Investigating the Biphasic Effect of Cannabidiol on $CD4^+$ T Cell Function and Rhythmicity

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Investigating the Biphasic Effect of Cannabidiol on $CD4^+$ T-cell Function and Rhythmicity

Norhan Mehrez

$CD4^+$ T cells enable adaptive immunity to pathogens, notably via cytokine secretion (IL2, IFNγ). Cannabidiol (CBD) has been shown to inhibit this secretion. However, with growing evidence suggesting cannabinoids behave biphasically, there is a need to re-evaluate the effect of CBD on $CD4^+$ T cells at a wider range of doses, including the role of CBD's target receptor, CB2. Additionally, while never investigated in $CD4^+$ T cells, CBD has been shown to moderate clock gene expression (BMAL1, PER2) in other immune cells, which may present an additional mechanism of regulating $CD4^+$ T cell activity. The present study therefore aimed to (1) investigate whether a biphasic dose-response relationship of CBD on $CD4^+$ T cell cytokine expression may exist, (2) whether CB2 activation is biphasic in response to selective stimulation, and (3) whether a biphasic effect on clock gene regulation exists. Primary $CD4^+$ T cells were stimulated *in-vitro*; CBD or a CB2-selective antagonist (AM630) were incubated at doses ranging 0.001-20 μM. ELISAs were performed to assess cytokine secretion (IL-2, IFNγ); BMAL1 and PER2 gene expression were measured via qPCR. A biphasic trend of cytokine secretion was indeed visualized for most participants, for both drugs. However, the doses at which these trends manifested varied highly across participants and drug vehicle used. No statistically significant, consistent trend was observed for either ELISA nor qPCR data. Still, it is recommended that future studies utilizing these compounds assess effects across a wide range of doses, with special attention to individual differences, vehicles used, and target rhythmicity.

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Abbreviations

APC – Antigen Presenting Cell

AM630 - 6-iodopravadoline

BMAL1 – Brain and Muscle ARNT-Like 1

CBD – Cannabidiol

CD3 – Cluster of differentiation 3

CD4 – Cluster of differentiation 4

cDNA – complementary DNA library

CR – Circadian Rhythm

DMSO – Dimethyl Sulfoxide

ELISA – Enzyme-Linked Immunosorbent Assay

IFNγ – Interferon-gamma

IL-2 – Interleukin-2

RNA – Ribonucleic acid

Sav-HRP – Streptavidin Horse Radish Peroxidase

IL-17 – Interleukin-17

NTC – No-Template Control

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffer Saline

PER2 – Period circadian clock 2

PPIA – Peptidylprolyl Isomerase A

RT-qPCR – Reverse-Transcription Quantitative Polymerase Chain Reaction

RPMI – Roswell Park Memorial Institute Medium

SCN – Suprachiasmatic nucleus

TCR – T Cell antigen Receptor

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The consumption of *Cannabis Sativa* marks an impressive history spanning several millennia. Dating back to at least the early bronze age, through to being documented in the earliest forms of written language, and up to current modern day, Cannabis remains poised as one of the longest and most commonly consumed psychoactive substances in the world (Pisanti & Bifulco, 2019). Indeed, its use is particularly pervasive in North America, with approximately one fifth of Canadians considering themselves current users (Government of Canada, 2019). The major active ingredient of Cannabis, delta-9-tetrahydrocannabinol (THC) largely popularized the plant for its psychoactive effects, ranging from appetite stimulation to euphoria (Dos Santos et al., 2021; Pisanti & Bifulco, 2019a). Cannabidiol (CBD), on the other hand, the second major active compound in Cannabis, is non psychogenic and remained comparatively obscured in public and academic interest (Mechoulam & Hanus, 2000). As its significant therapeutic properties —such as being an anxiolytic (Campos et al., 2013), an anti-inflammatory agent (Silvestro et al., 2020; White, 2019), and more—became uncovered and attributed to some of the therapeutic properties of Cannabis, CBD has seen a surge in academic interest, consumer production, sales, and off-label self-medicative consumption over recent years (Pisanti & Bifulco, 2019). While some of CBD's aforementioned therapeutic properties have been demonstrated, much investigation is still underway to elucidate the full breadth of CBD's effects, in particular in the context of immunity. One such avenue of investigation involves the role of CBD in adaptive immunity. Notably, while research has shown immunomodulatory effects of CBD, the pharmacological action of the drug remains to be fully elucidated, especially with respect to the

dose-response relationship which may exhibit a biphasic trend, that is, opposite effects on immunity at differing doses rather than a classical sigmoidal dose-response trend (Chaperon & Thiebot, 1999; Perisetti et al., 2020). Investigating such biphasic activity on immunity thereby becomes a crucial component of bridging current understanding of CBD's immunomodulatory effects in a comprehensive manner.

CBD and Adaptive Immunity

Adaptive immunity defines the ability to develop tailored, long-lasting immunity to specific pathogens, in contrast to innate immunity which employs a more immediate, less adaptable response to threats. Peripheral blood mononuclear cells (PBMCs), also known as white blood cells, comprise several subtypes of immune cells including T cells, comprising approximately 75% of PBMCs, B cells, Natural Killer cells and monocytes (Jr et al., 2001).

Crucial to adaptive immunity and embodying the predominant subpopulation of PBMCs, $CD4^+$ T cells help detect the presence of a pathogen and orchestrate an immune response amongst themselves and other PBMCs against the given pathogen. This process begins when an antigen-presenting cell (APC), such as a dendritic cell, internalizes an antigen and presents a cleaved fragment of it onto an APC surface receptor known as the Major Histocompatibility Complex Class II (MHC II). This antigen presentation binds the T-cell antigen receptor (TCR) on naïve $CD4^+$ T cells. At this occurrence, and to stabilize this binding, the APC's MHC complex also binds to a co-receptor on the surface the $CD4^+$ T cell, the cluster of Differentiation 4 (CD4), initiating the first event of cell activation known as Signal 1 (Jr et al., 2001). As a safeguard from erroneous activation against self-antigens which may bind to MHCs of antigen presenting cells, a second signal, Signal 2 is required for full T-cell activation. This signal involves the co-

stimulation of the T cell co-receptor Cluster of Differentiation 28 (CD28), typically done by the same APC. This second signal along with the initial antigen presentation triggers full activation: The $CD4^+$ T cell initiates cytokine secretion, notably Interleukin-2 (IL-2), and interferon-gamma (IFNγ), to promote cell survival, proliferation, and differentiation into specialized subtypes of $CD4^+$ T cells. This cytokine secretion also serves to invoke and activate additional $CD4^+$ T cells, as well as other types of immune cells, thereby launching a complex and multi-pronged immune response specific to the presented antigen (Jr et al., 2001).

CBD has been shown to moderate this immune function via various mechanisms. Specifically, it is reported to have immunosuppressive effects via general decrease of cytokine secretion and, naturally, resulting cell proliferation (Peyravian et al., 2020; Zgair et al., 2017). Despite the paucity of studies investigating CBD and cytokine release in $CD4^+$ T cell-only populations, CBD has been shown to decrease IL-2 in PBMCs, as well as IFNγ (Kaplan et al., 2008). Similarly, CBD decreases the expression of cytokines in the family of interleukin-6 (IL-6), providing additional indirect mechanisms that may contribute to decreased IL-2 activity (Kozela et al., 2016).

Hormesis

Touting CBD as an undisputed immunosuppressive agent, however, omits the growing body of work suggesting the drug, as a member of the cannabinoid family, may exhibit a paradoxical, biphasic effect. Anandamide, (AEA), an endogenous analog pharmacologically similar to CBD, has recently been shown to both stimulate and inhibit cell proliferation depending on the dosage (Miyato et al., 2009). While such outcomes of cannabinoid administration may seem conflicting, these differences may be reconciled through the lens of

hormesis. Hormesis, or a biphasic dose-response, is defined as a dose-response relationship where a given compound produces opposite effects in an organism or in a population of cells at different doses, rather than a classical sigmoidal dose-response (Mattson, 2008). Indeed, while there is scarcely any research investigating CBD in this manner, various other cannabinoids exhibit hormesis (Chaperon & Thiebot, 1999). For example, chronic cannabis consumption has long been considered an agent of neurological damage (Meier et al., 2012), leading to short-term memory deficits; alas more recently it has been shown that chronic low dose treatment with cannabis in fact promotes neurogenesis and improves cognition in older animals (Calabrese & Rubio-Casillas, 2018). A similar biphasic trend may be observed in the context of cannabinoids and anxiety with a low dose decreasing anxiety, and a high dose being anxiogenic; Petrie et al., 2021). Similar trends are seen with emesis, with an anti-nausea effect of cannabis at low doses, and cannabis-induced hyperemesis syndrome at higher doses (Perisetti et al., 2020) and body temperature, with low doses inducing hyperthermia, and higher doses inducing hypothermia in rats (Hodges & Ashpole, 2019). These mounting, recent findings of cannabinoids' tendency to act biphasically in various contexts render previous studies substantially incomplete. Previously documented effects of CBD, including its immunosuppressive activity, may not hold true in a linear fashion across doses. Verily, there is a need for researching this trend in the context of CBD and adaptive immunity.

Role of CB2 receptor

CBD's plural and variable effects have been theorized to stem, in part, from its plethora of receptor targets: cannabinoid receptors type 1 (CB1), cannabinoid receptors type 2 (CB2), vanillin receptors (HRPV-1), orphan receptor GPR-55, and serotonin receptors, to name a few

(Peyravian et al., 2020). Nevertheless, CB2 is mostly localized within immune tissues, notably PBMCs—including $CD4^+$ T cells (Klein et al., 2003). This dense presence places it auspiciously for modulating CBD's effect on T cell activity. Indeed, the CB2 receptor has been shown to modulate IL-2, IFNy, and related cytokines' release when stimulated with synthetically derived CB2-selective compounds (Cencioni et al., 2010; Robinson et al., 2013; Wang et al., 2013). While shown to modulate cytokine release, the exact mechanism of CB2 as well as CB2 ligands (including CBD and synthetic ligands), remains dubious. It is increasingly suggested that CB2's elusive mechanism is due, in part, to the variable responses it may produce based on dosage, receptor expression, and other cellular contexts (Basu & Dittel, 2011). As an example of the implications this poses for understanding the mechanism of action of CB2 ligands: AM630, a selective ligand for CB2, has traditionally been considered an antagonist, with some works reporting its action as an inverse agonist (Ross et al., 1999). However, more recently and comprehensively, it is deemed best classified as a protean ligand, by definition of which the drug may behave as an agonist, an antagonist, or an inverse agonist depending on the CB2 receptor's constitutive activity and cellular context (Bolognini et al., 2012). This flexibility in CB2's response to ligands further stresses the importance of assessing CB2-mediated cannabinoid effects through a lens of hormesis. Indeed, while AM630 is often used at a single dose to block studied effects of CBD, it is merited to assess whether AM630 may, independently administered, also have a biphasic modulatory effect on immune cells, which would improve understanding of CB2 activity and in turn shed light on how CBD may exert its effects through this receptor.

Circadian rhythms and $CD4^+$ T cells

Circadian rhythms (CRs) are 24-hour fluctuations in physiological processes that enable optimal alignment to a 24-hour-rhythm based environment. Such CRs manifest at various levels of the organism, spanning from a behavioural level (e.g. sleep-wake cycles) to the cellular level (rhythmic gene expression). Primarily, CRs synchronize to the environment via light entrainment: environmental light stimulates the retina, and this signal is relayed to the suprachiasmatic nuclei (SCN), also known as the "master clock". This signal allows the SCN to synchronize circadian rhythms in the brain, as well as in the rest of the body, through varied physiological cues.

In addition to entrainment, CRs are maintained inside and outside the SCN through rhythmic expression of a core set of genes, known as clock genes, including BMAL1, CLOCK, PER and CRY. The protein products of BMAL1 and CLOCK dimerize to promote transcription of PER and CRY. The protein products of PER and CRY then dimerize to prevent the BMAL1:CLOCK dimer from recruiting DNA transcription machinery. This cycle restarts when the PER:CRY dimers disintegrate, once again allowing BMAL1:CLOCK to promote expression of its targeted genes. This sequential, oscillatory process of positive and negative feedback occurs along a 24-hour cycle, and the genes involved in this loop have been shown to regulate the rhythmic transcription of many other genes, including those involved in immune (Labrecque & Cermakian, 2015; Takahashi, 2016).This oscillatory activity persists in tissues isolated from the body, as well as in cultured cells in-vitro (Bollinger et al., 2011)

An important instance of CRs in the immune system, at a functional level and at a molecular level, is the rhythmicity of $CD4^+$ T cells' activity. Indeed, $CD4^+$ T cells show robust diurnal rhythms of T cell proliferation, cytokine production, and clock gene expression.

(Bollinger, et al., 2011). Further, disruption of an individual's CR entrainment has been associated with the etiology of T helper cell-linked immune disorders, such as multiple sclerosis (Cermakian et al., 2014; Hedström et al., 2015). Conversely, challenges to the adaptive immune system have been shown to temporarily disrupt its CRs. For example, the administration of lipopolysaccharide (LPS), an endotoxin which stimulates T cells, has been shown to disrupt the expression of the PER2 clock gene in various tissues (Cermakian et al., 2014). Interestingly, the endocannabinoid system, comprising cannabinoids, their receptors and their degradative enzymes, seem implicated in CR modulation of cellular activity.

Does CBD modulate rhythmicity of $CD4^+$ T cells?

There are numerous indications of circadian rhythmicity in cannabinoid signalling and response. At the ligand level, Vaughn and colleagues have shown that serum concentrations of circulating endocannabinoids, including an endogenous analog of CBD, vary by time of day (Vaughn et al., 2010). In the same vein, at the receptor level, Bazwinsky-Wutschke and colleagues have shown that CB1 and CB2 expression in the rat liver exhibits robust circadian rhythms (2017). Further, the effect of THC on body temperature is time-of-day dependent (Hodges & Ashpole, 2019). More broadly, the SCN, responsible for overall synchronization of peripheral clocks in the organism, is found to be rich with cannabinoid receptors(Acuna-Goycolea et al., 2010). However, there is but one study to date, to our knowledge, that has examined the effect of CBD on circadian rhythms of the immune system. Conducted by Lafaye and colleagues, it was shown that CBD modulated clock gene expression in microglia, a subtype of immune cell which can behave as an APC (Lafaye et al., 2019). However, this work was done using a single dose, and at a single time point. Thus, a worthwhile exploration of CBD's

modulation of T-cell activity would involve investigating whether CBD similarly modulates clock genes in this cell population, at biphasic doses, and at additional time points. Indeed, proponents of the need for cannabinoid exploration stress the importance of considering cannabinoids' chronobiotic features through the lens of hormesis (Hodges & Ashpole, 2019).

Study Aims

The present aim of the study is therefore threefold. First and foremost, to investigate whether the effect of CBD on $CD4^+$ T cytokine secretion may constitute a biphasic dose-response relationship, whereby CBD is immunosuppressive or immune enhancing, by differing dosage. The second aim is to investigate the biphasic role of the CB2 receptor, by selectively stimulating it in a dose-response study. The third aim is to assess whether CBD modulates clock gene expression in a biphasic manner.

Methods

Ethics

The current project methods and guidelines were approved by and in accordance with the Concordia University Research Ethics committee, certificate #30009292.

Participants

Participants were recruited through the Psychology department's online participant pool at Concordia University, and through social media and word of mouth. Eligibility criteria consisted of being 18 years of age or older, having no known history of chronic illness or sleep disorders, not being a self-reported regular cannabis user, and not having received a vaccination in the two weeks prior to the blood draw. Upon arrival at Concordia University's PERFORM Centre, the participants were given the opportunity to provide their informed

consent for the study (Appendix A). With their consent, they were escorted into an examination room where 6-10 vials of venous blood were drawn by a licensed phlebotomist, using 10 mL Vacutainer sodium-heparin tubes to prevent coagulation prior to blood processing (BD Bioscience, Franklin Lakes, New Jersey). Participants were monitored for 15 minutes post-draw and offered refreshments prior to their dismissal. Participant samples and subsequent data were anonymously coded, whereby each participant was assigned a code "LCR###". Figure 1 depicts a simplified timeline of experimental workflow beginning with the blood draw.

Cell Culture Medium

10% FBS RPMI was prepared using RPMI 1640, 1X (Wisent, Saint-Jean-Baptiste, Quebec) or HyClone RPMI 1640 Media (Fisher Scientific, Saint-Laurent, Quebec). Penicillin-Streptomycin (HyClone, Fisher Scientific, Saint-Laurent, Quebec) was added to achieve a final 1% concentration and prevent pathogen growth. Similarly, Glutamax (HyClone, Fisher Scientific, Saint-Laurent, Quebec) was added to achieve a final 1% concentration. Finally, FBS (Wisent, Saint-Jean-Baptise, Quebec) was added to reach a final concentration of 10% or 20%, to create 10% FBS RPMI and 20% FBS RPMI, as needed.

PBMC processing

PBMC processing —and subsequent cell isolation and activation— was undertaken within a biological safety cabinet using sterile technique, materials and reagents. Within six hours of the blood draw, the blood was transferred from the sodium heparin collection vials into 50 mL conical tubes and diluted with PBS at a 1:1 ratio. Thirteen mL of Ficoll (VWR, Mont-Royal, Quebec) or lymphocyte separation media (Wisent, Saint-Jean-Baptise, Quebec) was added to a new set of 50 mL conical tubes, and up to 37 mL of the PBS-diluted blood was gently

layered onto the lymphocyte separation media with care to maintain separation of the layers. The tubes were centrifuged for 30 min at 400 g, at room temperature and at slow deceleration, leading to density-based separation of major blood fractions. The buffy coat from each tube was collected using a transfer pipette and transferred into a new 50 mL conical tube. The buffy layer was washed by adding PBS in the required amount to reach a final volume of 45 mL, and the tubes were centrifuged for 15 min, at 275 g, at room temperature. The tubes were decanted and pellets from all tubes combined and resuspended in 45 mL of PBS and gently mixed. For further washing, the tubes were centrifuged for 12 minutes at 175 g, at room temperature, resuspended and washed again in the same manner. Finally, obtained PBMC pellets were resuspended in 10 mL of 10% FBS RPMI warmed to 37°C and counted. To count the cells, 50 μL of the cell suspension was mixed with 50 μL of Trypan blue solution (Thermofisher, Saint-Laurent, Quebec), inserted into a hemocytometer and a manual count was performed. The PBMC suspension was topped up with additional 10% FBS RPMI such that a final concentration of approximately 3.33 x 10^6 cells / mL was achieved, and the suspension was transferred to a culture flask for resting in a humidified incubator at 37°C, and 5% CO_2.

$CD4^+$ T cell Isolation

For experiments performed on $CD4^+$ T cells, the PBMC cell suspension was centrifuged at 275g for 8 minutes, at room temperature and resuspended at a concentration of 50 x 10^6 cells/mL in a volume of 0.25-2 mL. The EasySep™ Human $CD4^+$ T cell Enrichment Kit (StemCell Technologies, Vancouver, British Columbia) was used to isolate $CD4^+$ T cells from the PBMCs and the manufacturer protocol was followed. Briefly, 50 μL/mL of the kit's enrichment cocktail was added to the PBMC cell suspension and incubated for 10 min. The kit's magnetic particles

were added to the suspension at 100uL/mL, and the suspension was mixed and incubated for 5 minutes. The suspension was topped up to 2.5 mL if applicable, placed into the EasySep™ magnet and incubated for 5 minutes. The magnet with the tube were inverted to pour the suspension of negatively-selected $CD4^+$ T cells into a new tube. Finally, isolated cells were counted as per the previously described Trypan™ count procedure, centrifuged at 275g for 8 minutes at room temperature and resuspended in 10% FBS RPMI at a concentration of approximately 3.33 x 10^6 cells for downstream applications.

$CD4^+$ T cell Purity Verification

To verify the purity of isolated $CD4^+$ T cells, antibody staining was performed. Four aliquots of approximately 0.1 x 10^6 cells were prepared in 1.5 mL microcentrifuge tubes, on ice, according to the following conditions: No stain, anti-CD3 stain, anti-CD4 stain, Double stain (anti-CD3 and anti-CD4). PerCP Mouse Anti-Human CD3 and APC Mouse Anti-Human CD4, or, alternatively, APC-Cy7 Mouse Anti-Human CD4 and PE-CF594 Mouse Anti-Human CD3 were used (BD Bioscience, Franklin Lakes, New Jersey). Each tube was centrifuged at 8,000 g for 30s and decanted. The cell pellet was resuspended in PBS with 4 µL of anti-CD3 and/or anti-CD4, depending on the condition, for a total volume of 50 uL per tube. The tubes were incubated on ice for approximately 20 min then washed by adding 100 uL of PBS and centrifuging at 8,100 g for 30 s. The tubes were decanted and the wash step repeated. Cells were resuspended in PBS for immediate flow cytometry acquisition, or fixed with formaldehyde and stored at 4°C for acquisition at a later date. Acquisition was performed using a FACSVerse flow cytometry system (BD Bioscience, Franklin Lakes, New Jersey) and acquisition parameters were adjusted according to the used fluorochromes. Gating was performed on the acquired signal to identify

the lymphocyte population, and further gating of cells positive for both $CD3^+$ and $CD4^+$ cells allowed for the calculation of percent $CD4^+$ T cells present in the sample.

Activation

Obtained cell suspensions were centrifuged at 275g for 8 minutes at room temperature. Resulting cell pellets were resuspended in 20% FBS RPMI, at a concentration of approximately 3.33 x 10^6 cells/mL, and placed in an incubator for approximately 24 hours to prevent stochastic subpopulation activity within the sample (serum shock). The cells were then similarly centrifuged and resuspended at the same concentration in 10% FBS RPMI. For each experiment, cells were plated according to their activation conditions into a 96-well U-bottom culture plate with a lid (VWR, Mont-Royal, Quebec), such that their final concentration was 0.5 x 10^6 cells per 200 µL, per well. All conditions except for the non-activated control condition (No Act) were stimulated using Immunocult™ Human CD3/CD28 T cell Activator (StemCell Technologies, Vancouver, British Columbia) at 12 or 6 µL per 0.5 x 10^6 cells. AM630, CBD, and vehicle controls (Sigma, St. Louis, Missouri) were administered in varying doses, in 2-6 technical replicates, according to experimental plan (Figure 1) and depending on cell yield. AM630 was dissolved in a DMSO vehicle, while CBD was dissolved in DMSO for one series of experiment, and in methanol in another series, to test the effect of chosen vehicle. Once prepared, the plates were transferred into a humidified incubator at 37°C, and 5% CO_2. Approximately 24-36 h after cell activation, culture plates were centrifuged at 275g, for 7 min, at 4°C. Cell supernatants were transferred into a new plate and frozen at -20°C until used for ELISA.

ELISA

ELISAs were conducted using IL-2, or IFN_{γ} kits and slightly adapted manufacturer protocols were used (BD Bioscience, Franklin Lakes, New Jersey). One follow-up exploratory experiment was conducted evaluating IL-17. One to three days prior to the assay, a solution of capture antibody in coating buffer was prepared at a ratio of 1:250, and 50 uL of this solution was added to each well of a 96-well flat-bottom ELISA plate and incubated at 4°C (Fisher Scientific, Saint-Laurent, Quebec). ELISA wash buffer was prepared by adding 1 mL of Tween-20 (Sigma, St. Louis, Missouri) to 1 L of 1 X PBS. On the day of the assay, the coating solution was aspirated and the plate was washed three times using ELISA blocking buffer (Thermofisher, Saint-Laurent, Quebec). Fifty µL of diluted sample supernatants or serially diluted kit-provided standard antibodies were added to duplicate wells according to experimental layout and incubated for two hours at room temperature. In the meantime, a working detector solution was prepared using kit-provided detection antibody and Sav-HRP, each diluted at 1:250, in ELISA blocking buffer. After incubation, the plate was washed five times, 50 uL of the working detector solution was added to all wells and incubated for one hour. The solution was then aspirated and the plate washed 7 times. One hundred µL of TMB substrate solution was prepared (Thermofisher, Saint-Laurent, Quebec) and added to all wells and incubated for 15-30 min in the dark. Finally, 50 µL of 2N sulfuric acid was used to stop the reaction and the plate was read using a microplate reader, at 450 nm with a 570 nm correction.

RNA extraction

To investigate the effect of CBD on clock gene expression, cell samples were collected approximately 6h and 18h after activation and incubation. Surfaces, micropipettes and tools were cleaned with RNAse Away (Fisher Scientific, Saint-Laurent, Quebec) to the extent possible

to mitigate RNA degradation. Approximately 2 x 10^6 cells per condition were pooled into RNAse-free microcentrifuge tubes, and centrifuged at 12,000 g, at 4°C, for 1 min. Supernatant was discarded and 300 μL of Trizol (Fisher Scientific, Saint-Laurent, Quebec) was added to each tube and incubated for 5 min to lyse the samples. Sixty μL of chloroform (Sigma, St. Louis, Missouri) was added to each tube and the tubes were shaken vigorously for 15 s, then incubated for 2 min. The samples were transferred to Phasemaker tubes™ (Thermofisher, Saint-Laurent, Quebec) and centrifuged at 12,000 g, at 4°C, for 15 min. The generated aqueous phase was collected, transferred to a new nuclease-free microcentrifuge tube, and frozen at -80°C for completion of RNA extraction at a later date. To complete the extraction, samples were thawed, and the Purelink RNA Mini Kit was used, with slight adaptations to the manufacturer protocol. An equivalent volume of freshly prepared 70% ethanol in nuclease-free water (Sigma, St. Louis, Missouri; Thermofisher, Saint-Laurent, Quebec) was added to the samples and the tubes were vortexed briefly to dissolve precipitate. The samples were transferred to the provided spin cartridges with collection tubes and centrifuged at 12,000 g, at room temperature, for 15 s. Flowthrough in the collection tube was discarded, and 700 μL of the kit's Wash Buffer I was added to each sample. The tubes were centrifuged at the same settings as the previous step, and flowthrough in the collection tube again discarded. Five hundred μL of Wash Buffer II was added and the samples were centrifuged at the same settings, flowthrough was discarded, and the step repeated. The tubes were left open and centrifuged at the same settings to dry the membrane in the spin cartridge. Finally, 30 μL of nuclease-free water was added to the center of the cartridge, incubated for 1 min, and the cartridges were placed into recovery RNAse-free tubes and spun at 12,000 g, at room temperature, for 2 min to elute RNA.

RNA purity and concentration was assessed using a NanoDrop 2000c spectrophotometer (Thermofisher, Saint-Laurent, Quebec). Purified RNA samples were stored at -80°C until thawed for cDNA conversion.

cDNA conversion

Purified RNA samples were converted to cDNA using iScript™ Reverse Transcription Supermix for RT-qPCR and the manufacturer protocol was followed (Bio-Rad, Saint-Laurent, Quebec). The following reaction setup was carried out on ice: 1 μg of RNA, 4 μL of iScript™ RT Supermix, and a variable amount of nuclease-free water were combined to a total volume of 20 μL per sample and transferred to nuclease-free 8-tube PCR strips with caps (Bio-Rad, Saint-Laurent, Quebec). The samples were placed into a CR CFX 96 Real Time System C1000 Thermal Cycler (Bio-Rad, Saint-Laurent, Quebec), set to the following protocol: 5 min at 25°C for priming, followed by 20 min at 46°C for reverse transcription, followed by 1 min at 95°C for reverse transcriptase inactivation. cDNA samples were stored at -80°C until used for RT-qPCR.

RT-qPCR

The expression of target clock genes BMAL1 and PER2, and reference housekeeping gene PPIA was measured by RT-qPCR using Taqman® Gene Expression Assay probes (Thermofisher, Saint-Laurent, Quebec). Reaction mixes for three technical triplicates of each sample were assembled on ice as follows: 23.1 μL of nuclease-free water was combined with 33 μL of SuperScript™ IV VILO™ Master Mix (Thermofisher, Saint-Laurent, Quebec), 3.3 μL of the appropriate gene probe, and 6.6 μL of sample cDNA. Twenty μL of this mix was transferred into 96-well PCR plates (Fisher Scientific, Saint-Laurent, Quebec). NTCs for each gene were prepared similarly by substituting nuclease-free water for the gene probes, and loaded similarly onto the

plate in technical triplicates of 20 μL. The plate was placed into a CR CFX 96 Real Time System C1000 Thermal Cycler (Bio-Rad, Saint-Laurent, Quebec) set to the following protocol: 40 repeated cycles of 50°C for 2 min (denaturing), followed by 95°C for 23 s (annealing), and 60°C for 30s (extension). Target gene expression was measured as a fold-change relative to the reference gene PPIA, using the delta-delta Ct method, on the CFX Maestro software (Bio-Rad, Saint-Laurent, Quebec).

Data Representation and Statistical Analyses

For ELISA data, raw data, transformed data, and corresponding statistical analyses were manually calculated then plotted as summary bar charts on Prism 9 (GraphPad Software, San Diego, California). Cytokine concentration was calculated from a standard-curve generated formula, and each the cytokine response for each dose was plotted as the percent change from the cytokine response produced by the dose's vehicle control. Error bars plotted correspond to a calculated standard error of the percent change, calculated as described by the United Stated Census Bureau (2015). Considering the unequal variances found in the small sample sizes comprised of each condition's biological and technical replicates grouped, a Welch's t-test was performed to determine whether the drug-elicited cytokine expression, at a given dose, was significantly different from the cytokine expression of the vehicle control.

For qPCR data, relative fold-change calculated by the delta-delta Ct method on the CFX Maestro software was plotted using Prism 9 (GraphPad Software, San Diego, California). The software was also used to run one-way ANOVAs for genetic expression across conditions, per target gene, per time point.

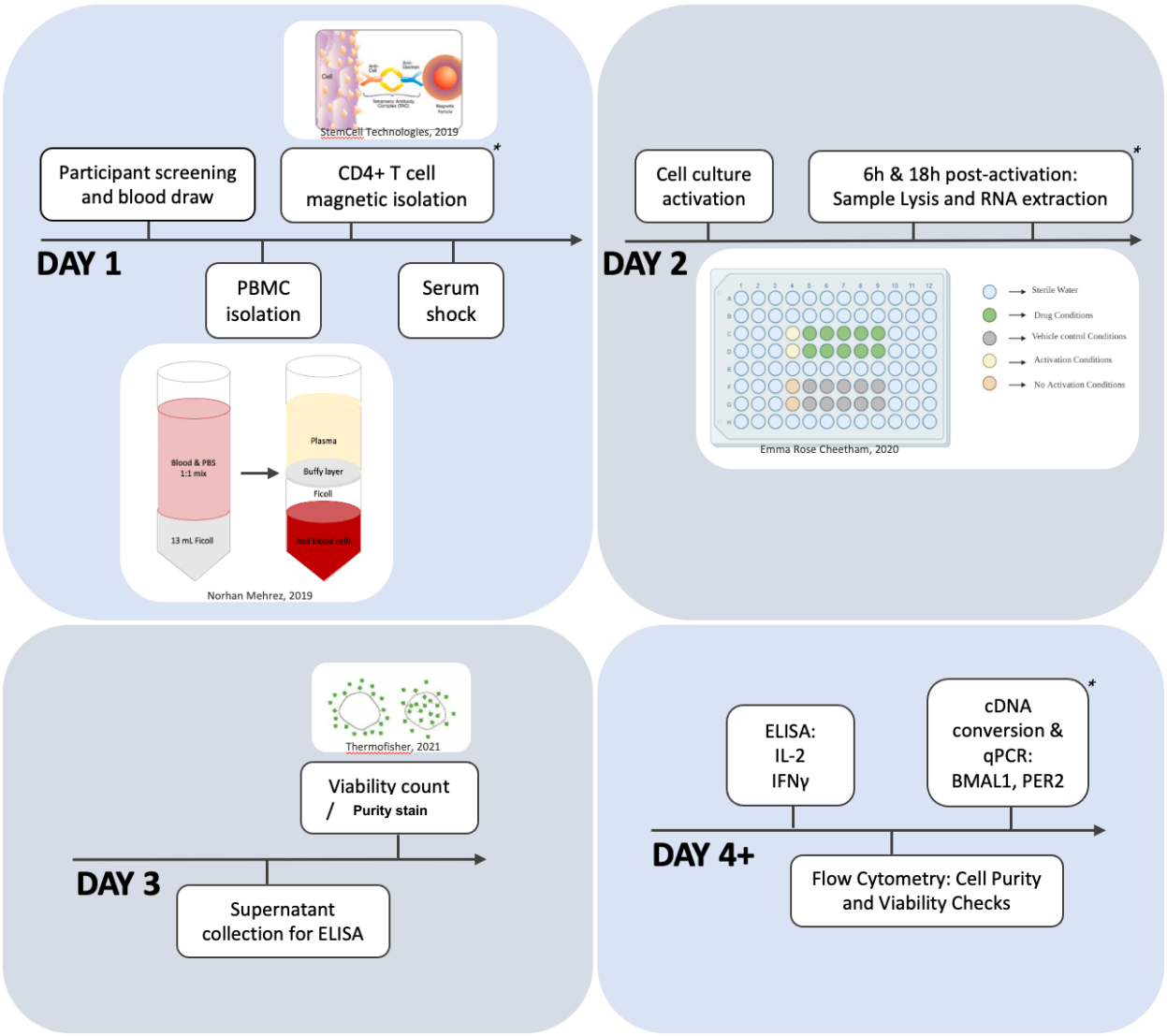


Figure 1. Schematic timeline of experimental workflow

** $CD4^+$ T cell isolation not performed for series of experiments using PBMC populations, refer to Results. Similarly, sample lysis, RNA extraction, cDNA conversion and qPCR only performed for circadian experiments; refer to Results.*

Results

Effect of CBD in methanol on $CD4^+$ T Cell Cytokine Release

In the first series of experiments, examining the effects of CBD using methanol as a vehicle, cytokine expression for IL-2 (N=6) and IFNγ (N=4) was assessed by ELISA. As shown in Figures 2 and 3, a general biphasic trend was observed across participants' expression of both cytokines, although the doses of the biphasic effect exhibited individual differences.

IL-2 Expression

In 5 out of 6 participants (all but LCR279), a 5 to 32 % decrease in IL-2 was observed at lower doses of CBD, followed by an increase of up to 240% of IL-2 at higher doses (Figure 2). Among these 5 participants, 4 saw decreased IL-2 in the nanomolar range of CBD dosing, switching to an increase above 1μM CBD. Decreased IL-2 was not statistically significant, however, and increased IL-2 was only statistically significant in two participants: in LCR277, at 2.5 μM ($t(4)=12.73$, $p<.01$, Δ=-41.94%, SEM =13.79) and 5 μM CBD ($t(4)=3.19$, $p=.03$, Δ=-27.55%, SEM=2.71); and in LCR283 at 0.1 μM ($t(4)=28.21$, $p<.01$, Δ=228.72%, SEM =12.37) and 5 μM ($t(3)=8.57$, $p<.01$, Δ=12.87%, SEM =1.68).

IFN-γ Expression

In 2 out of 4 participants (LCR284 and LCR285), contrary to expected though still biphasic in nature, IFNγ expression increased at lower doses, and decreased at higher doses. Increased expression was statistically significant for LCR285 at 0.01 μM, ($t(5)=3.17$, $p<.05$, Δ=16.19%, SEM=5.74), and decreased expression was statistically significant at 5μM for both LCR284 and LCR285 ($t(6)=-8.98$, $p<.01$, Δ=-50.29%, SEM=4.04; $t(4)=-3.9$, $p<.05$, Δ=-37.61%, SEM=6.29, respectively).

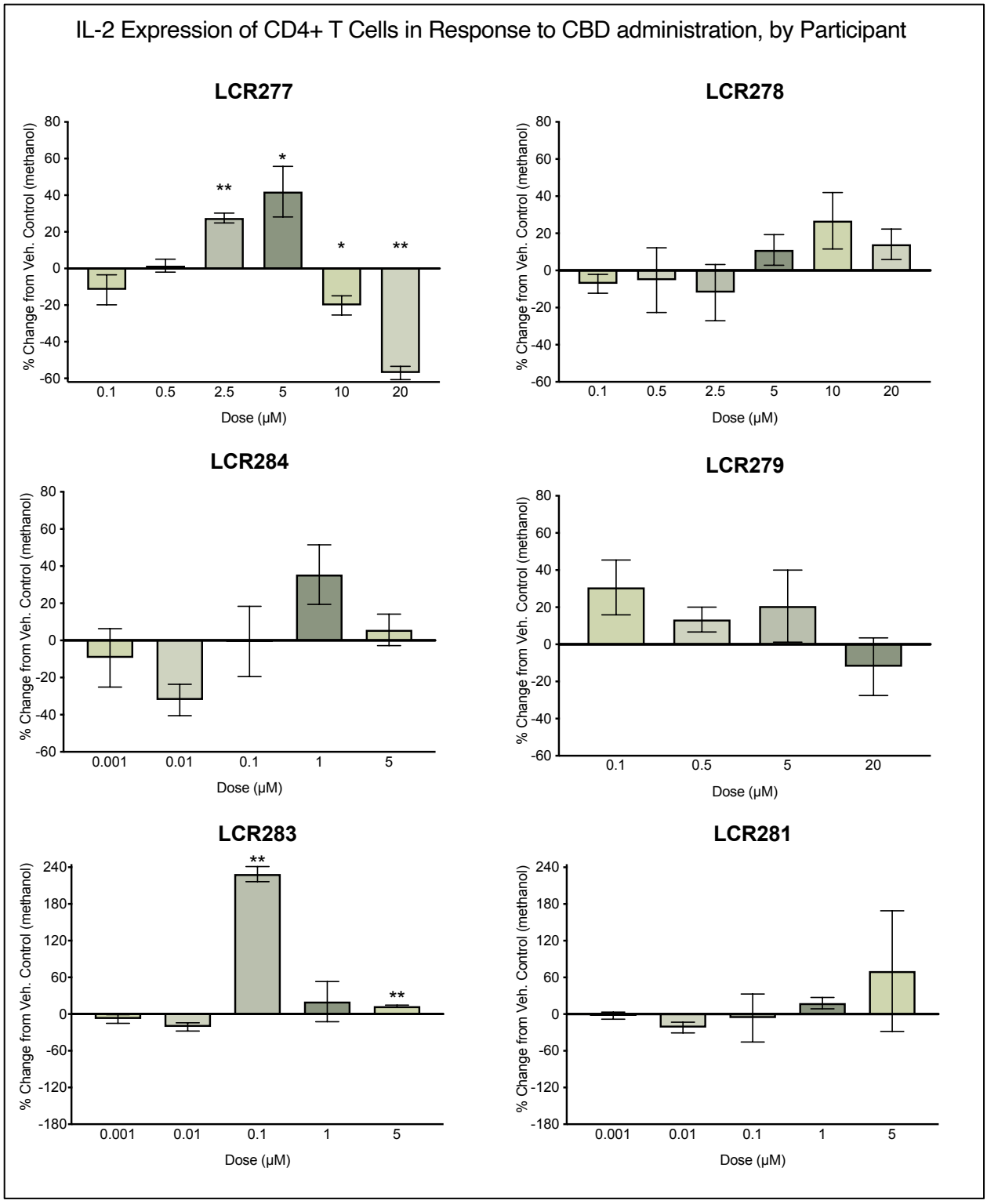


Figure 2. Effect of CBD, in methanol, on CD4⁺ T cell expression of IL-2 as measured by ELISA
*Note: * = p<.05; ** = p<.01*

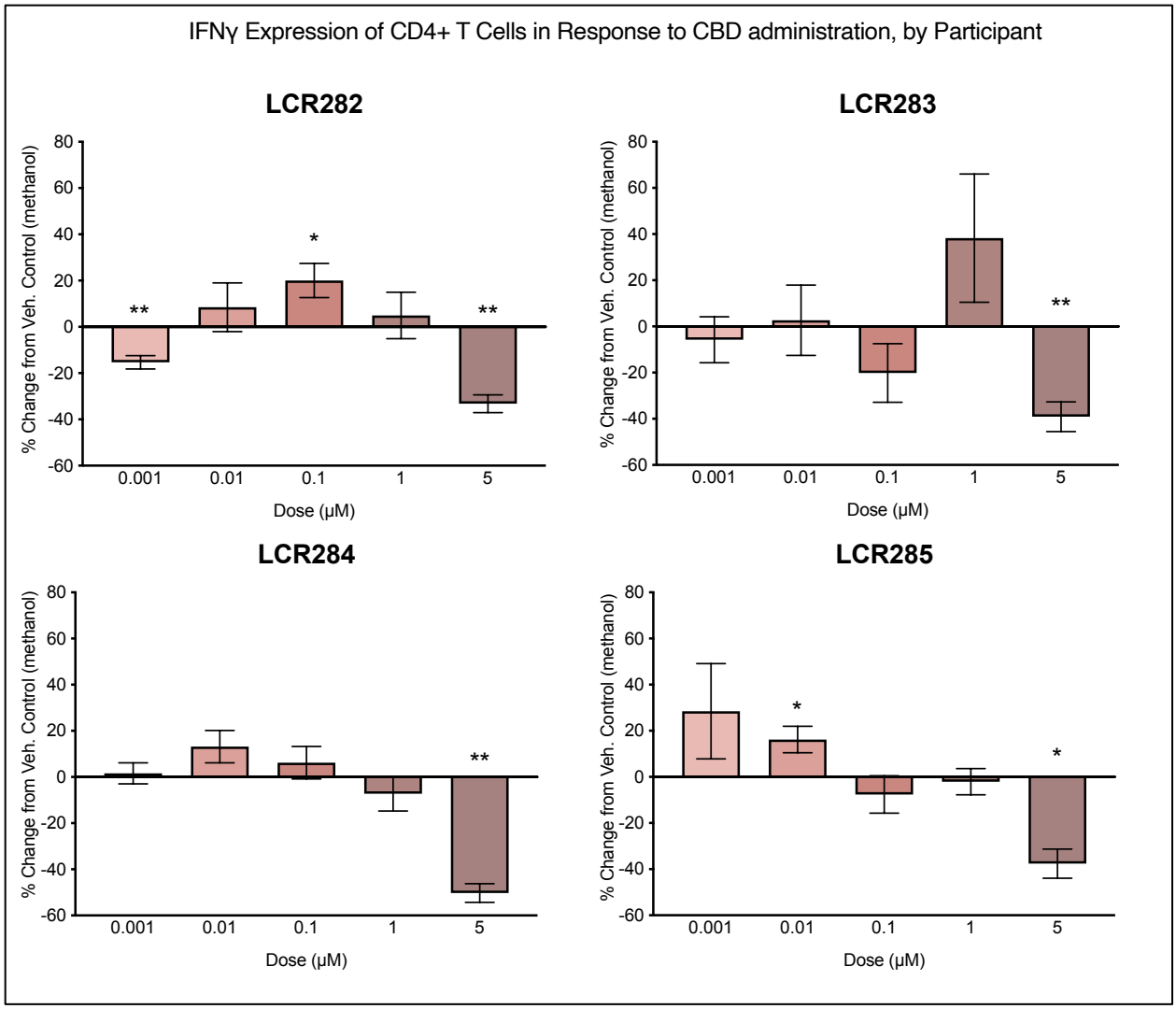


Figure 3. Effect of CBD, in methanol, on CD4⁺ T cell expression of IFNγ as measured by ELISA
*Note: * = p<.05; ** = p<.01*

In the remaining 2 out of 4 participants (LCR282 and LCR283), expected biphasic trends were observed, with decreased IFNγ expression at lower CBD doses, and higher expression at higher doses. Decrease and increase of IFNγ were statistically significant at 0.001 μM CBD for LCR282 ($t(4)=-5.18$, $p<.01$, Δ=-15.34%, SEM=2.89) and 0.1 μM CBD ($t(4)=3.11$, $p<.05$, Δ=20.03%, SEM=7.39), respectively. Interestingly, at the highest dose (5 μM CBD), both participants saw a return to decreased IFNγ expression that was statistically significant (LCR282: $t(4)=-6.34$, $p<.01$, Δ=-33.21%, SEM=3.83; LCR283: $t(4)=-4.13$, $p<.05$, Δ=-39.11%, SEM=6.42).

Effect of CBD in DMSO on $CD4^+$ T Cell Cytokine Release

Due to unforeseen shortages in cell yields, dosing conditions were dropped for 4 out of 5 participants (Figure 4). In addition, experiments were conducted using PBMC populations for participants LCR291 and LCR292 for the same reason. For all participants, CBD elicited opposing responses of both increasing and decreasing IL-2 expression at different doses, though data varied highly between participants making it difficult to elucidate a general biphasic trend for this set of data. However, 0.1 μM CBD did decrease IL-2 expression in 3 out of 5 participants, with this decrease being statistically significant for LCR178 and LCR2 ($t(6)=-3.29$, $p<.05$, Δ=-23.63%, SEM=6.37; $t(6)=-4.19$, $p<.05$, Δ=-29.01%, SEM=5.87, respectively). Additionally, in 3 out of 5 participants, 1 μM increased IL-2 expression, though this was statistically significant only for LCR178 ($t(6)=7.24$, $p<.01$, Δ=23.26%, SEM=3.58).

Effect of AM630 on $CD4^+$ T Cell Cytokine Release

A decrease in IL-2 expression was observed at lower doses for all participants (N=5), though doses at which this occurred varied between participants (Figure 5). Lower-dose CBD-induced decrease of IL-2 was statistically significant at 0.5 μM for participants LCR273, LCR271,

and LCR155 (respectively: $t(4)=-4.45$, $p<.05$, Δ=-23.75%, SEM=4.23; $t(6)=-7.71$, $p<.01$, Δ=-46.24%, SEM=5.07; $t(3)=-3.73$, $p<.05$, Δ=-72.78%, SEM=15.19). At doses ranging 2.5μM-10μM, following decreased IL-2 at lower doses, an increase in IL-2 was observed in all participants, though only statistically significant at 10μM for LCR155 ($t(2)=4.88$, $p<.05$, Δ=229.05%, SEM=51.77). Finally, at high doses, similarly to the effect of CBD noted in the previous paragraph, IL-2 expression switched to a decrease in 4 out of 5 participants. This high-dose decrease was statistically significant at 10 μM in LCR272 ($t(5)=-9.16$, $p<.01$, Δ=-39.39%, SEM=3.11) and at 20 μM in both LCR272 and LCR274 ($t(3)=-12.23$, $p<.01$, Δ=-79.01%, SEM=1.66; $t(5)=-3.55$, $p<.05$, Δ=-33.93%, SEM=17.47, respectively). Interestingly, as apparent in Figures 4 and 5, this trend of decreased-increased-decreased cytokine expression may allude to a triphasic response.

Circadian Experiments

ELISA data

Due to a shortage of $CD4^+$ T Cell yield, experiments exploring the effect of CBD on clock genes (N=3) were performed using PBMCs. To verify whether selected doses achieved a biphasic effect, ELISA for IL-2 was carried out on cell supernatant obtained from circadian experiments (aimed primarily at assessing the effect of CBD on clock gene expression). Three participants were examined, two of which demonstrated a verified biphasic response at the same doses. CBD increased IL-2 expression at 0.1 μM (LCR178: $t(6)=1.47$, $p=.19$, Δ=194.35%, SEM=138.21; LCR2: $t(5)=2.90$, $p<.05$, Δ=53.56%, SEM=1.81) and decreased IL-2 expression at 1 μM (LCR178: $t(10)=-1.41$, $p=.19$, Δ=-53.06%, SEM=25.59; LCR2: $t(12)=-0.36$, $p=.72$, Δ=-13.38%, SEM=33.61), see Appendix B.

qPCR findings

Gene expression levels varied greatly across genes, timepoints, and individuals, making it difficult to elucidate an overall trend. Consequently, no statistical significant main effect, nor pairwise comparisons were found for any of the participants. However, while not significant and highly variable, the effect of CBD on BMAL1 expression seemed influenced by time: in participant LCR178 (summary statistics available in Appendix C), BMAL1 was downregulated at 6h and 18h post activation compared to no-drug controls, but this downregulation was greater for 1μM at 6h, versus at 0.1 μM at 18h. CBD at 1 μM also seemed to downregulate PER2 expression compared to 0.1 μM at 6h, though the opposite occurred at 18h . For participant LCR274, CBD doses' effect on gene expression also seemed influenced by time, with 1μM showing opposite trends than 0.1 μM between time points, for both genes. The data obtained for the third participant, LCR2, was not interpretable due to variability. For brevity and due to the exploratory nature of the data, only one experiment is presented in Figure 6 (LCR178) with the remaining participants presented in Appendix D.

Cell Purity Verification

Flow cytometry analysis for $CD4^+$ T cell Isolation Purity revealed, as expected, high purity (>95%) of isolated $CD4^+$ T cells in experiments using these cells. For brevity, one experiment's representative diagram to this effect, depicting lymphocyte gating on a plot of forward scatter vs side scatter, as well as gated doubly-positive $CD3^+$ and $CD4^+$ T cell populations is presented in Appendix E.

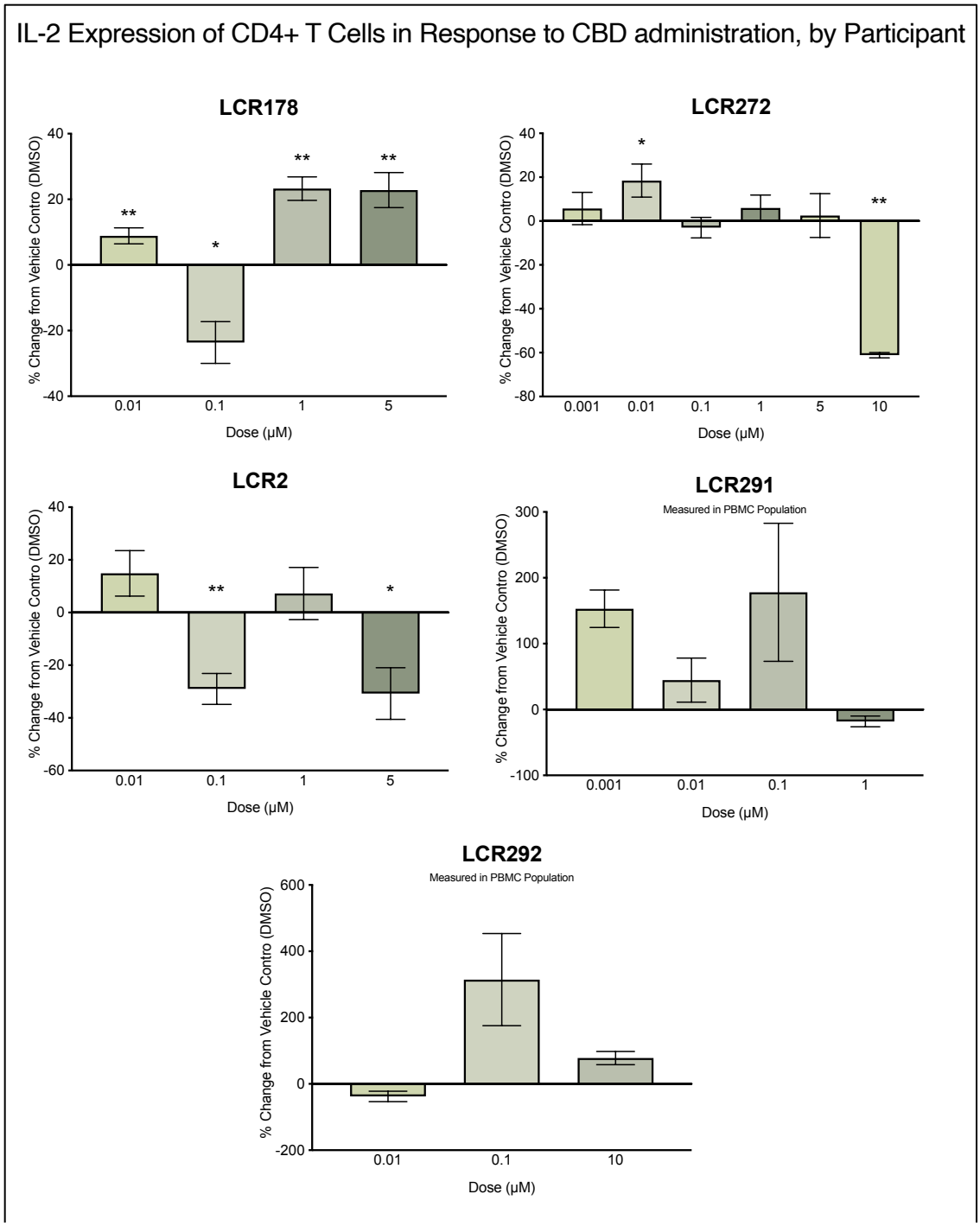


Figure 4. Effect of CBD, in DMSO, on CD4⁺ T cell expression of IL-2 as measured by ELISA
*Note: * = p<.05; ** = p<.01; y axes are adjusted for LCR291 and LCR292 to accommodate larger data and error bar span.*

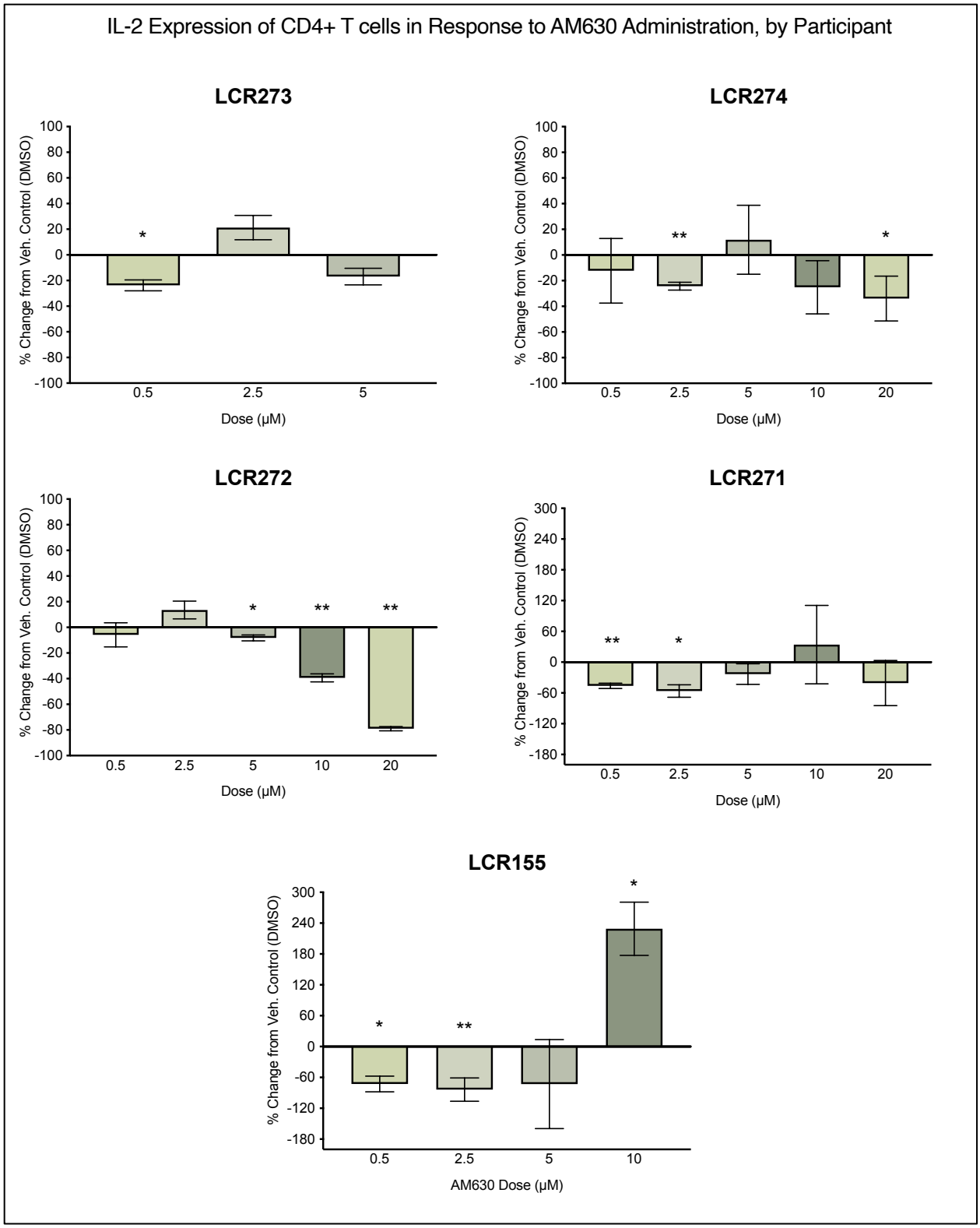


Figure 5. Effect of AM630 on CD4⁺ T cell expression of IL-2 as measured by ELISA
*Note: * = p<.05; ** = p<.01; y axes are adjusted for LCR271 and LCR155 to accommodate larger data and error bar span.*

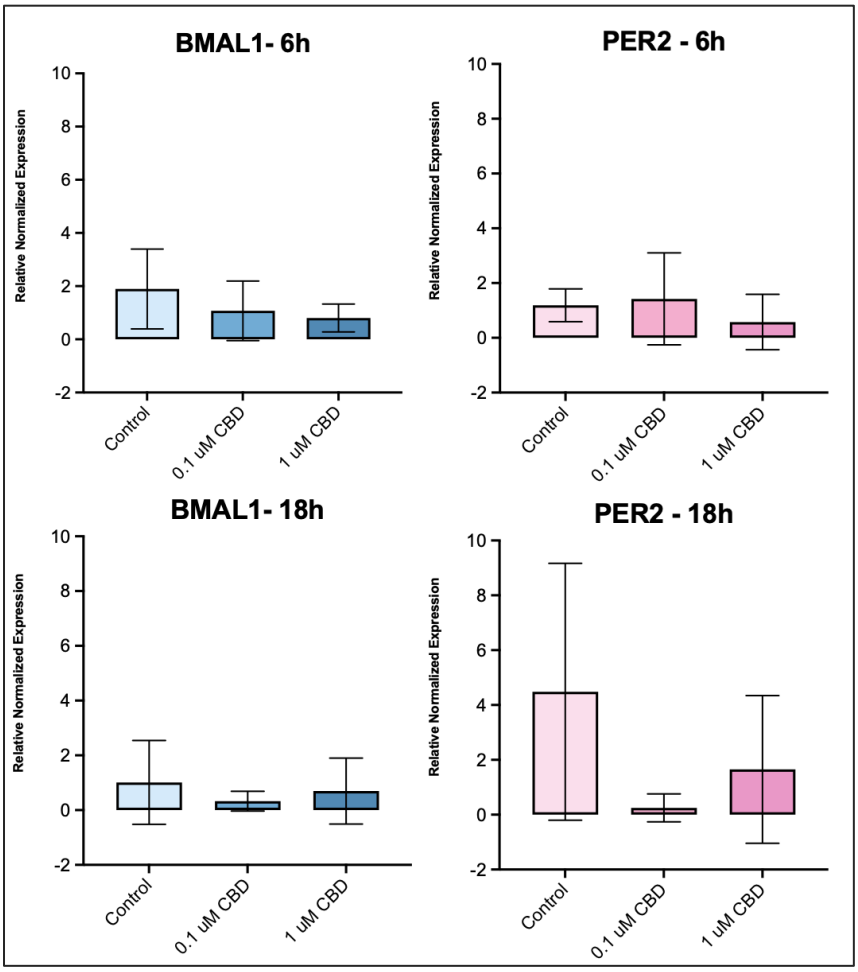


Figure 6. Gene expression of PBMCs at 6h and 18h post activation, in response to CBD administration

Note: Relative normalized expression is calculated in reference to a stable housekeeping gene, PPIA. No significant effects of time nor dose were found.

Discussion

Multiple inferences may be gleaned from the present work. First, there seems to be support for CBD as a biphasic agent in modulating $CD4^+$ T cell cytokine secretion. Decreased cytokine secretion was observed at lower doses in most participants, followed by increased secretion at higher doses, typically beginning around 1-5 μM. However, the doses at which such biphasic trends arise, if they arose, vary greatly across individuals. Second, the type of vehicle used for *in-vitro* delivery of CBD seems to influence cytokine secretion. Third, when selectively stimulated, CB2 seems to respond biphasically in $CD4^+$ T cells, though also under large influence of individual differences, suggesting a mechanism by which CBD may present biphasic effects. Fourth, at doses of CBD and AM630 beyond those inducing a biphasic response, our data suggests a potential reversion to a decrease in cytokine response, potentially due to off-target effects, that is, CBD binding to other receptors than CB2. Finally, CBD may have an effect in modulating clock gene expression in PBMCs that varies by time, implicating CBD as a potentially chronobiotic agent, however this requires further investigation as obtained data presented was limited and exploratory.

Out of the presented ELISA findings evaluating the effect of CBD on cytokine secretion (N=15), all experiments, including those on $CD4^+$ T cells, PBMCs, and those evaluating IL-2 as well as IFNγ exhibited traits of a biphasic dose-response, though variable and not always statistically significant. This may be due to a multitude of potential confounds. First of which may be the type of vehicle used. The data evaluating cytokine-secretion response of $CD4^+$ T cells to CBD administered using DMSO seemed the least consistent and most erratic (Figure 4). Indeed, when ELISA results were expanded and visualized, IL-2 secretion across varying doses of

DMSO vehicle controls were rather diverse, in comparison to the vehicle controls when methanol was used for CBD delivery (Appendix F). DMSO is a vehicle very commonly used throughout *in-vitro* studies, including in studies evaluating the effect of cannabinoids on cytokine production(Robinson et al., 2013; Zgair et al., 2017). However, it is rarely evaluated at varying doses, and often a control is presented at only the highest dose (Watzl et al., 1991). Further, it is often disregarded whether DMSO may influence immune activity. Indeed, it has been suggested that using DMSO as a vehicle should be carried out with care, as its direct interaction with ion channels on cells can lead to undesired confounding effects on immune cells cultured *in-vitro* (Rivers-Auty & Ashton, 2013). Considering CBD is highly lipophilic and similarly acts on a multitude of ion channels (Mangal et al., 2021), it remains to be investigated whether DMSO and CBD may interact. Future studies may remedy this gap by running competitive binding experiments as well as perform assays to evaluate various vehicles for the delivery of CBD on the same participant.

Another limitation of our study which may have contributed to confounding the obtained ELISA results, is the use of PBMC populations in experiments where sufficient $CD4^+$ T cell numbers could not be obtained. However, considering PBMCs are mostly composed of $CD4^+$ T cells, this is unlikely to cause a large difference in patterns of activation across doses, especially as only $CD4^+$ T cells are activated using our anti-CD3 anti-CD28 stimulator (Immunocult™). Naturally, the comparatively decreased quantity of $CD4^+$ T cells in culture wells may lead to increased variability and experimental error effects. Still, research investigating CBD and cytokine expression has often been carried out on populations of PBMCs with results not dissimilar to those done directly on $CD4^+$ T cells (Cencioni et al., 2010; Watzl et al., 1991).

Despite these confounds, a biphasic effect was indeed noted overall, as expected from our study aim. Confirming this property of yet another cannabinoid allows reconciliation between previous, conflicting findings. For example, as the literature largely proposed CBD as an immunosuppressive agent, Watzl, Scuderi and Watson showed that nanomolar-range doses, CBD and THC actually increased IFNγ and IL-2 secretion (Watzl et al., 1991). Establishing such a relationship is crucial given the rising interest in CBD, as well as its use in clinical trials (Pauli et al., 2020). Not only does this body of work confer caution to carefully assess dosing, it helps bridge the gap between *in-vitro* and in-vivo studies. In fact, upon investigation of cannabinoid serum levels following human consumption, it has become evident doses in the 10-μM range, most typical for *in-vitro* research, do not accurately reflect physiological doses in most contexts of medication and recreational use of cannabinoids, which are closer to the nanomolar *in-vitro* range (Pertwee, 2008).

Interestingly, our data suggested not only a biphasic response, but a tendency to revert to a decrease in cytokine production at high doses among several of our participants. A few potential explanations arise. Among them is simply that cannabinoids may in fact exhibit a more complex relationship than a biphasic dose-response. However, given the scarcity of evidence supporting this in the literature, other plausible reasons are worth exploring. Firstly, cytokines such as IL-2 do not serve only to signal and trigger activation in target immune cells; they also serve to provide feedback and maintain a controlled level of activation. Thus, in the presence of a very strong immune response, secreted IL-2 may bind to and be consumed by $CD4^+$ T cells, effectively transforming an increase in cytokine production into a measurable decrease in terms of ELISA findings. Secondly, at high doses of cannabinoids, in particular in

conjunction with cellular proliferation due to activation, there is a high likelihood that administered drugs are having a variety of off-target effects not occurring at lower doses where a simpler, biphasic trend may be observed. Indeed, CBD is known to bind to a variety of receptors with full effects on immunity still under investigation (Peyravian et al., 2020). In order to fully investigate this phenomenon, and considering the relative safety of cannabinoids, follow-up studies may increase the breadth of the dose-response by including a wider range of doses, and by increasing the number of increments between them. This would help determine whether a biphasic trend may be an oversimplification, and whether seemingly inverted trends for some participants may simply arise due to obscured differences that would be captured at a wider, more incremental range. This may also reconcile the few participants in our experiments that showed an inverse relationship: increased cytokine secretion at low doses, followed by a decrease in doses. These participants may have a shifted dose-response compared to their participating counterparts, such that a wider range of doses may have captured doses leading to decreased cytokine secretion.

In addition to biphasic trends seen with CBD administration, selective stimulation of CB2 using aM630 presented similar biphasic patterns of response. Indeed, such flexibility in responding to doses of a same stimulus suggests the mechanism of CBD's biphasic effects may in part be due to biphasic receptor response. Notably, observed hormesis of CB2 action provides further support for the dynamic nature of the endocannabinoid system as a regulator of immunity, in line with findings previously described. Indeed, this dynamic nature enables the endocannabinoid system to fulfill its role in maintaining one of its crucial functions; that to maintain homeostasis. This can be seen in various contexts, from fertility, to appetite regulation

and temperature control (Wenger & Moldrich, 2002). Unfortunately, the pervasive and dynamic nature of this system renders its investigation vastly more complicated.

An important limitation to this work is the small sample size used, particularly given the influence of individual differences in our findings. While it is a truism for most immunological studies that a large sample size may improve visibility of trends in the data, an easier remedy for counteracting individual differences' influence on data would be to draw larger amounts of venous blood from each participant, in addition to the use of higher throughput methods of $CD4^+$ T cell isolation, and to run more extensive assays within each same participant, as described in previous paragraphs. Similarly, the use of more refined and efficient methodology such as flow cytometry or microarray analysis may prove more helpful than the highly variable nature of ELISA data. Another avenue worth exploring is that of evaluating the response of other cytokines, in the hopes of uncovering a more complete profile of immune response to CBD. In fact, we conducted one follow-up study investigating interleukin 17 (IL-17), a cytokine secreted by a subset of $CD4^+$ T cells known as T-helper cells, and found a similar hormetic trend that seemed more robust and less variable than data obtained from IL-2 and IFNy assays.

While exploratory in nature, the novelty of exploring the effect of CBD on PBMC clock gene expression has far reaching, important implications that merit further analysis. While the preliminary nature of the data evidently did not support nor refute the idea that CBD behaves as a chronobiotic, a vague trend was observed suggesting that dosage of CBD may potentially have differing effects depending on time of day. Such circadian-dependent properties of immune modulation would be in line with previous findings suggesting the adaptive immune response may differ greatly to stimulation depending on the time of day, and would have

important implications for dosing in medicinal contexts. For example, one study showed that morning immunizations lead the adaptive immune system to develop an antibody titre that is nearly five times greater than from immunizations provided in the afternoon (Long et al., 2016), providing a basis for time-of-day consideration when administering vaccines. Similarly, cannabidiol administration may prove to be most effective at a given time of day. Another important consideration: aberrant clock gene expression is linked to disorders of the adaptive immune system, such as the development of autoimmunity (Lavtar et al., 2018), and cannabidiol seems promising in the treatment of such disorders (Jones & Vlachou, 2020). However, the mechanism of action in the improvement of symptoms and pathology in these disorders has yet to be fully established, and regulation of clock gene expression may be one way CBD can treat immune disorders with a circadian component. Thus, despite our variable qPCR data, in part due to small cell populations used and few replicates, further gene expression analysis is warranted, to better examine the effect of cannabinoids on clock gene expression. More target clock genes may be explored, in addition to more time points, ideally in a fashion similar to studies examining clock gene expression in $CD4^+$ T cells (Bollinger et al., 2011). Such timepoint analysis may importantly determine whether cannabinoids affect sinusoidal rhythms of gene expression, inducing shifts or ablations, rather than the static analysis of one or two time points. Another method of improving gene expression analysis is to use higher amounts of anti-CD3 anti-CD28 cell activators before incubating with cannabinoids, to improve RNA concentrations that may be extracted from these cells.

In conclusion, though variable, our data overall supports the growing body of literature indicating that cannabinoids behave in a biphasic manner. Specifically, CBD administration to

$CD4^+$ T cells increases or decreases cytokine secretion at differing doses. This effect is however influenced by a multitude of factors worth considering in the design of future experiments: individual differences, vehicle used for drug delivery and cytokine investigated. A potential mechanism by which CBD elicits varying responses is through the seemingly biphasic response of the CB2 receptor, as suggested by our data exploring dose-response effects of AM630. Additionally, CBD may act as a chronobiotic agent, whereby its effect on immune responses is mediated by time-of-day. Considering the widespread use of cannabis, including an increasing presence in the medicinal sphere, it is equally important to consider these elements in both future research as well as clinical and consumer contexts, where careful assessment of individual needs, as well as the measurement of an individual's responses at given doses may be required to adequately achieve intended effects.

References

Acuna-Goycolea, C., Obrietan, K., & Pol, A. N. van den. (2010). Cannabinoids Excite Circadian Clock Neurons. *Journal of Neuroscience*, *30*(30), 10061–10066. https://doi.org/10.1523/JNEUROSCI.5838-09.2010

Basu, S., & Dittel, B. N. (2011). Unraveling the Complexities of Cannabinoid Receptor 2 (CB2) Immune Regulation in Health and Disease. *Immunologic Research*, *51*(1), 26–38. https://doi.org/10.1007/s12026-011-8210-5

Bazwinsky-Wutschke, I., Zipprich, A., & Dehghani, F. (2017). Daytime-Dependent Changes of Cannabinoid Receptor Type 1 and Type 2 Expression in Rat Liver. *International Journal of Molecular Sciences*, *18*(9), 1844. https://doi.org/10.3390/ijms18091844

Bollinger, T., Leutz, A., Leliavski, A., Skrum, L., Kovac, J., Bonacina, L., Benedict, C., Lange, T., Westermann, J., Oster, H., & Solbach, W. (2011). Circadian Clocks in Mouse and Human CD4+ T Cells. *PLOS ONE*, *6*(12), e29801. https://doi.org/10.1371/journal.pone.0029801

Bolognini, D., Cascio, M. G., Parolaro, D., & Pertwee, R. G. (2012). AM630 behaves as a protean ligand at the human cannabinoid CB2 receptor. *British Journal of Pharmacology*, *165*(8), 2561–2574. https://doi.org/10.1111/j.1476-5381.2011.01503.x

Calabrese, E. J., & Rubio-Casillas, A. (2018). Biphasic effects of THC in memory and cognition. *European Journal of Clinical Investigation*, *48*(5), e12920. https://doi.org/10.1111/eci.12920

Campos, A. C., Ortega, Z., Palazuelos, J., Fogaça, M. V., Aguiar, D. C., Díaz-Alonso, J., Ortega-Gutiérrez, S., Vázquez-Villa, H., Moreira, F. A., Guzmán, M., Galve-Roperh, I., & Guimarães, F. S. (2013). The anxiolytic effect of cannabidiol on chronically stressed mice

depends on hippocampal neurogenesis: Involvement of the endocannabinoid system. *International Journal of Neuropsychopharmacology*, *16*(6), 1407–1419. https://doi.org/10.1017/S1461145712001502

Cencioni, M. T., Chiurchiù, V., Catanzaro, G., Borsellino, G., Bernardi, G., Battistini, L., & Maccarrone, M. (2010). Anandamide Suppresses Proliferation and Cytokine Release from Primary Human T-Lymphocytes Mainly via CB2 Receptors. *PLoS ONE*, *5*(1), e8688. https://doi.org/10.1371/journal.pone.0008688

Cermakian, N., Westfall, S., & Kiessling, S. (2014). Circadian clocks and inflammation: Reciprocal regulation and shared mediators. *Archivum Immunologiae Et Therapiae Experimentalis*, *62*(4), 303–318. https://doi.org/10.1007/s00005-014-0286-x

Chaperon, F., & Thiebot, M.-H. (1999). Behavioral Effects of Cannabinoid Agents in Animals. *Critical Reviews™ in Neurobiology*, *13*(3). https://doi.org/10.1615/CritRevNeurobiol.v13.i3.20

Dos Santos, R. G., Hallak, J. E. C., & Crippa, J. A. S. (2021). Neuropharmacological Effects of the Main Phytocannabinoids: A Narrative Review. *Advances in Experimental Medicine and Biology*, *1264*, 29–45. https://doi.org/10.1007/978-3-030-57369-0_3

Government of Canada, S. C. (2019, February 7). *National Cannabis Survey, 4th quarter 2018*. https://www150.statcan.gc.ca/n1/pub/11-627-m/11-627-m2019010-eng.htm

Hedström, A. K., Åkerstedt, T., Olsson, T., & Alfredsson, L. (2015). Shift work influences multiple sclerosis risk. *Multiple Sclerosis (Houndmills, Basingstoke, England)*, *21*(9), 1195–1199. https://doi.org/10.1177/1352458514563592

Hodges, E. L., & Ashpole, N. M. (2019). Aging circadian rhythms and cannabinoids. *Neurobiology of Aging*, *79*, 110–118. https://doi.org/10.1016/j.neurobiolaging.2019.03.008

Jones, É., & Vlachou, S. (2020). A Critical Review of the Role of the Cannabinoid Compounds Δ9-Tetrahydrocannabinol (Δ9-THC) and Cannabidiol (CBD) and their Combination in Multiple Sclerosis Treatment. *Molecules*, *25*(21), 4930. https://doi.org/10.3390/molecules25214930

Jr, C. A. J., Travers, P., Walport, M., Shlomchik, M. J., Jr, C. A. J., Travers, P., Walport, M., & Shlomchik, M. J. (2001). *Immunobiology* (5th ed.). Garland Science.

Kaplan, B. L. F., Springs, A. E. B., & Kaminski, N. E. (2008). The profile of immune modulation by cannabidiol (CBD) involves deregulation of nuclear factor of activated T cells (NFAT). *Biochemical Pharmacology*, *76*(6), 726–737. https://doi.org/10.1016/j.bcp.2008.06.022

Klein, T. W., Newton, C., Larsen, K., Lu, L., Perkins, I., Nong, L., & Friedman, H. (2003). The cannabinoid system and immune modulation. *Journal of Leukocyte Biology*, *74*(4), 486–496. https://doi.org/10.1189/jlb.0303101

Kozela, E., Juknat, A., Gao, F., Kaushansky, N., Coppola, G., & Vogel, Z. (2016). Pathways and gene networks mediating the regulatory effects of cannabidiol, a nonpsychoactive cannabinoid, in autoimmune T cells. *Journal of Neuroinflammation*, *13*, 136. https://doi.org/10.1186/s12974-016-0603-x

Labrecque, N., & Cermakian, N. (2015). Circadian Clocks in the Immune System. *Journal of Biological Rhythms*, *30*(4), 277–290. https://doi.org/10.1177/0748730415577723

Lafaye, G., Desterke, C., Marulaz, L., & Benyamina, A. (2019). Cannabidiol affects circadian clock core complex and its regulation in microglia cells. *Addiction Biology*, *24*(5), 921–934. https://doi.org/10.1111/adb.12660

Lavtar, P., Rudolf, G., Maver, A., Hodžić, A., Starčević Čizmarević, N., Živković, M., Šega Jazbec, S., Klemenc Ketiš, Z., Kapović, M., Dinčić, E., Raičević, R., Sepčić, J., Lovrečić, L., Stanković, A., Ristić, S., & Peterlin, B. (2018). Association of circadian rhythm genes ARNTL/BMAL1 and CLOCK with multiple sclerosis. *PLoS ONE*, *13*(1), e0190601. https://doi.org/10.1371/journal.pone.0190601

Long, J. E., Drayson, M. T., Taylor, A. E., Toellner, K. M., Lord, J. M., & Phillips, A. C. (2016). Morning vaccination enhances antibody response over afternoon vaccination: A cluster-randomised trial. *Vaccine*, *34*(24), 2679–2685. https://doi.org/10.1016/j.vaccine.2016.04.032

Mangal, N., Erridge, S., Habib, N., Sadanandam, A., Reebye, V., & Sodergren, M. H. (2021). Cannabinoids in the landscape of cancer. *Journal of Cancer Research and Clinical Oncology*, *147*(9), 2507–2534. https://doi.org/10.1007/s00432-021-03710-7

Mattson, M. P. (2008). Hormesis Defined. *Ageing Research Reviews*, *7*(1), 1–7. https://doi.org/10.1016/j.arr.2007.08.007

Mechoulam, R., & Hanus, L. (2000). A historical overview of chemical research on cannabinoids. *Chemistry and Physics of Lipids*, *108*(1–2), 1–13. https://doi.org/10.1016/s0009-3084(00)00184-5

Meier, M. H., Caspi, A., Ambler, A., Harrington, H., Houts, R., Keefe, R. S. E., McDonald, K., Ward, A., Poulton, R., & Moffitt, T. E. (2012). Persistent cannabis users show

neuropsychological decline from childhood to midlife. *Proceedings of the National Academy of Sciences*, *109*(40), E2657–E2664. https://doi.org/10.1073/pnas.1206820109

Miyato, H., Kitayama, J., Yamashita, H., Souma, D., Asakage, M., Yamada, J., & Nagawa, H. (2009). Pharmacological synergism between cannabinoids and paclitaxel in gastric cancer cell lines. *The Journal of Surgical Research*, *155*(1), 40–47. https://doi.org/10.1016/j.jss.2008.06.045

Pauli, C. S., Conroy, M., Vanden Heuvel, B. D., & Park, S.-H. (2020). Cannabidiol Drugs Clinical Trial Outcomes and Adverse Effects. *Frontiers in Pharmacology*, *11*, 63. https://doi.org/10.3389/fphar.2020.00063

Perisetti, A., Gajendran, M., Dasari, C. S., Bansal, P., Aziz, M., Inamdar, S., Tharian, B., & Goyal, H. (2020). Cannabis hyperemesis syndrome: An update on the pathophysiology and management. *Annals of Gastroenterology*, *33*(6), 571–578. https://doi.org/10.20524/aog.2020.0528

Pertwee, R. G. (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin. *British Journal of Pharmacology*, *153*(2), 199–215. https://doi.org/10.1038/sj.bjp.0707442

Petrie, G. N., Nastase, A. S., Aukema, R. J., & Hill, M. N. (2021). Endocannabinoids, cannabinoids and the regulation of anxiety. *Neuropharmacology*, *195*, 108626. https://doi.org/10.1016/j.neuropharm.2021.108626

Peyravian, N., Deo, S., Daunert, S., & Jimenez, J. J. (2020). Cannabidiol as a Novel Therapeutic for Immune Modulation. *ImmunoTargets and Therapy*, *9*, 131–140. https://doi.org/10.2147/ITT.S263690

Pisanti, S., & Bifulco, M. (2019a). Medical Cannabis: A plurimillennial history of an evergreen. *Journal of Cellular Physiology*, *234*(6), 8342–8351. https://doi.org/10.1002/jcp.27725

Pisanti, S., & Bifulco, M. (2019b). Medical Cannabis: A plurimillennial history of an evergreen. *Journal of Cellular Physiology*, *234*(6), 8342–8351. https://doi.org/10.1002/jcp.27725

Rivers-Auty, J., & Ashton, J. (2013). Vehicles for Lipophilic Drugs: Implications for Experimental Design, Neuroprotection, and Drug Discovery. *Current Neurovascular Research*, *10*. https://doi.org/10.2174/15672026113109990021

Robinson, R. H., Meissler, J. J., Breslow-Deckman, J. M., Gaughan, J., Adler, M. W., & Eisenstein, T. K. (2013). Cannabinoids inhibit T-cells via cannabinoid receptor 2 in an in vitro assay for graft rejection, the mixed lymphocyte reaction. *Journal of Neuroimmune Pharmacology: The Official Journal of the Society on NeuroImmune Pharmacology*, *8*(5), 1239–1250. https://doi.org/10.1007/s11481-013-9485-1

Ross, R. A., Brockie, H. C., Stevenson, L. A., Murphy, V. L., Templeton, F., Makriyannis, A., & Pertwee, R. G. (1999). Agonist-inverse agonist characterization at CB1 and CB2 cannabinoid receptors of L759633, L759656, and AM630. *British Journal of Pharmacology*, *126*(3), 665–672. https://doi.org/10.1038/sj.bjp.0702351

Silvestro, S., Schepici, G., Bramanti, P., & Mazzon, E. (2020). Molecular Targets of Cannabidiol in Experimental Models of Neurological Disease. *Molecules*, *25*(21), 5186. https://doi.org/10.3390/molecules25215186

Takahashi, J. S. (2016). Molecular Architecture of the Circadian Clock in Mammals. In P. Sassone-Corsi & Y. Christen (Eds.), *A Time for Metabolism and Hormones*. Springer. http://www.ncbi.nlm.nih.gov/books/NBK453183/

United States Census Bureau. (2015, March 24). *Percent Changes*. https://www2.census.gov/programs-surveys/acs/tech_docs/accuracy/percchg.pdf.

Vaughn, L. K., Denning, G., Stuhr, K. L., de Wit, H., Hill, M. N., & Hillard, C. J. (2010). Endocannabinoid signalling: Has it got rhythm? *British Journal of Pharmacology*, *160*(3), 530–543. https://doi.org/10.1111/j.1476-5381.2010.00790.x

Wang, Z.-Y., Wang, P., & Bjorling, D. E. (2013). Activation of cannabinoid receptor 2 inhibits experimental cystitis. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *304*(10), R846. https://doi.org/10.1152/ajpregu.00585.2012

Watzl, B., Scuderi, P., & Watson, R. R. (1991). Influence of marijuana components (THC and CBD) on human mononuclear cell cytokine secretion in vitro. *Advances in Experimental Medicine and Biology*, *288*, 63–70. https://doi.org/10.1007/978-1-4684-5925-8_7

Wenger, T., & Moldrich, G. (2002). The role of endocannabinoids in the hypothalamic regulation of visceral function. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *66*(2–3), 301–307. https://doi.org/10.1054/plef.2001.0353

White, C. M. (2019). A Review of Human Studies Assessing Cannabidiol's (CBD) Therapeutic Actions and Potential. *The Journal of Clinical Pharmacology*, *59*(7), 923–934. https://doi.org/10.1002/jcph.1387

Zgair, A., Lee, J. B., Wong, J. C. M., Taha, D. A., Aram, J., Di Virgilio, D., McArthur, J. W., Cheng, Y.-K., Hennig, I. M., Barrett, D. A., Fischer, P. M., Constantinescu, C. S., & Gershkovich, P.

(2017). Oral administration of cannabis with lipids leads to high levels of cannabinoids in the intestinal lymphatic system and prominent immunomodulation. *Scientific Reports*, *7*(1), 14542. https://doi.org/10.1038/s41598-017-15026-z

Appendix A– Consent Form

Dr. Peter J. Darlington
Department of Health Kinesiology and Applied Physiology
vMay 2018

CONSENT TO PARTICIPATE IN *"HORMONAL REGULATION OF T CELL GENOMICS"*

I understand that I have been asked to participate in a program of research being conducted by Dr. Peter J. Darlington of the Department of Exercise Science of Concordia University.

A. PURPOSE: I have been informed that the purpose of this research is to further understand how the immune system is affected by signals sent from the brain and expression of circadian (day night cycle) genes.

B. PROCEDURES: I understand that I will fill out questionnaires before the blood draw. These include the morningness-eveningness questionnaire self-assessment version (MEQ-SA), the Pittsburg sleep quality index (PSQI), and the personal drug use questionnaire (PDQ). I understand that I will give a blood of up to 90cc (equivalent to approx. 6 Tbsp). My blood is drawn from a vein by qualified personnel, and it should take about fifteen to twenty minutes in a private consult room with a comfortable chair. After the blood is drawn I will be asked to stay for about ten minutes and offered light snack and refreshments, and then allowed to leave. My blood sample will be processed in the laboratory to obtain immune cells and genetic information in the form of DNA. My immune cells will be kept alive in the laboratory for about five days, and some will be kept in the freezer for later use. My DNA will be kept in the freezer, and then it will be characterized in more detail at Concordia University and Genome Quebec Center.

C. RISKS AND BENEFITS: I understand the risks associated with this study may be slight discomfort or bruising at needle puncture site. It is possible I will feel the signs of fainting such as dizziness, sweating, closed field of view, upset stomach, and faint can happen. These events are temporary and not life threatening. If this should happen I will let the nurse know as soon as possible and the procedure will be stopped. There are no direct benefits to taking part of this research study. The benefit of this study will be to help to understand how stress and sleep disturbances may affect the function of the immune system.

D. CONDITIONS OF PARTICIPATION: I understand that the confidentiality of all data and records including my identity and personal information including my DNA, will be fully maintained except to the research team. I maintain ownership over my DNA. My information such as ancestry or health will not be determined from my DNA. I will not be eligible to participate if I am taking prescription medication, or have a chronic medical condition. The data collected may be published, in which case my identity will not be disclosed at any point. All biological samples will be securely discarded at the end of the study including my DNA. My consent to participate in this study is entirely voluntary, and at any time my refusal and/or withdrawal to participate will involve no prejudice or penalty.

E. SIGNATURES: I HAVE CAREFULLY STUDIED THE ABOVE AND UNDERSTAND THIS AGREEMENT. I FREELY CONSENT AND VOLUNTARILY AGREE TO PARTICIPATE IN THIS STUDY.

NAME (please print) _____

SIGNATURE _____ DATE _____

If at any time you have questions about the proposed research, please contact the study's Principal Investigator Dr. Peter J. Darlington, 514-848-2424 ext. 3306 or peter.darlington@concordia.ca. If at any time you have questions about your rights as a research participant, please contact the Research Ethics and Compliance Advisor, Concordia University, 514-848-2424 ext. 7481 or ethics@concordia.ca

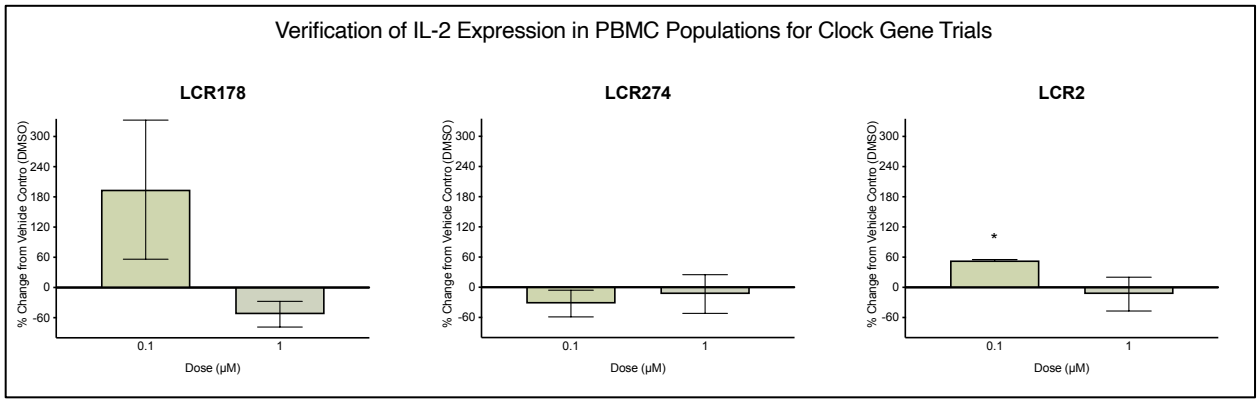
F. STATEMENT OF PERSON CONDUCTING INFORMED CONSENT: I have discussed the information contained in this document with the participant and it is my opinion that the participant understands the risks, benefits, and procedures involved with this research study.

NAME (please print)_____

Dr. Peter J. Darlington
Department of Health Kinesiology and Applied Physiology
vMay 2018

SIGNATURE _____ DATE _____

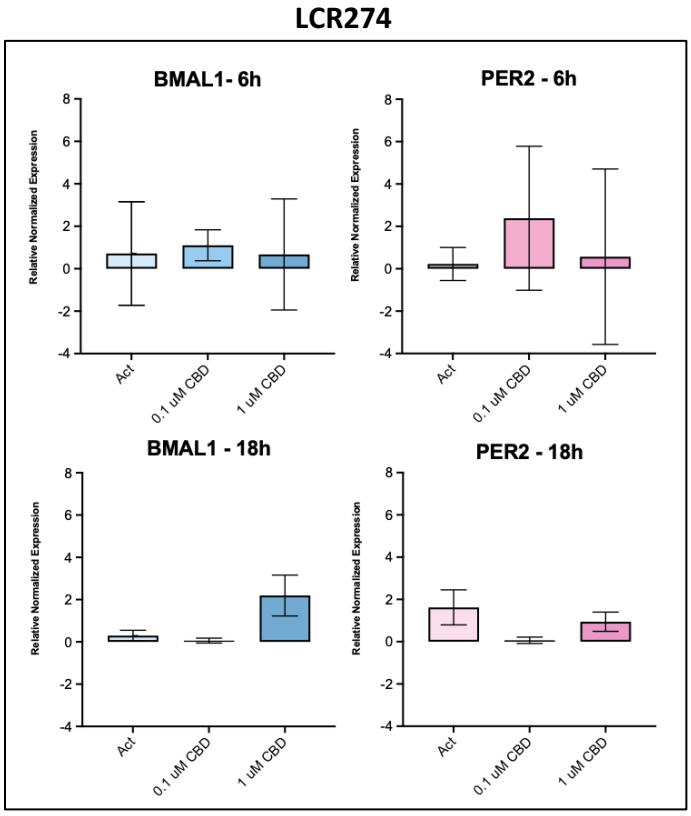
Appendix B – ELISA results, Circadian Experiments

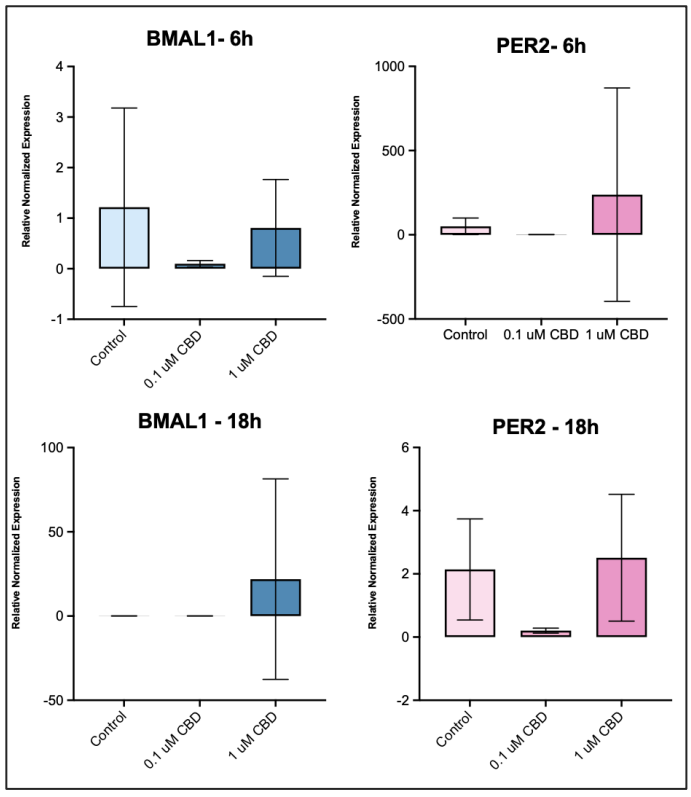


Appendix C- Statistical summary of ANOVA for Circadian Experiment

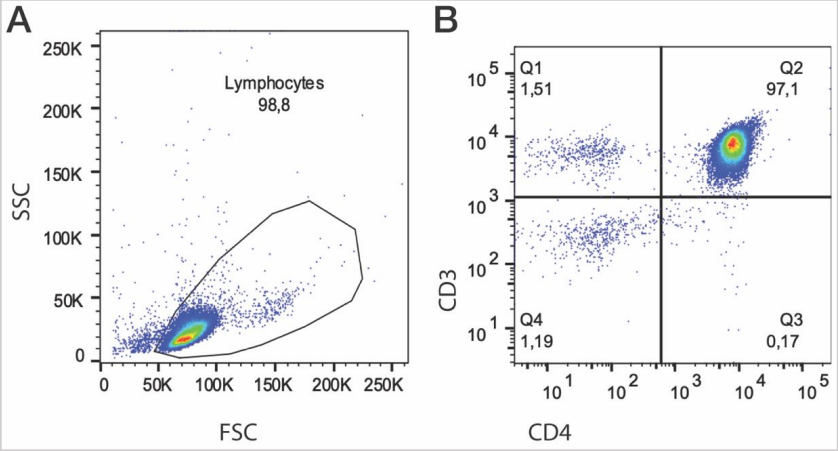
BMAL1 ANOVA	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.5143	2	0.2572	F (2, 12) = 0.06673	P=0.9358
Time Factor	1.498	1	1.498	F (1, 12) = 0.3886	P=0.5447
Dose Factor	2.125	2	1.063	F (2, 12) = 0.2757	P=0.7637
Residual	46.25	12	3.854		
PER2 ANOVA	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	8.396	2	4.198	F (2, 7) = 0.9381	P=0.4356
Time Factor	0.09409	1	0.09409	F (1, 7) = 0.02103	P=0.8888
Dose Factor	0.3953	2	0.1976	F (2, 7) = 0.04417	P=0.9571
Residual	31.32	7	4.475		

Appendix D- Gene expression, Circadian Experiments



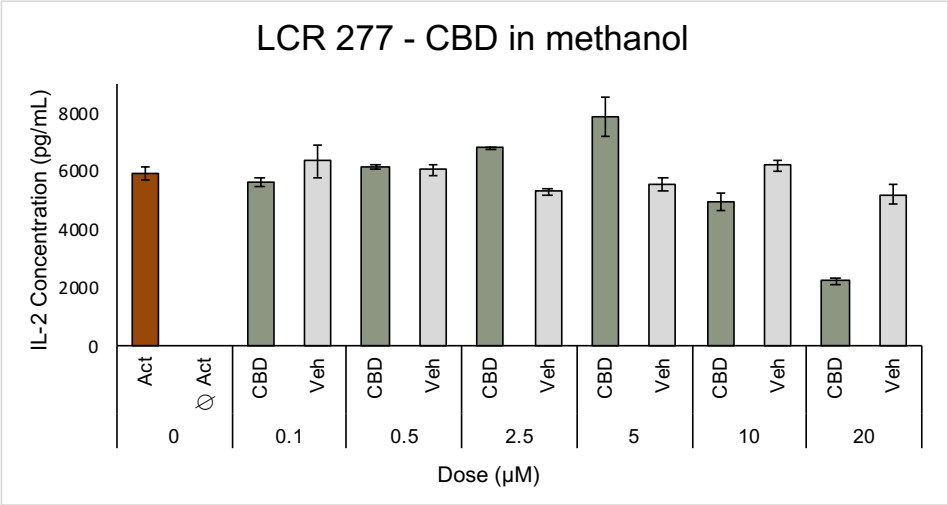


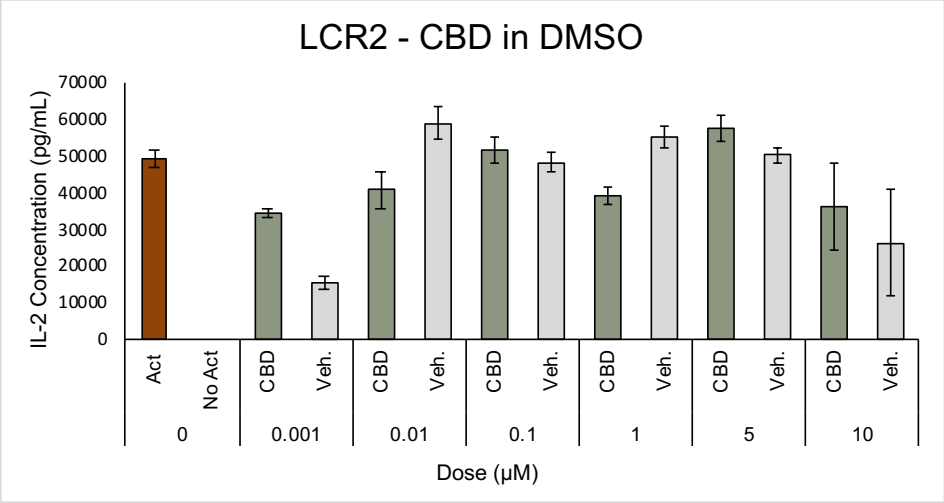
Appendix E – Flow cytometry analysis for $CD4^+$ T cell Isolation Purity



Note: $CD4^+$ T cell purity of 97.1% obtained as measured by gating of lymphocytes and gating of doubly positive anti-CD3, anti-CD4 fluorescent signal. An isolation purity of 95% or more was typical for experiments conducted.

Appendix F – Expanded ELISA data : DMSO vs methanol as a vehicle control





Note: Act = Activated Cell Controls, with no drug nor vehicle administered
No Act = Non Activated, i.e. unstimulated cell controls, (No Immunocult Added)

Appendix G - IL-17 versus IL-2