CONSUMPTION OF CANNABIS OIL DURING PREGNANCY DISRUPTS IMPLANTATION SITE REMODELLING AND CAUSES FETAL ABNORMALITIES AT TERM

CONSUMPTION OF CANNABIS OIL DURING PREGNANCY DISRUPTS IMPLANTATION SITE REMODELLING AND CAUSES FETAL ABNORMALITIES AT TERM

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Descriptive Note

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Abstract

The use of cannabis during pregnancy has increased dramatically in recent years, as legalization altered the accessibility and acceptability of cannabis use. However, maternal cannabis use has been found to substantially increase the risk of several adverse pregnancy outcomes like stillbirth and low birthweight. Despite the clear risk little is known about how cannabis causes these complications and whether all cannabis consumption methods pose a risk. For a successful pregnancy, the uterus must create a favourable environment to support fetal growth. This process is largely modulated by uterine Natural Killer (uNK) cells which are responsible for promoting tissue remodelling at the maternal-fetal interface. The current project aims to assess whether cannabis oil alters proper uNK cell functioning and in turn causes pregnancy complications. To assess pregnancy outcomes, we administered either CBD oil, THC oil or control oil via gavage from gestation day (GD) 6.5-11.5 in CD57BL/6J mice and assessed fetal outcomes on GD 18.5. Fetuses from pregnant mice receiving THC oil had significantly lower weights, lengths, and head size whereas mice treated with CBD oil had reduced fetal head size and a trend for elevated odds of being small for gestational age.

To pinpoint the cause of these abnormal fetal outcomes we examined implantation site remodelling earlier in pregnancy at GD10.5 and 12.5. CBD oil reduced placental area and caused a significant increase in the number of uNK cells within the decidua. Additionally, both CBD and THC oil impaired vascular remodelling. Lastly, we found that both THC and CBD reduced human NK cell production of the angiogenic factors IFN-γ and VEGF, which may account for the abnormal vessel remodelling. Thus, the present work highlights that both CBD and THC oil cause fetal abnormalities at term which may be a result of improper implantation site remodelling driven by uNK cell dysfunction.

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List of Abbreviations

2-AG – 2-arachidonoylglycerol

2-DG – 2-deoxyglucose

ACK – Ammonium-Chloride-Potassium

AEA – Anandamide

Aza – 5-Aza-2′-deoxycytidine

CB1 – Cannabinoid receptor 1

CB2 – Cannabinoid receptor 2

CBD – Cannabidiol

CO – Carbon monoxide

DAGL – sn-1-DAG lipase

DBA – Dolichos Biflorus Agglutinin

ELISA – Enzyme-linked immunosorbent assay

ER – Endoplasmic reticulum

ESCs – Uterine fibroblast-like endometrial stromal cells

EVT – Extravillous trophoblast

FAAH – Fatty acid amide hydrolase

FBS – Fetal bovine serum

FR α – Folate receptor α

FSH – Follicle stimulating hormone

GD – Gestation Day

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GnRH – Gonadotropin-releasing hormone

HGF – Hepatocyte growth factor

HIF – Hypoxia-inducible factor

HLA – Human leukocyte antigen

H&E – Hematoxylin and eosin

HPO – Hypothalamic pituitary ovarian axis

HUVEC – Human umbilical vein endothelial cells

IFN-γ – Interferon gamma

IL – Interleukin

IP-10 – IFN inducible protein 10

IUGR – Intrauterine growth restriction

LC-MS – High-performance liquid chromatography coupled to a mass spectrometer

LH – Luteinizing hormone

LIF – Leukemia inhibitory factor

MAGL – Cytosolic monoacylglycerol lipase

MAPK – Mitogen-activated protein kinase

MCT – Medium-chain triglyceride

MFI – Mean fluorescence intensity

MHC – Major histocompatibility complex

MIP – Macrophage inflammatory protein

MLAp – Major lymphoid aggregate of pregnancy

MMPs – Matrix metalloproteinases

NAPE-PLD – N-acyl-phosphatidylethanolamines-hydrolyzing phospholipase

NK – Natural killer

NKreg – Regulatory-like NK cell

PAS – Periodic acid-Schiff

PBMC – Peripheral blood mononuclear cells

pbNK – Peripheral blood NK cells

PBS – Phosphate buffered saline

PlGF – Placental growth factor

PPARs – Peroxisome proliferator-activated receptors

RANTES – Regulated on activation normal T cell expressed and secreted

ROS – Reactive oxygen species

RPL – Recurrent pregnancy loss

SGA – Small for gestational age

SMA – Smooth muscle actin

STAT – Signal transducer and activator of transcription

taNK – Tumour-associated NK cell

TGFβ – Transforming growth factor β

TNF-α – Tumor necrosis factor α

Treg – T regulatory

trNK – Tissue-resident uNK cell

TRPV1 – Transient receptor potential vanilloid-1 channel

uNK – Uterine natural killer cell

VEGF – Vascular endothelial growth factor

VEGFR-1 – VEGF receptor-1

VSMC – Vascular smooth muscle cells

α – Alpha

β – Beta

γ – Gamma

μ - Micro

Declaration of Academic Achievement

All experiments discussed in this body of work were performed by Tyrah M. Ritchie except for the following:

- H&E staining and PAS staining of GD 10.5 and GD 12.5 implantation sites as well as H&E staining of GD 18.5 placentas were performed by members of the McMaster Immunology Research Centre Histology Core Facility.
- The 31-plex mouse cytokine and chemokine array on GD 10.5 decidual supernatants was performed by Eve Technologies.
- The quantification of cannabinoid levels in the cannabis oils via high-performance liquid chromatography coupled to mass spectrometry was performed by members of the Centre for Microbial Chemical Biology at McMaster University.

Chapter 1: Introduction

1.1 Maternal Cannabis Use During Pregnancy

1.1.1 Prevalence of Cannabis Use

The prevalence of maternal cannabis use has increased dramatically in recent years, with one Canadian study reporting an alarming jump from 1.3% in 2003 to 7.5% in 2020 (1). The stark increase is likely due to the 2018 legalization of recreational cannabis in Canada which changed the accessibility and social acceptability of cannabis use (2; 3). Legalization tends to create the perception of safety, as one study found that over 60% of women reporting cannabis use during their pregnancy said they would increase their consumption if cannabis was legalized (4). Additionally, nearly 70% of the women who report cannabis use during their pregnancy perceive little to no risk of weekly cannabis exposure on the health of their pregnancy (4; 5). Women of lower socioeconomic status and young women are disproportionally impacted, as these women are roughly two and three times more likely to consume cannabis while pregnant, respectively (6). However, studies typically use self-reporting to assess cannabis use which may underestimate the true prevalence especially prior to legalization. One Californian study found that cannabis use among pregnant women was nearly doubled when assessed objectively as compared to self-report (7).

Typically, cannabis is consumed via smoke-inhalation, however accumulating evidence underpins the detrimental harm of cannabis smoke on lung health, as it contains similar carcinogens as tobacco smoke (8; 9). Thus, cannabis oil is becoming an increasingly popular alternative method of consumption as it provides the desired cannabis effects without the damage of smoke exposure (10). Furthermore, cannabidiol (CBD) oil is gaining attention as a broad-spectrum treatment for a variety of different conditions such as Parkinson's disease, anxiety, pain

management, and epilepsy with varying levels of evidence (11; 12; 13). Due to the perceived health benefits and lack of psychoactive effects, the perceptions surrounding CBD oil are generally positive, with one study finding that nearly 35% of all study respondents, including those who had not used CBD, felt that CBD oil was either "good" or "very good" for health (14). As studies do not typically tend to specify the method of cannabis consumption, pregnant women may be turning to cannabis oil as an alternative option.

1.1.2 Reasons for Maternal Cannabis Use

The use of cannabis during pregnancy is partially due to its natural anti-emetic properties, as pregnant women commonly report using it to self-medicate their nausea and vomiting symptoms (15; 16). Roughly 70% of women experience nausea and vomiting during their pregnancy, with symptoms typically peaking in the first trimester but sometimes lasting the entire pregnancy (17). Accordingly, cannabis use is highest in women who experience severe nausea during their pregnancy and used more frequently during the first trimester (18; 19). Cannabis users report cannabis to be very effective at treating their nausea symptoms and feel it is more natural than typical prescribed medications (16; 20). The use of cannabis as a natural anti-emetic treatment has been used in various cultures throughout history as well as in Western medicine (21). Its use in alleviating chemotherapy-induced nausea and vomiting has recently been assessed with cannabinoids having better acute anti-emetic effects than conventional drugs but inducing more side effects than control medications (22). As pregnant women appear to be turning to cannabis to self-medicate their nausea and vomiting symptoms, it is imperative to understand the overall impact of cannabis on pregnancy outcomes.

1.1.3 Risks of Maternal Cannabis Use

Despite the growing popularity of maternal cannabis use, numerous reports have identified increased complications and fetal abnormalities in pregnant women using cannabis. Cannabis has been shown to significantly reduce birthweight, increase the risk of preterm birth, and impair fetal brain development resulting in long-lasting cognitive deficits in the child (1; 23; 24). A recent Canadian study examining fetal abnormalities in pregnant women self-reporting cannabis use, found that infants exposed to cannabis *in utero* were more likely to be small for gestational age (SGA), have major anomalies, lower head circumference, a lower Apgar score, and require intensive care admission than controls (1). These findings were consistent with other reports, as a recent systematic review analyzing 16 different primary studies found similar adverse outcomes across all reports (24). Unfortunately, the effects of prenatal cannabis exposure extend beyond pregnancy, with children often experiencing altered cognitive processes later in life (25; 26; 27). Maternal cannabis use during pregnancy is associated with increased hyperactivity, inattention issues, anxiety, and aggression in the children, as well as a higher likelihood of being diagnosed with autism spectrum disorder and learning disorders later in childhood (25; 26; 27). Given the serious implications to the health of the pregnancy and child development coupled with the rising prevalence of maternal cannabis use, there is an urgent need to understand the mechanisms driving cannabis-induced pregnancy complications.

1.2 Cannabis: Receptors and Pharmacokinetics

1.2.1 The Endocannabinoid System

The two predominant cannabinoids derived from the cannabis plant are delta-9-tetrahydrocannabinol (THC), the major psychoactive chemical of cannabis, and CBD (28).

Investigation of THC binding sites identified the presence of two G protein-coupled receptors (GPCR) cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (29; 30). The CB1 receptors congregate within the central nervous system but are found in peripheral tissues such as the spleen, lungs, and vasculature (31). In contrast, CB2 is found predominantly in immune tissues and is thought to modulate the immune response by regulating cytokine production, cell activation, and cell proliferation (32). CB2 receptor expression has also been identified within the central nervous system, cardiovascular system, and respiratory system (31; 33). Thus, engagement of CB1 and CB2 can have far reaching effects on numerous body systems.

The natural ligands for CB1 and CB2 receptors are the endocannabinoids, most notably anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which are thought to be involved in a variety of physiological processes such as neurotransmission, cognition, and reproduction (31). The endocannabinoids are synthesized on demand from lipid precursors with N-acyl-phosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (NAPE-PLD) and sn-1-DAG lipase (DAGL) being the most prominent enzymes responsible for AEA and 2-AG synthesis, respectively (34; 35). The endocannabinoids will then engage with the CB receptors until they are transported into the cell and inactivated by fatty acid amide hydrolase (FAAH) for AEA or cytosolic monoacylglycerol lipase (MAGL) for 2-AG (36; 37). Thus, the action of the endocannabinoids is dependent upon their lifespan within the extracellular space (35).

Binding with the CB1 and CB2 receptors leads to the initiation of several different signalling cascades such as the inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase (MAPK), and engagement with different ionic currents (35).

1.2.2 Cannabinoid Receptors

Exogenous cannabinoids can signal through the CB1 and CB2 receptors of the endocannabinoid system but can also interact with peroxisome proliferator-activated receptors (PPARs), transient receptor potential vanilloid-1 (TRPV1) channels, and other G-protein-coupled receptors like GPR55 (38; 39). THC is a partial agonist for both CB1 and CB2 receptors but can also engage with TRPV1 channels, PPARs, GPR55, β-adrenoreceptors, and other channels for Na and K (40). THC elicits its psychoactive effects through interaction with CB1 receptors in the brain, which act to either reduce or stimulate neurotransmitter release from neurons (41). Conversely, CBD acts as an inverse agonist of CB1 and CB2 receptors, an agonist of TRPV1 channels and PPARs, and inhibits FAAH's ability to hydrolyze AEA leading to AEA accumulation (42; 43). The anti-inflammatory effects of CBD are thought to be partially mediated through its interaction with PPARs like PPARγ which has been found to promote anti-inflammatory cytokine production (44; 45). Additionally, the increase in AEA levels caused by CBD's inhibition of FAAH is also thought to mediate several of its effects (46; 43).

1.2.3 Pharmacokinetics of Cannabis – Different Routes of Administration

The rate of cannabis metabolism and potency of its effects are heavily dependent on the route of administration. In humans, inhalation of cannabis causes the highest bioavailability of THC at roughly 10-35% with peak levels experienced rapidly (6-10 minutes) following consumption (47; 48). In contrast, oral consumption of THC produces peak concentrations later at roughly 1-6 hours post-ingestion and only reaches a bioavailability of roughly 4-12% (49; 50). Interestingly, bioavailability tends to be higher for frequent users of cannabis, with heavy users having roughly double the systemic bioavailability than occasional users (47; 51). Similar rates of bioavailability are found for CBD when consumed either via inhalation or ingestion (50).

Extensive first-pass liver metabolism following ingestion also tends to result in higher levels of cannabinoid metabolites like 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THC-COOH) and hydroxylated 7-COOH derivatives of CBD (49).

However, the bioavailability of cannabinoids following oral consumption may be heavily dependent on the formulation of the carrier liquid and whether the individual is fasted (49; 52). One study found comparable serum bioavailability after oral or pulmonary exposure when fasted rats received cannabis via sunflower oil (49). Interestingly, the same group also found that while cannabinoid levels in the brain rapidly peaked and then declined after inhalation, the cannabinoid levels were more long-lasting and higher in the brain following oral consumption (49). Both THC and CBD are highly lipophilic causing them to accumulate within the brain, adipose tissue, and other organs (53). Hlozek et al. suggested that oral administration may cause cannabinoids to accumulate in the brain overtime.

Both THC and CBD can readily cross the placenta during pregnancy, though the concentrations in fetal circulation are lower than what is found in the mother (48). Oral administration of THC oil to pregnant animals causes the fetal THC level to be roughly one-tenth of the dam's serum THC concentration whereas inhalation results in higher levels at roughly one-third the maternal concentration (54; 55). After birth, cannabinoids are also able to pass into the breast milk resulting in a nursing infant being exposed (48; 56).

1.3 Endogenous and Exogenous Cannabinoids in Reproduction

1.3.1 The Impact of Cannabinoids on Implantation

The endocannabinoid system is involved in several stages of reproduction, as the human endometrium and placenta both express CB1 and CB2 receptors (57). Compared to other

mammalian tissues, the endogenous cannabinoid AEA is most abundant in the uterus (57). AEA is thought to be involved in several reproductive events such as synchronising the timing of implantation by coordinating endometrium susceptibility and embryo development (58). Levels of AEA seem to be the lowest at the implantation site but higher in the surrounding non-implantation areas, which corresponds to previous reports indicating that high AEA concentration impairs embryo implantation while lower levels stimulate differentiation via CB1 (58; 59). Furthermore, AEA concentrations are lower in the receptive murine uterus but increase during the non-receptive stage (58). A high affinity CB1 receptor has been identified on murine blastocysts and thus endocannabinoids may coordinate the crosstalk between the embryo and receptive uterus to promote implantation in the appropriate window (60). In agreement with this, embryos deficient in CB1 demonstrated asynchrony between embryo and uterine development compared to wild-type controls (59). Considering AEA and the cannabinoid receptors play a role in coordinating implantation, cannabis could potentially interfere with this early stage of pregnancy by disrupting AEA balance or directly acting on CB1 receptors.

Additionally, the cannabinoids have been implicated in altering the receptivity of the maternal endometrium to implantation and its ability to promote early movement of fetal-derived trophoblast cells (61). Decidualization is a process in the secretory phase of the menstrual cycle by which uterine fibroblast-like endometrial stromal cells (ESCs) create a favorable environment for embryonic development (62). Communication between the ESCs and trophoblast cells is essential to coordinate embryo attachment and subsequent trophoblast invasion (61). Neradugomma et al. demonstrated that treatment with THC or CBD delayed blastocyst attachment and initial spreading on the ESCs. Additionally, using a Boyden chamber assay to assess invasion, the authors showed culture of ESCs with THC or CBD for 7 days significantly decreased their

ability to promote trophoblast migration (61). Thus, cannabinoids may promote obstetrical pathology by impairing proper trophoblast implantation and subsequent migration by disrupting ESCs.

1.3.2 The Impact of Cannabinoids on the HPO Axis

Another point of involvement of the endocannabinoid system in regulating reproduction is its influence on the hypothalamic-pituitary-ovarian (HPO) axis. The hormones secreted by the three structures contained in this axis are responsible for regulating the ovarian cycle (63). The hypothalamus and anterior pituitary express CB1 and the ovaries express both CB1 and CB2 (63). THC has been found to suppress the secretion of several gonadotropins within the HPO axis such as gonadotropin-releasing hormone (GnRH) from the hypothalamus and follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (64; 65). As a result, chronic exposure to THC has been demonstrated to induce irregular estrous cycles in female rats and alter the structure of preovulatory follicles (66; 67). Repetitive administration of THC to rhesus monkeys induces abnormal menstrual cycles with variations in length and anovulation periods (68). Additionally, women who reported cannabis use experienced abnormal menstrual cycles, with a prolonged follicular phase, and a higher risk of fertility issues because of these abnormal cycles (69; 70). THC's ability to induce dysregulation of the menstrual cycle is thought to be partly caused by its impairment of the HPO axis.

1.3.3 The Impact of Cannabinoids on Trophoblast Functions

The early blastocyst stage of the developing embryo is divided into two distinct cell populations: the inner cell mass which eventually becomes the embryo, and the trophoblast which consists of cells that coordinate implantation and form the chorionic sac surrounding the fetus. Following implantation, the trophoblasts divide and migrate through the maternal tissue

establishing the unique structures of the maternal-fetal interface (71). The degree of trophoblast invasion has been associated with pregnancy outcome, with insufficient invasion being correlated with preeclampsia, preterm labor, and intrauterine growth restriction (IUGR) (72; 73). Several studies have investigated how CBD and THC are able to impair proper trophoblast functioning and subsequently induce pregnancy complications.

Cannabinoids have been shown to directly cause trophoblast dysfunction by impairing proliferation, reducing cell differentiation, and inducing apoptosis. Co-culture of THC with the BeWo trophoblast cell line impaired proliferation of these cells in a dose-dependent manner and altered expression of several genes involved in cell cycle progression (74). Additionally, THC has been shown to reduce differentiation of cytotrophoblasts to syncytiotrophoblasts demonstrated by decreases in biomarkers of trophoblast differentiation (75). The syncytiotrophoblasts form the cell layer that is in direct contact with maternal blood and are responsible for nutrient exchange and hormone secretion, therefore, reduced differentiation may impede these processes (75). Syncytiotrophoblasts are also responsible for folate uptake from the maternal blood stream as they express the high affinity folate receptor α (FRα) (76). Folate is an essential micronutrient for normal placental and fetal development, as women with folate deficiency have increased risks of neural tube defects and spontaneous abortion (77). Keating et al. demonstrated that 48-hour incubation of villous cytotrophoblasts, a model of syncytiotrophoblasts, with THC significantly impaired the ability of trophoblasts to uptake folic acid. Dysregulation of folate uptake by syncytiotrophoblasts cells could be another mechanism by which cannabis results in pregnancy complications. Lastly, THC has been indicated to affect the viability of trophoblasts by inducing apoptosis. Treatment of BeWo cells with THC for 36 hours induced increased activation of the effector caspases, caspase-3/-7, as well as caspase-9 compared to control cells (78). BeWo co-

culture with THC has also been shown to upregulate expression of pro-apoptotic genes (74). Trophoblast turnover is an essential process for placental development and relies on both proliferation and apoptosis (78). Cannabis thus seems to negatively regulate trophoblast turnover by dysregulating proliferation, differentiation, and apoptosis.

Potential Mechanisms Behind Cannabinoid-Induced Trophoblast Dysfunction

The cannabis-induced trophoblast dysfunction is thought to be regulated in part by STAT3 signalling and induction of endoplasmic reticulum (ER) stress. STAT3 is a member of the STAT family and is involved in many essential cell functions such as cell motility. Particularly, activation of STAT3 is essential for primary trophoblast invasion and seems to be induced by several soluble factors in the placenta such as IL-6, leukemia inhibitory factor (LIF), and hepatocyte growth factor (HGF) (79). Incubation of two trophoblast cell lines with cryptotanshinone, a selective STAT3 inhibitor, significantly reduced the migration ability of the trophoblast cells (80). Chang et al. looked at the phosphorylation of STAT3 in the placentas of mice treated with THC and women who used cannabis during pregnancy and found that activation of STAT3 is largely downregulated in these placentae. This group suggested that impairment in STAT3 signalling could explain how THC is capable of impairing trophoblast motility (80).

Another proposed explanation for the dysfunction of trophoblasts in response to THC exposure is induction of ER stress (81). Pregnant mice administered a single dose of tunicamycin to chemically induce ER stress experienced increased fetal growth restriction and lower placental weights (82). Considering maternal cannabis use during pregnancy is associated with fetal growth restriction, Lojpur et al. assessed whether THC induces ER stress in-0 trophoblast cells. In a dose-dependent manner, THC treatment activated the uncoupled protein response and significantly increased protein levels of ER stress markers, such as GRP78, in BeWo cells (81). Pre-treatment

with CB1 and CB2 antagonists completely abrogated this response indicating the ER stress induced by THC is dependent on cannabinoid receptors (81).

Lojpur et al. also examined the effect of THC on mitochondrial respiration in BeWo cells. 24-hour culture with THC lowered basal respiration by 50% and maximal respiration by 25% as measured via the XFe24 Seahorse analyzer (81). This result corroborates previous studies indicating the ability of THC to reduce mitochondrial function in various cell types (83). 48-hour exposure to 20 μM of THC reduced mitochondrial respiration in the human extravillous trophoblast (EVT) cell line HTR8/SVneo (84). Moreover, Walker et al. demonstrated that exposure of the BeWo trophoblast cell line to THC reduced mitochondrial membrane potential, induced mitochondrial stress, and increased reactive oxygen species (ROS) (85). Considering oxidative stress and elevated ROS are key features in several gestational pathologies such as preeclampsia and IUGR, metabolic insufficiency in trophoblast cells may be an important mechanism in THC-driven placental dysfunction and pregnancy complications (86). Additionally, given the physical proximity of the ER to the mitochondria, integrated signalling between the two organelles has been suggested and may explain how THC induces both ER stress and mitochondrial dysfunction (81). Nonetheless, THC may provoke IUGR via induction of ER stress and decreased mitochondrial respiration.

Overall, it is clear from previous research that the cannabinoids can affect several different processes during pregnancy. Moreover, as pregnancy is an amalgamation of several complex mechanisms and bodily systems working together, it is likely that the pregnancy complications associated with cannabis use are due to dysfunction in several different mechanisms.

1.4 The Maternal Immune System During Pregnancy

1.4.1 Changes to the Immune System During Pregnancy

For a successful pregnancy, the uterus must be receptive to the implanting embryo, tolerant to the semi-allogenic fetal cells, and undergo extensive modifications to promote placenta formation and decidual artery remodelling (87). These processes are largely modulated by a complex inflammatory system populating the maternal decidua surrounding the developing embryo. Towards the end of the progesterone-dominated luteal phase of the menstrual cycle the immune cells of the maternal endometrium proliferate and prepare for potential pregnancy (88). Of these cells, a unique form of natural killer (NK) cell called a uterine NK (uNK) cell predominates accounting for roughly 70% of leukocytes in the early pregnant decidua (89). Macrophages are the second largest population of leukocytes in the decidua comprising roughly 20% (90). Other immune cells like dendritic cells and Foxp3+ T regulatory (Treg) cells also play important roles in modulating inflammation and promoting development at the maternal-fetal interface (88). Additionally, the roles and proportions of these immune cells change throughout pregnancy as the inflammatory response alters depending on the stage of development (87).

The early stages of pregnancy, including implantation and placentation, are governed by pro-inflammatory processes that promote reorganization and increased expression of adhesion molecules, the removal of mucins at the uterine surface that block adhesion, and regulation of tissue remodelling (91). As the maternal-fetal interface is established and extensive fetal growth begins, the inflammatory response switches to more anti-inflammatory with promotion of immune tolerance and tissue repair (87). Lastly, a final switch to pro-inflammatory processes has been indicated as a necessary process for labour. Activation of the NF-κB pathway and recruitment of inflammatory cells into the myometrium has been shown to be crucial for the initiation of labour

and contraction of the uterus (87; 92). Thus, pregnancy requires an active immune response at the decidua that adapts depending on the needs of the specific stage of pregnancy.

The innate and adaptive immune systems also change systemically during pregnancy. There are increased numbers of circulating granulocytes, monocytes, and Treg cells in pregnancy, while NK cells and CD4+ T cells tend to decrease (93; 94; 95; 96). The monocytes and granulocytes also show evidence of increased activation during pregnancy, having elevated levels of activation markers and adhesion molecules as well as increased intracellular ROS production and phagocytosis (97). In contrast, peripheral blood NK (pbNK) cells and T cells from pregnant individuals show reduced secretion of cytokines like interferon (IFN)-γ (96). Some studies suggest that the reduced functions of circulating NK cells and T cells may account for the increased severity of infections in pregnant mothers like influenza (98). Nonetheless, pregnancy is a complex physiological process that changes both local and systemic immune landscapes to support the developing fetus.

1.4.2 Uterine NK Cell Function During Pregnancy

uNK cells are the predominate leukocyte of the first trimester decidua and are instrumental in promoting immune suppression, regulating trophoblast migration, secreting growth factors, and ensuring proper decidual vascular remodelling at the maternal-fetal interface. Unlike NK cells found in the blood, uNK cells are non-cytotoxic and promote immune suppression (99). The functions of uNK cell are tightly regulated by the balance of activating and inhibitory receptors, with most inhibitory receptors recognizing major histocompatibility complex (MHC) class 1 molecules (99). EVT cells do not express the classical MHC-1 molecules HLA-A and HLA-B allowing them to evade T cell attack but do express HLA-C and the non-classical HLA-G and HLA-E (99; 100). uNK cells express high levels of inhibitory receptors like KIR2DL2/3,

CD94/NKG2A, and ILT2 which recognize HLA-C, HLA-E, and HLA-G, respectively, preventing them from killing trophoblast cells (101). uNK cells also produce immunosuppressive cytokines like interleukin (IL)-10 and transforming growth factor β (TGF-β) which promote the anti-inflammatory functions of other immune cells at the maternal-fetal interface (102; 103).

Human uNK cells account for roughly 40% of leukocytes in the late secretory phase endometrium, rapidly proliferate during the first trimester of pregnancy, and then decline after placenta formation (89; 104). An important early function of uNK cells, prior to the formation of the placenta, is to produce essential growth-promoting factors. Specialized tissue-resident uNK (trNK) cells produce pleiotrophin, osteoglycin, and osteopontin which support development of fetal bone, cartilage, brain, vessel, and cardiac tissue (104). In *Nfil3*$^{-/-}$ mice, trNK cells are substantially reduced. These mice demonstrate impaired fetal growth and bone formation (104). However, the fetal growth restriction was alleviated upon adoptive transfer of *in vitro* generated trNK cells, indicating the connection between uNK cells and fetal growth (104).

uNK cells reside in close proximity to trophoblast cells and are capable of directly regulating trophoblast functions through secretion of various cytokines and growth factors (105). In an *in vitro* invasion assay, uNK cells, but not pbNK cells, were able to potently promote trophoblast migration (106). Moreover, administration of neutralizing antibodies for IL-8 and IFN-inducible protein 10 (IP-10) significantly reduced the ability of uNK cells to promote trophoblast migration indicating the potential role of these cytokines in uNK cell-mediated trophoblast migration (106). However, uNK cells have also been implicated in limiting trophoblast movement. IFN-γ and tumour necrosis factor (TNF)-α, both of which are produced by uNK cells, have been shown to inhibit trophoblast migration *in vitro* (107; 108). As the promotion and restraint of trophoblast

migration through the maternal tissue are equally important for proper placentation it seems uNK cells play an essential role in balancing this process (105).

An important objective of early pregnancy is to establish an adequate blood supply for the developing fetus to allow nutrient transport and gas exchange (109). Inadequate arterial remodelling can result in complications such as preeclampsia, IUGR, and recurrent pregnancy loss (110). Vascular remodeling is accomplished in part by EVT cells which invade maternal vasculature, remodel vessels, and create a villous space where chorionic villi can uptake nutrients (109). uNK cells are also thought to be heavily involved in regulating spiral artery remodelling by directly acting on vascular smooth muscle cells (VSMCs), as well as indirectly by producing angiogenic factors (111). Smith et al. demonstrated that human uNK cells infiltrate the VSMC layers of remodelling vessels and secrete matrix metalloproteinase (MMP)-7 and -9 which degrade the vascular extracellular matrix (112). Additionally, this leukocyte-mediated remodelling occurred during an early pregnancy stage when EVTs had not yet migrated to the maternal vessels indicating a clear trophoblast-independent role of uNK cells in vascular remodelling. Another mechanism of uNK cell-mediated angiogenesis is through the release of various mediators. uNK cells produce high amounts of the angiogenic factors vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and angiopoietin 2 (113). Supernatants of human uNK cells, but not pbNK cells, increased the migration of human umbilical vascular endothelial cells (HUVEC) and accelerated formation of complex network structures in the HUVECs (106). This effect was reduced upon addition of Flt1-Fc fusion protein, which neutralizes VEGF and PlGF, indicating that uNK cells are mediating angiogenesis partially through production of these factors (106). The production of IFN-γ by uNK cells has also been implicated in regulating vascular remodelling in both mice and humans (114; 115). In lymphocyte deficient mice, engraftment of bone marrow

cells unable to produce IFN-γ significantly impairs pregnancy-induced vascular remodelling and leads to necrosis and hypocellularity in the deciduae of these mice (116). However, treatment with IFN-γ can independently rescue arterial remodelling in these pregnant mice (116). Thus, murine uNK cells seem to modulate arterial modifications in an IFN-γ-dependent mechanism. Additionally, recent reports have identified "pregnancy trained" uNK cells in multigravid women that are capable of secreting significantly higher levels of VEGF and IFN-γ than uNK cells isolated from a first pregnancy (117). Pregnancy complications associated with defective placentation, like IUGR, appear at lower frequencies in repeated pregnancies, and thus improved uNK cell functioning may account for this (117).

The critical role of uNK cells in a successful pregnancy is highlighted as their dysfunction has been associated with various pregnancy complications. For instance, decidual tissue isolated from pregnancies experiencing fetal growth restriction had decreased proportions of uNK cells compared to healthy pregnancies. This was associated with aberrant vascular remodelling (118). Additionally, uNK cells isolated from women with an increased risk of developing preeclampsia were unable to effectively promote trophoblast migration in an *in vitro* assay (119). Thus, uNK cells are an integral player in ensuring a healthy pregnancy.

1.5 The Effects of Cannabinoids on Immune Cell Function

The two prominent receptors of the endocannabinoid system are CB1 and CB2. While CB1 primarily congregates within the nervous system and some peripheral tissues, CB2 is predominantly expressed on immune cells. B cells express the highest levels of CB2 followed by NK cells > monocytes > neutrophils > CD8+ T cells > CD4+ T cells (120; 121). Engagement of these receptors by cannabinoids has been demonstrated to potently modulate immune homeostasis

with a preference for immune suppression. This immune suppression is thought to be modulated by 1) inhibition of immune cell proliferation, 2) reduction in pro-inflammatory cytokine production, 3) recruitment of Treg cells, and 4) induction of apoptosis (122). Most of the research has addressed the effect of cannabinoids, particularly THC, on T cell function. Exposure of T cells to THC has been shown to decrease T cell proliferation and potently suppress pro-inflammatory cytokine production like IL-2, IL-12, IFN-γ, and TNF- α (123; 124; 125; 126). THC has also been shown to induce Treg cell development and promote IL-10 and TGFβ production (127; 128). Specifically, levels of IL-10 and TGFβ were significantly increased in murine lung tumours, both at the tumour site and in the spleen, following THC treatment. Moreover, mice had significant enhancement of tumour growth following THC treatment which was partially diminished by administration of anti-IL-10 and anti-TGFβ (128). CBD has also been shown to promote anti-inflammatory effects in T cells by reducing proliferation and production of pro-inflammatory cytokines (129). Incubation of T cells isolated from either rat spleens or lymph nodes with varying concentrations of CBD dose-dependently decreased IFN-γ production from the T cells (129). CBD has also been found to decrease IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) production in stimulated human T cells from healthy donor blood (129). Ultimately, the cannabinoids seem to induce anti-inflammatory functions in T cells through various mechanisms.

1.5.1 Cannabis and NK Cell Function

A few studies have assessed the influence of cannabinoids on pbNK cell function and have demonstrated changes in cytotoxicity as well as cytokine and chemokine production. Brief culture of human pbNK cells with THC caused a dose-dependent decrease in cytotoxicity against K562 cancer target cells (130). Similarly, pbNK cells isolated from mice that had been treated with THC showed a significant decrease in cytotoxicity against YAC-1 tumour cells (131). Pre-treatment

with CB1 and CB2 receptor antagonists attenuated this inhibition indicating a direct role of these receptors in the THC-mediated reduction in cytotoxicity (131). Cannabinoids have also been implicated in reducing cytokine production from pbNK cells. THC treatment greatly diminished the production of IFN-γ from murine pbNK cells and has also been shown to reduce TNF-α production in human pbNK cells (131; 132). Moreover, the reduction in IFN-γ production was rescued by pre-treatment with CB1 and CB2 receptor antagonists (131). pbNK cell chemokine production has also been altered by cannabinoids in previous research. THC seems to impair the ability of pbNK cells to recruit other inflammatory cells, as incubation with THC substantially decreases the production of the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and regulated on activation normal T cell expressed and secreted (RANTES) from a pbNK cell line (124). Lastly, THC has also been shown to inhibit proliferation of pbNK cells, as the proliferation of NKB61A2, a NK cell line, was inhibited following administration of THC (133). Thus, the cannabinoids, mainly THC, seem to affect NK cells similarly to T cells by reducing proliferation, cytotoxicity, and the production of cytokines. However, no work has currently been done to assess the effect of CBD on NK cell function.

1.5.2 Cannabis and Immune Cell Function During Pregnancy

Due to the sheer importance of proper immune cell functioning at the maternal-fetal interface during pregnancy, any disruption could result in pregnancy complications (118; 119). However, limited work has been done thus far to examine whether cannabis alters immune cell functions during pregnancy. A recent study that performed gene expression analysis on placental biopsies of women reporting cannabis use found that many immune-related genes involved in cytokine signalling (ex. *CXCL8* and *IL1B)* and the IFN pathway (ex. *IRF1* and IRF7) were substantially downregulated in mothers using cannabis (134). Additionally, these reduced immune-related gene

networks significantly correlated with abnormal neurobehavioral outcomes seen in the offspring of maternal cannabis users suggesting a dysregulated immune system as a potential driver of cannabis-induced pregnancy complications (134). On the other hand, exposure to THC during pregnancy has also been shown to disrupt immune development in the fetus. Thymic atrophy and reduced CD8+ T cells have been found in the fetuses of pregnant mice injected with THC, an effect that was partially blocked by pre-treatment with CB1 or CB2 antagonists (135). Additionally, this impaired immune development extends after birth, as pups exposed to THC *in utero* exhibited decreased response to HIV antigens five weeks after birth (135).

As of now, all studies investigating the effect of cannabis on NK cell function have been focused on NK cells found in the blood. However, one recent report has assessed the role of the natural endocannabinoid system in uNK cell function during miscarriage (136). Primary human uNK cells isolated from the first-trimester decidua of elective pregnancy terminations expressed both CB1 and CB2 receptors and contained the machinery capable of synthesizing the endocannabinoid AEA (136). However, the expression of these receptors and machinery was not different between uNK cells isolated from elective pregnancy terminations and uNK cells isolated from miscarriage. Interestingly, 18-hour co-culture of isolated uNK cells with AEA resulted in altered production of some angiogenic factors like angiogenin, angiopoietin-1 and -2, and fibroblast growth factor-2 (136). The researchers also found significantly higher concentrations of AEA in the decidual tissue of miscarriages compared to elective abortions of healthy pregnancies. Given that uNK cells express cannabinoid receptors and can be influenced by endocannabinoids, the exogenous cannabinoids found in cannabis could alter their function.

As mentioned earlier, preliminary reports have indicated that co-culture with THC or CBD decreases T cell and pbNK cell production of IFN-γ and TNF-α (131; 132). However, IFN-γ secretion by uNK cells in both mice and humans is a crucial mediator of the vascular remodelling process. Thus, cannabinoids may impair uNK cell cytokine production and disrupt their functions leading to pregnancy complications.

1.6 Project Rationale, Hypothesis and Thesis Objectives

1.6.1. Rationale

The legalization of recreational cannabis in Canada has drastically changed the accessibility, acceptability, and perceived safety of cannabis use (2; 3). As a result, the rates of maternal cannabis consumption have increased substantially with nearly a 6-fold increase in prevalence since 2003 (1). However, despite the growing popularity of maternal cannabis use, numerous reports have linked cannabis to several serious pregnancy complications such as low birthweight, stillbirth, and impaired fetal brain development (24; 137). The impact of cannabis extends beyond pregnancy with offspring developing attention issues, increased aggression, anxiety, and learning disabilities later in life (25; 26; 27).

Regardless of the growing prevalence and clear risks associated with maternal cannabis use, information remains limited on the cause of these complications. So far, work has primarily been done to uncover the effects of cannabis on placental development, as it appears THC disrupts trophoblast proliferation and differentiation (80). However, remodelling of the maternal decidua is a crucial process of early pregnancy which involves tissue and vascular changes to support fetal growth (138). The immune system populating the maternal-fetal interface primarily comprised of uNK cells, is the key driver of this remodelling process (106). The sheer importance of the maternal immune system during pregnancy is clear, as abnormal uNK cell function has been linked to several adverse pregnancy outcomes such as fetal growth restriction and preeclampsia (118; 139). A recent study found that many immune-related genes were substantially downregulated in the placentas of women using cannabis (27). Additionally, these reduced immune-related genes were significantly correlated with abnormal fetal outcomes suggesting a dysregulated immune system as a potential driver of cannabis-induced pregnancy complications. Preliminary work has also

found that THC significantly suppresses pbNK cell function and considering that uNK cells express the cannabinoid receptors their functions could also be impacted (131; 136).

While cannabis smoke inhalation remains the most popular method of consumption, alternative products like cannabis oil are becoming increasingly popular (10). Particularly CBD oil with its numerous reported health benefits is often perceived as safer by the public (14). However, despite the growing popularity of CBD oil limited research has evaluated its safety in pregnancy. Human studies of pregnant women typically do not specify cannabis consumption method and murine models tend to focus on smoke inhalation or injection of only THC, thus research is needed to evaluate the risk of CBD oil on pregnancy (140; 80).

The present work aims to uncover whether CBD and THC oil similarly impact pregnancy outcomes in a mouse model. We will also assess whether these complications are driven by abnormal decidual remodelling and impaired uNK cell functioning.

1.6.2. Hypothesis and Thesis Objectives

Hypothesis: Both THC and CBD oil cause pregnancy complications by disrupting implantation site remodelling and uNK cell function.

To test our hypothesis, we proposed the following specific project aims:

Aim 1: *Determine whether exposure to CBD or THC oil alters pregnancy outcomes in a mouse model.*

High CBD or high THC oil will be administered to CD57BL/6J mice from GD 6.5-11.5 and fetal outcomes such as fetal weight, length, head size, brain weight, and fetal resorption will be assessed one day before term on GD 18.5.

Aim 2: *Assess whether cannabis oil impairs implantation site remodelling in a mouse model.*

The implantation sites from mice exposed to either high CBD, high THC oil or control oil from GD 6.5-11.5, will be assessed on GD 10.5 and 12.5. The placental area, distribution of cells, and spiral artery remodelling will be analyzed through staining of the implantation sites.

Aim 3: *Pinpoint whether uNK cell functions are impacted by cannabinoid exposure.*

Objective 1: Assess the effect of exposure to either high CBD or high THC oil on murine uNK cell number, distribution, and ability to promote vascular remodelling in implantation sites from GD 10.5 and 12.5.

Objective 2: Assess the effect of incubation with either CBD or THC on both mouse and human NK cell production of angiogenic factors like IFN-γ and VEGF.

Chapter 2: Materials and Methods

2.1 Materials

Reagent	Company	Catalogue Number
Chemicals and Proteins		
5-Aza-2′-deoxycytidine	Sigma-Aldrich	A3656
Biotinylated Dolichos Biflorus Agglutinin	Vector Labs	B1035-5
CBD	Cerillant	C-045
Cytofix/Cytoperm Plus Fixation/Permeabilization Kit	BD Biosciences	554715
DAPI	ThermoFisher	D1306
DNase I	Sigma-Aldrich	10104159001
Fixable Viability Dye eFluor 780	ThermoFisher	65-0865-18
Fixable Viability Stain 510	BD Biosciences	564406
Golgi Stop	BD Biosciences	51-2092KZ
High CBD Oil	Symbl	N/A
High THC Oil	Symbl	N/A
Human IL-8/CXCL8 DuoSet ELISA	R&D Systems	DY208
Human IL-15	Peprotech	200-15
Human VEGF DuoSet ELISA	R&D Systems	DY293B
Liberase TM	Sigma-Aldrich	5401119001
Lymphoprep	Stemcell Technologies	07861
MACSxpress Whole Blood NK Cell Isolation Kit	Miltenyi Biotec	130-127-695
MCT Oil	Nutiva	N/A
Mouse IL-15	Peprotech	210-15
Mouse IL-12 p70	Peprotech	210-12
Percoll	Cytiva	17089101
ProLong Gold Anti-Fade Mounting Media	ThermoFisher	P36934
Streptavidin-Alexa 647	ThermoFisher	S32357
TGFβ	Peprotech	240-B-002
THC	Cerillant	T-093
Antibodies		
Alexa Fluor 700 Mouse Anti-Mouse CD45.2	ThermoFisher	56-0454-82
APC Mouse Anti-Mouse NK1.1	ThermoFisher	17-5941-82
APC Rat Anti-Mouse IFN-gamma	Biolegend	505810
BV421 Mouse Anti-Human IFN-gamma	BD Biosciences	564791
BV786 Hamster Anti-Rat/Mouse CD49a	BD Biosciences	740919
FITC Mouse Anti-Human CD3	Biolegend	300406

FITC Hamster Anti-Mouse CD3e	ThermoFisher	11-0031-82
FITC Rat Anti-Mouse CD19	ThermoFisher	11-0193-82
FITC Rat Anti-Mouse F4/80	ThermoFisher	11-4801-82
Pacific Blue Rat Anti-Mouse IFN-gamma	Biolegend	505818
PECF594 Rat Anti-Mouse CD11b	BD Biosciences	562287
PE-Cy7 Mouse Anti-Human CD14	BD Biosciences	557742
PE-Dazzle 594 Mouse Anti-Human CD56	Biolegend	300406
PerCP-eFluoro 710 Rat Anti-Mouse CD122	ThermoFisher	46-1222-82
Equipment		
Aperia ScanScope XT	Leica Biosystems	
Custom Gas Mixture	AirLiquide	
Hypoxia Incubator Chamber	Stemcell Technologies	27310
Eclipse Ni microscope and DS-Qi2 camera	Nikon	
LSRFortessa Flow Cytometer	BD Biosciences	
Experimental Mouse Models		
CD57BL/6J	The Jackson Laboratory	000664
Software		
FACSDIVA Software	BD Biosciences	
FlowJo Software	BD Biosciences	
ImageJ with Fiji Image Processing Package	NIH	
OlyVia	Olympus	
Prism Software (version 7.0)	GraphPad	
NIS-Elements AR Image Analysis Software	Nikon	

2.2 Methods: In Vivo Experiments

2.2.1. Generation of Timed Pregnancies

CD57BL/6J mice were obtained from the Jackson Laboratory (Strain #: 000664) and then bred and housed in specific pathogen-free conditions at McMaster's Central Animal Facility. A maximum of 5 mice were housed per cage in a temperature-controlled environment under a 12-hour light-dark cycle. Mice had access to water and irradiated Teklad global 18% protein diet (cat #: 2918). For generating timed pregnancies, one to three reproductively mature female CD57BL/6J

mice (9-11 weeks old) were paired with one male CD57BL/6J mouse (9-11 weeks old) overnight. The following morning, females were examined for the presence of a hard, occlusive vaginal copulation plug; females with a plug were designated at gestation day (GD) 0.5.

2.2.2. Cannabis Administration During Pregnancy

Starting on GD 6.5 mice received either 100 μL of high CBD oil (20 mg/kg body weight, Symbl), 100 μL of high THC oil (20 mg/kg body weight, Symbl), or 100 μL of control medium-chain triglyceride (MCT) oil (Nutiva) daily via gavage until GD 11.5. The high CBD and high THC oil were made and diluted in MCT oil. Dams were weighed at GD 0.5 and then daily from GD 6.5 up until GD 15.5. Mice were weighed and then euthanized by cervical dislocation at either GD 10.5, 12.5 or 18.5, depending on experiment outcome.

2.2.3. Mouse Tissue Processing

Fetal and Placental Tissue

At GD 18.5, dams were weighed and then euthanized via cervical dislocation. Their abdomens were opened, and uteri were excised via cuts at the cervix and utero-tubal junction. Intact implantation sites were then photographed and assessed for number of viable and resorbed fetuses. A fetus was deemed as resorbed if it had a black discoloration and was smaller in size. Then individual implantation sites were separated, and the myometrial tissue was removed. The placenta was then separated from the fetus, and then either transferred intact to 4% paraformaldehyde for histology or removed of excess tissue and weighed. The fetuses from each dam were photographed and weighed. To measure fetal brain weight, the skull cap was removed, and brain tissue collected and weighed. Crown-rump length was measured from the crown to base of the tail of each fetus using the Fiji image processing package on the ImageJ software with the evaluator blinded to treatment group. Fetal head length was also measured using Fiji on ImageJ from the nose to

posterior aspect of the head of each fetus with the evaluator blinded to treatment group. Both fetal head length and crown-rump length were measured on the images taken of GD 18.5 fetuses.

Implantation Sites for Histology

At GD 10.5 and 12.5, dams were euthanized, their abdomens opened, and uteri excised via cuts at the cervix and utero-tubal junction. Intact implantation sites (2-4 per dam) were separated and fixed in 4% paraformaldehyde for 48 hours, then stored in 70% ethanol. Fixed implantation sites were cut mid-sagittal to generate two symmetric sections and embedded in paraffin. For GD 18.5 implants, the uteri were excised, and placenta separated from the fetus and fixed in 4% paraformaldehyde for 48 hours, then stored in 70% ethanol. Fixed placentae were then cut mid-sagittal to generate two symmetric sections and embedded in paraffin.

Decidual Cell Isolation

On GD 10.5, the uteri were removed in a similar manner to isolating implants for histology. The individual implantation sites were then separated, and myometrial tissue removed to expose the decidua and fetal tissue. The fetal tissue, including the amnion and yolk sac were then removed and discarded. The major lymphoid aggregate of pregnancy (MLAp), decidua, and placenta from each implantation site were then pooled and weighed per dam. A small amount of RPMI media (200 μL) was added, and tissues were immediately homogenized, and cells pelleted via centrifugation at 800 x g for 5 minutes as previously described (116). After centrifugation, the supernatants were collected and stored at -80°C until use. The remaining cell pellet was then enzymatically digested in 2.5 mL of RPMI media containing 50 μg/mL Liberase TM (Sigma-Aldrich) and 50 μg/mL DNase I (Sigma-Aldrich) for 30 minutes at 37°C on a ThermoMixer. After digestion, the cell suspension was passed through a 70 μm nylon cell strainer and the collected cells were pelleted via centrifugation (1600 rpm for 10 minutes). The cells were then counted and

resuspended either for flow cytometry staining or further processing for cytokine stimulation. The cells used for cytokine stimulation were then subject to a 40%/80% Percoll gradient to enrich decidual leukocytes.

Splenocyte Isolation

Pregnant mice at GD 18.5 were euthanized by cervical dislocation, their abdomens opened, and spleens excised. The spleens were then mechanically digested into single cell suspension via the blunt end of a syringe plunger. Cells were then passed through a 70 μm nylon cell strainer, centrifuged at 1600rpm for 10 minutes, and then incubated in Ammonium-Chloride-Potassium (ACK) lysis buffer for 45 seconds to remove red blood cells. Cells were then washed with complete RPMI media (10% fetal bovine serum (FBS), 1% Hepes, 1% L-Glutamine, 1% Penicillin Streptomycin), counted, and then resuspended for cytokine stimulation.

2.2.4 Implantation Site Histology

H&E and PAS Staining

Cross-sections of paraffin embedded implantation sites or placentas were cut and stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS) by members of the McMaster Immunology Research Centre Histology Core Facility. Slides were scanned at 40x magnification with Aperia ScanScope XT (Leica Biosystems). Histology images were taken using Olympus OlyVia Software. Placental area (GD 12.5 and GD 18.5) and spiral artery diameter (GD 10.5) were analyzed using the Fiji image processing package on ImageJ software of H&E cross-sections (2x and 20x magnification, respectively) with the investigator blinded. Placental area was measured from the base of the placenta at the chorionic plate to the trophoblast giant cells and averaged per implantation site. Vessel to lumen diameter was measured by dividing the vessel diameter by the lumen diameter with measurements taken at the widest point of the vessel. The vessel to lumen

diameter was then averaged per implantation site. The number of vessel-associated uNK cells, deemed as PAS+ cells located within the vessel wall, were counted on PAS-stained GD 12.5 implantation sites with the investigator blinded. The number was then averaged per implantation site.

Immunofluorescent DBA Staining

Paraffin-embedded implantation sites were sectioned at 4 μm using a rotary microtome and dried overnight. Slides were incubated at 65˚C for 15 minutes, dewaxed in Xylene, and rehydrated through a series of ethanol solutions of decreasing concentration ending with distilled water. Slides were washed three times with phosphate buffered saline (PBS), and non-specific binding was blocked by incubation with 10% goat serum in 0.1% Tween20 buffered PBS for 1 hour. Next the slides were incubated with biotinylated Dolichos Biflorus Agglutinin (DBA, Vector Labs B1035-5), diluted 1:200 in blocking buffer, overnight at 4˚C. The next morning, sections were washed and incubated for 1 hour with Streptavidin-Alexa 647 (ThermoFisher; S32357, 1:500 in PBS with 0.1% Tween 20). Sections were counterstained with DAPI (1 mg/mL diluted 1:5000 in PBS) for 5 minutes. Slides were then washed, and cover slipped with ProLong Gold Anti-Fade Mounting Media (ThermoFisher; P36934). Fluorescence was imaged using a Nikon Eclipse Ni microscope and Nikon DS-Qi2 camera. Fluorescence was quantified using NIS-Elements AR image analysis software as area of image DBA+ (Cy5+) divided by total image area with the investigator blinded to treatment group.

2.2.5 Ex Vivo Cannabinoid Incubation and IFN-γ Production

Splenocyte Stimulation with Cannabinoids

After digestion and ACK lysis, the remaining cells were resuspended to $1x10^6$ cells/mL in complete RPMI media and stimulated with high dose IL-15 (100ng/mL Peprotech catalogue #

210-15) for 10 hours in the presence or absence of THC (Cerillant catalogue # T-093) or CBD (Cerillant catalogue # C-045) at 0.1-1 μg/mL. After 2 hours of the 10-hour incubation had elapsed, BD Golgi Stop (BD Biosciences catalogue # 51-2092KZ) was added. Following the incubation, cells were stained for viability, NK cell markers, and IFN-γ.

Decidual Leukocyte Stimulation with Cannabinoids

Decidual leukocytes isolated from GD 10.5 implantation sites were resuspended to $1x10^6$ cells/mL in complete RPMI media. Cells were stimulated with 100 ng/mL of IL-15 (Peprotech catalogue # 210-15) and 10 ng/mL of IL-12 (Peprotech catalogue # 210-12) for 18.5 hours as previously described (141). THC or CBD at 0.1 and 1 μg/mL were also added to the cell culture for the 18.5-hour incubation. 4 hours before the end of the incubation, BD Golgi Stop was added. After the incubation, cells were stained for viability, extracellularly for uNK cell markers, and intracellularly for IFN-γ.

2.2.6. Cytokine and Chemokine Array on Decidua Supernatants

A 31-plex mouse cytokine/chemokine array was conducted on decidual tissue supernatants from GD 10.5 implantation sites via Eve Technologies. The quantity of cytokine and chemokines were then calculated per gram of collected tissue.

2.3. Methods: Human Experiments

2.3.1. PBMC Isolation and Cannabinoid Incubation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human whole blood via density centrifugation using Lymphoprep (StemCell Technologies catalogue # 07861). Cells were then stimulated with high dose IL-15 (100ng/mL) (Peprotech catalogue # 200-15) for 24 hours in the presence or absence of THC or CBD (0.01-10 μg/mL) in complete RPMI media. 10 hours prior

to the end of incubation, BD Golgi Stop was added. After the 24-hour incubation, cells were stained for viability, NK cell identification markers, and for IFN-γ.

2.3.2. NK Cell Isolation and Production of Regulatory NK Cells

NK cells were freshly isolated from healthy human whole blood using MACSxpress Whole Blood NK Cell Isolation Kit (Miltenyi Biotec catalogue # 130-127-695). Following isolation, NK cells were incubated at $1x10^6$ cells/mL in complete RPMI media containing low dose IL-15 (10ng/mL), TGFβ (2ng/mL, Peprotech catalogue # 240-B-002) and 5-Aza-2′-deoxycytidine (Aza, 1μM) (Sigma-Aldrich catalogue # A3656), and cultures were maintained for 3 days under hypoxia (1% O_2) as previously described (142). For maintenance of cultures under hypoxia, cells were kept in a plastic chamber (Stem Cell Technologies catalogue # 27310) filled with a custom gas mixture of 1% O_2, 5% CO_2, and balance N_2 (Air Liquide). Following the 3-day incubation, cells were counted and then resuspended in the same culture conditions of IL-15, TGFβ, Aza and 1% hypoxia, but now with or without the addition of CBD or THC (0.1 and 1μg/mL). Following the 3-day incubation, cell-free culture supernatants were collected and stored in -80°C until use. The supernatants were then assessed for VEGF or IL-8 content via an ELISA (R&D Systems catalogue # DY293B and DY208, respectively). The cells were also stained with fixable viability dye after the incubation to assess viability.

2.4. Methods: General

2.4.1. Flow Cytometry

Human NK cells were identified first via Fixable Viability Dye (APCCy7) then via CD14 (PECy7), CD3 (FITC), CD56 (PE-Dazzle 594), and IFN-γ (BV421). Mouse splenocyte NK cells were identified first via Fixable Viability Dye (APCCy7) then via CD45.2 (Alexa Fluor 700), CD3e (FITC), NK1.1 (APC), and IFN-γ (Pacific Blue). Mouse uNK cells were identified first via

Fixable Viability Dye (BV510 or APCCy7) then via CD45.2 (Alexa Fluro 700), CD3e (FITC), CD19 (FITC), F4/80 (FITC), CD11b (PECF594), CD122 (PerCP eFluoro 710), CD49a (BV786), NK1.1 (BV421), and IFN-γ (APC). For IFN-γ intracellular staining, the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences catalogue # 554715) was used.

2.4.2. Statistical Analysis

All statistical analysis was performed using GraphPad Prism 7 software. All comparisons made between two conditions were analyzed using an unpaired t-test. A one-way ANOVA followed by a Tukey's multiple comparisons test was used when comparing more than two conditions. Analysis of two independent variables was conducted using a two-way ANOVA with Sidak correction for multiple comparisons. Odds ratios were calculated with Fisher's exact test. Correlations were calculated using a Pearsons correlation. Significance was defined as $P<0.05$.

Chapter 3: Results

3.1 Consumption of Cannabis Oil from Early to Mid-Gestation Impairs Fetal Growth

It is well established that exposure to cannabis during pregnancy is associated with several adverse pregnancy outcomes such as fetal growth restriction and low birthweight (24). However, the method of cannabis consumption is often not specified in human studies, and animal models typically focus on smoke inhalation or intraperitoneal injection of only THC (140; 80). Cannabis oil, and in particular CBD oil, is growing in popularity as it provides the desired cannabis effects while avoiding the harm of smoke inhalation (14; 10). Additionally, CBD oil is thought to provide numerous health benefits without the psychoactive effects of THC (11; 12; 13). Thus, we first aimed to assess whether oral consumption of either high CBD or high THC oil from early to mid-gestation leads to abnormal fetal outcomes in our mouse model. We chose to administer cannabis from early to mid-gestation as typically maternal cannabis use drops substantially during the third trimester (18). Additionally, we are interested in how cannabis may impact immune-mediated remodelling of the decidua which typically ends around GD 12.5.

Prior to assessing the effect of cannabis, we first validated whether oral gavage is a feasible delivery method. Female CD57BL/6J mice were co-housed with a male of the same strain overnight and if the following morning a copulation plug was apparent the mice were deemed at gestation day (GD) 0.5. Mice then received either 100 μL of control medium chain triglyceride (MCT) oil via gavage daily from GD 6.5-11.5 or were left untouched. Mice were euthanized on GD 12.5 and their implantation sites removed (Figure 1A). Compared to mice left untouched, mice receiving daily gavage had higher numbers of viable implantation sites, which might be due to the increased fat content in their diet from the daily gavage of oil (Figure 1B). Furthermore, mice receiving daily gavage had comparable numbers of resorbed fetuses and resorption rates to

untouched mice (Figures 1C and D). Thus, daily gavage of oil does not seem to increase fetal resorption or decrease the number of viable fetuses by GD 12.5 making it a feasible delivery method for cannabis oil. However, in all future experiments control mice will receive 100 µL of control MCT oil via gavage to standardize the gavage process across groups.

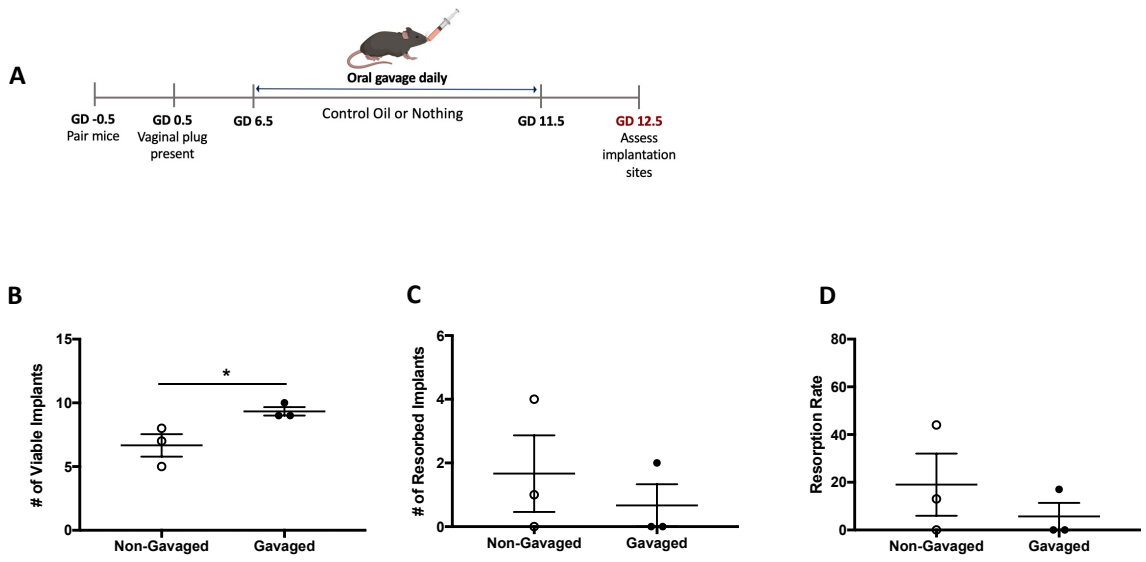

Figure 1: Daily Gavage of Pregnant Mice Does Not Increase Fetal Resorption by GD 12.5
A-D: Pregnant CD57BL/6J mice received either daily administration of 100 µL of control MCT oil from GD 6.5-11.5 or were left untouched. **A:** Schematic illustrating experiment timeline. **B-D:** Following euthanization on GD 12.5 the uteri were excised, and implantation sites observed. **B:** Number of viable implants. **C:** Number of resorbed implants. **D:** Resorption rate shown as percent of implants resorbed out of total implants. Data are means ± SEM of 3 replicates per condition. *P<0.05 (B-D, two-tailed t test).

For our animal experiments, we chose to use commercially available cannabis oil that pregnant women would have access to at their local retailer. The oils we used were deemed "high THC" and "high CBD" oil to represent the high ratio of either THC or CBD, respectively. For example, the high THC oil contained over 90% THC with less than 10% CBD. However, due to prior studies finding that the concentration of cannabinoids in some commercially available

cannabis products does not align with the labelled dosage, we first validated the levels of THC and CBD in our oils (143). The levels were quantified via high-performance liquid chromatography coupled to a mass spectrometer (LC-MS) with assistance from the Centre for Microbial Chemical Biology at McMaster University. The concentration of cannabinoids in the cannabis oils, as determined by LC-MS, are indicated in Table 1. Thus, for future experiments we calculated the dosage using the concentration determined by the LC-MS.

Sample ID	Description	[CBD] mg/mL	[THC] mg/mL	Avg [CBD] mg/mL	Avg [THC] mg/mL
Sample 1A	High CBD Oil	17	0.53	17 ± 0.41	0.52 ± 0.02
Sample 1B		17	0.50		
Sample 1C		17	0.53		
Sample 2A	High THC Oil	0.12	17	0.12 ± 0.002	17 ± 0.34
Sample 2B		0.12	18		
Sample 2C		0.12	17		

Table 1: Quantification of Cannabinoids in Cannabis Oil via LC-MS
Samples of the commercially available high THC and high CBD oil (Symbl) were measured for levels of cannabinoids via high-performance liquid chromatography coupled to a mass spectrometer (LC-MS) via the Centre for Microbial Chemical Biology at McMaster University.

To assess the effects of cannabis oil on fetal outcomes at term, pregnant mice received either high CBD, high THC, or control MCT oil from GD 6.5-11.5 via gavage, and were then euthanized at GD 18.5 (Figure 2A). There was no difference in maternal weight gain throughout the pregnancy or in the number of viable fetuses at term between the treatment groups (Figure 2B

and C). However, there was a trend for an increase in number of resorbed fetuses and in resorption rate in dams receiving high CBD oil (Figure 2D and E). Figure 2F shows a representative image of two fetal resorptions from a CBD treated mouse.

We next measured fetal weight and crown-rump length in mice receiving cannabis oil, as it appeared that fetuses from mice receiving high THC oil were smaller (Figure 2G). Mice receiving high THC oil had significantly lower fetal weights at GD 18.5 compared to controls with a 4.9% reduction in mean weight (1.16 ± 0.08 in high THC vs. 1.22 ± 0.07 in controls) which corresponded to a reduced crown-rump length (Figure 2H and I). This reflects what has been found in several human studies, as women using cannabis while pregnant exhibit significantly lower birthweights (24). While mice receiving high CBD oil did not show significantly lower fetal weight or crown-rump length at GD 18.5, there was a high variability in fetal weights with some fetuses being very small (Figure 2H). Given this, we quantified the percent of fetuses that would be considered small for gestational age (SGA) and fall below the 10^{th} percentile of control weights. It appeared that both mice treated with high THC and high CBD oil showed trends for elevated odds of having fetuses deemed as SGA (Figure 2J). The odds ratio is most likely not significant at this point due to the much lower n number within the control group (control n=22, high THC n=45, high CBD n=44) thus the experiment should be repeated in future to see if a similar trend occurs.

Children who experience *in utero* growth restriction and are SGA at birth exhibit significantly lower childhood cognitive outcomes than children with normal birthweight (144). Maternal cannabis use is also associated with abnormal brain development, reduction in fetal head circumference, and an increased likelihood of the child experiencing cognitive impairments (24; 145). Thus, we next assessed whether exposure to high CBD oil or high THC oil would affect brain weight and head size at GD 18.5. While there is a trend for decreased fetal brain weight in

mice receiving high CBD oil, the fetal head length was significantly smaller in both mice receiving high CBD or high THC oil (Figure 2K and L).

Lastly, we examined the placentas of pregnant mice treated with high THC or high CBD oil. There appeared to be no difference in the cross-sectional area or weight of the placentas between groups at GD 18.5 (Figure 2M-O).

These results show that consuming either high THC or high CBD oil during early to mid pregnancy can both cause several abnormal fetal outcomes at term. Previous studies have found intraperiotoneal injection of THC or cannabis smoke exposure to cause low birthweight in mice, however little work has been done on CBD (80; 140). As the popularity and positivity towards CBD oil as a self-treatment for a variety of different conditions continues to increase it is imperative to highlight the negative pregnancy outcomes associated with *in utero* exposure.

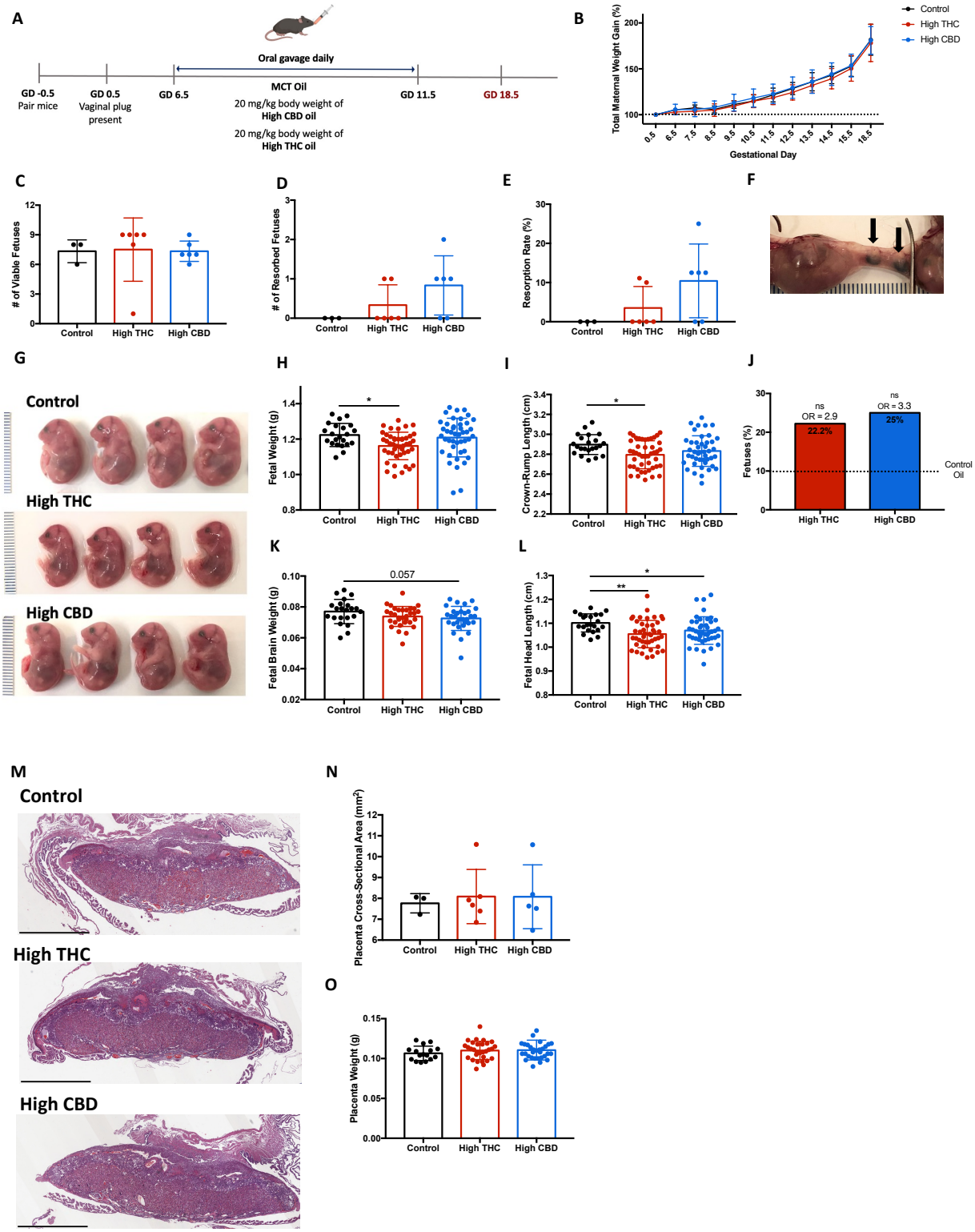

Figure 2: Oral Consumption of CBD or THC Oil from Early to Mid-Gestation Affects Fetal Development at Term

A-O: CD57BL/6J mice were gavaged with either high CBD, high THC, or control MCT oil from GD 6.5-11.5 and euthanized on GD 18.5 to assess fetal outcomes. **A:** Schematic illustrating experimental design. **B:** Maternal weight gain throughout the pregnancy shown as % weight gain. **C:** Number of viable fetuses. **D:** Number of resorbed fetuses. **E:** Resorption rate shown as percent of fetuses that were resorbed out of total fetuses. **F:** Image showing two resorbed embryos from a CBD oil treated mouse. **G:** Representative picture of fetuses. **H:** Fetal weight. **I:** Crown-rump length. **J:** Percent of fetuses deemed as small for gestational age (SGA). **K:** Fetal brain weight. **L:** Fetal head length. **M-O:** Placentas were separated from the fetus and fixed in paraformaldehyde prior to paraffin embedding. **M:** Representative images of placentas stained with H&E. **N:** Average placental cross-sectional area from each dam measured on placentas stained with H&E. **O:** Placental weight of freshly isolated placentas. Data are means ± SEM of 3 to 45 replicates per condition, *P<0.05, **P<0.01 (B, two-way ANOVA; C-I, K-O, one-way ANOVA; J, odds ratio with Fisher's exact test).

3.2 Cannabis Oil Disrupts Implantation Site Remodelling

During early pregnancy, the maternal decidua is extensively remodelled to support fetal development. This highly regulated process involves immune-mediated remodelling of decidual spiral arteries and trophoblast differentiation and invasion to establish the placenta (146). The significantly reduced fetal weights we observed in dams treated with high THC oil may be due to inadequate decidual remodelling being unable to support the extensive growth demands of late-stage pregnancy (147). Thus, we next sought to examine whether consumption of cannabis oil might be disrupting implantation site development earlier in the pregnancy. Pregnant mice received either high THC, high CBD, or control MCT oil via gavage from GD 6.5-11.5 and were euthanized either at GD 10.5 or 12.5 (Figure 3A). We first examined placental size in our THC and CBD oil treated pregnant mice as previous reports have shown that exposure to THC or CBD causes abnormal trophoblast proliferation *in vitro,* and aberrant endocannabinoid signalling *in vivo* leads to defective trophoblast invasion (148; 149; 80). While there may be a trend for lower placental area in THC oil treated mice, there is a significant reduction in placental area in pregnant mice treated with CBD oil (Figure 3B and C). Given the sheer importance of the placenta during

pregnancy, a reduction in size could account for some of the abnormal fetal outcomes we see in dams receiving CBD oil.

Next, we assessed decidual morphology in cannabis treated mice. Typically, by GD 12.5 the decidua has lower cell density as spiral artery remodelling is complete and uterine immune cells begin to decline in number (150). However, pregnant mice receiving high CBD oil appeared to have a higher density of cells located within their decidua compared to control treated or THC treated mice (Figure 3D-F).

The decidua in early murine and human pregnancy is populated by numerous immune cells, with uNK cells comprising nearly 70% of leukocytes in the first trimester human decidua (89). uNK cells contribute extensively to decidual remodelling by producing angiogenic factors to promote vascular remodelling, anti-inflammatory mediators to suppress immune activation, and chemokines to regulate trophoblast migration (106; 114; 116). To examine whether the dense population of cells within the decidua of CBD oil treated mice were uNK cells we first performed staining with Periodic Acid Schiff's (PAS) reagent on GD 10.5 and 12.5 implantation sites. PAS stains the cytoplasmic granules within uNK cells and classically any PAS+ cells within the decidua are defined as uNK cells (151). It appeared that PAS staining of GD 12.5 implantation sites showed a similar high density of PAS+ cells within the decidua of CBD treated mice compared to control and THC treated mice (Figure 3G-I). Thus, this high density of cells is likely an accumulation of uNK cells within the decidua which is abnormal for this point in gestation. We next looked at uNK cell morphology on GD 10.5 implantation sites, as GD 10.5 is typically when the decidua has peak uNK cell number (150). While the uNK cells were large and packed with numerous clearly defined cytoplasmic granules within the control mice, the uNK cells in CBD treated mice appeared to be less defined with large spaces devoid of granules within their cytoplasm (Figure 3J and L).

Additionally, on observation the uNK cells in both THC oil and CBD oil treated mice, but particularly in the THC oil treated mice, appeared smaller than the control uNK cells (Figure 3J-L). After decidualization, the maturation of uNK cells begins as uNK cells become progressively larger and acquire cytoplasmic granules (152). Thus, the smaller, less granular cells present in THC oil treated mice may be indicative of uNK cell immaturity (Figure 3K). Future work is required to quantify the cell size of PAS+ uNK cells within the treatment groups.

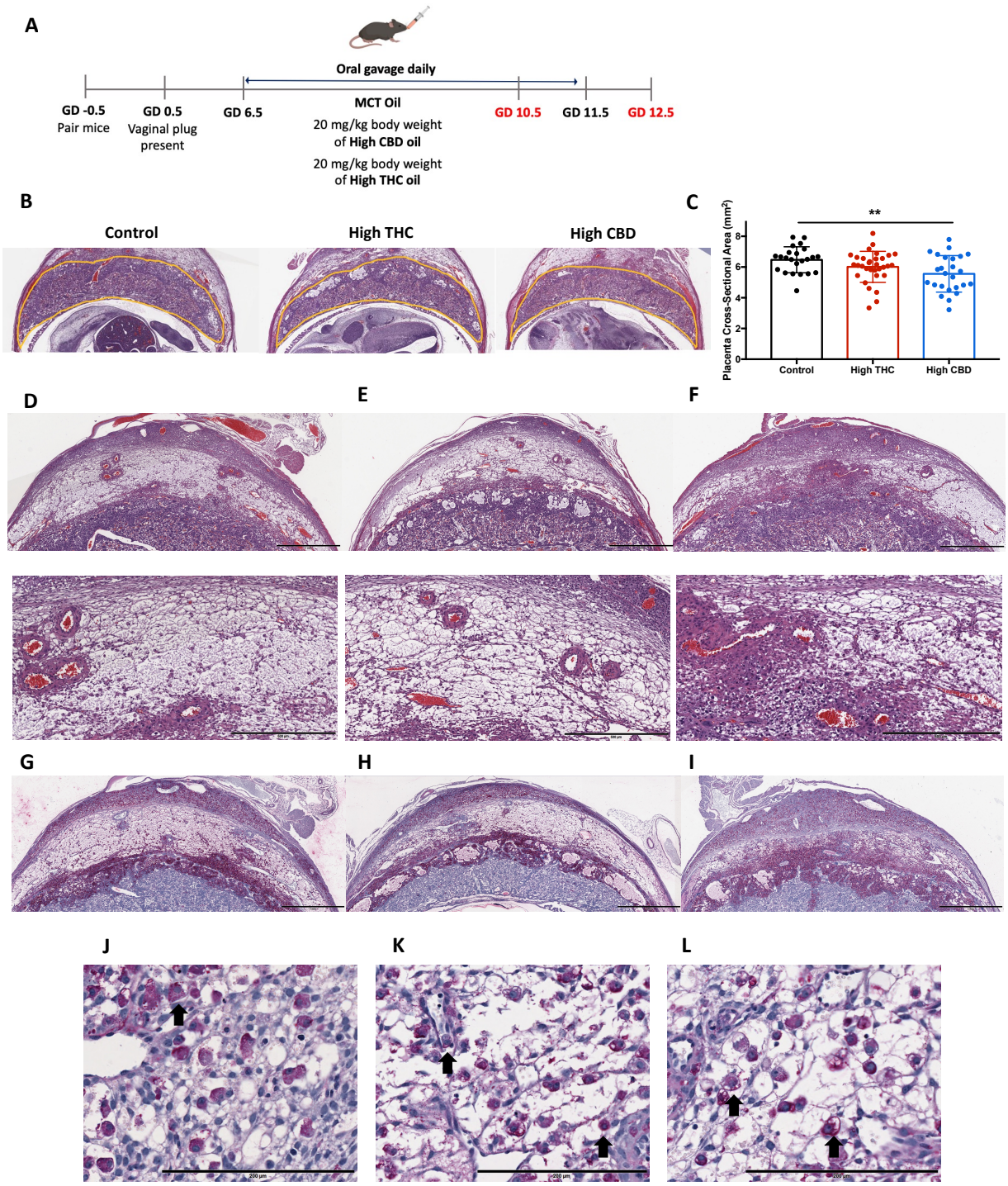

Figure 3: Consumption of Cannabis Oil Disrupts Placental Size and uNK Cell Morphology **A-L:** Implantation sites were collected on either GD 10.5 or 12.5 following gavage of high CBD, high THC, or control MCT oil from GD 6.5-9.5 or 11.5, respectively. Implantation sites were isolated, fixed in paraformaldehyde, and embedded in paraffin. **A:** Schematic illustrating experimental design. **B:** Representative H&E images of GD 12.5 placenta at 2x magnification. **C:** Quantification of placental cross-sectional area averaged per implantation site. **D-F:**

Representative H&E images of GD 12.5 decidua at 2x and 10x magnification from **D** control, **E** high THC and **F** high CBD oil treated mice. **G-I**: Representative PAS images of GD 12.5 decidua at 2x magnification from **G** control, **H** high THC and **I** high CBD oil treated mice. **J-L:** Representative images of PAS+ uNK cells within the GD 10.5 decidua of **J** control, **K** high THC, and **L** high CBD oil treated mice at 30x magnification. Black arrows point to PAS+ uNK cells. Data are means ± SEM of 23-32 replicates per condition, **P< 0.01 (C, one-way ANOVA).

To quantify the number of uNK cells within the decidua of cannabis oil treated mice we performed immunofluorescent *dolichos biflorus* agglutinin (DBA) staining on implantation sites isolated at GD 10.5 and 12.5. DBA is a popular marker for identifying uNK cells during murine pregnancy as it selectively stains for both uNK cell membranes and cytoplasmic granules (153). Compared to control mice, it appeared that mice receiving CBD oil had more abundant DBA+ uNK cells within their decidua at GD 10.5 and 12.5 while THC oil treated mice were more comparable to controls (Figure 4A-C, H-J). Quantification of DBA fluorescence (Area DBA+/Total Area) indicates that while not significant, there may be a trend for elevated DBA+ staining in GD 10.5 decidua (Figure 4D). However, there is a significant elevation in DBA+ staining in GD 12.5 decidua indicative of increased uNK cell number (Figure 4K). Typically, DBA+ uNK cells proliferate within the decidua and peak in number between GD 10.5-12.5 and then begin to steadily decline afterwards (152). It appears that there may be an increase in recruitment or local proliferation of uNK cells in CBD-oil treated mice. Interestingly, the uNK cells in the decidua from CBD oil treated mice and to a slightly lesser extent in THC oil treated mice also appeared to cluster together in large clumps which was not seen in control mice (Figure 4E-G). This clustering of cells is abnormal and could potentially indicate altered functioning of the uNK cells.

Nonetheless, an appropriate number of uNK cells within the decidua is essential for maintaining a healthy pregnancy. A higher number of uNK cells within the decidua has been linked to several pregnancy complications in women such as recurrent pregnancy loss (RPL) and may be a contributing factor to the pregnancy complications we see in CBD oil treated mice (154; 155). However, the high uNK cell number could also be a result of the decidua trying to compensate for impaired uNK cell function.

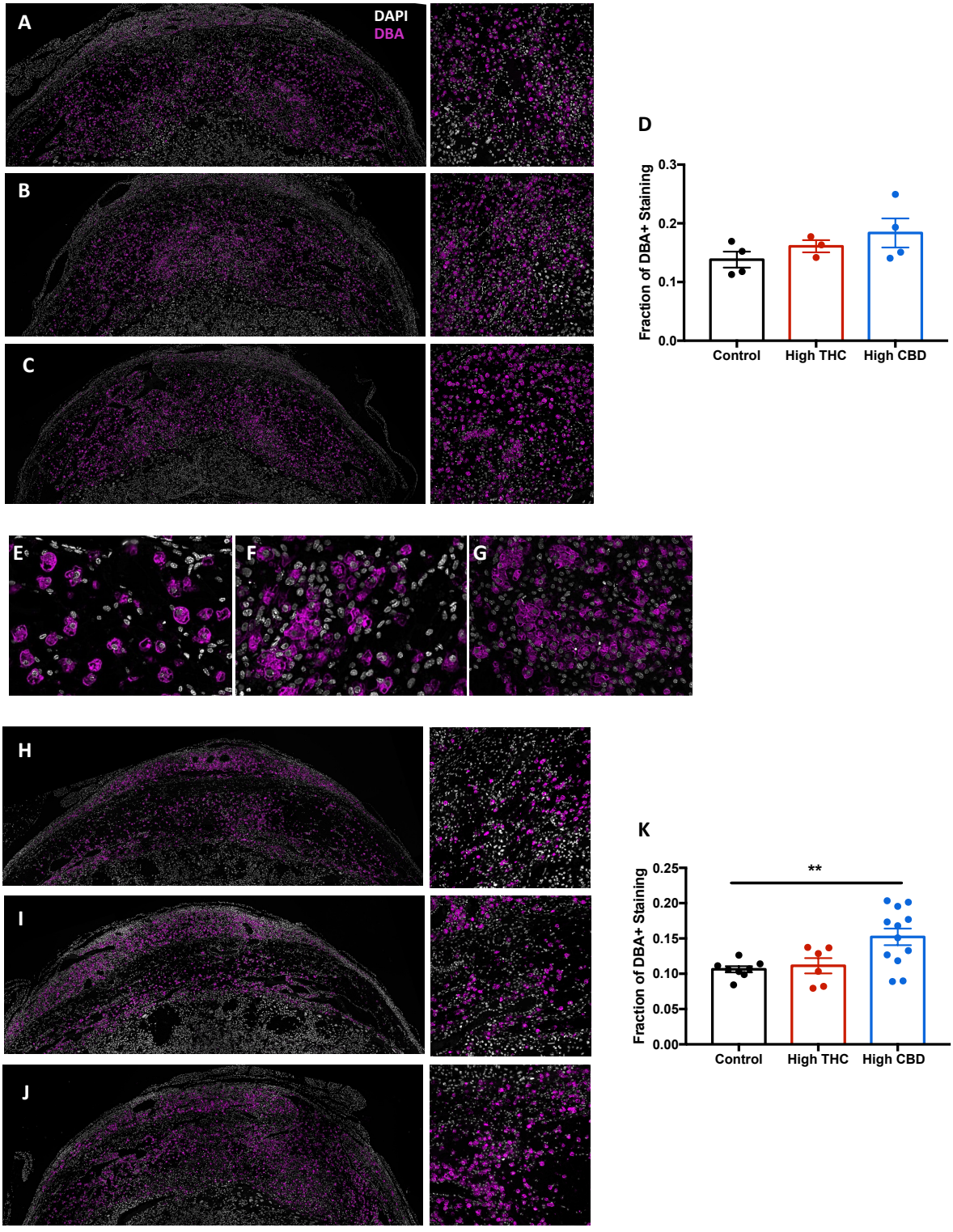

Figure 4: Exposure to CBD Oil Increases the Number of DBA+ uNK Cells Within the Decidua at GD 12.5

A-K: Implantation sites were collected and fixed on either GD 10.5 or 12.5 following gavage of high CBD, high THC, or control oil from GD 6.5-9.5 or 11.5, respectively. Fixed implantation sites were then sectioned and stained for DBA. **A-C:** Representative DBA/DAPI immunofluorescence images of GD 10.5 decidua at 10x and 20x magnification from **A** control, **B** high THC and **C** high CBD oil treated mice. **D:** Quantification of DBA+ area/whole area averaged per dam. **E-G:** Representative DBA/DAPI immunofluorescence enlarged images of GD 10.5 DBA+ uNK cells from **E** control, **F** high THC and **G** high CBD oil treated mice at 20x magnification. **H-J:** Representative DBA/DAPI immunofluorescence images from GD 12.5 decidua at 10x and 20x magnification form **H** control, **I** high THC and **J** high CBD oil treated mice. **K:** Quantification of DBA+ area/whole area averaged per dam. Data are means ± SEM of 3-12 replicates per condition, **P<0.01 (D&K, one-way ANOVA).

We also tried to quantify the proportion and populations of uNK cells via flow cytometry following enzymatic digestion of isolated decidual tissue. However, there were high variabilities within the populations and no clear difference between treatment groups potentially due to the difficulties with isolating the large, granular uNK cells from murine tissues (Figure 5A-G). uNK cell quantification via histology may be more representative as the implantation sites remain intact and we can see the number and distribution of uNK cells within the decidua.

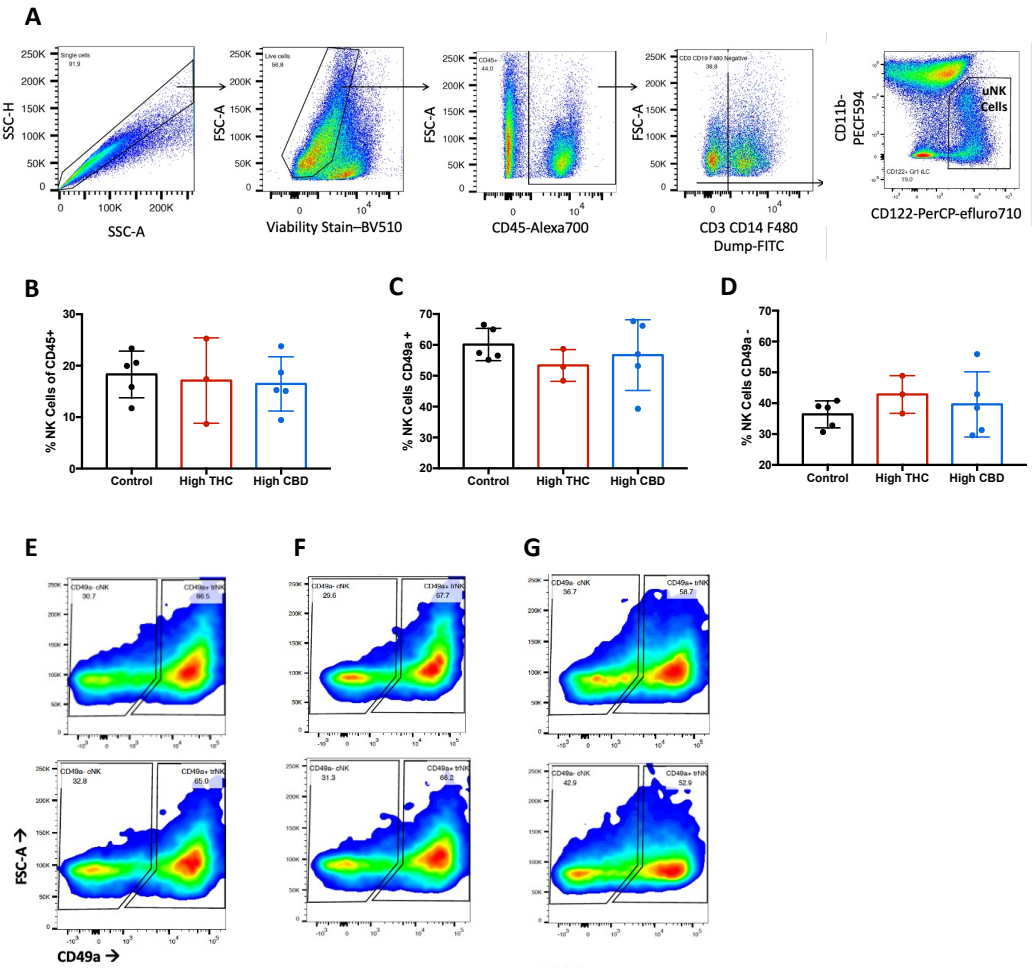

Figure 5: Cannabis Oil Does Not Affect Percent of NK Cells Detected via Flow Cytometry at GD 10.5

A-G: Implantation sites were excised from pregnant mice at GD 10.5 after treatment with either control MCT oil, high THC, or high CBD oil from GD 6.5-9.5. Decidual tissues were then digested into single cell suspension and stained for flow cytometry. **A:** Representative gating strategy to detect uNK cells which were deemed as CD45+ CD3- CD19- F4/80- CD122+. **B:** Percent of CD45+ leukocytes that are uNK cells. **C:** Percent of uNK cells that are trNK (CD49a+). **D:** Percent of uNK cells that are cNK (CD49a-). **E-G:** Representative flow plots of cNK and trNK cell populations. **E:** Control. **F:** High THC. **G:** High CBD. Data are means ± SEM of 3-5 replicates per condition (B-D, one-way ANOVA).

3.3 Cannabis Oil Disrupts Vascular Remodelling in Murine Pregnancy

Given the higher levels of uNK cells within the decidua of CBD oil treated mice and the potentially smaller uNK cells within THC oil treated mice, we next sought to examine whether uNK cell functions are impacted by cannabis. uNK cells are critical drivers of the vascular remodelling process during pregnancy, which is the production of dilated, elongated, low-resistance vessels that lack a smooth muscle layer to allow optimal blood flow to the maternal-fetal interface (156). Therefore, we measured spiral artery wall thickness in GD 10.5 implantation sites from cannabis oil treated mice. Compared to control, both THC oil and CBD oil treated mice had significantly increased vessel to lumen diameter indicating an abnormally thick vessel wall and reduced vascular remodelling (Figure 6A and B). We counted the number of uNK cells located within the vessel walls and found that while there were no significant differences there may be a trend for less uNK cells associated with vessels in pregnant mice receiving THC oil (Figure 6C and D).

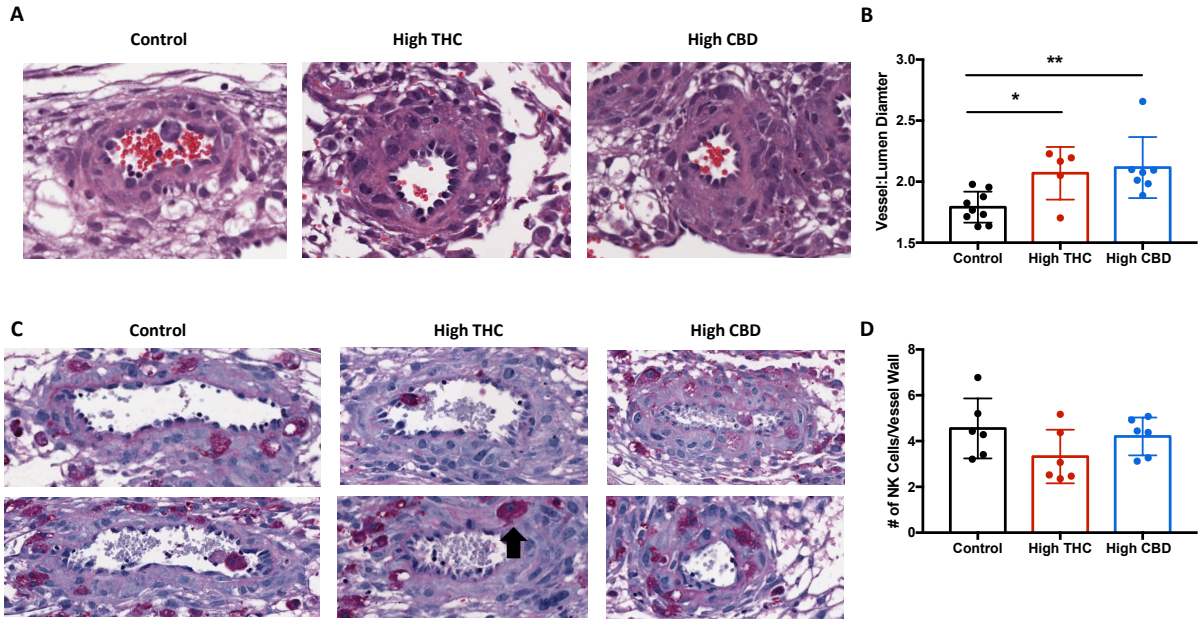

Figure 6: Both THC and CBD Oil Impair Spiral Artery Remodelling in Pregnant Mice

A-D: Implantation sites isolated from GD 10.5 or GD 12.5 mice treated with or without CBD or THC oil, were fixed and embedded. **A:** Representative images of spiral arteries from H&E-stained GD 10.5 implantation sites. **B:** Quantification of vessel to lumen diameter of spiral arteries from GD 10.5 mice averaged per implantation site. **C:** Representative images of spiral arteries stained with PAS from GD 12.5 implantation sites with an example of a uNK cell indicated by the arrow. **D:** Number of PAS+ uNK cells located within the vessel wall averaged per implantation site. Data are means ± SEM of 5-9 replicates per condition, *P<0.05, **P<0.01 (B & D, one-way ANOVA).

uNK cells are known to promote spiral artery remodelling by production of angiogenic factors as well as secretion of MMPs that directly degrade the vascular extracellular matrix (112). The production of IFN-γ by uNK cells is a major mediator in regulating vascular remodelling in both mice and humans (114; 115). Interestingly, preliminary reports have found that both THC and CBD are able to reduce IFN-γ production from murine T cells and THC treatment greatly diminishes the production of IFN-γ from murine pbNK cells (131; 129). Thus, we next aimed to assess if THC or CBD impaired NK cell IFN-γ production in stimulated murine splenocytes from our pregnant control mice (Figure 7A). THC appeared to decrease IFN-γ expression in NK cells by over 50% in the highest tested dose, without affecting cell viability, however as of now this is only a n=2 (Figure 7B-D). The effect of CBD on NK cell production of IFN-γ seemed more varied and would need to be repeated (Figure 7E).

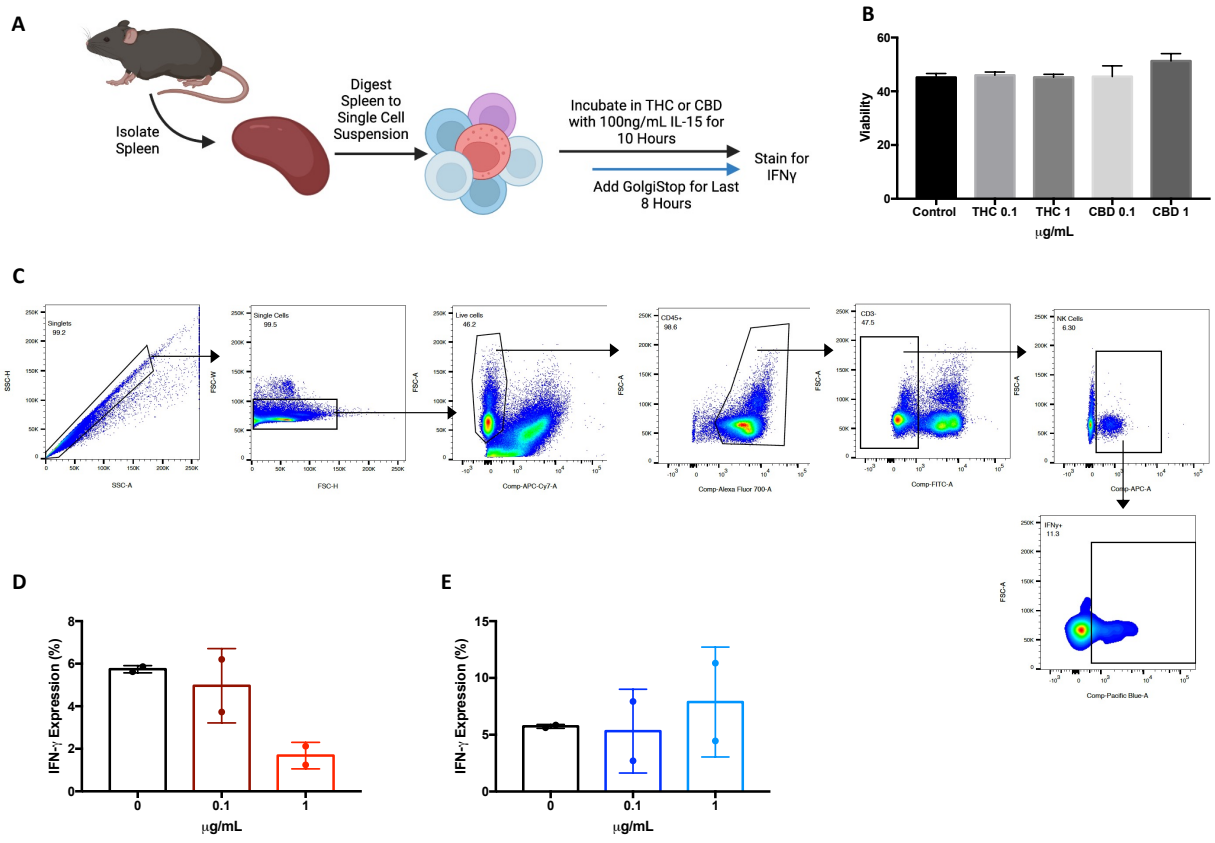

Figure 7: THC May Decrease IFN-γ Expression in NK Cells from Murine Splenocytes
A-E: Bulk splenocytes were isolated and stimulated with high dose IL-15 (100 ng/mL) in the presence or absence of THC or CBD (0.1 & 1 μg/mL) for 10 hours. After the incubation, cells were stained intracellularly for IFN-γ. **A:** Schematic of experimental design. **B:** Viability of splenocytes after incubation. **C:** Gating strategy to identify IFN-γ+ NK cells within bulk splenocytes. NK cells deemed as CD45+ CD3- NK1.1+. **D:** IFN-γ percent expression in NK cells following THC exposure. **E:** IFN-γ percent expression in NK cells following CBD exposure. Data are means ± SEM of 2 replicates per condition.

Due to the substantial involvement of IFN-γ in uNK cell-mediated vascular remodelling and the ability of THC to diminish IFN-γ production in murine spleen NK cells, we next sought to determine whether cannabinoids disrupted uNK cell production of IFN-γ. Uterine immune cells were isolated through enzymatic digestion and subsequent Percoll enrichment of GD 10.5 decidua from control mice. Then, the isolated immune cells were stimulated with IL-15 (100 ng/mL) and

IL-12 (10 ng/mL) for 18.5 hours in the presence or absence of THC or CBD. Following incubation, the cells were stained for uNK cell markers and stained intracellularly for IFN-γ expression (Figure 8A). While the results currently represent only an n of 1, THC may potentially reduce the expression of IFN-γ in murine uNK cells while CBD may not (Figure 8B-F). However, the experiment must be repeated with more mice to draw any conclusions. THC could be impairing uNK cell production of IFN-γ similarly to pbNK cells resulting in under-developed vessels that are unable to support the extensive growth demands of late-stage pregnancy leading to the abnormally small fetuses we see in the THC oil treated mice.

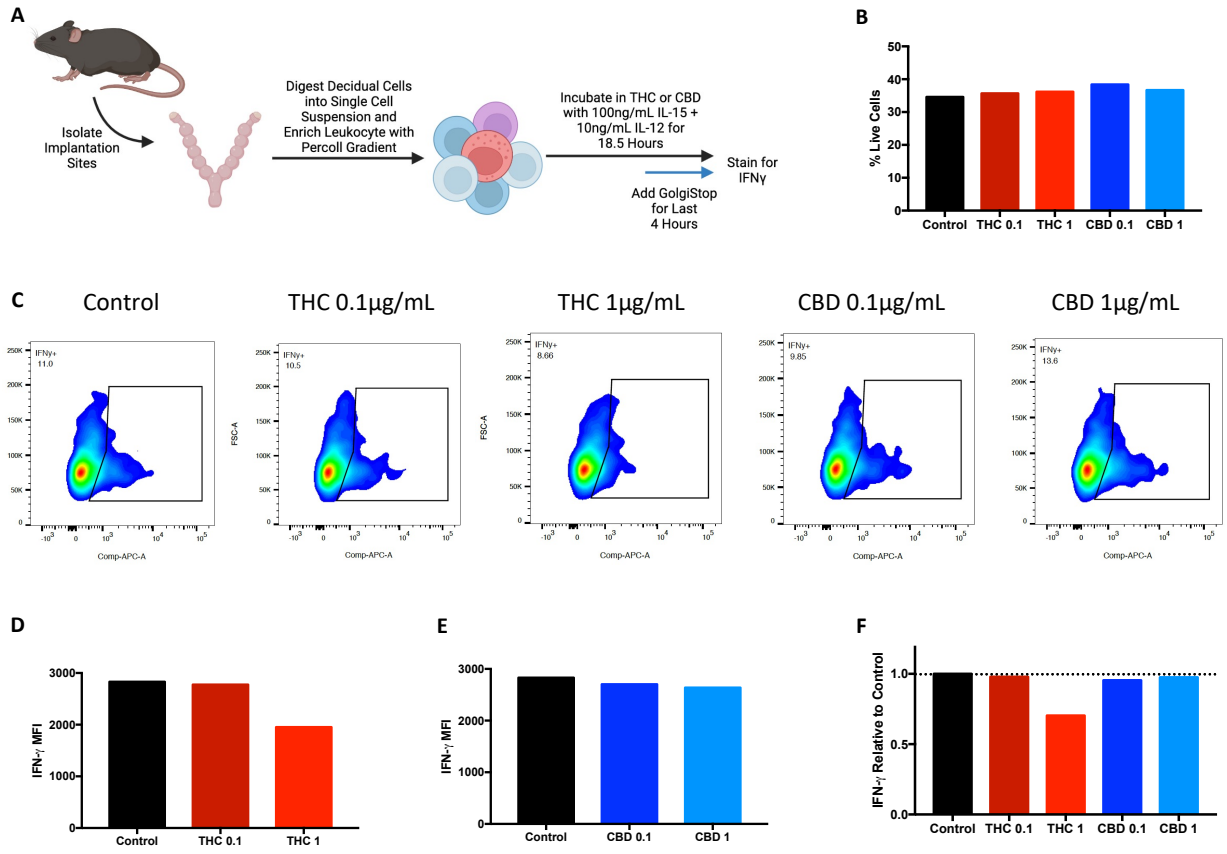

Figure 8: Incubation of Mouse uNK Cells with THC May Reduce IFN-γ Expression
A-F: Uterine leukocytes were isolated from GD 10.5 implantation sites and stimulated with IL-15 (100 ng/mL) and IL-12 (10 ng/mL) for 18.5 hours in the presence or absence of THC or CBD. **A:** Schematic representing experimental design. **B:** Viability of cells following the incubation. **C:**

Representative flow plots of IFN-γ expression in CD45+ CD3- CD19- F4/80- CD122+ uNK cells. **D:** Mean fluorescence intensity (MFI) of IFN-γ expression in uNK cells treated with THC. **E:** MFI of IFN-γ expression in uNK cells treated with CBD. **F:** IFN-γ MFI relative to control. Data is the mean of 1 replicate per condition.

3.4 CBD Oil May Increase uNK Cell Recruiting Cytokines

Inflammation is an important but tightly regulated process during pregnancy, thus deviations in several inflammatory mediators have been linked to pregnancy complications (157; 158; 159). As IFN-γ is only one of the many cytokines that are imperative during pregnancy, we next sought to perform a broad cytokine and chemokine array on the decidual tissue isolated from GD 10.5 mice. Although no cytokines or chemokines tested showed significant differences, many showed trends for higher levels in mice receiving high CBD oil (Figure 9A-N). Interestingly, the decidua of pregnant mice receiving high CBD oil had a trend for elevated levels of MIP-1α and IL-15 (Figure 9A and N). During pregnancy, IL-15 is imperative for uNK cell recruitment and development as *IL15*$^{-/-}$ mice lack uNK cells (160). Additionally, MIP-1α produced by cytotrophoblasts has been found to substantially promote NK cell migration *in vitro* (161). Thus, the elevated levels of MIP-1α and IL-15 may potentially explain the elevated number of uNK cells we see in the high CBD oil treated mice (Figure 4K). Moreover, a proper balance of IL-15 is essential for a healthy pregnancy, as altered levels of IL-15 have been associated with pregnancy complications (157). Here, we see that higher levels of IL-15 were significantly correlated with a lower number of viable fetuses (Figure 9O). Thus, the reduced spiral artery remodelling seen in high CBD oil treated mice may stimulate enhanced IL-15 and MIP-1α production to recruit more uNK cells to accommodate for their impaired functioning.

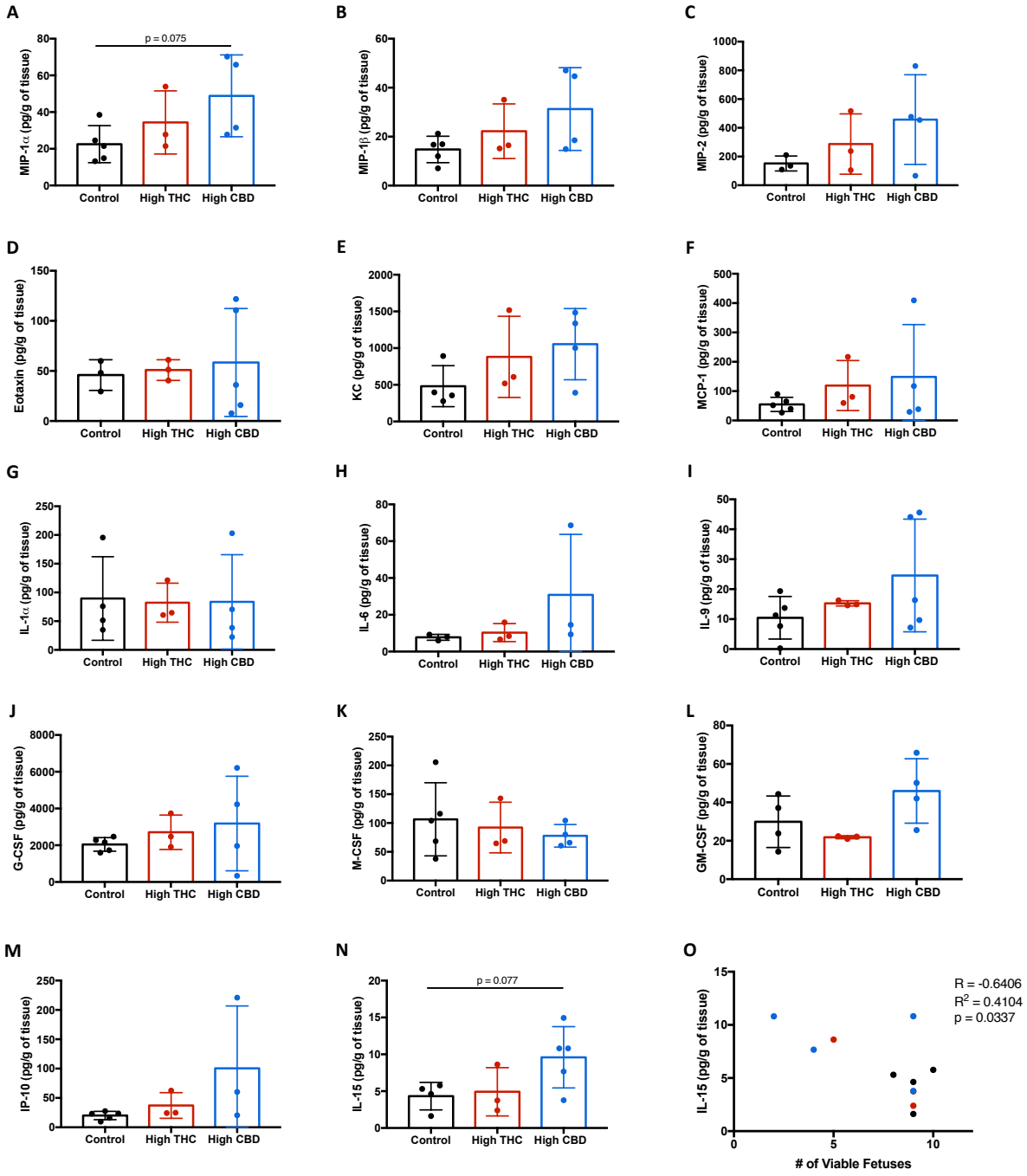

Figure 9: Cytokine and Chemokine Array on GD 10.5 Decidual Tissue

A-O: Decidual and placental tissue were separated from fetal tissue, pooled together and mechanically digested. Supernatants were collected and a cytokine/chemokine array was performed by Eve Technologies. **A-N:** Cytokine and chemokine levels per gram of harvested tissue. **A:** MIP-1α, **B:** MIP-1β, **C:** MIP-2, **D:** Eotaxin, **E:** KC, **F:** MCP-1, **G:** IL-1α, **H:** IL-6, **I:** IL-9, **J:** G-CSF, **K:** M-CSF, **L:** GM-CSF, **M:** IP-10, **N:** IL15. **O:** Correlation between number of

viable fetuses and level of IL-15 in the decidua. Data are means ± SEM of 3-5 replicates per condition (A-N, one-way ANOVA; O, Pearson correlation).

3.5 Cannabinoids Reduce Angiogenic Factor Production in Human NK Cells

As we saw THC reduce IFN-γ expression in mouse splenocytes, and other groups have seen similar results in mice pbNK cells, we next sought to determine whether cannabinoids impact human NK cell IFN-γ production. We isolated peripheral blood mononuclear cells (PBMCs) from healthy human blood and stimulated them with high dose IL-15 (100 ng/mL) for 24 hours in the presence or absence of various concentrations of THC or CBD (Figure 10A). THC significantly reduced IFN-γ expression in pbNK cells relative to control by over half, without affecting cell viability (Figure 10B and E). However, it took a higher concentration of CBD to reduce NK cell IFN-γ expression to a similar degree as THC (Figure 10C and F). Thus, THC clearly reduces IFN-γ expression in both murine and human NK cells, but the effect of CBD may require higher concentrations.

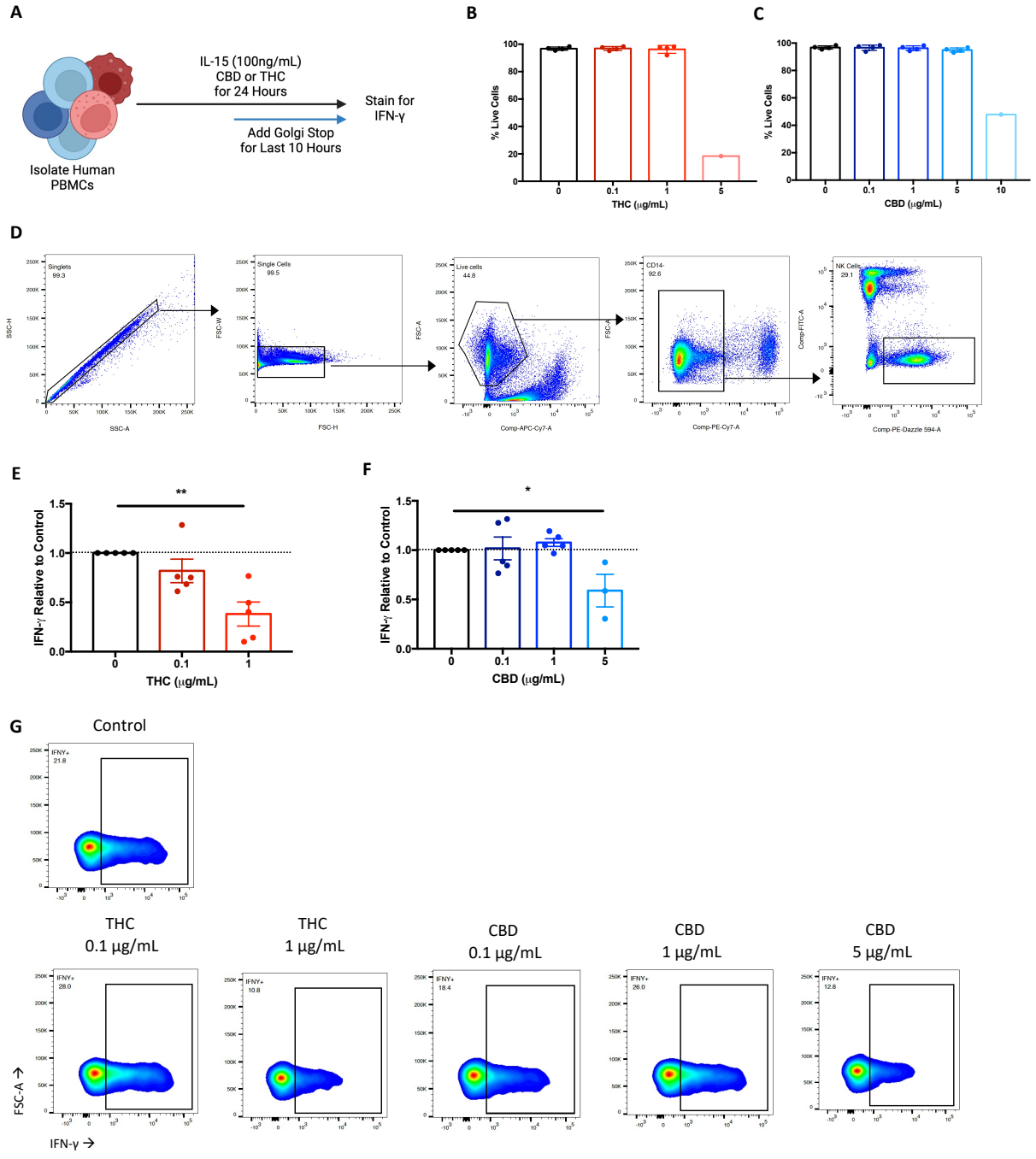

Figure 10: Cannabinoids Decrease IFN-γ Expression in Human pbNK Cells

A-G: PBMCs were isolated from healthy donor blood and incubated in the presence of absence of THC or CBD for 24 hours and then stained for IFN-γ. **A:** Schematic illustrating experimental design. **B:** Viability of NK cells treated with various concentrations of THC. **C:** Viability of NK cells treated with various concentrations of CBD. **D:** Representative gating strategy for NK cells identified as CD14- CD3- CD56+. **E:** IFN-γ expression in NK cells relative to control following incubation in THC. **F:** IFN-γ expression in NK cells relative to control following incubation in

CBD. **G:** Representative flow plots of IFN-γ expression in NK cells treated with THC or CBD. Data are means ± SEM of 1-5 replicates per condition, *P<0.05, **P<0.01 (E-F, one-way ANOVA).

pbNK cells, however, differ significantly from uNK cells in terms of their phenotype and function. pbNK cells are cytotoxic anti-tumour effector cells and uNK cells are non-cytotoxic and pro-tumour (150). Therefore, we aimed to assess the effects of THC and CBD on NK cells that are more similar in function to uNK cells. Based off a protocol previously published by Cerdeira et al. we generated *in vitro* induced regulatory-like NK cells (NKreg) from regular human cytotoxic pbNK cells (142). Freshly isolated pbNK cells were incubated with low dose IL-15 (10ng/mL), TGFβ (2ng/mL), and Aza (1μM) in hypoxic conditions (1% O_2) for 3 to 7 days. These cells are deemed "regulatory" as they exhibit low cytotoxicity and low IFN-γ production but secrete high levels of VEGF and promote tumour growth. We incubated the NKreg cells in THC or CBD for three days and then assessed cytokine levels in the culture supernatants (Figure 11A). So far, we only have an n of 2 but potentially both CBD and THC may reduce VEGF production, although it must be repeated to draw conclusions (Figure 11B-D). Like IFN-γ, VEGF is another angiogenic factor that is significantly implicated in uNK cell-mediated vascular remodelling (113). This could explain why we see significant impairments in vascular remodelling in both THC oil and CBD oil treated mice but varying levels of effects on IFN-γ production. The impaired spiral artery remodelling we see in pregnant mice receiving THC or CBD oil could be due to the cannabinoids disrupting different aspects of uNK cell-mediated angiogenesis.

Another function of uNK cells is to regulate trophoblast migration through the maternal tissue during pregnancy. Hanna et al. demonstrated that uNK cells promote trophoblast invasion via the production of IL-8 and IP-10. Considering we saw significantly smaller placental areas in CBD oil treated mice we questioned whether cannabinoids could be disrupting the ability of uNK

cells to modulate trophoblast invasion (Figure 3C). Following culture of our NKreg cells with CBD or THC we performed an IL-8 ELISA on culture supernatants. As of now, our results represent only an n of 1 but we see that the level of IL-8 is reduced by roughly 50% in the culture that received 1 μg/mL of THC (Figure 11E). Meanwhile, CBD at the lower concentration of 0.1 μg/mL may not reduce IL-8 production, but higher concentrations like those used in the VEGF and IFN-γ experiments should be tested (Figure 11E). The experiment must be repeated in future with more donors and higher concentrations of CBD but may indicate there is an effect of cannabinoids on NKreg production of IL-8. Considering Rompala et al. found that the *CXCL8* gene, which encodes IL-8, was significantly reduced in the placenta of maternal cannabis users it could be another factor impacted by cannabis (27).

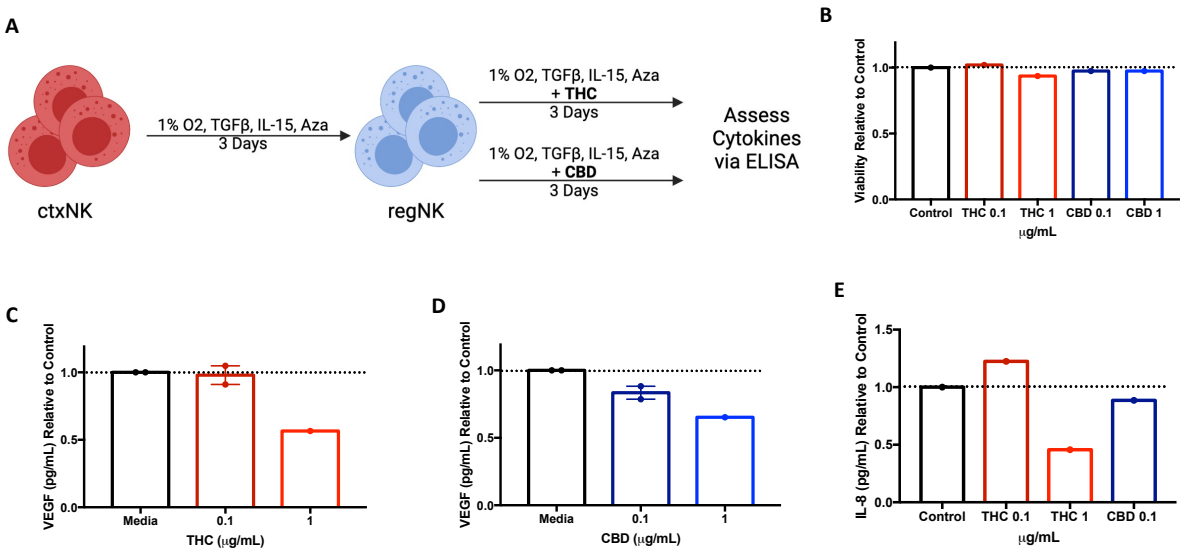

Figure 11: Cannabinoids May Reduce Cytokine Production by Regulatory-Like Human NK Cells

A-E: Cytotoxic pbNK cells (ctxNK) were isolated from healthy donor blood and incubated with TGFβ, IL-15, and Aza under hypoxia for 3 days. Cells were then cultured in the same condition for another three days but in the presence or absence of THC or CBD. **A:** Schematic representing experimental design. **B:** Viability of NKreg cells following 3-day culture with cannabinoids. **C:** Level of VEGF in culture supernatant after 3-day incubation with THC. **D:** Level of VEGF in culture supernatant after 3-day incubation with CBD. **E:** Level of IL-8 in culture supernatant after 3-day incubation with either THC or CBD. Data are means ± SEM of 1-2 replicates per condition.

Chapter 4: Discussion

The rate of maternal cannabis use is rising in Canada despite the clear risk of several adverse pregnancy outcomes such as low birthweight and preterm birth (1). Currently, the mechanisms driving these complications are not fully understood, as work has primarily been focused on placental development (80). However, there are many key processes that are essential for a successful pregnancy, such as the remodelling of the maternal decidua (138). Additionally, while cannabis smoke inhalation remains the most popular method of consumption, edible cannabis products like cannabis oil are gaining attention as alternative methods of cannabis use (10). Particularly CBD oil with its success as a treatment for various conditions like seizure disorders is often perceived as safer and good for one's health (11; 14). Despite the growing prevalence of maternal cannabis use and the increasing popularity of CBD, no work has directly compared the effects of CBD and THC oil on pregnancy outcomes. Thus, the present work compared fetal outcomes in mice exposed to either high THC or high CBD oil from early to mid-gestation. We found that THC oil reduced fetal weight, size, and fetal head length at term compared to control mice while CBD oil reduced fetal head size and showed a trend for increasing the rate of fetal resorption and risk of fetuses being considered as SGA. Therefore, our results suggest that consumption of either THC or CBD oil from early to mid-gestation leads to significant fetal abnormalities at term; highlighting that despite its 'safer' perception CBD oil also causes pregnancy complications.

A major objective of early pregnancy is to remodel the maternal environment to support fetal growth via the dilation of maternal vessels, proliferation of local immune cells, differentiation of stromal cells, and establishment of the placenta (138). Despite the significance of implantation site remodelling, little work has been done to examine how cannabis disrupts the maternal

adaptations to pregnancy. We next sought to investigate whether exposure to CBD or THC oil disrupts implantation site remodelling by analyzing histological sections from mice exposed to cannabis oil from GD 6.5-11.5. CBD oil significantly reduced placental area compared to control mice at GD 12.5. The implantation sites from CBD oil treated dams also displayed a higher density of uNK cells within their decidua. In addition to the increased number of uNK cells, there also appeared to be abnormal uNK cell morphology in both CBD oil and THC oil treated mice. Given that a major function of uNK cells in pregnancy is regulating vascular remodelling, we next assessed whether cannabis impaired this function. Compared to control, both THC oil and CBD oil treated mice demonstrated significantly thicker decidual spiral arteries indicative of reduced vascular remodelling. We attributed this in part to the ability of cannabinoids to reduce NK cell production of IFN-γ, a crucial mediator of NK cell-driven vascular remodelling (116). Our results demonstrate that THC, as well as CBD in higher doses, substantially reduces IFN-γ production in human pbNK cells, mouse splenocytes, and possibly mouse uNK cells isolated from GD 10.5 deciduae.

Ultimately, the present work highlights the detrimental harm of both THC and CBD oil on fetal outcomes and identifies impaired implantation site remodelling driven by abnormal uNK cell function as a potential mechanism.

4.1 Both CBD and THC Oil Lead to Pregnancy Complications at Term

THC Oil Reduces Fetal Weight

Numerous studies have found that maternal cannabis use significantly increases the risk of abnormal fetal development such as low birth weight, preterm birth, and reduced head circumference (24). Similar adverse outcomes have been identified in animal models of *in utero*

cannabis exposure. Exposure of pregnant mice to cannabis smoke from GD 5.5-17.5 resulted in significantly reduced birth weight and fetal organ weight (brain, lung, and liver) compared to room air exposed mice (140). Similarly, pregnant mice receiving an intraperitoneal injection of THC (5 mg/kg body weight) from GD 5.5-18.5 had significantly smaller pups and disorganized placenta (80). Our model using cannabis oil produced similar outcomes as these previous studies. Mice receiving THC oil from GD 6.5-11.5 exhibited significantly lower birthweights and smaller crown-rump length than control mice. We observed a 4.9% reduction in mean birth weight after exposure to THC oil whereas Benevenuto et al. observed a 9.9% reduction in mean birth weight in mice exposed to cannabis smoke (140). While this is over double the reduction we saw, we chose to only expose mice to cannabis oil from early to mid-gestation (GD 6.5-11.5) whereas the other group exposed mice to cannabis smoke from GD 5.5-17.5. This difference in treatment period could account for the difference in birth weights or it could be due to the difference in bioavailability following ingestion vs. inhalation. It could also be the effects of lung inflammation or exposure to other toxins following smoke inhalation. Nonetheless, we still observe a significant reduction in mean birth weight when mice receive THC oil from early to mid-gestation which may be a more relevant model given that maternal cannabis use drops substantially by the beginning of the third trimester. Additionally, since we see this decrease in birth weight at term despite stopping cannabis exposure at GD 11.5, the reduction in weight is likely due to changes occurring earlier in the pregnancy. One explanation may be the reduced vascular remodelling seen in THC oil treated mice being unable to support the rapid fetal growth occurring in the later stages of pregnancy.

Heightened Risk of SGA

While we saw a clear reduction in fetal weight in mice exposed to THC oil, there was only a non-significant reduction of 1.1% in fetal weights of mice receiving CBD oil. There was however

a high degree of variability in the weights of CBD oil exposed fetuses, with some being very small and others more comparable to control (standard deviation of CBD oil: 0.109g vs. control oil: 0.065g). Thus, we assessed how many fetuses would be considered as SGA and fall below the 10th percentile of control mice. While both THC oil and CBD oil treated mice exhibited a roughly three times higher odds of having SGA fetuses, the OR is not significant at this point most likely due to the small number in the control group causing large confidence intervals. Thus, this experiment should be repeated in future to see if a similar trend occurs. Nonetheless, the trend we observe matches what has been found in previous studies, as women who report cannabis use during their pregnancy have a significantly higher risk of having SGA fetuses (24).

One of the number one causes of SGA is pregnancy-induced hypertension, which affects numerous maternal vessels such as the decidual spiral arteries, umbilical arteries, and placental vessels (162). We observed that exposure to either high CBD or high THC oil from early to mid-gestation significantly impaired vascular remodelling by GD 10.5. The decidual spiral arteries were significantly thicker and appeared more circular and less elongated, all signs of reduced remodelling. We believe this is in part due to cannabinoids decreasing NK cell production of angiogenic factors like IFN-γ and VEGF. Nonetheless, the under-remodelled vessels in both CBD and THC oil treated groups may increase the odds of exposed fetuses being SGA, as the arteries are unable to provide adequate gas and nutrients to allow normal fetal growth.

The Risk of Preeclampsia in Maternal Cannabis Users

Another pregnancy disorder associated with abnormal spiral artery remodelling is preeclampsia. Preeclampsia is a pregnancy complication involving high blood pressure, proteinuria, and other symptoms that develops after 20 weeks of pregnancy (163). Severe preeclampsia can lead to fetal growth restriction and if left uncontrolled progress to eclampsia

which results in seizures (163). According to the World Health Organization, one-seventh of maternal deaths can be attributed to preeclampsia (164; 165). The underlying cause of preeclampsia remains unclear, however due to the substantial involvement of uNK cells in the vascular remodelling process their dysfunction has been investigated as a potential cause (163). Altered numbers of uNK cells have been found in both humans and mouse models with preeclampsia, with reports typically finding lower numbers of uNK cells within the decidua (166; 139). Additionally, women at higher risk of developing preeclampsia possess a specific KIR genotype that causes heightened inhibition of uNK cells (167). The suppressed uNK cells may then have a weakened ability to perform their essential functions like vascular remodelling and promoting trophoblast invasion.

However, while we observed impaired vascular remodelling in our pregnant mice that receive cannabis oil as well as evidence of impaired uNK cell function, preeclampsia is not typically a pregnancy complication associated with maternal cannabis use (168; 169). As mentioned previously, human studies often do not specify the method of cannabis consumption during pregnancy, but smoke exposure is the most common consumption method in the general population (10). Potentially, the reason why we observe impaired vascular remodelling is because we used cannabis oil rather than a smoking model.

Women who smoke tobacco during their pregnancy tend to have a lower risk of developing preeclampsia and exhibit increased levels of pro-angiogenic factors and decreased levels of anti-angiogenic factors in their blood (170). One reason tobacco smoke is believed to protect against preeclampsia is the exposure to carbon monoxide (CO). CO has been found to cause vasodilation of blood vessels within the placenta and uterus thereby enhancing utero-placental blood flow (171; 172). Like tobacco smoke, cannabis smoke also produces significant amounts of CO and thus may

counteract any hypertensive effects of THC or CBD (9). Therefore, the impaired vascular remodelling we see in mice exposed to cannabis oil may be apparent because the mice are not exposed to the several by-products of cannabis smoke like CO. This further supports the rationale for investigating the effects of different cannabis consumption methods on pregnancy outcomes, as the effects may be different. Additionally, in future it would be ideal to measure blood flow velocity and the resistance index of the uterine and umbilical arteries using micro-ultrasound Doppler between our cannabis oil groups and control mice to confirm that the altered vascular remodelling that we see does translate to increased blood pressure.

Cognitive Impairments After *in utero* Cannabis Exposure

The effects of *in utero* cannabis exposure go beyond pregnancy complications, as offspring often experience cognitive impairments and altered temperaments later in life (25). One study found that pups born from mice exposed to CBD oil before pregnancy, during pregnancy, and during lactation demonstrated increased anxiety behaviors and altered brain methylation (173). Additionally, offspring of women who report cannabis use during their pregnancy exhibit increased anxiety, aggression, and hyperactivity (134). Interestingly, these altered behaviours were correlated to impaired immune-related genes in the placenta, suggesting that immune dysregulation could be linked to the abnormal behavioural outcomes (134). Considering in our model we observe reduced fetal head size in both the CBD oil and THC oil exposed pups, the offspring may experience impaired cognitive development after birth. In future, it would be important to follow the pups after birth to observe their behaviour and cognitive development. One method is using the IntelliCage system which is an automated cage that assesses behaviours such as memory, navigation, anxiety, and impulsivity (174). It has been used previously to examine murine models of substance use disorders after exposure to drugs such as alcohol, morphine, and

THC (174; 175). Due to its automated nature, it also reduces the need for experimenter handling which could cause unnecessary stress on the pups. Ultimately, the reduced fetal head size that we observed in fetuses from both THC and CBD oil treated dams could lead to altered cognitive outcomes which should be studied in the future.

4.2 Cannabis Changes NK Cell Number and Size at the Maternal-Fetal Interface

In the pregravid murine uterus, small agranular immature uNK cells exist until implantation on GD 4.5 triggers their terminal differentiation. During this maturation process, the uNK cells proliferate and differentiate into large cells filled with numerous cytoplasmic granules and acquire DBA positivity (152; 153). These granules are filled with perforin and granzymes and are roughly triple the size of granules found in pbNK cells (176). uNK cells proliferate and reach peak numbers between GD 10.5-12.5 and then steadily decline afterwards with any remaining cells removed with the placenta at birth (152). When looking at the implantation sites of mice treated with either high THC or high CBD oil from early to mid-gestation, we saw several morphological differences within the uNK cell population.

CBD Oil Increases uNK Cell Number

First, we observed a high density of uNK cells within the decidua of GD 12.5 mice exposed to CBD oil via both PAS and immunofluorescent DBA staining. Typically, by GD 12.5 the decidua is not as densely packed with uNK cells as their responsibilities in vascular remodelling are complete (150). However, there was a significant increase in the number of DBA+ uNK cells located within the decidua at GD 12.5. Interestingly, the cytokine array on GD 10.5 supernatants revealed that there was a trend for elevated levels of IL-15 and MIP-1α in the decidua of CBD oil treated mice. IL-15 is a master regulator of uNK cell differentiation and proliferation, as *IL15*$^{-/-}$

mice lack uNK cells (160). Endometrial stromal cells begin to upregulate IL-15 production during the progesterone-dominated secretory phase of the menstrual cycle which is when uNK cells also begin to accumulate in preparation for pregnancy (177). After decidualization, the decidual stromal cells supply increasing amounts of IL-15 to promote local uNK cell proliferation (177). Previous studies have also showed that MIP-1α secretion by cytotrophoblasts promotes NK cell migration (161). Thus, potentially the elevation of IL-15 and MIP-1α explains the increased number of uNK cells within the CBD oil treated mice. Additionally, considering we observe impaired uNK cell functions in the CBD oil treated mice, the elevated number of uNK cells could be a compensatory mechanism in response to the impaired function.

In future, it would be beneficial to perform a dual immunofluorescent stain using DBA and Ki-67 to assess differences in proliferation between treatment groups. Additionally, it would be interesting to perform homing experiments by injecting labelled NK cells in the pregnant mice receiving CBD or THC oil, to identify if the increased uNK cell number in the CBD mice is due to local proliferation of cells or recruitment of circulating NK cells. The uNK cell population is thought to be comprised of both local proliferation of trNK cells and recruitment of conventional (cNK) cells from the periphery (178). trNK cells are abundant during early pregnancy and secrete growth promoting factors and vaso-active factors, whereas cNK cells accumulate in mid-pregnancy around placentation and are the major producers of IFN-γ (104; 176; 178). Thus, CBD might be promoting the proliferation of trNK cells, the recruitment of cNK cells, or both.

uNK Cells Appear Small in THC Oil Treated Mice

Another morphological difference within the uNK cell population, is that uNK cells in the decidua of high THC oil treated mice appeared to be smaller with less defined cytoplasmic granules. Small uNK cells with fewer cytoplasmic granules are typically considered less mature,

as uNK cells grow and acquire numerous granules during maturation (152). Several gene-modified mice strains that exhibit a higher proportion of immature uNK cells within their decidua exhibit abnormal implantation site development with impaired vascular remodelling (179). Additionally, THC has been found to reduce differentiation in other cell types like trophoblast cells, thus potentially THC is interfering with uNK cell differentiation during early pregnancy (75). These immature uNK cells may then be unable to perform essential NK cell functions resulting in the reduced spiral artery remodelling that we see in the THC oil treated mice. In future it is necessary to measure the diameter of these uNK cells to quantify if there is a significant difference in uNK cell size in THC oil treated mice.

uNK Cell Morphological Changes After Exposure to CBD Oil

While some of the uNK cells within the decidua of CBD oil treated mice appeared small like the cells in the THC mice, others looked more comparable in size to control. However, numerous of the larger uNK cells within the CBD mice lacked clearly defined cytoplasmic granules and exhibited large spaces void of granules. These irregularly shaped cells are similar in appearance to the subtype IV classification of DBA+ uNK cells which are thought to be senescent or apoptotic uNK cells (153). Thus, potentially the heightened number of DBA+ cells within the decidua of CBD oil treated mice are senescent or undergoing apoptosis. Cannabinoids have been found to increase apoptosis in other cell types, as treatment of trophoblast cells with THC caused activation of the effector caspases and upregulated expression of pro-apoptotic genes (74; 78). Additionally, THC is thought to cause immune suppression via induction of apoptosis in various immune cells (122). Treatment of T cells with THC has been shown to trigger apoptosis via caspase activity (122; 180). In future, we could do dual immunofluorescent staining with DBA and cleaved caspase-3 to assess the degree of apoptosis in our uNK cells exposed to CBD oil.

Nonetheless, there appears to be several morphological differences in uNK cells from either CBD oil or THC oil treated mice. These differences may account for the reduced effector functions we see, such as the impaired spiral artery remodelling.

4.3 Potential Mechanisms Driving Cannabinoid-Induced uNK Cell Dysfunction

In our pregnant mice that receive cannabis oil, we observe several morphological and functional differences that suggest uNK cell dysfunction. We also saw that exposure of human and mouse NK cells to CBD and THC *ex vivo* impairs their production of angiogenic factors like VEGF and IFN-γ. While it is likely that the cannabinoids disrupt NK cell functions through various mechanisms, below is a few potential avenues for future investigation.

Inhibition of STAT3 Signalling

Previous research has proposed that STAT3 suppression is a potential mechanism through which THC inhibits trophoblast invasion (80). STAT3 is a major transcription factor that regulates the migration and proliferation of many cell types, including trophoblast cells, and has been found to be essential for a successful pregnancy (80; 181). Chang et al. found that treatment of trophoblast cells directly with THC reduced STAT3 phosphorylation and subsequently trophoblast invasion. Additionally, they saw that phosphorylation of STAT3 was largely downregulated in the placenta of maternal cannabis users and in pregnant mice treated with THC (80). A similar phenomenon was found in T cells, as THC incubation inhibited STAT3 signalling via interactions with the CB2 receptor (182). Interestingly, the authors found that a downstream gene of the CB2-JAK-STAT3 pathway was *Ifng* which codes for IFN-γ (182). Thus, potentially THC is suppressing uNK cell IFN-γ production by binding to the CB2 receptor and inhibiting JAK/STAT3 signalling.

JAK1/STAT3 signalling is one of the ways through which IL-15 promotes the survival and various effector functions of NK cells (183). STAT3 is believed to be involved in NK cell IFN-γ production as inhibition of STAT3 phosphorylation via S31-201 diminishes NK cell IFN-γ expression following short term stimulation with IL-12, IL-15, and IL-18 (183). It is clear from both our data and work by previous groups that THC and possibly CBD reduce both mouse and human NK cell IFN-γ secretion (131). Potentially cannabinoids may be reducing IFN-γ by downregulating STAT3 phosphorylation in NK cells.

Additionally, STAT3 has been found to be essential for the accumulation of hypoxia-inducible factor 1-alpha (HIF-1α) in NK cells, which is a crucial mediator allowing NK cells to adapt to hypoxic environments (183). During early pregnancy, the uterus is a low oxygen environment with oxygen tension increasing once vascular remodelling is underway (184). The hypoxic nature of the uterus during early pregnancy is also thought to regulate immune cell phenotype and function, with NK cells exposed to hypoxia acquiring more regulatory functions like enhanced VEGF production (142). HIF-1α is the major inducer of VEGF expression in hypoxic conditions, so it's thought the hypoxic environment induces NK cells to secrete VEGF and promote angiogenesis (185; 176).

Cannabinoids may potentially be disrupting the ability of uNK cells to adapt to the low oxygen environment by suppressing STAT3 and subsequent accumulation of HIF-1α. In our model of converting cytotoxic pbNK cells to regulatory-like NK cells, we expose the NK cells to IL-15, TGF-β, Aza, and hypoxia (1% O_2). In preliminary experiments we see that culturing these NKreg cells with THC or CBD may impair their ability to secrete VEGF, albeit with only an n of 2 as of now. The cannabinoids may potentially be causing this inhibition by reducing HIF-1α and hampering the ability of the NK cells to thrive in the low oxygen environment. In future

experiments, it would be interesting to repeat the incubation but with addition of CB1 or CB2 receptor antagonists, as THC may disrupt STAT3 signalling via CB2 like with T cells. Additionally, we could perform western blotting on cell extracts to look at the level of STAT3 and HIF-1α. Ultimately, it would be beneficial to understanding the mechanism through which cannabinoids are impairing uNK cell functions to examine whether these functions could be restored.

Induction of ER Stress

Another proposed explanation for THC induced dysfunction is the induction of ER stress (81). ER stress occurs when there is an accumulation of misfolded proteins within the ER. If these misfolded proteins are not dealt with via the uncoupled protein response, apoptosis can be induced (186). Previous studies have suggested ER stress as a mechanism behind several adverse pregnancy outcomes such as IUGR. Administration of tunicamycin to chemically induce ER stress led to increased rates of growth restriction and lower placental weights in pregnant mice (82). This was in part due to the ER stress decreasing the expression of vascular-related genes within the placenta such as PlGF and VEGF receptor-1 (VEGFR1) (82). Another study found that the placentas from women who experienced IUGR or preeclampsia demonstrated signs of ER stress with expression of pro-apoptotic proteins and DNA damage (187).

THC has been previously shown to induce ER stress in neuronal cells, but Lojpur et al. also showed that treatment of trophoblast cells with THC increased ER stress markers and activated the uncoupled protein response (81). Moreover, pre-treatment with CB1 and CB2 receptor antagonists removed this response indicating THC-induced ER stress in trophoblast cells is driven by cannabinoid receptor engagement (81). Given that ER stress affects trophoblasts, it would be interesting to assess if uNK cells exposed to THC and CBD also experience ER stress.

We could assess for markers of the uncoupled protein response in uNK cells following exposure to either CBD or THC.

Impact on Cellular Metabolism

The importance of cellular metabolism in immune cell function is becoming increasingly clear, as it appears certain metabolic pathways not only support cell function but directly dictate functional output. Different classes of T cells have been found to utilize distinct metabolic fuel sources to elicit their specific functions (188; 189). For instance, activated T cells rely heavily on glucose and glutamine to fuel their robust proliferation and differentiation to the T_H1, T_H2, and T_H17 effector classes (188; 189). In contrast, inhibition of the glycolytic pathway with glucose analogue 2-deoxyglucose (2-DG) promotes differentiation into the Treg cell subset which relies primarily on exogenous fatty acids (189). Furthermore, metabolism has been greatly implicated in polarization of macrophage functionality, as different fuel sources support certain functions. The pro-inflammatory M1 subset relies primarily on glycolysis and the pentose phosphate pathway, while reducing oxidative phosphorylation (190). In contrast, fatty acid oxidation and oxidative phosphorylation fuel the more anti-inflammatory M2 macrophages (191).

In terms of NK cells, recent literature has demonstrated that glycolysis is a prerequisite for function (192). Keating et al. showed that a reduction in glycolysis, by culturing human NK cells in the presence of galactose, significantly decreased the secretion of IFN-γ compared to glucose treated controls. Additionally, NK cell antiviral function against cytomegalovirus infection was dependent on glycolysis, as treatment with the glucose inhibitor 2-DG resulted in higher viral burdens in infected C57BL/6 mice (193). Our lab has also shown that tumour associated NK (taNK) cells from ovarian cancer patients exhibit reduced glycolysis and oxidative phosphorylation, as well as exhibit fused mitochondria and reduced mitochondrial mass (194).

This reduction in metabolic activity directly corresponds to an impaired function, with taNK cells having decreased cytotoxicity and an inability to prevent tumour engraftment *in vivo* (194). Thus, immune cell functional output is critically linked to the cell's metabolic ability.

Cannabinoids have been shown to impair mitochondrial respiration and function in numerous cell types such as cancer and brain cells (195; 196). One proposed mechanism for THC induced trophoblast dysfunction is through inhibition of mitochondrial function. Treatment with THC significantly reduced maximal and basal cellular respiration measured via oxygen consumption rate in trophoblast cells (81). Additionally, THC reduced mitochondrial complex proteins and the mitochondrial mass of trophoblast cells (81). THC has also been shown to reduce mitochondrial membrane potential, increase mitochondrial stress, and increase ROS production in trophoblast cells (85). Lojpur et al. suggested that this ability of THC to disrupt mitochondrial function may be a key factor driving placental insufficiency in cannabis treated mice. Given the strong connection between cellular respiration and immune cell function, cannabinoids could be disrupting NK cell function via decreased oxidative metabolism. In future, it would be interesting to examine metabolic markers like nutrient receptors and mitochondrial mass as well as metabolic activity via Seahorse assays on NK cells following exposure to THC and CBD.

4.4 Conclusion

Despite the clear risk of several serious pregnancy complications, the prevalence of maternal cannabis use is rising (1). Little work has been done so far to uncover the mechanisms driving cannabis-induced pregnancy complications or assess whether all cannabinoids affect pregnancy outcomes. In the present work, we examined how exposure to either THC or CBD oil from early to mid-gestation led to abnormal fetal outcomes at term in a mouse model. We also assessed how cannabis exposure disrupted implantation site development and maternal immune cell function. Exposure to THC oil reduced fetal weight, length, and head size at term, corresponding to what has been found in previous studies (80; 140). Despite the often-positive view of CBD oil, we observed that its consumption also led to abnormal fetal outcomes such as a reduced head size and a trend for increased odds of being SGA.

In terms of implantation site remodelling, CBD oil caused substantial changes in uNK cell number and morphology, impaired vascular remodelling, and decreased placental area by mid-gestation. THC oil also reduced vascular remodelling and altered uNK cell morphology as they appeared to be smaller in size than control. As both THC and CBD oil resulted in impaired vascular remodelling, we assessed the ability of the cannabinoids to disrupt NK cell angiogenic factor production. Both THC and CBD substantially reduced human NK cell production of IFN-γ and may reduce NKreg production of VEGF. Ultimately, the impaired implantation site remodelling seen in both THC and CBD oil exposed dams may be unable to support the extensive growth demands of late-stage pregnancy resulting in the impaired fetal growth we see. The changes in decidual remodelling could be a result of cannabinoids disrupting uNK cell functions. Thus, cannabis oil consumption from early to mid-pregnancy results in substantial changes to immune cell function, implantation site remodelling, and ultimately fetal growth.

4.5 Future Directions

The following experiments are proposed as potential future directions of this project:

Aim 1: *Determine whether exposure to CBD or THC oil alters pregnancy outcomes in a mouse model.*

Thus far, we have examined changes to fetal development at term following exposure to CBD or THC oil. However, considering the effects of cannabis extend beyond pregnancy it would be interesting to follow the pups after birth to observe their cognitive development. We can examine whether *in utero* cannabis oil exposure leads to behavioural changes in the offspring such as heightened anxiety, inattention, and aggression which has been observed in human studies (25; 26; 27).

Aim 2: *Assess whether cannabis oil impairs implantation site remodelling in a mouse model.*

Our results indicate that cannabis oil results in reduced placental area, impaired vascular remodelling, and morphological changes within the uNK cells. To examine these changes further, we can perform additional histological analyses.

- Increased vessel wall thickness is one method to assess reduced vascular remodelling, however, we should also perform smooth muscle actin (SMA) staining on our implantation sites as the loss of SMA is an indicator for vessel remodelling (176). Additionally, to confirm if this impairment in vessel development corresponds to altered maternal blood flow, we should perform ultra-high frequency microultrasound Doppler to measure blood flow velocity of the uterine and umbilical arteries (147). Using a similar protocol to our term experiment, we can perform the ultrasound measurements at GD 12.5 and 14.5 when dilation of the uterine artery should be fully complete (147).

- There appeared to be morphological differences in the uNK cells from both mice treated with either CBD or THC oil. To quantify the cell size difference in THC oil treated mice, we can measure the cell size using ImageJ software and compare between the groups.
- One striking difference we observed was the significant elevation of DBA+ uNK cells within the decidua of CBD oil treated dams. As the uNK cell population within the decidua consists of both trNK cells and cNK cells, it would be interesting to see if this is a local proliferation of trNK cells or a recruitment of cNK cells. We can stain the implantation sites with Ki-67 to assess proliferation and perform homing experiments with fluorescently labelled NK cells injected during pregnancy.

Aim 3: *Pinpoint whether uNK cell functions are impacted by cannabinoid exposure.*

As of now, our results indicate that cannabinoids reduce human NK cell IFN-γ production and may reduce VEGF production by NKreg cells. However, angiogenic factor production is not the only function of uNK cells.

- The experiment assessing the ability of cannabinoids to suppress mouse uNK cell IFN-γ production must be repeated as it is currently only an n of 1. Additionally, we could also collect the supernatant following cannabinoid incubation and perform a broad cytokine and chemokine array to see if there are any other cytokines that may be impaired in uNK cells.
- We observe that THC and CBD reduce IFN-γ production in pbNK cells. It would be interesting to repeat this experiment but with the addition of either a CB1 or CB2 receptor antagonist to see if cannabinoid receptor engagement drives this inhibition.

- Our human data currently looks at cytokine production in pbNK cells or NKreg cells following cannabis exposure. However, pbNK cells differ substantially from uNK cells and while our NKreg cells behave similar they are still only a model of uNK cells. In future, it would be ideal to assess the effect of cannabinoid exposure on primary human uNK cells isolated from first trimester elective abortion procedures. We could look at uNK cell phenotype, expression of activating and inhibitory receptors, cytokine secretion, and ability to promote angiogenesis and trophoblast migration after exposure to cannabinoids *ex vivo*. If possible, we could also compare the functions of uNK cells isolated from either cannabis users or non-users. It would also be informative to perform RNA sequencing on the uNK cells isolated from users or non-users to help identify other functions that might be impacted or provide insight on the potential mechanism of action.

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