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Monocyte Phenotype and Whole Blood Immune Response in Physically Active Chronic Cannabis Users and Non-Users

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MONOCYTE PHENOTYPE AND WHOLE BLOOD
IMMUNE RESPONSE IN PHYSICALLY
ACTIVE CHRONIC CANNABIS
USERS AND NON-USERS

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of
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ABSTRACT

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Some physically active individuals are now advocating for the use of cannabis with exercise for its potential to optimize the immune response. Yet, it remains to be seen whether the chronic use of cannabis products is linked to alterations in immune characteristics, such as monocyte phenotype and function in physically active individuals.

The purpose of this cross-sectional study was to assess resting concentrations of c-reactive protein (CRP) and interleukin-6 (IL-6), monocyte phenotype and lipopolysaccharide (LPS) stimulated production of Interleukin-6 (IL-6) following pre-treated with synthetic cannabinoid 2 (CB2) receptor agonist and antagonist in physically active individuals using cannabis products at least 5-times per week for the past 6-months (CU) compared to physically active individuals who have not used any cannabis products in the past 6-months (NU).

Physically active participants (N=23; n=11 CU and n=12 NU) completed medical history, physical activity, and cannabis use surveys prior to assessment of their height, weight, body mass index (BMI), body fat percentage, resting heart rate and $\dot{V}O_2$max in their initial visit. In a subsequent visit, intravenous whole blood was collected following a 12-hour fast and 72-hours removed from last bout of vigorous exercise. Isolated serum was used to determine resting protein concentration of CRP and IL-6. Monocyte

phenotype was analyzed using an Attune NxT flow cytometer following co-staining of CD-14 and CD-16. Supernatant from whole blood samples diluted with culture medium and stimulated for 24-hours with LPS following 1-hour pre-treatment with CB2 agonist and antagonist at a concentration of 1μM. Collected supernatant was analyzed for IL-6 using an ELISA. Data are presented as mean ± SD and were analyzed using SPSS using unpaired t-tests and ANOVA (α=0.05). Pearson's correlations were calculated to determine meaningful relationships between primary outcome variables.

There were no differences between CU and NU with respect to age, height, weight, BMI, body fat percentage, resting heart rate, or relative $\dot{V}O_2$max. There were no differences between the groups with respect to resting concentrations of CRP or IL-6. Total monocytes per mL of blood was significantly greater in CU (5.08 x 105 ± 1.63 x 105 cells/mL) when compared to NU (3.23 x 105 ± 1.20 x 105 cells/mL) (p = 0.01). The number of classical (CU: 3.77 x 105 ± 1.36 x 105 cells/mL; NU: 2.56 x 105 ± 0.97 x 105 cells/mL, p=0.02) and intermediate monocytes (CU: 7.29 x 105 ± 5.19 x 105 cells/mL; NU: 2.32 x 105 ± 2.18 x 105 cells/mL, p=0.01) were significantly greater in CU compared to NU. Cannabis users had a significantly greater relative percent of intermediate monocytes (CU: 13.89 ± 8.43%; NU: 6.33 ± 4.91%; p=0.02), but there were no differences in classical (76.92 ± 8.48 %) or non-classical (12.73 ± 7.14 %) monocytes between the groups. There were no significant differences in stimulated production of IL-6 between CU and NU groups. Further, when the covariates of age, body fat percent, or monocytes/mL were used, there were no significant differences present between stimulated IL-6 production.

Results from this study suggest that the chronic use of cannabis in physically active individuals may alter monocyte phenotype and count, but this was not related to changes in resting concentrations of inflammatory markers CRP and IL-6, nor does it alter whole blood LPS stimulated IL-6 release.

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CHAPTER I

INTRODUCTION TO THE STUDY

Inflammation is the natural response of the body to invasion by a foreign object or tissue damage. Acute inflammation can last from a few hours to a few days and is commonly perceived as highly beneficial with minor symptoms of swelling, redness, heat and mild pain (1). On the other hand, chronic inflammation can result in pathological development of disease including: type 2 diabetes mellitus (2), cancer (3) and cardiovascular disease (CVD) (4).

Monocytes are immune cells implicated in the initiation and integration of both the innate and adaptive immune response and are recognized as key players in the development and progression of the inflammatory response (1). Monocytes are circulating precursors to tissue macrophages and dendritic cells and are further sub-divided into three separate phenotypes based on their expression of cluster of determination 14 (CD14) and 16 (CD16) (5). Classical monocytes are the most abundant sub-population at 85-95% of total monocytes followed by 5-10% non-classical monocytes and <5% intermediate monocytes (6, 7). Classical monocytes express the phenotype $CD14^{++}/CD16^{-}$ (5) and are the primary producers of cytokines, like interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) (8, 9). These cytokines are typically secreted in response to endotoxins, like lipopolysaccharide (LPS), binding to the CD14 and toll-like receptor 4 (TLR4) complex (6). Non-classical monocytes display the phenotype of $CD14^{+}/CD16^{++}$ (5) and primarily patrol the vasculature for nucleic acids and viruses

(48). Finally, intermediate monocytes display functions of both classical and non-classical monocytes (6, 10) and elevated numbers of these cells are linked to the development of diseases including CVD (10, 11).

There are several biomarkers commonly used in both basic science and clinical environments to characterize the inflammatory status of an individual. C-reactive protein (CRP) is an acute phase protein released from the liver (12) in response to IL-6 binding the IL-6 receptor (IL-6R) and glycoprotein 130 (gp130) activating mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) pathways (13). For nearly two-decades, circulating concentrations of CRP have been used for CVD risk stratification (14, 15). The cytokine IL-6 is also used as a biomarker of inflammatory health in cancer related cachexia and overall survival and quality of life in patients suffering from gastrointestinal cancers (16, 17).

Over the course of the past few decades, research efforts have been focused on identifying methods of controlling or optimizing the inflammatory response and chronic systemic inflammation. Regular exercise has emerged as an effective, long-term intervention. Aerobic and resistance exercise training programs lasting as little as 2 to 6-months lowered circulating CRP, and reduced risk for CVD (18, 19). In addition, these same types of exercise interventions are linked to improvements in the immune response with physically active individuals releasing fewer pro-inflammatory cytokines like IL-6 and TNFα in response to LPS stimulation and reduction of the percentage of intermediate monocytes (20, 21).

Recently, cannabis products have emerged as another potential modality for controlling inflammation; however, exploration of the effects of cannabis on

inflammation are limited due to the schedule 1 status of *Cannabis Sativa* within the United States (U.S.). Increasing localized medical and recreational availability of cannabis products across the U.S. necessitates the need to study the true implications of chronic cannabis use in health and inflammation. The active ingredients within cannabis are known as phytocannabinoids. While there are greater than 100 identified phytocannabinoids within *Cannabis Sativa*, delta-9-tetrahydrocannabidiol (THC) and cannabidiol (CBD) have received the most attention. THC is known for its intoxicating effects while CBD is sought after for its anti-inflammatory effects (22). The chemical structure of CBD mimics that of endogenous cannabinoids (endo-cannabinoids) anandamide (AEA) and 2-arachodonoylglycerol (2-AG) (23). Of the two primary cannabinoid receptors 1 (CB1) and 2 (CB2), CBD is a partial ligand of CB2, which is primarily expressed in immune cells, like monocytes, throughout the periphery (24-26). Because CBD is unable to activate the CB1 receptor (27), which is heavily localized in the central nervous system (CNS) (28, 29), it has few if any intoxicating effects.

Recent findings are fueling anecdotal accounts and advocation for use of cannabis products not only in diseased populations, but as part of the daily routine of individuals who are physically active (30). The majority of individuals claiming that the use of cannabis in conjunction with exercise improves not only their enjoyment, but their performance in that activity as well (30). While previous research does not support a link between chronic use of cannabis and increased cardiorespiratory function during maximal treadmill exercise in trained individuals (31), some individuals are under the impression that the use of cannabis following exercise improves their recovery through the mediation of inflammation (30). Chronic cannabis users that were physically active were at higher

risk for CVD when compared to an equally physically active, non-user control group based on circulating CRP (31); however, other studies suggest that recent cannabis users were more likely to be below the population average CRP (32). In vitro, pre-treatment of macrophages with a synthetic CB2 agonist at a 1 µM concentration resulted in a 25% reduction in released IL-6 in response to LPS stimulation (33). Cannabis use has also been linked to altered monocyte phenotype with decreased circulating CD16$^+$ monocytes in cannabis users (34). Isolated monocytes from cannabis users were also resistant to interferon α (IFNα) induced CD16$^+$ expression (34) and have altered migratory capacity (35); However, whole blood LPS induced IL-6 release and monocyte phenotype have not been assessed in physically active, chronic cannabis users.

Purpose

The purpose of this cross-sectional study was to assess monocyte phenotype in physically active cannabis users (CU) and non-users (NU) and whether the LPS induced release of IL-6 is different in the presence of CB2 agonists and antagonists in the whole blood of CU and NU. Finally, this study sought to explore whether resting, circulating concentrations of serum CRP and are different between CU and NU and related to number of intermediate monocytes. The specific aims for this study were:

A1   Explore monocyte phenotype (classical, non-classical, intermediate) in physically active CU and NU using cell surface expression of CD14 and CD16.

A2   Assess resting concentrations of serum CRP and plasma IL-6 in physically active CU and NU.

A3   Assess whole blood production of IL-6 in non-LPS-stimulated control (control), LPS-stimulated control (LPS), pre-treatment with synthetic CB2 agonist (Ag) LPS-stimulated (Ag+LPS), and pre-treatment with synthetic CB2 antagonist (Ant) LPS-stimulated (Ant+LPS) conditions.

A4   Explore whether any relationships are present between cannabis use, monocyte sub-populations (classical, non-classical, intermediate), resting CRP and IL-6 concentrations and LPS stimulated IL-6 release.

The hypotheses for the outcomes of these specific aims were:

H1   There will be no difference in overall monocyte number between CU and NU, but CU will have an increased number of intermediate monocytes compared to NU.

H2   Resting concentrations of CRP and IL-6 will be elevated in CU compared to NU.

H3   There will be no difference in IL-6 production in control, LPS, or Ant+LPS conditions between CU and NU, but CU will produce significantly more IL-6 than NU in the Ag+LPS condition.

H4   The number of intermediate monocytes will positively correlate with resting CRP and IL-6 concentrations, and the number of intermediate monocytes will positively correlate to measures of cannabis use. No other relationships will be significant.

CHAPTER II

REVIEW OF THE LITERATURE

Inflammation

Inflammation is initiated in response to trauma of tissue or invasion of a foreign

object, organism or pathogen. An inflammatory response is typically characterized by

local swelling, redness and heat due to increased blood flow to the site of injury or

infection (1). This acute response is intended to remove of the cause of the irritation and

promote remodeling and regeneration of the affected tissue. The localized effects of

inflammation can be attributed in part to proteins known as cytokines, which can mediate

both pro and anti-inflammatory actions (1). This acute inflammation, lasting less than a

couple of days, is highly beneficial and promotes removal of foreign pathogens and

damaged tissue while promoting growth and repair of local cells (1). However, chronic,

unresolved inflammation, lasting weeks to years, has been related to the progression and

development of chronic disease including: type 2 diabetes mellitus (2), cancer (3) and

cardiovascular disease (CVD) (4).

Monocytes

Monocytes are circulating leukocyte precursors to tissue macrophages and

dendritic cells and play a key role in initiation of the inflammatory response. Monocytes

can be further classified into three sub-categories known as classical, non-classical, and

intermediate based on their expression of CD14 and 16 (CD16) (36, 37). CD14 is a co-

receptor which can act in tandem with toll-like receptor 4 (TLR4) as pattern recognition

receptors for the bacterial endo-toxin LPS, which is the major component of the outer membrane of Gram-negative bacteria (38-40). The CD14-TLR4 receptor complex can bind LPS independently, but is accelerated in the presence of lipopolysaccharide binding protein (LBP) (38). Activation of this receptor binding complex activates nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) (8). Transcription of inflammatory cytokine genes, including interleukin 6 (IL-6), interleukin 1 beta (IL-1ß) and tumor necrosis factor-alpha (TNFα) are promoted by NF-κB activation (8, 9, 41, 42). In addition, activation of this signaling pathway through LPS increases the expression of TLR4 in a NF-κB dependent manner (43). The protein CD16, also known as fragment of crystallization-gamma receptor-III (FcγRIII), functions as part of the adaptive immune system by binding the Fc domain of immunoglobin g (IgG) antibodies (44) leading to antigen presentation (45). Activation of the CD16 receptor on monocytes can also promote monocyte mediated antibody-dependent cellular cytotoxicity and secretion of pro-inflammatory cytokines like TNFα (46). With the combined functions of pathogen recognition, cytokine secretion, antigen presentation, and cellular cytotoxicity, monocytes are viewed as key integrators of the adaptive and innate immune response.

In healthy individuals, 85-95% of circulating monocytes exhibit relatively high expression of CD14 on the cell surface, while the remaining 5-15% have relatively high cell surface expression of CD16 (6, 7, 47). Classical monocytes are characterized by high plasma membrane expression of CD14 and low expression of CD16, or CD14^{++}/CD16^{-} (37) and are the most abundant sub-population representing 85-95% of all monocytes (6, 7). Compared to non-classical and intermediate monocytes, classical monocytes release significantly more inflammatory cytokines in response to toll-like receptor (TLR)

stimulation (6). Non-classical monocytes represent the second most abundant of the three monocyte sub-types, typically consisting of 5-10% of the total monocyte population (6, 7). These cells exhibit relatively low expression of CD14 and high CD16 or $CD14^+/CD16^{++}$ (37), and patrol vascular tissue for nucleic acids and viruses via TLR7 and TLR8 (48). Intermediate monocytes are the least abundant of the monocyte sub-types (7) and express a phenotype of $CD14^{++}/CD16^+$ (37). These intermediate cells exhibit moderate functions of both classical and non-classical sub-types (6, 10).

Elevated numbers of intermediate monocytes have been implicated in multiple chronic diseases related to inflammation including: CVD (10, 11), rheumatoid arthritis (49, 50) and type 1 diabetes mellitus (51). For example, macrophages and dendritic cells can be found in atherosclerotic lesions, which, in turn, contribute to the inflammatory microenvironment through production and release of cytokines and reactive oxygen species (ROS) (52, 53). Inflamed vessels secrete monocyte chemokines C-C motif ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP1) and C-C motif ligand 5 (CCL5), promoting monocyte aggregation to the area followed by extravasation of the monocyte into the tissue through rolling adhesion (54). Following extravasation, these monocytes can differentiate into lipid rich foam cells that secrete ROS and inflammatory cytokines contributing to plaque formation which can rupture leading to stroke, thrombosis, or infarction (55, 56).

Cytokines

Immune cells secrete synthesized proteins known as cytokines, producing a variety of effects ranging from promotion to suppression of inflammation. One such multifaceted cytokine is (IL-6) which can be produced by muscle (57-59), fibroblasts (60,

61), adipocytes (62, 63) and monocytes/macrophages (64-66). Due to the variety of tissues that produce IL-6, this cytokine was initially identified as B-cell stimulatory factor 2 (BSF-2), hepatocyte stimulatory factor (HSF), and interferon beta 2 (IFN-ß2) until 1989, when it was realized they were all the same protein (67).

IL-6 and Exercise

Multiple studies have observed increased release of IL-6 following exercise (68-71). IL-6 in the circulation is highest immediately following exercise (68-70) and IL-6 returns to normal resting concentrations within 24-hours following intense exercise (69, 71, 72). IL-6 can be secreted from multiple tissues including monocytes/macrophages as a cytokine or skeletal muscle as a myokine. There are also observed increases of IL-6 mRNA in macrophages of regular exercisers compared to sedentary individuals following acute aerobic exercise (73). However, it is still unclear what portion of secreted IL-6 in response to sustained acute exercise is attributed to either immune or skeletal muscle activities.

IL-6 secreted from skeletal muscle is referred to as a myokine, with increased expression and release of IL-6 observed following exercise (58, 59, 74). The highest observed concentrations of IL-6 in the circulation are observed immediately following prolonged exercise (68-70). This increase in circulating IL-6 concentrations during and following exercise is independent of muscle damage as well as concentrations of TNFα (75) and often returns to normal concentrations within 24-hours following exercise (69, 71, 72). Others agree that IL-6 production is dependent on the availability of carbohydrate within the muscle (76, 77). Additionally, IL-6 release following exercise increases hepatic glycogenolysis and induces lipolysis (78). Skeletal muscle also

increases C-C Motif Chemokine Ligand 2 (CCL2) expression following acute exercise (71, 79). In CCL2 knockout mice, the migration of monocytes to muscle following acute injury was impaired and expression of insulin-like growth factor (IGF-1) was dependent on the presence of intramuscular macrophages (79). Increased intramuscular macrophages have been observed following exercise in addition to increased mRNA expression of CCL2 (71). Macrophages can be further classified into two sub types, macrophage 1 (M1) and macrophage 2 (M2). In general, M1 macrophages are typically activated by interferon gamma (IFNγ) or LPS initiating the immune response and release proinflammatory cytokines (1). While M2 macrophages are associated with tissue repair and wound healing through the release of anti-inflammatory cytokines (1). In humans, the number of M2 macrophages has been found to positively correlate with muscle fiber hypertrophy and numbers of muscle satellite cells following 12-weeks of cycle training (80). Further, new research suggests that microRNA-155 and microRNA-21 are key factors regulating macrophage activation and transition within skeletal muscle (81, 82). Together, these findings suggest that skeletal muscle repair following acute injury, like exercise, is dependent on recruitment of monocytes and regulation of M1 and M2 macrophages activity through myokines.

The stimulation of IL-6 production in monocytes occurs when pattern recognition receptors (PRRs), like TLR-4, bind pathogen-associated molecular patterns (PAMPs) like LPS (83). IL-6 can bind to both the soluble and membrane bound IL-6 receptor (IL-6R) which allows for homodimerization of the IL-6/IL-6R complex with glycoprotein 130 (gp130) activating the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) (13). Activation of these pathways leads to

transcription of acute phase proteins like C-reactive protein (CRP) (84) primarily released from the liver (12).

It is now theorized that the pro-inflammatory actions of IL-6 are attributed to the activation of cells by the soluble IL-6/IL-6R complex binding to unbound gp130 on the membrane of cells that do not express IL-6R. This action is also referred to as trans-signaling, IL-6 trans-signaling is implicated in reducing neutrophil migration and increasing monocyte recruitment and migration through down regulation of neutrophil specific chemokines, and up regulation of monocyte chemokines like CCL2 and adhesion molecules like vascular cell adhesion molecule (VCAM) (85-87). Conversely, the anti-inflammatory effects of IL-6 are initiated when IL-6 binds to the IL-6R/gp130 complex, which is expressed on the cell membrane. This action is also known as classic signaling (88).

Much like the trend for the monocyte numbers mentioned above, some suggest that elevations in skeletal muscle IL-6 following exercise are related to skeletal muscle hypertrophy and recovery through the stimulation of skeletal muscle satellite cell proliferation (89, 90). However, proliferation of satellite cells only occurred at low concentrations (0.01-1 ng/ml) of IL-6 and not high concentrations (10-100 ng/ml) (90). In fact, chronic, high skeletal muscle concentrations of IL-6 are implicated in muscle atrophy and degradation (91).

Adipose tissue IL-6 gene expression varies based on adipose tissue location. Subcutaneous adipose tissue has more IL-6 mRNA compared to visceral adipose tissue in individuals with and without metabolic disease (92). It is estimated that 15-35% of circulating IL-6 can be attributed to release from adipose tissue (93). However, much like

in muscle, IL-6 released from adipose tissue is not dependent on damage to the tissue and more linked to the metabolic state of the organism. Acute administration of IL-6 to adipocytes in culture significantly reduces lipoprotein lipase activity, which has led researchers to infer that increases in IL-6 in cancer patients may be responsible for loss of body fat in some cancer related cachexia (94). In IL-6 knockout mice, administration of intracerebroventricular IL-6 offset the occurrence of mature onset obesity when compared to animals that did not receive IL-6 replacement therapy (95). These observed effects might be attributed to injected IL-6 stimulated glucagon release and the resulting increases in circulating blood glucose concentration (96). With evidence that adipose secreted IL-6 may account for up to 35% of circulating IL-6 concentrations, it is possible that accelerated adipose tissue accumulation may result in immune disruption and increases in other circulating acute phase proteins such as CRP.

C-Reactive Protein (CRP)

C-reactive protein is a component of the innate immune system and plays a key role in response to *streptococcus pneumoniae* (97). As previously discussed, this protein is produced and released as part of the acute-phase response after induction by proinflammatory cytokines like IL-6 (84). In addition, a positive correlation exists between circulating CRP and body mass index (BMI), percent body fat and subcutaneous abdominal fat (98). Furthermore, elevated circulating concentrations of CRP >1.0 mg/L are implicated in increased risk for CVD and increased mortality in diabetic patients (15, 99).

Chronic exercise acts to decrease circulating CRP (19). Combined weight loss and aerobic training over a six-month period resulted in a greater reduction of CRP than six

months of weight loss alone (98). Conversely, when obese girls that underwent 12-weeks of aerobic training, but did not experience significant reductions in total body weight or body fat percentage, they did not experience significant reductions in CRP from pre to post intervention (3.21 ± 2.48 vs. 2.73 ± 1.88 mg/L) (100). In comparing the effects of an eight-week resistance training intervention between novice and experienced lifters, where both groups participated in three training sessions per week, CRP was significantly lower in both groups from pre to post intervention. However, experienced lifters demonstrated greater reductions in CRP by an average of 54.5% compared to novice lifters with an average reduction of 22.9% despite no overall changes in body mass or fat mass pre to post intervention (101). Finally, a combined resistance and aerobic training model of participants undergoing 24-weeks of training experienced significant reductions in CRP. In these participants, there was no change in pre to post body weight or BMI, but body fat percentage decreased from pre to post intervention (102). Together, these study findings suggest that reductions in CRP are not solely reliant on exercise but involve changes in body composition as well.

**Chronic Exercise and
Immune Function**

Immune dysfunction can promote a chronic inflammatory state. Repeated bouts of moderate exercise reduce systemic inflammation (103, 104) and reduce susceptibility to disease (105), which may be attributable to alterations in immune function. Physical inactivity has been related to accumulation of visceral fat mass, increased risk for diabetes, CVD, cancer, and depression, and a chronic low-grade inflammatory state (106, 107). The American College of Sports Medicine (ACSM) is using the phrase, "Exercise

is Medicine" in an effort to encourage the use of exercise to mediate help control inflammation and prevent disease risk (106, 108, 109).

Assessment of monocyte phenotype and immune function via LPS stimulation has been performed in individuals that were either physically active or physically inactive. Physically inactive individuals underwent a 12-week combined resistance and aerobic training program three times a week. From pre to post exercise training, physically inactive individuals experienced significant increases in strength and $\dot{V}O_2$max, with post intervention values similar to those of the physically active group. LPS stimulated production of TNFα in whole blood samples was significantly lower from pre to post in physically inactive individuals suggesting that training decreased the pro-inflammatory immune response. This effect could be attributed to the reduction from pre to post of the total percentage or intermediate monocytes in these physically active individuals (21). Similar effects were observed in comparison of LPS stimulated release of IL-6 in physically active females compared to non-physically active females, with significantly lower stimulated IL-6 in physically active individuals. However, unlike the previously described study, there were no observed difference in stimulated TNFα production or monocyte sub-populations between activity groups (110). These results suggest that changes in TNFα secretion may be dependent on changes in monocyte phenotype, specifically changes in the percentage of intermediate monocytes.

Conversely, excessive exercise training, also known as over training, is implicated in decreased performance and immune suppression (111, 112). Three weeks of repeated intense exercise bouts reduced CCL2 after the training period and remained suppressed following four weeks of recovery (113). In fact, this phenomenon is further explained by

the J-Shaped Curve, which is used to describe risk of upper respiratory tract infection depending on physical activity status. In this model, sedentary individuals are at higher risk for illness compared to those engaging in regular, moderate exercise, and individuals that are engaging in excessive exercise without adequate recovery at higher risk for illness than the other two groups (114).

Monocyte Phenotype and
Activity Following
Acute Exercise

Acute, maximal exercise elicits a proinflammatory response and phenotypic shift in monocyte populations (115). Following a maximal progressive exercise test on a treadmill, with exercise testing lasting 13.3 ± 2.8 minutes, circulating classical monocytes decreased ~8% from pre-exercise while intermediate and non-classical monocytes increased by 16% and 48% pre to post-exercise, respectively. Other studies reported increased $CD16^+$ monocytes in the circulation following intense exercise (116-118), and is believed to be linked to increased shear stress on the vascular endothelium with exercise and mobilization from the splenic reserve (119, 120). Expression of TLR4 pre to post exercise is also significantly lower, with classical and intermediate monocytes experiencing greater reductions than non-classical monocytes. Generalized monocyte expression of CD14 is also significantly lower pre to post exercise and is significantly lower across all three monocyte sub-sets. Exercise induced significantly greater expression of CD16 on intermediate monocytes pre to post exercise but was unaltered on non-classical monocytes. LPS induced IL-6 and IL-10 secretion was significantly lower from pre to post exercise while TNFα release was significantly greater following

exercise. Further, the ratio of IL-6 to IL-10 LPS stimulated release was significantly greater following maximal exercise (115).

Cannabis and Cannabinoids

Cannabis products derived from the seeds, leaves, and flowers are often sub-divided by their cultivar, or strain, including *Cannabis Indica*, *Cannabis Sativa*, Sativa/Indica Hybrids and Hemp (121). Anecdotal accounts suggest varying perceived effects that are dependent on strain of use, with users of *Cannabis Indica* reporting cannabis use for pain management and sedation, while users of *Cannabis Sativa* reporting cannabis use for its effects on euphoria and energy enhancing qualities (122). However, new research has failed to uphold these common beliefs. Genetic analysis of over 30-strains marketed as either *Cannabis Sativa, Cannabis Indica* or Hybrid obtained from recreational dispensaries showed there was no genetic evidence supporting the distinction of these three strains. In addition, recent research from the University of Northern Colorado suggests strong genetic variability within identically named products like "Purple Kush", "Blue Dream" and "Girl Scout Cookies" (123). Today, the current consensus is there are two distinct categories of cannabis-based genetics, *Cannabis Sativa* and hemp. Current research on both the genetic analysis of strains and developing a basic physiological understanding of the effects of cannabis is restricted; limited not only within the United States (U.S.), but around the world. Research within the U.S. is regulated by the Drug Enforcement Agency (DEA). Until 2016, researchers were required to utilize the raw cannabis flower supplied cannabis from the University of Mississippi. However, genetic analysis of research grade cannabis obtained from the University of Mississippi revealed that it was more genetically similar to hemp than

Cannabis Sativa (124). These new findings have implications that even studies using DEA supplied cannabis for research may not be applicable to recreational or medical grade cannabis.

According to a report by the National Institute on Drug Abuse (NIDA), 9% of adults within the United States have used cannabis products within the past month (125). Within the past decade, there has been increased enthusiasm for the medicinal and recreation legalization of cannabis products. This change is largely due to the psychoactive and medicinal properties of compounds, also known as phytocannabinoids, which are found within the *Cannabis Sativa* plant. There are more than 100 identified phytocannabinoids within cannabis (126), yet two of these cannabinoids delta-9-tetrahydrocannabidiol (THC) and cannabidiol (CBD) have received the majority of the attention from researchers and the general population alike. However, THC and CBD are not naturally found in large quantities within cannabis. Instead, THC and CBD are secondary products of the precursor's delta-9-tetrahydrocannabinolic acid A (THCA-A) and cannabidiolic acid (CBDA), respectively and can only be produced when decarboxylated through heat (127).

Active THC and CBD produce effects throughout the body through activation and utilization of the body's endocannabinoid system. The body's primary endogenous cannabinoids are anandamide (AEA) and 2-arachodonoylglycerol (2-AG) and bind to cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors expressed in specific tissues throughout the body (128). AEA is the primary endocannabinoid ligand of CB1, while 2-AG is the endogenous ligand for CB2 (128). Within the central nervous system (CNS), endocannabinoids are used as retrograde signaling molecules released from the post-

synaptic membrane to inhibit further neurotransmitter release through activation of cannabinoid receptors on the pre-synaptic terminal (129-131). CB1 and CB2 receptors share 48% amino acid sequence identity (132). Both CB1 and CB2 are trimeric, G-protein coupled receptors (133). The α sub-unit of the G-protein coupled to the receptor has demonstrated suppressive effects on cyclic adenosine monophosphate (cAMP) production through inhibitory actions on adenylyl cyclase (AC) (134) whose actions were blocked with pertussis toxin, a known inhibitor of the α sub-unit (135).

The CB1 receptor is heavily expressed in neural tissue of the CNS and to a lesser extent in the periphery (28, 29). Conversely, while the CB2 receptor has been observed to be expressed by cells in the CNS, like microglial cells (136), it is more abundantly expressed throughout the periphery (137, 138), especially in immune tissues like monocytes and macrophages (24-26). THC binds and activates both CB1 and CB2 receptors (27, 139) while the observed actions of CBD are acting as a CB2 agonist and antagonize CB1 receptor activation (27). The elevated receptor localization of CB1 within the CNS and its high affinity for THC have implicated THC as the main intoxicating ingredient in cannabis. On the other hand, CBD is viewed as a non-intoxicating compound.

Cannabis Use and Health

Phytocannabinoids like THC and CBD are highly lipophilic, and repeated subcutaneous injection of THC for 26-days causes an accumulation of THC and its metabolites 11-hydroxytetrahydrocannabinol and 8, 11-dihdroxytetrahydrocannabinol are ten times more likely to accumulate in adipose tissue compared to any other tissue; however, concentrations of all three compounds gradually dissipated following 2-weeks

of discontinued use (140). Furthermore, no differences in total body fat, or hepatic fat were present when cannabis users were compared with non-users; however, abdominal visceral fat was significantly greater in cannabis users when compared to age and body mass index (BMI) matched control participants (141).

There is limited research available as it pertains to the chronic effect of cannabis use on resting heart rate in physically active individuals when not under the acute influence of cannabis. Previously, cross-sectional research in our laboratory observing physically active males and females determined that there was no difference in resting heart rate when at least 12-hours removed from last cannabis use when comparing cannabis users to non-users (31). There have been mixed findings on the acute effects of cannabis use on resting systolic and diastolic blood pressure. There were no reported changes in systolic blood pressure (142-144) and one study reported a slight decrease in diastolic blood pressure (142) with no reported change in others (143, 144). Interestingly, when physical activity status is consistent in both cannabis and non-cannabis using groups, there are no observed differences in either male or females between users and non-users (31).

Acute use of cannabis exerts a bronchodilator effect on pulmonary tissue as measured by improvements in forced vital capacity (145-149). In our lab, male, physically active cannabis users exhibited no difference, when compared to non-cannabis using individuals, in pulmonary function as measured by forced expiratory volume in one second when at least 12-hours removed from last use (31). Recent, unpublished findings from our laboratory indicate that chronic cannabis use is not associated with impaired respiratory function in physically active females.

In terms of overall metabolic health, cannabis users had greater carbohydrate intake and HDL concentrations compared to non-users, but no differences in overall caloric intake, fasting glucose, LDL or free fatty acid concentrations. Cannabis users also demonstrated decreased adipocyte insulin resistance index and no overall dysfunction in ß-cell function or insulin sensitivity in response to an oral glucose tolerance test (141). Together, these results suggest that both acute and chronic cannabis use may affect overall health through altered body composition and cardiovascular, pulmonary and metabolic health, but more investigation is necessary.

Cannabis and Exercise

Exercise is a multifaceted stressor that requires integration and coordination of multiple systems throughout the body including cardiovascular, respiratory, and skeletal muscle systems that are heavily dependent on mode of exercise (i.e., endurance, strength, power). Acute cannabis use is associated with elevated resting heart rate following both inhalation and oral ingestion (142, 144, 150-152). When physical activity is considered, acute use of cannabis elevates resting heart rate, which persists throughout sub-maximal exercise and recovery duration when compared to control and inhaled placebo conditions (153), with similar observations in maximal exercise up to 80% of maximal work capacity (154). These effects may be in part due to the presence of CB1 receptors on vagal efferent and afferent neurons (155, 156), which could alter the vagal tone innervating cardiac tissue.

With respect to endurance exercise, early studies suggest that acute administration of THC decreases peak work capacity, maximal workload, and exercise duration on a bike (154, 157, 158). Moderate intensity exercise lasting 35-45 minutes on a bike has also

been observed to illicit release of THC from adipose tissue increasing circulating THC

from pre to post exercise (159, 160). However, neither of these studies reported if the

observed increases in circulating THC post exercise were associated with a psychoactive

effect in participants (159, 160). Very few studies have followed up on the potential

physiological and psychological implications of this response. Acute administration of

cannabis prior to maximal exercise does not appear to induce any significant changes in

$\dot{V}O_2$ or $\dot{V}CO_2$ (154). A recent study in our laboratory supports these earlier findings. In a

cross-sectional study of male, chronic users compared to non-users, there was no

difference in relative $\dot{V}O_2$max or perceived exertion at termination of exercise during a

progressive treadmill assessment when users were at least 12-hours removed from last

use (31). The observed lack of difference in relative $\dot{V}O_2$max among physically active

male cannabis users and non-users appears to be consistent in physically active females

as well (data unpublished).

Skeletal muscle expresses both CB1 and CB2 receptors (161), which suggests that

the acute and chronic use of cannabis have the potential to alter skeletal muscle

physiology and function. Interestingly, muscular strength as evaluated by grip strength

was not different between acute cannabis or acute placebo use conditions (157). A recent

cross-sectional study from our laboratory sought to build off of these findings and

assessed physically active male cannabis users and non-users for grip strength and lower

limb force production, finding no differences between groups (31). More recent

mechanistic studies have also explored muscle function. In an *ex vivo* model using mouse

muscle fibers, stimulation with cannabinoids increased muscle fatigue, but did not alter

peak contractile amplitude (162). In addition, cannabinoid treatment decreases the

calcium sensitivity of the contractile proteins, as well as decreases release of Ca^{2+} from the sarcoplasmic reticulum and reduces uptake of Ca^{2+} of the sarcoplasmic reticulum ATPase (162).

Cannabinoids and Immune Function

Although a number of other components within cannabis are currently being explored, the cannabinoid CBD is quickly gaining popularity for its proposed anti-inflammatory actions in diseased populations (163). Even physically active individuals are now using cannabis as part of their exercise routines in an effort to help mediate exercise associated inflammation and pain (30). However, current research on the effects of acute and chronic use of cannabis on immune health and function is limited, especially in human models. Lung associated macrophages were significantly greater in mice exposed to cannabis smoke twice daily for 40-minutes a session at 1, 2, 3, and 4-months following initial exposure. Lung epithelial damage was significantly greater in mice at 2, 3 and 4-months after cannabis exposure, with overall increased inflammatory cell (macrophage, lymphocytes, and granulocytes) infiltration at 1, 2, 3, and 4-months after exposure compared to control animals (164). This suggests that even four months of chronic cannabis exposure increases inflammatory cell migration into the lungs in response to cannabis associated tissue damage.

**Cannabis, Cannabinoids
and Monocytes**

While chronic cannabis use promotes immune cell migration and tissue damage in animal models, the potential medicinal effects of cannabis use in a diseased population is unclear. In a cross-sectional study assessing number of inflammatory CD16^{+} monocytes in individuals diagnosed with human immunodeficiency virus (HIV) using or not using

cannabis products, HIV cannabis users had significantly lower numbers of circulating $CD16^+$ monocytes compared to HIV infected non-users. Further, there was no significant difference in $CD16^+$ monocytes between HIV cannabis users and healthy, non-HIV infected non-users. Although other mechanisms are currently being investigated, Interferon-γ inducible protein 10, which is another proinflammatory protein released from monocytes that has been implicated in promotion of HIV related neuroinflammation, was significantly lower in cannabis users with HIV infection compared to non-users. Additionally, when monocytes harvested from HIV infected cannabis users were treated with interferon α (IFNα), the cells were unresponsive to inducible expression of $CD16^+$, whereas HIV infected non-users and non-infected healthy controls did express CD16 in response to IFNα. This action was further supported when IFNα induced $CD16^+$ was blunted with pre-treatment of THC (34). In another study assessing cannabinoids and immune related neurotoxicity, cultured THP-1 cells, a model for human monocytes, stimulated neurotoxicity was significantly reduced in cell culture supernatant when pre-treated with the synthetic CB2 ligand JWH-015. This effect was blocked when the cells were treated with the CB2 antagonist SR144528 prior to stimulation with JWH-015 (136). Early indications implicate that chronic use of cannabis may be detrimental in an apparently healthy population; however, results from clinical populations could prove to be beneficial in the future.

As of late, researchers are assessing how the chronic use of cannabis affects monocytes in the apparently healthy, general population. In human isolated monocytes, migratory capacity and CB1 and CB2 receptor mRNA expression were measured in cannabis users and non-users. In cannabis users, isolated $CD14^+$ monocytes had

significantly reduced migratory capacity in culture compared to isolated monocytes from non-users in response to treatment with CCL2 and isolated serum. When cells were pre-treated with cannabinoids THC and CBD monocytes from non-users, there was no change in migratory capacity compared to basal conditions when stimulated with CCL2. However, cannabis users demonstrated >25% reduction from basal migratory capacity when pre-treated with THC and CBD in response to CCL2. This suggests that chronic cannabis use in apparently healthy individuals could impair the ability of monocytes to migrate into affected tissue. Further, cannabis user monocytes had four times greater mRNA expression of CB1 compared to non-users. There was no significant difference between cannabis user and non-user expression of CB2 mRNA (165). These results imply that chronic cannabis users may be more sensitive to the effects of CB1 ligands like THC in cannabis products. Another study has reported a similar effect in patients suffering from multiple sclerosis (MS). When both healthy cannabis users and cannabis users with MS monocytes were stimulated with CCL2, stimulated monocyte migration was reduced by 50%, when compared to healthy, non-user controls and non-users with MS. There were no significant differences in circulating concentrations of CCL2 between any groups (166). Together, these results suggest that the chronic use of cannabis in both healthy and clinical populations significantly impairs circulating monocytes' ability to migrate into affected tissue.

Cannabinoids and Cytokines

While the migratory capacity of monocytes in clinical and healthy populations is just beginning to be explored, there is currently limited information available on how cannabis affects production and release of inflammatory cytokines. In BV-2 microglial

cells, a cell model for CNS macrophages, pre-treatment with varying concentrations of

THC (1, 5, 10 µM) and CBD (1, 5, 10 µM) prior to LPS stimulation resulted in dose

dependent decreases of IL-1ß and IL-6 when measured in culture supernatant. There was

no significant difference in THC or CBD induced suppression of IL-1ß at 1, 5 or 10 µM

concentrations of either cannabinoid; however, while LPS induced release of IL-6 was

significantly lower (~25% reduction) at 5 and 10 µM THC, treatment with 1 µM CBD

induced similar reductions in IL-6 release compared to 5 and 10 µM THC. The effect of

CBD was further exaggerated at 5 and 10 µM treatment with respective reductions of

85% and 91%. Further, treatment with 10 µM THC and 10 µM CBD inhibited release of

interferon (IFN) ß, and significantly reduced the LPS induced increase of IL-1ß and IFNß

mRNA expression within stimulated cells. When assessing what intracellular signaling

pathways were associated with these results, treatment with CBD but not THC decreased

activity of the proinflammatory NF-κB pathway with observed increases in CBD induced

phosphorylation of STAT3, 2 and 4 hours post LPS stimulation (167). This means that

the acute administration of a CB2 agonist, like CBD, prior to immune challenge,

produces a dose dependent suppression of pro-inflammatory cytokine release through

inhibition of the NF-κB signaling pathway.

  While the current available research has described the acute administration of

cannabinoids like CBD and THC to have suppressive effects on inflammation, the

associated release of cytokines from immune cells also play a key role in cell-to-cell

communication. In cannabis users and non-users that were either healthy or had MS, IL-

17 was significantly lower in both cannabis user groups compared to non-user groups. IL-

17 is a key pro-inflammatory cytokine released from T-helper 17 (Th17) cells that

initiates productions of inflammatory factors like TNFα, IL-6 and MCP-1 (1). In addition,

total T-helper cell 1 (Th1) cytokines in resting plasma samples (IFNγ, IL-2 and TNFα)

was significantly lower in both cannabis groups. A similar effect on T-helper cell 2

cytokine concentrations was also reported with cannabis users demonstrating

significantly lower resting plasma concentrations of IL-4, IL-10, and IL-13 (166). In mice

exposed to cannabis smoke for 40-minutes per session two times per day for 2-months,

lung homogenate IL-6 was significantly elevated compared to controls, as were lung

homogenate concentrations of IL-10, MCP-1, IFNγ, TNFα, and IL-12. Further

exploration of lung homogenate concentrations of inflammatory cytokines found that

cannabis-using mice had increased concentrations of IL-10, MCP-1, IFNγ, TNFα and IL-

12 compared to tobacco using mice that were using for the same duration. However, there

were no differences in cannabis or tobacco using mice concentrations of IL-6 (164).

Consequently, it is unclear if the reductions in release of inflammatory cytokines like IL-

6 and TNFα is a result of CB receptor mediated decreased activation of NF- κB in

monocytes, or the result of suppression of cytokines from other immune cells.

In cannabis users diagnosed with cannabis use disorder, using cannabis on

average of 56.67 ± 7.23 months, resting concentrations of serum cytokine concentrations

(IL-1ß, IL-6, IL-8, IL-12p70, IFNγ, and TNFα) were evaluated and compared to a control

group that had never used cannabis. There was no difference in resting concentrations of

IL-12p70 or IFNγ between cannabis users and non-users. Concentrations of IL-6, IL-1ß,

IL-8, and TNFα were significantly higher in cannabis users compared to non-users. This

suggests that the chronic use of cannabis in individuals with cannabis use disorder may

cause immune disruption and create a pro-inflammatory environment. However, physical

activity status, recent exercise, and acute use of cannabis were not taken into account in this study, which could have significantly altered many of these biomarkers (168). New data from our laboratory found that there are no significant differences between resting serum IL-6 in physically active cannabis users (1.28 ± 0.49 pg/mL) and non-users (1.28 ± 0.74 pg/mL), where users used an average of 4.5 times a week with an average duration of use of 6 years. Results from our laboratory showed that our observed resting concentrations of IL-6 were significantly lower than non-user control (12.15 ± 3.48 pg/ml) and cannabis user (17.75 ± 5.17 pg/ml) groups (168). This suggests that exercise may have protective effects over the possible immune disruption observed with chronic cannabis use. Contrary to the previously discussed findings on cannabis use promoting increases in pro-inflammatory cytokines like TNFα and IL-1ß, a more recent study has described a significant negative relationship between the number of cannabis using days and IL-1ß concentration in individuals using cannabis and alcohol (169). These findings suggest that cannabis use may increase resting concentrations of pro-inflammatory cytokines; however, exercise may attenuate this effect. Cannabis use may also decrease alcohol consumption associated increases in pro-inflammatory cytokines like IL-1ß.

Cannabis and C-Reactive Protein

There have been mixed findings on cannabis use and circulating CRP. In individuals with Crohn's Disease, 8-weeks of a cannabis smoking intervention did not cause any significant changes in circulating CRP (170). However, recent cannabis users were more likely to be below the population mean for CRP, suggesting that cannabis use may have acute anti-inflammatory effects (171). In physically active males, our laboratory observed no difference in resting CRP concentrations between cannabis users

and non-users, but based on average group concentrations, cannabis users (1.76 ± 2.81 mg/L) were at moderate risk for CVD compared to non-users (0.86 ± 1.49 mg/L) who were at low risk for cardiovascular disease (31). Preliminary, unpublished data from our laboratory show a similar effect in females with cannabis users (1.47 ± 2.50 mg/L) again at higher risk for CVD compared to non-users (0.50 ± 0.39 mg/L) based on CRP. Consequently, it is possible that the phytocannabinoids in cannabis could alter immune and monocyte function leading to this increased risk for CVD. Exploration into the potential mechanisms of CRP dysregulation in cannabis users is warranted to discern if these findings are the result of changes in monocyte phenotype or another mechanism.

Conclusions

Sustained, moderate exercise programs have been shown to improve immune function and reduce risk for cardiovascular disease in aerobic, resistance, and combined training models. Recent findings suggest that some physically active individuals are now regularly using cannabis in combination with their exercise as a potential means to improve recovery from exercise. Research has demonstrated that acute and chronic cannabis use suppresses monocyte and immune cell migration and decreases cytokine release. While there are new implications that the chronic use of cannabis increases rick for CVD, as defined by circulating concentrations of CRP, in physically active cannabis users; the mechanism for this observation is still unclear. It is possible that the chronic use of cannabis alters monocyte phenotype and whole blood immune function leading to this increased risk. No study to date has assessed monocyte phenotype or the LPS induced immune response in physically active cannabis users.

CHAPTER III

METHODOLOGY

Participants

Study participants were recruited from the surrounding area of the University of Northern Colorado through fliers, social media, and word of mouth. A total of N=23 male and female participants (n=12 cannabis users [CU], n=8 males; n=12 non-users [NU], n=8 males) were recruited and underwent health related measurements and blood collection over the course of three study visits as outlined in Figure 3.1. Study participants had to be between the ages of 18 and 40 years, apparently healthy, engaging in at least 150-minutes of moderate activity per week, and use cannabis products at least 5-times per week for the past 6-months (CU), or not have used any cannabis products within the past 6-months (NU). Participants were not currently using tobacco products, pregnant, or did not have a musculoskeletal or neurological injury. This study was approved by the Institutional Review Board (IRB) at the University of Northern Colorado (Appendix A).
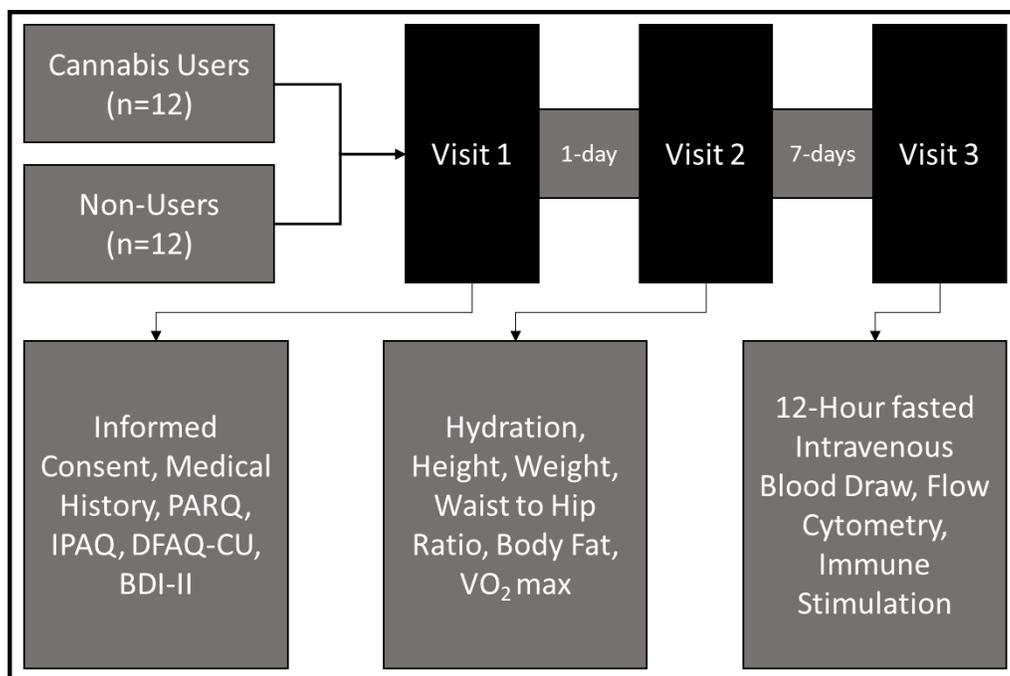
Figure 3.1. Study Layout and Progression. PARQ=Physical activity readiness questionnaire, IPAQ=International physical activity questionnaire, DFAQ-CU=Daily sessions, frequency, age of onset, and quantity of cannabis use questionnaire, BDI-II=Beck Depression Inventory-II, $\dot{V}O_2$max=Maximal volume of oxygen consumption.

Visit 1: Informed Consent and Surveys

When participants arrived at the exercise physiology laboratory (room 1610) in Gunter Hall, they were provided with the institutional approved informed consent and allowed adequate time to review the document. After obtaining informed consent and addressing any questions or concerns, participants were assigned a random participant number. Participants then completed Medical History and the Physical Activity Readiness Questionnaire (PAR-Q) forms. The International Physical Activity Questionnaire–Short Format (IPAQ) was then given to assess participant physical activity habits over the past seven days (172). For quantification of cannabis use, participants completed the Daily Sessions, Frequency, Age of Onset, and Quantity of Cannabis Use questionnaire (DFAQ-CU) (173). The Beck Depression Inventory-II (BDI-II) was

administered to assess participants depressive score and rating (174). Participants were excluded from partaking further in the study if they failed to meet the previously mentioned requirements.

Visit 2: Hydration, Anthropometric Measures and $\dot{V}O_2max$ Assessment

Prior to visit 2, participants were asked to refrain from vigorous physical activity for at least 48-hours, and abstain from alcohol, caffeine, cannabis and any non-prescription drug use for at least 12-hours prior to the visit.

Hydration Analysis

Prior to exercise testing, participants provided a urine sample in a urine collection container (Dynarex, Orangeburg, NY), and urine specific gravity was analyzed with a PAL-10S-4410 refractometer (ATAGO, Tokyo, Japan). Participants with a urine specific gravity greater than 1.025 were considered dehydrated and were asked to return to the lab at a later time when they were adequately hydrated.

Anthropometric Measures: Height, Weight, Waist to Hip Ratio and Body Fat Percent

Height was assessed without shoes using a wall-mounted stadiometer (Secca Precision for Health, Hamburg, Germany). Participant body mass was assessed using a digital platform scale (Detecto, Webb City, MO) without shoes, bulky clothing, and emptied pockets. Waist to hip ratio was taken by measuring the circumference of the waist, defined as the narrowest point between the umbilicus and xyphoid process, and the hips, defined as the widest point below the umbilicus using a Gulick measurement tape (Fabrication Enterprises Inc., White Plains, NY). Body fat percentage was assessed using a standard 7-site skinfold assessment (pectoral, triceps, sub-scapular, mid-axillary,

abdominal, suprailliac, and thigh). A spring-loaded Lange Skinfold Caliper (Cambridge Scientific Industries, Inc., Cambridge, MA, USA) was used to obtain measures of each site, in sequential order, for a total of two times. If the first two measurements differed by more than 2mm, a third measure was taken at that specific site. The average fold thickness for each site was used to calculate body density and body fat percentage using equations from Harrison et. al. 1988 (175)

$\dot{V}O_2max$ Assessment

Prior to the exercise assessment, participants were fitted with a Polar heart rate monitor (Polar Electro Inc., Bethpage, NY, USA) and asked to sit quietly for 5-minutes. Following the 5-minutes of rest, resting heart rate and blood pressure (American Diagnostic Corporation, Hauppaugge, NY) were obtained. Maximal oxygen consumption ($\dot{V}O_2max$) was assessed using the Bruce Ramp Protocol (176) and a TrueOne 2400 Metabolic Measurement System (Parvomedics, Model: MMS-2400; Sandy, UT, USA). This graded protocol had participants walk/jog as the speed and incline of the treadmill (Trackmaster, Model: TMX425CP, Full Vision Inc., Newton, KS, USA) was increased every 3-minutes until the participant reached volitional fatigue. The detailed protocol with treadmill speed and grade is presented in Table 3.1. In the final 30-seconds of each stage and immediately upon the termination of exercise, rate of perceived exertion (RPE) was obtained using the 0-10 Modified Borg Scale (177). For a Bruce assessment to be considered a true $\dot{V}O_2max$ any two of the four following criteria needed to be met: 1.) A plateau in $\dot{V}O_2$ despite an increase in workload, 2.) Heart rate within 10 bpm of age predicted heart rate max calculated by: 220-participant age, 3.) A respiratory exchange

ratio greater than 1.10, and 4.) A self-reported RPE greater than 8.5 on the Modified Borg

Scale immediately after termination of exercise.

Table 3.1: Bruce Protocol

	Treadmill Speed (mph)	Treadmill Grade (%)
Warm-up	1.7	0
Stage 1	1.7	10
Stage 2	2.5	12
Stage 3	3.4	14
Stage 4	4.2	16
Stage 5	5.0	18
Stage 6	5.5	20
Stage 7	6.0	22
Stage 8	6.5	24

Note: Stages are 3-minutes long and each successive stage starts immediately following
the previous stage

Visit 3: Blood Sample Collection, Flow Cytometry and Immune Stimulation

Blood Sample Collection

Blood draws took place in the Exercise Physiology Laboratory in Gunter Hall in

the morning between the hours of 5-9 am at least 7-days following visit 2 to allow for

adequate participant recovery. Participants were asked to arrive to the laboratory 12-

hours fasted (except water *ad libitum*), at least 72-hours removed from their last bout of

vigorous physical activity, and 12-hours removed from last use of alcohol, caffeine,

cannabis or any other non-prescription drug use. A trained phlebotomist collected a 40

mL blood sample via venipuncture. A total of 10 mL of blood was collected in serum

separation tubes (SST) (Thermo Fisher Scientific, Waltham, MA) and allowed to clot for

15-minutes at room temperature. The SST sample was then centrifuged at 800 rcf for 10-

minutes (Model: Marathon 21K, Thermo Fisher Scientific, Waltham, MA). The

remaining 30 mL of blood was collected in EDTA treated vacutainers (Becton Dickinson,

Franklin Lakes, NJ). EDTA treated blood was then aliquoted for whole blood, plasma, immune stimulation and flow cytometry analysis. Plasma was obtained by centrifuging the EDTA vacutainer for 10-minutes at 800 rcf. Whole blood for immune stimulations and flow cytometry was kept on ice for later analysis. Aliquoted samples of whole blood, plasma and serum were stored at -80°C for biomarker analysis.

Flow Cytometry Monocyte Phenotype

Whole blood collected in EDTA treated vacutainers was prepared for analysis of monocyte number and phenotype using flow cytometry. Whole blood, 150 µL, was stained for CD14 (anti-human CD14-FITC) and CD16 (anti-human CD16-PE/CY7) (eBioscince, San Diego, CA) following lysis of red blood cells with 1X red blood cell lysis buffer (BioLegend, San Diego CA) and blocking of Fc receptors with Fc-Blocker (eBioscience, San Diego, CA). Auto-fluorescent and anti-mouse IgG1 FITC and PE/CY7 isotype controls were also prepared for proper gating analysis (eBioscience, San Diego, CA). Prepared samples were run on an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA), stimulated with a 488 nm laser and detected in the FITC and PE/CY7 channels. Data were analyzed in FCS Express 6 (De Novo Software, Glendale, CA), setting gates for the monocyte sub-population, doublet exclusion and monocyte co-expression of CD14 and CD16 for $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{+}$ monocyte populations (Figure 3.2).
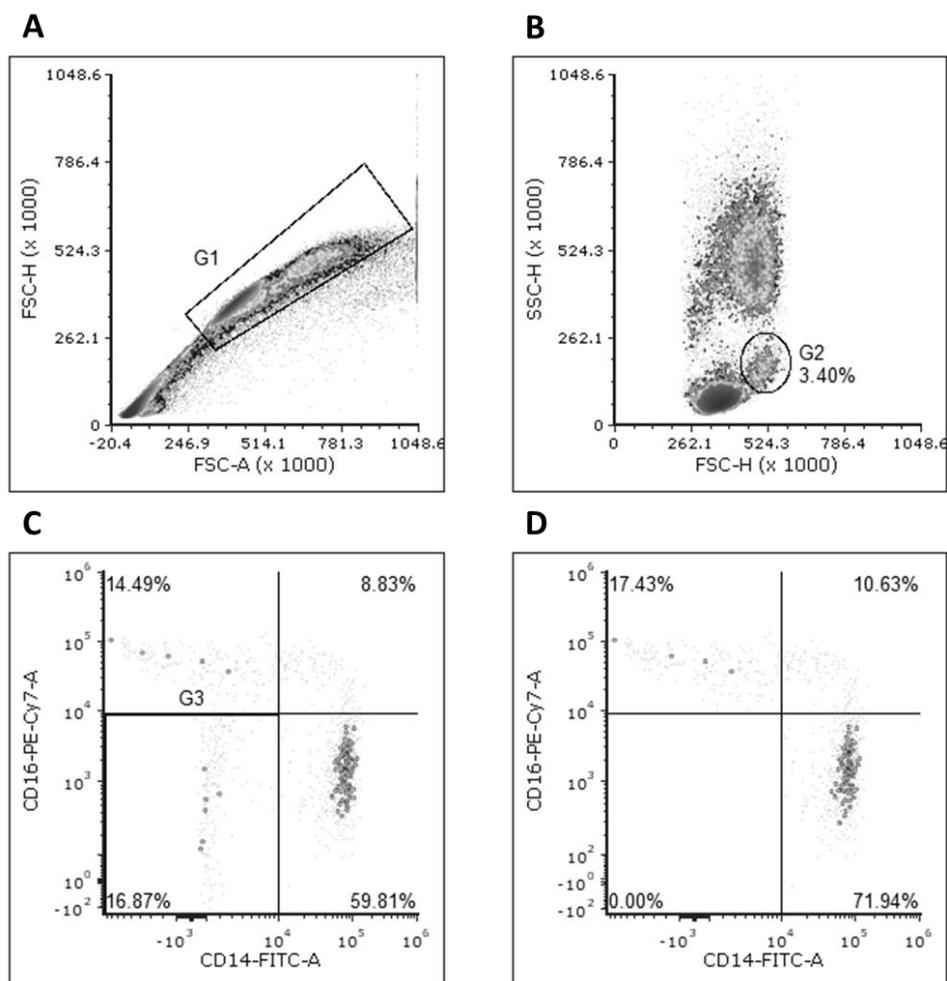
Figure 3.2. Flow Cytometry Monocyte Gating Strategy. Data were analyzed in FCS Express (De Novo Software, Glendale, CA). **A.** Data points were graphed using forward scatter area (FSC-A) and forward scatter height (FSC-H). Gate G1 was placed around all singly suspended cells, excluding for: doublets, dead cells, and any remaining red blood cells or particulate. **B.** Gate G2 was placed over the specific monocyte cell population. **C.** Cells from G2 were graphed based on expression of CD14 (x-axis) and CD16 (y-axis). Cells in gate G3 were excluded for low expression of both CD14 and CD16. Quadrant positioning was determined by FITC and PE-CY7 isotype controls for CD14 and CD16 respectively. **D.** Cells in the upper left quadrant were classified as non-classical monocytes, the upper right quadrant cells were classified as intermediate monocytes, and lower right quadrant cells were categorized as classical monocytes.

**Whole Blood LPS Immune
Stimulation**

Whole blood inflammatory response was measured after preparing EDTA treated

whole blood at a 1:10 dilution with prepared sterile Rowell Park Memorial Institute

(RPMI) cell culture media. Prepared RPMI consisted of sterile RPMI (Sigma Aldrich, St.

Louis, MO) treated with L-glutamine, streptomycin and penicillin (Sigma Aldrich, St.

Louis, MO) at a 1:100 dilution. For each treatment condition, control, LPS, Ag+LPS and

Ant+LPS, 2 mL of prepared blood was aliquoted into 24-well plates. The Ant+LPS wells

were treated with 10 µL of a 200 µg/mL SR144528, a synthetic CB2 antagonist (178),

stock solution for a final concentration of 1µg/mL in the well and incubated for 1-hour at

37°C and 5% CO_2. The Ag+LPS and Ant+LPS wells were then treated with 10 µl of a

200 µg/mL JWH-015, a synthetic CB2 agonist (179), stock solution for a final

concentration of 1 µg/mL and incubated for 1-hour 37°C and 5% CO_2. The LPS,

Ag+LPS and Ant+LPS wells were then treated with 50 µL of 1 mg/mL prepared LPS (S.

enteriditis; Sigma Aldrich, St. Louis, MO) for a final concentration of 25 µg/mL of LPS.

Samples incubated for 24-hours at 37°C and 5% CO_2 were then centrifuged at 800 rcf for

10-minutes at 4°C. Cell culture supernatant was harvested, aliquoted and stored at -80°C

for analysis of inflammatory proteins.

Protein Quantification

Stimulated cell culture supernatants were analyzed for protein concentration of

IL-6 using an enzyme linked immunosorbent assay (ELISA) (RayBiotech, Norcross,

GA). The inter-assay coefficient of variability (CV) for LPS stimulated IL-6 supernatants

was <10% and intra-assay CV was <6%. Isolated serum was analyzed for CRP using an

ELISA (ALPCO Diagnotics, Salem, NH). Resting concentrations of IL-6 were assessed

using isolated plasma and a high sensitivity ELISA (Abcam, Cambridge, MA). Analysis

for resting concentrations of CRP and IL-6 was done using a single plate and had intra-

assay CV's of <6%. All samples and ELISA kits were prepared according to manufacture

specifications and analyzed using an ELx800 BioTek microplate reader (BioTek

Instruments, Winooski, VT) at the recommended wavelength of 450 nm.

Statistical Analysis

All data were analyzed using SPSS (V. 24; IBM Analytics, Armonk, NY)

assessed for normalcy, distribution of variance and log transformed as necessary. A

standard unpaired t-test was used to compare descriptive measures, resting concentration

of CRP and IL-6, and monocyte populations between CU and NU groups. Differences

between males and females were assessed for all descriptive variables using an unpaired

t-test. For comparison of stimulated IL-6 production, a 2(group) x 4(treatment) ANOVA

was used to compare control, LPS, Ag+LPS, and Ant+LPS conditions with Bonferroni

post hoc testing to assess any potential differences between groups and within treatment

conditions. Cohen's D effect sizes were calculated to assess if any meaningful differences

were present between CU and NU groups (180). Pearson correlation was used to assess

significant correlations present between key variables of interest. The Pearson correlation

matrix for these key variables can be seen in Appendix B.

CHAPTER IV

RESULTS

Participants

A total of 26 participants were recruited into the study and a total of 23 participants completed all study visits. Of the three participants that were unable to complete all study visits, two were unable to provide blood samples and the remaining participant was excluded due to diagnosis of mononucleosis after completing the first study visit. Twelve were NU (n=8 male; n=4 female), and eleven were CU (n=8 male; n=3 female). The majority of participants were Caucasian (n=16) while the remaining participants were of Hispanic/Latino (n=5), Asian (n=1) or African American (n=1) decent. Participants did not report any current significant medical issues that would be considered counterproductive to their participation in this study. Only two participants reported minor current medical issues; one NU reported the presence of phenylketonuria and one CU reported non-alcoholic fatty liver. Of the n=7 female participants who completed the study, none were currently or had previously been pregnant. While all female participants had previously taken birth control, only one NU was currently taking birth control in pill form. None of the female participants had undergone surgery for total or partial hysterectomy or undergone hormone replacement therapy. There were no differences in the total amount of alcoholic beverages consumed on a weekly basis between groups (3.2 ± 3.1 drinks, p=0.26; ES=0.49).

Characteristics of Cannabis Use

All participants in the CU group met the minimum cannabis use requirement, which was 5 cannabis uses per week or more. Information pertaining to the cannabis use habits of the eleven CU can be found in Table 4.1. Of the eleven CU, nine (82%) were using cannabis products daily. Participant duration of cannabis use ranged from 1-14 years, with only two CU participants reporting the use of cannabis products prior to being 16 years of age. A total of six participants in the NU group reported previous use of cannabis products, but none of those participants reported any use of cannabis within the past 6-months. The remaining six NU reported no prior use of cannabis products.

Table 4.1: General Cannabis Use

	CU (n=11)
Days Used in the Past Week	5.9 ± 1.6
Days Used in the Past Month	26.4 ± 5.6
Daily Uses of Cannabis: Overall	3.0 ± 1.3
Daily Uses of Cannabis: Weekday	2.8 ± 1.3
Daily Uses of Cannabis: Weekend	3.7 ± 1.8

Primary Method of Use				
Method	**Bong**	**Joint**	**Hand Pipe**	**Vaporizer**
Number (n)	5	2	2	2
Total Years Using Cannabis (years)			7.5 ± 3.6	
Age at First Use (years)			16.8 ± 2.8	
Age Started Using Cannabis >2-times/month (years)			20.4 ± 6.9	
Age Started Using Cannabis on Daily or Near Daily Basis (years)			22.0 ± 6.5	

Note: Data are presented as mean ± SD. CU = cannabis user. Values are participant responses to the Daily Sessions, Frequency, Age of Onset, and Quantity of Cannabis Use Inventory. Primary method of use was defined as the method that participants used most often when using cannabis products.

Of the n=11 CU participants, 91% (n=10) reported that their primary form of

cannabis used was marijuana in the form of flower/bud (MU), while the remaining

participant primarily used products in the form of oil concentrates. Characteristics

describing the use habits of marijuana flower/bud of MU is provided in Table 4.2.

Table 4.2: Marijuana Flower/Bud Use

	MU (n=10)
Marijuana Used per Session (grams)	0.23 ± 0.12
Marijuana Used per Day of Use (grams)	0.94 ± 0.75
Marijuana Used Day Before 1st Visit (grams)	0.53 ± 0.69
Marijuana Used per Week (grams)	5.4 ± 4.2
Number of Sessions per Day of Marijuana Use	2.4 ± 1.1

Average THC Content of Marijuana Used			
THC Range	**10-14%**	**15-19%**	**20-24%**	**25-30%**
Number (n)	1	4	4	1

Note: Data are presented as mean ± SD. MU = Marijuana user, defined as: cannabis that was used in its unprocessed form, i.e., flower/bud. THC = delat-9-tetrahydrocannabinol. Values are responses to the Daily Sessions, Frequency, Age of Onset, and Quantity of Cannabis Use Inventory.

A total of six participants reported using cannabis in the form of concentrates at

least 25% of the time that they used, including the one individual that reported

concentrates as their primary form of use. None of these participants reported the use of

concentrates the day prior to the first study visit. When using cannabis concentrates,

individuals reported taking an average of 2.0 ± 1.3 hits per session, during an average of

1.7 ± 1.0 sessions per day, with a mean intake of 3.4 ± 2.9 hits per day. They also

reported using an average of 0.10 ± 0.05 grams of concentrate per day. Of the six

participants that were using cannabis concentrates, four of these individuals reported that

the concentration of THC in the products they used was at least 60%, while the remaining

two were unsure of the concentration. The use of edibles was not reported as the primary

method of use for any participants, but almost half (n=5) of the eleven CU reported that

they used edibles at least 25% of the time that they used cannabis products. The average

THC content of the edibles was 14.0 ± 6.5 mg and ranged from 10 mg to 25 mg of THC.

Physical Characteristics

There were no significant differences in age, height, body mass, BMI, body fat,

waist and hip circumference and ratio, when NU and CU were compared (Table 4.3).

While no significant difference was detected between NU and CU groups for body fat,

waist or hip circumference, there were moderate effect sizes for each variable.

Participants averaged 27.7 ± 5.5 years of age; NU ranged in age from 18-37 years old and

CU ranged from 20-39 years old. Mean BMI and body fat measures were 24.61 ± 4.30

kg/m^2 and $11.2 \pm 5.4\%$ and ranged from 19.33 to 37.91 kg/m^2 and 4.9 to 21.2%,

respectively.

Female participants (31.1 ± 7.1 years) were significantly older than male

participants (26.2 ± 4.1 years) and ranged from 18 to 39 and 20 to 35 years of age

respectively (p=0.04). Male participants had significantly higher systolic (p=0.03) and

diastolic (0.02) blood pressure, with an average blood pressure of $113.8 \pm 10.5 / 74.1 \pm$

7.8 mmHg compared to $103.7 \pm 7.1 / 65.1 \pm 7.0$ mmHg in females. Females had a

significantly higher mean body fat percent of $17.0 \pm 2.6\%$ when compared to males with

a mean body fat percent of $8.7 \pm 4.1\%$ ($p<0.001$).

Table 4.3: Descriptive Characteristics and Performance

	Overall (N=23)	**NU (n=12)**	**CU (n=11)**	**P-value**	**Cohen's D**
Age (years)	27.7 ± 5.5	28.3 ± 6.0	27.0 ± 5.1	0.58	0.24
Height (cm)	172.90 ± 9.89	173.25 ± 10.81	172.51 ± 9.30	0.86	0.07
Mass (kg)	74.20 ± 18.85	77.33 ± 22.96	70.78 ± 13.31	0.42	0.35
BMI (kg/m^2)	24.61 ± 4.30	25.42 ± 5.02	23.72 ± 3.35	0.36	0.40
Body Fat (%)	11.2 ± 5.4	12.9 ± 5.3	9.3 ± 5.0	0.11	0.72
Waist (cm)	77.0 ± 8.3	79.3 ± 10.3	74.5 ± 4.8	0.17	0.60
Hips (cm)	96.7 ± 8.10	99.0 ± 10.1	94.2 ± 4.4	0.17	0.61
Waist to Hip ratio	0.79 ± 0.05	0.80 ± 0.05	0.79 ± 0.04	0.66	0.19

Note: Data are presented as mean $\pm$ SD. NU = non-user of cannabis; CU = cannabis user; BMI = body mass index.

Physical Activity Habits

All participants met the inclusion criteria for weekly physical activity of at least

1.25-hours (75-minutes) of vigorous intensity activity or 2.5-hours (150-minutes) of

moderate intensity activity. There was no significant difference in the number of days or

average time engaged in vigorous or moderate intensity activity when NU and CU were

compared (Table 4.4). Overall, the number of days per week that participants engaged in

vigorous intensity physical activity ranged from 0-6 days and moderate activity ranged

from 0-6 days. Only one participant, a CU, reported not engaging in any vigorous

intensity activity. A total of four participants (NU; n=2 and CU; n=2) reported not

engaging in any moderate intensity activity. Although, CU did not statically spend less

time walking on a daily basis (p=0.06, effect size of 0.88) CU spent significantly less time sitting each day than NU (p<0.01), which was supported by a large effect size. There were no sex differences between any of the self-reported measures of physical activity.

Table 4.4: Self-reported Physical Activity Within the Past Week

	Overall (N=23)	NU (n=12)	CU (n=11)	P-value	Cohen's D
Days vigorous activity	3.5 ± 1.8	3.6 ± 1.8	3.4 ± 1.80	0.77	0.13
Time engaging in vigorous activity per day (hours)	1.5 ± 1.0	1.6 ± 0.8	1.3 ± 1.20	0.56	0.25
Days moderate activity	3.0 ± 1.9	3.4 ± 1.8	2.6 ± 2.0	0.28	0.48
Time engaging in moderate activity per day (hours)	1.3 ± 1.6	1.4 ± 1.9	1.1 ± 1.4	0.57	0.25
Days walked ≥ 10-minutes	5.3 ± 2.0	5.3 ± 2.0	5.3 ± 2.1	0.98	0.01
Time spent walking (hours)	1.7 ± 1.9	2.4 ± 2.3	0.9 ± 0.8	0.06	0.88
Time spent sitting per day (hours)	6.6 ± 2.6	8.0 ± 3.0	5.1 ± 0.7	<0.01	1.36

Note: Data are presented as mean ± SD. NU = non-user of cannabis; CU = cannabis user. Responses are based on the 7-question format of the International Physical Activity Questionnaire.

Cardiovascular and Performance Measures

There were no differences with respect to resting heart rate, systolic or diastolic blood pressure, hydration status, or relative $\dot{V}O_2$max between groups (Table 4.4). In addition, all variables with respect to cardiovascular and performance measures demonstrated small effect sizes (Table 4.5). Resting heart rate, systolic, and diastolic

blood pressure ranged from 43 to 63 bpm, 90 to 128 mmHg and 56 to 84 mmHg, respectively. All participants were considered hydrated at the time of performance testing; with hydration measured by urine specific gravity ranged from 1.003 to 1.025. All participants met at least two of the following four established criteria for attainment of a true $\dot{V}O_2$max: 1.) Plateau in $\dot{V}O_2$ with increased intensity, 2.) Heart rate within 10 bpm of age predicted max heart rate (220-age), 3.) Respiratory exchange ratio greater than 1.10, 4.) RPE $\geq$ 8.5 on the modified Borg scale.

There were no differences between NU and CU within sexes with respect to resting HR, systolic/diastolic BP, urine specific gravity, relative $\dot{V}O_2$max, or RPE at termination of maximal exercise. Average resting HR for male participants was 55.8 ± 6.4 bpm and was 55.0 ± 6.6 bpm for females. Males had higher systolic (p=0.03) and diastolic (0.02) blood pressure than females with an average of $113.9 \pm 10.5/74.1 \pm 7.8$ mmHg and $103.7 \pm 7.1/65.1 \pm 7.0$ mmHg, respectively. Female participants also had significantly lower urine specific gravity of 1.007 ± 0.005 compared to males 1.015 ± 0.007 (p<0.01). $\dot{V}O_2$max for male participants averaged 47.8 ± 4.1 ml/kg/min and 41.1 ± 7.5 ml/kg/min in female participants. There were no significant differences between male and female $\dot{V}O_2$max (p=0.06, with Levene's test for equality of variances=0.02). Male $\dot{V}O_2$max values ranged from 40.8 to 56.6 ml/kg/min and female $\dot{V}O2$max ranged from 35.5 to 52.5 ml/kg/min. There was no difference in RPE at termination of exercise between males (8.9 ± 0.8) and females (8.9 ± 0.7).

Table 4.5: Resting Heart Rate, Blood Pressure, Hydration and Cardiovascular

Performance

	Overall (N=23)	NU (n=12)	CU (n=11)	P-value	Cohen's D
Resting HR (bpm)	55.5 ± 6.3	55.5 ± 6.0	55.5 ± 6.9	0.99	0.01
Systolic BP (mmHg)	110.8 ± 10.6	110.2 ± 11.5	111.5 ± 10.0	0.78	0.12
Diastolic BP (mmHg)	71.4 ± 8.4	71.2 ± 9.7	71.6 ± 7.4	0.90	0.06
Urine specific gravity	1.013 ± 0.007	1.012 ± 0.008	1.014 ± 0.007	0.56	0.26
Relative $\dot{V}O_2$ max (ml/kg/min)	45.8 ± 6.1	45.4 ± 6.1	46.2 ± 6.2	0.75	0.14
RPE at Termination	8.9 ± 0.7	8.9 ± 0.9	8.9 ± 0.6	0.98	0.01

Note: Data are presented as mean ± SD. NU = non-user of cannabis; CU = cannabis user; HR = heart rate; BP = blood pressure; RPE = Rating of Perceived Exertion.

Psychological Characteristics

None of the participants reported being hospitalized for depression within the past 6-months; however, n=4 CU participants had previously received treatment for depression but were no-longer receiving treatment. A total of n=5 participants (n=4 CU) reported current feelings of anxiousness in their daily lives. On average, CU had a significantly greater total depressive score with a large effect size on the BDI-II with an average of 5.1 ± 4.8 points compared to NU at 0.8 ± 1.0 points (p<0.01; ES=1.29). This difference persisted when controlling for participant age and BMI (F[3,19] = 8.105, p=0.01). Across all participants there were no correlations between total BDI-II score, age, BMI, resting IL-6 or CRP concentration. Total BDI-II score in CU was positively correlated to the number of times individuals used cannabis on weekend days (r=0.620, n=11, p=0.04), but no other measures of cannabis use.

Resting Concentrations of Markers of Inflammation

Resting concentrations of CRP were not statistically different between NU (0.87 ± 1.51 mg/L) or CU (0.78 ± 1.11 mg/L) groups (p=0.88; ES=0.07). This lack of difference between groups with respect to CRP persisted even when controlling for age and body fat percentage (F[3,19] = 0.075, p=0.79). There were no significant correlations between resting CRP concentrations and age, height, mass, BMI or IPAQ generated measures of physical activity. Within CU who used marijuana flower/bud as their primary form of cannabis (MU; n=10), concentrations of CRP were positively correlated to total amount of marijuana flower/bud (grams) used per week (r=0.705, n=10, p=0.02). Concentrations of CRP were not related to any other measures of cannabis use.

Resting IL-6 concentrations were not different between NU (0.89 ± 0.71 pg/mL) and CU (1.33 ± 1.37 pg/ml) (p=0.34; ES=0.42) and remained insignificant despite adjusting for age and body fat percent (F[3,19] = 1.263, p=0.28). Resting concentrations of CRP and IL-6 were positively correlated to each other (r=0.524, n=23, p=0.01). No significant correlations existed between resting IL-6 concentrations and age, height, mass, BMI, or physical activity habits reported on the IPAQ. Similar to CRP, resting IL-6 concentrations were positively correlated to the amount of marijuana (grams) used per week (r=0.690, n=10, p=0.03) in MU, but not with other measures of cannabis use.

Monocyte Phenotype

The proportion (%) of monocytes circulating in the blood was not significantly different between groups (Table 4.6). Further assessment of the distribution of the three monocyte sub-types of classical, non-classical and intermediate monocytes, showed that there was no difference in the percent of classical or non-classical monocytes between

groups. However, the proportion of intermediate monocytes, which was accompanied by a large effect size, potentially suggesting that CU had a significantly greater proportion of intermediate monocytes compared to NU (Table 4.6). The findings on the total percent of circulating monocytes ($F[1,18] = 0.014$, $p=0.91$), percent classical ($F[1,18] = 4.401$, $p=0.05$), non-classical ($F[1,18] = 0.023$, $p= 0.88$) and intermediate ($F[1,18] = 6.238$, $p=0.02$) monocytes remained consistent when controlling for participant age and body fat percentage.

When standardizing the number of circulating monocytes per milliliter of blood, CU had significantly higher monocytes with an effect size of 1.31 suggesting a large meaningful difference between groups (Table 4.7). The number of monocytes per milliliter positively correlated to participant BDI-II score ($r=0.445$, $n=22$, $p=0.04$), the amount of marijuana flower/bud used the day before ($r=0.744$, $n=10$, $p=0.01$), and the average number of times participants used marijuana flower/bud per day used ($r=0.729$, $n=10$, $p=0.2$). The number of classical, non-classical, and intermediate monocytes per milliliter of blood is presented in Table 4.7. Cannabis users had significantly greater classical and intermediate monocytes per milliliter. The number of classical ($r=0.448$, $n=22$, $p=0.04$) and intermediate ($r=0.475$, $n=22$, $p=0.03$) monocytes both positively correlated to BDI-II score. The number of classical monocytes per milliliter did not correlate with any measures of cannabis use. In CU, the number of intermediate monocytes per milliliter positively correlated to the average number of times participants used marijuana flower/bud per day ($r=0.864$, $n=10$, $p<0.01$), the number of hits participants took when using cannabis concentrates ($r=0.916$, $n=6$, $p=0.01$) and the amount of milligrams of THC in edibles used ($r=0.934$, $n=5$, $p=0.02$). Finally, the number

of non-classical monocytes positively correlated to resting CRP (r=0.569, n=22, p<0.01),

resting IL-6 (p=0.712, n=22, p<0.01) and the amount (grams) of marijuana flower/bud

used per week.

Table 4.6: Relative Proportions of Whole Blood Monocytes

	Overall (N=22)	NU (n=11)	CU (n=11)	P-value	Cohen's D
Total Monocytes (%)	3.12 ± 1.55	2.97 ± 1.23	3.27 ± 1.87	0.63	0.56
Classical (%)	76.93 ± 8.48	79.49 ± 6.74	74.37 ± 9.54	0.16	0.63
Non-Classical (%)	12.74 ± 7.14	13.82 ± 6.12	11.65 ± 8.18	0.49	0.31
Intermediate (%)	10.11 ± 7.77	6.33 ± 4.92	13.89 ± 8.43	0.02	1.12

Note: Data are presented as mean $\pm$ SD. NU = non-user of cannabis; CU = cannabis user. Classical, Non-classical, and Intermediate sub-types were determined by relative expression of cell surface CD-14 and CD-16 expression using flow cytometry.

Table 4.7: Number of Monocytes per Milliliter of Whole Blood

	Overall (N=22)	NU (n=11)	CU (n=11)	P-value	Cohen's D
Monocytes per mL of blood	$4.12 \times 10^5 \pm 1.69 \times 10^5$	$3.23 \times 10^5 \pm 1.20 \times 10^5$	$5.08 \times 10^5 \pm 1.63 \times 10^5$	0.01	1.31
Classical Monocytes per mL of blood	$3.15 \times 10^5 \pm 1.30 \times 10^5$	$2.56 \times 10^5 \pm 0.97 \times 10^5$	$3.77 \times 10^5 \pm 1.36 \times 10^5$	0.02	1.04
Non-Classical Monocytes per mL of blood	$4.95 \times 10^4 \pm 3.50 \times 10^4$	$4.27 \times 10^4 \pm 2.38 \times 10^4$	$5.72 \times 10^4 \pm 4.38 \times 10^4$	0.31	0.41
Intermediate Monocytes per mL of blood	$4.72 \times 10^4 \pm 4.69 \times 10^4$	$2.32 \times 10^4 \pm 2.18 \times 10^4$	$7.29 \times 10^4 \pm 5.19 \times 10^4$	0.01	1.26

Note: Data are presented as mean $\pm$ SD. NU = non-user of cannabis; CU = cannabis user. Classical, Non-classical, and Intermediate sub-types were determined by relative expression of cell surface CD-14 and CD-16 expression using flow cytometry.

Lipopolysaccharide Stimulated IL-6 Production

There were no group or treatment effects with respect to the LPS induced IL-6 release over a 24-hour period ($F_{[1,63]} = 0.332$, p=0.567) (Figure 4.1). Despite adjusting for participant age, BMI, body fat percent and monocyte percent, there were still no differences present in the amount of LPS induced IL-6 release. The relative amount of IL-6 released per monocyte (Figure 4.2) was not significantly different between groups within treatment conditions. The number of non-classical monocytes per milliliter significantly correlated to the amount of IL-6 released in the LPS (r=0.681, n=22, p<0.01), Ag+LPS (r=0.757, n=22, p<0.01) and Ant+LPS (0.681, n=22, p<0.01) treatment conditions. The number of classical and intermediate monocytes per milliliter did not correlate to the amount of IL-6 release in any of the treatment conditions. The resting concentrations of IL-6 were strongly correlated to the amount of IL-6 released in in each of the LPS stimulated conditions LPS control (r=0.569, n=23, p<0.01), Ag+LPS (r=0.641, n=23, p<0.01) and Ant+LPS (r=0.601, n=23, p<0.01) conditions.
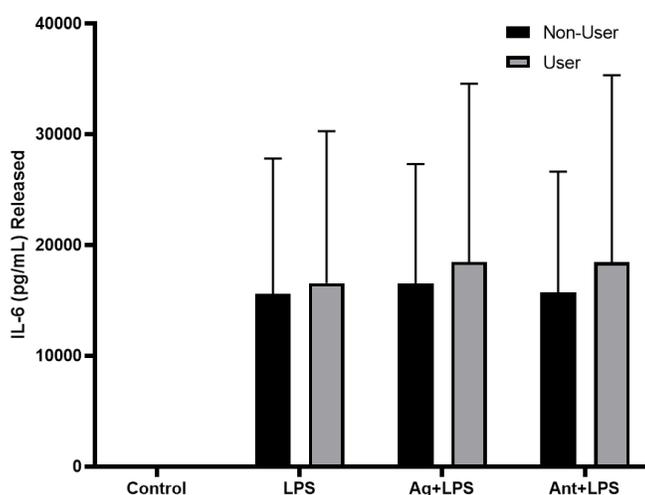
Figure 4.1. Lipopolysaccharide Stimulated IL-6 Release. Data are depicted as mean ± SD. LPS=Lipopolysaccharide, Ag+LPS=CB2 agonist with LPS, Ant+LPS=CB2 antagonist with LPS. Control samples were unstimulated with LPS.
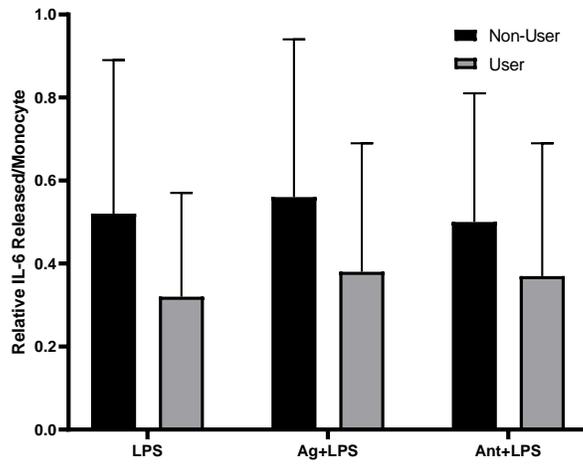
Figure 4.2. Relative Lipopolysaccharide Stimulated IL-6 Released per Monocyte. Data are depicted as mean ± SD. LPS=Lipopolysaccharide, Ag+LPS=CB2 agonist with LPS, Ant+LPS=CB2 antagonist with LPS. Stimulated concentrations of IL-6 were standardized to the number of monocytes per participant.

CHAPTER V

DISCUSSION AND CONCLUSIONS

This was the first study to assess the effects of chronic cannabis consumption in individuals that regularly engage in physical activity on circulating monocyte phenotype and immune response to LPS. The hypothesis in response to our first specific aim was supported, as there was no difference in total circulating blood monocytes between NU and CU, but CU demonstrated significantly elevated concentrations of intermediate monocytes when compared to NU. The second specific hypothesis of this study stated that resting concentrations of serum CRP and IL-6 would be greater in CU, which was rejected as there were no differences in the circulating concentrations of these two inflammatory markers. In assessment of LPS induced IL-6 release from whole blood samples, there was no difference found between the total amount of IL-6 released between NU and CU in any of the treatment conditions. Finally, the number of intermediate monocytes per milliliter was positively associated with the number of times CU were using marijuana flower or bud products per day.

Characteristics of Cannabis Use

The inclusion criteria for cannabis use in this study required at least 5 cannabis uses a week for the past 6-months. All CU participants exceeded this criterion using, on average, slightly less than 6-days per week with an average number of cannabis uses of 3-times per day. This equates to an average of slightly less than 18 uses per week. Further,

participants reported using an average of 26.4 ± 5.6 days over the past 30 days. In the present study, CU were using an average of 1.4 more days per week and almost twice as many times per day compared to a previous study performed by our lab aimed at evaluating circulating bio-markers of neural and immune health in physically active cannabis users and non-users (181). The cannabis users from this previous study were using an average of 1.67 ± 0.72 uses per day and reporting an average of 18 days of use within the last 30 days (181); however, it should be mentioned that the inclusion requirement for the aforementioned study was only one use per week for the past 6-months. It is likely that this difference may have led to the disparity in reported cannabis use.

In the current study, all participants reported that their primary method of use was through inhalation with 46% using a bong, 18% using a joint, 18% using a hand pipe, and 18% using vaporizer. Comparing this information to two previous studies performed in our lab assessing physically active individuals using cannabis products, all but one participant, who used edibles, also used cannabis primarily through inhalation (31, 181). Additionally, a survey study published from our lab found that 80% of individuals that used cannabis with exercise reported that their primary method of use was also through inhalation (30). Even though other methods of use including topicals, tinctures and edibles are other options for use, it appears that, for someone who is physically active, inhalation is the primary method of choice. This could be due to the fact that the use of cannabis through inhalation increases blood concentrations of cannabinoids, like THC, more rapidly when compared to edibles (182).

The majority (80%) of participants reported that the average content of their marijuana flower or bud was 15 to 24% THC. This reported content of THC reflects a concentration range that is recreationally available in most commercial cannabis dispensaries (183, 184). Only one individual reported average marijuana THC content between 10-14% and another individual reported average marijuana THC content between 25-30%. In addition, of the four participants that claimed to know the THC content of their cannabis concentrates, no one reported a THC content less than 60% which is similar to what has been measured previously (183); however, it is important to note that the participant reported THC percentages might not be reflective of the actual THC content of the product. More specifically, THC concentrations may vary over time (183, 185), and the reported contents of cannabis products may be inaccurately labeled (186) as well as to vary between strains (183). The inconsistency in the self-report of cannabis product THC/cannabinoid content, cannabinoid content changes over time, and labeling inaccuracies are major factors that should be better controlled for in future studies.

Recently, there has been increased interest in the use of cannabis by physically active individuals. One study from our lab found that 90.5% physically active individuals, who reported using cannabis in conjunction with structured exercise, were using cannabis products at least once per week, with nearly 69% using on a daily basis (30). In a second survey study in individuals who were engaging in an average of 160-minutes of aerobic exercise a week, individuals reported using cannabis an average number of 5.5 days per week (187). In the present study, all participants were required to be physically active and reported using cannabis products at least once per week with

82% of participants using on a daily basis. This suggests that the CU participants in this study should be classified as heavy users. It is important to note that while 91% of CU had used cannabis products the day prior to testing, none reported that they were currently high on the DFAQ-CU, as they were specifically asked to refrain from cannabis use for at least 12-hours prior to testing.

Physical Characteristics

Overall average BMI was 24.61 kg/m^2, which would place individuals in this study within normal ranges (188). While there was no significant difference between groups with respect to BMI, it is important to note that 3 participants in NU and 3 in CU fell into the overweight or obese BMI categories. Average NU BMI of 25.42 kg/m^2 classified this group as overweight, while average CU BMI of 23.72 kg/m^2 were considered normal (188). Interestingly, BMI within individuals using marijuana flower or bud was positively associated with the amount of marijuana used per day, but no other correlations between BMI or cannabis use were present. Prior research has reported mixed effects with respect to BMI and cannabis use. In high school teens and HIV-positive patients using cannabis, there were no reported relationships between BMI and cannabis use (189, 190). Yet, in support of our current findings, multiple studies have reported that prolonged cannabis use was associated with lower BMI and decreased likeliness to be obese (191-193). In contrast, there was no difference in average BMI or group BMI classification between cannabis users or non-users who were equally physically active (181). It should be noted that while BMI is a simple way to provide insight into the body size of the general population it is often considered inaccurate in athletic populations as it does not take fat or fat-free mass into consideration.

While there was no significant difference between groups with respect to body fat percent, there was a moderate effect size accompanying this variable suggesting that there was a moderately meaningful difference present between groups. A combined average body fat percent for the present study of $11.2 \pm 5.4\%$ is significantly lower than the average body fat of male and female cannabis users and non-users of 28% observed in a study assessing the metabolic health, total body fat and abdominal fat distribution (141). Male participants had an average body fat of $8.7 \pm 4.1\%$, and with an average age of 26.2 years old, these participants would be classified in the 85-90th percentile for body fat percentage of similarly aged males (188). However, the average values for male body fat percentage of the present study were relatively lower than those observed in a previous study which reported an average body fat of $11.9 \pm 5.8\%$ in physically active, college aged, male cannabis users and non-users (31). The female participants in the present study had an average body fat of $17.0 \pm 2.6\%$ and, with an average age of 31.1 years old, corresponds to the 80-85th percentile for body fat percentage of similarly aged females (188). While the aforementioned study by Muniyappa et al. 2013 described no difference in total body fat when comparing cannabis users to non-users, which is similar to our study, authors reported that cannabis users had significantly lower total abdominal fat and subcutaneous abdominal fat as measured by magnetic resonance imaging (141). While our study did not measure abdominal fat area or subcutaneous fat area, the average skin-fold thickness at the abdominal site was 33% lower (p=0.04) in CU, which was accompanied by a large effect size of 0.93. Despite these findings, there were no significant relationships between abdominal skin-fold thickness and measures of cannabis

use. This effect warrants deeper exploration into the possible physiological effects that may be leading to this difference.

Physical Activity Habits

The American College of Sports Medicine recommends that individuals engage in at least 2.5-hours of moderate physical activity per week or at least 1.25-hours of vigorous physical activity per week (188). All participants exceeded these recommendations and were engaged in an average of 5.01-hours of moderate physical activity and 5.6-hours of vigorous physical activity on a weekly basis. There was no observed difference in the days, duration, or overall average time that NU and CU engaged in either moderate or vigorous physical activity. These values are comparable to the self-reported total amount of hours of moderate (5.2-hours) and vigorous (5.5-hours) physical activity engaged in by male cannabis users and non-users in a previous study (31). On average, CU in the present study engaged in a total of 8.6 hours of moderate or vigorous activity per week. This was is significantly greater than the total amount of moderate or vigorous physical activity that was reported by physically active cannabis users in two recent survey studies of 4.3 and 6.7 hours per week (30, 187).

Previous research on cannabis use and sedentary activity has provided mixed findings and has mostly focused on the youth or young adult populations. One recent study found that in adolescents aged 12 to 15 years old, more frequent use of cannabis was associated with increased sedentary activity (194), but in a similarly aged population of 14-16-year-old adolescents, there was no relationship between these individuals with respect to their cannabis use or sedentary behavior (195). In another recent study, authors reported that in emerging, young adults, the frequent use of cannabis was associated with

an increased likelihood of meeting physical activity requirements (191). A self-reported,

survey-based finding that assessed physical activity habits of adults who used cannabis

reported that participants who used cannabis with exercise engaged in significantly

greater minutes of physical activity per week compared to cannabis users who did not use

cannabis with their exercise (187). In our study, we found that our physically active

cannabis users spent 37% less time sitting per day when compared to non-users. Given

that there was no difference in the amount of time CU spent engaging in moderate/

vigorous activity or walking on a daily basis, it is possible that CU spend more time

standing or sleeping on a daily basis or engage in more intermittent light activity. Further

research is needed assessing the activity habits of cannabis users throughout the entire

day with more accurate, real-time, objective measures.

Cardiovascular and Performance Measures

The acute use of cannabis products increases resting heart in adults and

adolescents (196-199). There is additional support that even the chronic use of cannabis

alters the sympathovagal balance, with cannabis users displaying increased heart rate

variability compared to individuals that were matched for age and BMI who were not

using illicit drugs or pharmaceuticals (200). While the acute use of cannabis on resting

heart rate has been assessed in multiple studies, there were no differences in NU and CU

resting heart rate in the present study. This is conflicting with data previously reported by

our lab in which physically active male and female cannabis users had significantly

higher resting HR than NU (181) but is in support of our previous findings in physically

active male cannabis users and non-users (31).

There are mixed effects of acute cannabis use on systolic blood pressure with both increases (201) and no-changes (198) observed; however, in both of these studies, diastolic blood pressure increased immediately following use (198, 201). We have not observed any differences in our lab with respect to either systolic or diastolic blood pressure (31, 181). The average systolic blood pressure of 110.8 mmHg and diastolic blood pressure of 71.4 mmHg would place them into the normal classifications of 100-119 mmHg and 60-79 mmHg, respectively (202).

According to ACSM standards, the male participant average $\dot{V}O_2$max in the present study would place them into the excellent category for $\dot{V}O_2$max based on a mean age of 26.2 years and $\dot{V}O_2$max of 47.8ml/kg/min (188). Female participants would also be categorized in the excellent category based on a mean age of 31.1 years and $\dot{V}O_2$max of 41.1ml/kg/min (188). There were no participants who received a classification lower than "good," which would be considered above average in relative terms (188). In comparison to a previous study in our lab which explored $\dot{V}O_2$max in male and female chronic cannabis users and non-users, the mean $\dot{V}O_2$max in the present study was significantly lower compared to a mean of 50.3 ± 7.4ml/kg/min (181). Despite this difference, there was no difference between NU and CU in the present study. In the two other studies assessing cardiorespiratory fitness in physically active cannabis users and non-users, findings from the current study are consistent with both studies with no reported difference in cardiorespiratory performance in cannabis users or non-users not currently under the influence of cannabis (31, 181). In a more acute exercise setting, cannabis inhalation prior to a maximal cycling test, revealed no difference in $\dot{V}O_2$max in cannabis and placebo trials (154). The results of this study may no-longer be applicable,

as the cannabis flower/bud currently available at recreational dispensaries has an average THC content of 15 to 24% which is significantly greater than the 1.7% THC cannabis participants smoked in their in their study (183, 184).

Psychological Characteristics

Previous research on depressive score measured by the BDI-II in physically active cannabis users has not detected differences when cannabis users were compared to non-users (181). In the present study, CU had a significantly higher average BDI-II score compared to NU even after controlling for age and BMI, and BDI-II score. Additionally, scores correlated to the frequency of cannabis usage on weekend days. Four participants in the CU group reported that they had received treatment for depression in the past, however, none of these participants reported that they were currently suffering from depression nor had they been hospitalized for depression in the past six-months or currently receiving treatment. In another study conducted in our lab, physically active CU had significantly lower circulating brain derived neurotrophic factor (BDNF), which was negatively correlated to total BDI-II score and positively correlated to CRP (181).

In recent years, cannabis use has been associated with an increased incidence of depression in adolescents (203) and frequent cannabis users (191, 204). Further, in individuals prescribed opioids for pain, depression positively correlated to self-reported percent of THC and CBD in cannabis products, particularly those high in CBD (205), however, it was unclear in this study if the increase in depressive symptoms associated with cannabis use were the result of cannabis use itself or the result of an interaction effect between the co-use of opioids and cannabis. One possible explanation for the observed effects of cannabis on depression could be the result of the interaction between

CBD and the serotonin 1a (5-HT1a) receptor. The non-intoxicating cannabinoid, CBD, is a moderate agonist of the 5-HT1a receptor, displacing [3H]8-OH-DPAT, which is another known agonist of the receptor in a concentration dependent manner (206). Similar to the CB1 and CB2 receptors, the 5-HT1a receptor is a 7-transmembrane alpha-helical G-protein coupled receptor that inhibits activation of adenylyl cyclase (207). Both dysfunction and dysregulation of the 5-HT1a receptor have been implicated as an underlying cause in depressive disorders (207, 208). Consequently, it is possible that the chronic use of cannabis products causes disruption and dysregulation of the 5-HT1a receptor leading to increases of depressive symptoms. However, it is still unclear if the chronic use of cannabis products is leading to these increased depressive symptoms or depressive symptoms are leading to cannabis use.

Resting Concentrations of Markers of Inflammation

The average resting concentration for circulating CRP in the present study was not significantly different between groups and the group averages would place them both at low risk for cardiovascular disease (CVD) (14). These findings contradict previous research from our lab in which average CRP concentrations placed cannabis users at moderate risk for CVD and non-users at low risk for CVD (31, 181). While there was no relationship in the previous studies with respect to CRP and cannabis use, the present study found that there was a positive relationship between CRP and the amount of marijuana used per week in individuals using marijuana flower or bud. Although the present study was cross-sectional in design, results may be similar to a longitudinal study in which, over an 8-week treatment period, patients suffering from Crohn's disease who used cannabis did not have any changes or differences in pre to post CRP concentrations

compared to a placebo control (170). However, a more recent epidemiological study using the U.S. National Health and Nutrition Examination Survey data, found that the use of cannabis within the past 30-days was associated with lower concentrations of CRP than those who had not used cannabis within that same time frame (32). Although these results are intriguing, the authors did not account for participant physical activity status. It is also possible that there is no observable difference with respect to CRP between physically active CU and NU because regular exercise acts powerfully to decrease circulating CRP (18, 19). This physical activity effect may be eliminating the difference in CRP observed within the general population.

It is important to note that no participants had CRP concentrations greater than 10 mg/L, which suggests that none of the participants were experiencing an acute infection. The majority of participants (78%) were classified as low risk for cardiovascular disease based on circulating CRP. Three individuals, two NU and one CU, were classified as moderate risk and two other participants, one NU (5.51 mg/L) and one CU (3.96 mg/L), were classified as high risk. Finally, when compared to the general population average CRP concentration of 3.9 mg/L, both groups were significantly lower than the national average. This effect may be attributable to the physical activity habits of the subjects in the present study, which was discussed previously (32).

Circulating CRP and IL-6 concentrations are related (13) with CRP production being dependent on IL-6 (13, 84). Therefore, it is not surprising that a significant, positive relationship between CRP and IL-6 concentrations was observed when groups were combined in the current study. Even though a positive relationship was present between the amount of marijuana flower/bud used per week and resting concentrations of

IL-6, this trend did not result in a significant difference in IL-6 concentrations between NU and CU groups. This result supports previous findings from our lab in which physically active cannabis users and non-users had an average resting concentration of 1.26 pg/mL, which was similar to the total participant average of 1.10 pg/mL, which was observed in the present study (181). Furthermore, a separate study observed no differences in IL-6 concentrations when recent, previous and never users of cannabis were compared (209). Conversely, in another study, where activity status was not accounted for, the long-term use of only cannabis was related to lower concentrations of circulating IL-6 compared to a non-user group and a group of individuals who used cannabis in conjunction with other drugs (210). In comparison to the values reported by Keen et al. 2014, the average IL-6 values of participants in our study was 1.10 pg/mL which is significantly lower than the group average for both the only cannabis use and the non-user group of 2.20 ± 1.93 pg/mL and 3.73 ± 6.28 pg/ml respectively (210). The long-term effects of exercise have produced mixed effects on resting IL-6. Some studies report no effect of long-term exercise training on resting IL-6 (211, 212) while others have observed a reduction in circulating IL-6 (213, 214). Some suggest that this effect may be dependent on the presence of the SNP-174C, IL-6 gene variant (214). Given all of these findings, it is possible that the immune modulatory effects of sustained exercise are greater than those of the chronic use of cannabis and nullified any possible observable effects between groups in this study.

Monocyte Phenotype

To the researcher's knowledge, this is the first study to describe circulating monocyte populations in healthy participants that are using cannabis. Chronic cannabis

users displayed altered monocyte phenotype and count compared to non-using participants. Specifically, CU had a significantly greater relative percent of intermediate monocytes. In healthy individuals, the expected percent of intermediate monocytes circulating in the blood is 5% (37). Cannabis users in this study had nearly three-times the expected percent of intermediate monocytes at 13.8% and had more than double the percent of NU at 6.33%. This difference in the relative percent of intermediate monocytes was supported by a large effect size between groups. Further, when standardizing the number of intermediate monocytes per milliliter of blood, CU had a significantly greater number of intermediate monocytes when compared to NU, which was also accompanied by a large effect size. These results suggest that CU have a significantly greater relative percentage of intermediate monocytes and a greater number of intermediate monocytes per milliliter. In terms of relating cannabis use to the number of intermediate monocytes per milliliter of blood, the number of hits per day of cannabis concentrates and the THC content of edible cannabis products was positively correlated to the number of intermediate monocytes in CU; however, both of these correlations are based on small sample sizes of n=6 and n=5, respectively, limiting the validity of these observations. Yet, in the 10 individuals that reported using marijuana flower or bud, the number of times these participants used per day was positively associated with the number of intermediate monocytes per milliliter. Previous research observed that the administration of THC reduced monocyte ability to differentiate (34, 215). Intermediate monocytes display functions and cell surface markers of classical and non-classical monocytes. The increased number of intermediate monocytes could be the result of THC limiting the ability of chronic user monocytes to fully differentiate into either the classical or non-

classical sup-populations. Finally, even though previous research has related the number of intermediate monocytes to increased risk for chronic disease including CVD, there was no correlation between the number of intermediate monocytes and the resting concentrations of either CRP or IL-6.

The findings from the present study conflict with previous results in which cannabis use was associated with a significant reduction of CD16$^+$ monocytes in individuals diagnosed with HIV (34, 216). However, HIV is an immunocompromising disease, and, in that same study, HIV infected non-users had a significantly greater number of CD16$^+$ monocyte when compared to both the non-HIV infected, non-cannabis user control group and the HIV infected cannabis users (34). In that study, there was no non-HIV infected, cannabis user group so it is plausible that in a diseased population, like those with HIV, cannabis use suppresses CD16$^+$ monocyte numbers, but may promote CD16$^+$ monocyte numbers in a relatively healthy population. Across all participants in the present study, the average percent of circulating classical monocytes, which was 77%, was slightly below the expected norm of 85% in a healthy population (37). However, when accounting for classical monocyte group average percentages, NU were closer to the expected norm at 80% while CU were still significantly lower at 74%. Although, CU were below the expected norm with respect to percent classical monocytes, there was no difference between NU or CU groups.

Clinically, the average number of circulating monocytes per milliliter of 4.12 x 10^5 is similar to that reported in college aged males and females of average physical activity status (3.60 x 10^5 cells per milliliter) and physically active females (3.74 x 10^5 cells per milliliter) (110, 217). When controlling for monocytes per milliliter, CU had

significantly more classical monocytes when compared to NU, which was accompanied by a large effect size between groups. Similar effects have been reported when heavy cannabis users were compared to non-users (216). In our study, we believe the reported differences in the number of classical monocytes per milliliter are most likely the result of CU also having a significantly greater number of total monocytes per milliliter. When the number of classical monocytes was relativized with the other two monocyte populations, this difference was no-longer present. Further, the large effect size that was observed between the number of classical monocytes per milliliter between groups was absent in the relative percent of classical monocytes. It is difficult to ascertain whether this finding is consistent with previous research as the numbers of circulating CD14^{++} monocytes in HIV-infected cannabis users was not presented or discussed (34). The non-classical monocytes were the only sub-population that was not different between NU and CU groups in terms of either relative percent or the number of cells per milliliter. In spite of this, our findings with respect to classical and intermediate monocytes suggest that there are significant alterations in monocyte phenotype between NU and CU.

Lipopolysaccharide Stimulated IL-6 Production

Previous research has shown that acute treatment with cannabinoids like THC and CBD results in immune suppressive effects on pro-inflammatory cytokine production in response to an immune stimulus (167). In the 2010 study, authors used a concentration of 1μM CBD to pretreat BV-2 microglial cells, which was the same concentration of the CB2 agonist and antagonist used in our study, prior to stimulating cells with LPS. The pretreatment with CBD resulted in a 25% decrease in stimulated IL-6 release compared to their LPS stimulated control (167). This CBD induced suppression of IL-6 was not

observed in the present study as there was no differences in LPS induced IL-6 production

in either CU or NU groups when pretreated with the either the CB2 agonist or antagonist.

This lack of differences in stimulated IL-6 persisted despite standardizing for the number

of total monocytes per milliliter. It is possible that this response may be the result of time

course dependent effects. For example, in the aforementioned study, control and

stimulated samples were collected 4-hours post addition of LPS in which non-

cannabinoid stimulated samples had significantly greater concentrations of IL-6 (167).

Yet, in the present study, stimulated supernatant was collected 24-hours post LPS

stimulation. Consequently, it is plausible that CB2 agonists like CBD or JWH-015 have

acute suppressive effects, and the longer samples are exposed to LPS, the more likely

these samples are able overcome these suppressive effects.

Another explanation for the lack of difference between stimulated treatments and

groups could be the lack of difference in both the circulating percent and the number of

non-classical monocytes per milliliter. When all participants were considered, only the

non-classical monocyte subtype was positively associated with the total amount of IL-6

release in any of the conditions. Classical monocytes are considered largely phagocytotic

with limited inflammatory attributes; while intermediate monocytes display moderate

functions of both classical and non-classical sub-sets. It is largely the non-classical

monocytes that are attributed to pro-inflammatory cytokine release (218-220). The

number of classical and intermediate monocytes was significantly greater in CU

compared to NU, but there was no difference in either the percent or number per milliliter

of non-classical monocytes. This lack of difference in the intermediate monocyte sub-set

may be accountable for the lack of difference in LPS induced cytokine release. Further,

there is a lack of literature pertaining to the distribution of both the CB1 and the CB2 receptor expression between the different monocyte populations. If the CB2 receptor is expressed to a lesser extent in non-classical monocytes, it may result in a reduced response to LPS.

Limitations

This study was cross-sectional in design and compared physically active NU to physically active CU. As such, we cannot be certain that reported differences in monocyte phenotype and number are strictly the result of cannabis use status. All participants met the inclusion criteria for use and non-use status; however, half of the participants in the NU group had previously used cannabis to some extent throughout their life. It is possible that the previous use status of these participants could be confounding factors of these results. Further, while all CU were primarily using cannabis by inhalation methods, there was variation as to the primary method of inhalation as well as their secondary method of use. The type of cannabis product, the frequency of use, and timing of cannabis use was also not standardized across CU. Future research assessing these parameters should aim to standardize these variables to minimize variation between participants in terms of cannabis use. Further, in the future, research should focus on comparing not only physically active NU and CU but also non-physically active NU and CU. This will help elucidate if similar observations are observed regardless of physical activity status causes different effects.

At the time of this study, the phytocannabinoids THC and CBD were still considered schedule one substances and researchers were forced to use synthetically derived cannabinoids to mimic the effects of the aforementioned phytocannabinoids.

These synthetic cannabinoids are designed specifically to stimulate or suppress activation of the CB2 receptor; however, THC and CBD act on more than just the CB2 receptor. In the future, not only should the effects of purified CBD and THC extracts be fully explored, but the effects of full spectrum cannabis extracts containing all the active ingredients within the cannabis plant may be valuable. This approach would be more reflective of products available on the market for recreational and medicinal consumption. Finally, immune stimulations in this study were reflective of how an immune response would proceed in response to non-sterile inflammation, like an infection. Future studies should also assess the immune response in the presence of a sterile stimulus, like adenosine, that would be reflective of tissue damage like resulting from trauma or intense exercise.

Conclusions

Findings from this study provide novel insight into the effects of chronic cannabis use in physically active individuals. Although there were no differences between CU and NU groups with respect to age, mass, height, body fat percent, BMI, resting heart rate, blood pressure or $\dot{V}O_2max$ there was evidence to suggest there might be differences in the fat distribution, with CU having a significantly lower abdominal skinfold thickness. Cannabis users displayed altered monocyte phenotype compared to NU, with CU displaying more than a 2-fold increase in the relative circulating percentage of intermediate monocytes. In addition to altered monocyte phenotype, CU had significantly greater total monocytes, classical and intermediate monocyte sub-populations per milliliter. Non-classical monocytes were the only cell population to be similar in both groups in terms of both relative percent and cells per milliliter. It is believed that this lack

of difference in the non-classical cell population is potentially accountable for the lack of difference observed in LPS stimulated IL-6 release in the presence of CB2 agonist and antagonist pre-treatment. In conclusion while previous research suggests that individually exercise and cannabis use are associated with beneficial effects on monocyte phenotype and immune function there is not a combinatorial effect between the two. Monocyte phenotype and count did differ between physically active NU and CU, but this was not related to changes in resting concentrations of inflammatory markers CRP and IL-6, nor did it alter whole blood LPS stimulated IL-6 release.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL

I n s t i t u t i o n a l   R e v i e w   B o a r d

DATE: February 11, 2019

TO: Laura Stewart
FROM: University of Northern Colorado (UNCO) IRB

PROJECT TITLE: [1185722-5] Metabolism and Activity with Cannabis (MAC) Study
SUBMISSION TYPE: Continuing Review/Progress Report

ACTION: APPROVED
APPROVAL DATE: February 11, 2019
EXPIRATION DATE: February 20, 2020
REVIEW TYPE: Expedited Review

Thank you for your submission of Continuing Review/Progress Report materials for this project. The University of Northern Colorado (UNCO) IRB has APPROVED your submission. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on applicable federal regulations.

Please remember that informed consent is a process beginning with a description of the project and insurance of participant understanding. Informed consent must continue throughout the project via a dialogue between the researcher and research participant. Federal regulations require that each participant receives a copy of the consent document.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure.

All UNANTICIPATED PROBLEMS involving risks to subjects or others and SERIOUS and UNEXPECTED adverse events must be reported promptly to this office.

All NON-COMPLIANCE issues or COMPLAINTS regarding this project must be reported promptly to this office.

Based on the risks, this project requires continuing review by this committee on an annual basis. Please use the appropriate forms for this procedure. Your documentation for continuing review must be received with sufficient time for review and continued approval before the expiration date of February 20, 2020.

Please note that all research records must be retained for a minimum of three years after the completion of the project.

If you have any questions, please contact Nicole Morse at 970-351-1910 or nicole.morse@unco.edu. Please include your project title and reference number in all correspondence with this committee.

APPENDIX B

PEARSON CORRELATION MATRIX

Correlations

		CRP	IL-6: Resting	IL-6: LPS Control	IL-6: Ag+LPS	IL-6: Ant+LPS	Classical Monocytes/mL	Non-classical Monocytes/mL	Intermediate Monocytes/mL	Monocytes/mL	Total BDI-II Score	DFAQ-CU Question 10	DFAQ-CU Question 11	DFAQ-CU Question 18a	DFAQ-CU Question 19a	DFAQ-CU Question 20a	DFAQ-CU Question 16b	DFAQ-CU Question 17b	DFAQ-CU Question 21c
CRP	Pearson Correlation	1	.524*	.323	.386	.415*	.079	.569**	-.037	.168	-.184	-.128	.413	.611	.705*	.111	-.359	-.142	.550
	Sig. (2-tailed)		.010	.132	.069	.049	.728	.006	.869	.454	.401	.707	.207	.061	.023	.760	.485	.788	.337
	N	23	23	23	23	23	22	22	22	22	23	11	11	10	10	10	6	6	5
IL-6: Resting	Pearson Correlation	.524*	1	.569**	.641**	.601**	-.053	.712**	-.094	.085	-.031	-.321	.218	.464	.690*	-.109	-.427	-.204	-.082
	Sig. (2-tailed)	.010		.005	.001	.002	.814	.000	.677	.708	.889	.335	.520	.177	.027	.764	.399	.699	.896
	N	23	23	23	23	23	22	22	22	22	23	11	11	10	10	10	6	6	5
IL-6: LPS Control	Pearson Correlation	.323	.569**	1	.955**	.941**	.072	.681**	.035	.208	-.096	-.065	.274	.501	.740*	.182	-.678	-.230	-.076
	Sig. (2-tailed)	.132	.005		.000	.000	.752	.000	.878	.354	.664	.849	.415	.140	.014	.614	.139	.661	.903
	N	23	23	23	23	23	22	22	22	22	23	11	11	10	10	10	6	6	5
IL-6: Ag+LPS	Pearson Correlation	.386	.641**	.955**	1	.951**	-.015	.757**	-.026	.140	-.129	-.096	.180	.408	.763*	.049	-.702	-.341	-.523
	Sig. (2-tailed)	.069	.001	.000		.000	.949	.000	.910	.534	.557	.779	.597	.242	.010	.893	.120	.508	.366
	N	23	23	23	23	23	22	22	22	22	23	11	11	10	10	10	6	6	5
IL-6: Ant+LPS	Pearson Correlation	.415*	.601**	.941**	.951**	1	.129	.681**	.022	.249	-.037	-.098	.322	.482	.747*	.113	-.671	-.372	-.282
	Sig. (2-tailed)	.049	.002	.000	.000		.567	.000	.922	.264	.866	.775	.334	.158	.013	.756	.144	.468	.646
	N	23	23	23	23	23	22	22	22	22	23	11	11	10	10	10	6	6	5
Classical Monocytes/mL	Pearson Correlation	.079	-.053	.072	-.015	.129	1	.075	.640**	.962**	.448*	.295	.475	.627	.039	.597	.690	-.229	.313
	Sig. (2-tailed)	.728	.814	.752	.949	.567		.740	.001	.000	.037	.379	.140	.052	.915	.069	.129	.662	.608
	N	22	22	22	22	22	22	22	22	22	22	11	11	10	10	10	6	6	5
Non-classical Monocytes/mL	Pearson Correlation	.569**	.712**	.681**	.757**	.681**	.075	1	-.064	.248	-.151	-.266	.103	.439	.711*	-.146	.556	-.305	-.370
	Sig. (2-tailed)	.006	.000	.000	.000	.000	.740		.779	.266	.502	.429	.762	.205	.021	.687	.252	.556	.540
	N	22	22	22	22	22	22	22	22	22	22	11	11	10	10	10	6	6	5
Intermediate Monocytes/mL	Pearson Correlation	-.037	-.094	.035	-.026	.022	.640**	-.064	1	.753**	.475*	.745**	.403	.374	-.201	.864**	.117	.916*	.934*
	Sig. (2-tailed)	.869	.677	.878	.910	.922	.001	.779		.000	.025	.009	.220	.288	.577	.001	.825	.010	.020
	N	22	22	22	22	22	22	22	22	22	22	11	11	10	10	10	6	6	5
Monocytes/mL	Pearson Correlation	.168	.085	.208	.140	.249	.962**	.248	.753**	1	.445*	.410	.553	.744**	.144	.729*	.459	.242	.426
	Sig. (2-tailed)	.454	.708	.354	.534	.264	.000	.266	.000		.038	.210	.078	.014	.691	.017	.360	.644	.474
	N	22	22	22	22	22	22	22	22	22	22	11	11	10	10	10	6	6	5
Total BDI-II Score	Pearson Correlation	-.184	-.031	-.096	-.129	-.037	.448*	-.151	.475*	.445*	1	.434	.620*	.218	.205	.430	.055	-.290	-.315
	Sig. (2-tailed)	.401	.889	.664	.557	.866	.037	.502	.025	.038		.182	.042	.546	.569	.215	.918	.577	.606
	N	23	23	23	23	23	22	22	22	22	23	11	11	10	10	10	6	6	5
DFAQ-CU Question 10	Pearson Correlation	-.128	-.321	-.065	-.096	-.098	.295	-.266	.745**	.410	.434	1	.797**	.373	.205	.823**	-.140	.130	-.413
	Sig. (2-tailed)	.707	.335	.849	.779	.775	.379	.429	.009	.210	.182		.006	.289	.570	.003	.791	.805	.490
	N	11	11	11	11	11	11	11	11	11	11	11	11	10	10	10	6	6	5
DFAQ-CU Question 11	Pearson Correlation	.413	.218	.274	.180	.322	.475	.103	.403	.553	.620*	.797**	1	.600	.202	.215	-.404	.625	.192
	Sig. (2-tailed)	.207	.520	.415	.597	.334	.140	.762	.220	.078	.042	.006		.067	.576	.551	.427	.185	.757
	N	11	11	11	11	11	11	11	11	11	11	11	11	10	10	10	6	6	5
DFAQ-CU Question 18a	Pearson Correlation	.611	.464	.501	.408	.482	.627	.439	.374	.744**	.218	.373	.600	1	.522	.643*	-.254	-.237	-.425
	Sig. (2-tailed)	.061	.177	.140	.242	.158	.052	.205	.288	.014	.546	.289	.067		.122	.045	.681	.702	.575
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	5	5	4
DFAQ-CU Question 19a	Pearson Correlation	.705*	.690*	.740*	.763*	.747*	.039	.711*	-.201	.144	.205	.205	.202	.522	1	.717*	-.837	-.531	-.265
	Sig. (2-tailed)	.023	.027	.014	.010	.013	.915	.021	.577	.691	.569	.570	.576	.122		.020	.077	.358	.735
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	5	5	4
DFAQ-CU Question 20a	Pearson Correlation	.111	-.109	.182	.049	.113	.597	-.146	.864**	.729*	.430	.823**	.215	.643*	.717*	1	-.296	.671	.408
	Sig. (2-tailed)	.760	.764	.614	.893	.756	.069	.687	.001	.017	.215	.003	.551	.045	.020		.628	.215	.592
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	5	5	4
DFAQ-CU Question 16b	Pearson Correlation	-.359	-.427	-.678	-.702	-.671	.690	.556	.117	.459	.055	-.140	-.404	-.254	-.837	-.296	1	-.296	1.000**
	Sig. (2-tailed)	.485	.399	.139	.120	.144	.129	.252	.825	.360	.918	.791	.427	.681	.077	.628		.628	.
	N	6	6	6	6	6	6	6	6	6	6	6	6	5	5	5	6	6	2
DFAQ-CU Question 17b	Pearson Correlation	-.142	-.204	-.230	-.341	-.372	-.229	-.305	.916*	.242	-.290	.130	.625	-.237	-.531	.671	-.296	1	1.000**
	Sig. (2-tailed)	.788	.699	.661	.508	.468	.662	.556	.010	.644	.577	.805	.185	.702	.358	.215	.628		
	N	6	6	6	6	6	6	6	6	6	6	6	6	5	5	5	6	6	2
DFAQ-CU Question 21c	Pearson Correlation	.550	-.082	-.076	-.523	-.282	.313	-.370	.934*	.426	-.315	-.413	.192	-.425	-.265	.408	1.000**	1.000**	1
	Sig. (2-tailed)	.337	.896	.903	.366	.646	.608	.540	.020	.474	.606	.490	.757	.575	.735	.592	.		
	N	5	5	5	5	5	5	5	5	5	5	5	5	4	4	4	2	2	5

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).