THE IMPLICATIONS OF DELTA-9-TETRAHYDROCANNABINOL ON LOCALIZED IMMUNE AND HORMONAL RESPONSES MEDIATED BY TROPHOBLASTS OF THE HUMAN PLACENTA

By HARMEET GURM, B.Sc.

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AUTHOR: Harmeet Gurm, B.Sc.

SUPERVISOR: Dr. Sandeep Raha

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LAY ABSTRACT

The human placenta is a pregnancy-specific organ that supports the health of the mother-to-be and fetus. Stem cells known as cytotrophoblasts undergo differentiation and fusion to support the establishment of the syncytium, which creates a boundary that separates the maternal and fetal circulations. In the case of cannabis consumption during pregnancy, its biologically active components can travel to the placenta, cross the syncytium, and enter fetal blood. Our primary objective was to determine how cannabis exposure can impact the formation and maintenance of the syncytium. While maternal use has been linked to short- and long-term consequences for child health, existing research lacks a complete understanding of the underlying mechanisms. We demonstrate that cannabis exposure alters the production of important immune and hormonal factors during cytotrophoblast fusion, which may play a role in mediating poor placental development. Ultimately, it is critical to explore the implications of cannabis use for female reproductive health due to a rising trend in its use.

ABSTRACT

Over the approximate nine months of its intrauterine existence, the development of the fetus is supported by the human placenta. This transient organ is central to pregnancy success as it facilitates maternal-fetal exchange, immunological tolerance, and hormone production. Villous trophoblasts mediate placental formation by engaging in a continuous turnover process of proliferation, differentiation, fusion, and apoptosis. In doing so, cytotrophoblasts and syncytiotrophoblasts maintain the integrity of the outer placental lining known as the syncytium. Exposure to drugs, however, can compromise placental establishment, which can in turn adversely impact pregnancy and fetal health. Specifically, cannabis is widely used by women of reproductive age and during pregnancy. While maternal cannabis use is linked to poor outcomes such as preterm birth and neurodevelopmental delays in exposed children, the underlying mechanisms are not well-defined. First, we characterized a functionally relevant cell line to model differentiation and fusion. In a comparison of the BeWo and BeWo b30 cell lines, our findings demonstrated that both models similarly undergo fusion. We then explored the implications of exposure to delta-9-tetrahydrocannabinol (Δ^9-THC) on the immunological roles of villous trophoblasts. We observed that cytotrophoblast differentiation and fusion were associated with localized inflammation due to elevated interleukin-2 (IL-2) and tumour necrosis factor-alpha (TNF-α) but inhibited interleukin-4 (IL-4) and interleukin-10 (IL-10) production. Δ^9-THC exposure impaired this T helper 1/2 cytokine balance through decreased IL-2 and TNF-α as well as increased IL-4 and IL-10 levels. Subsequently, we investigated the effects of Δ^9-THC in TNF-α- and IL-10-dominant environments, to represent inflammatory and

immunomodulatory microenvironments, respectively. Coincident with inflammation, Δ^9-THC attenuated trophoblast fusion and the biosynthesis of steroid hormones, progesterone and cortisol, through perturbed cytochrome P450 regulation. This thesis ultimately lays a foundation for understanding how cannabis use during pregnancy may compromise the fusogenic, immune and endocrine functions of villous trophoblasts in the placenta.

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LIST OF ABBREVIATIONS AND SYMBOLS

2-AG	2-arachidonoyl glycerol
$2^{-\Delta\Delta Ct}$	Delta-delta comparative cycle times analysis
ACTH	Adrenocorticotrophin hormone
AEA	Anandamide
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBD	Cannabidiol
CB1R	Cannabinoid receptor 1
CB2R	Cannabinoid receptor 2
CDH-1	Cadherin-1
COVID-19	Coronavirus disease 2019
CRH	Corticotrophin-releasing hormone
CTB	Cytotrophoblast
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CYP17A1	Cytochrome P450 family 17 subfamily A member 1
CYP21A1	Cytochrome P450 family 21 subfamily A member 1
CYP11B1	Cytochrome P450 family 11 subfamily B member 1
DAPI	4’,6-diamidino-2-phenylindole
dUTP	Deoxyuridine triphosphate
EGF	Epidermal growth factor
ERVFRD-1	Endogenous retrovirus group FRD member 1

ERVW-1	Endogenous retrovirus group W member 1
EVT	Extravillous trophoblast
FSK	Forskolin
GCM-1	Glial cells missing homolog 1
GPR18	G protein-coupled receptor 18
GPR55	G protein-coupled receptor 55
GPR119	G protein-coupled receptor 119
hCG	Human chorionic gonadotrophin
HERV	Human endogenous retrovirus
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenal
HSD3β1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1-beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-13	Interleukin-13
MeOH	Methanol
NR3C1	Nuclear receptor subfamily 3 group C member 1

PBS	Phosphate-buffered saline
PGR	Progesterone receptor
PKA	Protein kinase A
PMDD	Premenstrual dysphoric disorder
PMS	Premenstrual syndrome
PPAR	Peroxisome proliferator activated receptors
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SOGC	Society of obstetricians and gynaecologists of Canada
SP	Sodium phosphate
STB	Syncytiotrophoblast
TGF-β	Transforming growth factor-beta
Th1	T helper 1
Th2	T helper 2
Δ^9-THC	Delta-9-tetrahydrocannabinol
TNF-α	Tumour necrosis factor-alpha
TRP	Transient receptor potential
VIM	Vimentin

DECLARATION OF ACADEMIC ACHIEVEMENT

Harmeet Gurm contributed to the writing, experimental design, conducting of experiments, all aspects associated with data analyses, and figure/table generation in this thesis with the assistance of Dr. Sandeep Raha.

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CHAPTER 1: INTRODUCTION

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The Human Placenta

Anatomical development

In human pregnancy, the development of the placenta is transiently initiated within the first week following fertilization to establish a communicative and protective barrier between the mother and fetus [1]. The beginning of placentation is marked with the formation of the trophectoderm (4-5 days post-fertilization), which is the outer epithelial layer of the blastocyst [2]. The trophectoderm is then separated from the inner cell mass and interacts with the maternal endometrium (6-7 days post-fertilization) [2,3]. Thereafter, the endometrium is transformed into the decidua through the differentiation of endometrial stromal cells and migration of peripheral leukocytes [2,3]. The extraembryonic mesoderm also contributes to the placental structure by forming its stromal core that contains the vascular network, fibroblasts and resident Hofbauer macrophages [4].

During the implantation period, mononuclear cytotrophoblasts (CTBs) are derived from trophectoderm stem cells and begin to further expand into the decidua by forming primary villi projections (2 weeks post-fertilization) that will eventually transform into a vascular network over the first trimester for fetal-placental hemodynamics [1,2]. CTBs also undergo

proliferation, differentiation and cell-cell fusion to give rise to a second trophoblast cell lineage, the multi-nucleated syncytiotrophoblasts (STBs) (2 weeks post-fertilization to term) [1,2,5]. CTBs and STBs are collectively referred to as villous trophoblasts [5]. By the end of the first trimester, the maternal-fetal interface is fully formed, with STBs localized to the outer placental lining, known as the syncytium, directly above a layer of CTBs [1,2]. A third lineage of trophoblasts, known as the extravillous trophoblasts (EVTs) is also generated by the differentiation of CTBs (3 weeks post-fertilization) [1,2]. EVTs are primarily responsible for invasion into the maternal decidua and subsequent migration into the spiral arteries to establish low resistance blood flow to the uterus [1,2].

Importance of villous trophoblast turnover

Since STBs are terminally differentiated and form the primary site of maternal-fetal exchange of gases and nutrients, the integrity of the placental syncytium is dependent on continuous villous trophoblast turnover (Fig. 1) [5]. CTBs undergo proliferation, differentiation and fusion in this process to replace aged STBs, which will then aggregate and be shed into the maternal uteroplacental veins as syncytial knots [5]. Following their release, syncytial knots become trapped in the capillary beds of the maternal lungs and are engulfed by resident macrophages [6].

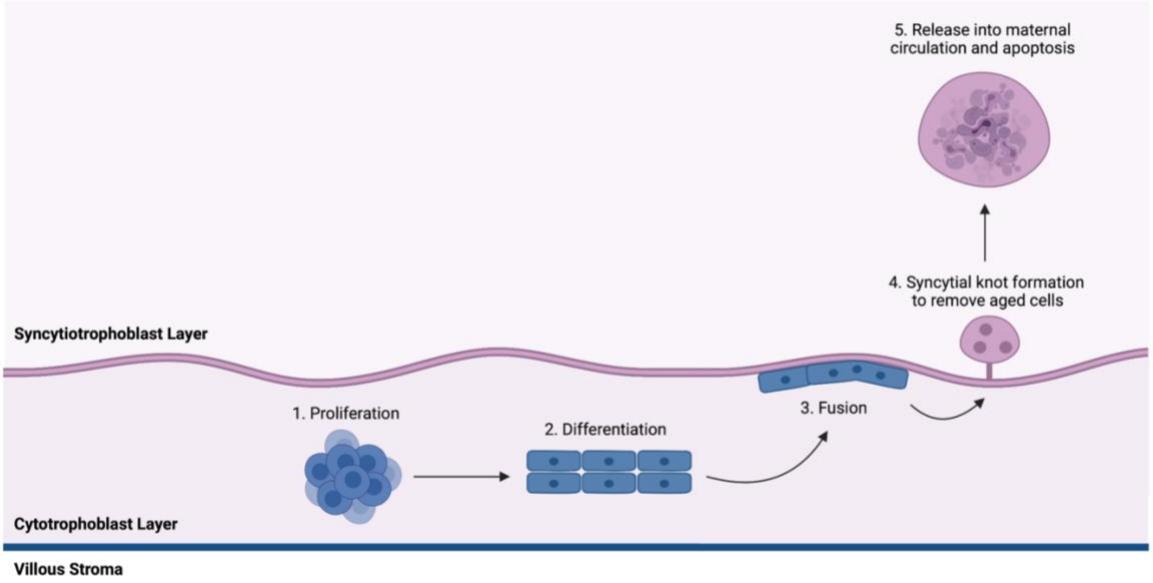

Figure 1. Schematic represention of villous trophoblast turnover. During pregnancy, CTBs undergo a continuous process of proliferation, differentiation and fusion to replaced STBs in the syncytium. The aged STBs are removed in the form of syncytial knots and released into the maternal circulation. Created with BioRender.com.

Investigations using the cell proliferation marker Ki-67 to stain human placentae and terminal deoxynucleotidyl transferase dUTP nick end labelling to identify DNA fragmentation have reported that an estimated 600 mitotic events per 10,000 μm^2 of villous surface occur in the first trimester of pregnancy [5,7]. Since only 10-15% of these nuclei will undergo fusion to be incorporated into the syncytium, the proliferative capacity of CTBs exceeds the physiological requirements in early pregnancy [5,7]. While the rates of CTB proliferation, differentiation and fusion decrease up to 90% by the third trimester, it is estimated that a single STB nucleus remains in the syncytium for 3-4 weeks [5,7]. Overall, the maintenance of the placental syncytium in healthy pregnancy is sustained by

trophoblast turnover beginning as early as 2 weeks post-fertilization and continuing until term [5].

Human endogenous retroviruses as fusogenic regulators

An estimated 8% of the human genome is comprised of human endogenous retroviruses (HERVs) with a genomic structure containing the *gag*, *env* and *pol* genes, which encode structural (matrix, capsid, nucleocapsid), envelope and polymerase proteins, respectively [8,9]. HERVs, particularly HERV-W and HERV-FRD, possess a critical role in mediating trophoblast fusion [8,10]. The *env* gene of HERV-W encodes the syncytin-1 protein, which interacts with the sodium-dependent neutral amino acid transporters A and B [10]. Similarly, HERV-FRD encodes syncytin-2 that binds to a sodium-dependent lysophosphatidylcholine transporter known as major facilitator superfamily domain 2A [10]. The transcription of both syncytin-1 and syncytin-2 is mediated by glial cells missing homolog 1 (GCM-1), which in turn is upregulated by activation of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway [11]. If levels of GCM-1 are disrupted, the associated decrease in syncytin-1 and syncytin-2 has been shown to contribute to the pathogenesis of preeclampsia [9,12,13].

Syncytin-1 and syncytin-2 are both synthesized as inactive 538 amino acid long polyproteins, after which they are cleaved into surface and transmembrane subunits by a furin protease [10]. The surface subunit allows for receptor recognition and binding while the transmembrane subunit directly induces fusion between two cells [9,10]. A fusion

peptide, which is part of the transmembrane subunit, then drives the joining of intercellular membranes after being inserted on syncytin-expressing target cells [8,10]. Within human placental tissues and cell types, including villous trophoblasts, syncytin-1 and syncytin-2 are expressed and play a direct role in inducing CTB cell-cell fusion [10,14,15].

Metabolic, endocrine, and immune functions of the syncytium

The syncytium forms a barrier as close as 2-4 μm between the maternal and fetal circulations [4,16]. As a result, the placenta is a highly metabolically active organ that fulfill the energetic demands of itself and the developing fetus through the transfer of gases, nutrients and waste products [17]. Respiratory gases, such as oxygen and carbon dioxide, can readily diffuse across the syncytium due to their small and hydrophobic nature [4,16]. Nutrients, however, require the use of transporter proteins due to their hydrophilicity [16]. The primary source of energy for the fetus is glucose, which is transferred across the placenta via facilitated diffusion using glucose transporters [4,16]. Although the majority of fetal energy demands are maintained by maternal glucose transfer, the fetus can also minimally generate glucose through gluconeogenesis [16]. Other sources of fuel for the fetus include lactate, amino acids and fatty acids [16].

A plethora of hormones that are necessary for pregnancy maintenance, including human chorionic gonadotropin (hCG), progesterone and the estrogens, are also produced by the syncytium [18]. hCG is a heterodimeric glycoprotein with an α-subunit that is shared by luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone and a

unique β-subunit [18]. The initial production of hCG occurs during the 8-cell stage of embryonic development and then exponentially increases until about 10 weeks of gestation [18]. Levels of hCG thereafter experience a steady decline until term [18]. In terms of functionality, hCG promotes the production of progesterone by the corpus luteum, differentiation and fusion of CTBs, angiogenesis, and uterine expansion [19]. Unlike hCG, progesterone is a steroid hormone that is synthesized by the corpus luteum until about 12 weeks of gestation, after which the syncytium increases its production over the duration of pregnancy [20]. Progesterone functions to support blastocyst implantation, decidualization and mammary gland development as well as modulate immune responses at the maternal-fetal interface [18,21]. Estrogen is another steroid hormone that contributes to pregnancy success [22]. Three major forms exist and are elevated as pregnancy progresses, including estrone, estradiol and estriol, however estradiol is the most abundant [22]. While the corpus luteum produces estrogen until about 9 weeks of gestation, the placenta interacts with the maternal and fetal adrenal glands to take over as the primary source [22]. The estrogens play a role in endometrial growth, angiogenesis, and induction of labour [18]. Additional critical hormones during pregnancy are placental lactogen, placental growth hormone, leptin, adiponectin and resistin [18].

The syncytium also contributes to the local immune milieu (Fig. 2) [23]. The maternal-fetal interface is an immunologically privileged environment since the fetus, a semi-allogenic graft containing both maternal (self) and paternal (non-self) antigens, evades attack and rejection by the maternal immune system [24]. Decidual immune cell populations, which

are primarily uterine natural killer cells, macrophages and T cells, play necessary roles in the balance between maternal immunity and fetal tolerance [25]. Villous trophoblasts can also contribute to the immunological responses in pregnancy through the production of inflammatory T helper 1 (Th1) and modulatory T helper 2 (Th2) cytokines [23]. CTBs and STBs are reported to produce various Th1 cytokines, including interleukin-1-beta (IL-1β), interleukin-2 (IL-2), interleukin-8 (IL-8), interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) [23,26]. The Th2 cytokines interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13) and transforming growth factor-beta (TGF-β) are also secreted by villous trophoblasts [26–28].

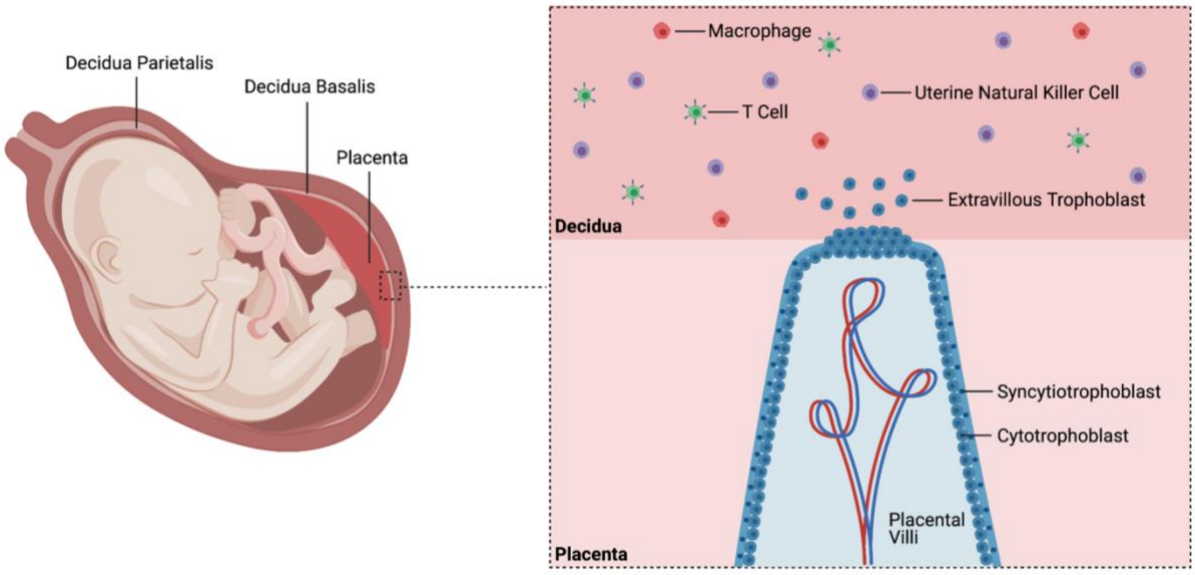

Figure 2. Decidual immune cells and placental trophoblasts at the maternal-fetal interface. The point of contact between the decidua basalis and placenta forms the maternal-fetal interface. While three lineages of trophoblasts are found in the placenta, only EVTs come into direct contact with the cell types in the decidua. The major populations are immune cells, specifically uterine natural killer cells, T cells and macrophages. Created with BioRender.com.

Immunology of Healthy Pregnancy

Early stages of pregnancy: Implantation and placentation

Human pregnancy is characterized by tightly orchestrated cytokine responses, with the first and early second trimesters being dominated by Th1 cytokines (Fig. 3) [29]. Implantation possesses three primary phases, including apposition, attachment and invasion, which create an environment that resembles an "open wound" [30]. During apposition, the blastocyst loosely interacts with endometrial stromal cells, following which adhesion molecules increase its affinity to allow for attachment [30]. This local injury to the endometrium facilitates receptivity to prime for implantation [31]. In the final step of invasion, EVTs penetrate the epithelium and invade the maternal vasculature to establish utero-placental blood flow [29,30]. An inflammatory response is then induced to repair the damaged epithelial lining, remove cellular debris and replace maternal endothelial cells with invasive EVTs [29,30]. Following implantation, early placentation is dependent on maternal spiral artery remodelling to establish exchange between the maternal and fetal circulations [32]. Besides being regulated by angiogenic factors, such as vascular endothelial growth factor and angiopoietin, vascularization during placental development is associated with an increased production of inflammatory mediators (IL-8, IFN-γ, TNF-α) [30,33]. This state of controlled inflammation is mediated by elevated Th1 cytokine production by trophoblasts, endometrial stromal cells and immune cells, of which uterine natural killer cells are the most abundant, at the maternal-fetal interface [29,30].

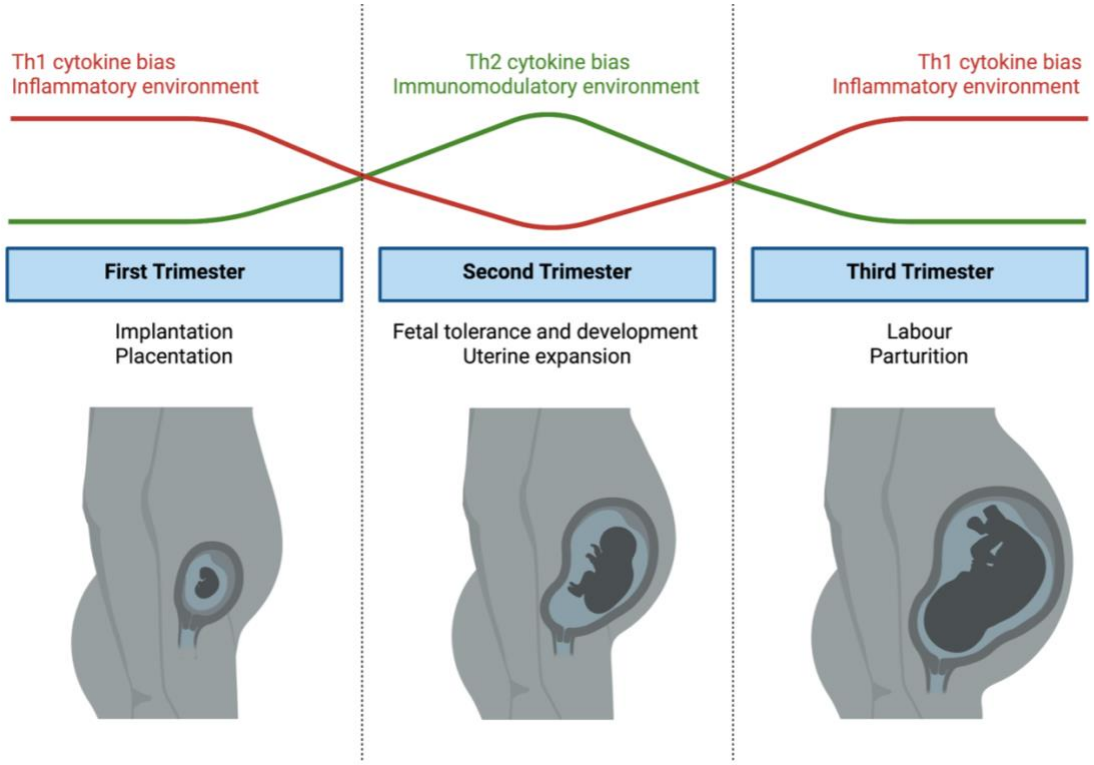

Figure 3. The three immunological stages of healthy pregnancy. Human pregnancy can be divided into distinct stages depending on the Th1/Th2 cytokine balance. In the first trimester, a Th1 cytokine bias is necessary for implantation and placentation. As pregnancy progesses, a shift toward increased Th2 cytokines supports fetal toleranace as well as fetal and uterine development. Finally, pregnancy reaches completion with a final rise in Th1 cytokine production to induce labour and parturition. Created with BioRender.com

Mid-late stages of pregnancy: Fetal tolerance and uterine expansion

The majority of pregnancy, ranging from the second to late third trimester, is distinguished by a Th2 cytokine bias to support maternal tolerance of the fetus (Fig. 3) [30]. Th2 cytokines (IL-4, IL-10, TGF-β) play a role in tissue repair, preventing immune activation against paternal antigens present in the fetus and accommodating uterine expansion, all of which promote successful fetal growth [29,30,34]. Organ development, particularly the

brain and lungs, is also reported to be modulated by Th2 cytokines [35]. All of these outcomes are promoted by the downregulation of Th1 cytokine production as exacerbated inflammation during the period of rapid fetal development is associated with pregnancy complications such as intrauterine growth restriction, recurrent miscarriage, preterm birth and preeclampsia [35,36].

Final stages of pregnancy: Labour and parturition

The final immunological phase of human pregnancy is defined by the completion of fetal development [30]. A switch to an elevated Th1/Th2 cytokine balance is now required to facilitate labour and delivery [30]. At this stage, inflammation is triggered by the cell types already present at the maternal-fetal interface alongside an influx of recruited immune cells to the myometrium [29,30]. An array of Th1 cytokines is produced that, paired with decreased progesterone and increased prostaglandins and oxytocin, promote uterine smooth muscle cell contraction to initiate delivery and placental expulsion [29,30]. Successful human pregnancy is thus governed by three carefully coordinated immunological phases that fluctuate from heightened production of Th1 to Th2 to Th1 cytokines.

Cannabis

Effects of cannabis on female reproductive health

Since the legalization of cannabis in October 2018, the Canadian Cannabis Survey annually collects information regarding patterns of use [37]. In 2020, 54% of Canadians reported using cannabis in the previous 12 months, with females accounting for 21% [37]. The

predominant route of administration was reported to be smoking, followed by ingestion and vapourization [37]. While 96% of females (ages 16-50) indicated cessation of use during pregnancy, cannabis remains one of the most commonly used drugs among females of reproductive age (ages 16-50) [37]. Given that cannabis use has also followed an increasing trend over recent years in post-legalization Canada, it is necessary to understand its effects over the female reproductive lifespan [38].

Beginning with ovulation, the reported effects of cannabis exposure are conflicting [38]. While some investigations indicate no correlation between cannabis use and adverse outcomes for female fertility, others have demonstrated changes in ovarian function and the menstrual cycle, particularly through altered levels of luteinizing hormone and follicular phase length [38,39]. Cannabis has also been implicated in ameliorating symptoms of the premenstrual syndrome (PMS) and premenstrual dysphoric disorder (PMDD) such as irritability, depression, and joint or muscle pain [40]. However, an investigation with women suffering from PMS/PMDD, who also consumed cannabis in the previous year, suggests that positive expectations about cannabis-induced relief reinforce increased substance use [40]. Similarly, the implications of cannabis during menopause and the menopausal transition are not well defined [38]. Since menopause and PMS/PMDD share common symptoms, specifically those listed earlier, cannabis is considered an alternative treatment for easing the transition to menopause and assisting with menstrual pain [40,41]. Due to the lack of empirical findings that confirm self-reported benefits, the

effects of cannabis use on reproductive events ranging from ovulation to menopause require further exploration to evaluate its potential as a therapeutic.

During pregnancy, cannabis is one of the most commonly used substances with a self-reported incidence of 1.4-11.3% in Canadian women [42–45]. Of these cannabis users, up to 50% are under the age of 24 and come from low socioeconomic status households [45]. Women belonging to these demographics have displayed an increasing trend in cannabis use during pregnancy over recent years [43]. The majority of pregnant women further indicate use of cannabis primarily in the first trimester during their routine prenatal care visits [46]. Increased consumption in early pregnancy may be attributed to the perceived effects of diminished nausea and vomiting [46]. Despite the reported anecdotal benefits, conflicting clinical data suggests that chronic cannabis use is linked to cannabinoid hyperemesis syndrome [47]. This condition is characterized by episodes of acute nausea, vomiting and abdominal pain persisting for 24-48 hours post-cannabis use [47]. However, only 6 case studies of cannabinoid hyperemesis syndrome have been published with pregnant patients to-date [48]. This may be the result of individuals not disclosing cannabis use to health care providers when patient history is recorded due to the perceived social and medical stigma, desirability bias, and/or fear of child protective social services [42,48].

Maternal cannabis use has been associated with an increased risk of preterm birth at less than 37 weeks of gestation, small for gestational age status due to a birth weight below the 10^{th} percentile, stillbirth, increased admissions to the neonatal intensive care unit, placental

abruption and miscarriage [42,49]. These obstetrical and neonatal outcomes are reported following adjustments for confounding variables such as maternal age, socioeconomic status and ethnicity between users and non-users [42,45]. Controlling for polysubstance use, however, represents a challenge in investigations as many cannabis users report tobacco smoking and alcohol consumption, which makes it difficult to determine the effects of only cannabis on pregnancy outcomes [42]. In addition, prenatal exposure has been linked to lower scores in verbal reasoning, language comprehension, reading tasks, memory, impulse control and tasks requiring sustained attention during infancy and adolescence [49]. Child exposure to cannabis through breastfeeding may contribute to these long-term consequences for neurodevelopment, however this has not been adequately studied to date [49]. Since research examining the mechanistic effects of cannabis on pregnancy and fetal development remains limited, there is a requirement to develop clear safety data to help inform patients who consider using cannabis during pregnancy [44,49]. This knowledge may assist health care providers in developing clinical strategies to mitigate maternal and fetal health complications, particularly in the high-risk demographics of young and socioeconomically disadvantaged women.

Complexity of cannabis

Cannabis is a complex plant composed of over 400 chemical compounds that range from terpenes to phenols to plant-derived cannabinoids [50,51]. Terpenes are phytochemicals that are responsible for the aromaticity and flavour of cannabis while phenols function as antioxidants [51]. Cannabinoids are arguably the most investigated class of compounds

[50,51]. Of over 90 entities, delta-9-tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive cannabinoid, however other abundant forms are cannabidiol (CBD), cannabinolic acid and cannabinodiolic acid (Fig. 4) [51]. The biosynthesis of these cannabinoids occurs via the polyketide and 2-C-methyl-D-erythritol-4-phosphate pathways [51]. Common to all cannabinoids is the cannabigerolic acid precursor, which is then catalyzed to delta-9-tetrahydrocannabinolic acid, cannabidiolic acid and cannabichromenic acid [51]. All other derivations are then generated from these three cannabinoids [51].

The endocannabinoid system

The endocannabinoid system plays a necessary role in a variety of physiological processes in the human body, including modulation of immune responses and reproductive events [52]. These effects are exerted through endogenous cannabinoids, referred to as endocannabinoids, the enzymes involved in their biosynthesis and degradation, and cannabinoid receptors [53]. Anandamide (AEA) was the first identified endocannabinoid in 1992, following which its main biosynthetic and hydrolytic enzymes were identified as *N*-acyl-phosphatidylethanolamine-specific phospholipase D and fatty acid amide hydrolase, respectively [54–57]. Shortly after, the second major endocannabinoid was identified as 2-arachidonoyl glycerol (2-AG), which is synthesized by diacylglycerol lipase and degraded by monoacylglycerol lipase [58–62]. The targets of these endocannabinoids are cannabinoid receptors 1 (CB1R) and 2 (CB2R), which are the primary receptors of the endocannabinoid system (Fig. 4) [13,23]. CB1R is most abundant in the central nervous system, although it is also expressed in the peripheral nervous system and peripheral cell

types [64,65]. CB2R, however, is predominantly localized in peripheral cells of the hematopoietic, immune and reproductive systems [66,67]. In fact, localization of CB1R and CB2R has been reported in the human placenta and trophoblast cells [68].

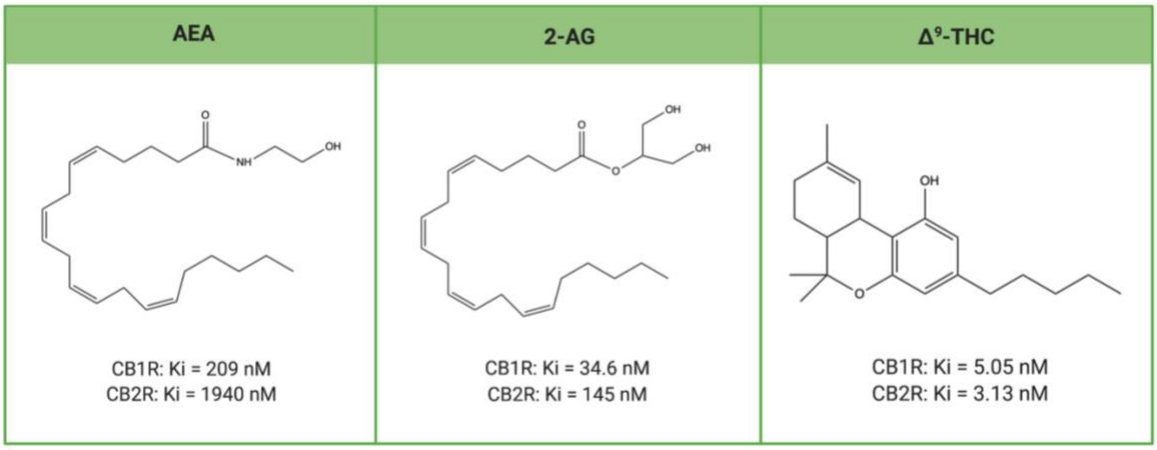

Figure 4. Cannabinoid structures and receptor binding affinities. The structures of AEA, 2-AG and Δ^9-THC are displayed, with the corresponding inhibitory constant Ki values for CB1R and CB2R [101,112–114]. Created with BioRender.com

CB1R and CB2R regulate an array of signal transduction cascades, particularly the adenylyl cyclase pathway [69]. Cannabinoid receptor activation is associated with attenuated adenylyl cyclase through guanine nucleotide-binding protein complex-mediated actions that inhibit cAMP levels and PKA activity [70–72]. The functional responses impacted by cannabinoid-dependent inhibition of adenylyl cyclase include cellular apoptosis, differentiation, proliferation, and survival [73–76]. Other receptors of the endocannabinoid system are the transient receptor potential (TRP) ion channels, peroxisome proliferator activated receptors (PPARs), and G protein-coupled receptors 18 (GPR18), 55 (GPR55)

and 119 (GPR119) [77–81]. Unlike CB1R and CB2R, the roles of these non-classical receptors are poorly characterized.

Endocannabinoid signalling during pregnancy

In healthy pregnancy, plasma levels of AEA are tightly regulated from ovulation to labour [82]. During ovulation, an increase in AEA is favoured, suggesting a role for this endocannabinoid in follicular maturation [83]. Once pregnancy is established, low AEA is necessary for fertilization, implantation, decidualization, and placentation [82,84]. AEA levels then experience a surge to assist in labour and parturition [82,84]. In fact, a cross-sectional investigation following healthy singleton pregnancies in humans reported mean AEA levels of 0.89, 0.44 and 0.42 nM in the first, second and third trimesters, respectively [84]. These levels increased 6-fold in labouring women, reaching a plasma concentration of 2.5 nM [84]. Relatively low and stable AEA levels are therefore present over the course of pregnancy, with imbalances linked to gestational complications, such as miscarriage and preeclampsia [84,85]. In comparison, the regulation and roles of 2-AG in human pregnancy remain undefined.

Endocannabinoid signalling during pregnancy also plays a role in modulating trophoblast functions [86]. In the human choriocarcinoma-derived BeWo cells, treatment with AEA (10 μM) attenuated cellular proliferation [87]. 2-AG (10-25 μM) also inhibited proliferation in primary human CTBs as well as induced apoptosis through chromatin condensation and fragmentation, presence of apoptotic bodies, and generation of reactive oxygen and

nitrogen species [88]. Furthermore, the cannabinoids Δ^9-THC (20 μM), AEA (10 μM) and 2-AG (10 μM) have all been shown to impair the biochemical and morphological differentiation of CTBs, leading to impaired cell-to-cell fusion [89–91]. Δ^9-THC (20 μM) exposure also decreased the secretion of hCG, placental lactogen, and insulin-like growth factors in BeWo cells, all of which are important for fetal development and pregnancy success [90]. Finally, Δ^9-THC (10 mM) has been associated with decreased trophoblast invasion in the HTR-8/SVneo cell line that models human EVTs [92].

Δ^9-THC

The most common mode of delivery for cannabis is smoking, which results in rapid diffusion to perfuse organs such as the brain and liver [93]. Unfortunately, the half-life of Δ^9-THC is dependent on the frequency of use and ranges from about 1 hour to 4 days as a result of its accumulation and delayed elimination from adipose tissue [93]. Due to its lipophilic nature, Δ^9-THC can also readily cross the placenta [94]. While human investigations of Δ^9-THC transfer across the placental barrier have not been evaluated, animal models indicate that 10-28% of its maternal plasma concentrations are found in the fetal circulation [95,96]. Similar outcomes may be observed in humans, however the transplacental passage of Δ^9-THC is dependent on the route of administration, placental permeability and individual variations in usage [94,97]. Although the pharmacokinetics of maternal-fetal transfer of Δ^9-THC require further evaluation, smoking cannabis with a Δ^9-THC content of 6.8% is associated with a mean peak whole blood concentration of 50 μg/mL (range: 13-63 μg/mL) and plasma concentration of 76 μg/mL (range: 18-110

μg/mL) [98]. Other investigations using 1.8% and 3.6% Δ^9-THC have reported mean peak plasma levels of 79 ng/mL and 150 ng/mL, respectively [99]. An important consideration is that products containing up to 32% Δ^9-THC are sold through the Ontario Cannabis Store [100].

In terms of pharmacological activity, Δ^9-THC is a partial agonist for CB1R (Ki = 5.05 nM) and CB2R (Ki = 3.13 nM) [101,102]. While AEA is also a partial agonist for CB1R (Ki = 209 nM), it is largely inactive at CB2R (Ki = 1940 nM) whereas 2-AG is a full agonist for both CB1R (Ki = 34.6 nM) and CB2R (Ki = 145 nM) [102,103]. Besides differences in cannabinoid receptor affinity, only endocannabinoids possess the ability to initiate a negative feedback mechanism via retrograde signalling to suppress further neurotransmitter release [104]. Since exogenous cannabinoids lack this regulatory feedback, exposure to cannabis can perturb endocannabinoid signalling through persistent cannabinoid receptor stimulation [104,105]. Plant-derived cannabinoids also possess multi-systemic effects, making it particularly relevant for pregnancy due to the crosstalk between the maternal immune and reproductive systems [105].

Gaps in scientific research: Δ^9-THC as an immunomodulatory agent

In the immune system, the expression of CB1R and CB2R is reported in major cell types that mediate innate and adaptive responses [106–108]. Δ^9-THC-dependent (5-60 μM) activation of cannabinoid receptors located on immune cells is further associated with immunomodulatory effects, including decreases in cytotoxicity of natural killer cells, nitric

oxide production in macrophages and inflammatory cytokine production by $CD8^+$ effector T cells [109–111]. The effects of Δ^9-THC on immune cell regulation are well-established, however a critical knowledge gap remains to determine whether similar outcomes are observed for immune roles, specifically the production of Th1/Th2 cytokines, carried out by trophoblasts in the placenta. We therefore aimed to understand if Δ^9-THC impairs the ability of villous trophoblasts to contribute to the local immune milieu at the maternal-fetal interface during placental development.

Research Hypothesis

We hypothesized that exposure to Δ^9-THC would induce a shift toward enhanced Th2 cytokine production by villous trophoblasts, which may in turn adversely impact the greater immune microenvironment required for cell differentiation and fusion.

Research Objectives

The primary research objectives of this research project were:

1. To compare differentiation and fusion in the BeWo and BeWo b30 cell lines to assess if one serves as a better *in vitro* model for the development of the syncytium.
2. To investigate the effects of Δ^9-THC on the production of Th1 and Th2 cytokines by villous trophoblasts.
3. To investigate the effects of Δ^9-THC on hormone biosynthesis and secretion in localized inflammatory and immunomodulatory microenvironments during villous trophoblast differentiation and fusion.

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CHAPTER 2: CHARACTERIZATION OF DIFFERENTIATION AND FUSION IN IMMORTALIZED HUMAN TROPHOBLAST CELL LINES

This chapter addresses research objective 1: To compare differentiation and fusion in the BeWo and BeWo b30 cell lines to assess if one serves as a better *in vitro* model for the development of the syncytium.

Introduction

To investigate placental syncytium formation and the required transition from a CTB to STB-dominant phenotype, the human choriocarcinoma-derived BeWo cell line is the most frequently used *in vitro* model [1]. BeWo b30 cells, which are a cloned derivative, can similarly recapitulate trophoblast differentiation and fusion [2]. Both immortalized cell lines satisfy the four defining criteria of the human trophoblast [3]. BeWo and BeWo b30 cells express various trophoblast markers, including hCG, cytokeratin 7 and GATA binding protein 3, and are null for classical human leukocyte antigen (HLA) loci [3,4]. Of the three trophoblast lineages (CTBs, STBs, EVTs), only EVTs express HLA-I molecules, including HLA-C, HLA-E and HLA-G [5]. The final conditions include a hypomethylated E74-like factor 5, which is a transcription factor that regulates trophoblast lineation and stem cell renewal, and expression of the placenta-specific chromosome 19 microRNA cluster [3,6,7].

BeWo and BeWo b30 cells exhibit a low spontaneous fusogenic capacity at baseline, however enhanced cAMP signalling can promote differentiation and fusion [1]. This occurs

in response to treatment with cAMP, 8-bromo-cAMP or forskolin (FSK) [1,2,8]. In the case of FSK stimulation, activation of adenylyl cyclase leads to increased intracellular cAMP levels [1]. Activation of PKA then increases GCM-1 expression, which in turn is linked to the transcription of endogenous retrovirus group W member 1 (*ERVW-1*) and endogenous retrovirus group FRD member 1 (*ERVFRD-1*) [1]. *ERVW-1* and *ERVFRD-1* encode syncytin-1 and syncytin-2, respectively [9]. The resulting cell-cell fusion then upregulates hormonal markers of differentiation, particularly hCG-β [1,2]. While the reported fusion percentage in BeWo and BeWo b30 cells varies depending on the type, concentration and exposure length of the cAMP-stimulating agent, it ranges from 10-80% [8,10–12].

Furthermore, RNA-sequencing demonstrates that syncytium formation in BeWo b30 cells and primary trophoblasts isolated from term human placentae is similarly regulated [2]. STBs from both models display an overlap in the expression of factors involved in fusion such as hCG, cysteine-rich intestinal protein 2, inhibin-alpha, and follistatin-like-3 [2]. The underlying mechanisms are not entirely identical as several genes, including those that regulate histone variants and cytochrome oxidases, are differentially regulated [2]. Additional points of contrast are that primary human trophoblasts spontaneously undergo fusion over a 48-72 hour period and display up to a 3-fold increase in their fusogenic capacity relative to immortalized cell lines [10]. However, primary human trophoblasts are incapable of *in vitro* proliferation and show a decline in cell viability with increasing length of time in culture [4,13]. Since the availability of human placentae represents a limiting

factor for placental research, the use of BeWo and BeWo b30 cells is invaluable to advance our understanding of the establishment and functionality of the maternal-fetal interface.

While differences in choriocarcinoma- and placental-derived trophoblasts continue to be explored, a comparative analysis of differentiation and fusion between the BeWo and BeWo b30 cell lines has not been performed. An understanding of these trophoblast model systems is necessary to ascertain their relevance to *in vivo* placentation, particularly since information surrounding the origination of BeWo b30 cells is not readily available. The present chapter provides insight into whether one of these cell lines is a better model to study *in vitro* trophoblast differentiation and fusion. To investigate this, we explored the CTB to STB transition at the transcript and protein levels using various fusogenic mediators, including syncytin-1, syncytin-2, hCG-β and E-Cadherin. We also examined the effects of Δ^9-THC exposure, over a range of 0-50 μM, on cell viability and fusion to determine an appropriate drug concentration for further in-depth investigation (Chapter 3).

Materials and Methods

Cell culture

BeWo cells (ATCC® CCL-98) were cultured in Ham's F-12K Nutrient Mixture (Kaighn's Modification; Corning, 10-025-CV) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. BeWo b30 cells (AddexBio, C0030002) were cultured in Dulbecco's Modified Eagle's Medium (DMEM with 4.5 g/L glucose; Corning, 10-013-CV) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2 and seeded at a density of $1x10^5$ cells/cm^2. The media of undifferentiated cells, representative of CTBs, was replenished every 48 hours. BeWo and BeWo b30 cells were treated with epidermal growth factor (EGF; 50 ng/mL) for a 48-hour period to induce monolayer formation [14]. Cells were subsequently induced to differentiate and undergo fusion, to represent STBs, using EGF (50 ng/mL) and forskolin (FSK; 50 μM) for another 48 hours [1]. In BeWo b30 cells, the addition of Δ^9-THC (5-50 μM; Sigma Aldrich, T4764) or the methanol (MeOH) vehicle was concomitant with EGF and FSK.

RNA extraction and quantitative RT-PCR

BeWo and BeWo b30 cells were cultured on 6-well polystyrene plates. Total RNA was extracted from cells using TRIzol™ Reagent (500 μL; Thermo Fisher Scientific, 15596026) and isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research, R2050), according to the manufacturer's instructions. Quantification of gene expression using quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described by our research group [15]. Relative mRNA expression was calculated using the delta-delta comparative cycle times ($2^{-\Delta\Delta Ct}$) analysis, normalized to the endogenous control genes, *18S* and *β-actin*, and expressed as the fold change to the vehicle control. Primers sequences designed for all target genes of interest are listed in Table 1.

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
18S	CACGCCAGTACAAGATCCCA	AAGTGACGCAGCCCTCTATG
β-actin	TTACAGGAAGTCCCTTGCCATC	GCAATGCTATCACCTCCCCTG
ERVW-1	TCATATCTAAGCCCCGCAAC	CGCCAATGCCAGTACCTAGT
ERVFRD-1	CCTTCACTAGCAGCCTACCG	GCTGTCCCTGGTGTTTCAGT
hCG-β	ACCCCTTGACCTGTGAT	CCTTATTGTGGGAGGATCGG
CDH-1	GTGCCTGAGAACGAGGCTAA	TGCATCTTGCCAGGTCCTT

Table 1. Forward and reverse primer sequences used for RT-PCR

Immunofluorescence

BeWo and BeWo b30 cells were cultured on 4-well glass culture slides. Cells were fixed using ice-cold MeOH for 5 minutes at 20°C. Non-specific binding was blocked using Tween-20 (0.01%), goat serum (10%) and bovine serum albumin (BSA; 1%) in Dulbecco's Phosphate-Buffered Saline (PBS) for 2 hours at room temperature. Cells were then incubated with Anti-E-Cadherin primary antibody (1:500 dilution; Abcam, ab40772) overnight at 4°C. Subsequently, cells were incubated with Goat Anti-Rabbit IgG Alexa Fluor® 488 (1:1000 dilution; Abcam, ab150077) secondary antibody for 45 minutes at room temperature. Both antibodies were diluted using Tween-20 (0.01%) and BSA (0.1%) in PBS. Cells were counterstained and mounted using Fluormount-G™ Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, 00-4959-52). Slides were then visualized using an Eclipse Ti-E Inverted Fluorescence Microscope (Nikon Instruments), and images were captured using 20x magnification at five randomly selected, non-overlapping fields per sample. To visualize fusion, E-Cadherin and DAPI staining identified plasma membranes and cell nuclei, respectively [16]. The cell counts of two

blinded individuals were averaged to quantify fusion using the following equation: Fusion percentage (%) = (Number of nuclei in fused cells/Number of total nuclei) x 100%.

Cell viability

BeWo b30 cells were cultured on 96-well polystyrene plates. Cells were treated with EGF (50 ng/mL), FSK (50 μM) and Δ^9-THC (0.1-50 μM) or MeOH for 48 hours. The effects of Δ^9-THC on cell viability were determined with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS Assay; Promega, G5421), according to the manufacturer's instructions. Absorbance was measured at 490 nm using the Spark® Multimode Microplate Reader (Tecan Trading AG). The fold change in cell viability was calculated relative to treatment with only EGF and FSK.

Statistical analyses

All experiments were replicated at least three times ($n \geq 3$). Each biological replicate was performed using independently generated samples to account for variation. Data are presented as the mean ± standard error of mean. To determine significant differences across treatment groups, paired t-tests or one-way ANOVA followed by Tukey's multiple comparisons tests were utilized. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.

Results

Fusogenic mediators are similarly regulated during fusion in BeWo and BeWo b30 cells at the transcript and protein levels.

To explore whether cellular fusion is similarly regulated in BeWo and BeWo b30 cells, the transcriptional profiles of *ERVW-1*, *ERVFRD-1* and *hCG-β* were investigated. Changes in mRNA expression were assessed following 48 hours of exposure to 50 ng/mL EGF and 50 μM FSK to induce a STB-predominant phenotype. The control population, representative of CTBs, was left untreated. BeWo cells undergoing differentiation and fusion showed an increase in the expression of *ERVW-1* (3.1-fold), *ERVFRD-1* (18.2-fold) and *hCG-β* (89.3-fold) (Fig. 1A-C). Similarly, BeWo b30 cells demonstrated an increase in *ERVW-1* (1.9-fold), *ERVFRD-1* (9.8-fold) and *hCG-β* (61.6-fold) (Fig. 1D-F). Visualization of fusion using immunofluorescent staining with E-Cadherin and DAPI further demonstrated an increase in the number of fused nuclei within a single plasma membrane boundary in both cell lines (Fig. 2A and 3A). Fusion quantification based on cell counts supported these findings as the fusion percentage rose from 10.1% to 15.8% following BeWo cell exposure to EGF and FSK (Fig. 2B). BeWo b30 cells exhibited a similar trend, with the fusion percentage increasing from 4.9% in CTBs to 16.4% in STBs (Fig. 3B). An interesting observation is that the BeWo b30 cell line displayed a greater change in fusion from baseline (CTBs) to the induction of fusion (STBs) (Fig. 2 and 3). Finally, we examined the mRNA expression of cadherin-1 (*CDH-1*), which encodes E-Cadherin. As expected, the expression of *CDH-1* was decreased in BeWo and BeWo b30 STBs by 1.0- and 3.0-fold, respectively (Fig. 2C and 3C).

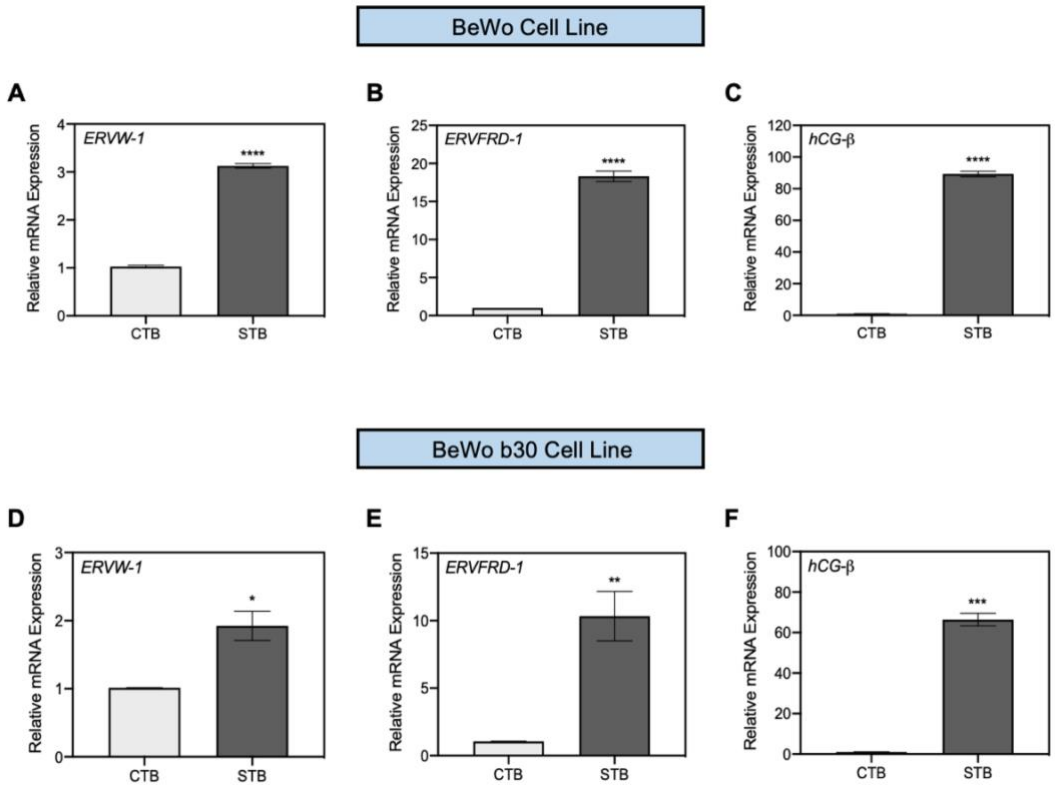

Figure 1. Comparison of transcriptional profiles of differentiation and fusion markers in BeWo and BeWo b30 cells. (A-C) Relative mRNA expression of (A) *ERVW-1*, (B) *ERVFRD-1*, (C) *hCG-β* in BeWo cells. (D-F) Relative mRNA expression of (D) *ERVW-1*, (E) *ERVFRD-1*, (F) *hCG-β* in BeWo b30 cells. Significant differences between groups were determined using paired t-tests. Significant differences are indicated by **p*<0.05, ***p*<0.005, ****p* <0.001 and *****p*<0.0001; n=4.

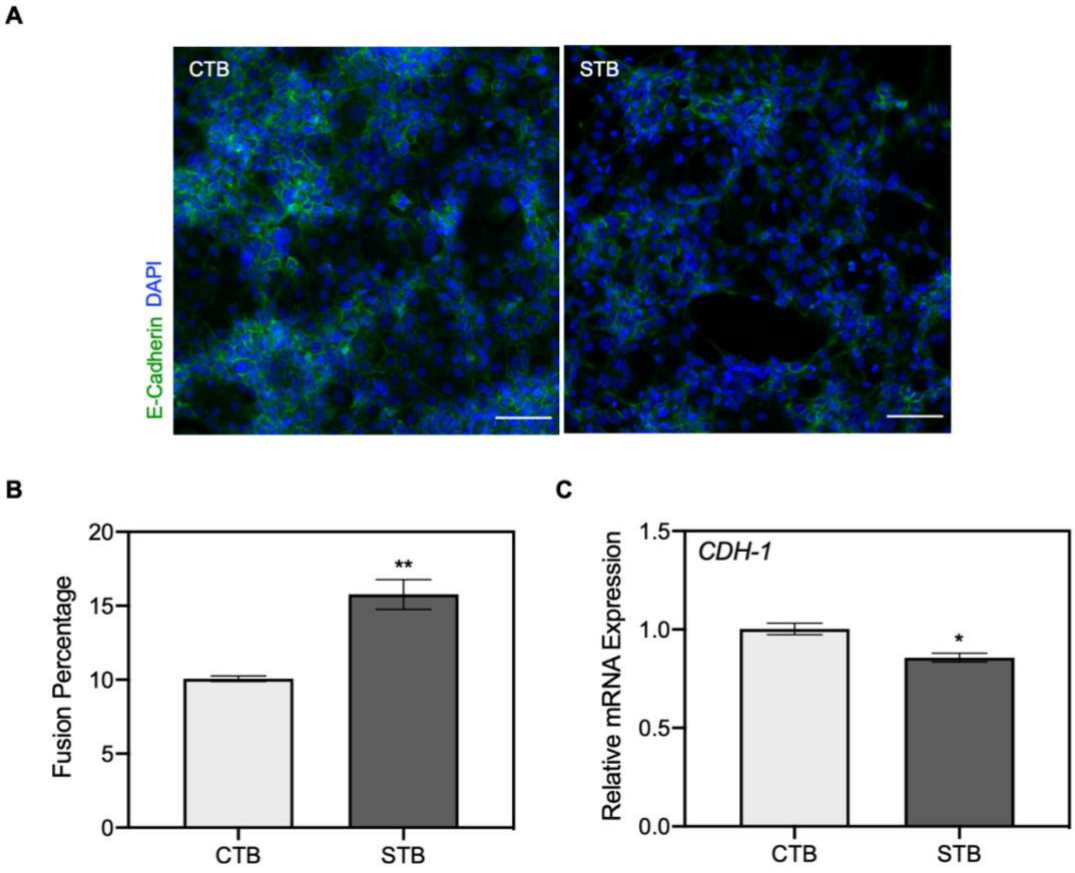

Figure 2. Visualization and quantification of BeWo cell-cell fusion. (A) Immunofluorescent staining in which green fluorescence indicates E-Cadherin staining for plasma membranes and blue fluorescence indicates DAPI staining for cell nuclei in BeWo cells. Representative fluorescent microscopy images are shown for the CTB and STB phenotypes. Images were captured at 20X magnification; scale bars indicate 100 μm. (B) Fusion percentage. (C) Relative mRNA expression of *CDH-1*. Significant differences between groups were determined using paired t-tests. Significant differences are indicated by *p<0.05 and **p<0.005; n=4.

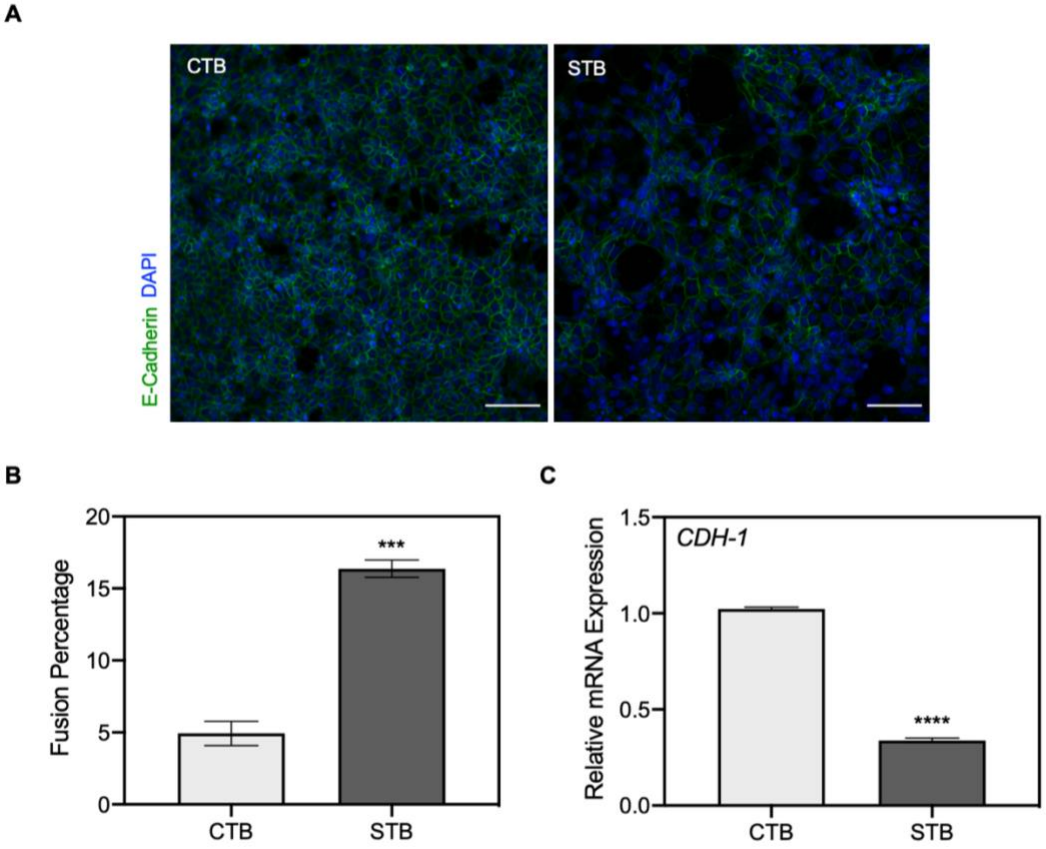

Figure 3. Visualization and quantification of BeWo b30 cell-cell fusion. (A) Immunofluorescent staining in which green fluorescence indicates E-Cadherin staining for plasma membranes and blue fluorescence indicates DAPI staining for cell nuclei in BeWo b30 cells. Representative fluorescent microscopy images are shown for the CTB and STB phenotypes. Images were captured at 20X magnification; scale bars indicate 100 μm. (B) Fusion percentage. (C) Relative mRNA expression of *CDH-1*. Significant differences between groups were determined using paired t-tests. Significant differences are indicated by ***p<0.0005 and ****p<0.0001; n=4.

Effects of Δ^9-THC on cell viability in BeWo b30 cells.

To determine an appropriate concentration of Δ^9-THC to utilize for *in vitro* experiments, BeWo b30 cells were treated with 0.1, 1, 5, 10, 15, 20 or 50 μM Δ^9-THC, or the vehicle control (MeOH). Cells were concurrently exposed to 50 ng/mL EGF and 50 μM FSK. Although treatment with both 5 and 10 μM Δ^9-THC enhanced cell viability, higher concentrations (20 and 50 μM) were associated with attenuated cell viability by least 1.5-fold (Fig. 4). Relative to the control, 20 μM Δ^9-THC was associated with a 67.8 ± 3.2% decrease, which was then further diminished to 54.8 ± 2.1% with 50 μM Δ^9-THC (Fig. 4).

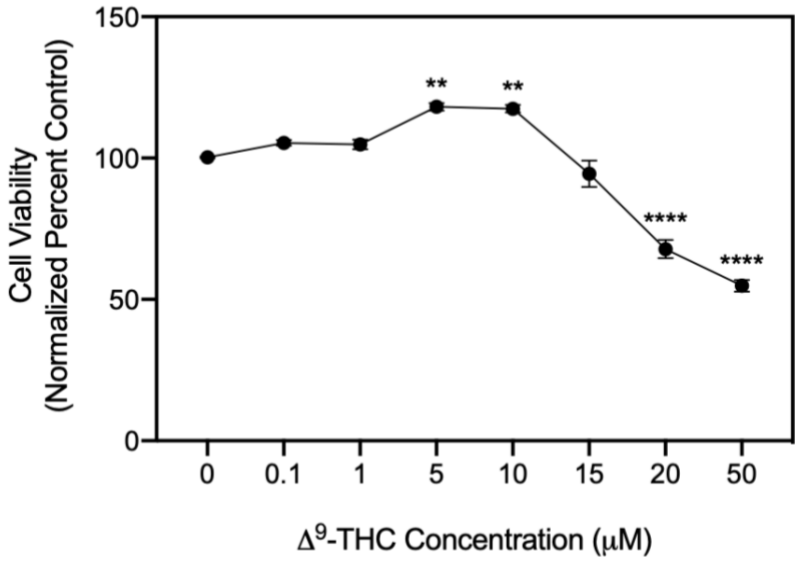

Figure 4. Effects of Δ^9-THC on cell viability in BeWo b30 cells. Normalized percent cell viability in response to various concentrations of Δ^9-THC for 48 hours with EGF and FSK to induce fusion. Significant differences relative to the control, as represented by no Δ^9-THC treatment, were determined using one-way ANOVA, followed by Tukey's multiple comparisons test. Significant differences relative to the control are indicated by $^{**}p<0.001$ and $^{****}p<0.0001$; n=3.

Δ^9-THC alters the expression of transcripts involved in regulating trophoblast fusion.

To investigate if Δ^9-THC exhibits dose-dependent effects on transcripts involved in trophoblast fusion, BeWo b30 cells were treated with 50 ng/mL EGF, 50 μM FSK and 0-50 μM Δ^9-THC. Out of the 6 concentrations of Δ^9-THC investigated, exposure to only 20 or 50 μM significantly altered levels of *ERVW-1*, *ERVFRD*-1, *hCG-β* and *CDH-1* (Fig. 5). In particular, 20 μM Δ^9-THC attenuated *ERVW-1*, *ERVFRD*-1 and *hCG-β* by 2.2-, 1.9- and 3.6-fold, respectively (Fig. 5A-C). At this concentration, there was also a 2.0-fold increase in *CDH-1* (Fig. 5D). Treatment with 50 μM Δ^9-THC further reduced the expression of *ERVW-1* (5.7-fold), *ERVFRD*-1 (18.2-fold) and *hCG-β* (64.4-fold) as well as increased the expression of *CDH-1* (2.4-fold) (Fig. 5).

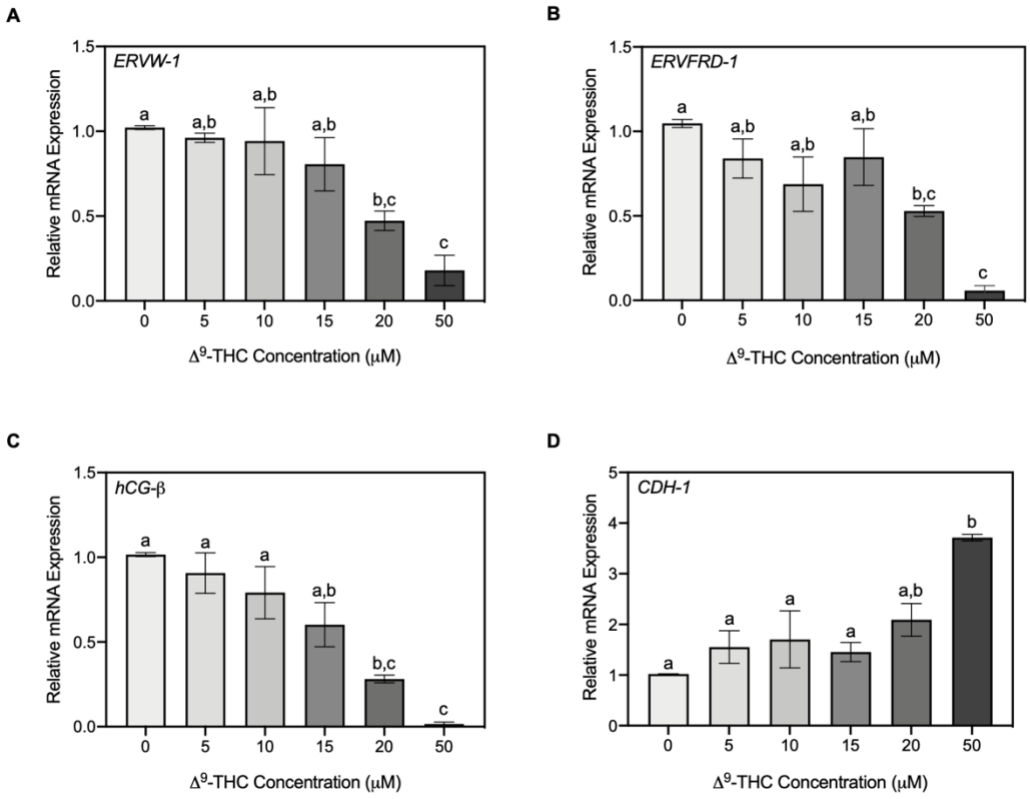

Figure 5. BeWo b30 cell exposure to Δ^9-THC alters the transcriptional profiles of genes involved in trophoblast differentiation and fusion. Relative mRNA expression of (A) *ERVW-1*, (B) *ERVFRD-1*, (C) *hCG-β*, (D) *CDH-1* after 48 hours of the displayed treatments with EGF and FSK to induce fusion. Significant differences between groups were determined using one-way ANOVA, followed by Tukey's multiple comparisons test. Different letters represent significant differences across treatment groups (p<0.05); n=3.

Discussion

The human placenta undergoes a series of morphological and functional changes over pregnancy to support the intrauterine development of the fetus [17,18]. One of these defining events include the continuous differentiation and cell-cell fusion of CTBs to form the multi-nucleated syncytium [17,18]. In comparison to other physiological frontiers, such as the blood-brain and blood-testis barriers, the mechanisms underlying trophoblast differentiation and fusion, and thus the establishment of the maternal-fetal interface, are not entirely understood [19,20]. This knowledge is required to advance our understanding of the structure and roles of the placenta in healthy and complicated pregnancies [21]. Therefore, we aimed to determine if there are differences in the ability of the BeWo and BeWo b30 cell lines to serve as *in vitro* models for studying syncytium development.

In BeWo and BeWo b30 trophoblasts, exposure to EGF (50 ng/mL) and FSK (50 μM) is reported to induce differentiation and fusion through a cAMP-dependent mechanism [1,8]. Our findings demonstrate that the cellular transition from mononucleated CTBs to multinucleated STBs is associated with enhanced transcription of the fusogenic markers *ERVW-1, ERVFRD-1* and *hCG-β*. While similar outcomes are observed for both BeWo and BeWo b30 cells, the parent cell line exhibits more dramatic increases in *ERVW-1*, *ERVFRD-1* and *hCG-β*. Cell fusion is also characterized by a decrease in E-Cadherin, which has been demonstrated in both cell lines at the gene level using *CDH-1* as well as protein level using immunofluorescent staining for E-Cadherin and DAPI. Despite both cell lines having a similar fusion percentage following stimulation with EGF and FSK,

BeWo b30 cells display a greater fold increase between the CTB to STB populations. Previous research also demonstrates that primary human trophoblasts exhibit a low baseline fusion capacity of about 5%, which then spontaneously rises to 60-70% after 48-hours in cell culture. Taken together, our results suggest that BeWo b30 cells may be able to better recapitulate primary human trophoblast fusion due more significant differences observed before and after the induction of fusion. Moving forward in this thesis, experiments have been performed using the BeWo b30 cell line.

The BeWo and BeWo b30 cell lines have transformed reproductive research in the field of placental biology due to their time- and cost-effectiveness as well as ability to form cell monolayers and undergo fusion [1,22]. However, their derivation from a gestational choriocarcinoma, relatively low fusogenic capacity compared to primary human trophoblasts and inability to recapitulate the dynamic microenvironment of the maternal-fetal interface are considerable limitations. As a result, primary trophoblasts that are isolated and purified from human placentae, either after termination or parturition, can also serve as *in vitro* models for syncytium development [4]. We have completed preliminary experiments using villous trophoblasts obtained from healthy term placentae following caesarean deliveries. The associated methods and results are presented in the Appendix.

While primary human trophoblasts exhibit greater functional relevance to *in vivo* differentiation and fusion compared to immortalized cell lines, several challenges are associated with their use for experimentation [4]. A thin layer of villous trophoblasts

covering the placenta villi is left by term, thus cell isolations contain an increased proportion of stromal, decidual and red blood cells [4]. First- and second-trimester trophoblasts therefore serve as more suitable models for investigating the formation of the syncytium as these gestational time-points are defined by elevated rates of CTB turnover [4]. Following the removal of contaminating cell types, primary trophoblasts exhibit problems in culture, including an inability to proliferate and requirement of an extracellular matrix due to poor adherence to plastic [4]. Primary CTBs also require full cell confluency to form aggregates and undergo fusion, and display an estimated 45% decrease in cell viability after 24 hours of culture [13]. Finally, reproducibility of placental research is an additional difficulty due to variations in maternal age, gestational age, parity and/or presence of pregnancy complications [4].

In this chapter, we also examined how BeWo b30 trophoblasts respond to the principal psychoactive component of cannabis, Δ^9-THC, during differentiation and fusion. We set out to create a simplistic model to investigate the isolated effects of Δ^9-THC on the predominant cell type in the placenta, the trophoblasts. Greater physiological relevance can be obtained in future studies by utilizing cannabis extracts and/or co-culture model systems with decidual immune or stromal cell populations. Based on an assessment of cell viability and the expression of fusogenic genes following exposure to Δ^9-THC (0-50 μM), our findings support the use of 20 μM Δ^9-THC in BeWo b30 cells. This concentration correlates to a classification of weak cytotoxicity as 67.8% of cells remained viable after 48 hours of exposure [23]. Within the range of 60-80% cell viability, alterations observed in functional

outcomes can be attributed to drug exposure as opposed to collateral damage that results from cell death [24]. In addition, the mean peak plasma concentrations after smoking cannabis with a 1.8-6.8% content of Δ^9-THC are reported to be 0.079-76 μg/mL (Outlined in Chapter 1) [25,26]. Since this is equivalent to approximately 0.25-250 μM Δ^9-THC, the use of 20 μM is within the range of what has been reported in humans following smoke exposure.

Overall, this chapter demonstrates that BeWo b30 cells exhibit a greater fold increase in fusion as cells transition from CTBs to STBs when compared to the parent cell line. Using this model, we also provide rationale for cell treatment with 20 μM Δ^9-THC to explore how responses during trophoblast differentiation and fusion may be altered.

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CHAPTER 3: Δ^9-THC MODULATES THE IMMUNE MICROENVIRONMENT AND COMPROMISES STEROID HORMONE BIOSYNTHESIS DURING HUMAN CTB FUSION

This chapter addresses research objectives 2 and 3:

2. To investigate the effects of Δ^9-THC on the production of Th1 and Th2 cytokines by villous trophoblasts.
3. To investigate the effects of Δ^9-THC on hormone biosynthesis and secretion in localized inflammatory and immunomodulatory microenvironments during villous trophoblast differentiation and fusion.

Abstract

Maternal cannabis use during pregnancy can adversely impact placental and fetal development, with sustained implications extending into adulthood. Despite the high prevalence of cannabis use in pregnant women and ability of its bioactive component Δ^9-THC to compromise trophoblast fusion, its biological mechanisms remain poorly understood. Therefore, the present study aimed to elucidate whether Δ^9-THC disrupts immune responses mediated by trophoblasts during fusion. We also characterized the effects of Δ^9-THC on fusogenic and steroidogenic factors in localized inflammatory Th1 cytokine- and immunosuppressive Th2 cytokine-dominant microenvironments through TNF-α and IL-10 stimulation, respectively. Using the human trophoblast BeWo b30 cell line, we demonstrate that Δ^9-THC behaves as an immunomodulatory agent by favouring Th2 cytokine production. Furthermore, Δ^9-THC alters the production of steroid hormones that participate in immune regulation, resulting in decreased progesterone and increased cortisol at the transcriptional and protein levels. These immune and endocrine outcomes are further shown to be mediated by CB1R activation. Taken together, our findings suggest that prenatal exposure to Δ^9-THC may disrupt cytokine and hormone regulation at the maternal-fetal interface, which may contribute to altered placental development and reduced fetal growth.

Introduction

In human pregnancy, the fetus experiences a period of rapid intrauterine development that is dependent on the dynamic morphological and functional transformation of the placenta [1]. The generation of CTBs from the trophectoderm marks the beginning of placental formation, which is followed by their differentiation and fusion into STBs [1,2]. These multinucleated STBs are localized to the outer epithelial lining of villous trees and give rise to the placental barrier known as the syncytium [3,4]. STBs lack the capacity to proliferate, thus maintenance of the integrity of the syncytium requires continuous trophoblast turnover throughout gestation [5]. In terms of function, the syncytium modulates the placental transfer of nutrients, gases and waste products [3,4,6]. This placental barrier is also the predominant source of hormones required for the maintenance of pregnancy, such as hCG and progesterone, and contributes to fetal protection against excessive cortisol in cases of maternal psychosocial stress, glucocorticoid treatment and/or drug use [7–10].

In addition to serving as a central site for metabolic and endocrine functions, the syncytium contributes to the uteroplacental cytokine network [11]. Human pregnancy is sustained by tightly orchestrated cytokine responses that balance maternal immune suppression to accept the semi-allogenic fetus with immune activation to protect against infection [12,13]. While a Th1 cytokine bias is required for implantation, invasion, labour and parturition, pregnancy predominantly favours a Th2 cytokine shift for fetal growth and uterine expansion [13]. These immunological responses are regulated by decidual immune cells, placental Hofbauer cells, and trophoblasts [14–16]. In fact, CTBs and STBs, collectively known as

villous trophoblasts, are reported to express and secrete various Th1 cytokines, including IL-2, TNF-α and IFN-γ [17–19]. Production of Th2 cytokines, such as IL-4, IL-10 and TGF-β, is also documented [17,19,20]. Villous trophoblasts can therefore modulate gestational immune responses, however the roles of Th1 and Th2 cytokines in maintaining the syncytium during healthy pregnancy remain unclear. Furthermore, alterations in trophoblast-mediated cytokine production, potentially due to placental drug exposure, can lead to impaired maternal immune adaptations, which may then contribute to pregnancy complications such as preterm birth and preeclampsia [12,21,22].

Although the placenta transiently develops to protect the fetus during its in utero existence, it is not a perfect barrier as drugs can cross the placenta to enter the fetal bloodstream [6]. Research on transplacental drug transfer gained significant traction following the thalidomide tragedy from the 1950-1960s that resulted in thousands of severe congenital birth defects [23]. Like thalidomide, which was marketed to alleviate nausea and vomiting in early pregnancy, cannabis is often used by expectant mothers partially due to its anti-emetic effects [23,24]. An important point of contrast, however, is that thalidomide is classified as a teratogen whereas cannabis has not been associated with teratogenic risk to date [23]. Despite the self-reported benefits, adverse outcomes following prenatal cannabis exposure have been reported in three major longitudinal investigations: the Ottawa Prenatal Prospective Study (Ottawa, Canada), the Maternal Health Practices and Child Development Study (Pittsburgh, United States), and the Generation R Study (Rotterdam, Netherlands) [25–27]. Clinical complications were observed from the time of fetal development and

followed until early adulthood, however these primarily focused on neurodevelopment outcomes such as impaired memory, hyperactivity and heightened impulsivity [28,29]. Additional investigations have further linked cannabis use during pregnancy with low fetal birth weight, small for gestational age status, and preterm birth [30–33].

Moreover, our recent *in vitro* research demonstrates that the primary psychoactive component of cannabis, Δ^9-THC, impairs the fusion of human placental BeWo cells [34]. The BeWo cell line, along with its BeWo b30 clone, are representative of villous trophoblasts and serve as cell culture models for syncytium formation [35,36]. While Δ^9-THC can interact with the CB1R and CB2R of the endocannabinoid system, its inhibitory effects on trophoblast fusion have been shown to be primarily mediated by CB1R activation [34,37,38]. Δ^9-THC is also reported to exert immunomodulatory effects in immune cell types, leading to outcomes such as altered cytokine and chemokine production, apoptosis and cell proliferation [39,40]. Exposure to this cannabinoid may therefore compromise the regulation of Th1/Th2 cytokines by placental trophoblasts as well. A bias toward Th1 responses and inflammation in human pregnancy is associated with preterm labour, preterm premature rupture of membranes and preeclampsia whereas exaggerated Th2 responses may lead to an increased risk of maternal infection and decreased fetal birth weight [41–43].

In the present study, our objective was to investigate the effects of Δ^9-THC exposure on the synthesis and secretion of Th1/Th2 cytokines during trophoblast differentiation and

fusion in BeWo b30 cells. We also explored whether Δ^9-THC, concomitant with cytokine stimulation, compromises the hormonal biosynthesis and secretion of progesterone and cortisol in villous trophoblasts. Finally, we provide insight into the role of CB1R activation in regulating trophoblast cell-cell fusion and steroidogenesis, which are two important contributors to the establishment of the multi-nucleated syncytium of the human placenta.

Materials and Methods

Cell culture

BeWo b30 cells (AddexBio, C0030002) were cultured in Dulbecco's Modified Eagle's Medium (DMEM with 4.5 g/L glucose; Corning, 10-013-CV) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained in a humified atmosphere at 37°C and 5% CO_2. Cells were seeded at a density of $1x10^5$ cells/cm^2 in 6-well polystyrene plates, unless otherwise stated. Undifferentiated cells, representative of CTBs, were treated with Δ^9-THC (20 μM; Sigma Aldrich, T4764) or the MeOH vehicle for a 48 hour period [34]. In cells representative of STBs, monolayer formation was promoted using EGF (50 ng/ml) for a 48 hour period [44]. Cells were subsequently induced to differentiate and undergo fusion using EGF (50 ng/ml) and FSK (50 μM) for another 48 hours [35]. The addition of Δ^9-THC (20 μM) or MeOH was concomitant with EGF and FSK.

Cytokine stimulation

To evaluate the effects of exogenous cytokine exposure on trophoblast differentiation and fusion, BeWo b30 cells were treated with EGF (50 ng/ml), FSK (50 μM) and TNF-α (10

ng/ml; Sigma Aldrich, H8916) or the Dulbecco's Phosphate-Buffered Saline (PBS) and BSA (0.1%) vehicles [45–47]. Similarly, cells were treated with EGF, FSK and IL-10 (10 ng/ml; Sigma Aldrich, SRP3071) or the sodium phosphate (SP; 5 mM) and BSA (0.1%) vehicles [48,49]. The addition of Δ^9-THC (20 μM) was concomitant with TNF-α or IL-10 for 48 hours.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells using TRIzol™ Reagent (500 μL; Thermo Fisher Scientific, 15596026) and isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research, R2050), according to the manufacturer's instructions. Quantification of gene expression using RT-PCR was performed as previously described by our research group [50]. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ analysis, normalized to the endogenous control genes, *18S* and *β-actin*, and expressed as the fold change to the vehicle control. Primers sequences designed for all target genes of interest are listed in Table 1.

Gene	**Forward sequence (5' → 3')**	**Reverse sequence (5' → 3')**
18S	CACGCCAGTACAAGATCCCA	AAGTGACGCAGCCCTCTATG
β-actin	TTACAGGAAGTCCCTTGCCATC	GCAATGCTATCACCTCCCCTG
IL-2	AACCTCAACTCCTGCCACAA	GCATCCTGGTGAGTTTGGGA
IFN-γ	GCCAAGGTGGATGTGTTTCG	GTCCAATGGGGCCTTCAGAT
TNF-α	GCTGCACTTTGGAGTGATCG	GGGTTTGCTACAACATGGGC
IL-4	GCTATGACTAAAGCAGGGACAA	CACTCTCCTCAGCAAACACAGA
IL-10	TGAGAACCAAGACCCAGACA	TCATGGCTTTGTAGATGCCT
TGF-β	CTACGCCGCCTACTACTGTG	TCAGGATGACGTTGGAGCTG
ERVW-1	TCATATCTAAGCCCCGCAAC	CGCCAATGCCAGTACCTAGT
ERVFRD-1	CCTTCACTAGCAGCCTACCG	GCTGTCCCTGGTGTTTCAGT
hCG-β	ACCCCTTGACCTGTGAT	CCTTATTGTGGGAGGATCGG
CYP11A1	CATTTCTGGAGGGAGACGGG	GCGGGATGAGGAATCGTTCT

HSD3β1	CCCGCAGGAGTCTATATTCTGAT	GGATACTCACCAGTCCCAGC
PGR	GGGCTACGAAGTCAAACCCA	TGTGAGCTCGACACAACTCC
CYP17A1	GCCCCATCTATTCGGTTCGT	CAGAGTCAGCGAAGGCGATA
CYP21A1	CGACCTCCCCATCTATCTGC	TGGCTTCCTCAATGGTCCTC
CYP11B1	GTGAACATCCCCAGAAGGCA	GGTTGCGACCCAGAGAGTAG
NR3C1	ACGTTACCACAACTCACCCC	TTTGCCCATTTCACTGCTGC

Table 1. Forward and reverse primer sequences used for RT-PCR.

Immunofluorescence

BeWo b30 cells were cultured on 4-well glass culture slides. Cells were fixed using ice-cold MeOH for 5 minutes at 20°C. Non-specific binding was blocked using Tween-20 (0.01%), goat serum (10%) and BSA (1%) in PBS for 2 hours at room temperature. Cells were then incubated with Anti-E-Cadherin primary antibody (1:500 dilution; Abcam, ab40772) overnight at 4°C. Subsequently, cells were incubated with Goat Anti-Rabbit IgG Alexa Fluor® 488 (1:1000 dilution; Abcam, ab150077) secondary antibody for 45 minutes at room temperature. Both antibodies were diluted using Tween-20 (0.01%) and BSA (0.1%) in PBS. Cells were counterstained and mounted using Fluormount-G™ Mounting Medium with DAPI (Invitrogen, 00-4959-52). Slides were then visualized using an Eclipse Ti-E Inverted Fluorescence Microscope (Nikon Instruments), and images were captured using 20x magnification at five randomly selected, non-overlapping fields per sample. To visualize fusion, E-Cadherin and DAPI staining identified plasma membranes and cell nuclei, respectively [51]. The cell counts of two blinded individuals were averaged to quantify fusion using the following equation: Fusion percentage (%) = (Number of nuclei in fused cells/Number of total nuclei) x 100%.

Protein quantification and ELISA

Total protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 23227), according to the manufacturer's instructions. Absorbance was measured at 562 nm using the Spark® Multimode Microplate Reader (Tecan Trading AG). Cell culture supernatant was collected for ELISA to assess the secretion of progesterone (Invitrogen, EIAP4C21), TNF-α (Invitrogen, BMS223HS), IL-10 (Abcam, ab100549) and cortisol (Cusabio, CSB-E05111h), according to the manufacturer's instructions. Secreted protein levels from the ELISAs were then normalized to the total cellular protein concentrations.

Cannabinoid receptor antagonism

To determine the role of CB1R, BeWo b30 cells were treated with the selective antagonist AM281 (1 μM; Santa Cruz Biotechnology, 202463-68-1) [52]. Cells were first incubated with AM281 for 30 minutes at 37°C, which was followed by treatment EGF (50 ng/ml), FSK (50 μM) and the vehicle control (MeOH) or Δ^9-THC (20 μM), as previously described, for 48 hours.

Statistical analyses

All experiments were replicated at least three times. Data are presented as the mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons tests were utilized to compare treatment groups. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.

Results

Fusion of trophoblasts is characterized by an increase the Th1 cytokine TNF-α and decrease in the Th2 cytokine IL-10 at the transcript and protein levels.

To determine whether BeWo b30 cellular fusion is a Th1 cytokine- or Th2 cytokine-dominant process, the transcriptional profiles of *IL-2*, *IFN-γ*, *TNF-α*, *IL-4*, *IL-10* and *TGF-β* were investigated. Changes in mRNA expression were assessed following 48 hours of exposure to EGF and FSK, which are chemical agents that induce differentiation and fusion, or the vehicle control (MeOH). BeWo b30 cells demonstrated a 2.7- and 6.7-fold increase in *IL-2* and *TNF-α*, respectively (Fig. 1A,C). The other Th1 cytokine, *IFN-γ*, showed a decrease (Fig. 1B). Transcripts for the Th2 cytokines *IL-4* and *IL-10* also exhibited a 1.6- and 2-fold decrease, respectively, whereas *TGF-β* was not significantly altered during fusion (Fig. 1D-F). We then selected the Th1 cytokine TNF-α and Th2 cytokine IL-10 for further investigation. Changes in the secretion of TNF-α and IL-10 by BeWo b30 cells undergoing fusion were assessed using cell culture supernatant. Normalized protein levels of TNF-α were increased by 1.4-fold, although this difference was not statistically significant, while those of IL-10 decreased by 2.4-fold (Fig. 2).

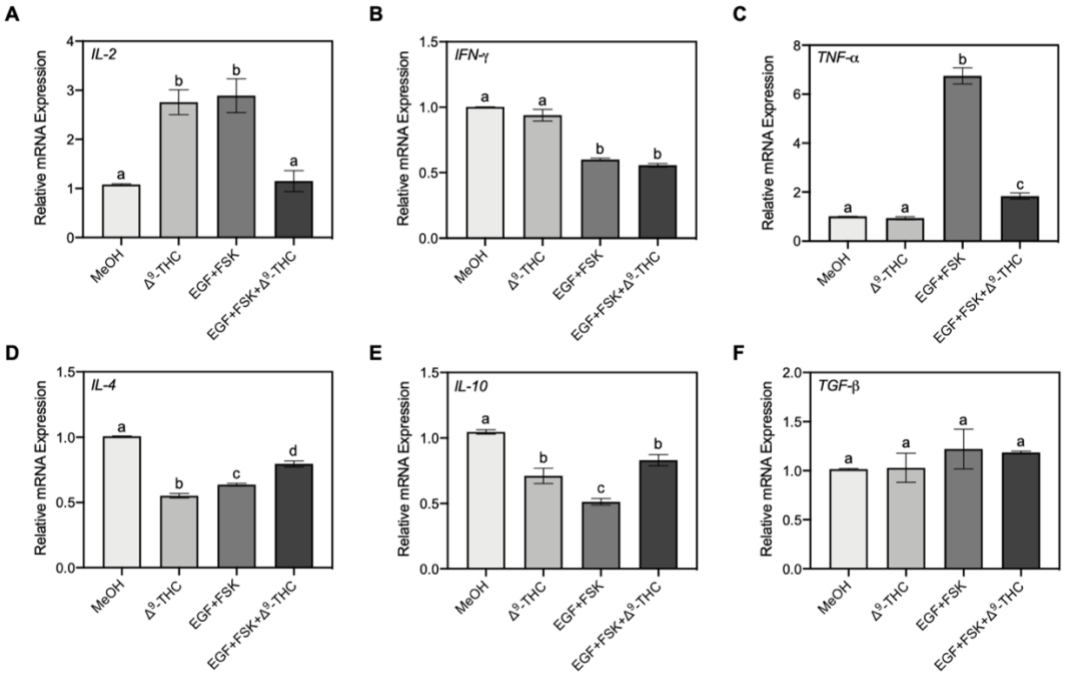

Figure 1. Exposure to Δ^9-THC alters the transcriptional profiles of various Th1 and Th2 cytokines during cellular fusion. Relative mRNA expression of (A) *IL-2*, (B) *IFN-γ*, (C) *TNF-α*, (D) *IL-4*, (E) *IL-10* and (F) *TGF-β* after 48 hours of the displayed treatments in BeWo b30 cells. The MeOH and EGF+FSK groups are representative of unfused CTBs and fused STBs, respectively. Δ^9-THC treatment was performed in the CTB and STB populations. Different letters represent significant differences across treatment groups. One-way ANOVA followed by Tukey's multiple comparisons test were utilized to compare treatment groups; n=4; $p<0.05$.

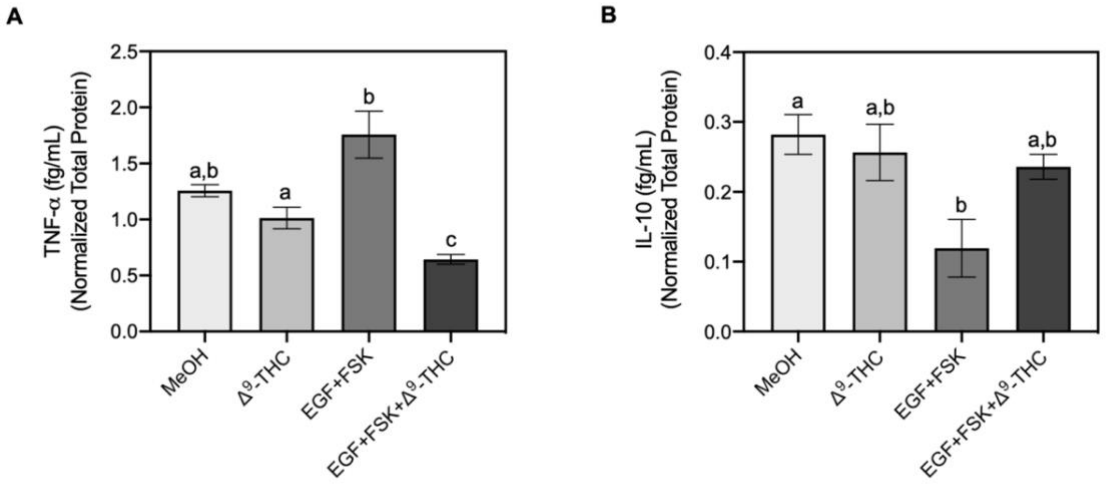

Figure 2. Exposure to Δ^9-THC inhibits the secretion of TNF-α, not IL-10, during cellular fusion. Protein secretion of (A) TNF-α and (B) IL-10 in cell culture supernatant after 48 hours of the displayed treatments in BeWo b30 cells. Values are normalized to total protein content from the respective wells. The MeOH and EGF+FSK groups are representative of unfused CTBs and fused STBs, respectively. Δ^9-THC treatment was performed in the CTB and STB populations. Different letters represent significant differences across treatment groups. One-way ANOVA followed by Tukey's multiple comparisons test were utilized to compare treatment groups; n=3; $p<0.05$.

Effects of Δ^9-THC on the Th1/Th2 cytokine profile during trophoblast fusion.

To assess the effects of 20 μM Δ^9-THC treatment on Th1/Th2 cytokine regulation, the mRNA expression of *IL-2*, *IFN-γ*, *TNF-α*, *IL-4*, *IL-10* and *TGF-β* was evaluated in unfused and fused cells. In comparison to the vehicle control, Δ^9-THC increased the expression of *IL-2* (2.6-fold) as well as decreased the expression of *IL-4* (1.8-fold) and *IL-10* (1.5-fold) in unfused cells (Fig. 1). Exposure to Δ^9-THC during fusion, however, was associated with contrasting outcomes wherein the mRNA expression of *IL-2* (2.5-fold) and *TNF-α* (3.7-fold) was downregulated while *IL-4* (1.3-fold) and *IL-10* (1.6-fold) were upregulated (Fig. 1). Similar results were observed for the normalized protein levels of TNF-α and IL-10, however Δ^9-THC only significantly altered TNF-α levels in cells undergoing fusion through a 2.7-fold decrease (Fig. 2). Therefore, our results indicate that Δ^9-THC treatment leads to altered Th1/Th2 cytokine responses in villous trophoblasts by attenuating TNF-α and enhancing IL-10.

Δ^9-THC diminishes the effects of TNF-α stimulation on factors involved in mediating trophoblast fusion.

To further investigate how Δ^9-THC impacts the regulation of Th1/Th2 cytokines by trophoblasts, BeWo b30 cells undergoing fusion were treated with 20 μM Δ^9-THC, 10 ng/ml TNF-α or Δ^9-THC+TNF-α. We assessed changes in mRNA expression and fusion percentage after 48 hours of each treatment condition. TNF-α was associated with at least a 1.5-fold increase in the mRNA expression of *ERVW-1* and *ERVFRD-1*, which are genes that encode the fusogenic proteins syncytin-1 and syncytin-2, respectively (Fig. 3A,B).

Interestingly, TNF-α showed contrasting results for the hormonal regulation of fusion as it was associated with a 1.5-fold decrease in *hCG-β* (Fig. 3C). We then visualized cell-cell fusion using immunofluorescent staining with E-Cadherin and DAPI, which displayed a decrease in the number of fused nuclei within one plasma membrane boundary (Fig. 3D). Quantification of fusion based on the captured images further demonstrated a 1.9-fold reduction in fusion following TNF-α treatment (Fig. 3E). In comparison to TNF-α or Δ^9-THC, BeWo b30 cell treatment with Δ^9-THC+TNF-α restored the levels of *ERVW-1* and *ERVFRD-1* (Fig. 3A,B). No significant alterations were observed in *hCG-β* levels with Δ^9-THC+TNF-α relative to either treatment alone (Fig. 3C). In contrast to these findings, Δ^9-THC+TNF-α treatment reduced the fusion percentage in comparison to TNF-α alone (Fig. 3E).

IL-10 stimulation does not significantly alter trophoblast fusion.

To evaluate the effects of Δ^9-THC and IL-10, BeWo b30 cells undergoing fusion were treated with 20 μM Δ^9-THC, 10 ng/ml IL-10 or Δ^9-THC+IL-10. No significant differences were observed in the transcriptional markers of fusion (*ERWV-1*, *ERVFRD-1*, *hCG-β*) or fusion percentage following IL-10 stimulation relative to its vehicle controls (Fig. 4). Treatment with Δ^9-THC+IL-10 did not significantly alter *ERWV-1*, *ERVFRD-1* and *hCG-β* expression compared to only IL-10 (Fig. 4A-C). However, the addition of Δ^9-THC further reduced the fusion percentage (Fig. 4E).

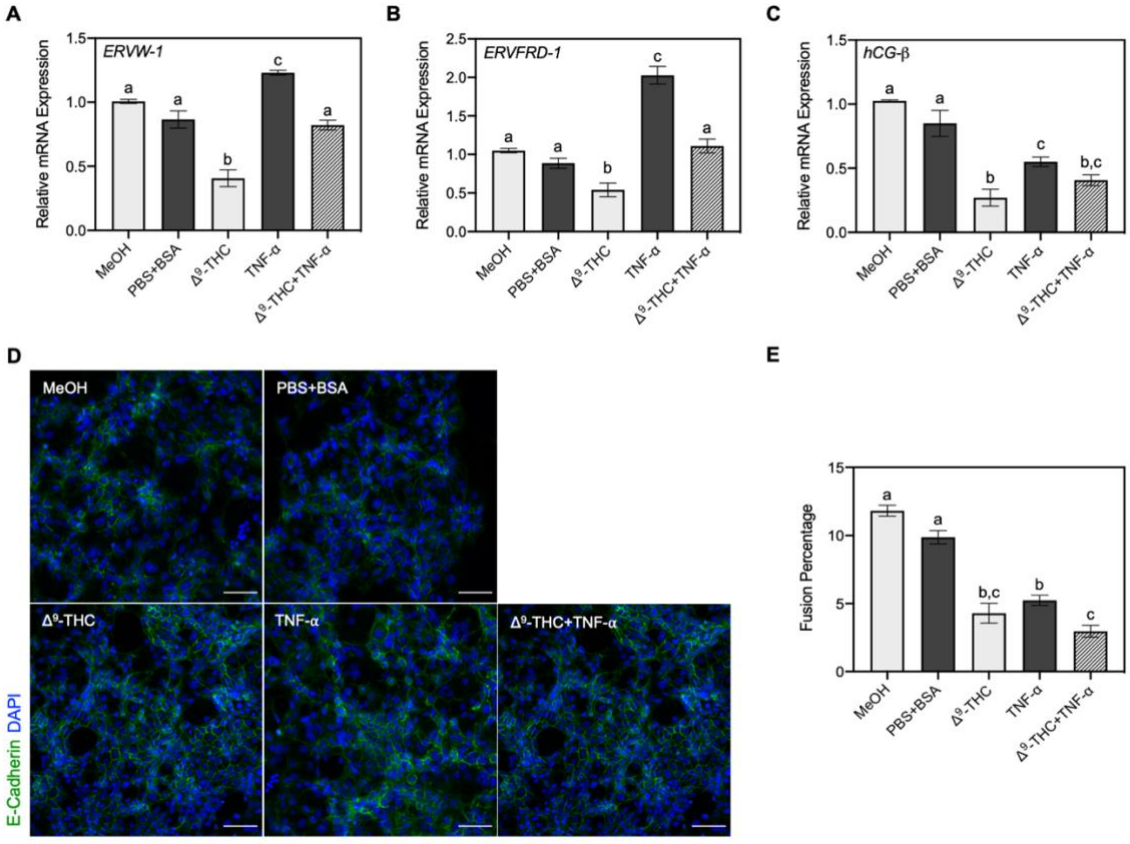

Figure 3. Effects of TNF-α on factors that regulate trophoblast fusion are altered by Δ⁹-THC. Relative mRNA expression of (A) *ERVW-1*, (B) *ERVFRD-1* and (C) *hCG-β* after 48 hours of the displayed treatments alongside EGF and FSK to induce fusion in BeWo b30 cells. (D) Immunofluorescent staining in which green fluorescence indicates E-Cadherin staining for plasma membranes and blue fluorescence indicates DAPI staining for cell nuclei in BeWo b30 cells. Representative fluorescent microscopy images are shown for MeOH, PBS+BSA, Δ^9-THC, TNF-α and Δ^9-THC+TNF-α. Images were captured at 20X magnification; scale bars indicate 100 μm. (E) Fusion percentage. Each treatment condition is shown in the same colour as its corresponding vehicle. Different letters represent significant differences across treatment groups. One-way ANOVA followed by Tukey's multiple comparisons test were utilized to compare treatment groups; n=6 (A-C) and n=4 (D-E); $p<0.05$.

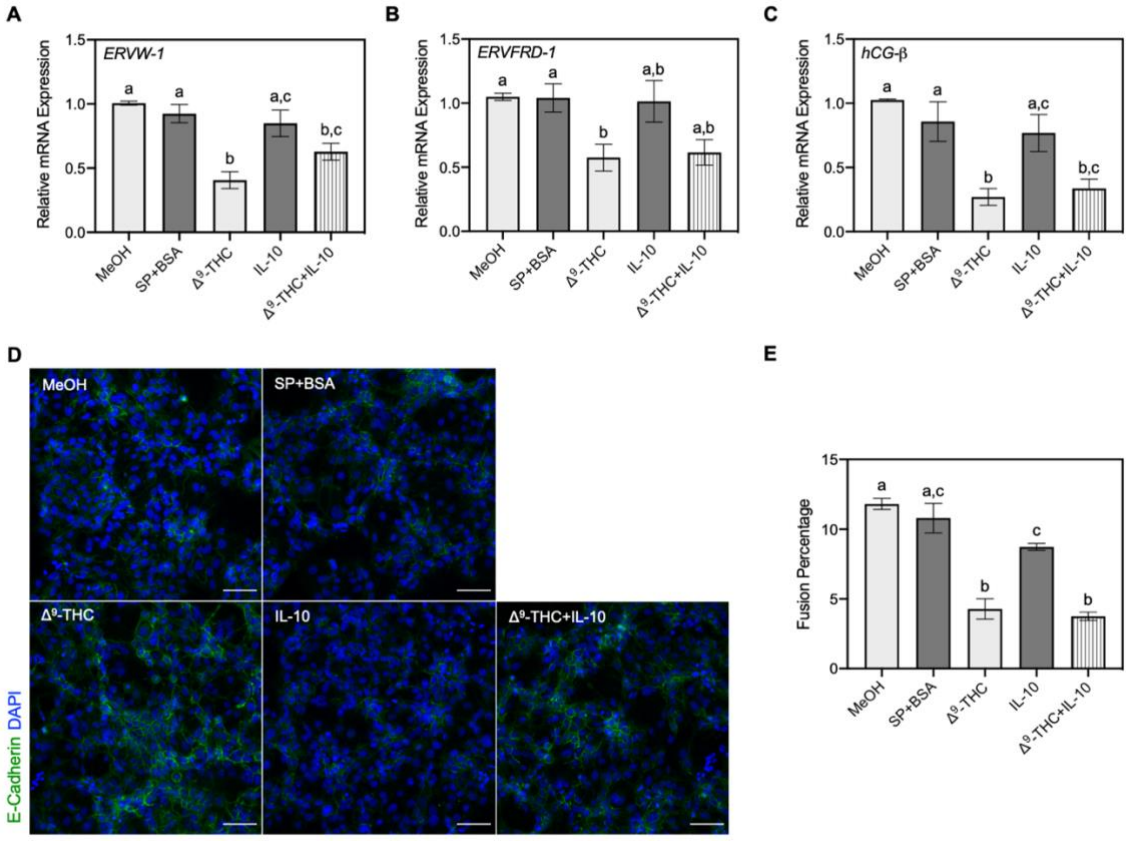

Figure 4. IL-10 does not significantly alter factors involved in trophoblast fusion. Relative mRNA expression of (A) *ERVW-1*, (B) *ERVFRD-1* and (C) *hCG-β* after 48 hours of the displayed treatments alongside EGF and FSK to induce fusion in BeWo b30 cells. (D) Immunofluorescent staining in which green fluorescence indicates E-Cadherin staining for plasma membranes and blue fluorescence indicates DAPI staining for cell nuclei in BeWo b30 cells. Representative fluorescent microscopy images are shown for MeOH, SP+BSA, Δ^9-THC, IL-10 and Δ^9-THC+IL-10. Images were captured at 20X magnification; scale bars indicate 100 µm. (E) Fusion percentage. Each treatment condition is shown in the same colour as its corresponding vehicle. Different letters represent significant differences across treatment groups. One-way ANOVA followed by Tukey's multiple comparisons test were utilized to compare treatment groups; n=6 (A-C) and n=4 (D-E); $p<0.05$.

Trophoblast steroidogenesis is inhibited by Δ^9-THC and enhanced by TNF-α.

To explore if progesterone synthesis is influenced by Δ^9-THC-mediated alterations in the production of TNF-α and IL-10, the transcriptional profiles of cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*) and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3β1*) were investigated. No significant alterations in the levels of our target genes were observed for the vehicle controls. These included MeOH for Δ^9-THC, PBS+BSA for TNF-α and SP+BSA for IL-10, no significant alterations in the levels of our target genes were observed. Moreover, expression of *CYP11A1*, which regulates the conversion of cholesterol to pregnenolone, was reduced 2.7-fold by Δ^9-THC and increased 1.9-fold by TNF-α (Fig. 5A,B). The subsequent gene that converts pregnenolone to progesterone in the placenta, *HSD3β1*, was significantly altered by only Δ^9-THC (2.5-fold decrease) (Fig. 5A,C). Treatment with Δ^9-THC+TNF-α did not further reduce *CYP11A1* or *HSD3β1* levels relative to TNF-α alone (Fig. 5B,C). Normalized total protein levels of progesterone followed the same trend as its transcriptional regulation wherein Δ^9-THC enhanced and TNF-α attenuated its secretion by at least 1.6-fold (Fig. 5F). To further look into the mechanisms by which Δ^9-THC and TNF-α disrupt progesterone synthesis, we examined the mRNA expression of the progesterone receptor (*PGR*). Δ^9-THC (2.2-fold decrease) and TNF-α (1.6-fold increase) had contrasting effects on *PGR*, which remained unchanged with Δ^9-THC+TNF-α treatment relative to just TNF-α (Fig. 5D). IL-10 stimulation displayed no significant effects on progesterone production (Fig. 5).

Next, we explored whether cortisol synthesis is influenced by Δ^9-THC-mediated alterations in the production of TNF-α and IL-10. We assessed the transcriptional profiles of cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*), cytochrome P450 family 21 subfamily A member 1 (*CYP21A1*) and cytochrome P450 family 11 subfamily B member 1 (*CYP11B1*). The roles of these genes are outlined in Fig. 6A. *CYP17A1* was reduced by Δ^9-THC and Δ^9-THC+TNF-α by over 5-fold (Fig. 6B). *CYP21A1* was significantly increased 1.7-fold by TNF-α and remained unchanged by Δ^9-THC or Δ^9-THC+TNF-α (Fig. 6C). Interestingly, Δ^9-THC and TNF-α alone upregulated levels of *CYP11B1* by 1.9-fold, however the effects of Δ^9-THC+TNF-α were not significantly different from the vehicle controls (Fig. 6D). The normalized protein secretion also corroborated that both Δ^9-THC (3.6-fold) and TNF-α (1.9-fold) are associated with elevated cortisol levels (Fig. 6F). Finally, we assessed the mRNA levels of nuclear receptor subfamily 3 group C member 1 (*NR3C1*), which encodes the glucocorticoid receptor. Expression of *NR3C1* was upregulated by Δ^9-THC (1.5-fold) and downregulated by TNF-α (1.8-fold), with no significant changes following the addition of Δ^9-THC+TNF-α (Fig. 6E). Similar to progesterone, IL-10 had no significant effects on cortisol production (Fig. 6). These results are indicative of perturbed hormonal regulation in response to Δ^9-THC or the Th1 cytokine TNF-α contributing to altered trophoblast cell-cell fusion.

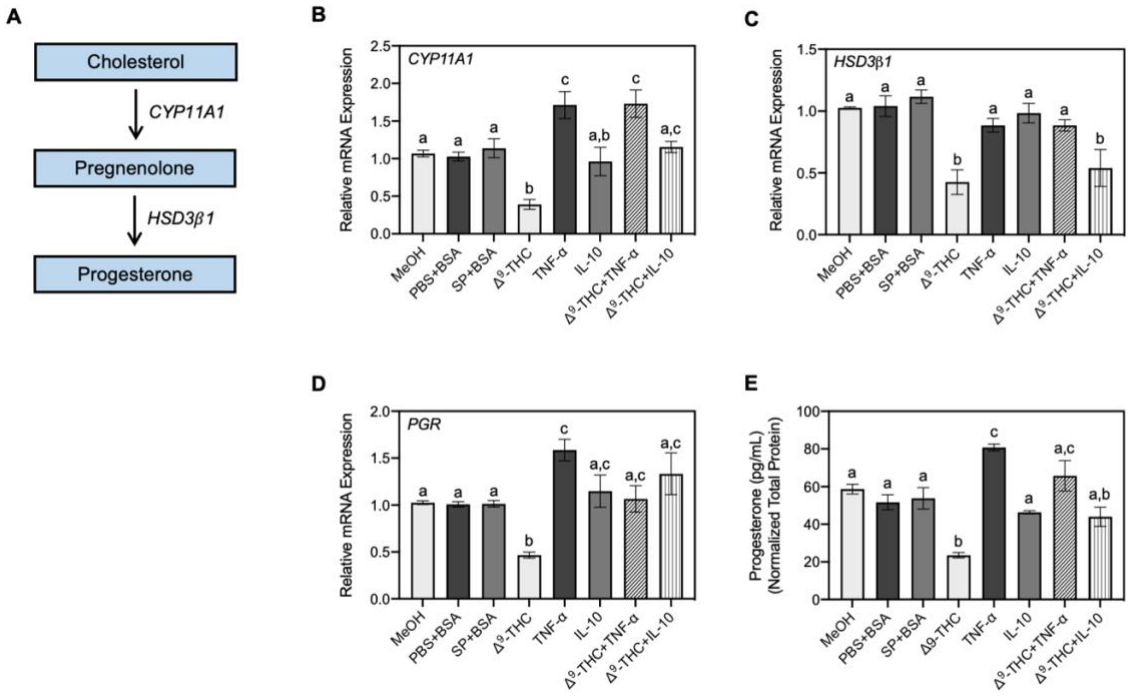

Figure 5. Effects of TNF-α, IL-10 and Δ^9-THC on the transcriptional regulation and protein secretion of progesterone. (A) Schematic representation of progesterone biosynthesis from cholesterol in the human placenta, with the genes responsible for each conversion. (B-D) Relative mRNA expression of (B) *CYP11A1*, (C) *HSD3β1* and (D) *PGR* in BeWo b30 cells. (E) Protein secretion of progesterone in the cell culture supernatant of BeWo b30 cells. All treatments were performed for 48 hours alongside EGF and FSK to induce fusion. Each treatment condition is shown in the same colour as its corresponding vehicle. Different letters represent significant differences across treatment groups. One-way ANOVA followed by Tukey's multiple comparisons test were utilized to compare treatment groups; n=6 (B-E) and n=3 (E); $p<0.05$.

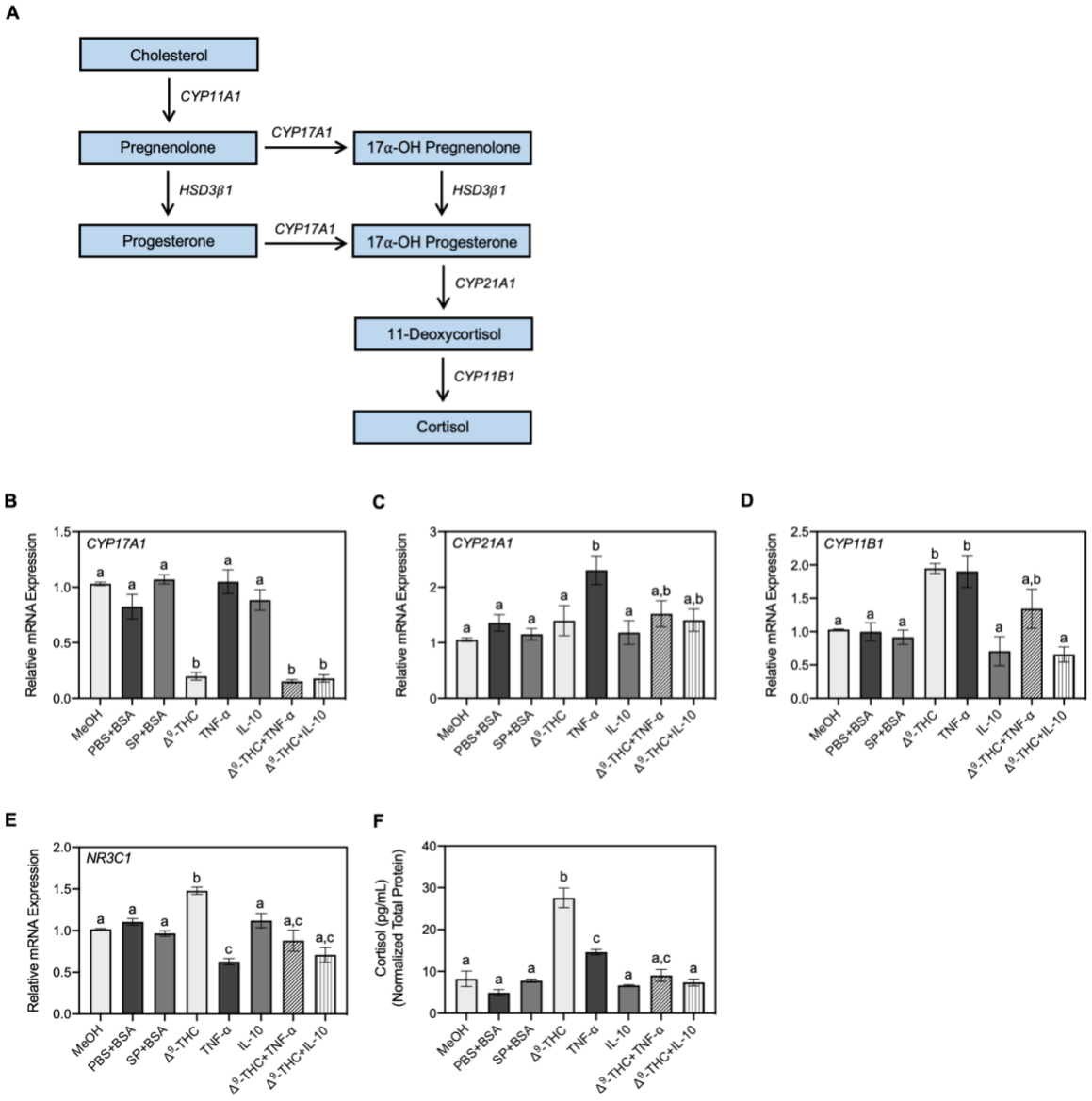

Figure 6. Effects of TNF-α, IL-10 and Δ^9-THC on the transcriptional regulation and protein secretion of cortisol. (A) Schematic representation of cortisol biosynthesis from cholesterol in the human placenta, with the genes responsible for each conversion. (B-D) Relative mRNA expression of (B) *CYP17A1*, (C) *CYP21A2*, (D) *CYP11B1* and (E) *NR3C1* in BeWo b30 cells. (F) Protein secretion of cortisol in the cell culture supernatant of BeWo b30 cells. All treatments were performed alongside EGF and FSK to induce fusion for 48 hours. Each treatment condition is shown in the same colour as its corresponding control. Different letters represent significant differences across treatment groups. One-way

ANOVA followed by Tukey's multiple comparisons test were utilized to compare treatment groups; n=6 (B-E) and n=3 (F); $p<0.05$.

CB1R antagonism restores the expression of fusogenic and steroidogenic transcripts.

To examine if Δ^9-THC disrupts trophoblast fusion and steroidogenesis via activation of CB1R, BeWo b30 cells were treated with 1 μM AM281, a selective CB1R antagonist, followed by EGF, FSK and the vehicle control (MeOH) or 20 μM Δ^9-THC. We assessed changes in mRNA expression after 48 hours of each treatment condition. Consistent with our earlier findings, Δ^9-THC reduced levels of fusogenic (*ERVW-1*, *ERVFRD-1*, *hCG-β*) and steroidogenic (*CYP11A1*, *HSD3β1*) transcripts, as well as increased *CYP11B1*, by at least 1.4-fold (Fig. 7). Antagonism of CB1R then restored the expression of all markers, with the exception of *ERVFRD-1* which remained statistically different from the vehicle control (Fig. 7).

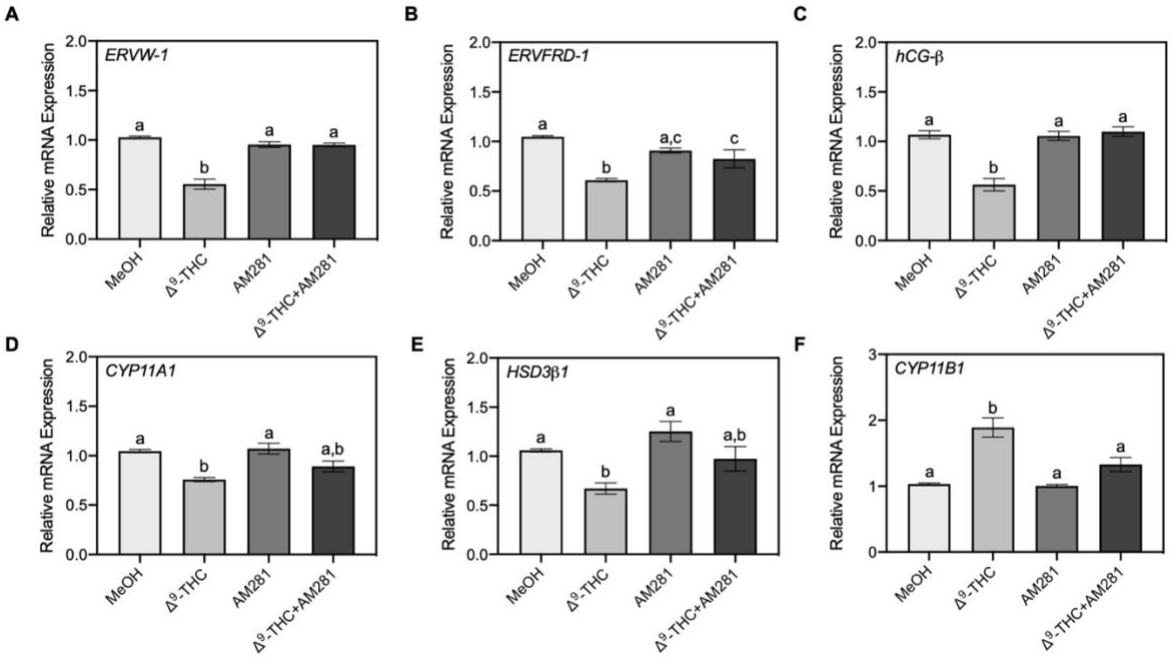

Figure 7. Activation of CB1R by Δ^9-THC contributes to inhibited fusion and steroidogenesis in trophoblasts. Relative mRNA expression of (A) *ERVW-1*, (B) *ERVFRD-1*, (C) *hCG-β*, (D) *CYP11A1*, (E) *HSD3β1* and (F) *CYP11B1* after 48 hours of the displayed treatments alongside EGF and FSK to induce fusion in BeWo b30 cells. Different letters represent significant differences across treatment groups. One-way ANOVA followed by Tukey's multiple comparisons test were utilized to compare treatment groups; n=6; $p<0.05$.

Discussion

CTB differentiation and fusion are necessary functions for maintaining the integrity of the human syncytium as these mononucleated cells reside subjacent to an layer of terminally differentiated STBs [5,53]. As a result, villous trophoblasts undergo a process of turnover wherein CTBs proliferate, differentiate, fuse, and replace aged STBs that are then shed into the maternal circulation as syncytial knots through apoptosis or necrosis [5]. Critical proteins that participate in trophoblast fusion include syncytin-1 and syncytin-2, both of which are upregulated through a cyclic adenosine monophosphate-dependent mechanism [35]. STBs also secrete the pregnancy hormone hCG, with its β-subunit commonly used as a fusogenic marker as it differentiates hCG from luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone [54].

In vitro exposure to Δ^9-THC (10-30 μM) can disrupt trophoblast turnover by inhibiting cell proliferation, differentiation, and fusion [34,55,56]. Δ^9-THC can also readily cross the placental barrier due to its lipophilic nature, with 10-28% of its maternal plasma concentrations crossing over into fetal circulation where Δ^9-THC will accumulate in fetal adipose tissue [57,58]. An important consideration, however, is that these investigations of placental cannabinoid transfer have been conducted in murine and non-human primate models. Δ^9-THC (5-20 μM) is also associated with suppressing responses initiated by various immune cell types, including dendritic cells, macrophages, natural killer cells and T cells, however its effects on trophoblast immunology during cellular differentiation and fusion have yet to be elucidated [39,59–63]. Given that maternal cannabis use may also

alter the contribution of trophoblasts in shaping the immune microenvironment at the maternal-fetal interface, it is necessary to understand the immunological changes that may occur following placental exposure to Δ^9-THC. Therefore, the present study was designed to explore how Δ^9-THC, in combination with the Th1 cytokine TNF-α or the Th2 cytokine IL-10 to represent either an inflammatory or immunomodulatory microenvironment, respectively, alter trophoblast immune and endocrine functions during cell-cell fusion.

The development of the placenta begins at approximately two weeks post-fertilization, with complete formation of the syncytium occurring by the end of the first trimester [5]. Trophoblast turnover, however, continues throughout gestation to maintain placental barrier integrity [5]. Our work demonstrates that CTB fusion may contribute to the inflammatory response that is characteristic of the early stages of syncytium development. We observed an increase in the transcription of *TNF-α* and *IL-2* and a decrease in *IL-4* and *IL-10* after stimulation with EGF and FSK, in comparison to the MeOH vehicle [13]. Similar trends were observed for TNF-α and IL-10 protein secretion, although TNF-α was not statistically different from its vehicle controls. When cellular fusion was disrupted with 20 μM Δ^9-THC, we observed attenuated TNF-α and enhanced IL-10 transcription and protein levels, thus implicating Δ^9-THC in the modulation of trophoblast immune responses.

Alterations in the orchestration of immune adaptations during pregnancy can manifest as clinical consequences for the mother and fetus [64]. While TNF-α is necessary for

gestational events such as blastocyst implantation, invasion and labour, aberrant maternal serum levels are reported in pregnancies complicated by intrauterine growth restriction and preeclampsia [65,66]. In this study, we provide further insight into the roles of TNF-α, used at a concentration that stimulates an inflammatory response, during trophoblast fusion [45]. We observed conflicting evidence for the effects of exogenous TNF-α (10 ng/ml) as it enhanced levels of the syncytin protein-encoding genes, *ERVW-1* and *ERVFRD-1*, yet attenuated *hCG-β* transcription and morphological fusion in trophoblasts. A possible explanation is that these outcomes may result from autocrine signalling that functions to control inflammation, however placental and decidual immune cell types also produce TNF-α and the associated paracrine interactions were not explored in this study [67,68]. While TNF-α (10 ng/ml) has also been reported to increase the protein expression of syncytin-1 in a human squamous choriocarcinoma cell line (SCC-9), this translated to enhanced cell fusion [69]. However, CTBs isolated from term human placentae following caesarean delivery that were treated with TNF-α (10 ng/ml) likewise displayed attenuated hCG-β production and fusion, thus supporting our observed results [49]. We also indicate that the addition of Δ^9-THC attenuates the effects of TNF-α in BeWo b30 cells by decreasing syncytin gene expression as well as the fusion percentage. Δ^9-THC can therefore interfere with trophoblast fusion by altering immune responses both at baseline and conditions mimicking exacerbated inflammation, as demonstrated through TNF-α stimulation.

Our results also suggest that IL-10 does not play a direct role in CTB fusion as it did not significantly alter any of the investigated fusogenic characteristics. In the presence of Δ^9-THC, however, the morphological fusion of BeWo b30 cells was attenuated compared to only IL-10. This indicates that IL-10 regulation can be perturbed by Δ^9-THC, which may then have consequences for gestation as plasma levels of IL-10 are significantly elevated in pregnant women compared to age-matched non-pregnant women [70]. While the functions of IL-10 during placental development have not been explored, increased levels are shown to contribute to successful pregnancy whereas decreased levels are associated with complications such as spontaneous abortion and preeclampsia [71,72].

Maternal immune responses during pregnancy, which are necessary to support successful tolerance of the semi-allogenic fetus and overall pregnancy, are partly regulated by reproductive steroid hormones [73]. Steroidogenesis in the fetal-placental unit is responsible for the synthesis of pregnancy-associated hormones such as estrogen and progesterone from cholesterol [74]. While the placenta does not independently produce estrogen as it cannot synthesize the dehydroepiandrosterone precursor, STBs are a major source of progesterone in pregnancy [75,76]. Clinically, the Society of Obstetricians and Gynaecologists of Canada (SOGC) recommends vaginal progesterone administration to prevent spontaneous preterm birth [77]. Progesterone therapy is supported for women who have a history of previous spontaneous preterm birth or a short cervical length, which is defined as less than 25 mm between 16-24 weeks of gestation [77]. Moreover, progesterone is important for the maintenance of pregnancy as it regulates immune responses by leading

to decreased Th1 cytokine production and increased Th2 cytokine production in immune and reproductive cell types [76]. Other functions of progesterone include supporting blastocyst implantation, pregnancy maintenance and mammary gland development [76,78]. Our results further indicate that progesterone may have a role in trophoblast fusion as its transcription (*CYP11A1* and *HSD3β1*) and protein secretion were attenuated by Δ^9-THC and enhanced by TNF-α in trophoblasts. Treatment with Δ^9-THC+TNF-α did not further disrupt progesterone production relative to either compound alone. These observations may be the result of altered expression of the nuclear progesterone receptor (*PGR*), which is a transcription factor [76]. Two isoforms exist for the progesterone receptor, however only isoform A displayed consistent gene expression across controls in BeWo b30 cells. Although progesterone (3 μg/mL) is reported to inhibit TNF-α secretion in human trophoblasts, no associations have been made concerning the effects of exogenous TNF-α on progesterone production [79]. Disruption of progesterone following Δ^9-THC exposure in trophoblasts has similarly not been explored to date. Our research suggests that Δ^9-THC and TNF-α can alter progesterone production, which may then play a role in compromising trophoblast fusion.

In addition to the synthesis of reproductive steroid hormones, steroidogenesis in the fetal-placental unit sustains the production of glucocorticoids [74]. Cortisol is a major glucocorticoid that plays a necessary role in fetal development, with total plasma levels rising three-fold by the third trimester [80]. The synthesis of cortisol during pregnancy is regulated by the hypothalamic-pituitary-adrenal (HPA) axis wherein release of

corticotrophin-releasing hormone (CRH) from the hypothalamus stimulates adrenocorticotrophin hormone (ACTH) from the pituitary gland and in turn, cortisol from the adrenal cortex [81]. Elevated cortisol then stimulates placental CRH, which participates in a positive feedback loop by acting on the maternal pituitary and releasing ACTH [81]. Despite the increase in cortisol during pregnancy, the fetus is protected from excessive exposure as the placenta converts up to 90% of circulating cortisol into inactive cortisone [81]. In cases of maternal stress and drug use during pregnancy, however, the fetus can be exposed to high levels of cortisol due to disruptions in the HPA axis [9,82]. Our investigation of cortisol production in trophoblasts revealed that both Δ^9-THC and TNF-α can increase levels of the *CYP11B1* transcript, which regulates the conversion of 11-deoxycortisol into cortisol as well as secreted cortisol. However, the Δ^9-THC+TNF-α combination did not significantly alter the transcript or protein expression levels of cortisol relative to either compound. Δ^9-THC also enhanced the expression of the glucocorticoid receptor (*NR3C1*) in trophoblasts, suggesting that Δ^9-THC can stimulate this transcription factor to potentially control the synthesis of cortisol [83,84]. Although the effects of Δ^9-THC exposure on cortisol production by trophoblasts have not been evaluated, adolescents who have been prenatally exposed to cannabis display characteristics of a hypoactive HPA axis response, which is associated with prolonged exposure to cortisol and negative behavioural outcomes such as externalizing problems and aggression [82,85].

Due to its activity as a partial agonist, Δ^9-THC can mediate its cellular effects through CB1R signalling [37]. Previous work by our research group demonstrates that exposure to

the CB1R antagonist AM281 restores the Δ^9-THC-induced reduction in fusogenic gene expression whereas a CB2R antagonist does not [34]. In support of this, the present study indicates that interactions between Δ^9-THC and CB1R contribute to disrupted cellular fusion and steroidogenesis in BeWo b30 cells. Since the endocannabinoid system plays a role in regulating reproductive events that range from ovulation to labour, exposure to Δ^9-THC may interfere with placental development through compromised signalling in trophoblasts [86,87].

Our findings also indicate that within a localized inflammatory microenvironment mediated by TNF-α stimulation, Δ^9-THC can modulate fusion through the transcriptional regulation of the syncytins [13]. However, Δ^9-THC does not display any synergistic actions alongside IL-10, another immunomodulatory agent. We further demonstrate that exposure to Δ^9-THC or TNF-α can perturb the production of progesterone and cortisol, thereby providing support for a tightly regulated cytokine and hormonal network contributing to trophoblast fusion in BeWo b30 cells. The effects of Δ^9-THC on fusogenic and steroidogenic markers are also shown to be mediated through Δ^9-THC-CB1R binding. Since maternal cannabis use is associated with low fetal birth weight, small for gestational age status and preterm birth, the activation of CB1R by Δ^9-THC may play a role in mediating these adverse outcomes [30–33]. Ultimately, Δ^9-THC compromises the cytokine and hormonal responses in villous trophoblasts, which may be a consequence of its immunomodulatory role in shifting from a Th1 to Th2 cytokine-dominant state during differentiation and fusion.

An understanding of the implications of a Th2 cytokine bias in gestation is necessary as it leaves pregnant women immunologically disadvantaged when it comes to fighting off infections [88]. In fact, pregnancy is historically linked to an elevated risk of contracting viral outbreaks, with the 1964 rubella and 2009 influenza A virus subtype H1N1 pandemics serving as the most documented [88]. Reported clinical complications associated with rubella and influenza infections during pregnancy include fetal growth restriction, stillbirth, preterm birth, and maternal mortality [89,90]. Pregnant women are postulated to be at a disproportionate risk of developing viral infections due to reductions in Th1 cytokine production by decidual immune cells and placental trophoblasts, which in turn translates to diminished inflammatory responses and cell-mediated immunity that are necessary for pathogenic clearance [64,91]. This is particularly relevant to the novel coronavirus disease 2019 as cannabis use is associated with a 5-fold greater risk of infection in comparison to the general population due to respiratory comorbidities [92]. We speculate that prenatal consumption of cannabis may increase maternal susceptibility of developing viral infections, such as coronavirus disease 2019 (COVID-19), as Δ^9-THC can impair the Th1/Th2 cytokine balance in villous trophoblasts. However, additional investigations are required to explore the effects of Δ^9-THC on cytokine responses mediated by immune cells at the maternal-fetal interface before associations between cannabis use and risk of viral infection in pregnancy can be made.

Although the present study contributes to a cellular understanding of the implications of cannabis use in pregnancy, our human trophoblast model is limited in that we have

investigated the role of an isolated cannabinoid in only one cell type. To extrapolate our results to *in vivo* placental development, further investigations are necessary to elucidate the regulation of trophoblast immune and endocrine responses in the presence of decidual immune cell types given the dynamic nature of the maternal-fetus interface [14]. Ultimately, an understanding of how Δ^9-THC impacts female reproduction is critical as the prevalence of cannabis use has steadily increased during pregnancy over recent years, particularly during the first trimester when the placenta is undergoing formation [29].

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CHAPTER 4: CONCLUSIONS

Summary of Findings

In the present thesis, we first aimed to select a functionally relevant trophoblast cell line to model trophoblast differentiation and fusion. Given that there is a lack of clarity pertaining to differences in the traditionally employed human choriocarcinoma-derived BeWo and BeWo b30 cell lines, we questioned whether one of these models is better able to recapitulate syncytium development *in vitro*. In Chapter 2, we compared the ability of the BeWo and BeWo b30 cell lines to model fusion by examining the transcriptional regulation of *ERVW-1* (syncytin-1), *ERVFRD-1* (syncytin-2) and *hCG-β* (Fig. 1). As expected, all genes displayed an increase following treatment with EGF (50 ng/mL) and FSK (50 μM) to induce FSK-stimulated fusion. The listed concentrations of EGF and FSK have previously been used for fusion stimulation in BeWo cells [1]. Further assessment of fusion using immunofluorescent staining for E-Cadherin, an epithelial protein that is inversely correlated with fusion, quantification using cell counts and transcription of *CDH-1* (E-Cadherin) demonstrated that the parent and cloned cell lines similarly model villous trophoblasts (Figs. 2 and 3). We ultimately chose the BeWo b30 cell line for subsequent investigations due to a greater fold difference from baseline to the induction of fusion using EGF and FSK (CTB versus STB populations). Next, we set out to perform drug-dose experiments to ascertain an appropriate concentration of Δ^9-THC to investigate its effects on trophoblast immune and endocrine roles during fusion. Based on the observed outcomes

for viability (Fig. 4) and mRNA levels of fusion-related markers (Fig. 5), we moved forward with implementing 20 μM Δ^9-THC alongside EGF and FSK [1].

As an extension of the research in Chapter 2, we outline our preliminary work with successfully isolating and purifying primary trophoblasts from term human placentae in the Appendix. Following negative selection for trophoblasts, cells exhibited an increase in the mRNA levels of the trophoblast marker *hCG-β* as well as decrease in the non-trophoblast marker *VIM* (Fig. 1). This leads us to the ask the question: Do the mechanisms underlying trophoblast fusion differ between choriocarcinoma-derived cell lines and primary human trophoblasts isolated from term placentae? Progression of this work should therefore explore the expression of fusogenic genes (*ERVW-1*, *ERVFRD-1*, *hCG-β*, *CDH-1*) and quantify fusion in primary human trophoblasts. This will provide a foundation for investigating and comparing how BeWo b30 cells versus primary human trophoblasts respond to Δ^9-THC during differentiation and fusion.

Based on our early work in selecting an appropriate cell model and drug concentration, we evaluated the effects of Δ^9-THC on the Th1/Th2 cytokine balance in villous trophoblasts in Chapter 3. Our findings are suggestive of trophoblast differentiation and fusion requiring an inflammatory Th1 cytokine-dominant environment, which is like other early pregnancy events such as implantation and invasion [2] (Figs. 1 and 2). We then recapitulated a localized inflammatory and immunomodulatory microenvironment through trophoblast stimulation with TNF-α (10 ng/mL) and IL-10 (10 ng/mL) as these cytokines showed the

greatest alterations during fusion and in response to Δ^9-THC. At a concentration of 10 ng/mL, TNF-α stimulated an inflammatory response in trophoblasts, which has previously been demonstrated via an increase in the expression of activating mediators that include intercellular adhesion molecule 1 and IL-8 [3]. The use of 10 ng/mL IL-10 may similarly function to modulate exacerbated inflammation [4]. We show that IL-10 had no direct impact on syncytium establishment, however exogenous TNF-α was associated with compromised fusion and biosynthesis of progesterone and cortisol, both of which are tightly regulated throughout pregnancy (Figs. 3 and 4) [5,6]. Concomitant treatment with Δ^9-THC and TNF-α also perturbed the regulation of genes involved in fusion (*ERVW-1*, *ERVFRD-1*, *hCG-β*) and steroid hormone synthesis (*CYP11A1*, *CYP17A1*) (Figs. 3, 5 and 6). Lastly, we suggest that the observed outcomes may be the result of, at least in part, Δ^9-THC-mediated activation of the classical CB1R of the endocannabinoid system (Fig. 7).

Bench to Bedside: Clinical Implications

Villous trophoblast turnover in placental pathology

Successful formation of the human placenta is central to the maintenance of pregnancy due to its roles in hormone production and maternal-fetal transport of nutrients, gases and wastes [7]. Alterations in trophoblast turnover can perturb placental development, with increased CTB proliferation leading to exaggerated fusion and apoptosis [8]. In the opposite situation wherein CTB proliferation is decreased, the syncytium is thinned due to the release of aged STB cell components into the cytoplasm [8,9]. Our findings demonstrate that a reduction in trophoblast fusion can be mediated by exposure to exogenous stressors

such as Δ^9-THC (20 μM) and TNF-α (10 ng/mL). Previous investigations by our research group also indicate that Δ^9-THC can disrupt trophoblast turnover at the organelle level, specifically the mitochondria which contribute to the regulation of placental oxidative stress [1]. In particular, STBs treated with Δ^9-THC (20 μM) exhibit mitochondrial dysfunction through elevated heat shock protein and superoxide dismutase production as well as attenuated membrane potential [1]. As a consequence of impaired syncytial activity, transplacental passage can become limited and systemic maternal immune responses may be initiated due to an increase in necrosis [8,9]. Abnormalities commonly associated with perturbed trophoblast turnover include preeclampsia, fetal growth restriction, placental abruption and stillbirth [7,9,10].

Susceptibility to viral infections in pregnancy

During pregnancy, women possess a higher risk of developing infections compared to non-pregnant healthy counterparts due to a Th2 cytokine bias in the second and third trimesters [11]. Cell-mediated immunity is necessary to effectively fight against infection and is primarily stimulated by Th1 cells [12,13]. Due to the decrease in Th1 cytokine production, pregnancy is associated with an increased susceptibility to viral infections [12,13].

In addition to immune cells at the maternal-fetal interface, trophoblasts participate in the activating response against pathogens [14]. Using the hepatitis C virus as an example, viral detection promotes the production of IFNs and migration of uterine natural killer cells via activating chemokines such as interferon-inducible protein-10 and C-C motif chemokine

ligand 5 [15]. This demonstrates that exposure to viruses during pregnancy shifts the required Th1/Th2 balance to favour inflammation [14]. Moreover, hepatitis C virus has a transmission rate of 3-6% and is associated with adverse outcomes such as preterm birth, fetal growth restriction, fetal growth and development of pediatric cirrhosis [15]. In fact, vertical transfer of pathogens across the placenta can occur through a variety of mechanisms that include infection of endothelial cells in the maternal vasculature, EVTs or decidual immune cells as well as compromised integrity of the placental syncytium [16]. The notorious ZIKA virus can also be transmitted vertically, with a rate of 25-47% [17]. ZIKA virus infection is linked to congenital microcephaly, which results in cognitive and physical delays in exposed neonates [16]. Other outcomes that have been associated with antenatal ZIKA infection include placental insufficiency, fetal growth restriction and fetal death [16].

Historically, some of the most well-documented outbreaks that disproportionately impacted pregnant women include rubella (1964-1965) and H1N1, an influenza A strain (2009-2010) [18,19]. Beginning with rubella, maternal infection manifested as congenital rubella syndrome in exposed neonates [20]. Clinical outcomes associated with fetal rubella infection include cardiac, ophthalmic, audiologic and central nervous system defects [20]. Transmission of rubella to the fetus also primarily occurred if the mother was exposed at less than 16 weeks of gestation and was associated with an infection rate of up to 80% [20]. The advent and introduction of the rubella vaccine in 1969, however, assisted in reducing the rate of transmission and development of congenital rubella syndrome to 8% [20].

Furthermore, influenza pandemics have plagued humans since at least 1918, the time of the Spanish flu, and most recently, during 2009 [21]. Pregnant women are 5-fold more likely to contract influenza, however rates of vertical transmission are not readily available [21]. H1N1 is associated with pregnancy complications such as spontaneous abortion and preterm premature rupture of membranes as well as consequences for fetal health, including neural tube defects, tachycardia, encephalopathy and cerebral palsy [21].

One of the risk factors for maternal-to-fetal viral transmission is drug use during pregnancy [22]. The effects of drug use have been most investigated in the case of hepatitis C virus, which has a vertical transmission rate of 8-11% if mothers have engaged in prenatal intravenous drug use versus 5% in non-users [22,23]. While research has not explored how maternal cannabis use in particular impacts vertical transmission, a damaged syncytium can be breached by viruses and lead to fetal infection [24]. Viruses that cannot cross the maternal-fetal interface can also impact fetal development through placental infection [24]. Based on our findings, it is plausible that maternal cannabis use may increase the risk of fetal infection as Δ^9-THC is associated with perturbed Th1/Th2 cytokine responses during fusion [24]. This may occur through increased viral passage to the fetus due to poor development or damage of the syncytium [24]. To explore this avenue, research must focus on elucidating how Δ^9-THC influences placental immune responses during infection.

Maternal and fetal cannabis exposure during the COVID-19 pandemic

Following the first report of COVID-19 (December 2019), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, the turbulent global impact resulted in hundreds of publications surrounding the effects of COVID-19 in pregnancy [25]. A systematic review that compiled data from 192 studies found that 7-28% of pregnant women were suspected or confirmed to be positive for COVID-19 from December 2019 to June 2020 [25]. This large range of prevalence may be the result of variance across sampling strategies [25]. Adverse outcomes have been commonly reported in mothers whereas the risks to the fetus appear to be minimal and require further investigation [25]. Compared to healthy pregnant women, those with COVID-19 have a higher incidence of being admitted to an intensive care unit, requiring ventilation or extracorporeal membrane oxygenation, preterm birth and in rare cases, preeclampsia [25,26]. Besides the increased likelihood of being admitted into an intensive care unit, current literature suggests that neonates do not possess a high risk of developing complications [25]. This is attributed to rare vertical transmission of SARS-CoV-2 due to low rates of transplacental movement of the viral load and a lack of placental infection [27]. However, conflicting evidence also suggests that there is reduced transfer of anti-SARS-CoV-2 antibodies across the placenta [27]. Additional research is therefore required to ascertain the potential risks of SARS-CoV-2 vertical transmission and infection in neonates.

Another factor that contributes to a high risk of contracting COVID-19 is cannabis use [28]. A retrospective case-controlled study of 80 non-pregnant patients diagnosed with both

cannabis use disorder and COVID-19 observed an elevated prevalence of respiratory risk factors, including asthma and chronic obstructive pulmonary disease [28]. Lockdown measures further contributed to a rise in cannabis use. For example, one investigation reported that 40% of individuals endorsed greater consumption during this period in a survey [29]. Similar statistics are not available for changes in patterns of cannabis use during pregnancy due to COVID-19. Given that pregnant women and cannabis users represent two vulnerable populations, an investigation of the implications of maternal cannabis use during the COVID-19 pandemic, particularly for pregnancy success and fetal health, is necessary.

Limitations

In vitro to *in vivo* translatability

While FSK-stimulated BeWo b30 cells are well-validated in their ability to model villous trophoblast fusion, they display genetic and morphological differences in comparison to primary human trophoblasts [30]. The limitations of BeWo b30, and the parent BeWo, cells are outlined in detail in Chapter 2. In addition to primary trophoblasts that can be isolated and purified from human placentae, most often after caesarean delivery at term, animal models can be utilized for greater extrapolation to *in vivo* trophoblast functionality [30]. The use of mouse models has transformed placental biology as they allow researchers to investigate cell-cell communication at the whole organ level [31]. Like humans, mice have a hemochorial placenta that allows for direct contact between trophoblasts and maternal blood, and possess a similar blastocyst structure [30,31]. However, mouse models come

with unique caveats as they have a short gestational length of about 20 days and display significant differences, compared to humans, in placental structure following implantation [32]. For example, mouse placentae have trophoblast giant cells at the outer lining instead of CTBs and STBs [32]. Non-human primates, sheep and rats can also be used to study the development of the placenta [32]. Outside of animals, human embryonic stem cells, placental explants and trophoblast organoids are commonly published model systems [30].

Consideration of low placental oxygenation

The development of the human placenta occurs in a relatively hypoxic environment, beginning at a partial pressure of 20 mmHg in the first trimester and thereafter increasing to 60 mmHg [33,34]. 20-60 mmHg corresponds to an oxygen concentration of about 2.5-7.5% [33,34]. Despite this, the majority of cellular experiments, ours included, are performed under 21% oxygen, which ultimately creates another limitation for *in vitro* models of the human trophoblast and placenta [33,35]. In a comparison of primary trophoblast culture at 2.5% and 21% oxygen, no significant differences were observed in viability or apoptosis during differentiation and fusion [33]. However, contrasting outcomes have been reported for trophoblast fusion under low oxygenation as hypoxia has been shown to both downregulate and have no significant effect on syncytin expression [9,33]. To better mimic the *in vivo* placental environment, future cell culture work can be performed under 2.5-7.5% oxygenation.

Dynamic architecture of the maternal-fetal interface

At the maternal-fetal interface, there is an intricate network of trophoblasts, Hofbauer cells, endothelial cells, smooth muscle cells and immune cells [30]. While the present thesis investigated cytokine responses mediated by villous trophoblasts during differentiation and fusion, decidual leukocytes are the primary contributors to the Th1/Th2 cytokine balance that supports fetal tolerance [36]. Of the immune cell populations localized at the maternal-fetal interface, uterine natural killer cells the most prevalent and well-studied [37,38]. Their roles include the production of important cytokines and growth factors such as the granulocyte and macrophage colony-stimulating factors [39]. Uterine natural killer cells also closely interact with invasive EVTs to support invasion and vascular remodelling by producing angiogenic mediators, particularly vascular endothelial growth factor [40,41]. Since uterine natural killer cells express the classical cannabinoid receptors CB1R and CB2R, an understanding of whether these cells contribute to cannabis-mediated complications in pregnancy is warranted [42]. Although we did not explore the paracrine responses mediated by the studied Th1/Th2 cytokines, we examined how trophoblast-specific immune responses are regulated during fusion and altered following Δ^9-THC exposure. Since trophoblasts represent the major cell type in the placenta, this work demonstrates that immune dysregulation in CTBs and STBs can have consequences for syncytium formation during pregnancy.

Future Directions

Co-culture model with villous trophoblasts and uterine natural killer cells

In the present thesis, we provide insight into the immune and endocrine roles of villous trophoblasts during differentiation and fusion to better understand placental development in health and disease. A natural progression of this research is to explore the paracrine effects of immune cells in response to Δ^9-THC-dependent alterations in Th1/Th2 cytokine production by trophoblasts. The answer to this may lie in the development of a co-culture model system with STBs and uterine natural killer cells. While published models are more commonly available for uterine natural killer cell and EVT co-culture, only a handful have utilized STBs [43–46]. This discrepancy is likely attributable to the lack of direct communication and contact between these cell types. Nonetheless, BeWo or BeWo b30 cells can be cultured with human NK-92 cells to create a model wherein the effects of Δ^9-THC treatment on placental-decidual interactions can be explored [45]. Research questions that can potentially be addressed using this co-culture model system include:

1. How do immune responses mediated by uterine natural killer cells contribute to the development of the syncytium?
2. Does Δ^9-THC similarly impact Th1/Th2 cytokine production in villous trophoblasts and uterine natural killer cells?

Multi-cytokine cellular treatments

To determine the effects of Δ^9-THC in localized inflammatory and immunomodulatory settings, we stimulated differentiating and fusing BeWo b30 cells with TNF-α or IL-10.

TNF-α was employed to recapitulate a simplistic and localized inflammatory microenvironment whereas IL-10 was representative of an immunomodulatory-dominant state (Chapter 3). Additional experiments should develop and utilize a concoction of Th1 and Th2 cytokines to better model the architecture of the *in vivo* placenta. The production of Th1 cytokines is upregulated during early (implantation, placentation, invasion) and late pregnancy events (labour, parturition) [2,14]. Therefore, a combination of important cytokines such as TNF-α, IFN-γ, IL-1β, IL-2 and IL-8 can be employed to induce inflammation [2,14]. Similarly, a more tolerant immune setting can be mediated through multi-cytokine treatment with the Th2 cytokines TGF-β, IL-4, IL-10 and IL-13 [2,14].

Putting the spotlight on CBD

CBD is another major biologically active component of cannabis that differs from Δ^9-THC due to its non-psychoactive nature [47]. An increasing amount of CBD-based products are now available for recreational and self-medication use, with about 16% of Canadians reporting use of CBD in 2019 [48]. Moreover, the International Cannabis Policy Study reported that CBD oil or liquid drops were most frequently used in Canada, followed by topicals and food-based products [48]. Consumers are also reported to perceive products containing only CBD as beneficial for their health [48]. Unfortunately, relatively little is known about the effects of CBD use on placental development and pregnancy in general. The increasing prevalence of cannabis use among women of reproductive age and in pregnancy, paired with the increase in accessibility and availability of products, calls for further research to develop informed counselling strategies [49,50].

Conclusions

From counterculture to medicalization, societal perceptions of cannabis have proved to be fluid [51,52]. Research must therefore maintain a steady stride to develop clear safety data to inform current users, which is particularly relevant to post-legalization Canada [50]. An understanding of the effects of cannabis during pregnancy is necessary due to the increase in maternal use over previous years and associations with short- and long-term consequences for child health [50]. We contribute to this field by showing that Δ^9-THC impairs the integrity of the placental syncytium by compromising various trophoblast functions, including fusion, cytokine responses and hormone production. Finally, we hope that this research assists scientists, health care providers and political leaders in creating safe public health guidelines for mothers and mothers-to-be.

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APPENDIX: ISOLATION AND PURIFICATION OF TROPHOBLASTS FROM TERM HUMAN PLACENTAE

Rationale

Although the use of human choriocarcinoma-derived BeWo and BeWo b30 cell lines has revolutionized research on placental development, these models are not completely functionally and morphologically reflective of *in vivo* villous trophoblasts (Chapter 2) [1,2]. Since the underlying differences remain unclear, it is often argued that key results obtained using immortalized trophoblasts must be verified in primary trophoblasts to ascertain the extent of their applicability to placental functions, including the development of the syncytium [3]. Therefore, we worked to optimize this challenging technique to isolate, purify and culture trophoblasts from human term placentae to eventually compare the regulation of fusion at the transcript and protein levels. While characterizing the levels of fusogenic genes (*ERVW-1*, *ERVFRD-1*, *hCG-β*, *CDH-1*) as well as quantifying the fusion percentage remains to be completed, we have demonstrated successful purification of primary trophoblasts that were isolated from term human placentae.

Materials and Methods

Cell culture

Term placentae were collected following caesarean deliveries at the McMaster University Medical Centre, with approval from the Hamilton Integrated Research Ethics Board (HiREB Study #1877) and informed patient consent. All inclusion and exclusion criteria were temporarily waived during this refinement process. Patient contact occurred under the supervision of Dr. Dustin Costescu. In brief, trophoblast isolation and purification from term human placentae involves enzymatic dissociation of villous tissue, Percoll density gradient centrifugation (5-70%; Sigma-Aldrich, P4937) as well as negative selection using the mouse anti-human HLA-ABC clone W6/32 primary antibody (Bio-Rad, MCA81) and goat anti-mouse immunoglobulin G microbeads (Miltenyi Biotec, 130-048-401) as the secondary antibody. The complete protocols are published by Petroff et al. (2006) and Clabault et al. (2018) [4,5].

Purified primary human trophoblasts were cultured in Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 Mix (Corning, 10-090-CV) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. Cells were seeded on 24-well polystyrene plates coated with 5 μg/cm^2 human fibronectin (Sigma-Aldrich, FC010) at a density of 1x10^6 cells/cm^2 for cell-cell contact.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells using TRIzol™ Reagent (500 μL; Thermo Fisher Scientific, 15596026) and isolated using the PicoPure™ RNA Isolation Kit (ThermoFisher Scientific, KIT0204), according to the manufacturer's instructions. Quantification of gene expression using RT-PCR was performed as previously described by our research group [6]. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ analysis, normalized to the endogenous control gene, *18S*, and expressed as the fold change to the control. Primers sequences designed for all target genes of interest are listed in Table 1.

Gene	**Forward sequence (5' → 3')**	**Reverse sequence (5' → 3')**
18S	CACGCCAGTACAAGATCCCA	AAGTGACGCAGCCCTCTATG
hCG-β	ACCCCTTGACCTGTGAT	CCTTATTGTGGGAGGATCGG
VIM	GGACCAGCTAACCAACGACA	AAGGTCAAGACGTGCCAGAG

Table 1. Forward and reverse primer sequences used for RT-PCR

Statistical analyses

All experiments were replicated three times. Data are presented as the mean ± standard error of mean. To determine significant differences across isolated and purified groups to verify successful trophoblast collection, two-way ANOVA followed by Tukey's multiple comparisons tests were utilized. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.

Results and Discussion

To demonstrate that primary trophoblasts were successfully separated from endothelial cells, leukocytes and fibroblasts, we completed a transcriptional analysis of isolated and purified cells. Isolated cells are representative of a mixture of all cell types found in the placental villous tissue while purified cells should, in theory, only contain trophoblasts [1]. hCG-β was utilized as a trophoblast-specific marker as STBs are the major producers of this hormone during pregnancy [7]. On the other hand, the cytoskeletal protein vimentin (VIM) served as a non-trophoblast marker and identified the presence of contamination in purified samples [1,8]. Changes in the mRNA expression of *hCG-β* and *VIM* were assessed following isolation and purification after 24, 48, 72 or 96 hours of cell culture. Since primary trophoblasts spontaneously undergo cell-cell fusion over a 72-hour period when seeded at high densities, we completed a time-course experiment to assess if and how the transcriptional profiles are altered with increasing length of time in cell culture. We observed that the mRNA expression of *hCG-β* and *VIM* signifcantly decreased after only 24 hours, after which it stabilized (Fig. 1). This outcome may be the result of an excess number of cells that underwent apoptosis due to a lack of adherence to the plate's surface [9]. Other investigations have similarly reported a loss in cell viability by 45% after 24 hours of culture that is then normalized over the following days [9].

Our results also show that the mRNA levels of *hCG-β* are significantly increased from purification to isolation after 48 and 72 hours of culture ($*p<0.05$) (Fig. 1). This is indicative of a rise in the relative number of STBs due to the cell differentiation and fusion.

In contrast, *VIM* expression significantly decreased after 24 hours of cell culture (***$p<0.0005$) following purification, demonstrating a decrease in the presence of non-trophoblast cells. Given the expression of *VIM*, albeit low, it is evident that the purified trophoblast populations were not entirely devoid of contaminating endothelial cells, leukocytes and/or fibroblasts.

Due to the challenges we experienced in terms of primary trophoblast cell culture, we implemented several adaptations to the protocols by Petroff et al. (2006) and Clabault et al. (2018) to optimize this technique within our laboratory [4,5]. The primary changes included a shift from harvesting 40-50 to 90-100 grams of villous tissue to maximize cellular yields as we observed inconsistencies in viability following purification. Furthermore, we noted that purified trophoblasts must be plated immediately after purification as another round of cryopreservation results in cellular death and low viability. These highly sensitive cells also do not adhere well to polystyrene, therefore plates were coated with the extracellular matrix protein fibronectin to maximize attachment. In conclusion, further investigations are necessary to define the mechanisms of differentiation and fusion in trophoblasts isolated from term human placentae to create a comparison with the BeWo and BeWo b30 cell lines.

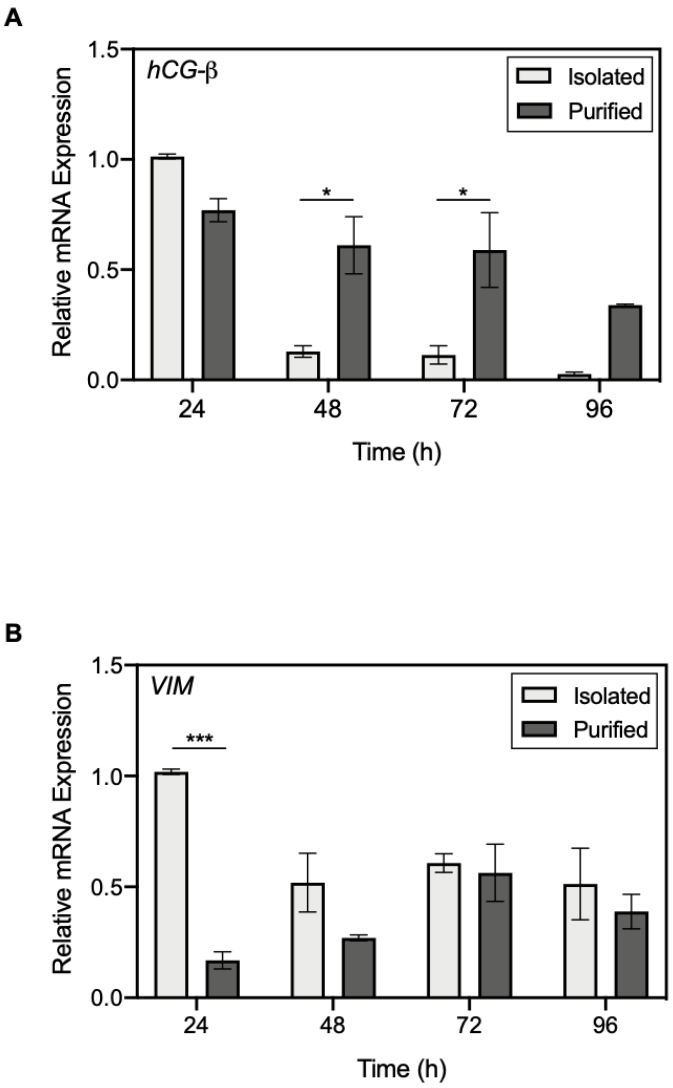

Figure 1. Characteristics of isolated versus purified primary human trophoblasts. Relative mRNA levels of (A) *hCG-β* and (B) *VIM* after the displayed length of time (hours) in cell culture. Significant differences between the isolated and purified groups are indicated by *p<0.05 and ***p<0.0005; n=3.

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