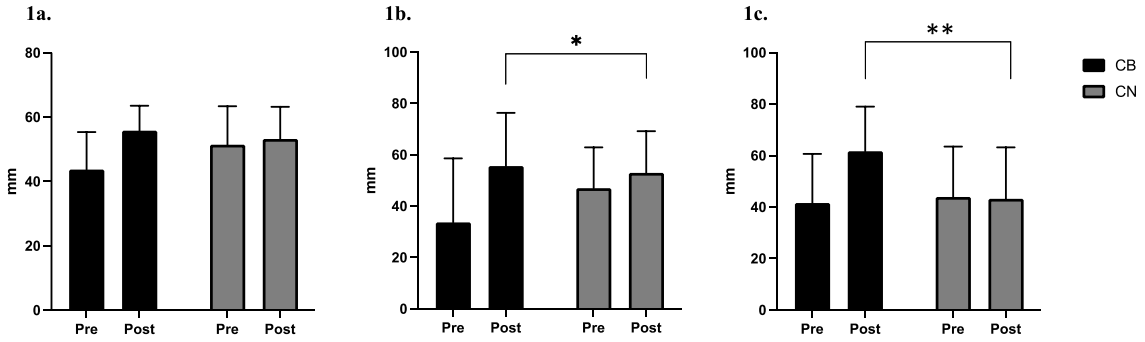
Measures of Sleep Quantity and Quality

	CB-Pre	*CB-Post*	*CN-Pre*	*CN-Post*
Minutes Asleep (min)	388 ± 55	379 ± 57	385 ± 83	400 ± 59
Minutes Awake (min)	54 ± 11	55 ± 12	49 ± 12	51 ± 7
Wake Episodes	27 ± 6	26 ± 9	25 ± 9	27 ± 7
Time in Bed (min)	442 ± 63	435 ± 67	435 ± 94	451 ± 62
Sleep Efficiency (%)	8.0 ± 1.5	87.5 ± 2.1	88.9 ± 1.3	88.7 ± 1.6
LSEQ	43.7 ± 11.6	55.8 ± 7.8**	51.4 ± 12.0	53.3 ± 10.0**
GTS	50.4 ± 13.6	55.8 ± 12.4	58.4 ± 13.8	57.7 ± 15.5
QOS	33.7 ± 24.9	55.6 ± 20.7**	47.0 ± 15.9	53.0 ± 16.2**
AFS	41.6 ± 19.1	61.7 ± 17.3*	43.9 ± 19.6	43.2 ± 20.0*
BFW	45.2 ± 21.7	51.9 ± 17.1	52.4 ± 17.2	55.7 ± 14.1

Note. Values presented are mean ± SD. Abbreviations: *LSEQ* Leeds sleep evaluation questionnaire, *GTS* getting to sleep, *QOS* quality of sleep, *AFS* awake following sleep, *BFW* behavior following wakening, *mm* millimeters, *min* minutes, *%* percent, *CB-Pre* Cannabidiol Pre Intervention, *CB-Post* Cannabidiol Post Intervention, *CN-Pre* Control Pre Intervention, *CN-Post* Control Post Intervention. *significant group x time interaction (p=0.05), **significant time effect (LSEQ p=0.023; QOS p=0.018)

Figure 1

Sleep Analysis



Note. CB (n=15) and for CN (n=12)1a: *LSEQ:* Leeds Sleep Evaluation Questionnaire, 1b: Quality of Sleep (subscale of LSEQ), 1c: Awake Following Sleep (subscale of LSEQ), *mm:* millimeters, *Values presented are mean ± SD.* *Significant main effect of time (*p=0.018), **Significant group x time interaction(**p=0.05)

Peripheral Blood Mononuclear Cell Populations

Percent of PBMCs that were NKT cells was 4.56 ± 2.68% when CB and CN groups were combined at the pre intervention time point and ranged from 1.18-15.40%, with no significant difference between the groups. There was no main effect of group or group x time interaction. There was a significant time effect with decreased NKT cell percent from pre intervention to post intervention in both CB (19.0% decrease) and CN (21.6% decrease), (p=0.040; Table 5.). Percentage of PBMCs that were NKC was 9.01 ± 5.33% when CB and CN groups were combined at the pre intervention time point and ranged from 2.38-24.9%. There was no main effect of group or time, or group by time interaction with respect to NKC percentage of PBMC. However, NKC percentage in PBMCs decreased in CB group (1.1%) and increased in CN (2.5%) pre to post intervention.

Percentage of PBMCs that were T cells was 44.64 ± 10.61% when CB and CN groups were combined at the pre intervention time point and ranged from 19.7-67.9%. There was no main effect of group or time, or group by time interaction with respect to T cell percentage of PBMC. However, T cell percentage in PBMC increased in CB group (1.5%) and in CN (8.3%) pre to post intervention.

Table 5

Peripheral Blood Mononuclear Cell Frequency

	CB-Pre	*CB-Post*	*CN-Pre*	*CN-Post*
NKT (CD3+/CD56+; %)	4.16 ± 1.48	3.33 ± 1.79*	5.07 ± 3.70	3.79 ± 1.33*
NKC (CD3-/CD56+; %)	9.46 ± 6.07	9.35 ± 4.93	8.46 ± 4.44	8.68 ± 3.13
T Cell (CD3+/CD56-; %)	46.98 ± 11.76	47.69 ± 7.57	41.71 ± 8.54	45.48 ± 10.62

Note. Values presented are mean ± SD. Abbreviations: *NKT* Natural Killer T Cell, *NKC* Natural Killer Cell, *%* percent, *CB-Pre* Cannabidiol Pre Intervention, *CB-Post* Cannabidiol Post Intervention, *CN-Pre* Control Pre Intervention, *CN-Post* Control Post Intervention. *significant time effect (p=0.048)

Natural Killer Cell Cytotoxicity

A visual representation of cytotoxicity assay gating strategy is presented in Figure 2 and a representation of the differences in fluorescence intensity analysis between mean and median fluorescence intensity is presented in Figure 3. At the pre intervention time point, there were no differences between CN and CB with respect to median fluorescence intensity measures of NKC cytotoxicity (Table 6). There was a significant main effect of time, where pre values were 42.8% lower than post values at the 1:1 PBMC: K562 ratio (p=0.047) when NKC cytotoxicity was

evaluated using mean fluorescence intensity. While not statically significant, post intervention CN median fluorescence intensity values at E:T ratios of 5:1 and 10:1 were increased 19.9% and 8.5% respectively, with 20:1 value decreasing by 5.1% when compared to pre intervention CN values. When data were normalized to K562 only controls, a significant group by time interaction was found at the 5:1($p=0.006$; Post: CB 52.6 ± 18.9% CN 71.9 ± 23.7%) and 10:1 E:T ratio ($p=0.033$; Post: CB 34.0 ± 10.7% CN 51.4 ± 20.0).

Table 6

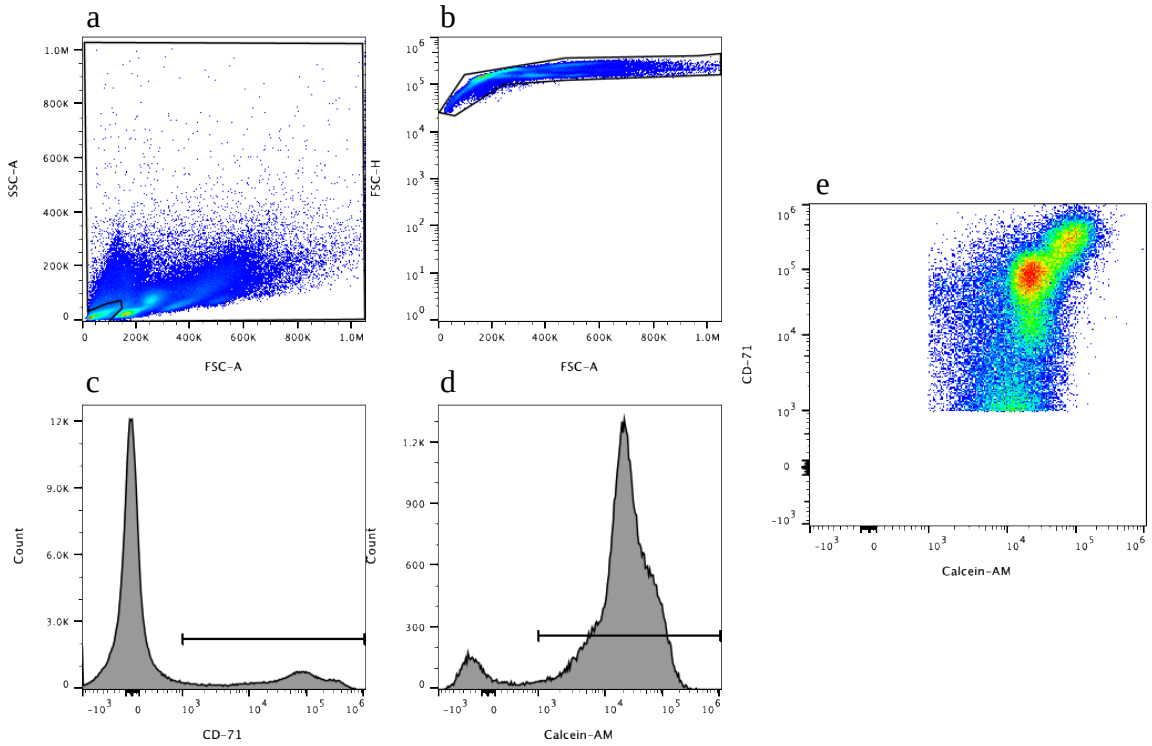
Natural Killer Cell Cytotoxicity Median Fluorescence Intensity

	CB-Pre	*CB-Post*	*CN-Pre*	*CN-Post*
K562 Only	94,573 ± 40,179	117,318 ± 98,453	127,306 ± 75,410	112,166 ± 81,798
1:1	77,688 ± 11,083	117,275 ± 118,030	84,561 ± 13,361	121,427 ± 108,834
5:1	66,281 ± 18,130	53,503 ± 25,954	61,400 ± 20,949	73,642 ± 53,680
10:1	46,902 ± 18,503	36,815 ± 25,420	46,848 ± 24,185	50,847 ± 35,891
20:1	36,531 ± 15,674	29,738 ± 16,552	34,657 ± 16,820	32,904 ± 21,850

Note. Median fluorescence values are presented as mean ± SD. Abbreviations: *CB-Pre* Cannabidiol Pre Intervention, *CB-Post* Cannabidiol Post Intervention, *CN-Pre* Control Pre Intervention, *CN-Post* Control Post Intervention.

Figure 2

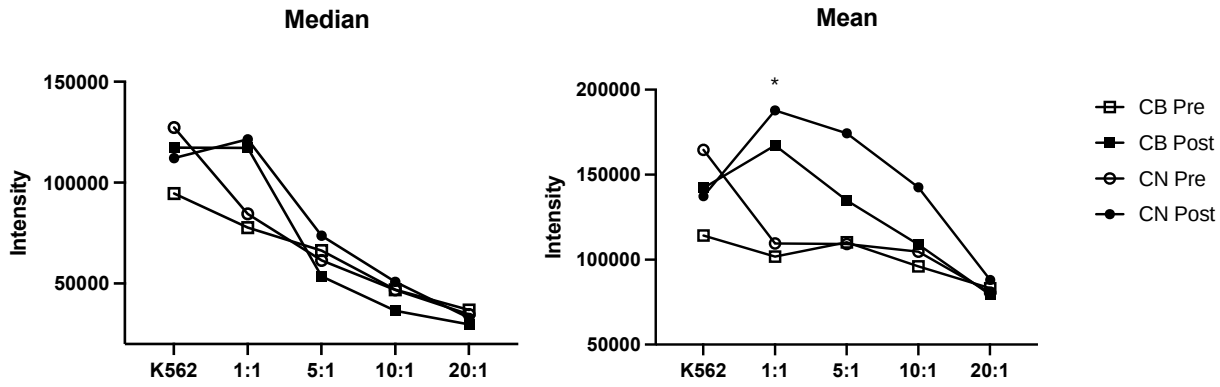
Natural Killer Cell Cytotoxicity Assay Gating Strategy Using a Representative Sample



Note. a) gated with debris removed, b) singlets gate, c) CD71+ gate, d) Calcein-AM+ gate, e) Calcein-AM+/CD71+ for analysis

Figure 3

Differences in Natural Killer Cell Cytotoxicity



Note. Presented As (a) Median and (b) Mean Fluorescence Intensity. *Abbreviations: K562* K562 leukemia cells only, *CB-Pre* Cannabidiol Pre Intervention, *CB-Post* Cannabidiol Post Intervention, *CN-Pre* Control Pre Intervention, *CN-Post* Control Post Intervention. *significant main effect of time in 1:1 E:T Ratio Pre-Post (p=0.047)

CHAPTER V

DISCUSSION AND CONCLUSION

Over the course of this investigation, we observed no significant changes in mental health parameters. Additionally, no alterations were found in quantitative measures of sleep quantity and quality; however CB experienced improvements in perceived sleep quality as defined by measures of LSEQ. Furthermore, no significant changes between groups were found in PBMC population percentages. Finally, no changes in NKC cytotoxic function were observed in the raw median fluorescence intensity values; however when data was normalized to control and expressed as % cell viability, there were significantly fewer (%) viable cancer cells in individuals consuming CB when compared to CN. Therefore, the anxiolytic action of CBD that has been shown by previous investigations (García-Gutiérrez et al., 2020; Zuardi, Cosme, et al., 1993) were not found in the current investigation. Additionally, CBD has been previously hypothesized to improve sleep quality (Shannon et al., 2019), and while the device measured parameters of sleep quantity and quality were unchanged, measures of perceived sleep quality significantly improved in CB throughout this investigation. Furthermore, changes in immune cell population percentages of PBMC were smaller in magnitude in CB when compared to CN which may be linked to the proposed anti-inflammatory effects of CBD and/or the effects mental health and sleep have on immune cell population percentages of PBMCs (Atalay et al., 2020). Finally, CB experienced no change in NKC cytotoxic function pre intervention to post intervention, whereas CN experienced a non-significant decrease in NKC cytotoxic function as represented by increased K562 cell viability. This change may suggest a potential reduction in NKC function in

CN when compared to CB which can be further exasperated when cytotoxicity data is normalized. In this situation, CB had significantly less K562 cancer cell viability when compared to CN.

Mental Health Assessment

Over 260 million people worldwide suffer from mood disorders leading to reductions in quality of life and activities of daily living (García-Gutiérrez et al., 2020). Depression and anxiety are types of mood disorders, which in 2020, effected 33.7% and 31% of the population, respectively (Salari et al., 2020). Common side effects of depression and anxiety are increased fatigue and decreases in QOL which can result in overall lower mental health status. Given the increased prevalence of depression and anxiety, and the hypothesized anti-depressant and anxiolytic properties of CBD, we sought to investigate whether 8 weeks of daily CBD (50mg/d) was sufficient to change depression and anxiety scores, in addition to other outcomes related overall health effects, in healthy college aged individuals. The average dosage of 1.43mg/kg used for the current investigation was far below the FDA approved dosage for Epidiolex (2.5-25mg/kg) (Epidiolex, 2022). As stated earlier, the dosage used for this investigation was to ensure participant safety and limit side effects; however, the lower dose used when compared to Epidiolex may have caused lack of alterations in the following outcomes. All pre intervention scores for all mental health measures, including assessments for depression, anxiety, fatigue, and quality of life were not significantly different as assessed by an independent-sample t-test, indicating a homogenous mental health status for all participants at the start of the intervention.

Depression

Participants completed the BDI, which provided an assessment for depression. This survey contains 21 questions, and each is scored 0-3 for a maximum total score of 63 points.

Participant total score corresponded to a depression category, with the lowest depression category ranging from 1-10 described as “These ups and downs are considered normal,” to the highest depression category ranging >40 described as “Extreme depression.” Mean pre intervention and post intervention BDI scores for CB and CN were within the lowest depression category labeled “These ups and downs are considered normal” with a mean combined pre intervention score of 4.6 ± 3.8, and post intervention score of 4.4 ± 3.7. Cannabidiol supplementation has previously been found to reduce measures of depression (García-Gutiérrez et al., 2020), although here, CBD failed to further improve depression scores.

This lack of a measurable anti-depressive effect may be attributed to the low depression scores at the pre intervention time point thereby allowing very little room for improvement. As an inclusion criterion, participants were required to meet the ACSM physical activity guidelines of 150min of moderate intensity physical activity per week, or 75min of vigorous intensity physical activity per week (Pescatello et al., 2013). This inclusion criteria may have played a role in the low overall prevalence of depressive symptoms in our current population. For instance, when investigators prescribed 45min of moderate intensity aerobic exercise 3 days per week to a clinically depressed population, improvements in their depressive symptoms were similar when compared to anti-depressant medication (venlafaxine XR) (Gujral et al., 2019). Furthermore, populations with no symptoms of depression who were currently preforming physical activity on a regular basis during the Covid-19 pandemic had a 12-32% lower chance of presenting with depressive symptoms (Wolf et al., 2021).

The dose of CBD and duration of CBD administration may have also played a part in the unchanged depression scores. When healthy subjects consumed 200mg CBD daily for 10 weeks, a 75% reduction in BDI scores were found pre intervention to post intervention (Beale et al.,

2018). Conversely, in humans, a lower dose of 80mg CBD for 6 days resulted in no change in measures of depression (Allsop et al., 2014). Based on previous literature demonstrating changes in depressive symptoms following CBD consumption, we believe that the lack of change in CB when compared to CN likely stems from a combination of low baseline depression scores, and the lower dose of CBD administered throughout this intervention.

Anxiety

Unlike the anti-depressive effects of CBD, the anxiolytic function of the compound has been thoroughly investigated, and is shown to act in an inverted U-shaped dose-response manner (Zuardi et al., 2017). Humans were given 100, 300, and 900mg of CBD, prior to a simulated public speaking intervention. Subjects given 300mg experienced significant decreases in anxiety scores during the public speaking event, with scores matching those of clonazepam, an approved anti-anxiety drug. Subjects given 100 and 900mg of CBD experienced no reduction in anxiety during the public speaking event, which further supports the inverted U-Shaped dose response relationship between CBD and anxiety. In the current investigation, a much lower dose of CBD at 50mg/d elucidated no change in anxiety scores. However, at the pre intervention time point, CB began the intervention at a higher state of anxiety, as defined by the mean group anxiety score, compared to the CN (CB: 7.9 ± 6.6; CN: 4.3 ± 4.7). More specifically, CB group's mean pre intervention score placed this group into a mild classification of anxiety category while the mean for CN placed this group in a no to low risk of anxiety category (Spitzer et al., 2006). Following the intervention, levels of anxiety were reduced 26.5% in CB which moved the mean of this group closer to the scores of CN (CB: 5.8 ± 4.2; CN: 3.7 ± 4.3), while anxiety in CN group remained unchanged. Anxiety scores in CB did not decrease enough to change their classification from mild anxiety to no to low risk of anxiety categories; however, the scores were

reduced to the lower range of the mild anxiety classification compared to the upper range of the category at the start of the intervention. Previous literature has demonstrated anxiolytic benefits of CBD following both acute and chronic administration conditions (Gujral et al., 2019; Salari et al., 2020; Specter et al., 1989); however due to the lack of significant change in the current investigation the current dose of 50mg/d was likely insufficient to alter anxiety levels.

While cannabidiol follows an inverted U-shaped curve with low and high doses of CBD resulting in a less effective anxiolytic response (Almeida et al., 2013; Guimarães et al., 1990; Onaivi et al., 1990), significant reductions in anxiety have been shown following CBD administration in doses ranging from 25mg/d-600mg/d (Freeman et al., 2019; Zuardi, Guimarães, et al., 1993), however even with 50mg/d falling within the range of 25-600mg/d no changes in CB were found. Furthermore, CBD has a relatively low bioavailability ranging from 6-19% (García-Gutiérrez et al., 2020); however, previous literature has demonstrated that chronic administration of CBD at lower doses can still produce anxiolytic effects when compared to acute CBD administration (García-Gutiérrez et al., 2020). It is possible that the dosing conditions may have affected CBD bioavailability, which may have ultimately played a role in the variability of these anxiety-related responses.

To ensure optimal CBD bioavailability in the current investigation, various steps were taken. The vehicle used in this study was a coconut derived MCT and chosen as a method to increase CBD's bioavailability. In addition, participants consumed the CBD in a fed state, which also causes up to 3 fold increases the CBD bioavailability (Millar et al., 2018; Zgair et al., 2016, 2017). Given that an acute dose of 300mg CBD is sufficient to induce anxiolytic effects, we took these steps to ensure the optimal bioavailability of CBD in addition to administering the CBD daily for 8 weeks. These efforts in the present study were still insufficient to induce optimal

anxiolytic effects. Furthermore, the change in anxiety caused by CBD may also be less pronounced due to the slightly higher rates of other comorbidities associated with anxiety in CB.

Previous literature demonstrates at least a 50% prevalence of other comorbidities, including, depression, irritability, impaired memory, loss of motivation, disrupted sleep, tiredness, and withdrawal from others in patients suffering from anxiety (García-Gutiérrez et al., 2020). The presence of one or more of these comorbidities reduces the effectiveness of anxiety treatment (García-Gutiérrez et al., 2020). Consequently, one common comorbidity of anxiety is depression, in which CB experienced a slightly higher increase in depression scores (5%) compared to the decrease in CN (15%) from pre intervention to post intervention; however this difference was non-significant. Furthermore, disrupted sleep is also a comorbidity of anxiety and results in less effective anxiolytic treatments. It is possible that the 10min decrease in CB TST from pre intervention to post intervention potentially led to a less effective anxiolytic effect. Therefore, while the reductions in anxiety were nonsignificant in CB, potential comorbidities such as slightly higher levels of depression and reductions in TST may have blunted the potential for CBD to affect anxiety.

Even though the current dose of 50mg/d CBD falls within a dosage range in which anxiolytic effects have been previously shown and the CBD was delivered using methods to increase bioavailability, the decreases in anxiety scores which occurred over the course of the intervention remained non-significant. While there was a reduction in anxiety lowering scores from mild anxiety to the cusp of low-risk anxiety, it is possible that lower baseline scores of anxiety and slightly higher rates of comorbidities may be the cause of non-significant changes found in CB when compared to CN.

Fatigue

One of the main side effects of CBD use in a variety of populations is lethargy or fatigue; however, its prevalence is relatively low. The majority of studies observing fatigue as a side effect used 10-20mg/kg of CBD (Epidiolex) with fatigue reported in only 12% of the participants in these clinical trials (Brown & Winterstein, 2019; Epidiolex, 2022). In the current investigation, changes in fatigue were not observed in CB compared to CN. Along these same lines, 24 female NCAA athletes consuming an acute dose of 5mg/kg CBD, equating to approximately 350mg of CBD, experienced no changes in fatigue measured by the visual analog fatigue scale (Crossland et al., n.d.). While the results of the current investigation failed to detect changes in fatigue, previous clinical trials using a higher dosage of CBD ($\geq$10mg/kg) reported changes in fatigue. In the present study, 8 weeks of 50mg/d of CBD failed to produce changes in fatigue. This phenomenon may be associated with to the low occurrence of fatigue symptoms observed in previous clinical trials in addition to the lower dosage of CBD administered in the current investigation.

Quality of Life

Quality of life was assessed using the 66-item, 1-6 scale Ferrans and Powers QOL index. In the current investigation, we failed to observe any change in QOL between groups (CBD: pre: 19.0 ± 1.9 post: 19.4 ± 1.3; PLA: pre: 20.0 ± 1.5 post: 20.2 ± 1.3). This was the first investigation evaluating the effects of CBD on a healthy, active population. The high quality of life, with little room for improvement, reported by study participants at the pre intervention time point may be the reason for the lack of change in QOL. Most studies that have observed QOL following a CBD intervention have utilized populations with chronic illnesses, which are actively hindering QOL (Capano et al., 2020).

Mental Health Conclusion

Overall, this investigation found no changes in depressive symptoms which may have been likely caused by the combination of participants actively exercising, low levels of depressive symptoms, and a lower dose of CBD when compared to previous investigations. Furthermore, CBD did not produce a reduction in measures of anxiety, even with the lowering of anxiety levels from mild anxiety towards the lowest category of no to low risk of anxiety. This lack of reduction in anxiety is likely due to the lower dose of CBD used, the low bioavailability of CBD, slightly higher comorbidities found in CB group, and the low baseline levels of anxiety reported at the start of the intervention. Additionally, fatigue and QOL were unchanged from pre intervention to post intervention in both CB and CN which was likely due to the low prevalence of fatigue prior to CBD consumption in addition to the high quality of life scores allowing for little improvement.

Sleep Quality and Quantity Outcomes

Cannabidiol's effects on sleep vary. Following its administration, acute doses of CBD have biphasic effects. More specifically, CBD is stimulatory at lower doses and sedating at higher doses (Chagas et al., 2013; Zuardi, 2008). Furthermore, many studies in which CBD modulates sleep have extreme variability in the populations used, CBD doses, routes and conditions of CBD administration and reported study outcomes. In the current investigation, sleep was measured using 3 common outcomes used in reporting for sleep quantity and quality assessment. These outcomes include TST measured as minutes asleep per night, WE measured as the number of awakenings per night, and SE measured as minutes asleep divided by minutes in bed multiplied by 100 to get SE as a percentage. We found no statistically significant results

with respect to sleep quantity or sleep quality in this study; however TST and WE slightly decreased in CB (2.2% and 5.4%) and increased in CN (3.8% and 4.3%) .

A common method for sleep assessment is wrist actigraphy, with measurement accuracy of >80% when compared to PSG (Marino et al., 2013). Slightly higher TST was observed in CB at the pre intervention time point when compared to CN though this difference was not statistically significant (CB:388 ± 55min; CN: 385 ± 83min). At the post intervention time point, CB and CN experienced a 10min reduction in TST (379 ± 57min) and a 15min increase (400 ± 59min), respectively. This slight decrease in TST in CB may be attributed to the aforementioned stimulatory effect of lower doses of CBD (Chagas et al., 2013; Zuardi, 2008). For instance, when CBD was acutely administered at 15mg/d as an oromucosal spray to healthy college aged individuals, study participants experienced alerting properties and increased wakefulness (Nicholson et al., 2004). Furthermore, when CBD is administered in healthy volunteers at higher doses (600mg), and in populations with insomnia (160mg/d), CBD has sedative effects as defined by increases in TST and less frequent awakenings (Carlini & Cunha, 1981; Zuardi, Guimarães, et al., 1993). These outcomes support the findings of the present study.

As mentioned previously, the present study used a dose of 50mg/d which was likely too low to elicit any response, stimulatory or otherwise, which was corroborated by our observation of no changes in WE. Furthermore, when examining time in bed, CB experienced a 10min decrease (Pre: 442 ± 63min; Post 435 ± 67min), which coincides with the 10min reduction in CB TST. Therefore, it is possible that TST was reduced following the 8-week CBD intervention due to less time spent in bed from pre intervention to post intervention and not due to a lower dose of CBD, which may have resulted in stimulatory effects.

When observing WE in the current investigation, CB experienced a slight decrease from pre to post measurement (pre: 27 ± 6; post: 26 ± 9), with a similar minute increase pre to post in CN (pre: 25 ± 9; post: 27 ± 7); however, these differences were not statistically significant. A lack of change in WE during this investigation is supported by previous literature observing WE following acute administration of CBD at 5 and 15mg. Specifically, investigators reported no changes in WE following acute oromucosal administration of 5 (WE: 12.50) and 15mg (WE: 14.25) CBD when compared to placebo (WE: 13.63) (Nicholson et al., 2004). It should be mentioned that this study used EEG for the evaluation of sleep quantity and quality, which remains the gold standard for sleep variable assessment. Even with differences in measurement tools, the variations in WE in the current investigation, when compared to Nicholson et al., were consistent, thereby further corroborating the findings from the present study in addition to validating the use of wrist actigraphy. In summary, the slight decrease in WE from pre to post intervention in the present study provides support for the idea that CBD at 50mg/d does not cause a stimulating effect that would lower TST, as previously mentioned. The lack of change in sleep quantity and quality variables is likely due to the lower dose of CBD, which fails to produce the sedative effects of higher doses of 160mg and 600mg of CBD (Almeida et al., 2013; Gaston et al., 2019), and manages to avoid the stimulatory effects of lower dose of CBD.

In similar fashion, the 8-week CBD intervention had no effect on SE when compared to CN. Even though TST decreased by approximately 10min in CB when compared to CN, SE did not change (CB: pre: 88.0 ± 1.5% post: 87.5 ± 2.1%; CN: pre: 88.9 ± 1.3% post: 88.7 ± 1.6%). Together, these results suggest that the decrease in TST in CB when compared to CN was likely due to less time spent in bed compared to a low dose of CBD causing a stimulatory response. If a low dose of CBD caused a stimulatory response as measured by a decrease in TST, there would

have been differences in SE with an increase in WE. In fact, SE remained unchanged, there was a slight decrease in WE and time spent in bed, which likely resulted in the 10 min decrease in TST in CB.

Sleep was also measured using the LSEQ. The LSEQ is a 10 question, VAS that measures overall perceived sleep quality, or can assess sub-measures of sleep, separating sleep into 4 categories (Tarrasch et al., 2003). These 4 categories are GTS, QOS, AFS, and BFW. Throughout this investigation there were no discernable changes in GTS, larger changes measured in QOS and AFS, no changes in BFW, and changes in mean LSEQ scores (Figure 1). The intervention period resulted in a larger (65.1%) increase post intervention in CB group when compared to CN (12.7%) in QOS scores as measured by the LSEQ with AFS increasing by 48.3% in CB compared to a 1.5% decrease in CN, and LSEQ increasing 27.5% in CB compared to a 3.5% increase in CN following the 8-week intervention. A significant effect of time was found in QOS ($p=0.018$) indicating an improvement in QOS in both groups. A significant group x time interaction was found in AFS ($p=0.05$), with the LSEQ having a significant effect of time ($p=0.023$). While no significant change in device measured sleep was detected, the significant change in subjective sleep measures indicates CBD use is beneficial for improvements in sleep quantity and quality. Interestingly, 42.5% of CBD users report benefits in their subjective sleep quality, with 54% of users taking 50mg/d or less (Moltke & Hindocha, 2021).

In the current investigation, participants recorded their perceived benefits of supplementation with respect to sleep and reported “deeper sleep”, “more sound sleep” in addition to a few responses of “woke up around 2-3am each morning”. These responses coincide with the improvements found in subjective sleep quality, but not with the results of the device measured sleep. Both CBD and THC have the ability to effect sleep architecture such as time

spent in REM sleep and various sleep stages(Cohen-zion et al., 2010; Liguori et al., 2021). These changes could have remained undetected by the broader measures of sleep quantity and quality utilized in the current investigation. Due to the inability of the present study's sleep measurement tools to assess sleep stages and other variables related to sleep architecture, further exploration is required to fully elucidate this question.

Our results indicate that CBD did not affect device measured sleep quantity and quality but influenced perceived measures of sleep quality through LSEQ. Previous investigations have shown improved sleep quantity and quality enhances various aspects of mental health; however, without device measured changes in TST, WE, and SE, perceived sleep measures may be helpful. Previous literature investigating perceived sleep quality using the Pittsburgh Sleep Quality Index (PSQI) in Korean men reported significant associations with perceived sleep quality and mental health. Authors found that perceived sleep quality was strongly correlated with depression ($r=0.35$, $p<0.001$), with sleep duration ($r=0.13$, $p<0.001$) and sleep efficiency ($r=0.13$, $p<0.001$) being weakly correlated with depression (Chang et al., 2014). These results suggest that perceived sleep quality is strongly related to outcomes of mental health. In the current investigation, a linear regression analysis was conducted between variables previously thought to have a linear relationship, to investigate whether perceived measures of sleep quality were related to outcomes of mental health. We found that LSEQ responses were significantly correlated with both depression ($R^2=.308$, $p=0.003$) and anxiety ($R^2=.296$, $p=0.003$). Additionally, R^2 values for the linear regression analysis between LSEQ and depression as well as anxiety indicate that perceived sleep quality measured using LSEQ accounted for 30.8 and 29.6 % of the variation in depression and anxiety scores respectively. These linear regression results may be slightly inflated due to various social and lifestyle factors, which can act as

covariates effecting mental health. These factors may include, emotional stress brought on by examination stress or stress linked to the covid pandemic, which our study population consistently endured throughout the academic semester. Nonetheless, these results represent a clear association between perceived sleep quality and measures of mental health. To further this analysis, we also examined whether device measured sleep quantity and quality had a similar relationship with measures of depression and anxiety but did not find significant results.

Overall, CBD supplementation did not influence measures of TST, WE, and SE. Furthermore, CBD effected perceived measures of sleep quality. These results indicate that even though CBD failed to effect device measured sleep quantity and quality, it may have increased participants perceived sleep quality. When taken together CBD at 50mg/d for 8 weeks is likely too low to evoke change in device measured sleep quantity and or quality. However, this regimen may have a positive effect on perceived sleep quality.

Peripheral Blood Mononuclear Cell Populations

Peripheral blood mononuclear cells (PBMCs) are a fraction of whole blood in which the red blood cells and granulocytes are removed by density gradient centrifugation leaving T cells, B cells, NKC, dendritic cells, and monocytes (Verhoeckx et al., 2015). Percentage of cells included in PBMCs are 10-20% monocytes, 1-2% dendritic cells, 5-20% NKC (CD3-CD56+), 40-60% T cells (CD3+ CD56-) and 1-5% NKT (CD3+CD56+) (Gritzapis et al., 2002; Koreck et al., 2002; Verhoeckx et al., 2015). In the current investigation, mean percentages of NKT, NKC, and T cells were within normal ranges based on average cell distribution in PBMCs (Table 5) (Gritzapis et al., 2002; Koreck et al., 2002; Verhoeckx et al., 2015).

Interestingly, while the mean percentage of immune cell types within PBMC fell within the normal ranges (NKT: 4.56 ± 2.68%; NKC: 9.01 ± 5.33%; T cell: 44.64 ± 10.61%), NKT had

4 samples greater than 5%, NKC had 4 samples below 5% and one sample above 20%, and T cells had 6 samples below 40%. These ranges that fell outside of normal ranges found in PBMC and could be attributed to a multitude of variables including acute exercise training, caffeine consumption, and alcohol consumption. For example, following an acute exercise training session, lymphocyte percentage of PBMC increases within 90min of exercise, then falls below pre-exercise levels resulting in immunodepression (Shaw et al., 2018). Furthermore, circulating lymphocyte populations were found to increase by up to 35% following the consumption of caffeine (Bassini-Cameron et al., 2007). Additionally, acute and chronic consumption of alcohol causes redistribution of PBMC, specifically within NKC and T cell populations (Díaz et al., 2002). To combat the known effects of exercise, caffeine, and alcohol on immune cell population distribution, participants were required to arrive 72h fasted of strenuous activity, and 24h fasted from caffeine and alcohol. While these measures were put into place to help control the variability of immune cell percentage in PBMC, there was no way to ensure our participants were fasted other than word of mouth.

In the current investigation, a significant main effect of time was found in NKT cell population distribution. There was a significant, decrease in NKT population from pre (4.56 ± 2.68%) to post (3.62 ± 1.60%) intervention regardless of supplementation group. Additionally, all immune cell percentages of PBMC for CN had a greater degree of change (NKT: 21.6% decrease; NKC: 2.5% increase; T cell: 8.3% increase) than in CB group (NKT: 19% decrease; NKC: 1.1% decrease; T cell: 1.5% increase), which could possibly be linked to CBD action as an anxiolytic, sleep-aid, and anti-inflammatory supplement aiding to resist change in immune cell percentage of PBMC (Burstein, 2015a; Mantur & Vasudeva, 2010; H. Suzuki et al., 2018).

Psychological stressors such as academic examinations, bereavement, public speaking, daily hassles and life events have been previously found to cause psychoneuroendocrinological alterations leading to increases in immune cell populations in peripheral blood (Mantur & Vasudeva, 2010; Schmid-Ott et al., 2001). Populations such as NKT, NKC, neutrophils, monocytes, B cells, and CD4+ T cells increase in their percent distribution in PBMC following incidences of psychological stressors in both a laboratory and field settings structured to explore the effects of real-life stressors (Biselli et al., 1993; Brosschot et al., 1992; Gerritsen et al., 1996; Mantur & Vasudeva, 2010; Schmid-Ott et al., 2001). In the current investigation, there were increases in NKC and T cell, and decreases in NKT percentage distributions in PBMCs in CN which may be attributed to acute and chronic psychological stress (Mantur & Vasudeva, 2010; Schmid-Ott et al., 2001) brought upon by academic stress as well as the stress of the Covid-19 global pandemic. In CB, NKT decreased their percentage by 19% compared to a 21.6% decrease in CN, NKC decreased their percentage by 1.1% compared to a 2.5% increase in CN, and T cells increased by 1.5% in CB compared to an 8.3% increase in CN. These attenuated changes in NKT, NKC and T cell percentage of PBMCs may be attributed to the anxiolytic effects of CBD reducing levels of psychological stress in turn reducing the percent change in NKT, NKC and T cell populations, however no significant decreases were found between CB and mental health status in the current investigation. When college students were assessed for levels of stress, 52.1% of students of the 145 individuals surveyed indicated relatively high levels of stress during the course of a “typical semester,” with 55.6% of the same population responding to feeling stressed “all or most of the time” (Hudd et al., 2000). Data collection for the current investigation occurred between the spring semester of 2021 through the spring semester of 2022, during the Covid-19 global pandemic. Semesters throughout this time were not considered a

"typical semester". When 2031 students were surveyed during the Covid-19 global pandemic, 71.26% indicated their stress/anxiety levels had increased during the pandemic, with 48.14% having moderate-to-severe levels of depression measured using the Patient Health Questionnaire-9, and 38.48% showed a moderate-to-severe level of anxiety measured using the GAD-7 (Wang et al., 2020). While stressors such as academics and global pandemics cause changes in NKT, NKC and T cell percentages of PBMCs, CBD may have helped to attenuate the changes in immune cell percentages. Moreover, ratings of anxiety in CB compared to CN were higher at baseline and had a greater degree of change from pre intervention to post intervention with CB reducing anxiety scores by 26.5% when compared to 14% in CN. While CB still had a higher rating of anxiety post intervention, the larger reduction in anxiety from baseline may be linked to CBD attenuating changes in NKT, NKC and T cell distributions when compared to CN. However, the lack of significance in anxiety scores from pre to post intervention suggests that more research should explore this relationship in the future.

In addition to the potential for anxiolytic effects of CBD to changes in immune cell percentages of PBMC, sleep quantity and quality also cause alterations in the percentage of immune cell populations in PBMC (Cover & Irwin, 1994). As previously discussed, there were no changes in device measured sleep quantity and quality variables TST, WE, and SE between groups; however, there were significant changes in perceived sleep quality as assessed through the LSEQ, between CB and CN groups. It is possible that the improvements in sleep quality through the actions of CBD also contributed to the variations found in NKT, NKC and T cell percentages of PBMC between groups over the course of this investigation. For example, perceived sleep quality as assessed by the PSQI in healthy populations and populations with major depressive disorder were evaluated on their percentage of NKT, NKC and T cells in

peripheral blood. Investigators found that participants who scored lower on the PSQI, indicating lower sleep quality, had increased percentages of NKC and T cells in peripheral blood, but NKT percentages did not differ (H. Suzuki et al., 2018). In the current investigation, CB experienced significant increases in perceived sleep quality when compared to CN. The CB also experienced a decrease in NKC percentage of PBMC (1.1%) whereas CN experienced an increase in PBMC percentage (2.5%). Furthermore, CB experienced an increase (1.5%) in T cell percentage of PBMC while CN experienced a larger increase (8.3%). The decreases in NKC percentage in CB group, along with the smaller increase in T cell percentage, coincides with previous literature indicating that a lower sleep quality results in an increase in NKC and T cell percentages in PBMC. However, reduced sleep quality was not linked to changes in percentage of NKT population in PBMC, yet CB experienced a more modest decrease in NKT populations from pre (4.16 ± 1.48%) to post (3.33 ± 1.79%) when compared to CN (Pre: 5.07 ± 3.70%; Post: 3.97 ± 1.33%). These findings corroborate our findings that subjects consuming CBD had improved perceived sleep quality which may have helped to resist changes more effectively in immune cell population percentages of PBMC when compared to control.

Further justification for the differences observed in immune cell population percentages of PBMC in CB compared to CN are likely related to the anti-inflammatory properties of CBD. No previous literature has assessed immune cell population percentages of PBMC in humans consuming CBD; however, CBD supplementation in humans elicits anti-inflammatory effects (Burstein, 2015b) which may be related to the changes shown in the current investigation. Previous investigations in animal models suggests that CBD supplementation elicits similar effects on immune cell population percentages of whole blood (Burstein, 2015b). Cannabidiol, when administered i.p. at 5mg/kg to adult Wistar rats, reduced the total number of leukocytes in

peripheral blood as well as reduced the total number of T cells, B cells, and CD4+ T cells; however, NKC and NKT were unchanged (B. Ignatowska-Jankowska et al., 2009). Furthermore, 46d of CBD supplementation at 10mg/kg/d administered i.p., in 40 male A/J mice resulted in decreased CD3+ and CD4+ T cell percentages in PBMC, in addition to large attenuations in CD3+ and CD4+ T cell mediated inflammatory responses. More specifically these inflammatory responses were associated with reductions in IL-6, INF-γ, and IL1β gene expression in CD3+ and CD4+ cell types (Lee et al., 2016). In another study, human PBMCs were extracted from populations after an acute dose of 30mg of CBD, and then stimulated with LPS to observe the cells' immune response. The PBMCs from subjects consuming CBD experienced a significant suppression of the pro-inflammatory biomarker TNF following LPS stimulation when compared to control (Hobbs et al., 2020). Results of these previous investigations illustrate that CBD elicits reductions in immune cell migration into the peripheral blood as well as anti-inflammatory properties in both human and animal models.

College students are under large amounts of psychological stress now, more than ever before, due to the typical stressors of higher education in combination with navigating a global pandemic. This stress can lead to reductions in sleep quality (Van Laethem et al., 2015). Stress and poor sleep quality has been shown to alter immune cell population distributions in PBMCs. Therefore, it is possible that 8-weeks of CBD supplementation may have led to improved measures of mental health and sleep quality, and reductions in inflammation resulting in the attenuation of NKT, NKC and T cell populations in PBMCs when compared to CN.

Cytotoxicity Analysis

Cellular cytotoxicity was assessed through co-cultures of PBMC: K562 cell ratios at 1:1, 5:1, 10:1, and 20:1, and measured using median fluorescence intensity. Post intervention mean

fluorescence intensities for CN at the 1:1 ratio were significantly greater than mean fluorescence intensity values at the pre intervention time point for the same ratio, and, while not statistically significant, this same phenomena is observed at a 5:1 ratio (Figure 3). However, when assessing the same ratios and time points using median fluorescence intensity, a less dramatic change in intensity was discovered. Mean and median fluorescence intensity is measuring the same events in flow cytometry, but their values differ due to the calculations for finding mean and median (Best, 2019). As stated above, mean fluorescence intensity will lead to increased skewedness of values due to the measurement on a log scale exponentially shifting the mean higher when compared to how the median is calculated. For this reason, median fluorescence intensity was used to ensure non-biased analysis of the functional assay for the duration of this discussion.

Our results indicate that at control K562, 1:1, 5:1, 10:1 and 20:1 E:T cell ratios, median fluorescence intensity was not different between CB and CN. While these results are not statistically significant, upon visual inspection of the changes in cytotoxicity between CB and CN groups, both CB and CN at the 1:1 ratio had a greater median fluorescence intensity when compared pre intervention measures. As the ratios increased above 1:1, the post intervention CB median fluorescence intensity reduced to levels observed in the pre intervention analysis. The CN group remained elevated in the 5:1 and 10:1 ratio, until returning to near baseline at the 20:1 ratio. The higher median fluorescence intensity for post intervention CN indicates more viable K562 cells and in turn, reduced cellular cytotoxicity. This reduction in cellular cytotoxicity, while non-significant may be attributed to changes in stress, mental health, and sleep quality in CN and all of these responses may have been blunted by the consumption of CBD in CB.

In terms of sleep quality, reductions in sleep quality both increase and decrease NKC cytotoxicity (Cover & Irwin, 1994; Dinges et al., 1994). Moderate reductions in sleep quality and

extreme reductions in sleep quality have varying impacts on NKC function as assessed through NKC killing of K562 leukemia cells. One investigation found that increased sleep disturbance scores correlated with lytic units (r=.33), a measure of NKC cytotoxicity, with an R^2 value of 0.16, indicating that sleep disturbances accounted for 16% of the variation found in NKC activity (Cover & Irwin, 1994). On the more extreme side of sleep quality reductions, when individuals were kept awake for 64h, NKC populations in whole blood increased and NKC cytotoxicity increased by 13.9% (Dinges et al., 1994). While these two investigations contradict each other, the results of our investigation seem to be more significantly linked to increased sleep disturbances and reductions in NKC cytotoxicity. At more extreme levels of sleep deprivation, there may be larger physiological alterations at play that may act in a similar fashion to "flight or fight." In previous investigations, when healthy volunteers given a continuous intravenous infusion of adrenaline, an immediate increase in circulating NKC was found and this was accompanied by an increase in NKC activity (Tønnesen et al., 1987). Based on the previous literature and the results of the current investigation, the more positive changes in sleep quality observed in CB when compared to control may have blunted the effects of other daily, external stressors experienced by college aged individuals resulting in a slight, but non-significant decrease in NKC cytotoxicity in CN.

Previous investigations assessing stress and mental health on NKC cytotoxicity link improvements in mental health to an increase in NKC cytotoxicity while declines in mental health are associated with reductions in NKC cytotoxicity. Interestingly, in 33 adult women who participated in an intervention of mirthful laughter in which the laughter group watched a humorous video and the control group watched a tourism video, the laughter group reported reductions in overall stress levels which were linked to improved NKC cytotoxicity, which was

evaluated using the chromium release NKC cytotoxicity assay (Bennett et al., 2003). Conversely, depressive patients have significantly reduced NKC cytotoxicity when compared to non-depressed individuals (Caldwell et al., 1991). In a less extreme example of the link between reductions in mental health and NKC cytotoxicity, 144 patients from an oral surgery outpatient clinic were given the QOL questionnaire and their whole blood was evaluated for NKC cytotoxicity. Patients with greater stress and anxiety about cancer had significantly lower NKC cytotoxicity when compared to patients with a more positive outlook (Koga et al., 2001). Overall, results from previous investigations on mental health and stress indicate that a more positive mental health status as assessed through less symptoms of depression and overall stress result in improvements in NKC cytotoxicity. This finding is in agreement with our current investigation, in that CB experienced non-significant, but larger reductions in anxiety compared to CN, which may have been attributed to the anxiolytic effects of CBD. These larger reductions in anxiety and stress observed in CB may have blunted the negative effects of stress and anxiety experienced by college students.

Overall, the influence of CBD on NKC cytotoxicity remains unclear due to lack of statistical significance between CB and CN. It is important to note that CN post values for NKC cytotoxicity had increased values of median fluorescence intensities when compared to CB pre and post. This represents increased K562 cell viability in CN, through a reduced NKC cytotoxic function. Furthermore, when cytotoxicity data is normalized to control, CB at E:T cell ratios of 5:1 and 10:1 had significantly less K562 % viability when compared to CN. This finding suggests that CBD may be linked to greater cellular cytotoxicity. Given the previous literature showing that moderate reductions in sleep quality can lead to decreased NKC cytotoxicity, the lower perceived sleep quality in CN could be the reason for variation observed in post

intervention CN when compared to CB. Furthermore, while CN scores for depression and anxiety were not significantly different than CB, CB experienced larger reductions in overall mental health status which may be associated with the anxiolytic actions of CBD. This action could have blunted the overall influence of daily stressors observed in college aged individuals, thereby retaining NKC cytotoxic function when compared to CN.

We understand that most of the changes in mental health, sleep quantity and quality, and immune cell number and function presented in the current investigation were not statistically significant. Our discussion of these non-significant differences was not intended to mislead the reader, but to help understand potential reasoning for the differences observed between groups. In the current investigation, dosing of CBD may have been too low to see significant changes or CBD may have had no actions in the measured variables. This is important information for consumers and clinicians alike. More questions related to the appropriate dosing, timing and duration of CBD administration must be explored in the future. The present study was a step in the right direction.

Conclusion

Overall, college students experience their own trials and tribulations, many of which can result in decreases in mental health and reductions in sleep quality, both of which can lead to increased susceptibility to illness through alterations in the immune cell populations and overall function (Mantur & Vasudeva, 2010). Over the course of this 8-week CBD intervention, participants consuming CBD experienced non-significant, reductions in mental health measures when compared to participants taking a vehicle control. Furthermore, measures of perceived sleep quality were found to improve to a larger degree in CB when compared to CN. Decreases in mental health and sleep quality increase the percentage change of NKT, NKC, and T cell

populations in PBMC as well as reduce NKC cytotoxic function (Bennett et al., 2003; Cover & Irwin, 1994; Dinges et al., 1994; H. Suzuki et al., 2018; Wang et al., 2020). Non-significant observations between CN and CB groups indicate that CN had larger percent change in NKT, NKC, and T cell populations in the peripheral blood. Furthermore, non-significant reductions in NKC cytotoxic function were seen in CN, and when data was normalized a significant improvement in NKC cytotoxic function was seen in CB following the 8-week supplementation intervention. Results of the current investigation were predominantly non-significant. While we discuss the changes in mental health, sleep, and immune cell quantity and function and their relationship to CBD, we understand that low doses of CBD may have made the changes non-significant, and/or CBD may not have an effect on these outcome measures. We believe that CBD supplementation may have helped to attenuate decreases in mental health predominantly through its anxiolytic actions, improve perceived measures of sleep, and by doing so, acted to reduce immune cell population changes and retain NKC cytotoxic function. This is valuable information and can be explored in future studies.

Limitations

There are several study limitations that are worth mentioning. Participants were asked to arrive 72h fasted from exercise, and 24h fasted from caffeine, and alcohol. Furthermore, participants were asked to consume the same diet prior to both pre and post intervention blood draws; however, researchers had to rely on verbal confirmation only.

Additionally, the dose of 50mg CBD per day was on the lower end of CBD dosages to elicit mental, physical, and immunological responses. This was the first CBD intervention study at UNC and this dose was chosen with participant safety in mind. Previous literature commonly reports 100mg per day or more which acts to increase the amount of CBD acting on the body due

to the low bioavailability of the compound. Furthermore, the length of the intervention may have been too long to detect changes caused by CBD. The longer intervention time likely caused a tolerance to participants consuming CBD, and the time point at which optimal changes in mental and physical parameters occurred may have been missed. Also, although capsule containers were returned and refilled every 2 weeks, capsule consumption was not directly supervised by a member of the research team.

Commercially available fitness trackers and sleep surveys were used in this study. These measures are not equivalent to the gold standard EEG and PSG sleep measurement devices and only provide a glimpse of the effects that CBD may have on sleep. Furthermore, assessment of NKC cytotoxic function was assessed using PBMCs rather than purified NKC. Although this method is commonly used in scientific research (Chien et al., 2011), a purified cell line would have reduced variability been NKC percentages between subjects.

Lastly, data was collected between the spring semester of 2021 and spring semester of 2022 at the University of Northern Colorado. During this time, the Covid-19 global pandemic had affected the campus community. This situation caused psychological and physiological stress which may have caused alterations in just about all of the primary outcomes associated with this study. In combination with the Covid-19 global pandemic, we were required to take extra precautions when scheduling participants. We found that in participants who had Covid or the Covid-19 within a week of their blood draw, blood properties and immune cell populations were altered. Once the blood was collected in EDTA tubes following the draw, it would clot at extremely high rates, making the blood unusable. Furthermore, if blood was processed prior to it clotting, populations of PBMCs would be denser than the density gradient used for blood separation, again rendering the blood unusable. With these issues in mind, the research team

made efforts to control for the effects of vaccines and COVID-19 by having participants fill out a Covid-19 symptom survey prior to their visits, checking participants temperature at the start of every visit, and recorded participants vaccination dates to ensure all visits were at least 2-weeks after their last vaccination or COVID diagnosis.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL

UNIVERSITY OF
NORTHERN COLORADO

Institutional Review Board

Date:	06/23/2021
Principal Investigator:	Laura Stewart
Committee Action:	**APPROVED – Renewal with Amendment**
Action Date:	06/23/2021
Protocol Number:	2005001624R001
Protocol Title:	CBD, Inflammation, & Natural Killer Cell Study (CINS)
Expiration Date:	06/22/2022

Based on the information submitted, your study is currently: Active - Open to Enrollment. The University of Northern Colorado Institutional Review Board (IRB) for the protection of human subjects has reviewed and approved your renewal with amendment application.

As a reminder, all research must be conducted in accordance with the procedures outlined in your approved protocol.

This approval extends your expiration to the date listed above and approves the following amendments to your protocol:

We are requesting a renewal for this protocol. We have recruited 8 subjects into this project and have not had any serious adverse events. We have slightly adjusted the number of study visits (just to allow for equipment pick up and drop off). We have also changed the compensation. After consulting with Drs. Pullen and Haughian, they have agreed to list our study as an option (among others, as well as a not research opportunity) for extra credit in their courses. We have highlighted all changes to the consent form and study protocol in blue.

- Add/Modify Attachments
- Protocol Permissions

This project will continue to require renewal on an annual basis for as long as the research remains active. The investigator will need to submit a request for Continuing Review at least 30 days prior to the expiration date. If the study's approval expires, investigators must stop all research activities immediately (including data analysis) and contact the Office of Research and Sponsored Programs for guidance.

UNIVERSITY OF
NORTHERN COLORADO

Institutional Review Board

As principal investigator of this research project, you are responsible to:

- Conduct the research in a manner consistent with the requirements of the IRB and federal regulations 45 CFR 46.
- Obtain informed consent and research privacy authorizations using the currently approved forms and retain all original, signed forms, if applicable.
- Request approval from the IRB prior to implementing any/all modifications.
- Promptly report to the IRB any unanticipated problems involving risks to subjects or others and serious and unexpected adverse events.
- Maintain accurate and complete study records.
- Report all Non-Compliance issues or complaints regarding the project promptly to the IRB.

Please note that all research records must be retained for a minimum of three (3) years after the conclusion of the project. Once your project is complete, please submit the Closing Report Form.

If you have any questions, please contact Nicole Morse, Research Compliance Manager, at 970-351-1910 or nicole.morse@unco.edu. Please include your Protocol Number in all future correspondence. Best of luck with your research!

Sincerely,

Michael Aldridge
IRB Co-Chair, University of Northern Colorado: FWA00000784

Silvia Correa-Torres
IRB Co-Chair, University of Northern Colorado: FWA00000784

APPENDIX B

STUDY TIMELINE

Week 0 (Pre-Intervention)	8-week Intervention Weeks 1-7	Week 8 (Post-Intervention)
• Consent Form • Fasted Blood Draw • Sleep Actigraphy • Body Composition • Questionnaires	• Weekly supplement refill • Sleep Actigraphy (week 7)	• Fasted Blood Draw (Visit 5) • Questionnaires (Visit 5) • Body Composition (Visit 5)