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Study on Water-soluble Cannabidiol (CBD) Effects in Experimental Models of Type 1 Diabetes (T1D)

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Study on Water-soluble Cannabidiol (CBD) Effects in Experimental Models of Type 1 Diabetes (T1D)

by:

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Abstract

Type 1 Diabetes (T1D) is an autoimmune disease in which immune cells called T cells initiate an attack on the insulin-producing beta cells of the pancreas, resulting in chronic hyperglycemia. Oil-based cannabidiol (CBD) has been used in previous research of T1D, showing beneficial effects in lowering diabetes incidence, glycemia, and pro-inflammatory cytokines. However, recently emerging water-soluble CBD products claiming an increased bioavailability, have never been tested in T1D. Therefore, the goal of this study was to evaluate the effects of water-soluble CBD on the development and severity of T1D in two mouse models: the non-obese diabetic (NOD) and low-dose streptozotocin-induced C57BL/6 (STZ-B6) mice. We expected to see a reduction in disease incidence, glycemia levels, along with altering T cell composition, function, and cytokine profiles of CBD-treated mice. For the NOD model, 5-6-week-old female mice were injected intraperitoneally (IP) for 6 weeks with 5 or 10 mg/kg of commercially available water-soluble CBD. They were then monitored weekly for glucose and body weight measurements up to 24 weeks of age. Mice were sacrificed at an early (11 weeks of age) and late (24 weeks of age or when confirmed hyperglycemic) timepoint in disease development, when immune parameters, such as spleen weights, cell counts, viability, immunophenotypes, and cytokine profiles were assessed. For the B6 model, 5-7-week-old male mice were injected with 5 mg/kg water-soluble CBD IP, for 4 weeks. While one cohort of B6 mice received IP injections of low-dose-streptozotocin (STZ) post CBD administration, another cohort received STZ and CBD simultaneously during the fourth week of CBD treatment. Following STZ administration, the mice were followed up to 33 days post first STZ injection for glucose and body weights measurements. An early and late time point was also assessed, day 11 and day 33 post first STZ injection. In the NOD model, dosage of 5 mg/kg of water-soluble CBD did not exhibit a beneficial effect of delaying disease development or reducing hyperglycemia. However, 10 mg/kg CBD treatment appeared to exhibit detrimental effects on T1D development, with significantly increased glycemic levels. In the STZ-B6 mouse model, a dosage of 5 mg/kg CBD was used both before and during STZ administration. When CBD was given before STZ, a non-significant lowering of hyperglycemia and a significant reduction in pro-inflammatory cytokines at an early time point was seen. However, when mice received CBD during STZ administration, we noted that CBD-treated mice exhibited significantly increased T1D incidence, along with a trend of higher glycemia. In conclusion, our findings highlight the need for further investigation of long-term water-soluble CBD usage and its effective dosages, as well as necessity for overall standardization of commercially available CBD formulations.

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Chapter 1: Introduction

Type 1 Diabetes

T1D is an autoimmune disease in which immune cells called T cells mediate an attack of the insulin-producing beta cells of the pancreas. Insulin is a hormone that stimulates cells to uptake blood glucose which is important for overall cell function and energy production. If the production of this hormone is reduced or halted, blood glucose levels will rise leading to hyperglycemia which can lead to severe complications and if left untreated even death. T1D primarily affects children, teens, and young adults, but it can develop at any age. Globally there has been an increase in the incidence and prevalence of T1D, with 1.5 million children and adolescents under the age of 20 with T1D (Gregory et al., 2022). In the United States, about 1.7 million people are affected by T1D, or 5.7% of all US adults (CDC, 2021). There is no cure for T1D, currently, symptoms are treated with insulin injections for the patient's lifetime. However, insulin injections pose a monetary burden, especially in the United States where they can cost almost 10 times more than any other developed country. This has led to some patients being forced to go either without treatment or having to ration the amount of insulin they use (Furst, 2020). Even with treatment, patients can suffer from complications such as heart and blood disease, blindness, and kidney failure (Fang et al., 2020). A recent study highlights the need for exploring alternative treatments for T1D due to the overall economic burden of patients with T1D estimated to be $813 billion more than those without the disease in the United States (Sussman et al, 2020).

Basic Immunology and Immunopathology of T1D

T1D is an autoimmune T cell-dependent disease that develops in individuals who possess susceptible genetic backgrounds and are exposed to particular environmental stressors. Human major histocompatibility molecules (MHC) genes, called human leukocyte antigens (HLA) genes, are strongly linked to the autoreactivity found in T1D, especially HLA-DQA1, HLA-DQB1, and HLA-DR genes (Noble & Valdes, 2011). Viral infections (enterovirus and rotavirus), food toxins such as nitrates, and vitamin D deficiency are some of the most cited environmental factors that contribute to the development of T1D (Quinn et al, 2021; Esposito et al, 2019). To understand the process of autoimmunity, it is necessary to explain the basics of the immune responses, involved cells, and the proteins they secrete.

The immune system responses can be divided into two types: innate immunity and adaptive immunity. Innate immunity is the body's first line of defense and produces a non-specific initial inflammatory response. Cells of the innate branch of the immune system include phagocytes such as macrophages and dendritic cells, which are often referred to as antigen-presenting cells (APC). These APCs can activate the adaptive immune response. Adaptive immunity is highly specific and is composed of T and B lymphocytes (T cells and B cells) (Murphy et al., 2022). T cells can be further classified into subpopulations, such as T helper (Th), T cytotoxic (Tc), and T regulatory (Treg) cells. Each population can be identified by their surface markers called clusters of differentiation (CD): Th by CD4 marker, Tc by CD8 marker, Treg by CD4, CD25, and intracellular Foxp3 markers. Th cells can be further divided into subtypes such as Th1, Th2, and Th17, to name a few, which are identified based on the cytokines they secrete (Bonilla & Oettgen, 2010). A healthy immune system involves the balance between

pro-inflammatory responses (Th1, Tc, and Th17) that secrete pro-inflammatory cytokines and anti-inflammatory responses (Th2 and Treg cells) that secrete anti-inflammatory cytokines.

Pathology of self-recognition can occur when adaptive immune cells start to recognize own proteins (autoantigens) and produce an inflammatory response. Autoimmunity starts when the autoimmune Th clones, which escaped thymic negative selection, recognize autoantigens, presented on the MHC class II on APCs (Rock et al., 2016), by their T cell receptors (TCRs) (Wieczorek et al., 2017). Another MHC molecule, MHC class I, which is expressed on almost all nucleated cells, presents autoantigens to Tc. T1D starts developing when APCs uptake and process beta cells damaged by some environmental stressor previosly mentioned. Once these beta cells are uptaken by APCs, they are processed and then present beta cell proteins (autoantigens) in the groove of MHC class II molecules to Th cells, which then become activated (Zirpel & Roep, 2021). Glutamate decarboxylase (GAD), islet-glucose-6-phosphatase catalytic subunit-related protein (IGRP), islet antigen 2 (IA-2), and insulin, to name a few, are beta cell-specific molecules recognized as autoantigens (Roep & Peakman, 2012). After recognition of autoantigens, the autoreactive Th cells preferentially differentiate into Th1 and Th17 subtypes, which induce beta cell destruction by the secretion of pro-inflammatory cytokines, interferon-γ (IFNγ), interleukin 17a (IL-17a), and tumor necrosis factor-α (TNFα) (Wagner , 2011), as well as by activation of Tc cells and macrophages (Spellberg & Edwards, 2001). Tc cells recognize autoantigens presented by MHC class I on beta cells, and initiate and propagate their direct destruction (Zirpel & Roep, 2021). While pro-inflammatory Th cells are becoming more predominant at the inflammatory site, there is a downregulation of the anti-inflammatory immune cells, such as Th2 and Treg cells. Th17 cells are also responsible for the inhibition of Treg cells (Lee, 2018). Th2 and Treg cells are thought to correlate with the protection of T1D, if

they become activated and overcome pro-inflammatory T cells (Kikodze, 2013). As the autoimmune response is induced, it leads to beta cell islet infiltration by macrophages and T cells, referred as insulitis, that promote and perpetuate beta cell killing until all the beta cells are destroyed (Rewers & Ludvigsson, 2016).

Mouse Models

Experimental mouse models are commonly used in the study of a variety of human diseases. When studying T1D development there are two models commonly used: female NOD/LtJ (NOD) mice, and chemically-induced T1D in male C57Bl/6J (B6) mice. The NOD/LtJ mouse model spontaneously develops T1D, making it the ideal model as it most closely resembles the disease development in humans. It is regarded as the standard model for studying T1D (Thayer et al., 2010). NOD mice have been shown to develop spontaneous insulitis at 4 weeks of age: female mice start becoming diabetic from about 12 to 24 weeks of age, with an incidence of 60% to 100%, depending on colony (Mullen, 2017).

The B6 mouse model needs a chemical induction by multiple low doses of streptozotocin (STZ) intraperitoneally (IP) in order to develop T1D (Cetkovic-Cvrlje et al., 2017). STZ is a glucosamine-nitrosourea compound derived from S*treptomyces achromogenes*, commonly used in the treatment of pancreatic cancers, due to its beta cell cytotoxicity. It is a glucose analog, allowing it to enter beta cells through the glucose transporter 2 (GLUT2), where it can accumulate. Upon entering, STZ can cause DNA damage due to the DNA alkylating activity of its methyl nitrosoureas moiety leading to DNA fragmentation. DNA fragmentation leads to the activation of poly ADP-ribosylation, resulting in the depletion of NAD + and ATP along with the increase of free oxygen radicals. The increase of these radicals causes oxidative *stre*ss in beta cells, thereby halting their ability to synthesize insulin (Wszola et al., 2021).

The multiple low-dose STZ approach in B6 mice is favored for induction of autoimmune T1D over the single high-dose, due to its ability to induce only partial damage to pancreatic beta cells. In contrast, direct toxicity to the majority of beta cells is observed when a single high dose is used, which leads to the induction of toxic, not autoimmune T1D (Deeds et al., 2011). The initial low-dose STZ-induced damage of beta cells induces an inflammatory response that leads to further loss of beta cell activity, resulting in decreased insulin production. Once insulin deficiency appears, hyperglycemia follows. This inflammatory response to low-dose STZ in B6 mice more closely resembles the pathogenesis of disease seen in both the standard NOD mouse model and in humans (Furman, 2021). Male B6 mice are used over female mice as females are resistant to multiple low doses of STZ (Saadane et al., 2020). In addition, low doses of STZ are used to prevent tissue damage, such as acute liver and kidney toxicity seen when high doses of STZ have been used. STZ has a short half-life, and when administered at low doses can be easily eliminated before causing liver and kidney failure, while still inducing small-scale beta cell damage (Yan & Wu, 2015). Mouse strains that are the most sensitive to low-dose STZ action are B6 and DBA/2J (DBA). The low-dose STZ-induced B6 model has been widely used to study the development of T1D and possible preventative therapies for T1D, while the DBA strain has been used for research of diabetic complications, such as nephropathy (Gurley et al., 2006).

Endocannabinoid System and Cannabidiol Interaction with Immune cells

The endocannabinoid system (ECS) is an important neuromodulation system. The ECS has been found to be a crucial modulator of the physiological functioning of the human body. It is made up of endocannabinoids, cannabinoid receptors, and the enzymes responsible for the synthesis and degradation of endocannabinoids. Endocannabinoids are endogenous lipids that

interact with cannabinoid receptors to induce a variety of responses (Lu & Mackie, 2016). There are two types of cannabinoid receptors which are CB1 and CB2 receptors. While CB1 receptors are found to be expressed in high levels in the central nervous system (CNS), CB2 receptors are not. CB2 receptors are found in multiple lymphoid organs, where they regulate immune and inflammatory pathways, while CB1 receptors regulate neurotransmission in the CNS (Turcotte et al., 2016). CB2 receptors have been found on APCs and T cells where they participate in an immune response when activated by an endocannabinoid. When cannabinoids bind to CB2 receptors, inhibition of T-cells was observed, along with a reduction in a pro-inflammatory response (Robinson et al., 2013). Some studies have researched the effects of CB2 binding on immune cell functions such as Th cells, where TNF-α and Il-17 levels decreased, and Tc cells, where CB2 binding led to a decrease in TNF-α as well (Turcotte et al, 2016). The inhibition of T cells is an important aspect of treatment in prevention of T1D development. Autoreactive T cells are the main effectors in the etiopathogenesis of T1D. Therefore, an inhibition of autoreactive T cells would be beneficial, which might be accomplished through the activation of their CB2 receptors using CBD.

Cannabidiol (CBD) is one of the major components of the *Cannabis sativa* marijuana plant. It can be classified as an endocannabinoid, as it can bind to both CB1 and CB2 receptors, however, CBD does not produce psychotropic effects as it has not been seen to bind as readily to CB1 receptors (Klein et al., 1998). Multiple studies have been performed to study the different immune-modulating effects of CBD on polymorphonuclear cells (PMNs, or neutrophils), T cells (more specifically Th cells), and macrophages (Martini et al., 2023; Furgiuele et al., 2023). All these immune cells play a crucial role in promoting inflammation by secreting pro-inflammatory cytokines, such as TNF-α, and by producing reactive oxygen species (ROS). ROS is produced by

all immune cells and is a major cause of cellular and tissue injury when overproduced (Mittal et al., 2014). Researchers have investigated the CBD's effects on different cellular receptors present on immune cells (T cells, PMNs, macrophages, etc.). Although CB2 receptors are a widely known endocannabinoid receptors that are present on immune cells, research has shown that CBD can also bind to other targets such as transient receptor potential vanilloid 2 (TRPV2) channels (Muller et al., 2019), and peroxisome proliferator-activated receptor gamma (PPAR-γ) (O'Sullivan, 2016). TRPV2 is expressed in lymphocytes (T and B cells) and macrophages where it mediates functions such as cytokine release, phagocytosis, and inflammation (Santoni et al., 2013). CBD can activate TRPV2, which both directly and indirectly reduces ROS production (Atalay et al., 2019). PPAR-γ activation by CBD inhibits the nuclear factor-κB (NF-κB) pathway, which normally promotes inflammation by activating the transcription of pro-inflammatory genes such as nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2) (Atalay et al., 2019). PPAR-γ has also been shown to reduce oxidative stress by interacting with the transcription factor erythroid 2-related factor 2 (Nrf-2), which controls genes associated with oxidative stress, in other words reducing ROS production (De Fillips et al., 2011). CBD interaction with CB2 receptors, TRPV2, and PPAR-γ has shown anti-inflammatory effects that make it ideal to study its use as treatment in inflammatory-based pathologies.

Cannabidiol Studies in Experimental Models of Autoimmunity

CBD has been extensively studied in experimental models of multiple sclerosis (MS) (Kozela et al, 2011; Kozela et al., 2013; Elliott et al., 2018; Rahimi et al., 2015; Mecha et al., 2013) and rheumatoid arthritis (RA) (Malfait et al., 2000; Lowin et al., 2019; Hammell et. al., 2015; Costa et al., 2004). MS is an autoimmune neurodegenerative disease which involves

autoreactive T cells that migrate into the CNS and initiate demyelination and oligodendrocyte cell death. Autoreactive T cells are activated by local APCs in the CNS, which triggers an inflammatory response that leads to the release of pro-inflammatory cytokines (TNF-α and IFN-γ), and recruitment of more pro-inflammatory cells (Yamout & Alroughani, 2018). Ultimately, the increase in a pro-inflammatory response leads to activation of autoreactive T cells that attack central neurons, their myelin sheaths, and their axons (Fletcher et al., 2010). Many researchers have aimed to advance treatment of MS by studying diverse ways to suppress or modulate the auto-reactive response associated with the disease. Kozela et al. (2011) performed a study on the effects of CBD on T cells in a myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), a B6 mouse model of MS, using 5 mg/kg IP CBD treatment for three days. CBD treatment led to amelioration of disease signs during the CBD administration, as well as delaying disease progression. Also, CBD decreased T cell and microglia infiltration of the spinal cord, and the MOG-induced proliferation of T cells (Kozela et al., 2011). Another study, exploring in-vitro CBD effects on MOG-specific T cell lines quantified cytokines and found a decrease of IL-6 and Il-17, but not of TNF-α and IFN-γ (Kozela et al., 2013). The other study showed beneficial effects of a higher dose of CBD in EAE B6 model of MS; mice were treated for two weeks by 20 mg/kg CBD and an attenuation of clinical signs of EAE, with a decreased infiltration of spinal cord by Th and Tc cells, and reduced production of IFN-γ and IL-17, and increased IL-10 by the cultured spinal cord and splenic T cells, was observed (Elliott et al., 2018). Even when CBD was given later during the onset of disease, rather than prophylactically, 5 mg/kg dose reduced the severity of MOG-induced EAE and, and the expression of proinflammatory cytokines (Rahimi et al., 2015). Data collected in different, virus-induced experimental mouse model of MS, using a 7-10-day-long treatment with

5 mg/kg IP administration of CBD, showed a positive action of treatment on leukocyte infiltration in the brain and spinal cord of CBD-treated mice (Mecha et al., 2013).

CBD has also been studied for its therapeutic potential in treating RA, an autoimmune disease in which synovial joint membranes are destroyed by the immune system. In a study conducted by Malfait et al., CBD purified from hashish and administered either IP (5 and 10 mg/kg) or via oral gavage (25 and 50 mg/kg), was investigated in the collagen-induced arthritis mouse model. Researchers performed histological analyses of the hind paws of mice to assess for synovial damage by clinically scoring. Whereas mice treated IP with 5 mg/kg had the lowest clinical scores for synovial damage, comparable to the lowest scores of mice treated orally with 25 mg/kg, only 5 mg/kg IP treatment led to a significant decrease in TNF production by synovial cells (Malfait et al., 2000). Lowin et al. (2019) found that CBD treatment *in vitro* reduced IL-6, IL-8, and metalloproteinase 3 production by snovial fibroblasts. The effect of CBD was also studied in a rat, Freund's adjuvant-induced, experimental model of inflammatory arthritis in which transdermal application of CBD, at a dosage of 6.2 and 62 mg/kg for four consecutive days, led to a decerease in joint inflammation, and a reduction of TNF-alpha (Hammell et. al., 2015). CBD was also given orally in a doses of 5-40 mg/kg in a rat carrageenan-induced RA model. Results showed that three doses of CBD reduced the paws edema, and plasma levels of prostoglandin E2, tissue COX activtiy, and production of both oxygen free radicals and NO (Costa et al., 2004)

Cannabidiol Studies in Experimental Models of T1D

Regarding T1D, two different studies tested the immunological effects of oil-based CBD in the non-obese diabetic (NOD) mouse model. A 2006 study conducted by Weiss et al. investigated whether administration of 10-20 IP injections of 5 mg/kg oil-based CBD to pre-diabetic, 6-12-week-old, female NOD mice affect the development of disease. The CBD was extracted in their laboratory from Cannabis resin (hashish), and the emulsifier Cremophor EL was added to aid in the absorption of CBD. CBD-treated mice showed a significant delay of disease development, and reduction of diabetes incidence, along with a decrease in insulitis score. A decrease in the cytokines IFN-γ and TNF-α, and an increase in IL-10 and IL-4 was observed as well (Weiss et al. 2006). Another study on the effects of CBD on T1D using the NOD mouse model was conducted in 2008 (Weiss et al. in 2008), in which "older pre-diabetic or nearly diabetic", 11-14-week-old, female NOD mice were injected with IP injections of 5 mg/kg CBD for four weeks. Mice were then observed up to 24 weeks of age for diabetes incidence, and at the end point for insulitis, and cytokine levels (Weiss et al., 2008). CBD-treated mice a decrease in diabetes incidence and a reduction of insulitis. A decrease in TNF-α, and an increase in IL-4 concentrations were observed in CBD-treated mice compared to controls, suggesting CBD's anti-inflammatory properties (Weiss et al., 2008).

The third study investigated the effects of abnormal-CBD (abn-CBD) on beta cell damage in both NOD and a high-dose STZ-induced B6 mouse models. The abn-CBD was purchased as a premade synthetic regioisomer of CBD. It is known that abn-CBD acts on both CB1 and CB2 receptors, along with a proposed atypical cannabinoid receptor, G protein-coupled receptor 55 (GPR55). NOD mice were treated IP with 0.1-1 mg/kg of abn-CBD, while B6 mice received 1mg/kg abn-CBD. In both models cytokine analysis and insulitis were studied. In the

NOD model, both 0.1 and 1 mg/kg dosages of abn-CBD led to lower glucose levels, a delay in the onset of diabetes, reduced insulitis grade, and a noticeable decrease in TNF-alpha. The pancreases of the abn-CBD-treated B6 mice showed a reduction of beta cell apoptosis, and decreased TNF-α (González-Mariscal et al., 2022). This study provided insight into the effects of abn-CBD on T1D development in NOD model, however, studies on glycemia and diabetes development were lacking.

Although Weiss (Weiss et al., 2006, Weiss et al., 2008) and González-Mariscal's (González-Mariscal et al., 2022) studies in general indicated positive effects of CBD on T1D, the use of the researchers' own purified CBD (Weiss's studies), and abn-CBD (González-Mariscal's study) makes it difficult to replicate the study, or comprehensively conclude about the "ideal" dose of CBD in preventative treatment of experimental T1D. In addition, these three studies provided some insight on CBD's efects in NOD mouse model, while there is no data on preventative action of CBD on T1D incidence and glycemia in the autoimmune low-dose STZ B6 model. Weiss's research group (Weiss et al., 2006, Weiss et al., 2008) studied in-house prepared oil-based CBD. Such CBD formulation is hydrophobic, leading to decreased absorption. Oil-based CBD has questionable efficacy due to it undergoing extensive first-pass metabolism degradation and a majority of it is excreted by the kidneys before it can exhibit cellular effects (Millar et al., 2018).

Chapter 2: Aim and Hypothesis

Water-soluble CBD is an emerging CBD formulation that has been chemically made to easily dissolve in water, therefore, allowing more rapid and effective absorption (Gokavi, 2022). The goal of this study was to evaluate the effects of water-soluble CBD on the development and severity of autoimmune experimental T1D in NOD and low-dose STZ-treated B6 mice. In recent years, researchers have been trying to find alternative treatments for those suffering from autoimmune disease. Cannabidiol (CBD) has been shown to have anti-inflammatory effects in varying autoimmune diseases (Giorgi et al., 2021). It has been shown that CBD might delay the onset and reduce the severity of T1D in NOD mouse model. However, the previous studies on CBD effects on T1D used pure oil-based CBD that has either been altered structurally or conjugated in the researcher's laboratory with an emulsifier. We aimed to investigate the effects of pre-made, commercially available, water-soluble CBD, which effects on T1D development have never been investigated. Water-soluble CBD might serve as an prophylactic treatment for T1D patients. The hypotheses of this study were as follows:

H_o: Water-soluble CBD will not affect the development of T1D and glycemic levels in the NOD or STZ-B6 mouse models nor alter T cell composition and function.

H_1: Water-soluble CBD treatment will affect the development of T1D in the NOD and/or STZ-B6 mouse models by delaying the onset of the disease, decreasing glycemia, and altering T cell composition and function.

Chapter 3: Materials and Methods

Mice

Prior to conducting any work on experimental mice, the Saint Cloud State University Institutional Animal Care and Use Committee approved the study (Protocol #5-142). NOD/LtJ (NOD) and C57Bl/6 (B6) mice used for this study were bred in-house in the St. Cloud State's Vivarium., where they were housed during the duration of the experiments. NOD and B6 mice were originally purchased from the Jackson Laboratory in Bar Harbor, ME, and then were continuously bred in the Vivarium at St. Cloud State University. The mice were housed in BPA-free NexGen Lo-Profile cages in a temperature and humidity-controlled room with a 12-hour light/dark cycle. They received water and casein-based phytoestrogen-free diet *ad libitum* (AIN-93G Rodent Diets, Research Diet, Inc., Harlan research laboratories). Once mice met sexual maturity they were sexed and then lodged in same-sex cages until they were chosen for experiments. There were limited numbers of mice available at different times due to their in-house breeding. Therefore, multiple experiments were performed to reach the desired number of mice per experimental group, with each experiment consisting of 4-6 mice per group. Mice were always divided into experimental groups in a way that the same number of mice from the particular litter was placed in each experimental group.

Experimental Design for NOD-mouse model:

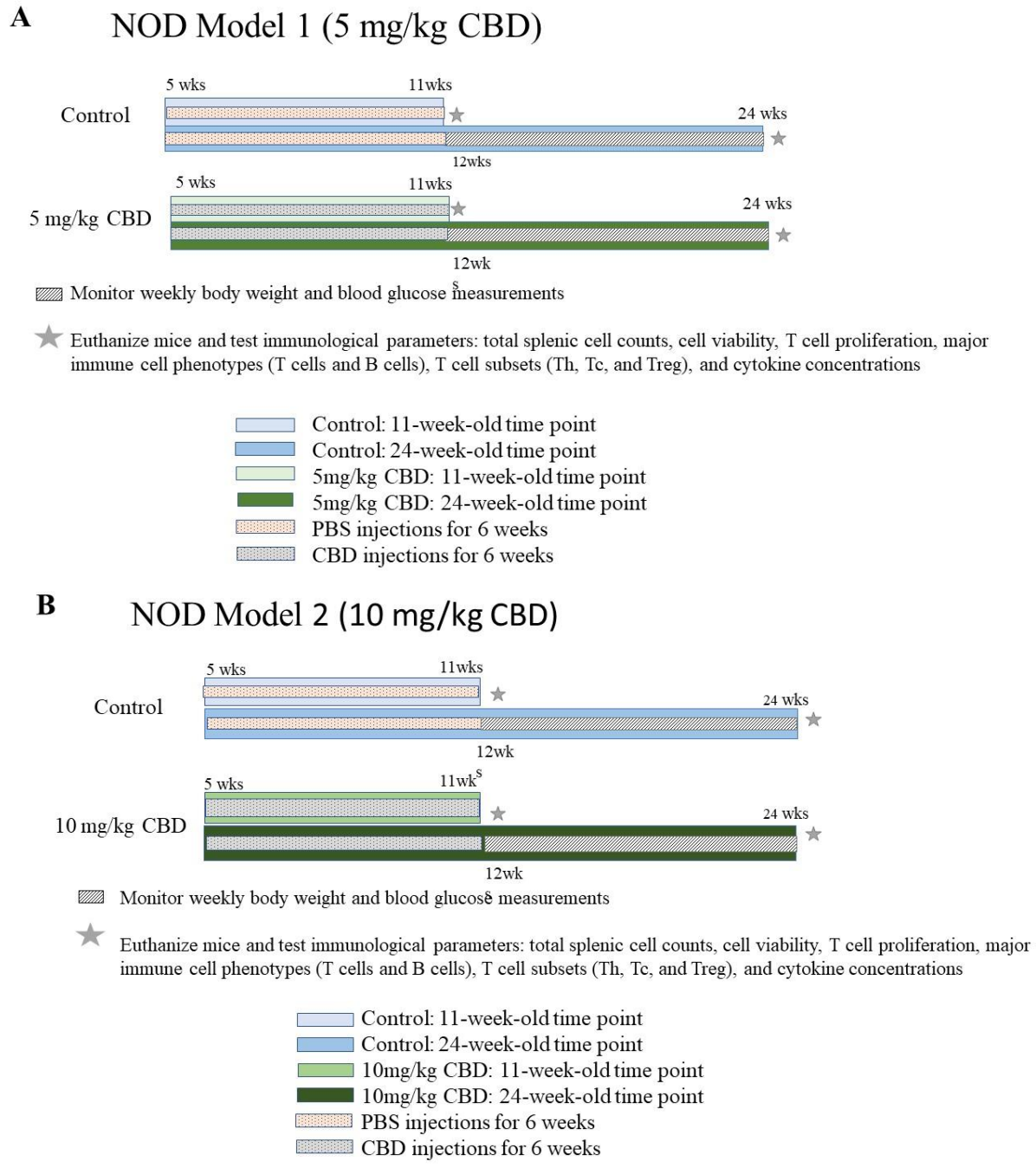
In the first set of experiments, 5-6-week-old female NOD mice were used. They were randomly divided into a control and CBD treatment group, ear notched for identification, and their initial body weights (BW) and blood glucose (BG) levels were taken. The control group received intraperitoneal (IP) injections of phosphate-buffered saline (PBS), while the CBD group

received IP injections of water-soluble CBD at a dosage of 5 mg/kg. Both groups received either CBD or PBS injections for six consecutive weeks. At the end of the injection period, the mice were 11 weeks of age. Once the NOD mice reached 12 weeks of age, their BW and BG levels were measured weekly up to 24 weeks of age to study the development and severity of disease. Two time points were assessed to observe the effects of water-soluble CBD on immune parameters, the 11 and 24 weeks of age (Figure 1a), at which the mice were euthanized by carbon dioxide asphyxiation, as described in our IACUC-approved protocol.

In the second set of experiments, 5-6-week-old female NOD mice were divided into control and 10 mg/kg water-soluble CBD-treated groups. The experimental design, CBD administration, follow-up of T1D development, and euthanasia time points for the study of immunological parameters were completely identical to those described above for the set of experiments in which 5 mg/kg CBD treatment was used (Figure 1b).

Figure 1

Experimental Design for NOD Mouse Model: An initial experiment (Model 1) studying 5 mg/kg CBD treatment for 6 weeks (A), and the second experiment (Model 2) studying 10 mg/kg CBD treatment for 6 weeks (B).



Experimental Design for B6 mouse model:

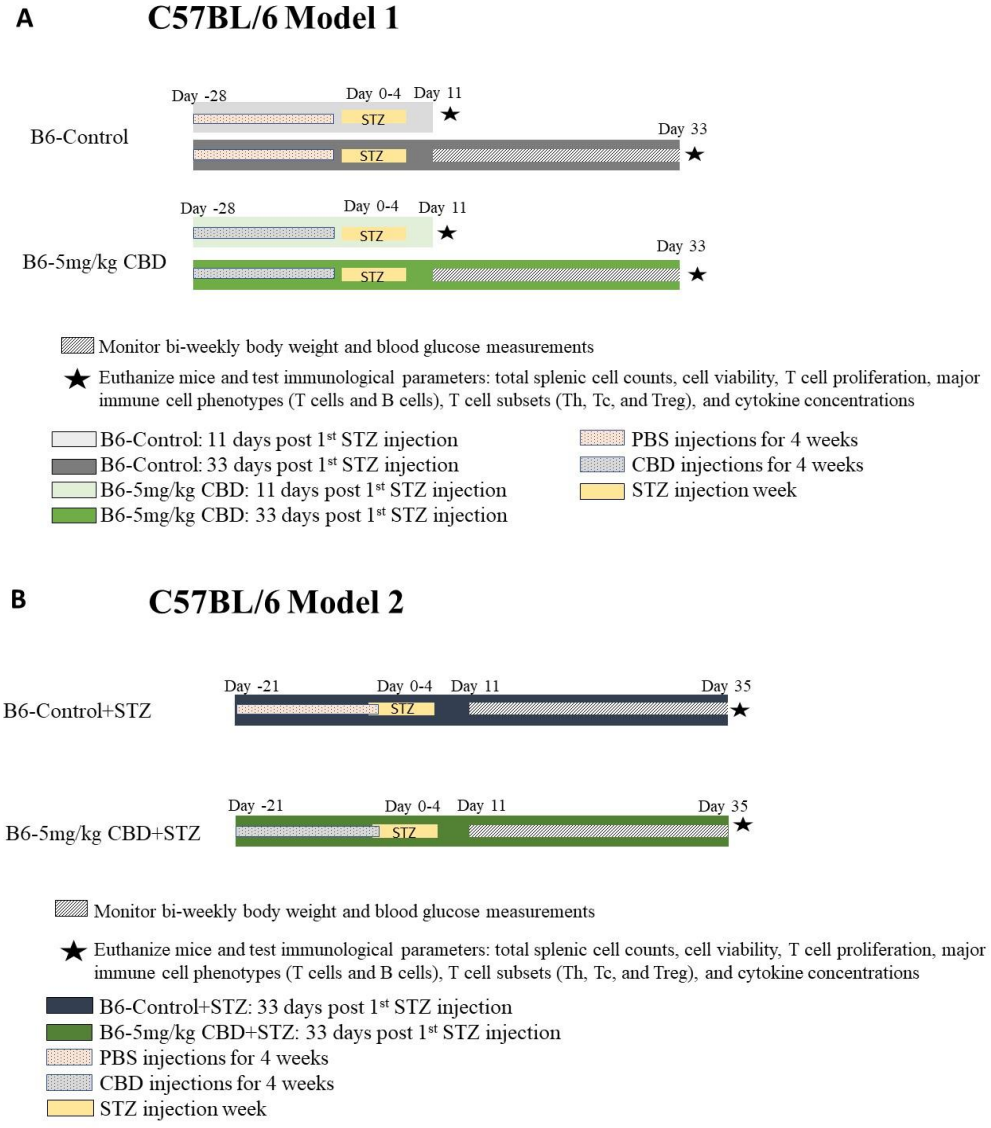
B6 male mice, 5-7 weeks old, were randomly divided into a control and a CBD treatment groups, ear notched for identification, and their initial body weights (BW) and blood glucose (BG) levels were taken. While the control group received IP injections of PBS for four weeks, the CBD group received IP injections of water-soluble CBD at a dosage of 5 mg/kg for four weeks. Following the fourth week of CBD/PBS injections mice then received STZ. One week post-first STZ injection (day 8), both BW and BG levels were taken twice weekly up to 33 days post-first STZ injection. Two time points, day 11 and 33 post-first STZ injection, were studied to assess the effects of water-soluble CBD on immune parameters (Figure 2a). At the end of each time point, the spleens of the B6 mice were dissected, and cellular parameters were tested: total cell counts, cell viability, T cell proliferation, major immune cell phenotypes (B cells and T cells), T cell subsets phenotypes (Th, Tc, and Treg), and cytokine concentrations. Early time points for each experimental group were used to identify and assess the differences in cell populations along with their activity at different points of T1D development (Figure 2a). The mice were euthanized by carbon dioxide asphyxiation, as described in our IACUC-approved protocol.

Initial data, collected for a small number of B6 mice (n=11 per group) receiving STZ following CBD treatment, showed a significant decrease in incidence, and BG levels of CBD-treated mice compared to controls. It brought to question the interaction between the CBD and STZ, possibly leading to a decreased effectiveness of STZ on inducing T1D. To answer this question, we decided to extend the CBD treatment during the STZ administration of B6 mice in

the second set of experiments. During the final week of CBD injections, STZ was administered simultaneously with CBD. Mice received CBD injections in the mornings, while STZ injections in the afternoons. Both BW and BG levels were measured twice weekly, starting one week post-first STZ injection (day 8), up to 35 days post-first STZ injections. Only one-time point was studied for immunological parameters in this experiment, the end point – day 35 post-first STZ injection, to obtain glucose values throughout the entire experimental period (Figure 2b). The mice were euthanized by carbon dioxide asphyxiation, as described in our IACUC-approved protocol. The experimental design, besides the execution of STZ administration in parallel with CBD administration, and a singular endpoint for examination of immunological parameters, was similar to the one described above for the initial set of experiments in B6 model.

Figure 2

Experimental Design for B6 Mouse Model: An initial experiment (Model 1) studying 5 mg/kg CBD treatment, performed for 4 weeks prior to the STZ induction of T1D (A), and the second experiment (Model 2) studying 5 mg/kg CBD treatment, performed for 4 week s with a STZ administration during the last week of CBD treatment (B).



CBD Preparation and Administration

A water-soluble CBD powder was obtained from Stirling CBD (https://www.stirlingcbdoil.com/product-category/pure-cbd-isolate-for-sale/). It was diluted in sterile PBS. Since CBD water-soluble powder contains 10% of CBD, we prepared a CBD stock suspension at a concentration of 5 mg/mL and 10 mg/mL (see Figure 3) for the 5 mg/kg and 10 mg/kg mouse body weight dosage, respectively. Stock CBD suspensions were prepared daily and administered immediately after preparation. Once the proper amount of CBD was weighed, it was then dissolved in PBS and vortexed for three minutes. While we initially expected to dissolve the water-soluble CBD stock into a solution, the desired stock concentration was not able to be dissolved in solution, but in suspension. Pharmaceutical suspensions can be favored over solutions, as they may allow higher concentrations of drugs (solute) to be incorporated into medications (Yagnesh & Rada, 2016).

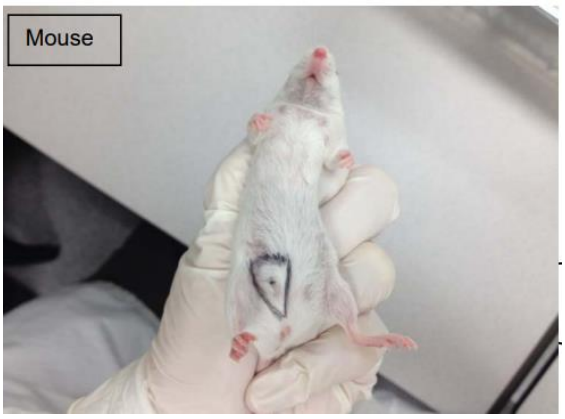
Figure 3

Preparation of water-soluble CBD powder diluted in PBS into suspension.

The CBD suspensions were administered to mice via IP injections (Indiana University IACUC, 2017) for five days a week for up to six weeks for NOD mice, and for four weeks for B6 mice. For IP injections mice were restrained using one hand as can be seen in Figure 4, and the injection site was disinfected with 70% ethanol. The 27-gauge needle was then inserted with the bevel facing "up" into the lower-right quadrant of the abdomen towards the head at a 30–40-degree angle (University of British Columbia IACUC, 2014).

Figure 4

Restraint of a mouse for IP injections (University of British Columbia, 2014).



Low-Dose Streptozotocin Injections of B6 Mice

STZ was prepared daily in fresh citrate buffer (0.05 M, pH 4.5, trisodium citrate dihydrate with 4°C distilled and autoclaved water), and administered IP to both B6 control and CBD-treated groups at a dose of 40 mg STZ/kg body weight in a volume of 6.52 mL buffer/g body weight at a concentration of STZ (74mg of STZ in 1,200 µl of PBS) for five consecutive days (Cetkovic-Cvrlje et al., 2017).

Blood Glucose and Body Weight Measurements

Body weights and BG measurements in both strains of mice were taken and analyzed to determine the effects of CBD on the incidence and severity of diabetes. BG was measured in both strains at the start of experiments. Bi-weekly BG measurements were taken in B6 mice for up to 33 days after the administration of the first STZ injection. Measurements were not taken during STZ injections to reduce additional manipulations with mice and alleviate added stress. For NOD mice, their BG levels were taken weekly from 12 weeks of age, which is the typical

point at which insulitis progresses sufficiently to induce clinical hyperglycemia. Their BG and body weights were taken weekly up to 24 weeks of age.

For BG measurements, the lateral vein of each mouse was punctured with a 26GX5/8 needle to produce a single blood drop which was then placed on an Accu-check Aviva Plus glucose strip purchased from Diabetic Warehouse. The blood sample was then analyzed with an Accu-check Aviva glucometer to obtain a BG measurement (mg/dL) (Roche Diagnostics, Indianapolis, IN, USA). Mice were restrained for BG sampling using a 50 mL Falcon tube modified with drilled breathing holes. Immediately after the reading was complete, pressure was applied to the puncture site to halt bleeding and aid in clotting. The mice were then gently returned to their respective cages. A BG reading of 250 mg/dL or higher was considered a diabetic value. The first of two or more consecutive diabetic BG readings was considered the time of diabetes onset (Cetkovic-Cvrlje et al., 2017).

Preparation of a Single Cell Suspension from the Spleen

Immediately following euthanasia, mouse spleens were aseptically harvested in a biosafety cabinet, subsequently weighed in grams (g), and placed in Petri dishes containing 5mL of phosphate-buffered salt solution (PBS, Sigma-Aldrich). Single-cell suspensions were then prepared by mashing the spleens with a plunger from a 10 mL syringe and a passage through 70 µm nylon strainers (BD Falcon, USA) atop a 50 mL Falcon tube, to disrupt the pulp of the spleen and release splenocytes. PBS totaling 4 mL was added to pass cells through the mesh of the cell strainer and aid in preserving the cells. The resulting mixture was then transferred to a 15 mL Falcon tube and 1mL of PBS was used to rinse the original 50 mL Falcon tube to capture as many splenocytes as possible, which was then transferred into the 15 mL Falcon tube containing

the additional 4 mL of the PBS and splenocyte mixture. The suspensions were then centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was decanted, the cell pellet was resuspended, and then 750 µL of ACK lysis buffer was added (NH4Cl 8.29g/L, KHCO3 1.0g/L, EDTA Na2· 2H2O 0.0375g/L; 18 Lonza Bio Whittaker, Walkersville, MD, USA) for 1-minute for complete hemolysis. An additional 5 mL of ice-cold PBS was added to stop the action of the lysis buffer, and the suspensions were centrifuged, their supernatants decanted, and the pellets resuspended. Three additional washes with PBS and subsequent centrifugation were done to remove cell debris and wash out the ACK buffer. Following the final wash, cells were resuspended in 5 mL PBS, and cell counts, and viability were conducted via Trypan Blue (Lonza Bio Whittaker Walkersville, MD, USA) exclusion method. Briefly, the isolated splenocytes were prepared in a 1:20 dilution with Trypan Blue and then loaded in a hemocytometer; the loaded hemocytometer was put under 40x magnification objective of a microscope to determine the number of live and dead cells by counting all cells in two opposing quadrants. Dead cells appeared blue while live cells appeared yellow or white in color. For each sample, the calculated total number of live cells for each suspension was used to determine the quantities needed for further procedures.

T-Cell Proliferation Assay

Proliferation assays were done to determine the *ex vivo* effects of water-soluble CBD on T cell function. Single-cell suspensions were obtained from mouse spleens and then diluted in the "complete" culturing medium (CM) [RPMI-1640 with 5 units Penicillin and Streptomycin, and 10% Fetal Bovine Serum (Sigma, St. Louis, MO, USA)]. Cells were then plated in a 96-well plate in a concentration of $4x10^5$ in a total volume of 100 µL. Each mouse sample had a triplicate of wells for control and a triplicate with the addition of T-cell specific mitogen Concanavalin A

(ConA) at a concentration of 3µg/mL (ConA, Sigma-Aldrich). The first triplicate of wells was deemed non-stimulated wells, as they contained only cells and medium, but not ConA. The last triplicate of wells was deemed as stimulated wells as T cells were exposed to ConA to stimulate their proliferation. Plates were incubated for 72 hours at 37°C with 5% CO_2. After the incubation period, 10µl of Alamar Blue (Invitrogen) was added to each well and incubated for 4-6 hours more. Alamar Blue is a cell-permeable blue dye containing resazurin. Resazurin is water-soluble and non-toxic reagent, allowing it to be permeable through cell membranes. It can be reduced by the metabolic byproducts of cells and converted to the pink-colored, highly fluorescent resorufin (Rampersad, 2012). If resorufin is present in culture, it changes the color of the media surrounding the T cells, allowing for changes of color to be detected by optical densities using the ELISA spectrophotometer at 570nm (GeneMate).

Immunophenotyping

To determine the immune cell composition of mouse spleens post CBD treatment, flow cytometry was conducted. The immune cells investigated are T cells, their subtypes, and B cells. From obtained single-cell suspensions, two aliquots of $1x10^6$ splenocytes were transferred to labeled polystyrene tubes in 1 mL of FACS buffer (1xPBS, 0.1% w/v NaN_3 w/v, and 1% FCS v/v in PBS). Samples were centrifuged for 5 minutes at 1200 rpm at 4°C, the supernatant decanted, tubes were blotted to rid excess buffer, and their pellets were resuspended. Splenocytes were stained with fluorochrome-conjugated monoclonal antibodies that bind to specific clusters of differentiation (CD) markers on immune cells (Table 1). Antibodies were prepared with FACS buffer in a 1:100 ratio, then 100µL was added to splenocyte samples. Tubes were vortexed and then incubated in the dark for 45 mins at 4°C. After incubation the samples were washed with

500µL of FACS buffer and then centrifuged for 5 minutes at 1200 rpm at 4°C three times. After centrifugation their supernatants were decanted, tubes blotted, and pellets resuspended. Then, cells were acquired using the Accuri C6 Plus (BD Biosciences) flow cytometer and analyzed using the Accuri C6 Plus software (BD Biosciences).

Table 1

Cells identified during flow cytometry with unique CD markers and appropriate fluorochrome-conjugated monoclonal antibodies (BD Biosciences)

Cell	CD	Antibody Fluorochrome
All T cells	CD3	Anti-CD3 PE (clone 145-2C11)
T helper (Th)	CD4	Anti-CD4 PerCP (clone RM4-5)
T cytotoxic (Tc)	CD8	Anti-CD8 FITC (clone 53-6.7)
T regulatory (Treg)	CD4/CD25	Anti-CD25 APC (clone 3C7)
B cells	CD45R/B220	Anti-B220 APC (clone RA3-6B2)

Note. FITC-fluorescein isothiocyanate, PE-phycoerythrin, PerCP-peridinin chlorophyll, and APC-allophycocyanin

Cytokine Analysis

Splenocytes obtained from each mouse were cultured in a 24-well plate at a concentration of $4x10^6$/mL, with each mouse sample seeded into two wells: one non-stimulated (cells + CM) and one stimulated by ConA (cells + ConA in CM). Each well had a total volume of 1mL. Then plates were incubated for 48 hours at 37°C and 5% CO_2, after which they were centrifuged for 10 mins at 1200 rpm at 4°C and their supernatants were collected. The supernatant was then collected in 500 and 250µL aliquots and stored at – 80°C, to be analyzed later for cytokine levels.

Cytokine quantification was completed using the CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA) to detect the cytokines of interests IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α by the flow cytometry. Lyophilized mouse cytokine standards were reconstituted with 2.0 mL of assay diluent and followed by a serial dilution that was performed in a ratio of 1:2 to a final dilution of 1:256, to draw calibration curves. The total bead capture solution was prepared by adding 10 µL of each cytokine into a single tube labeled "mixed capture beads". Then the 50 µL of capture beads were added to each assay tube with the addition of 50 µL of the sample, along with 50µL of the fluorochrome PE. The samples were then incubated for 2 hours at room temperature (20°C) in the dark. Samples were acquired with the Accuri C6 Plus flow cytometer and analyzed by CBA Analysis software (BD Biosciences).

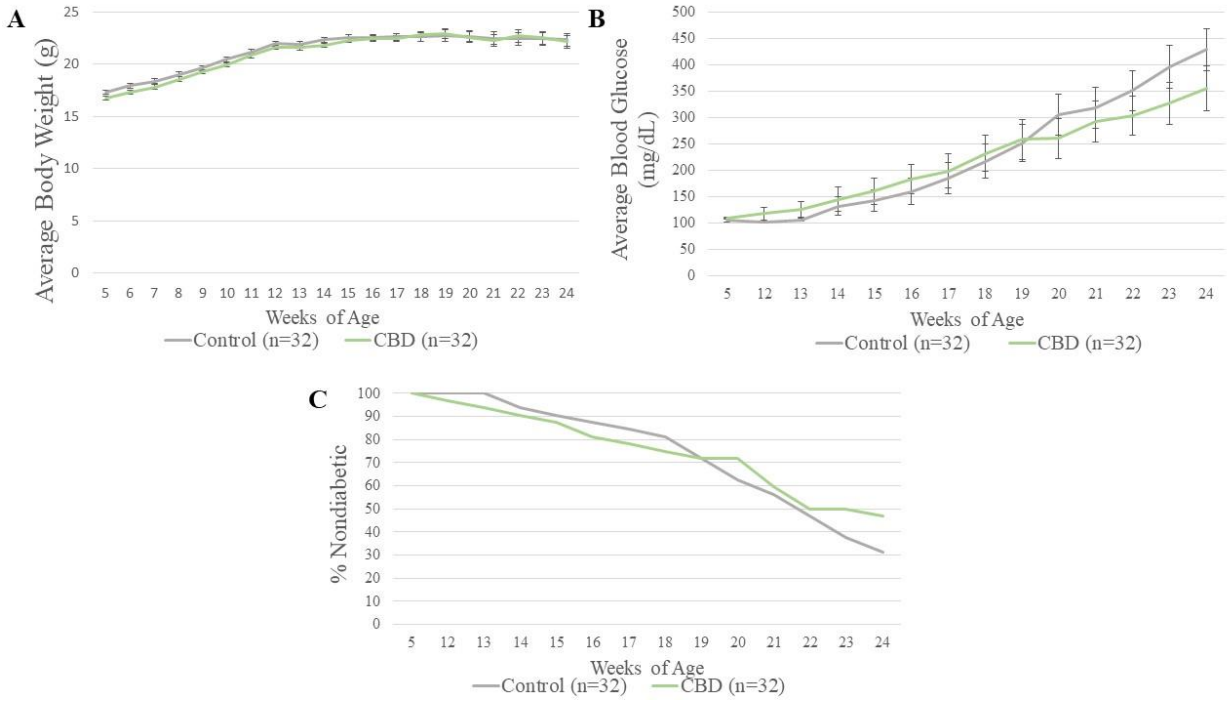
Chapter 4: Results

In vivo Studies on 5 mg/kg CBD Treatment in NOD Model of T1D: Diabetes Incidence, Glycemia and Body Weight

NOD mice were treated with 5 mg/kg CBD or PBS IP injections for six weeks, starting at 5-6 weeks of age, after which their body weights and BG levels were measured weekly until the mice were 24 weeks of age. There were no significant differences between the body weights of the control and treatment mice, both remained similar from 5 to 24 weeks of age (Figure 5a). Glycemia levels of the control group and NOD CBD treatment group (NOD-5mgCBD) rose at about the same rate throughout the duration of the experiment until about 19 weeks of age, when control mice began to have higher glucose levels than the NOD-5mg CBD treatment group. At the endpoint, glycemia levels of the control group averaged 428±39 mg/dL (*Mean* ± *SD*) while the NOD-5mgCBD treatment group averaged 355±41 mg/dL (*Mean*± *SD*). However, the statistical significance by using MANOVA was not obtained (Figure 5b). BG levels were further analyzed selectively from 19 to 24 weeks of age to test for significance between groups, as the control group began to have higher BG levels at 19 weeks of age. However, no statistical significance was obtained (Figure 5b). CBD treatment in a dose of 5 mg/kg did not prevent T1D development, nor decreased the frequency of overall diabetic cases in comparison to controls (Figure 5c).

Figure 5

Body weights (A), blood glucose levels (B), and incidence of T1D (C) of female NOD mice treated with 5 mg/kg CBD from 5-6 weeks of age to 24 weeks of age.



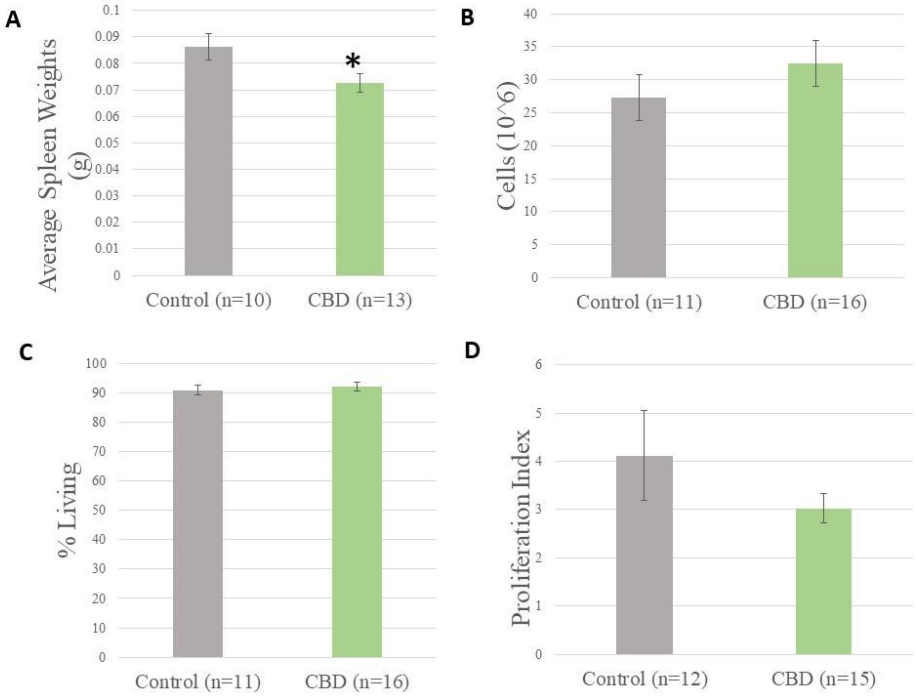
Note. Initial body weights and blood glucose were taken the first day of 5 mg/kg treatment and then monitored weekly from 12 to 24 weeks of age. Incidence of T1D is presented as a percentage of nondiabetic (diabetes-free) mice. Diabetes onset was defined by the first of two consecutive glucose readings of ≥ 250 mg/dL. Values are shown as means ±SEM. No statistical significance was evident (MANOVA: A-B, Survival analysis: C).

Ex vivo Immunological Studies in NOD-5mgCBD Model at Early Timepoint: 11 Weeks of Age

To analyze the immunological effects of CBD on NOD mice before the onset of T1D, mice were sacrificed at 11 weeks of age, following the sixth week of CBD injections. There was found a significant difference between the average spleen weights of the control group compared to the CBD treatment group (p=0.0378) (Figure 6a). However, cell counts obtained from single-cell suspensions showed no statistical significance between the control and CBD treatment group (Figure 6b). Cell viability was also no different between the two groups (Figure 6c). Whereas T cell proliferation index in CBD-treated mice did exhibit a trend of decrease, it did not reach a statistical significance (Figure 6d). Flow cytometry did not show any significance in the different immune cell types between the control and CBD treatment groups (Figure 7a). Cytokine analysis showed a trend of decrease in both pro-inflammatory (TNF-α, IFN-γ, and IL-6) and anti-inflammatory cytokine IL-10, in the CBD-treated mice compared to the controls at the early time point, however, none reached statistical significance (Figure 7b).

Figure 6

Spleen weights (A), cell counts (B), cell viability (C), and T cell proliferation indices (D) obtained from the spleens of NOD mice treated with 5 mg/kg CBD for six weeks at the early endpoint of 11 weeks of age.



Note. NOD mice were treated with 5 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 11 weeks of age the spleens of the mice were removed, weighed, and the single-cell suspension were made. Spleen weights were obtained (A). Cell counts (B) and viability (C) were determined by Trypan blue exclusion tests using a hemocytometer. T cell proliferation was induced by using ConA during a 72-hour incubation period. Then, AlamarBlue reagent was added, and optical densities (OD) were determined. The proliferation index was calculated by dividing the OD of ConA-stimulated cells by the OD of non-stimulated cells (D). Values shown as means± SEM, * $p<.05$ compared to controls using Student's t-test.

Figure 7

Immunophenotypes (E) and cytokine concentrations (F) obtained from the spleens of NOD mice treated with 5 mg/kg CBD for six weeks at the early endpoint of 11 weeks of age.

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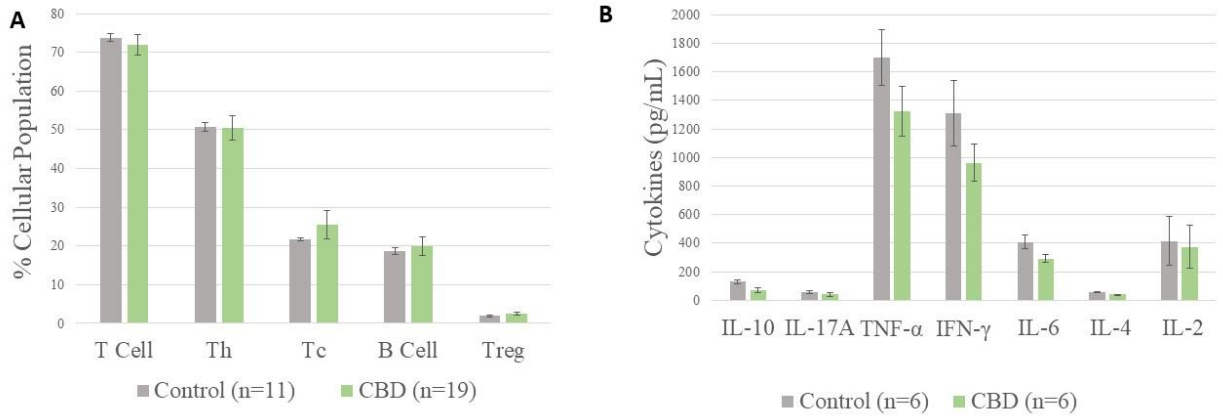


Note. NOD mice were treated with 5 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 11 weeks of age the spleens of the mice were removed and used to perform immunophenotyping on splenocytes stating with fluorochrome-conjugated monoclonal antibodies listed in Table 1 and analyzed by flow cytometry (A) CBA Mouse Th1/Th2/Th17 cytokine kit was used to determine cytokine concentrations from the supernatants of splenocytes stimulated by ConA during a 48 hour incubation period (B). Values shown as means ± SEM, * $p<.05$ compared to controls using Student's t-test.

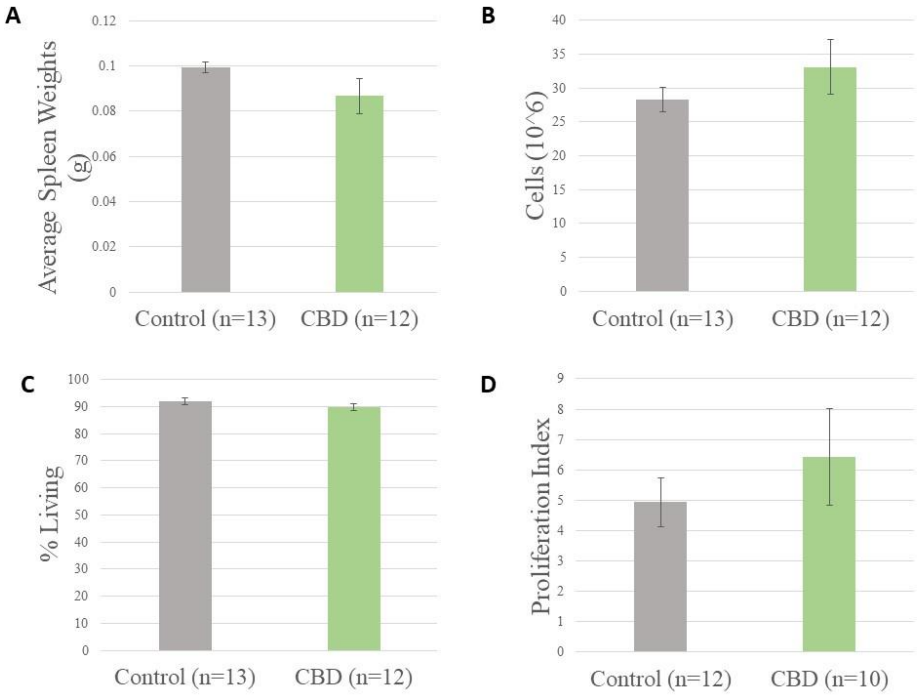
***Ex vivo* Immunological Studies in NOD-5mgCBD Model at End Timepoint: 24 Weeks of Age**

Ex-vivo data were collected at the endpoint of 24 weeks of age. Spleens were dissected and weighed, with no statistical significance found between the control and CBD treatment

groups (Figure 8a). The spleens single-cell suspensions were made, and cell counts, and viability were measured. There was no statistical significance found between the cell counts or viability of the control and CBD treatment groups (Figure 8b and Figure 8c, respectively). T cell proliferation indices did not vary significantly between the control and CBD treatment groups (Figure 8d). Flow cytometry did not show a difference between the types of immune cells found in the spleen (Figure 9a). Cytokine analysis revealed that there was no statistical difference found between the control and CBD-treated groups (Figure 9b).

Figure 8

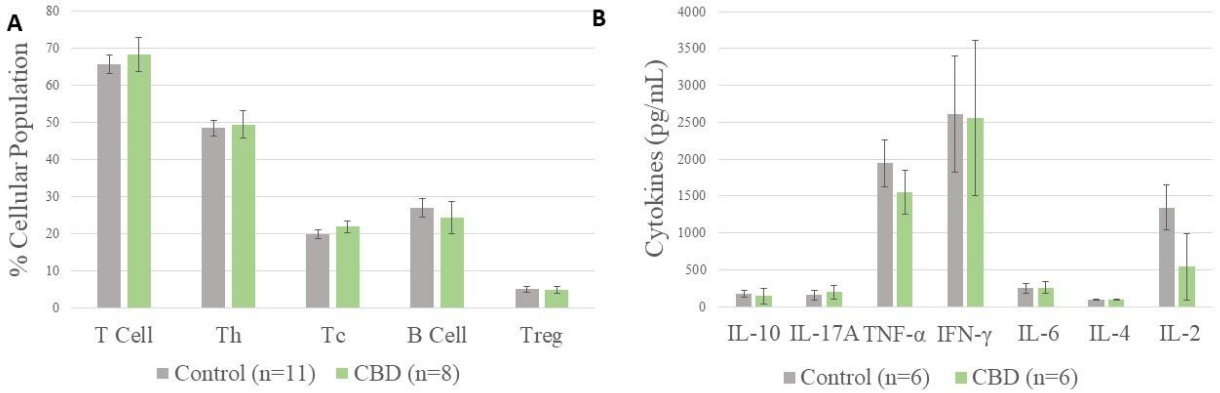
Spleen weights (A), cell counts (B), cell viability (C), and T cell proliferation indices (D)obtained from the spleens of NOD mice treated with 5 mg/kg CBD for six weeks and sacrificed at 24 weeks of age.



Note. NOD mice were treated with 5 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 24 weeks of age the spleens of the mice were removed, weighed, and the single-cell suspension were made. Refer to note from Figure 6 (A-D). Values shown as means ± SEM. No statistical significance was evident (Student's t-test).

Figure 9

Immunophenotypes (A) and cytokine concentrations (B) obtained from the spleens of NOD mice treated with 5 mg/kg CBD for six weeks and sacrificed at 24 weeks of age.



Note. NOD mice were treated with 5 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 24 weeks of age the spleens of the mice were removed, weighed, and the single-cell suspension were made. Refer to note from Figure 7 (A-B). Values shown as means ± SEM. No statistical significance was evident (Student's t-test).

In vivo Studies on B6-5mgCBD Model of T1D: Diabetes Incidence, Glycemia and Body Weight

Male B6 mice were treated for four weeks with IP injections of 5 mg/kg CBD or PBS, which were followed by IP injections of low-dose STZ in the fifth week over five consecutive days (day 0-4). Then, starting one week post-first STZ injection, body weights and BG were measured bi-weekly until day 33 post-first STZ injection (day 33). There were no significant differences between the body weights of the control and CBD-treated B6 mice, from day -28 to

the end point of day 33 (Figure 10a). CBD-treated mice exhibited persistently lower glycemia throughout the observed period, from day 8 to 33 post-first STZ injection, BG averaging 420 ±26 mg/dL (*Mean* ± *SD*) for the control mice, and 366±19 mg/dL (*Mean* ± *SD*) for the CBD treatment mice at the endpoint (Figure 10b). However, a statistically significant difference was not obtained, even when comparing groups specifically from day 11 to day 33 post-STZ, when the difference between BG levels seemed the most prominent. The incidence of T1D was not significantly different between the CBD-treated and the control group (Figure 10c).

Figure 10

Body weights (A), blood glucose levels (B), and incidence of T1D (C) of male B6 mice treated with 5 mg/kg CBD for 4 weeks prior the STZ induction of T1D.

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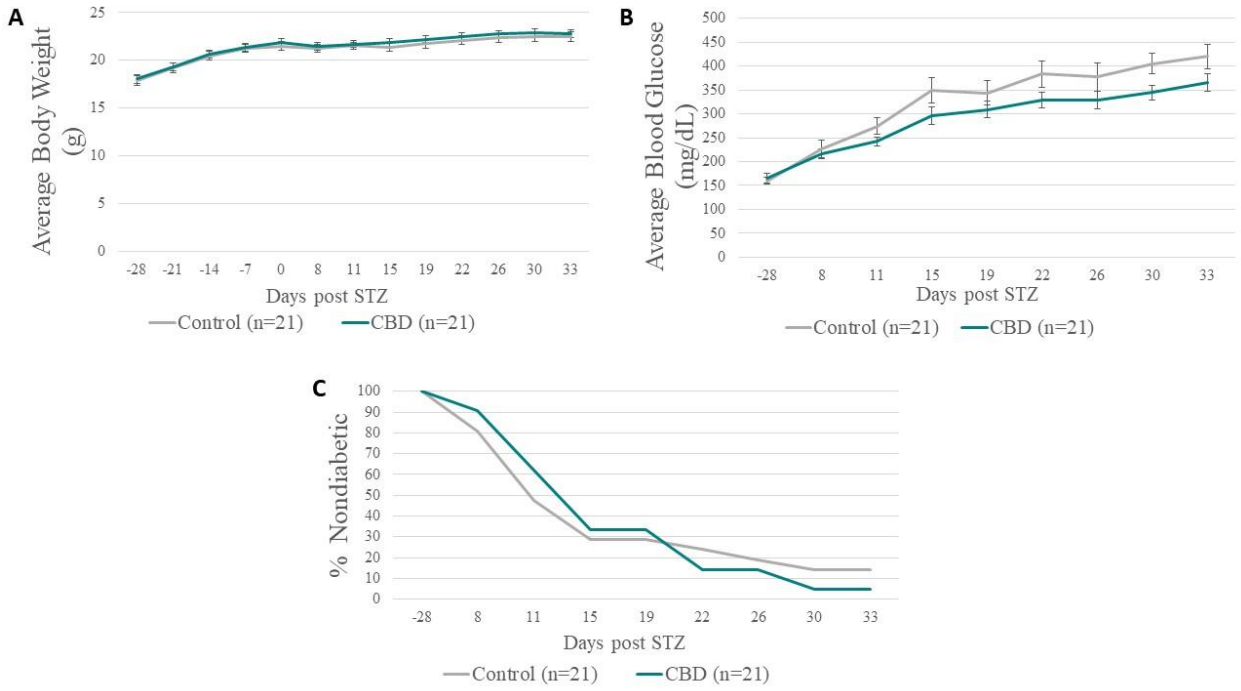


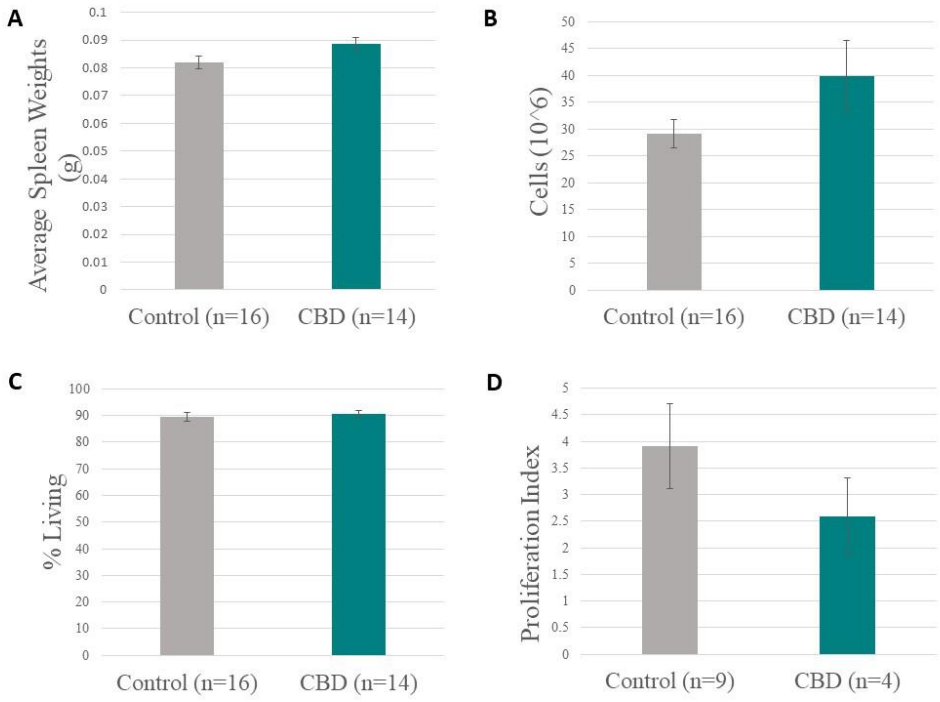
Note. Initial body weights and blood glucose were taken the first day of 5 mg/kg treatment (day -28), and then bi-weekly one week after the first STZ injection (day 8). Incidence of T1D is presented as a percentage of nondiabetic (diabetes-free) mice. Diabetes onset was defined by the first of two consecutive glucose readings of ≥ 250mg/dL. Values are shown as means ±SEM. No statistical significance was evident (MANOVA: A-B, Survival analysis: C).

Ex vivo Immunological Studies in the B6-5mgCBD Model of T1D: Early Timepoint - 11 Days Post-first STZ

Ex vivo data was collected at the early time point post first STZ injection (day 11) to assess the effects of CBD on immunological parameters in the beginning of T1D development in B6 mouse model. Spleen weights were obtained after the mice were sacrificed and their spleens removed; there was no significant difference between the average spleen weights of the control and CBD treatment groups (Figure 11a). After the spleens were processed into single-cell suspensions, cell counts, and viability were measured; they did not differ between the control and CBD treatment groups (Figure 11b: cell counts, Figure 11c: viability). Due to technical difficulties, resulting in a low number of samples collected for T cell proliferation assays, the data for both early (day 11) and late (day 33) time points were combined for analysis. T cell proliferation indices did not vary significantly between the control and CBD treatment group (Figure 11d). Immunophenotyping via flow cytometry did not reveal a significant difference between the immune cell types in the spleens of control mice and CBD-treated mice (Figure 12a). Interestingly, cytokine analysis showed a significant decrease in the pro-inflammatory cytokines, TNF-α, INF-γ, and IL-6 levels in the CBD-treated mice compared to the control mice, without differences in anti-inflammatory cytokines (IL-4 and IL-10) (Figure 12b).

Figure 11

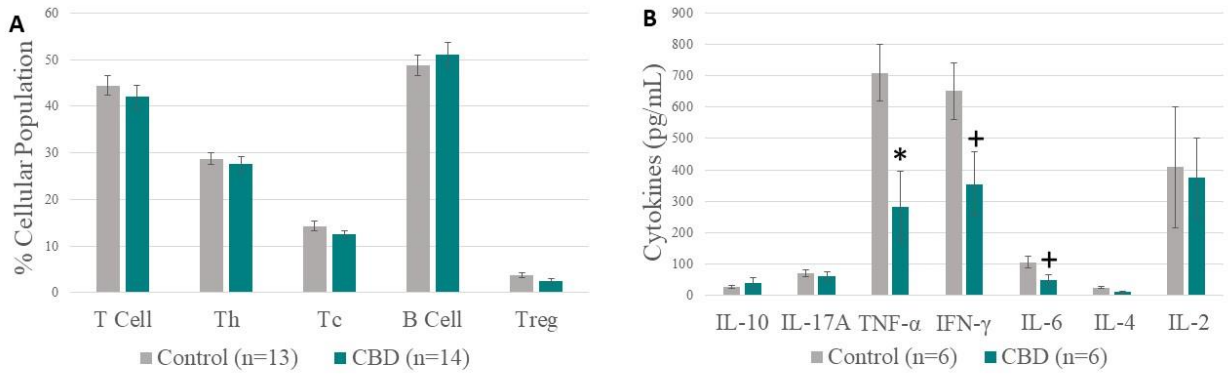
Spleen weights (A), cell counts (B), cell viability (C), and T cell proliferation indices (D) of male B6 mice treated with 5 mg/kg CBD for 4 weeks prior the STZ induction of T1D; early time point – day 11 post STZ.



Note. B6 mice were treated with 5 mg/kg CBD for four weeks, starting at 5-6 weeks of age. After the fourth week of CBD injections B6 mice received STZ injections. They were then sacrificed at an early time point of 11 days post first STZ injection, where their spleens were removed, weighed, and the single-cell suspension made. Refer to note from Figure 6 (A-D). Values shown as means ± SEM, * $p<.05$ and +$p=0.05$ compared to controls using Student's t-test.

Figure 12

Immunophenotypes (A) and cytokine concentrations (B) of male B6 mice treated with 5 mg/kg CBD for 4 weeks prior the STZ induction of T1D; early time point – day 11 post STZ.



Note. B6 mice were treated with 5 mg/kg CBD for four weeks, starting at 5-6 weeks of age. After the fourth week of CBD injections B6 mice received STZ injections. They were then sacrificed at an early time point of 11 days post first STZ injection, where their spleens were removed, weighed, and the single-cell suspension made. Refer to note from Figure 7 (A-B). Values shown as means ± SEM, * $p<.05$ and +$p=0.05$ compared to controls using Student's t-test.

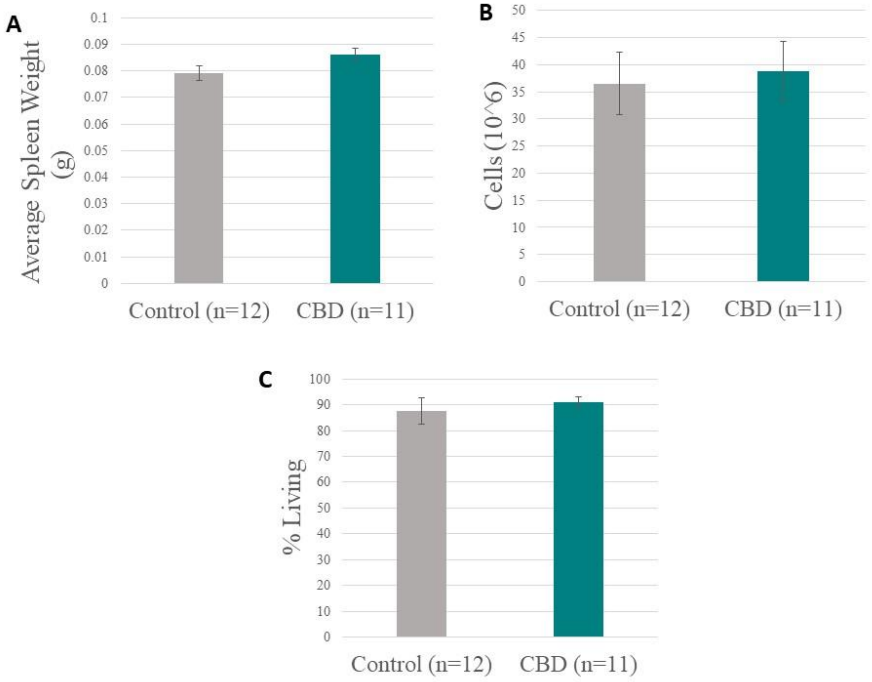
***Ex vivo* Immunological Studies in the B6-5mgCBD Model of T1D: End Point - 33 Days Post-first STZ**

There were no differences found in the spleen weights (Figure 13a), cell counts (Figure 13b), and viability (Figure13c) of splenocytes obtained from CBD-treated and control mice at the end of the experimental period, on day 33 post-first STZ injection. For T cell proliferation data please refer to Figure 9d, as data for both early and end time points were combined due to small sample size. Flow cytometry did not show a significant difference between the immune cell types of the control and CBD treatment group (Figure 13d). Cytokine acquisition of samples

obtained from CBD-treated mice did not show a significant decrease of pro-inflammatory cytokines, observed at the early time point, besides a trend of decreased INF-γ (Figure 14a). There was no difference seen in the anti-inflammatory cytokine levels, IL-4 and IL-10, between the two groups (Figure 14b).

Figure 13

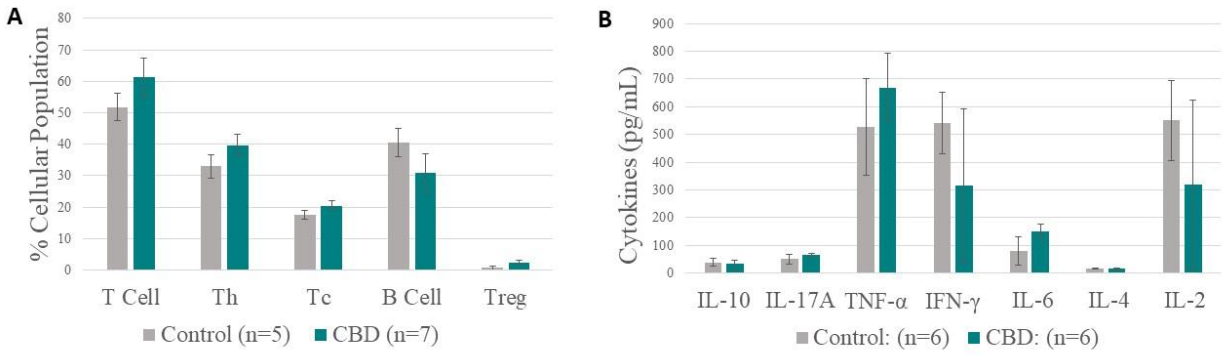
Spleen weights (A), cell counts (B), and cell viability (C) of male B6 mice treated with 5 mg/kg CBD for 4 weeks prior the STZ induction of T1D; endpoint – day 33 post STZ.



Note. B6 mice were treated with 5 mg/kg CBD for four weeks, starting at 5-6 weeks of age. After the fourth week of CBD injections B6 mice received one week of STZ injections. They were then sacrificed at an endpoint of 33 days post first STZ injection where their spleens were removed, weighed, and the single-cell suspension were made Refer to note from Figure 6 (A-C). Values shown as means ± SEM No statistical significance was evident (Student's t-test).

Figure 14

Immunophenotypes (A) and cytokine concentrations (B) of male B6 mice treated with 5 mg/kg CBD for 4 weeks prior the STZ induction of T1D; endpoint – day 33 post STZ.



Note. B6 mice were treated with 5 mg/kg CBD for four weeks, starting at 5-6 weeks of age. After the fourth week of CBD injections B6 mice received one week of STZ injections. They were then sacrificed at an endpoint of 33 days post first STZ injection where their spleens were removed, weighed, and the single-cell suspension were made Refer to note from Figure 7 (A-B). Values shown as means ± SEM No statistical significance was evident (Student's t-test).

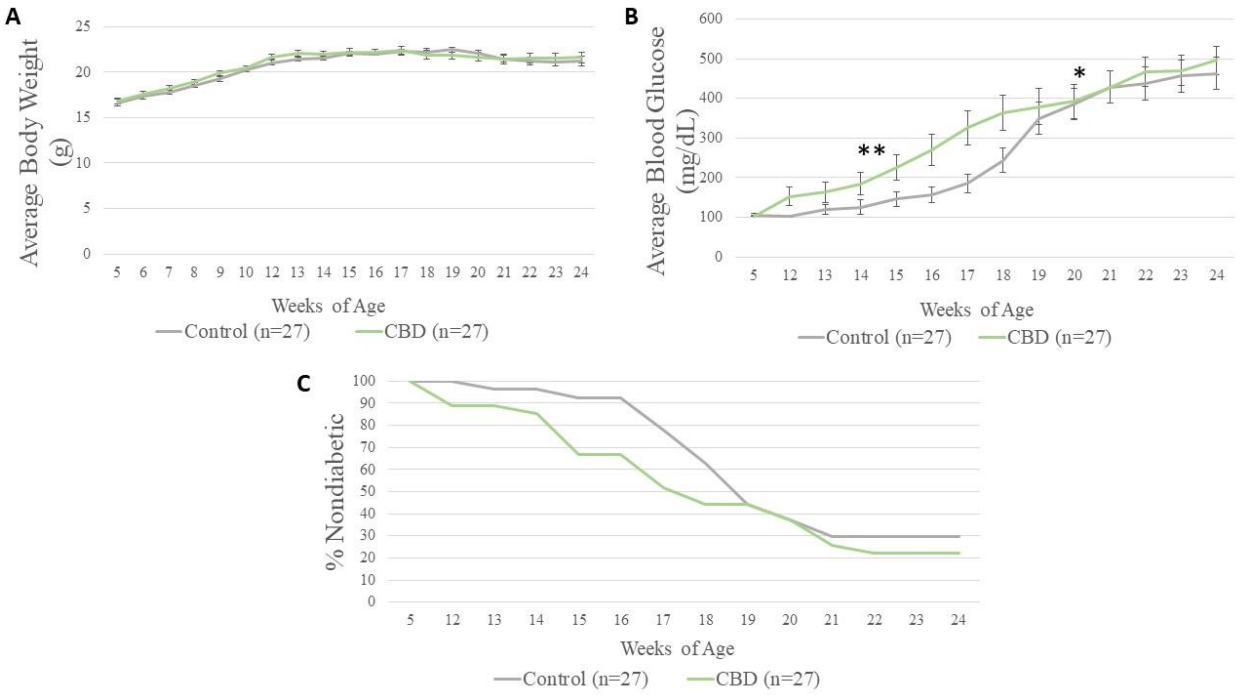
In vivo Studies on 10 mg/kg CBD Treatment in NOD Model of T1D: Diabetes Incidence, Glycemia and Body Weight

Since the prophylactic treatment with 5 mg/kg CBD in NOD mice did not result in the desired effects on T1D incidence and glycemia levels, we decided to increase the treatment dose to 10 mg/kg CBD. Thus, NOD females were treated with 10 mg/kg CBD or PBS IP injections for six weeks, starting at 5-6 weeks of age, after which their body weights and blood glucose levels were measured weekly until the mice were 24 weeks of age. The body weights of the control and CBD-treated NOD mice were comparable during the entire experimental period (Figure 15a). Interestingly, glycemia levels of CBD-treated mice were significantly higher

throughout the entire experimental period (p=0.0058), especially in the period from 12 to 19 weeks of age (p=0.002), than those of the control mice (Figure 15b). In addition, the incidence of T1D appeared to be higher in the CBD treatment group than the control group, especially in the period between 12 and 19 weeks of age, however, a significant difference was not reached. At 12 weeks of age, 100% of control mice were nondiabetic, compared to 88.9% in the CBD treatment group. By 15 weeks of age, the percentage of nondiabetic mice decreased in the CBD treatment group to 66.7%, while 92.6% were not diabetic in the control group. By 24 weeks of age, the control group had more nondiabetic mice (8 out of 27, 29.6%), while 6 out of the 27 CBD treatment mice were nondiabetic (22.2%) (Figure 15c).

Figure 15

Body weights (A), blood glucose levels (B), and incidence of T1D (C) of female NOD mice treated with 10 mg/kg CBD from 5-6 weeks of age until 24 weeks of age.



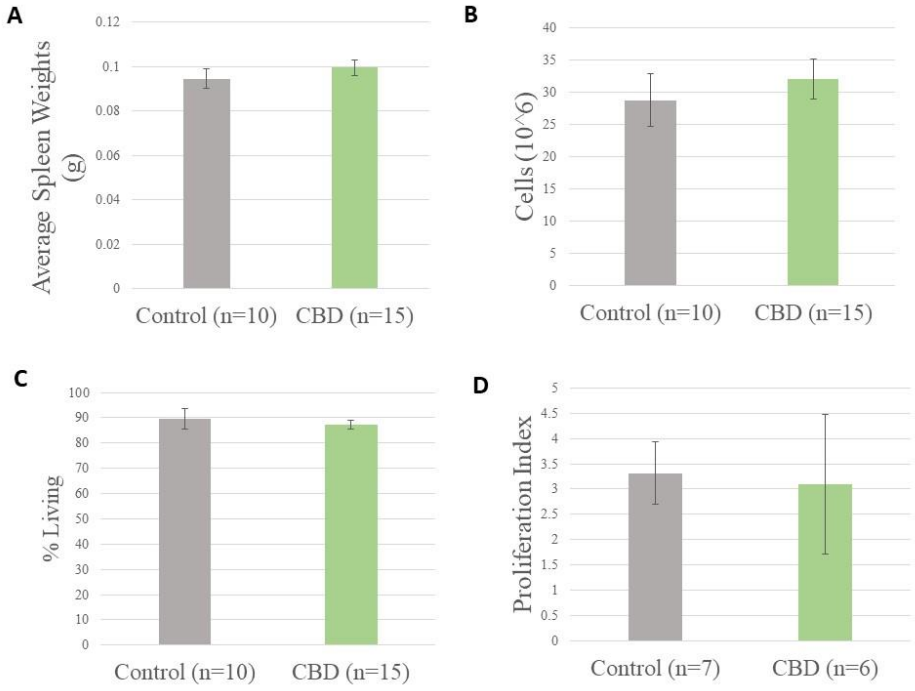
Note. Initial body weights and blood glucose measurements were taken the first day of 10 mg/kg treatment, and then monitored weekly from 12 to 24 weeks of age. Incidence of T1D is presented as a percentage of nondiabetic (diabetes-free) mice. Diabetes onset was defined by the first of two consecutive glucose readings of ≥ 250mg/dL. Values are shown as means ±SEM. * $p<.05$ compared to controls from 5-24 weeks of age, ** $p<.05$ compared to controls from 5-19 weeks of age (MANOVA: A-B, Survival analysis: C).

Ex vivo Immunological Studies in NOD-10mgCBD Model at Early Timepoint: 11 Weeks of Age

NOD mice treated with 10 mg/kg CBD IP injections for six weeks were sacrificed at 11 weeks of age; their spleens were dissected, weighed, and then used to make single-cell suspensions. Spleen weights, cell counts, and viability did not vary between the control and CBD treatment group (Figure 16a: spleen weights, Figure 16b: cell counts, Figure 16c: viability). Due to the low sample number of controls in the early time point group, T cell proliferation data for the controls from both the early time point of 11 weeks of age and the end point of 24 weeks of age were combined. T cell proliferation indices did not differ between the CBD treatment and control groups (Figure 17a). There also was no significant difference between the immune cell types of both groups (Figure 17b).

Figure 16

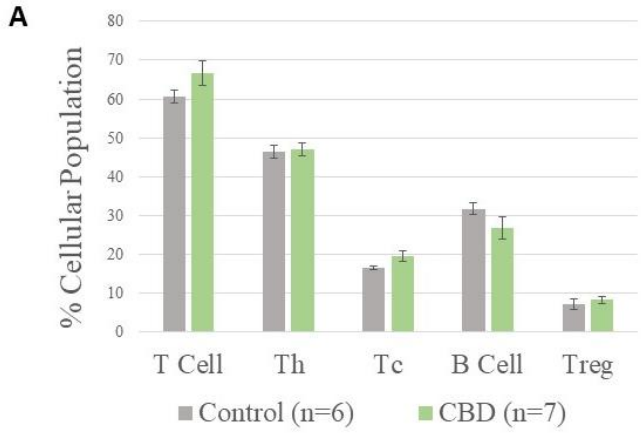
Spleen weights (A), cell counts (B), cell viability (C), and T cell proliferation indices (D) obtained from the spleens of NOD mice treated with 10 mg/kg CBD for six weeks at 11 weeks of age.



Note. NOD mice were treated with 10 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 11 weeks of age the spleens of the mice were removed, weighed, and the single-cell suspension were made. Refer to note from Figure 6 (A-D). Values shown as means ± SEM. No statistical significance was evident (Student's t-test).

Figure 17

Immunophenotypes (A) obtained from the spleens of NOD mice treated with 10 mg/kg CBD for six weeks at 11 weeks of age.



Note. NOD mice were treated with 10 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 11 weeks of age the spleens of the mice were removed, weighed, and the single-cell suspension were made. Refer to note from Figure 7 (A). Values shown as means ± SEM. No statistical significance was evident (Student's t-test).

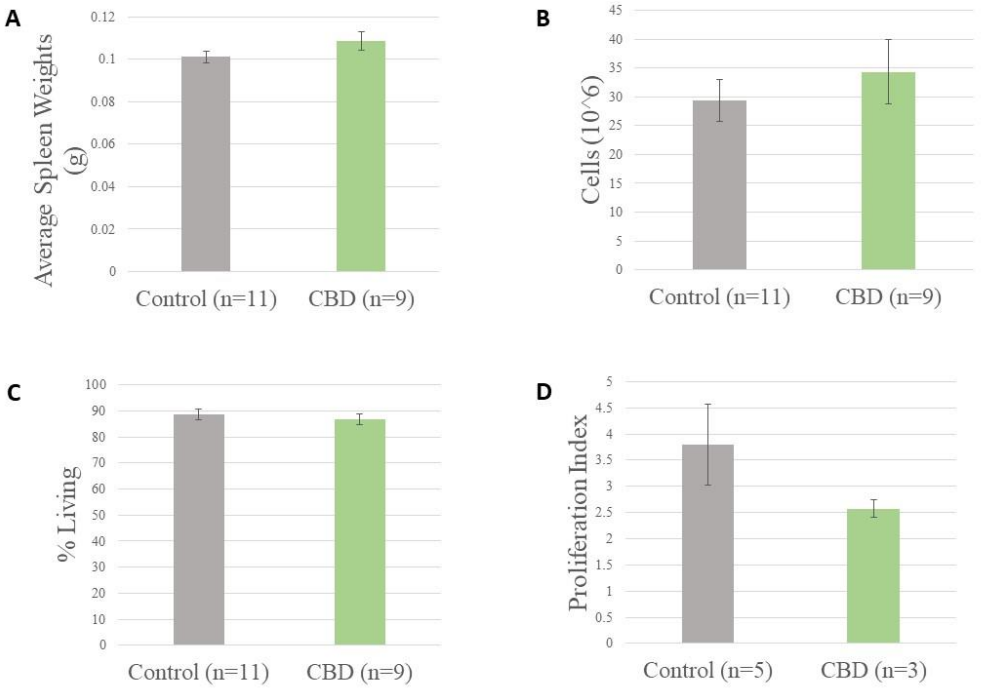
***Ex vivo* Immunological Studies in NOD-10mgCBD Model at the Endpoint: 24 Weeks of Age**

NOD mice treated with 10 mg/kg CBD injections for six weeks, and then euthanized at 24 weeks of age, had their spleens removed for weight measurements and to make single-cell suspensions. The spleen weights, cell counts, and viability did not differ between the control and 10 mg/kg CBD-treated mice (Figure 18a: spleen weights, Figure 18b: cell counts, Figure 18c: viability). T cell proliferation indices of the control mice did not significantly differ from those of the CBD-treated NOD mice (Figure 18d). Flow cytometry did not show any significant

difference between the different immune cell types of the control and CBD treatment groups (Figure 19a).

Figure 18

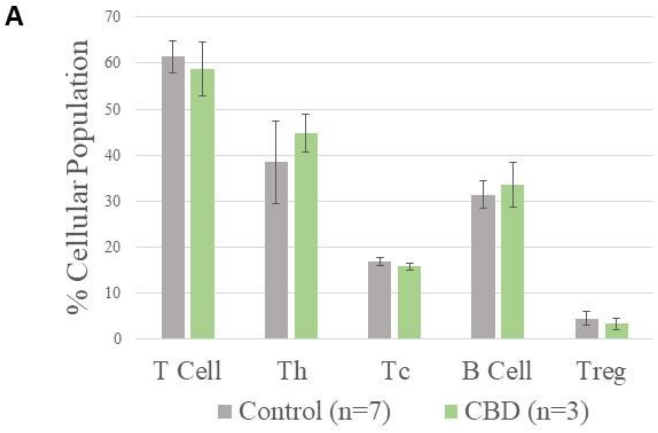
Spleen weights (A), cell counts (B), cell viability (C), and T cell proliferation indices (D obtained from the spleens of NOD mice treated with 10 mg/kg CBD for six weeks and sacrificed at 24 weeks of age.



Note. NOD mice were treated with 10 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 24 weeks of age the spleens of the mice were removed, weighed, and the single-cell suspension were made. Refer to note from Figure 6 (A-F). Values shown as means ± SEM. No statistical significance was evident (Student's t-test).

Figure 19

Immunophenotypes (A) obtained from the spleens of NOD mice treated with 10 mg/kg CBD for six weeks at 24 weeks of age.



Note. NOD mice were treated with 10 mg/kg CBD for six weeks, starting at 5-6 weeks of age. At 24 weeks of age the spleens of the mice were removed, weighed, and the single-cell suspension were made. Refer to note from Figure 7(A). Values shown as means ± SEM. No statistical significance was evident (Student's t-test).

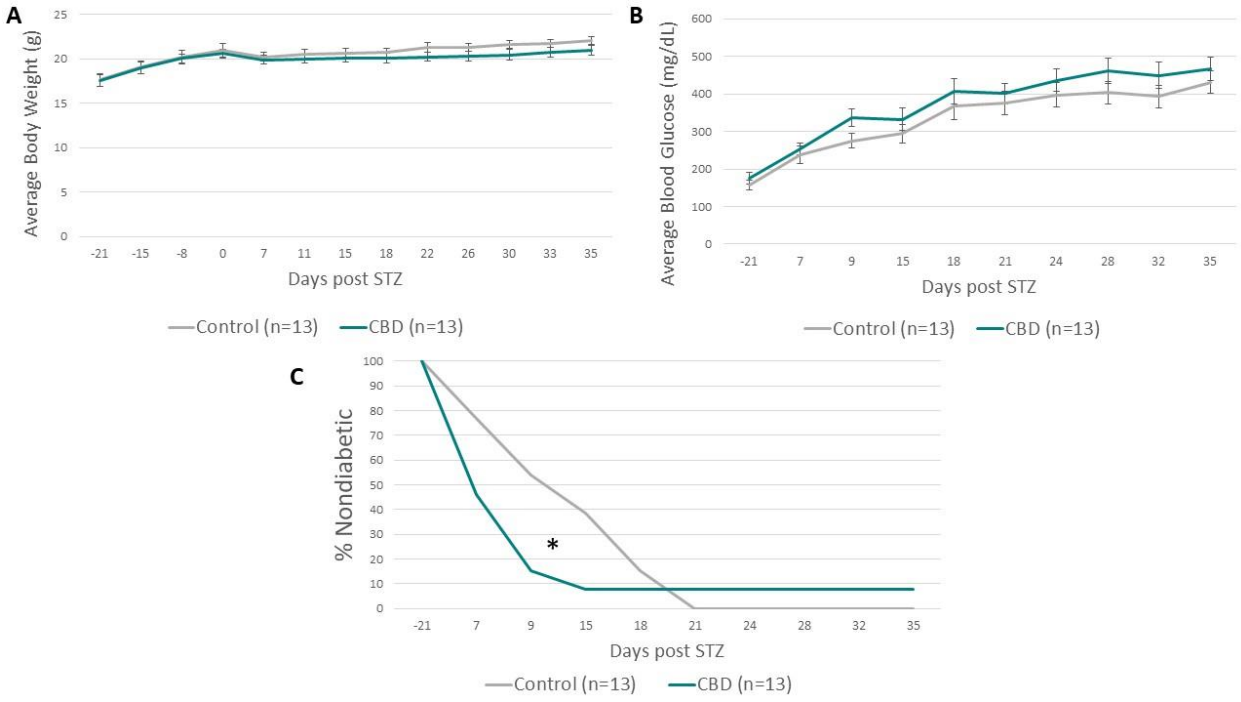
***In vivo* Studies in B6-5mgCBD Model Injected Simultaneously with CBD and STZ: Diabetes Incidence, Glycemia and Body Weight**

Since CBD treatment might interact with STZ's effects in a chemically induced model of T1D, we decided to extend the CBD treatment during the STZ administration in B6 mice. Thus, male B6 mice were treated for four weeks with IP injections of 5 mg/kg CBD or PBS. During the fourth week of CBD/PBS administration, mice simultaneously received low-dose STZ injections over five consecutive days (day 0-4). The same parameters, as described previously, were followed in mice for 35 days post-first STZ injection in this experiment. The body weight did not differ in the control and CBD treatment groups (Figure 20a). Interestingly, glycemia levels of the

CBD treatment group appeared higher than in the controls throughout the entire experimental period; CBD treatment mice had blood glucose levels averaging 467 ± 32 mg/dL (*Mean* ± *SD*), while control mice had an average of 431 ± 30 mg/dL (*Mean* ± *SD*) at the endpoint (day 35) (Figure 20b). However, no significant difference was observed between the CBD treatment and control mice. In addition, there was a significant increase in the incidence of T1D in the CBD-treated group compared to the control group (p=0.0141). On day 7 post-first STZ injection, there were 53.8% diabetic mice in the CBD treatment group compared to 23.1% in the control group. By day 9 the percentage of diabetic mice increased to 84.6% in the CBD-treated group, and to 46.2% in the control group. Starting on day 15, the CBD-treated group had 92.5% of diabetic mice, which remained diabetic till the endpoint, while the control group had 61.5% diabetic mice, which increased to 100% by day 35 (Figure 20c).

Figure 20

Body weights (A), blood glucose levels (B), and incidence of T1D (C) of male B6 mice treated with 5 mg/kg CBD for 4 weeks and STZ in the last week of CBD treatment.



Note. Initial body weights and blood glucose measurements were taken the first day of 5 mg/kg treatment (day -21), and then bi-weekly from one week after the first STZ injection (day 8). Incidence of T1D is presented as a percentage of nondiabetic (diabetes-free) mice. Diabetes onset was defined by the first of two consecutive glucose readings of ≥ 250mg/dL. Values are shown as means ±SEM. * p<.05 compared to controls (MANOVA: A-B, Survival analysis: C).

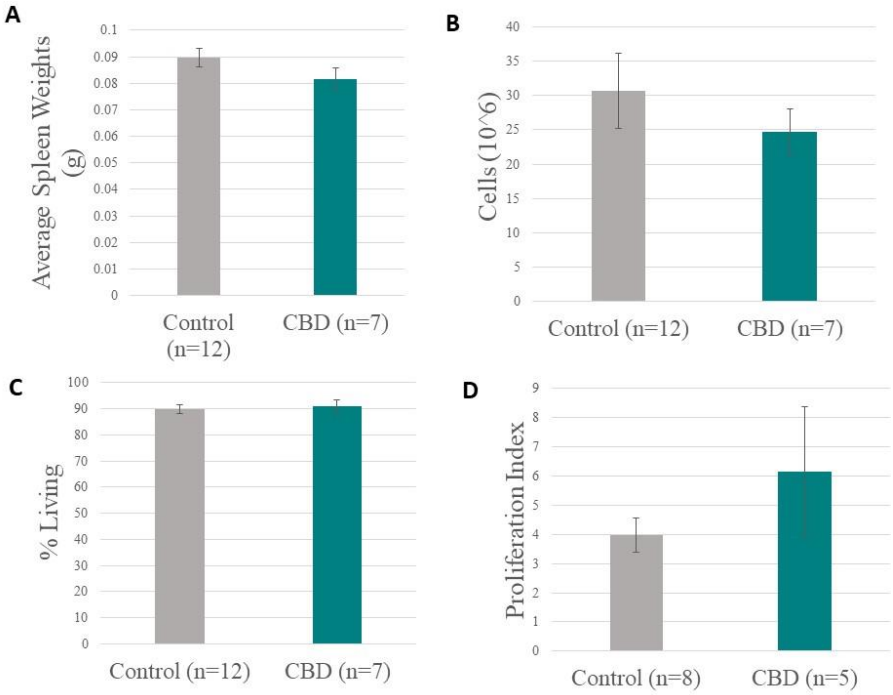
***Ex vivo* Immunological Studies in B6-5mgCBD T1D Model with Simultaneous Treatments by CBD and STZ: End Point - 35 Days Post-first STZ Injection**

B6 mice treated with 5 mg/kg CBD or PBS for four weeks and given STZ during the last week of CBD/PBS treatment, were then sacrificed on day 35 post-first STZ injection, their

spleens dissected, weighed, and used for a preparation of single-cell suspensions. The spleen weights, cell counts, and viability did not differ between the control and CBD treatment groups (Figure 21a: spleen weights, Figure 21b: cell counts, Figure 21c: viability). There were no significant differences between the proliferation indices (Figure 21d), or the immune cell types (Figure 22a) in the CBD-treated and control groups. Cytokine analysis showed no significant differences in either pro- or anti-inflammatory cytokine concentrations between the control and CBD treatment mice (Figure 22b).

Figure 21

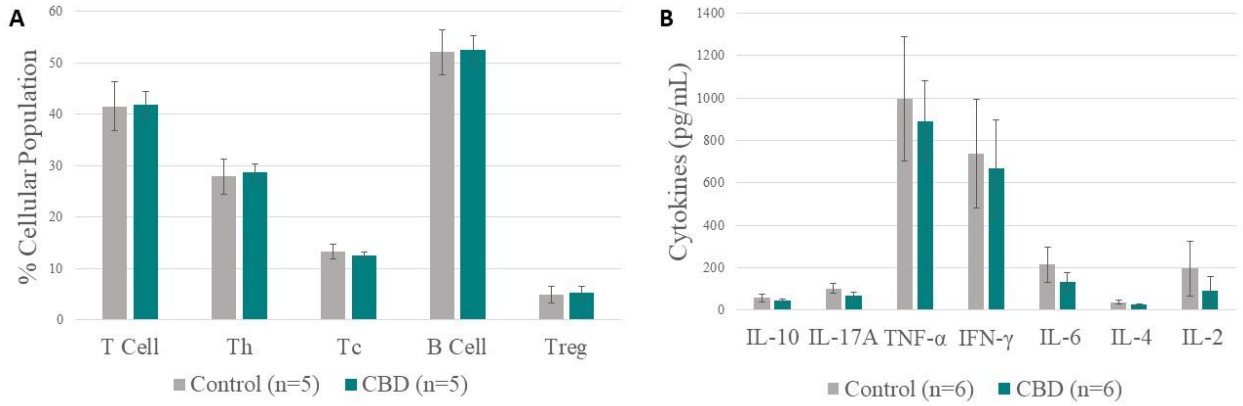
Spleen weights (A), cell counts (B), cell viability (C), and T cell proliferation indices (D) of male B6 mice treated simultaneously with 5 mg/kg CBD and STZ sacrificed at the end of the experiment, on day 35 post first STZ injection.



Note. B6 mice were treated with 5 mg/kg CBD for four weeks, starting at 5-6 weeks of age. Mice were also given STZ during the final week of CBD injections. They were then sacrificed at the endpoint, day 35 post-first STZ injection, their spleens removed, weighed, and the single-cell suspension made. Refer to note from Figure 6 (A-D). Values are shown as means ± SEM. No statistical significance was evident (Student's t-test).

Figure 22

Immunophenotypes (A) and cytokine concentrations (B) of male B6 mice treated simultaneously with 5 mg/kg CBD and STZ sacrificed at the end of the experiment, on day 35 post first STZ injection.



Note. B6 mice were treated with 5 mg/kg CBD for four weeks, starting at 5-6 weeks of age. Mice were also given STZ during the final week of CBD injections They were then sacrificed at the endpoint, day 35 post-first STZ injection, their spleens removed, weighed, and the single-cell suspension made. Refer to note from Figure 7 (A-B). Values are shown as means ± SEM. No statistical significance was evident (Student's t-test).

In summary, 5 mg/kg water-soluble CBD treatment in the NOD model did not lead to a delay in T1D development nor reduce hyperglycemia. At both early and late time points, CBD treatment at the dosage of 5 mg/kg dosage, did not affect the overall splenic parameters studied in the NOD model such as: spleen weights, cell counts, viability, the immunophenotypes, nor the cytokine levels. In the B6 model, when mice were given STZ following 5 mg/kg CBD administration, they did not show CBD influencing T1D incidence. However, the mice did exhibit a trend of persistently lower glycemic values. At an early timepoint in disease development CBD treatment significantly reduced the pro-inflammatory cytokines TNF-α, IFN-

γ, and IL-6 in the B6 mice. However, this effect was not noted at the study's end point. The other studied spleen parameters showed no beneficial effects of CBD treatment in our B6 model receiving 5 mg/kg CBD. Based on initial data collected for the NOD model, we also decided to investigate treatment with the dosage of 10 mg/kg of CBD which led to a significant increase in hyperglycemia and exhibited a trend of increased T1D incidence. However, 10 mg/kg CBD administration did not affect any of the splenic parameters studied at both the early and late time points. Further along in our study we decided to investigate the possible interaction of water-soluble CBD affecting the potency of STZ in our B6 model. Therefore, we administered STZ simultaneously during the final week of CBD treatment. Our data showed that when STZ was administered at the same time as CBD, it led to a significant increase in T1D incidence along with a trend of persistently higher glucose levels. Both the early and late timepoint assessments of immune cell parameters did not show a beneficial effect of 10 mg/kg CBD in the NOD model.

Chapter 5: Discussion

T1D is a chronic inflammatory autoimmune disease in which autoreactive T cells attack the insulin-producing beta cells of the pancreas. Insulin injections have been the primary treatment option for T1D; however, insulin is not a prophylactic treatment option, and has a high financial burden. Potential preventative interventions have been a Holy Grail of T1D treatment. Thus, a search for a medication that might act in an anti-inflammatory fashion, reducing or suppressing the activity of autoreactive T cells, has been imperative. Marijuana plant, and its derivatives, THC and CBD have been known for a while for their anti-inflammatory and antioxidant properties (Atalay et al., 2019). However, CBD, known as a non-psychotropic compound, gained in popularity since 2018, when the Farm Bill was signed into law that removed hemp and cannabis from the Controlled Substance Act, if they contain < 0.3% THC (Abernethy, 2019).

Previous research has centered around CBD in its hydrophobic fat-soluble form which therapeutic applications are greatly limited by its strong lipophilicity and poor oral bioavailability. Recent market has experienced a surge in water-soluble formulations of CBD, believed to be able to overcome oil-based CBD's limitations, and boost its bioavailability, prolong half-life, and improve efficacy (Zhang et al., 2023) However, limited data exists on the effects of these water-soluble CBD formulations and their improved clinical effects. One recent study showed that water-soluble CBD treatment did not significantly affect overall osteoarthritis in dogs when compared to CBD oil (Sims et al., 2022). Numerous studies, which investigated the effects of oil-based CBD in autoimmune pathologies (Kozela et al, 2011; Kozela et al., 2013; Elliot et al., 2018; Rahimi et al., 2015; Mecha et al., 2013; Malfait et al, 2000), even in experimental models of T1D (Weiss et al., 2006; Weiss et al., 2008; González-Mariscal et al.,

2022), generally observed beneficial effects of CBD treatments. However, water-soluble CBD's effects on T1D have never been studied so far. In addition, CBD action has never been studied in a low-dose STZ mouse model of T1D. Therefore, we aimed to investigate the effects of water-soluble CBD on T1D development in two mouse models, the NOD and low-dose-STZ-induced B6 model, in which we anticipated to observe beneficial effects on delayed disease development, lowered glycemia levels, and positively impacted immunological parameters.

Firstly, we investigated 6 week-long prophylactic IP treatment of prediabetic NOD mice with 5 mg/kg water-soluble CBD. This dose and route were chosen as previous studies, which used the oil-based CBD delivered IP in the same dose, showed beneficial effects on T1D development in NOD mice (Weiss et al., 2006; Weiss et al., 2008). Our results showed that treatment with 5 mg/kg water-soluble CBD did not prevent disease development nor significantly reduced hyperglycemia, although a trend of lower glycemia was observed between 19 and 24 weeks in treated NOD mice. Original Weiss and colleagues' study (2006) has been widely cited, not only by T1D, but even by T2D researchers, as a staple study that described anti-diabetic effect of CBD. However, looking closely, there are several crucial limitations of Weiss's published experimental design: NOD mice introduced into study exhibited overtly wide age-range (they were 6-12-wk-old), CBD treatment regimen differed in the CBD-treated group of mice (10 to 20 injections over the two or four weeks; thus, some mice received 2-week-long, and the others 4-week-long treatment), and even the length of the study was not clearly defined, as mice were followed up to 20 weeks, which was not disclosed whether this referred to mouse age or the length of time post initiation of treatment (Weiss et al., 2006). Regardless of the criticism, this study remains the standard for conclusion regarding the beneficial effects of oil-based CBD on T1D development in the NOD mouse model (Weiss et al., 2006).

Although even second Weiss's group paper suggest beneficial effects of 5 mg/kg oil-based CBD treatment in lowering T1D incidence in NOD mice, there remains an inconsistency in chosen experimental subjects; 11-14-week-old prediabetic female NOD mice with both impaired and normal glucose tolerance were grouped into vehicle-control and CBD-treated groups, without disclosure about the number of each per group, which might heavily impact the observed results (Weiss et al., 2008). In addition, urine glucose measurements, not blood glucose level, were used as a main parameter for confirmation of disease occurrence in both Weiss's manuscripts, without notification of a glucose level considered as positive. Thus, a firm conclusion on CBD's hypoglycemic potential cannot be drawn from these studies (Weiss 2006 and 2008).

In both studies conducted by Weiss et al., (Weiss et al., 2006; Weiss et al, 2008) the beneficial effects of oil-based 5 mg/kg CBD treatment on delaying T1D incidence, was based on urine glucose monitoring for diabetes occurrence, which might be misleading. According to the Mayo Clinic and the American Diabetes Association, diagnosis of T1D is based on plasma glucose levels or hemoglobin A1C (Mayo Clinic, 2024; American Diabetes Association, 2016; ElSayed et al., 2022). A review manuscript in 2000 reported six studies that found that blood glucose measurements were preferred over urine glucose measurements due to the low sensitivity found in urine glucose testing (Engelgau, 2000). Another review described five studies in which the urine glucose testing exhibited a wide range of sensitivity (Wei & Teece, 2006). Both reviews support the reasoning of blood glucose measurements being more reliable and preferred in assessing disease development in comparison to urine glucose monitoring. Therefore, our data on disease occurrence, based on collection of blood glucose, seems to be more stringent in assessing the effects of CBD treatment on T1D development.

Ex-vivo studies of 5 mg/kg water-soluble CBD treatment in the NOD model, at both the early and late time points of disease development, did not show beneficial effects of treatment on cell counts, cell viability, immune cell composition, nor cytokine profiles. Even though there were significant differences in the spleen weights of CBD-treated mice and control mice, this was suspected to be the result of technical mistakes when obtaining some of the spleen weights. Although there was a trend in decreased pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-6, in the CBD treatment group at the early time points, the observed trends were not observed at the end point of the study. Previous research conducted by Weiss et al., showed that treatment with 5 mg/kg oil-based CBD in 6–12-week-old NOD mice led to a significant decrease in IFN-γ production, and a significant increase in IL-4 by splenocytes obtained from treated mice (Weiss et al., 2006). Another study, in 11-14-week-old NOD mice treated with 5 mg/kg oil-based CBD, found significantly lowered levels of the pro-inflammatory cytokines IL-6 and IL-12, and an increase in the anti-inflammatory cytokine IL-10 (Weiss et al., 2008).

Overall, numerous studies in different models of autoimmunity found reduced levels of proinflammatory cytokines post treatment with oil-based CBD (Al-Ghezi et al., 2019; Giacoppo et al., 2017; Malfait et al., 2000; Tsiogkas et al., 2024). One such study found that CBD treatment led to a decrease of IL-17 and IFN-γ, while also increasing the anti-inflammatory cytokine IL-10 (Al-Ghezi et al., 2019). Another found the CBD treatment suppressed IFN-γ and IL-17 levels (Giacoppo et al., 2017). Therefore, this led us to believe that we would see similar effects using a water-soluble form of CBD.

The basis of using the dosage of 5 mg/kg water-soluble CBD centered around the beneficial effects that were noted when using 5 mg/kg of oil-based CBD in research on T1D development (Weiss et al. 2006; Weiss et al., 2008), as well as in studies in other auto-immune

diseases such as MS and RA (Kozela et al., 2011; Malfait et al, 2000). Therefore, we expected to observe similar or better effects using the dosage of 5 mg/kg of water-soluble CBD. However, as previously stated, we did not observe a decrease in diabetes incidence nor a lowering of a pro-inflammatory response at this dosage. We even questioned whether a CBD formulation we decided to test worked at all? Did chemical manipulation with a CBD compound, in order to make it a water-soluble, diminish its efficacy? Based on a personal communication with chemistry professors at SCSU, Drs. Mike Mechelke and Nathan Brunder, it does not seem to be a case. Since results in other autoimmunities, such as MS and RA, showed that treatment with oil-based CBD dosages of 10 and 20 mg/kg effectively delayed onset of symptoms and reduced disease progression of MS (Elliott et al., 2018), and that 10 mg/kg CBD had a therapeutic effect in reducing the severity of clinical scores of arthritis (Malfait et al., 2000), we decided to test a higher dosage of 10 mg/kg of water-soluble CBD in the NOD mouse model. Completely opposite to our expectation, it is found that increasing the dosage from 5 to 10 mg/kg of CBD led to a trend of increased T1D incidence, in addition to a significant increase in hyperglycemia in treated mice. CBD-treated mice exhibited higher blood glucose levels in the period from the beginning of the experiment to 19 weeks of age, which would suggest that a higher dose of CBD potentiates the disease development. These findings are in line with observation obtained in a study conducted on the effects of 10 mg/kg on diabetic nephropathy in the STZ-induced B6 model (Carmona-Hidalgo et al., 2021), which found that CBD treatment did not prevent STZ-induced hyperglycemia, nor did it protect beta cell from damage by STZ (Carmona-Hidalgo et al., 2021). Although we did not perform cytokine analysis in NOD mice treated with 10 mg/kg CBD, due to budget and time constraints, one can speculate an increase in pro-inflammatory cytokines that promote a pro-inflammatory response with its detrimental effects on beta cells.

Also, based on Carmona-Hidalgo's results (Carmona-Hidalgo et al., 2021), it is possible that 10 mg/kg of CBD dosage was even directly toxic to beta cells, promoting beta cell apoptosis and leading to increased hyperglycemia.

It is worth noticing that our experimental design involved a relatively longer, 6-weeks-long, CBD treatment of prediabetic NOD mice. In contrast, Weiss and colleagues treated NOD mice for a period of 2- 4 weeks (Weiss et al., 2006; Weiss et al., 2008). The vast majority of studies in other auto immunities used length of 3 days (Kozela et al., 2011; Kozel et al., 2013; Rahimi et al., 2015), 5 days (Dopkins et al., 2021), and 2 weeks (Elliott et al., 2018) for their respective treatments. Thus, our prolonged CBD treatment might be a cause of not observed beneficial effects by dosage of 5 mg/kg, and detrimental effects by 10 mg/kg CBD in NOD mouse model of T1D.

Since CBD has never been studied in a low-dose STZ-induced model of autoimmune T1D, we intended to research the effects of a water-soluble CBD in this chemically initiated T1D in B6 mice. We found that water-soluble CBD treatment of 5 mg/kg did not affect T1D incidence, while a trend of lowered glycemic values was observed in B6 mice developing T1D post low-dose STZ administration. Although a recent study provided evidence that 10 mg/kg CBD treatment did not prevent high-dose STZ-induced hyperglycemia in a diabetic nephropathy model in B6 mice (Carmona-Hidalgo et al., 2021, there is no study, besides ours, that studied the effect of CBD treatment in the pre-diabetic stages of low dose STZ-induced T1D in the B6 model.

It was observed at the early time point of disease development (11 days post first STZ injection) that CBD treatment significantly reduced the pro-inflammatory cytokines TNF-α, IFN-γ, and IL-6; although, this was not noted at the end of the study when disease was fully

developed (33 days post STZ). This brings awareness to the variability in the overall immune modulating effect that long term CBD treatment might exhibit.

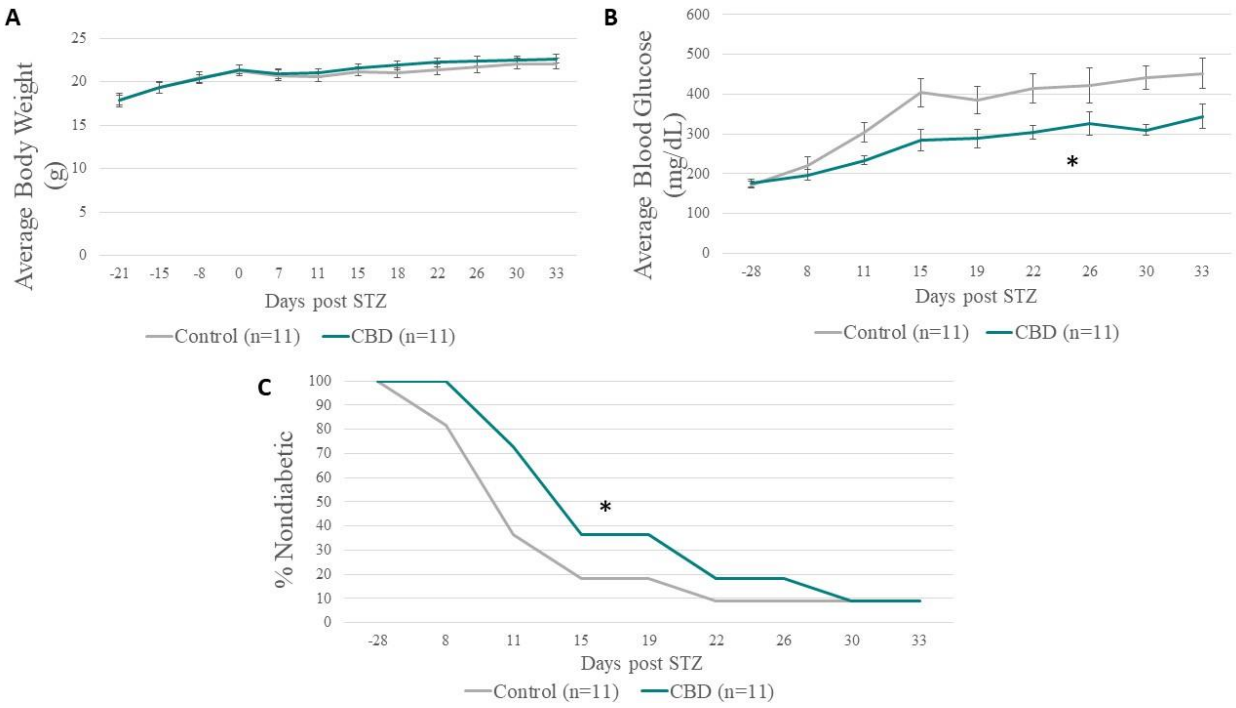
There have been many reported studies on the effects of CBD on inflammatory responses, specifically on inflammatory cytokine responses to CBD. In a systematic review on the effects of CBD on pro- and anti-inflammatory cytokines in *in-vivo* studies, it was concluded that CBD administration broadly decreased TNF-α, IL-6 and IFN-γ levels (Henshaw et al., 2021). Although, there is a gap of knowledge on the effects of CBD on cytokine levels in the low-dose STZ-induced experimental T1D model, there have been some studies performed on close analogs of CBD, such as different forms of Abn-CBD; one study showed its anti-inflammatory effects by decreasing TNF-α and IL-6 levels in acute pancreatitis in B6 mice (Li et al., 2013), while the other study described that Abn-CBD treatment led to decrease in plasma pro-inflammatory cytokines, such as TNF-α and a reduction in activation of the pro-inflammatory NF-κB pathway (González-Mariscal et al., 2022). In the same study conducted by González-Mariscal et al, it was seen that treatment with 1 mg/kg Abn-CBD reduced high-dose STZ-induced islet cells apoptosis, and protected small-size islets from damage (González-Mariscal et al., 2022).

Based on our initial data collected in STZ-induced B6 model with a small number of mice (n=11/group), CBD treatment appeared to significantly lower incidence (p= 0.0476) and hyperglycemia (p= 0.0305) (Figure 23b and Figure 23c, respectively). This data suggested a possible interaction between CBD and STZ that could reduce the potency or effectiveness of STZ to induce T1D in the B6 mice. To investigate the direct effect of CBD on STZ, another set of experiments was performed, in which we extended the CBD treatment in B6 mice during the STZ administration. Data showed that when CBD was administered simultaneously with STZ, it

led to an accelerated disease onset, and a trend of increased glycemia. These results bring to question the potential cytotoxic effects that CBD may be exhibiting on beta cells. In support of our results, it has been reported that 10 mg/kg administration of CBD led to a significant increase in blood glucose levels and decrease in insulin staining and total beta cell mass of pancreatic islets in high-dose STZ-treated B6 mice (Carmona-Hidalgo et al., 2021). Further research provided interesting results that the activation of CB1 receptors induces interaction with insulin receptors on beta cells, leading to beta cell apoptosis. In particular, activated CB1 receptors can form a heteromeric complex with insulin receptors, which inhibits insulin receptor kinase activity; inhibition occurs as the CB1 heteromeric complex binds to the activation loop in the tyrosine kinase domain of the insulin receptor, which results in the reduction of phosphorylation of the proapoptotic BH3-only protein (BAD), and BAD-induced activation of beta cell apoptosis (Kim et al. 2012). Although previous research has shown beneficial effects of CBD on suppressing pro-inflammatory responses when binding to CB2 receptors and other intracellular receptors (Martini et al., 2023; Furgiuele et al., 2023; Muller et al., 2019; O'Sullivan, 2016), there still is a possibility that CBD can bind to CB1 receptors *in-vivo*. This binding could possibly be potentiated when STZ and CBD are administered simultaneously, leading to an increase in beta cell apoptosis and a rapid decline in insulin production, and accounting for the drastic increase in T1D incidence that was seen in our second model with CBD and STZ co-administration in B6 mice. Interestingly, a case report on type 2 diabetic patients observed that CBD treatment did not have the desired effect in reducing hyperglycemia, but there was no overall potentiation of disease (Mattes et al., 2021).

Figure 23

Initial study (n=11/group) of male B6 mice treated with 5 mg/kg CBD for 4 weeks prior the STZ induction of T1D. Body weights (A), blood glucose levels (B), and incidence of T1D (C).



Note. Initial body weights and blood glucose were taken the first day of 5 mg/kg treatment (day -28), and then bi-weekly one week after the first STZ injection (day 8). Incidence of T1D is presented as a percentage of nondiabetic (diabetes-free) mice. Diabetes onset was defined by the first of two consecutive glucose readings of ≥ 250mg/dL. Values are shown as means ±SEM. No statistical significance was evident. (MANOVA: A-B, Survival analysis: C).

Recently, the CBD market has being experiencing an explosion in emerging CBD products. However, there are many limitations to the different forms of CBD. CBD remains to be highly hydrophobic and when administered orally, it is primarily absorbed through passive diffusion in the gastrointestinal tract. Absorption is facilitated by the small intestine by bile salts

that form micelles around CBD that are then up taken (Takano et al., 2008). Once absorbed CBD binds to plasma carrier proteins for transportation throughout the body. However, oral CBD has limitations in the fact that it undergoes extensive first hepatic metabolism, resulting in a decrease in CBD's bioavailability (Gonçalves et al., 2019). Although the consumption of CBD with high lipid content can improve bioavailability, there is still room to investigate other forms and routes of CBD administration. One review article highlighted the different routes and forms of CBD administration in which lipid-based, polymer-based, and solid-based CBD formulations were discussed (Hossain et al., 2023). Intravenous, inhalation, and subcutaneous administration were found to result in the highest concentrations of CBD in the blood, therefore having the most bioavailability and bypassing the liver first-pass metabolism. These routes, however, vary in their overall effectiveness as some are considered invasive and may shorten the half-life of oil-based CBD in the blood (Hossain et al., 2023).

A new way of CBD administration involving nanocarriers has also begun to be studied in order to increase CBD's bioavailability. Various lipid-based nanocarriers have been reported such as nanoliposomes, nano emulsions, nanostructured lipid carriers, and solid lipid nanoparticles. Some studies have reported that IP administration of CBD using a nanoparticle delivery system in ovarian cancer showed rapid absorption of CBD and provided high availability (Fraguas-Sánchez et al., 2020). In our study, the CBD we used was expected to have an increase in bioavailability due to the use of a nano emulsifier (tartaric acid), and consequently see an increase in the anti-inflammatory effects that were seen oil-based CBD studies. However, our results do not fully support the claim that the usage of a water-soluble form of CBD will lead to an overall decrease in inflammation associated with T1D. Another study found that the usage of water-soluble CBD did not have a significant effect on inflammation associated with arthritis

in dogs when compared to the usage of oil-based CBD (Sims et al., 2022). Our results along with the previously mentioned canine arthritis study, bring to question the “enhanced bioavailability” that many water-soluble CBD products claim to have that enable it to “exert its potential health benefits” (stirlingcbdoil.com).

We also found that CBD might exhibit negative effects either by a prolong administration, high(er) dose (in our case 10 mg/kg), or interaction with other drugs (STZ in our case). Although many beneficial effects of immunomodulation by short term usage of CBD have been noted in inflammatory diseases, more research is needed on the effects of long-term exposure to CBD. One study investigated the effects of prolonged THC exposure in adolescent rats, which led to an increase in inflammation in both the brain and periphery (Moretti et al., 2015). Although THC does vary from CBD, and has more of a psychotropic effect, both have been studied for supposed immunosuppressive and anti-inflammatory properties. There have been multiple studies showcasing the anti-inflammatory properties of CBD, however, more attention is being brought to the immune dysregulation that may occur after cannabis administration. Researchers have begun to investigate immune dysregulation that may led cannabis users to be more susceptible to viral and bacterial infections (Maggirwar & Khalsa et al., 2021). Although there are described beneficial effects of cannabis on suppressing inflammation, without negative effect on glucose metabolism (Permutt et al., 1976), cannabis as whole can have possible negative effects on self-management through the appetite-stimulating effects that may lead to an increase in calorie intake and poor disease control (Smit & Crespo, 2001).

Another cause for concern is the unreliability of the purity and dosage of CBD in different products. A recent study of 84 CBD products bought online showed that more than a

quarter of the products contained less CBD than labeled. In addition, THC was found in 18 products (Bauer, 2022). Due to the significant prevalence in CBD use, there remains a need for standardization of CBD extraction processes and dosages. Some of the limitations in standardizing cannabis is due to the complex clinical and pharmacological nature of cannabis, since *Cannabis sativa* consists of more than 100 different cannabinoids, of which THC and CBD are the most studied and used (Jugl et al., 2021). There is also variation in extraction methods used to extract the different cannabinoids from the plant, which may lead to varying results in cannabis-related research. There has been no study to date that has reported the absolute oral bioavailability of CBD in humans, and there have been limited dose-determination studies surrounding desired plasma concentrations to achieve minimum effective doses (Millar et al., 2018). Overall, the research surrounding the effects of oil-based CBD can be difficult to interpret due to the variation of extraction methods and to the fact there is no therapeutic dosage noted in humans to compare it to. This also highlights the lack of research in the commercially available water-soluble form of CBD, claimed to have enhanced bioavailability. In conclusion, our current study brings awareness to the need for further testing on both oil-based and water-soluble CBD effects and the standardization of formulations of both products as they continue to be proposed in treatments of many different ailments, including T1D.

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