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The Role Of Ppar γ In Placental Development And Disease

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THE ROLE OF PPAR γ IN PLACENTAL DEVELOPMENT AND DISEASE

by

LEENA KADAM

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

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Advisor

Date

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LIST OF ABBREVIATIONS

PPAR γ : Peroxisome proliferator- activated receptor gamma

IUGR: Intra-uterine growth restriction

GDM: Gestational diabetes mellitus

PE: Pre-eclampsia

PTB: Preterm birth

PTL: Preterm labor

NF- κ B: Nuclear factor kappa B

sFLT: Soluble fms-like tyrosine kinase

IL: Interleukin

TNF α : Tumor necrosis factor alpha

STAT: Signal transducer and activator of transcription

AP1: Activator protein 1

GCM1: Glial cell missing 1

CG- β : Chorionic gonadotropin beta

TLR4: Toll like receptor 4

CHAPTER 1 – INTRODUCTION

(This chapter contains previously published material. See Appendix C)

Anatomy of the human placenta

Placenta in mammals is a transient organ that acts as the primary source of nutrition for the growing fetus during pregnancy (1, 3). A fully developed human placenta is a circular disc-like organ that is around 3-4cm in thickness and weighs approximately 500 grams. The gross structure of the placenta is depicted in Figure 1. The margins/ends of the placenta are marked by

the chorionic plate (covered by the amnion) on the fetal side and the base plate on the maternal side. These plates fuse at the end of the disc structure of the placenta and form the fetal membranes that envelope the growing fetus. The space between these plates is called the inter-villous space which is

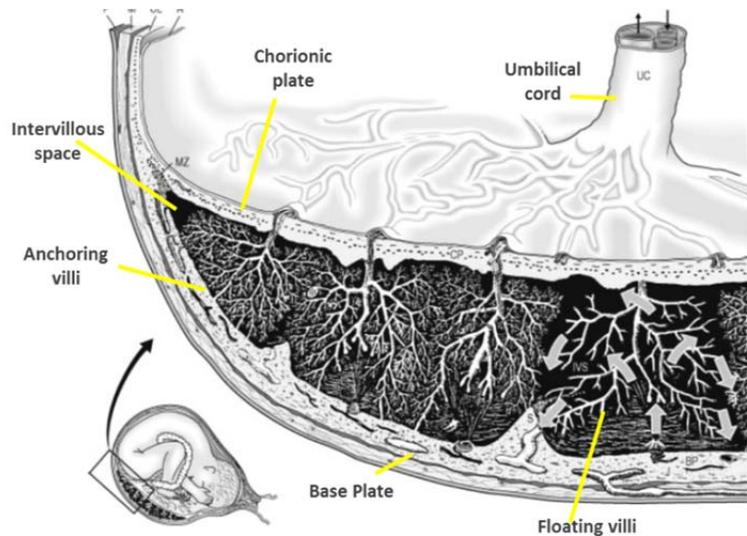


Figure 1: The anatomy of human placenta. Image adapted from Benirschke & Kaufmann, 2005 (1)

filled with maternal blood and consists of the placental villi – tree-like structures that stem out from the chorionic plate. The placenta villi comprise the functional unit of the placenta and are primarily of two types: (i) the anchoring villi - that connected to the basal plate at their tips and anchor the placenta and (ii) the floating villi – that are not attached to the basal plate and remain floating in the intervillous space. The core of the placental villi consists of capillary vessels that unite at the base of the placenta to form the umbilical artery and vein. The villi bathe in the maternal blood that fills the intervillous space and thus absorb nutrients and exchange gases with the maternal blood.

Development of the human placenta

The placenta develops from the outer cells of the blastocyst known as trophoblast cells (Figure 2A). The trophoblast cells initiate implantation by attaching and eventually invading into the maternal endometrium (4). These cells proliferate and form the first wave of syncytiotrophoblast cells that invade into the maternal endometrium and embeds the blastocyst inside the uterine tissue on approximately 1 week after fertilization (Figure 2B). This layer of cells continues to proliferate.

Around week 2, it starts forming fluid filled spaces called lacunae (Figure 2B). Over the next few weeks of development, these lacunae merge to form one continuous intervillous space. The walls of the lacunae stretch from fetal side to the maternal decidual tissue and are called trabeculae (Figure 2D). The trabeculae are essentially finger like projections of cytotrophoblast cell columns emerging from the embryonic side and are referred to as primary villi. Over the course of weeks 3-7, these villi are penetrated by the extra-embryonic mesenchyme pushing the cytotrophoblast cells towards the periphery, giving rise to secondary villi. The secondary villi also show presence of mesenchymal cells. These cells give rise to the connective tissue inside the villous core and are precursors of the endothelial cells required for vascularization of the villi. The secondary villi finally develop into tertiary (mesenchymal) villi that are covered with cytotrophoblasts and have a connective tissue core with developed placental blood vessels. The

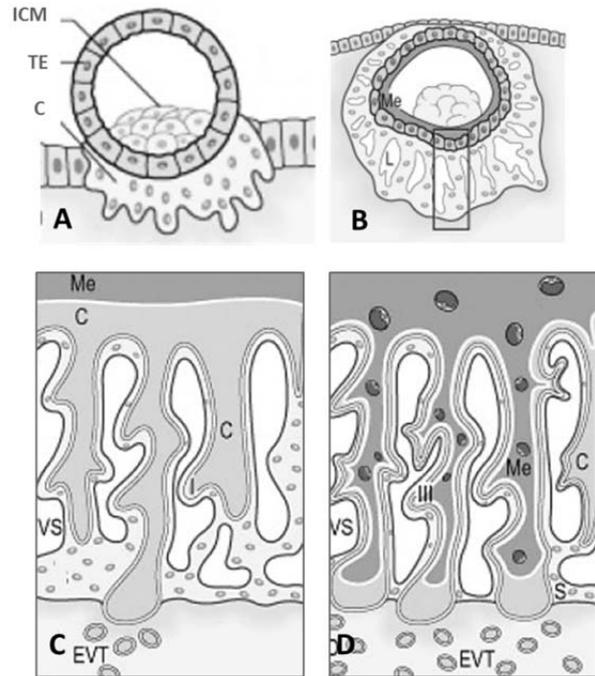


Figure 2: Development of human placental structures. Image adapted from (2). (A) Images shows the attachment to and invasion of the uterine tissue, (B) development of trophoblast lacunae, (C) development of primary villi and (D) formation of secondary villi. ICM: inner cell mass, TE: trophoblast, C: cytotrophoblast, Me: extraembryonic mesenchyme, VS: intervillous space, EVT: extra-villous trophoblast.

basic structure of human placenta is thus established by week 8 (Figure 2D). In the coming weeks, the tertiary villi further undergo rounds of trophoblast sprouting and branching angiogenesis to form a network of terminal villi – the most prominent form of villi found in term placenta.

Human placental trophoblast lineages and their functions

The cytotrophoblast cells proliferate and differentiate towards two major lineages. The extra-villous trophoblast lineage that develops from the cytotrophoblast cell columns at the tips of anchoring villi and the villous trophoblast lineage that cover the floating villi (5, 6).

Extra-villous trophoblast lineage (EVT)

These cells are primarily invasive in nature. The proximal cells of the cytotrophoblast columns proliferate and differentiate towards this invasive lineage (7, 8). They secrete several extracellular matrix degrading enzymes such as - matrix metalloproteinase 9,3,7, gelatinases - that help to breakdown of extracellular matrix and aid in their invasion into the maternal endometrial tissue (9, 10). The EVT lineage differentiates into subtypes with specialized functions: the interstitial subtype invades the decidua and secretes extracellular matrix components that help in anchoring the placenta whereas the endovascular subtype is involved with remodeling of uterine arteries (11). The endovascular EVT's migrate into the artery lumen and displace the endothelium and degrade the smooth muscle cells in the artery wall, which enables regulation of blood flow at the-placental site (10, 12).

The EVT cells also help evade the maternal immune mechanisms at the maternal- fetal interface (13, 14). They refrain from expressing major histocompatibility complex proteins HLA-A, HLA-B and HLA-D- which are major stimulators of T-cell mediated graft rejection process. They further express HLA-E which binds to CD94 receptors on the uterine natural killer (uNK) cells resulting in recognition of trophoblast cells as 'self', thus rendering the uNK

cells inactive (15-19). They are also implicated in preventing fetal rejection by suppressing the local immune responsiveness and impairing responses to immune-activating cytokines present in the placental bed (19).

Villous trophoblast lineage (VT)

The VT cells are in direct contact with the maternal blood and form the exchange surface (1). The VT must adapt to environmental changes to secure growth throughout pregnancy and ensure nutrient, gas and waste exchange, while generating the bulk of the placental hormones essential to facilitate growth throughout pregnancy (6). The cytotrophoblasts under the surface of the villi can undergo either a symmetrical or an asymmetrical cell division. Asymmetrical division results in the formation of a proliferative cell and a differentiating cell (20). These differentiating cells exit the cell cycle and undergo cell fusion to form the multinucleate syncytiotrophoblast/syncytium, the exchange surface(21). Over the course of the pregnancy, this layer undergoes apoptosis, is sloughed/shed off as syncytial knots and is constantly replenished by the underlying proliferative cytotrophoblast (22, 23). The VT comprises of two compartments, an outer multinucleate fused layer that acts as the exchange surface and a lower proliferating cytotrophoblast layer that maintains a balance of proliferative: differentiating cells (24). The process of proliferation, differentiation, fusion, apoptosis and shedding within the trophoblast layer is termed as “trophoblast turnover” which is crucial for VT function. In addition to nutrient and gas exchange, the VT cells also participate in immune functions by expressing pattern recognition receptors like TLR (toll-like receptors) and NLR (Nod-like receptors) that recognize and bind to sequences unique to, and expressed on, the surface of microorganisms (25). Thus, they also play an active role in control of pathogens that may compromise fetal wellbeing (26).

Diseases associated with placental development

Proper development of the VT and EVT trophoblast lineages is crucial for placental function and dysregulation in both lineages has been associated with placental insufficiency syndromes. Shallow invasion and reduced remodeling of uterine arteries will result in under-perfusion of the placenta. The reduced perfusion often translates into reduced exchange of nutrients resulting into growth restricted fetuses observed in intra-uterine growth restriction (IUGR). Incomplete remodeling of uterine spiral arteries results in high velocity-unsteady blood flow which leads to hypoxia-reperfusion injury in the placenta. These events expose the placental tissue to oxidative stress, trophoblast damage from shear stress and local thrombosis all associated with pathologies like PE (27, 28). These features can result in various disease phenotypes; one hallmark being the release of proteins such as soluble fms-like tyrosine kinase (sFLT) into the maternal circulation. sFLT is an anti-angiogenic factor secreted by trophoblast cells and elevated level of sFLT have been observed in both human and animal models of PE and is often used as a marker for PE (29, 30). In addition to abnormal trophoblast differentiation, elevated inflammation is a common feature observed in these pathologies (31-33). Increased placental inflammation due to oxidative stress and elevated placental cytokine release are both features associated with PE and IUGR placentae (31, 34, 35). Severe forms of these pathologies lead to preterm birth which is a leading cause of neonatal mortality and morbidity (2, 36, 37).

Currently, there is a lack of clear understanding the molecular pathways contributing to abnormal placental function. Despite a strong correlation between elevated inflammation and placental pathologies, the effects of inflammation on trophoblast differentiation have not been explored (33, 34, 38, 39). ***Factors common to both processes, inflammation and trophoblast differentiation, could serve as molecular targets for therapeutic use.*** In the current study, we evaluate the potential of the nuclear hormone receptor Peroxisome proliferator-activated receptor

(PPAR γ) for development as a molecular target. PPAR γ was chosen because it has been shown to be involved with multiple aspects of pregnancy and placental function, as outlined in the upcoming sections.

Peroxisome proliferator-activated receptor transcription factors family

Peroxisome proliferator-activated receptors (PPARs), a moniker owed to the early observation that stimulation of these proteins could induce the proliferation of peroxisomes in rodent hepatocytes, are transcription factors belonging to the nuclear hormone receptor superfamily (40). The spectrum of ligands which effectively target these receptors include endogenously expressed lipid-soluble molecules (e.g. prostacyclin, lysophosphatidic acid), and the thiazolidinedione family of pharmaceutical compounds (e.g. Rosiglitazone, Pioglitazone) (41, 42). Like other members of this receptor superfamily, the interaction of PPARs with their corresponding ligands elicits their activation via a change in protein conformation, resulting dimerization with retinoid X receptor (RXR) and recruitment of co-activators like histone deacetylases (HDAC's), p300/CBP or members of the steroid receptor co-activator (SRC) family (43, 44). This is followed by either direct binding to a consensus sequence on the DNA or to enable binding to other transcription factors like NF-kappa β to regulate gene expression (45, 46).

To date, three isotypes of the receptor have been identified - PPAR α , β and γ . All three subtypes, possess the canonical domain structure common to other nuclear receptor family members: the amino-terminal AF-1 trans activation domain, followed by a DNA-binding domain, and a dimerization and ligand-binding domain with a ligand-dependent trans activation function AF-2 located at the carboxy-terminal region (47). Although, each subtype is a product of a distinct gene and they have organ specific expression pattern. PPAR's and their functions in different systems have been widely studied. Briefly, **PPAR α** is expressed primarily in brown adipose tissue, in liver, kidney, heart and skeletal muscle. It has been implicated in

mitochondrial fatty-acid (FA) oxidation, which provides energy for peripheral tissues (48). It's involvement in antioxidant pathways is suggested to contribute to the pathogenesis of age-related macular degeneration (AMD) (48, 49). **PPAR β** is expressed predominantly in the gut, kidney and heart; and involved in lipid metabolism, cell survival, wound healing, embryonic implantation and development of the central nervous system (50). **PPAR γ** is mainly expressed in adipose tissue and to a lesser extent in colon, the immune cells – macrophages and is known to be involved with processes such as adipogenesis and macrophage differentiation (51-53). The three PPAR's also show expression in both male and female reproductive tissues. They are widely expressed in interstitial Leydig cells, Sertoli and germ cells, however their action in the testis is not completely clear (54, 55). In the female reproductive organs PPAR α and PPAR β isoforms are expressed primarily in the theca and stroma tissues whereas PPAR γ shows strong expression in the granulosa cells. Mice lacking PPAR β and γ are sub fertile suggesting an important role for these receptors in fertility (56).

Interestingly, all three subtypes show expression in the placenta also in the rodent placenta, they are expressed in the trophoblast cells of junctional zone as well as the labyrinth zone (57). They also show expression in the human placental trophoblast cells (57).

Peroxisome proliferator-activated receptor-gamma (PPAR γ)

Regarding placental function and pregnancy, PPAR γ has emerged as the crucial isoform owing to the embryonic lethality of the knockout mice due to gross placental abnormalities. While, the PPAR α null mice show increased fetal loss (20% over wild type), surviving pups develop normally (58). PPAR β knockout mice also show an embryonic lethality due to defects placental morphogenesis, but some pups do survive (lethality >90%) (59, 60). PPAR γ knockouts on the other hand show a 100% lethality rate due to defects in development of placental vasculature and thinning of the myocardial lining of the ventricles. Interestingly, correcting for

the mutation in these embryos (via aggregation with tetraploid embryos which contribute only to the extra-embryonic lineages) rescued the placental as well as the cardiac phenotype suggesting an important role for PPAR γ in placental function. All three PPAR isotypes are expressed in the human trophoblast and a subset of endothelial cells (57). The role of the different molecules in human development though is only poorly understood. Aspects of PPAR γ function in placental development have therefore been extensively explored in the human placenta. In the current chapter, we outline the current and potentially new roles of PPAR γ in placental development and other functions at the maternal-fetal interface (Figure 3).

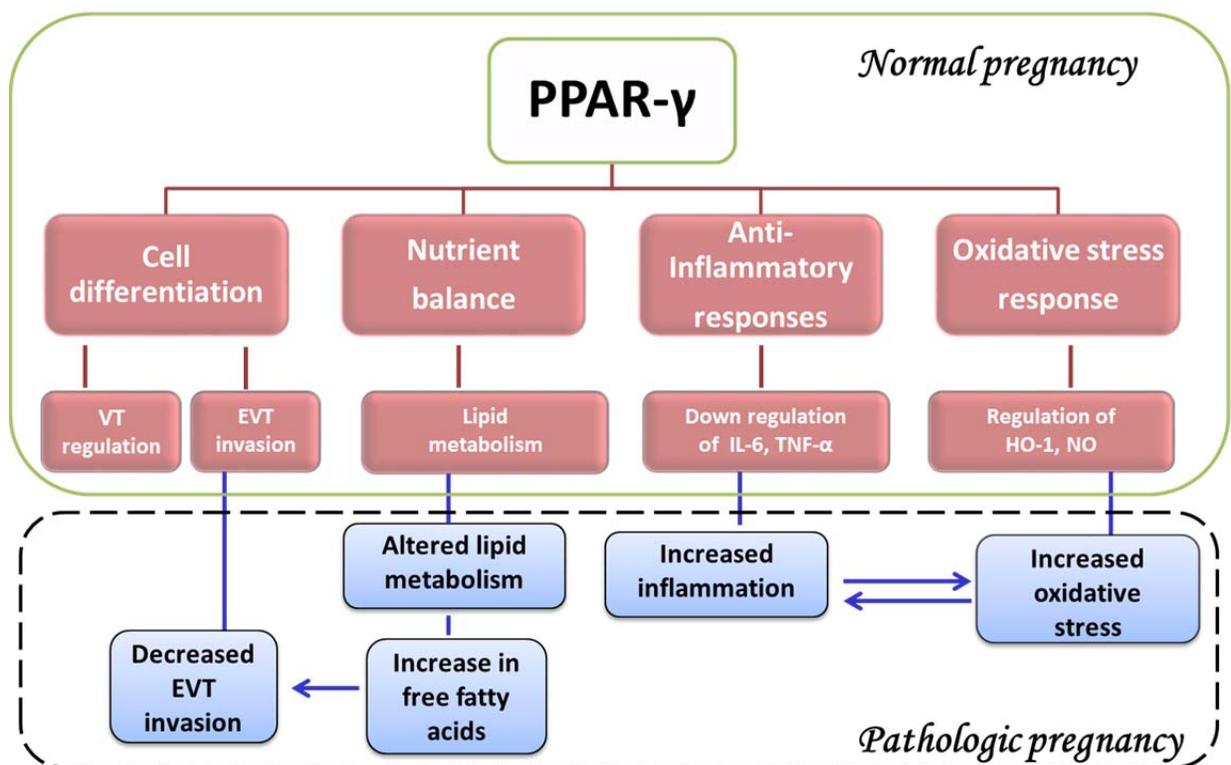


Figure 3: PPAR γ has been shown to be involved in multiple key metabolic pathways in placentation and pregnancy. These include trophoblast differentiation, inflammatory and oxidative response and nutrient sensing - specifically fatty acid metabolism. Placental disorders such as PE often show changes in these pathways which are partially regulated by PPAR γ . Changes in activity and not necessarily expression may result in altered fatty acid metabolism which in turn may influence villous and extra villous trophoblast differentiation. Similarly, altered activity may cause changes in oxidative stress and inflammation due to regulation and release of inflammatory cytokines such as TNF- α , IL-6 and others which have shown to be associated with conditions like pre-term labor, miscarriage and pre-eclampsia. The observation that all these factors are at least in part regulated by PPAR γ supports its potential critical role in placental physiology and disease. (EVT: extra villous trophoblast, VT: villous trophoblast, HO-1: heme-oxygenase 1, NO: Nitric oxide)

PPAR γ in trophoblast differentiation

Trophoblast differentiation is a critical process in establishment of placental lineages that govern the placental development, maintenance and function. We distinguish the human trophoblast cells into the decidua invading extra villous trophoblast (EVT) and the placenta residing villous trophoblast cells (VT) which are covered by the syncytium. EVT's invade the maternal decidua to establish pregnancy and secure blood-flow to the implantation site which provides nutrients and oxygen to the fetus. The villous trophoblast forms the main maternal-fetal exchange surface, which is critical for fetal development and must adapt to environmental changes to secure growth throughout pregnancy. Trophoblast differentiation is a tightly regulated process in human-and is implicated to be abnormally regulated in placental dysfunction disorders (61-63). As mentioned previously, PPAR's are expressed human trophoblast cells. However, while the expression of alpha and beta subtypes nuance as the cells differentiate, PPAR γ continues to be expressed strongly in these cells (57, 64-67).

In vitro studies with 1st trimester EVT's showed that treatment with PPAR γ antagonists increased invasion whereas agonists hampered it, implicating involvement of PPAR γ in regulating invasion of decidua (65). Similar study with isolated term villous trophoblast showed, induction of differentiation upon treatment with agonists (66). The VT has to balance cell proliferation and differentiation to sustain nutrient and gaseous exchange throughout pregnancy (68). Involvement of PPAR γ in regulation of functions of both EVT's and VT PPAR γ not only suggest its crucial role in trophoblast differentiation but these studies also highlight the differences in response of the trophoblast subtypes to PPAR γ induction.

Interestingly, the studies on term villous trophoblast also observed differential behavior of these cells in response to synthetic and naturally occurring ligands of PPAR γ . Treatment with synthetic ligand - Troglitazone induced differentiation, whereas the natural ligand PGJ2 hindered

it, even inducing apoptosis in cells (66). Thus, PPAR γ seems to have different roles depending upon 1) trophoblast subpopulations 2) the gestational age and type and 3) the stimulating ligand (69). However, one needs to bear in mind that isolated trophoblast lack their natural environment and tend to differentiate directly in culture which is a critical limitation in some of these studies (70, 71). Additionally, while we have substantial evidence for involvement of PPAR γ in trophoblast differentiation, we still lack the understanding of the molecular regulation. Few studies focusing on downstream targets of PPAR γ exist (72, 73). Glial cell missing 1 (Gcm-1) has emerged as an interesting candidate in this respect. It regulates differentiation of chorion into labyrinth trophoblast populations and controls syntiotrophoblast differentiation and mice lacking Gcm1 die at E10.5 due to absence of placental labyrinth (74). PPAR γ deficiency in mouse trophoblast stem cells was shown to affect labyrinth cell lineages via Gcm-1 (75). Gcm1 has been shown to be also present in human trophoblast tissue and altered levels of Gcm-1 have been associated with PE placentas (76-78). Recently, Levystka K , *et al* showed that Gcm-1 levels could be altered by PPAR γ agonists or antagonists in BeWo choriocarcinoma cells suggesting that PPAR γ via Gcm-1 (79) may play a role in human trophoblast differentiation.

PPAR γ and Fatty Acid metabolism in the placenta

Placental fatty acid (FA) transfer from the mother to the fetus is crucial for adequate development (80). PPAR γ has been classically known for its role in lipid metabolism. The observation that the PPAR γ knockout placentas showed less accumulation of lipid droplets suggested that it may have some similar function in the placenta. Schaiff T, et al in 2007 showed that PPAR γ could alter the fatty acid uptake in the placenta by increasing the expression of fatty acid transport proteins (FTP's) in mice (81).

Several fatty acid transporters (FABP, FATP and CD-36) have also been identified at the microvillous and basal membranes of the human placenta. Similar to observations in mice,

increase in PPAR γ activity has been shown to increase fatty acid uptake and accumulation in primary human trophoblast cells by regulating the expression of proteins such as fatty acid binding proteins (FABP family) (71). In turn, oxidized LDL's (low density lipoproteins) were shown to be capable of activating PPAR γ in primary cytotrophoblast cells and even cause inhibition of trophoblast invasion (70, 82). Thus, PPAR γ appears to be regulating and it self being regulated by lipid metabolites. PPAR γ might act as a nutritional sensor and co-ordinate fatty acid uptake and trophoblast differentiation in the placenta to ensure growth and function. This helps to explain the placental insufficiency disorder phenotype such as PE which is associated by increased lipid peroxidation and defective trophoblast invasion (83, 84).

PPAR γ in placental oxidative stress

Pregnancy is a state of physiological stress including oxidative stress. The initial hypoxia followed by reoxygenation (ischemic reperfusion) are the major contributors of oxidative stress during early pregnancy (85). In later stages, increased placental mitochondrial activity and production of reactive oxygen species (ROS) further contribute to the oxidative stress (8). In moderation, this normally does not cause a problem but excess oxidative stress has been observed in pregnancy complications like IUGR, diabetes and PE (8, 85, 86).

Nitric oxide (NO) is a vasodilator at normal levels, but at elevated levels it reacts with ROS to cause lipid peroxidation and nitrosylation of tyrosine residues affecting many signaling pathways. High levels of NO are also observed in GDM with cases shown to have higher levels of oxidative stress (87). Interestingly, blocking PPAR γ greatly increases endogenous NO production suggesting that PPAR γ might be involved in its regulation (8).

Heme oxygenase-1 (HO-1) is known for its anti-oxidant properties and cytoprotective effects. In the placenta, HO is found in vascular endothelium and villous and extra villous trophoblast (88). HO metabolites - carbon monoxide (a potent vasodilator) and bilirubin

negatively regulate endothelin 1 and ROS; and anti-angiogenic proteins such as soluble fms-like tyrosine kinase 1 (sFLT-1) (89, 90). Evidence showing regulation of HO-1 expression by PPAR γ further emphasizes its potential role in managing oxidative stress and hypertension during pregnancy (91, 92).

Immune mediated effects of PPAR γ

Pregnancy in general is a state dynamic inflammatory phases, wherein the immune status of the mother varies from being pro-inflammatory in the start of pregnancy, to anti-inflammatory in the middle phase to again being pro-inflammatory towards the end (93). There is also growing body of evidence suggesting that parturition at term is an inflammatory process with pro-inflammatory cytokines like IL-8 and TNF- α playing a role (94-96). Thus, a balance between the pro and anti-inflammatory modulators at the fetomaternal interface is crucial for maintenance of pregnancy. Indeed, a pre-mature (or untimely) shift towards the pro-inflammatory conditions has been associated with cases of spontaneous abortion and preterm delivery (97). Increased levels of pro-inflammatory cytokines like TNF- α , IL-6 and IL-8 have also been reported in PE cases (98, 99).

Interestingly, PPAR γ has been known for its anti-inflammatory effects (100). It has also been shown to down regulate expression of pro-inflammatory cytokines like IL-6, IL-8, and TNF- α in human gestational tissue (101). Reduction in the levels of PPAR γ during labor has also been documented (102). These observations together suggest that reduction in levels of PPAR γ at term might contribute to parturition by regulating the expression of inflammatory cytokines. A premature reduction would thus ensure an inflammatory response which may result in loss of pregnancy.

PPAR γ expression levels and activity

Studies assessing the protein levels of PPAR γ associated aberrant levels of PPAR γ with

placental disorders. Decreased levels of PPAR γ were reported in cases of GDM, whereas increased levels were observed in IUGR associated PE (103, 104).

On the other hand, placentas from women with only PE did not show any changes in the levels of PPAR γ protein expression (103, 104). These women showed significantly lowered levels of the activators (implicated to be a fatty acid derivative) of the receptor in their serum when compared to age matched controls (105). During a normal gestation, PPAR γ activators increase as the pregnancy progresses to term (64). Women with PE showed a drop in these activating factors levels 10-15 weeks before presentation of symptoms (105). Lowered levels of the activators may lead to decreased activation of PPAR γ which in turn may contribute to the pathology (105). While these studies emphasize the importance of PPAR γ in placental pathologies, they also suggest that balance between the absolute levels of PPAR γ protein and the level of activity is crucial.

Furthermore, Levystka *et al.* recently showed that that PPAR γ underlies an auto-regulatory mechanism (79). The authors showed using the BeWo choriocarcinoma cell line that inhibition of PPAR γ activity using an antagonist T0070907 led to an up-regulation of expression whereas activation by an agonist Rosiglitazone had the opposite effect. Thus, in-case of PPAR γ increased levels of protein may not always correlate with increased activity. These factors need to be considered when investigating pathologic cases for the role of PPAR γ . Studies on pathologic placentas, similar to those in cell lines will add on to our knowledge about molecular regulation of PPAR γ in such pathologies.

PPAR γ as a therapeutic option in placental disorder

Involvement of PPAR γ with key aspects of pregnancy and the fact that it can be specifically modulated by a vast array of drugs already available makes it an attractive therapeutic option (30). McCarthy et al in 2011 showed that pregnant rats treated with synthetic

PPAR γ antagonists developed pre-eclamptic phenotypes comparable to what has been described in human suggesting a relevant role in physiology and disease (106). More interestingly the same authors showed in a separate study that activation of the receptor by administration of its synthetic agonist Rosiglitazone, to the (reduced uterine perfusion pressure) RUPP rats showed considerable improvement in hypertension with no adverse effects on the litters or placental vasculature (30, 106). The RUPP model of rats resembles in parts the human PE condition, and administration of Rosiglitazone significantly reduced hypertension and improved vascular function in a HO-1 dependent manner (106). The authors did not observe any adverse effects on placental morphology which contrasts with some other studies (81, 107). The group argued that the low dosage used in the study and the time of administration of the drug contributed to the contrasting results. The study demonstrates the ease and effectiveness of PPAR γ modulation using drugs with so far no observed side-effects, albeit in an animal model.

These parameters of low dosage and exposure times to the drugs seem to hold true in human cases too. A few human case reports assessed the effects of use of synthetic PPAR γ agonists (Rosiglitazone and Pioglitazone) during pregnancy, although the drugs were shown to cross the maternal- fetal barrier but no harm to the fetus or induction of PE was noted (108-110). The dosage in these studies was not as high (dose 4 mg) as recommended for diabetic patients (8mg) and the duration of treatment was between 7 – 17 weeks of pregnancy. Additionally, while these drugs were previously thought to increase the risk of cardiovascular diseases in humans, recent reports do not support these claims and the drugs have been recently approved by the FDA for widespread use (111).

While further studies and risk assessment would be necessary to ensure safety, PPAR γ is a strong candidate for development of therapies managing pregnancy related disorders. This dissertation project explores the role of PPAR γ in placental development and pregnancy per the

following aims:

Aim 1: To determine the molecular mechanism of PPAR γ in prevention of preterm birth in the mouse model for endotoxin induced PTB. (Outlined in Chapter 2 & 3)

Aim 2: To determine the effects of endotoxin exposure and PPAR γ induction on human trophoblast physiology. (Outlined in Chapter 4)

Aim 3: To determine the role of PPAR γ in differentiation of extra-villous and villous trophoblast lineages. (Outlined in Chapter 5 & 6)

CHAPTER 2 - PPAR γ PREVENTS INFLAMMATION INDUCED PRETERM BIRTH BY ATTENUATING BOTH SYSTEMIC AND LOCAL INFLAMMATORY PATHWAYS

(This chapter contains previously published material. See Appendix D)

Introduction

Preterm birth (PTB), defined as delivery before 37 weeks of gestation, is the leading cause of neonatal morbidity and mortality (112-114). Premature neonates are at high risk of short-term complications such as respiratory distress syndrome, inter-ventricular hemorrhage, neonatal sepsis, and necrotizing enterocolitis. Long-term complications include neurodevelopmental disorders such as cerebral palsy, chronic lung disease, blindness, and deafness (115-117). In the United States, preterm births accounted for 11.39% of all documented births in 2013 (118). Therefore, it is critical to elucidate the mechanisms that lead to preterm labor (PTL) to provide strategies for the prevention of PTB and the improvement of neonatal outcomes.

PTL is a syndrome caused by multiple pathological processes (119). Of all the potential causes, intra-amniotic infection/inflammation is the only direct link to PTL that accounts for about 30% of all PTBs (120-122). Infection results in a transition from an anti-inflammatory to a pro-inflammatory physiological state in the mother. This involves the infiltration of innate immune cells, such as macrophages, and an increased expression of inflammatory mediators at the uterine-placental unit. Activation of this pro-inflammatory response triggers PTL and consequently PTB (121, 123-125).

In order to study the mechanisms of inflammation-induced PTL, animal models have been developed using mice (126-129), rats (130, 131), rabbits (132), sheep (133), guinea pigs (134, 135) and monkeys (136, 137). The most common model is the murine model, in which PTL can be induced by the systemic administration of bacterial lipopolysaccharides (LPS) (138). LPS administration to pregnant mice triggers an inflammatory response, activating key signal

transduction pathways such as NF- κ B and TNF- α that ultimately will lead to PTB (139, 140). These pathways are similarly activated in inflammation-induced PTL in humans (141).

The ligand-activated transcription factor PPAR γ while mostly known for its role in lipid metabolism and insulin sensitivity has a proven anti-inflammatory role (142-146). Upon activation, PPAR γ has been shown to suppress the transcription of inflammatory signal transduction pathways, such as NF- κ B and TNF- α (100, 147). The anti-inflammatory role of PPAR γ has also been recognized in diseases like multiple sclerosis (MS) and atherosclerosis (148, 149). However, its potential role in inflammation induced PTB remains unexplored. Our group recently showed in a human trophoblast cell line (150), and in a rat model of PE, that PPAR γ activation *via* Rosiglitazone induced HO-1 expression, improved vascular function and pregnancy outcomes (30, 106). PPAR γ has also been implicated in human parturition and dramatically reduced levels were reported during labor as compared to early gestation (102). Activation of PPAR γ was also shown to reduce expression of inflammatory cytokines from human gestational tissues (101, 151). These observations suggest that reduced PPAR γ activity might regulate the onset of parturition by suppressing inflammatory cytokines. Further, availability of approved drugs like Rosiglitazone that can specifically target PPAR γ make it an attractive target for therapeutic interventions.

In the current study, we aimed to study the potential anti-inflammatory role of PPAR γ in inflammation induced PTB. Using the LPS induced preterm birth model in mice, and Rosiglitazone to modulate PPAR γ activity we aimed to: (i) determine whether treatment with RSG reduces the rate of LPS-induced preterm birth and improves neonatal outcomes; (ii) evaluate whether treatment with RSG reduces the systemic proinflammatory response in pregnant mice injected with LPS.

Recently, St Louis D *et al.* demonstrated that PPAR γ activation reduces the expression of inflammatory genes in activated uterine macrophages in a model of PTB induced by iNKT-cell activation (152). Given that during pregnancy local inflammatory cytokines are mostly produced by innate immune cells such as macrophages and neutrophils (153, 154), and the depletion of macrophages, but not neutrophils, prevents inflammation-induced PTB (155, 156) we also evaluated if PPAR γ activation has a direct effect on macrophages at the uterine-placental unit.

Materials and Methods

Animal treatments

C57BL/6J mice were bred in the animal care facility at the C.S. Mott Center for Human Growth and Development at Wayne State University, Detroit, Michigan, USA, and housed under a circadian cycle (light: dark=12:12 h). Females 8-12 weeks old were mated with male mice of proven fertility. Female mice were examined daily between 8:00 a.m. and 9:00 a.m. for the presence of a vaginal plug, which denoted 0.5 days post coitum (dpc). Upon observation of vaginal plugs, female mice were then separated from the males and housed in different cages. Procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University (Protocol No. A09-08-12).

Pregnant mice were categorized into four different groups and injected intraperitoneally on 16.5 dpc with: 1) 10 μ g of LPS (*Escherichia coli* O111:B4; Sigma-Aldrich, MO) in 200 μ L of 1X PBS (n=10); 2) 200 μ L of PBS as a control; 3) 10 μ g of LPS in 200 μ L of 1X PBS followed by 10mg/kg RSG 6 hours after the initial injection (n=10); and 4) 10mg/kg of RSG as a control (Figure 2A). RSG was dissolved in 1:10 DMSO; therefore, pregnant mice were also intraperitoneally injected on 16.5 dpc with 1:10 DMSO as a control (n=6). Pregnancy parameters including gestational age and the rates of preterm birth and stillbirth were recorded

via video camera (Sony Corporation, China). Preterm birth was defined as delivery before 18 dpc, and its rate was defined as the percentage of dams delivering preterm among all births. The stillbirth rate was defined as the percentage of pups born dead out of the total litter size. All DMSO control mice delivered at term (19.5 ± 0.5 dpc); therefore, the gestational age is shown instead of the rate of preterm birth. Gestational age was calculated from the presence of the vaginal plug (0.5 dpc) until the observation of the first pup born.

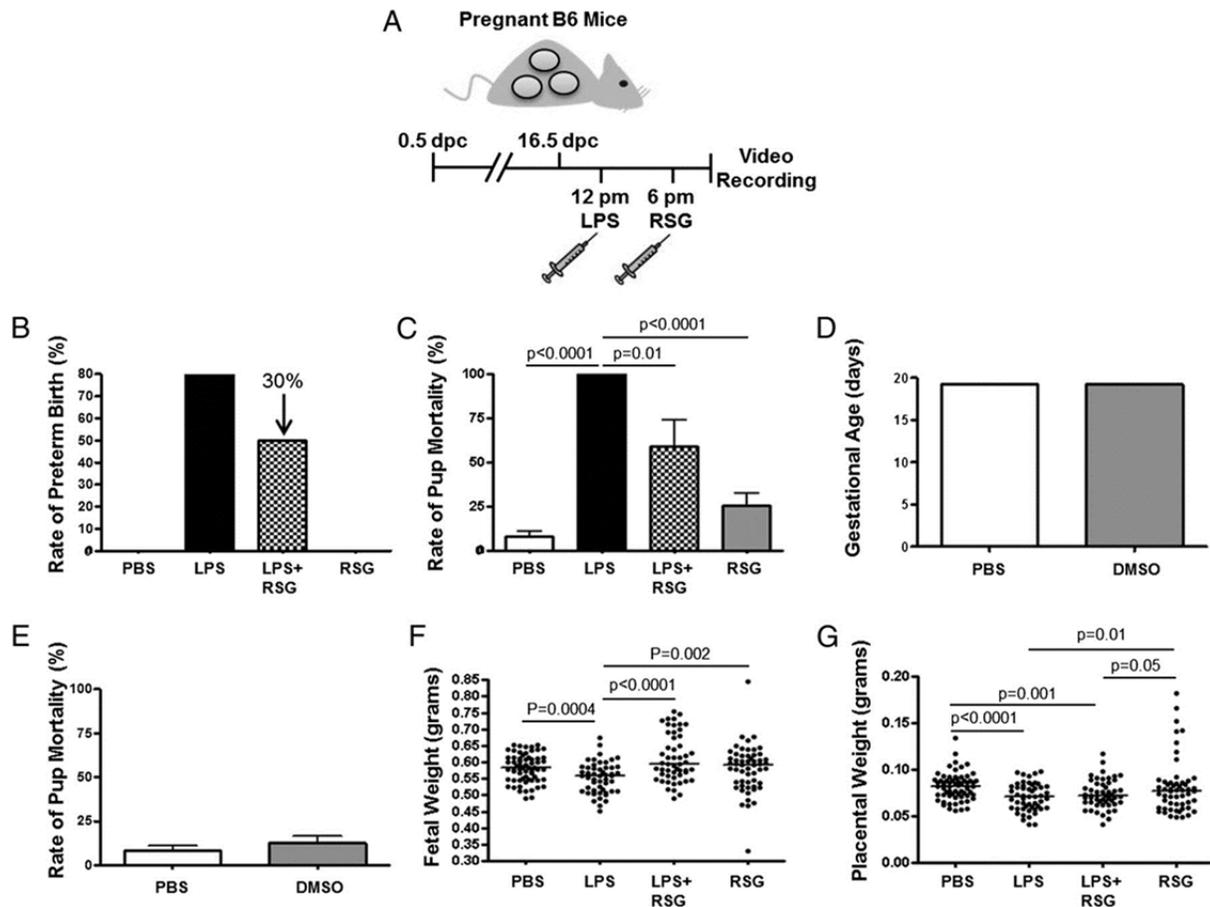


Figure 4: Rosiglitazone treatment reduces the rate of LPS-induced PTB and improves neonatal outcomes. A) On 16.5 dpc, pregnant mice were i.p. injected with LPS and treated 6 hours after with rosiglitazone (RSG; i.p.) and video-monitored. Control mice were injected with LPS, PBS, or RSG alone. (B) The rate of PTB was defined as the percentage of dams delivering at <18.0 dpc among all births. (C) The rate of stillbirth for each litter was defined as the proportion of born pups found dead out of the total litter size. Data are shown as bar plots (mean \pm SEM). Mann-Whitney U tests: $n = 10$ each. Fetal weights (D) and placental weights (E) two hours following PBS, LPS, LPS+RSG, or RSG injection. Data are shown as scatter plots (median). T-tests: $n = 8$ dams each.

Fetal and placenta weights

A second cohort of pregnant mice was injected intraperitoneally on 16.5 dpc with LPS, PBS, LPS + RSG or RSG (n=8 each), as described previously. Two hours after injection, mice were euthanized, and peripheral blood was collected by cardiac puncture and placed into a 1.5 safe-lock Eppendorf tube (Fisher Scientific, MA) (Figure 4A). Animal dissection and tissue collection (myometrial and decidual tissues) were performed as previously described (125). The pup and placental weights were determined using a weight scale (DIA-20, American Weight Scales, GA).

Chemokine/cytokine serum concentrations

Peripheral blood samples were centrifuged at 491 x g for 10 min at 4°C, and serum was separated and stored at -20°C until analysis. The Milliplex MAP Mouse Cytokine/Chemokine Kit (MCYTOMAG-70K-PX32, EMD Millipore, MA) was used to measure the concentrations of G-CSF, GM-CSF, IFN γ , IL1 α , IL1 β , IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL10, IL12p40, IL12p70, IL13, IL15, IL17, CCL11, CXCL10, CXCL1, LIF, CXCL5, CCL2, M-CSF, CXCL9, CCL3, CCL4, CXCL2, CCL5, and TNF α in the serum samples, per the manufacturer's instructions. Plates were read using the Luminex 100 System (Luminex Corporation, TX), and analyte concentrations were calculated using the xPONENT3.1 software (Luminex). The sensitivities of the assays were: 1.7pg/ml (G-CSF), 1.9pg/ml (GM-CSF), 1.1pg/ml (IFN γ), 10.3pg/ml (IL1 α), 5.4pg/ml (IL1 β), 1.0pg/ml (IL2), 1.0pg/ml (IL3), 0.4pg/ml (IL4), 1.0pg/ml (IL5), 1.1pg/ml (IL6), 1.4pg/ml (IL7), 17.3pg/ml (IL9), 2.0pg/ml (IL10), 3.9pg/ml (IL12p40), 4.8pg/ml (IL12p70), 7.8pg/ml (IL13), 7.4pg/ml (IL15), 0.5pg/ml (IL17), 1.8pg/ml (CCL11), 0.8pg/ml (CXCL10), 2.3pg/ml (CXCL1), 1.0pg/ml (LIF), 22.1pg/ml (CXCL5), 6.7pg/ml (CCL2), 3.5pg/ml (M-CSF), 2.4pg/ml (CXCL9), 7.7pg/ml (CCL3), 11.9pg/ml (CCL4), 30.6pg/ml (CXCL2), 2.7pg/ml

(CCL5), and 2.3pg/ml (TNF- α). Inter-assay and intra-assay coefficients of variation were below 15% and 4.9%, respectively.

Macrophage isolation from murine myometrial and decidual tissues

Immediately after collection, myometrial and decidual tissues were mechanically disaggregated in the Accutase cell dissociation reagent (Life Technologies, CA) using scissors for approximately 1-2 min, as previously described (125). Samples were then incubated at 37°C for 35 min with gentle shaking (MaxQ™ 4450 Benchtop Orbital Shaker, Thermo Fisher Scientific, MA). The cell suspensions were filtered using a 100 μ m cell strainer (Fisher Scientific, MA) and washed with staining buffer [Bovine-serum albumin 0.1% (Sigma Aldrich, MO), sodium azide 0.05% (Fisher Scientific Bioreagents, MA), 1X PBS (Fisher Scientific Bioreagents)]. The resulting cell pellet was re-suspended in 96 μ l of staining buffer, and 4 μ l of an anti-mouse F4/80 antigen biotin (clone BM8, eBioscience) were added. The cell suspension was then incubated for 15 min at 4°C. After incubation, the cells were washed with 2 ml of staining buffer and centrifuged at 1250 x g for 7 min at 4°C. The cell pellet was re-suspended in 90 μ l of staining buffer, and 10 μ l of Streptavidin microbeads (Miltenyi Biotec, Germany) were added. This cell suspension was incubated at 4°C for 15 min. Following incubation, the cells were washed with 2 ml of staining buffer, and the cell pellet was re-suspended in 500 μ L of MACS buffer. F4/80⁺ cells (macrophages) were separated by positive selection using MS columns and a magnetic MACS separator. Macrophages were then washed with MACS buffer at 1250 x g for 7 min at 4°C, and an aliquot was taken to confirm purity by flow cytometry using an anti-mouse F4/80-PE (Clone BM8, eBioscience; Figure 6A). The cell pellet was re-suspended in 400 μ L of RNeasy Lysis Buffer (Qiagen, Germany) and placed into a 1.5mL safe-lock Eppendorf tube (Fisher Scientific, MA), which was stored at -80°C until RNA isolation.

RNA isolation, cDNA synthesis, and qRT-PCR

Total RNA was isolated from myometrial and decidual macrophages using the RNeasy mini kit (Qiagen, Germany), following the manufacturer's instructions. RNA concentrations and purity were assessed with the NanoDrop 1000 spectrophotometer (Thermo Scientific, MA), and RNA integrity was evaluated with the 2100 Bioanalyzer system (Agilent Technologies, CA) using the Agilent RNA 6000 Pico Kit (Agilent). cDNA was synthesized by using iScript Reverse Transcription Supermix for RT-qPCR kits (Bio-Rad Laboratories, CA) on the Applied Biosystems GeneAmp PCR System 9700 (Life Technologies, MA), following the manufacturer's instructions. cDNA was amplified using the SsoAdvanced PreAmp Supermix (Bio-Rad Laboratories, CA) on the Applied Biosystems GeneAmp PCR System 9700. mRNA expression of *Nfkb1*, *Tnf*, and *Il10*, and housekeeping genes (*Actin*, *Gapdh*, and *Tbp*) was determined by qPCR using the LuminoCt[®] SYBR Green qPCR ReadyMix (Sigma Aldrich, MO) on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, CA), per the manufacturer's instructions. Primers are described in Table 1.

Table 1: List of primers sequences used for gene expression analysis in mouse decidual and myometrial macrophages

Gene Name	Gene Symbol	Sequence
Glyceraldehyde 3-phosphate dehydrogenase	<i>Gapdh</i>	5'-AAT GGT GAA GGT CGG TGT G-3'
		5'-GTG GAG TCA TAC TGG AAC ATG TAG-3'
β -actin	<i>Actin</i>	5'-GCG AGC ACA GCT TCT TTG-3'
		5'-ATG CCG GAG CCG TTG TC-3'
TATA Box Binding Protein	<i>Tbp</i>	5'-TTC ACC AAT GAC TCC TAT GAC C-3'
		5'-CAA GTT TAC AGC CAA GAT TCA CG-3'
Interleukin-10	<i>Il-10</i>	5'-TCA GCC AGG TGA AGA CTT TC-3'
		5'-GGC ATC ACT TCT ACC AGG TAA-3'
Tumor Necrosis Factor- α	<i>TNF-α</i>	5'-AGA CCC TCA CAC TCA GAT CA-3
		5'-TCT TTG AGA TCC ATG CCG TTG-3'
Nuclear Factor of κ Light Polypeptide Gene Enhancer in B-Cells 1	<i>NF-κB1</i>	5'-AGT CAC ATC TGG TTT GAT CTC TG-3'
		5'-CCT CTA CTA CAT CTT CCT GCT TG-3'

Immunofluorescence

Myometrial and decidual tissues from mice injected with LPS, PBS, LPS + RSG, or RSG (n = 5 each) were immediately frozen in Tissue-Tek O.C.T Compound (Sakura Finetek, CA). Ten-micrometer-thick cryosections were cut and placed on Fisherbrand Superfrost Plus microscope slides (Thermo Scientific, MA). After fixation with 4% paraformaldehyde (Electron Microscopy Sciences, PA), the slides were washed with 13 PBS containing 0.1% Tween 20 and permeabilized with 13 PBS containing 0.25% Triton X-100 for 10 min. Nonspecific Ab interaction was blocked using serum (KPL, MD) at room temperature for 1 h. Slides were then incubated with the following rabbit anti-mouse Abs: 10 mg/ml IL-10 (Abcam, UK), 33 mg/ml TNF, or 2 mg/ml NF- κ B1 (Novus Biologicals, CO) at 4°C overnight. After washing with PBS containing 0.1% Tween 20, the slides were incubated with 2 mg/ml goat anti-rabbit secondary Ab conjugated to Alexa Fluor 594 (Invitrogen Molecular Probes, MA) at room temperature for 1 h, washed again with 13 PBS containing 0.1% Tween 20, and incubated with 2 mg/ml rat anti-mouse F4/80 Ab directly conjugated to FITC (Abcam, UK) at room temperature for 2 h. After mounting with ProLong diamond antifade mountant with DAPI (Life Technologies, CA), immunofluorescence was visualized using an Olympus BX 60 fluorescence microscope (Olympus, Japan) at 1000x original magnification. The pictures were taken using an Olympus DP71 camera and DP Controller Software (Olympus, Japan). The images were merged using ImageJ 1.44p.

Statistical analysis

The qRT-PCR data analysis was performed in R (<http://www.R-project.org/>), and for all other data analyses, IBM SPSS Version 19.0 (IBM Corporation, NY) was used. For qRT-PCR data analysis, the statistical significance of group comparisons was assessed using the Mann-

Whitney U test. For qRT-PCR data, negative ΔC_t values were determined using two reference genes (*Gapdh* and *Actb* or *Tbp*) averaged within each sample to determine gene expression levels. For the rate of stillbirth, the statistical significance of group comparisons was assessed using the Mann-Whitney U test. For fetal and placental weights, the statistical significance of group comparisons was assessed using T-tests. For cytokine concentrations, multiple comparisons were performed using analysis of variance and the Sidak post-hoc tests. For human demographic data, the statistical significance of group comparisons was performed using the Chi-square test for proportions as well as the Kruskal-Wallis test for non-normally distributed continuous variables. A p-value < 0.05 was used to determine statistical significance.

Results

Rosiglitazone treatment reduced the rate of LPS-induced preterm birth and stillbirth

To study the *in vivo* effect of PPAR γ activation on preterm birth, we determined whether administration of RSG could rescue LPS-induced preterm birth (Figure 4A). As expected, LPS injection induced 80% of preterm births (128) (Figure 4B). When pregnant mice were injected with LPS followed by treatment with RSG, a 30% reduction in the rate of preterm birth was observed (LPS versus LPS + RSG; (Figure 4B). No preterm births were observed in mice injected with PBS or RSG alone (Figure 4B). The rate of pup mortality in mice injected with LPS was 100% (Figure 4C) yet, this was reduced by 41% after the treatment with RSG (LPS versus LPS + RSG; Figure 4C). Because RSG was dissolved in DMSO, it was essential to investigate whether DMSO alone had adverse effects in pregnancy. All mice injected with DMSO delivered at term, as in mice injected with PBS (19.5 ± 0.5 d; Figure 4D). Also, mice injected with DMSO had a low rate of pup mortality, similar to those injected with PBS (8–13%; Figure 4E). Next, we examined the effects of RSG treatment on fetal and placental weights. LPS

injection reduced fetal weight (LPS versus PBS; Figure 4F), but body mass was restored following treatment with RSG (LPS+RSG versus LPS; Figure 4F). The reduced placental weight (LPS versus PBS; Figure 4G), and this was not restored by treatment with RSG (LPS + RSG versus RSG; Figure 4G). The administration of RSG alone did not alter placental weight (RSG versus PBS; Figure 4G). These data demonstrate that treating mice with RSG reduces the rate of LPS-induced preterm birth, as well as improves adverse neonatal outcomes

Rosiglitazone treatment attenuated the systemic pro-inflammatory response induced by LPS

Preterm labor/birth is associated with a systemic pro-inflammatory response (122, 124, 125, 157-165). Therefore, we determined whether treatment with RSG reduced the elevated systemic concentrations of cytokines/chemokines induced by LPS. As expected, LPS increased the systemic concentration of several cytokines and chemokines when compared with the PBS control group (LPS vs. PBS; Figure 5A-C). Treatment with RSG attenuated the LPS-induced pro-inflammatory response by reducing the concentrations of several cytokines and chemokines including TNF α , IL1 β , IL3, IL4, IL9, IL10, IL12 (p40 and p70), IL13, IL15, GM-CSF, CCL2, CCL3, CCL4, CXCL2, CXCL5, and CXCL10 (LPS+RSG vs. LPS, Figure 3A & 3B). In addition, treatment with RSG increased the systemic concentrations of IL5 and CXCL9 in mice injected with LPS (LPS+RSG vs. LPS, Figure 5A and B). However, treatment with RSG did not reduce the systemic concentration of LPS-induced IFN γ , IL1 α , IL2, IL6, LIF, G-CSF, M-CSF, CCL5, and CCL11 (, Figure 3C). The administration of RSG alone did not alter the basal concentrations of cytokines/chemokines (RSG vs. PBS, Figure 5A-C).

These results demonstrate that treatment with RSG attenuates the LPS-induced pro-inflammatory response in the mother, which provides insight into the systemic immune mechanisms whereby this PPAR γ agonist prevents LPS-induced PTB.

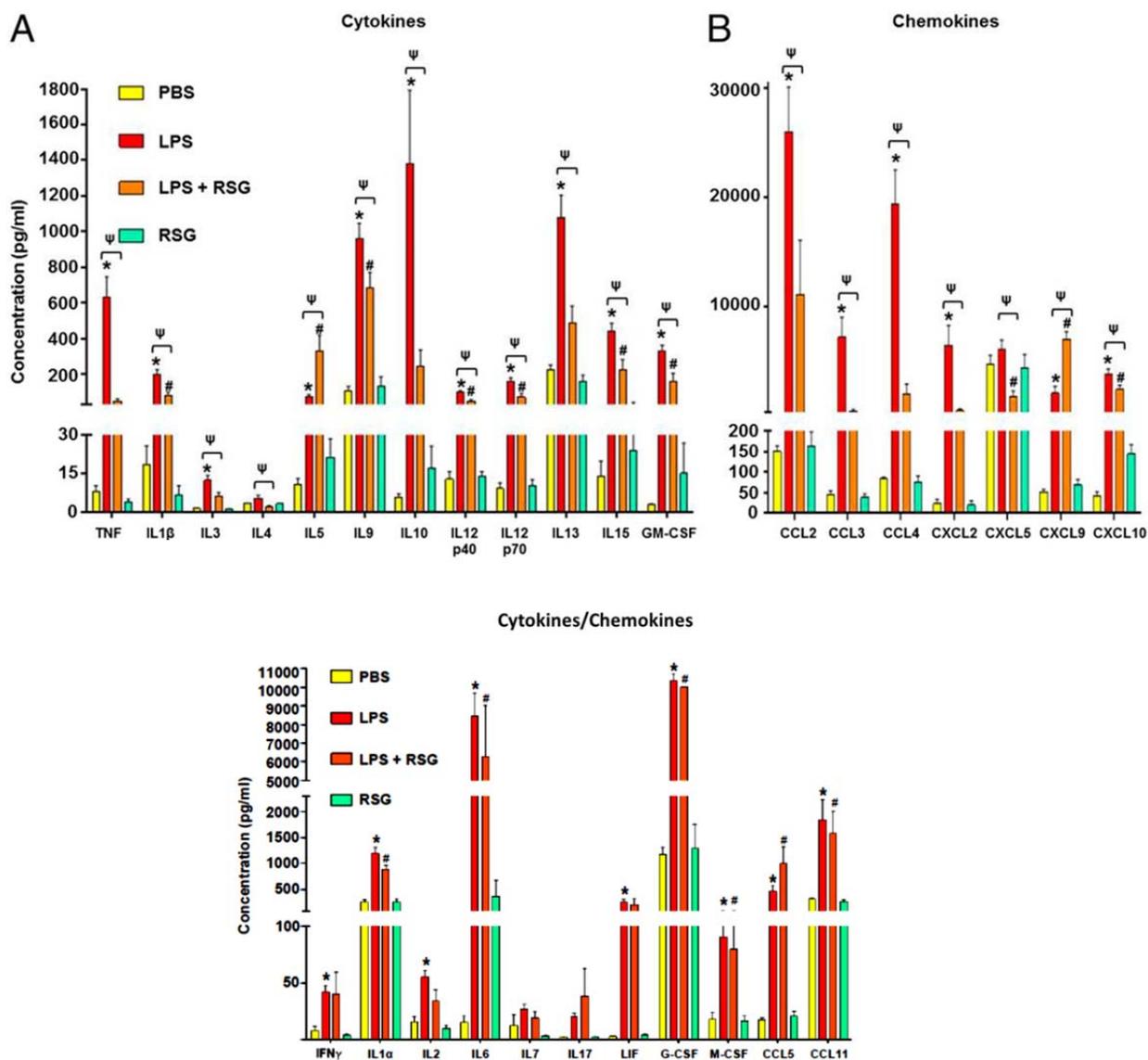


Figure 5: Rosiglitazone treatment attenuates the systemic inflammatory response induced by LPS.

A) Serum concentrations of cytokines in mice injected with PBS, LPS, LPS + rosiglitazone (RSG), and RSG alone. B) Serum concentrations of chemokines in mice injected with PBS, LPS, LPS + RSG, and RSG alone. Data are shown as bar plots (mean \pm SEM). Sidak T-tests: n = 8 each. * $p \leq 0.05$ LPS vs. PBS; # $p \leq 0.05$ LPS+RSG vs. PBS; $\psi p \leq 0.05$ LPS vs. LPS + RSG.

Rosiglitazone treatment attenuated LPS induced macrophage activation in myometrial and decidual macrophages

PPAR γ activation suppresses gene transcription by interfering with signal transduction pathways, such as the NF- κ B, STAT, and AP-1 pathways (166-168), and induces an M2 macrophage polarization (169). Therefore, we investigated whether PPAR γ activation *in vivo* through treatment with RSG alters the NF- κ B pathway, and the expression of M1 and M2 cytokines in decidual and myometrial macrophages from pregnant mice injected with LPS (Figure 6A). Decidual and myometrial macrophages (F4/80⁺ cells) were isolated, and their purity was confirmed by flow cytometry (88%-97%; Figure 6A). mRNA expression of *Nfkb1* (a pathway regulated by PPAR γ activation (166)), *Tnf* (an M1 cytokine (170)), and *Il10* (an M2 cytokine (170)) were determined in isolated macrophages. LPS injection up-regulated the expression of *Nfkb1*, *Tnf*, and *Il10* in decidual and myometrial macrophages (LPS vs. PBS; Figure 6B-6G); however, the mRNA abundance of these genes was down-regulated upon treatment with RSG (LPS+RSG vs. LPS; Figure 6B-G). In decidual macrophages, the LPS-induced expression of *IL10* was partially reduced upon treatment with RSG (LPS+RSG vs. LPS; Figure 6D). The administration of RSG alone did not alter the expression of any of these genes (RSG vs. PBS; Figure 6B-6G). These results demonstrate that treatment with RSG down-regulates the LPS-induced expression of *Nfkb1*, *Tnf*, and *Il10* in decidual and myometrial macrophages, which provides insight into the local immune mechanisms whereby this PPAR γ agonist prevents LPS-induced PTB.

The protein expression of NF- κ B, TNF, and IL-10 was also determined via immunofluorescence in myometrial and decidual macrophages. Consistent with our mRNA data, LPS-induced expression of NF- κ B, TNF, and IL-10 in myometrial macrophages was reduced upon treatment with RSG (Figure 7A-C). Similar results were found in decidual

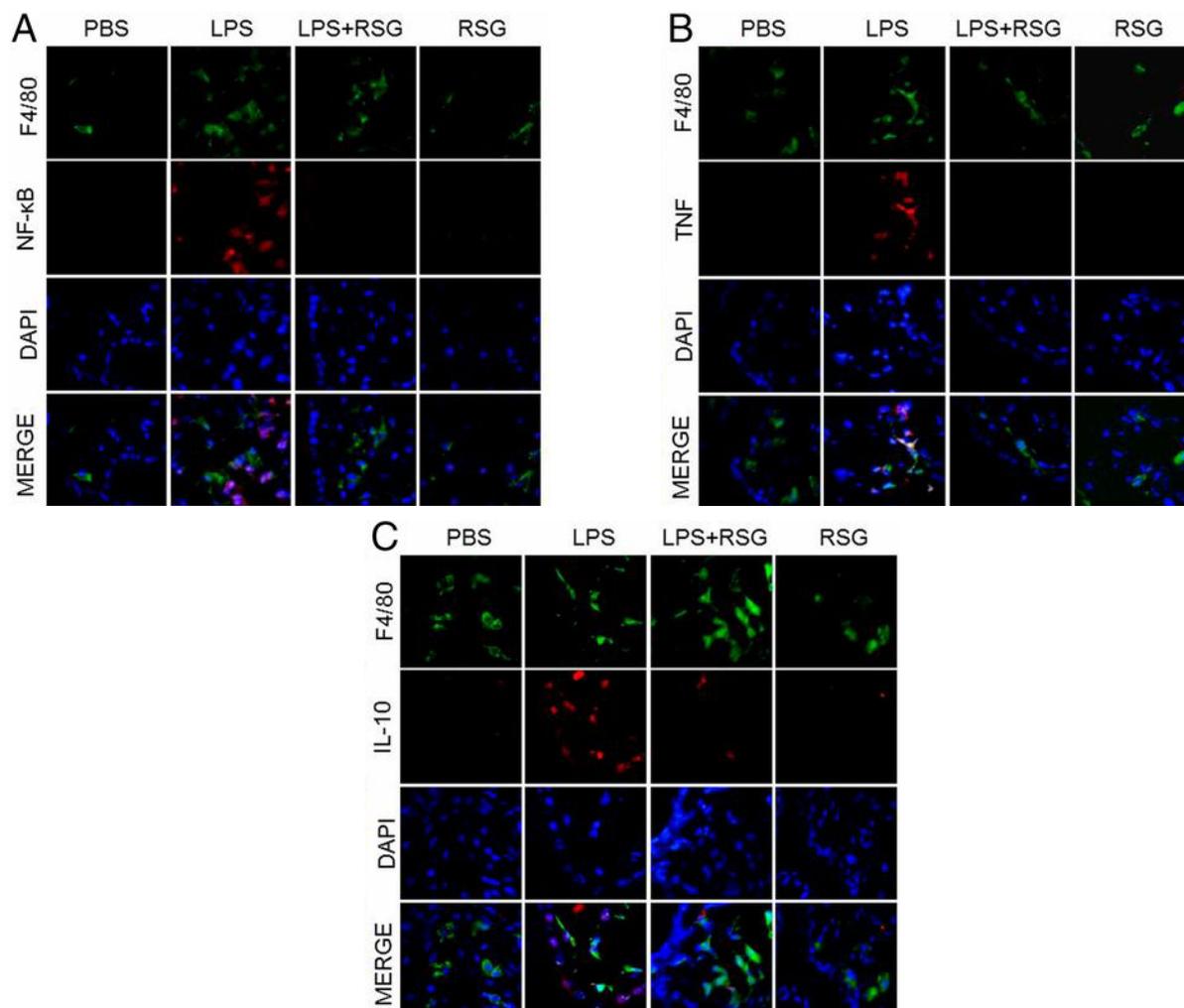


Figure 7:RSG treatment altered the protein expression of NF- κ B, TNF, and IL-10 in myometrial macrophages. On 16.5 dpc, pregnant mice were injected with LPS and treated with RSG 6 h after. Two hours after injection, myometrial tissues were processed for histology. Control mice were injected with LPS, PBS, or RSG alone. NF- κ B (A, red signal), TNF (B, red signal), and IL-10 (C, red signal) expression in myometrial macrophages (F4/80+ cells, green signal) was determined by immunofluorescence. Nuclei are blue (DAPI). Original magnification = 31000. n=5 each.

Discussion

In the current study, we evaluated if activation of PPAR γ could rescue inflammation-induced PTB. PPAR γ is a hormone nuclear receptor (40, 171) that binds lipid metabolites including eicosanoids, polyunsaturated fatty acids, and oxidized phospholipids as well as synthetic thiazolidinedione's (e.g., RSG and GW1929) (172). PPAR γ is expressed in the fetal membranes and placenta (173, 174) as well as in the reproductive tissues (175-177). PPAR γ has

an essential role in placental development as its depletion interferes with terminal differentiation of the trophoblast and placental vascularization, causing fetal death (178). In later stages of gestation, the PPAR γ pathway is linked to the inflammatory process of parturition in both term and preterm stages (101, 151, 174, 177, 179-183). Defective PPAR γ signaling is associated with pregnancy complications (184, 185) including GDM (186), IUGR (187, 188), PE (30, 105, 189, 190), and preterm birth (182, 191). Conversely, PPAR γ activation via 15-deoxy- Δ -^{12,14}-Prostaglandin J₂ delays LPS-induced PTB and reduces neonatal mortality by promoting the resolution of inflammation (192). However, the administration of 15-deoxy- Δ -^{12,14}-Prostaglandin J₂ alone has negative effects on gestational length, causing late PTB (192). In this study, PPAR γ activation via RSG reduced the rates of LPS-induced PTB by 30% (Figure 4B) and stillbirth by 40% (Figure 4C) Further, the administration of RSG alone did not have adverse effects on the mother or offspring (Figure 4E).

To assess the effects of RSG administration on systemic inflammatory process, we analyzed the levels of pro and anti-inflammatory cytokines and chemokines in sera from mice LPS and LPS + RSG group. Administration of LPS induced an inflammatory response in the mice and we observed a significant increase in serum levels of pro-inflammatory cytokines (Figure 5A-C). The systemic inhibitory activity of RSG on inflammatory cytokines has been previously demonstrated in non-pregnant mice (193-195) and rats (196, 197) injected with inflammatory stimuli, as well as in patients with diabetic and nondiabetic coronary artery disease (198) or obesity (199). Similar to the observations in these reports, administration of RSG significantly reduced the expression of pro-inflammatory cytokines in our model (Figure 5A-C). These results demonstrated that PPAR γ mediated reduction in PTB acts via attenuation of the systemic inflammatory response. Interestingly, in our study we observed that treatment with

RSG increased the serum concentration of LPS-induced IL5 and CXCL9. IL5 is a Th2 cytokine that is implicated in eosinophilic responses and B-cell proliferation (200), and CXCL9 is involved in T-cell trafficking (201) in chronic inflammatory processes (202, 203). Previous reports, however, have demonstrated that treatment with RSG reduces bronchoalveolar lavage fluid or serum concentrations of IL5 in rodent models of asthma (204, 205) and attenuates tissue eosinophilia induced by this cytokine (206). In the same fashion, treatment with RSG inhibits the release of CXCL9 induced by the IFNs (α, γ, β) in primary cultures of human thyroid follicular cells (207) and by IFN γ and TNF α in primary cultures of thyrocytes, retrobulbar fibroblasts, and retrobulbar preadipocytes obtained from Graves' ophthalmopathy patients (208). These disparities may be due to our study being performed in pregnant mice, and the serum sampling conducted shortly after LPS injection. However, further research is needed to investigate whether increased serum concentrations of IL5 and CXCL9 contribute to the effectiveness of RSG in preventing LPS-induced PTB.

In addition to reproductive organs, PPAR γ is expressed by adipose tissue and immune cells (54) including monocytes/macrophages (100, 166, 167, 209). Activation of PPAR γ has been shown to induce an anti-inflammatory profile in macrophages, which is impaired in macrophage-specific PPAR γ knockout mice, suggesting a critical role for PPAR γ in macrophage anti-inflammatory responses (210). Interestingly, LPS induced PTB is completely abrogated in pregnant mice with depleted macrophages, suggesting these are the crucial cell type propagating the effects of LPS(155). To understand the mechanism for RSG mediated PTB prevention, we decided to study the inflammatory gene expression in macrophages isolated for animals in the LPS and LPS + RSG treated animals. Since PTB is characterized by infiltration of macrophages at the maternal fetal unit, macrophages for the current study were isolated from these tissues.

Treatment with RSG significantly reduced the expression of *Nfkb1*, *Tnf*, and *Il10* by myometrial and decidual macrophages from mice injected with LPS (Figure 6B-G). The suppressive role of PPAR γ activation in macrophages has been previously demonstrated in the NF- κ B pathway (166, 167, 211), and in the expression/secretion of TNF α (100, 211-213) and IL10 (214, 215). Similarly, in our study the macrophages from the LPS + RSG group had significantly lower expression of *Nfkb1*, *Tnf*, and *Il10*. These changes in mRNA expression level were also reflected at the protein level (Figure 7A-C). Taken together, these data demonstrate that RSG prevents LPS-induced PTB by suppressing the local pro-inflammatory response mediated, at least in part, by decidual and myometrial macrophages.

In summary, the study herein demonstrates that PPAR γ activation via RSG can attenuate the LPS-induced systemic and local pro-inflammatory responses mediated by macrophages, preventing PTB and improving neonatal outcomes. These findings suggest that the PPAR γ pathway is a new molecular target for future preventative strategies for spontaneous preterm labor/birth.

CHAPTER 3 - ROSIGLITAZONE REGULATES TLR4 AND RESCUES HO-1 AND NRF2 EXPRESSION IN MYOMETRIAL AND DECIDUAL MACROPHAGES INFLAMMATION-INDUCED PRETERM BIRTH

(This chapter contains previously published material. See Appendix E)

Introduction

Preterm birth (PTB) is defined as delivery before 37 weeks of gestation and affects approximately 9.62% of births in 2015 (37). Premature neonates are at risk for short and long term health problems; making PTB one of the leading causes of neonatal mortality and morbidity worldwide (216). Approximately 70% of all PTBs are preceded by spontaneous preterm labor (PTL) (120, 217-220), a syndrome of multiple pathological processes (119). Of all the putative causes associated with spontaneous preterm labor, only intra-amniotic infection/inflammation has been causally linked to PTB (221-223). Inflammation-associated PTB is characterized by increased expression of pro-inflammatory proteins and infiltration of immune cells in the maternal-fetal interface (154), as well as elevated oxidative stress (122, 123, 224). Several animal models have been established to study the mechanisms whereby inflammation induces PTB (126, 130, 132). Lipopolysaccharide (LPS)-induced PTB is the most widely used model (138). In pregnant mice, administration of LPS induces an inflammatory response resulting in premature delivery (225). LPS binds to Toll-like receptor-4 (TLR4), which recruits the adaptor protein myeloid differentiation factor 88 (Myd88) (226) in order to initiate the activation of pro-inflammatory proteins such as the transcription factor - $\text{Nf-}\kappa\text{B}$ (227, 228). Activation of the $\text{Nf-}\kappa\text{B}$ pathway results in the expression of inflammatory cytokines such as $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ (226), triggering an inflammatory cascade that ultimately leads to PTL and PTB. Like the human syndrome, LPS-induced PTB increases oxidative stress and promotes the infiltration of neutrophils and macrophages at the maternal-fetal interface (124, 229-231). Therefore, targeting pro-inflammatory macrophages at the maternal-fetal interface may represent a new strategy to

prevent inflammation-associated PTB.

Pro-inflammatory macrophages are present at the maternal-fetal interface in term (i.e. physiological inflammation) and preterm (i.e. pathological inflammation) parturition (154, 232-234). Such innate immunity cells have a pro-inflammatory or M1-like phenotype in both processes of labor, which can be attenuated by administering Rosiglitazone (Rosi) (235). Rosiglitazone belongs to the thiolidazone family of compounds and acts as a specific agonist for the nuclear hormone receptor, Peroxisome proliferator activated receptor-gamma (PPAR γ) (235). PPAR γ is known for its role in lipid metabolism, adipocyte differentiation as well as for regulating the genes involved in inflammation and oxidative stress (52, 142, 236, 237). Interestingly, PPAR γ knockouts die *in-utero* due to placental abnormalities suggesting a pivotal role in placentation (178). Altered levels of PPAR γ levels or its activators have been associated with pregnancy-related pathologies such as GDM, IUGR and pre-eclampsia (103-105); however, its potential role in preterm birth remained unexplored. Recently, we showed that treatment with Rosiglitazone 1) reduced the rate of LPS-induced preterm birth by 30%, 2) reduced the rate of stillbirth by 41%, and 3) significantly downregulated the systemic inflammatory response in mice (235). Therefore, by targeting the PPAR γ pathway we could reduce the inflammatory effects of LPS.

In addition to inflammation, elevated oxidative stress is a major contributor to LPS-induced preterm birth (224, 238). Indeed, anti-oxidant supplementation has been demonstrated to improve pregnancy outcomes in different models of preterm birth (230, 231, 239). We therefore aimed to evaluate whether Rosiglitazone had similar actions in our model of preterm birth. In the study herein, we focused on Nuclear factor (erythroid-derived 2)-like 2 (NRF2) and its downstream target Heme oxygenase-1 (HO-1) due to their dual role as anti-inflammatory and

anti-oxidative regulators (240, 241). NRF2 is a transcription factor that regulates the expression of proteins involved in the detoxification of oxygen radicals by binding to anti-oxidant response element (ARE) in gene promoters (242). Additionally, it has been reported to block inflammatory signaling in bone marrow derived macrophages and reduce inflammation in a thrombin induced PTB model (243, 244). HO-1, is the rate limiting enzyme catalyzing the breakdown of heme, producing carbon monoxide and biliverdin. Biliverdin is then degraded further into bilirubin which is a strong anti-oxidant (245). HO-1 has been shown to play a critical role in placental function and reduced levels have been associated with pre-eclampsia and cases of spontaneous abortions (246, 247). HO-1 has also been shown to reduce myometrial contractility and may thus play a role in PTB (248).

Rosiglitazone has been shown to upregulate the expression of both NRF2 and HO-1 in hepatocytes (249), but its effects on expression in decidual and myometrial macrophages is unknown. Herein, we hypothesized that treatment with Rosiglitazone would improve pregnancy outcomes in an LPS-induced model of preterm birth by indirectly affecting the inflammatory cascade by regulating TLR4 receptor and reducing oxidative stress by upregulating the anti-oxidant factors NRF2 and HO-1.

Materials and Methods

Animal treatments

Pregnant C57BL/6J mice were divided into 4 groups and received intraperitoneal injections on 16.5 dpc: Group I: LPS – received 10µg of LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, MO) in 200µL of 1X PBS; Group II: PBS - 200µL of 1X PBS as a control; Group III: LPS+Rosi - received 10µg of LPS in 200µL of 1X PBS followed by 10mg/kg of Rosiglitazone 6 hours after the initial injection; and Group IV: Rosi - 10mg/kg of Rosiglitazone as a control. All

procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University (Protocol No. A 09-08-12).

Macrophage isolation from murine myometrial and decidual tissues

Eight hours after the first injection, the mice were euthanized and decidual and myometrial tissues were collected and processed immediately for (i) macrophage isolation and (ii) cryo-sectioning.

Macrophage isolation

Macrophages were isolated from the decidua and myometrium, as previously described (152). Briefly, the tissues were mechanically disaggregated using the Accutase cell dissociation reagent (Life Technologies, CA) and filtered using a 100 μ m cell strainer (Fisher Scientific, MA) to obtain single cell suspensions. The cells were then washed once with staining buffer [1X PBS (Fisher Scientific Bioreagents) containing 0.1% Bovine-serum albumin (Sigma Aldrich, MO) and 0.05% Sodium Azide (Fisher Scientific, MA)]. The cells were re-suspended in 96 μ L of staining buffer, with 4 μ L of an anti-mouse F4/80 antigen biotin (clone BM8, eBioscience, CA) and incubated at 4⁰C for 15 mins. After incubation, the cells were washed by centrifugation at 1250 x g for 7 min at 4⁰C. The cell pellet was re-suspended in 90 μ L of staining buffer containing 10 μ l of Streptavidin microbeads (Miltenyi Biotec, Germany) and again incubated at 4⁰C for 15 mins followed by 1 wash with 2ml of staining buffer. The resulting pellet was re-suspended in 500 μ L of MACS (Miltenyi Biotech, Germany) buffer and F4/80⁺ cells (macrophages) were separated by positive selection using MS columns and a magnetic MACS separator. Macrophages were then washed with MACS buffer at 1250 x g for 7 min at 4⁰C. The resulting cell pellet was lysed using 400 μ L of RNeasy Lysis Buffer (Qiagen, Germany) and used for RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was extracted (RNAeasy, Qiagen, Germany) following the manufacturer's instructions. All the samples were simultaneously reverse transcribed using the iScript Reverse Transcription Supermix RT synthesis kit (Bio-Rad Laboratories, CA), per the manufacturer's instructions. As the number of macrophages isolated from the murine tissues is very low, the amount of RNA extracted was also low. To increase the sensitivity and efficiency of qPCR, the target genes *Tlr4*, *Ho-1*, *Nrf2* and housekeeping genes *Gapdh*, *Tbp*, *Top1* were enriched by pre-amplification. The primers were obtained from IDT, and reconstituted with Tris-EDTA buffer (10mM Tris, 0.1mM EDTA, pH 8.0) to a 500 μ M working stock. 2.5 μ L from of each primer was mixed and the final volume was adjusted to 500 μ L to create the pre-amplification primer cocktail (0.5 μ M). 10 μ L of the cDNA (25 ng) was mixed with 5 μ L of primer cocktail, 10 μ L of nuclease free water, and 25 μ L of pre-amplification master-mix (Sso – Advanced PreAmp Supermix, Bio-Rad Laboratories, CA). The mix was then cycled for 3 mins at 95⁰C, 15 seconds at 95⁰C and 4 mins at 58⁰C for 10 cycles.

Real time PCR and data analysis

The target genes *Tlr4*, *Ho-1*, *Nrf2* and the housekeeping genes *Gapdh*, *Tbp*, *Top1* were run in triplicates for each sample using 3 μ L of pre-amplified cDNA (diluted 1:30) per PCR reaction. The primers used are outlined in Table 2. Briefly, 3 μ L of cDNA template was mixed with 1 μ L primer (500nM), 1 μ L nuclease free water, and 5 μ L of Sybr-green master mix (LuminoCT, Sigma-Aldrich, MO). The PCR protocol used was: initial 95 °C for 5 min followed by 38 cycles of 95 °C for 15s and 60°C for 20s. The qPCR data was analyzed by the Pfaffl method using housekeeping genes as an internal reference (250, 251). Briefly, the mean C_T values of housekeeping genes per tissue sample was used to calculate the geometric mean C_T

value (Geo-mean C_T) and used as reference for the respective sample. The relative expression of target genes was calculated by using the formula $2^{-\Delta C_T}$, where $\Delta C_T = \text{Mean } C_T (\text{Target gene}) - \text{Geo-mean } C_T$. The mean relative expression (MRE) of the target gene for each treatment groups was calculated by taking an average of the relative expression of individual tissue samples from the respective groups.

Table 2: Sequences of primer used for gene expression in macrophages

Gene Name	Gene Symbol	Sequence
Glyceraldehyde 3-phosphate dehydrogenase	<i>Gapdh</i>	5'-AAT GGT GAA GGT CCG TGT G-3'
		5'-GTG GAG TCA TAC TGG AAC ATG TAG-3'
Topoisomerase-1	<i>Top1</i>	5'-CTT TAA TTC GTG GCG GAC TAG A -3'
		5'- AGA CAA GGA AAG ACG AAA GGA G-3'
TATA Box Binding Protein	<i>Tbp</i>	5'-TTC ACC AAT GAC TCC TAT GAC C-3'
		5'-CAA GTT TAC AGC CAA GAT TCA CG-3'
Toll like receptor - 4	<i>Tlr4</i>	5'-GAA GCT TGA ATC CCT GCA TAG-3'
		5'-AGC TCA GAT CTA TGT TCT TGG TTG-3'
Heme oxygenase -1	<i>Ho-1</i>	5'-ACA CTC TGG AGA TGA CAC CT-3'
		5'-TTG TGT TCC TCT GTC AGC ATC-3'
Nuclear factor (erythroid-derived 2)-like 2	<i>Nrf2</i>	5'-CCT TGT ACT TTG AAG ACT GTA TGC-3'
		5'-GAG GGA CTG GGC CTG AT-3'

Immunofluorescence staining

Myometrial and decidual tissues (n = 5 each) were immediately frozen in Tissue-Tek O.C.T Compound (Sakura Finetek, CA). The tissues were sectioned into 10 μ m sections and placed on Fisherbrand Superfrost Plus microscope slides (Thermo Scientific, MA). For staining, the tissues were fixed in 4% paraformaldehyde for 10 mins and washed in 1X PBS containing 0.1% Tween-20. The tissues were then permeabilized with 0.25% Triton X-100 in 1X PBS for 10 min. Nonspecific antibody interactions were blocked using donkey serum (Jackson Immunoresearch, PA) at room temperature for 1 hour. Slides were then incubated with the following: anti- TLR4 antibody, anti-HO-1 antibody (Abcam, UK), anti-NRF2 antibody (Cell Signaling, MA) at 4°C overnight. The following day, the slides were washed 3 times (5 minutes/wash) with 1X PBS containing 0.1% Tween 20. The slides were incubated with 2

mg/mL of goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (Jackson ImmunoResearch, PA) at room temperature for 1 hour. The slides were washed again with 1X PBS containing 0.1% Tween 20, and incubated with 2 mg/mL of rat anti-mouse F4/80 antibody conjugated to FITC (1:100, Abcam, UK) at room temperature for 2 hours. Post incubation, the slides were again washed 3 times and stained with DAPI (Room temperature for 20 mins). The slides were given a final wash and mounted in Vectashield Mounting Medium (Vector Laboratories, CA). Immunofluorescence was visualized using the Leica DM IRB epifluorescence microscope (Leica, Germany), and images were captured using a Hamamatsu Orca digital camera (Hamamatsu Corp, Japan).

Results

Macrophage populations

Macrophages were isolated from the decidual and myometrial tissues of mice treated with LPS only (LPS), LPS + Rosiglitazone (LPS + Rosi), Rosiglitazone only (Rosi), and PBS only (PBS). The purity of the isolated F4/80+ macrophage cells was assessed by flow cytometry. The percentage of macrophages isolated from the decidual and myometrial tissues was 84.9% and 88.6% respectively (Figure 8A and B).

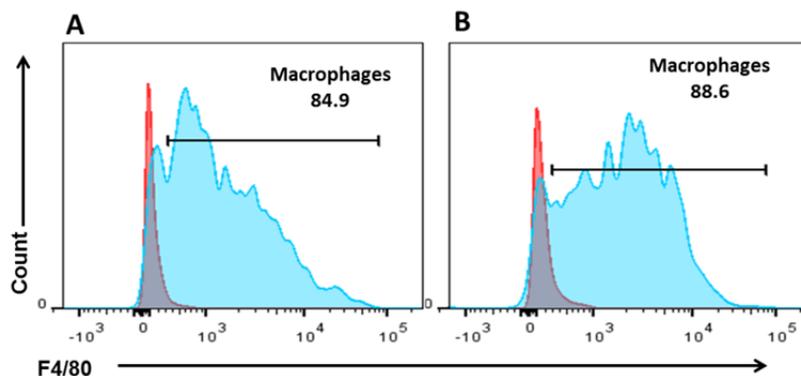


Figure 8: Purity of isolated macrophages from decidual and myometrial tissues. Decidual (A) and myometrial (B) macrophages (F4/80+ cells) were isolated by magnetic cell sorting, and purity was evaluated by flow cytometry. The red histogram represents the auto-fluorescence control and the turquoise histogram represents isolated macrophages from decidia or myometrial tissues.

Rosiglitazone downregulated LPS-induced Tlr4 expression

Total RNA was extracted from decidual and myometrial macrophages isolated from mice from LPS, LPS + Rosi, Rosi, and PBS groups and converted to cDNA. The expression of the *Tlr4* receptor was assessed using qPCR.

The mean relative expression (MRE) of *Tlr4* in decidual macrophages isolated from mice injected with endotoxin alone was significantly higher compared to those isolated from mice injected with PBS only (1.81 ± 0.23 vs 1.23 ± 0.10 ; $p=0.031$). The MRE of decidual macrophages from mice injected with Rosiglitazone only was significantly reduced compared to those isolated from mice injected with endotoxin alone (0.96 ± 0.08 vs 1.81 ± 0.23 ; $p=0.007$). The MRE of decidual macrophages isolated from LPS injected mice treated with Rosiglitazone was significantly lower compared to those isolated from mice injected with endotoxin alone (1.14 ± 0.18 vs 1.81 ± 0.23 ; $p=0.048$). However, no significant differences were observed in the *Tlr4* expression of isolated macrophages from mice injected with PBS and Rosiglitazone alone (Figure 9A).

In line with the observation in decidual macrophages, the MRE of *Tlr4* in myometrial macrophages isolated from mice injected with endotoxin alone was significantly higher compared to those isolated from mice injected with PBS only (2.29 ± 0.24 vs 1.25 ± 0.11 ; $p=0.007$) and Rosiglitazone only (2.29 ± 0.24 vs 1.44 ± 0.10 ; $p=0.031$). The MRE of *Tlr4* in myometrial macrophages isolated from LPS injected mice treated with Rosiglitazone was significantly lower compared to those isolated from mice injected with endotoxin alone (1.20 ± 0.19 vs 2.29 ± 0.24 ; $p=0.015$) (Figure 9B).

Typically, the number of isolated macrophages is too low for conventional quantitative protein assessment by western blotting. Hence, we decided to assess TLR4 protein expression

by immunohistochemistry (235). The cryo-fixed decidual and myometrial tissues from mice injected with LPS only, LPS + Rosiglitazone, Rosiglitazone only, and PBS only were sectioned and stained with F4/80+ and TLR4 antibodies. The presence of F4/80 and TLR4 was observed in both decidual and myometrial tissues. The relative expression of TLR4 was higher in myometrial and decidual tissues from mice injected with endotoxin compared to controls injected with PBS and Rosiglitazone only (Figures 9C and D).

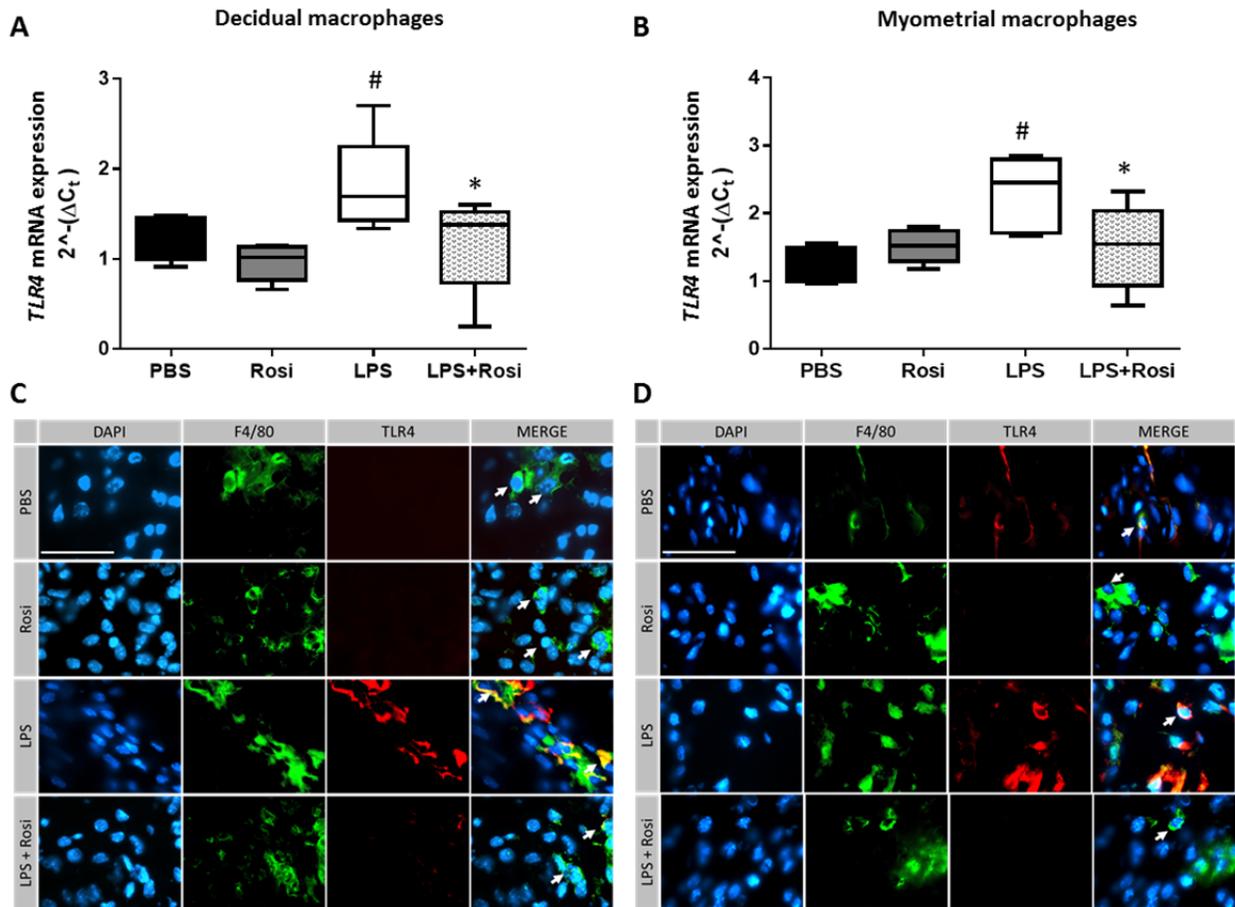


Figure 9: Rosiglitazone downregulates LPS induced TLR4 expression. Mean relative expression of Tlr4 mRNA in decidual (A) and myometrial (B) in macrophages isolated from animals (n=4-7) in the 4 treatment groups. Macrophages from the LPS treated group showed increased expression of TLR4 which was significantly downregulated when Rosiglitazone was administered. Representative images show immune-staining for TLR4 protein expression (red) in (C) decidual and (D) myometrial macrophages (green, arrows). The nuclei were stained with DAPI (blue). Data are shown as box plots (median). ‘*’, ‘#’ indicates significance at $p < 0.05$ when compared to the LPS group and PBS group respectively. Scale bar: 50 μ M, Magnification is 1000X, n=3.

Treatment with Rosiglitazone inhibited the LPS mediated downregulation of Nrf2 expression

NRF2 is the master transcription factor that regulates oxidative stress via the expression of anti-oxidant factors (252)-(253). Therefore, to evaluate the oxidative stress signaling pathway in an endotoxin-induced model of preterm birth, we assessed NRF2 expression in isolated myometrial and decidual macrophages from mice injected with LPS only, LPS + Rosiglitazone, Rosiglitazone only, and PBS only.

The MRE of *Nrf2* in decidual macrophages isolated from mice injected with endotoxin was significantly lower compared those isolated from mice injected with PBS (2.10 ± 0.22 vs 3.28 ± 0.30 ; $p=0.028$) and Rosiglitazone alone (2.10 ± 0.22 vs 3.28 ± 0.37 ; $p=0.028$). The MRE of *Nrf2* in decidual macrophages isolated from LPS injected mice treated with Rosiglitazone was significantly increased compared to those isolated from mice injected with endotoxin alone (3.15 ± 0.2 vs 2.10 ± 0.22 ; $p=0.028$). The MRE of *Nrf2* was comparable between decidual macrophages isolated from mice treated with LPS + Rosiglitazone, PBS, and Rosiglitazone alone (Figure 10A).

A similar trend in *Nrf2* expression was observed in myometrial macrophages. The MRE of *Nrf2* in myometrial macrophages isolated from mice injected with endotoxin was significantly lower compared to those isolated from mice injected with PBS (1.37 ± 0.16 vs 2.31 ± 0.18 ; $p=0.028$) and Rosiglitazone only (1.37 ± 0.16 vs 2.51 ± 0.29 ; $p=0.015$). In addition, the expression of *Nrf2* in myometrial macrophages isolated from LPS injected mice treated with Rosiglitazone was, significantly higher than those injected with endotoxin alone (2.80 ± 0.49 vs 1.37 ± 0.16 ; $p=0.031$) (Figure 10B). *Nrf2* expression levels were comparable between myometrial macrophages isolated from LPS injected mice treated with Rosiglitazone and those isolated from mice injected with PBS and Rosiglitazone alone.

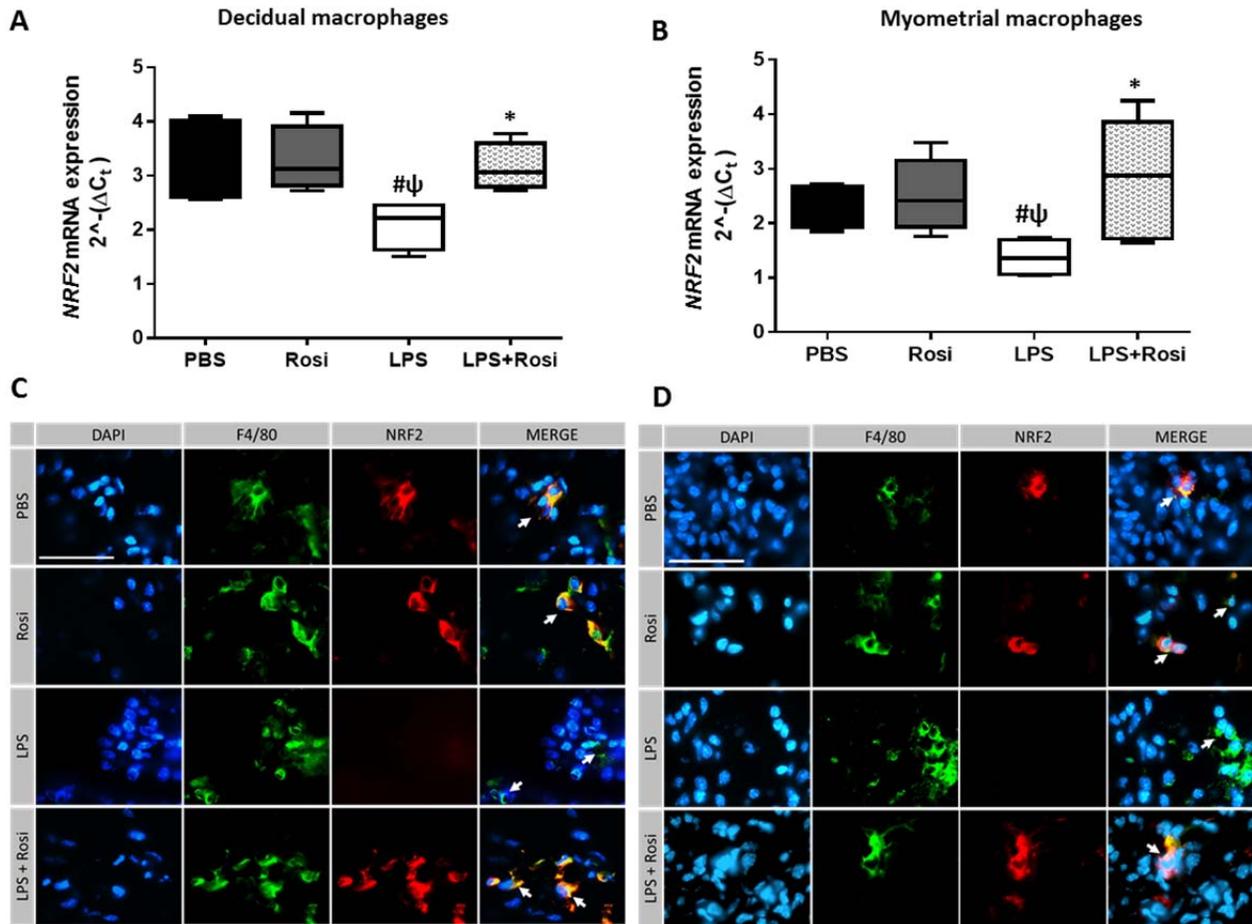


Figure 10: Rosiglitazone rescues LPS mediated downregulation of NRF2 expression. Mean relative expression of Nrf2 mRNA in decidual (A) and myometrial (B) macrophages isolated from animals (n=4-7) in the 4 treatment groups. Macrophages from the LPS treated group showed a significantly lower expression of Nrf2, which was rescued to control levels when Rosiglitazone was administered. Representative images show immune-staining for NRF2 protein expression (red) in (C) decidual and (D) myometrial macrophages (green, arrows). The nuclei were stained with DAPI (blue). Data are shown as box plots (median). ‘*’, ‘#’, ‘ ψ ’ indicates significance at $p < 0.05$, when compared to the LPS group, PBS group and ROSI group respectively. Scale bar: 50 μ M, Magnification is 1000X, n=3

Visualization of the NRF2 protein was performed using immunohistochemistry. Neither decidual nor myometrial tissues from mice injected with LPS endotoxin expressed the NRF2 protein; however, a comparable expression of NRF2 was observed in decidual and myometrial tissues from LPS injected mice treated with Rosiglitazone and those from mice injected with PBS and Rosiglitazone alone (Figures 10C and D).

Treatment with Rosiglitazone inhibited the LPS mediated reduction of Ho-1 expression

The mRNA expression of *Ho-1*, a known downstream target for NRF2 signaling and a critical anti-oxidant mediator in cells, was assessed in decidual and myometrial macrophages (253). The MRE of *Ho-1* in decidual macrophages isolated from mice injected with endotoxin was significantly lower compared to those isolated from mice injected with PBS (0.11 ± 0.02 vs 0.21 ± 0.004 ; $p=0.028$) and Rosiglitazone only ($0.11 + 0.02$ vs 0.42 ± 0.09 ; $p=0.015$).

The HO-1 expression in decidual macrophages isolated from LPS injected mice treated with Rosiglitazone was significantly higher compared to those isolated from mice injected with endotoxin alone (0.50 ± 0.10 vs $0.11 + 0.02$; $p=0.015$). Interestingly, the HO-1 expression of decidual macrophages isolated from LPS injected mice treated with Rosiglitazone was significantly elevated compared to those isolated from PBS controls ($0.50 + 0.10$ vs $0.21 + 0.004$; $p=0.0159$). The HO-1 expression in isolated decidual macrophages from mice injected with Rosiglitazone alone was also significantly higher compared to PBS controls ($0.42 + 0.09$ vs $0.21 + 0.004$; $p=0.015$) (Figure 11A).

In contrast to decidual macrophages, HO-1 expression in myometrial macrophages isolated from mice injected with endotoxin was not significantly different compared to PBS controls (0.26 ± 0.020 vs 0.22 ± 0.04 ; $p=0.45$). However, HO-1 expression in isolated myometrial macrophages from LPS injected mice treated with Rosiglitazone was comparable to those isolated from the decidua. The MRE of *Ho-1* in isolated myometrial macrophages from mice injected with LPS+ Rosiglitazone was significantly higher compared those isolated from mice injected with endotoxin (0.53 ± 0.06 vs $0.26 + 0.020$; $p=0.03$) and PBS alone ($0.53 + 0.06$ vs $0.22 + 0.04$; $p=0.015$). The MRE of *Ho-1* in myometrial macrophages isolated from mice injected with Rosiglitazone alone was elevated compared to those isolated from LPS-injected mice ($0.46 \pm$

0.06 vs 0.26+ 0.020) and PBS controls (0.46 + 0.06 vs 0.22+ 0.04); yet, these values were not statistically significant (Figure 11B).

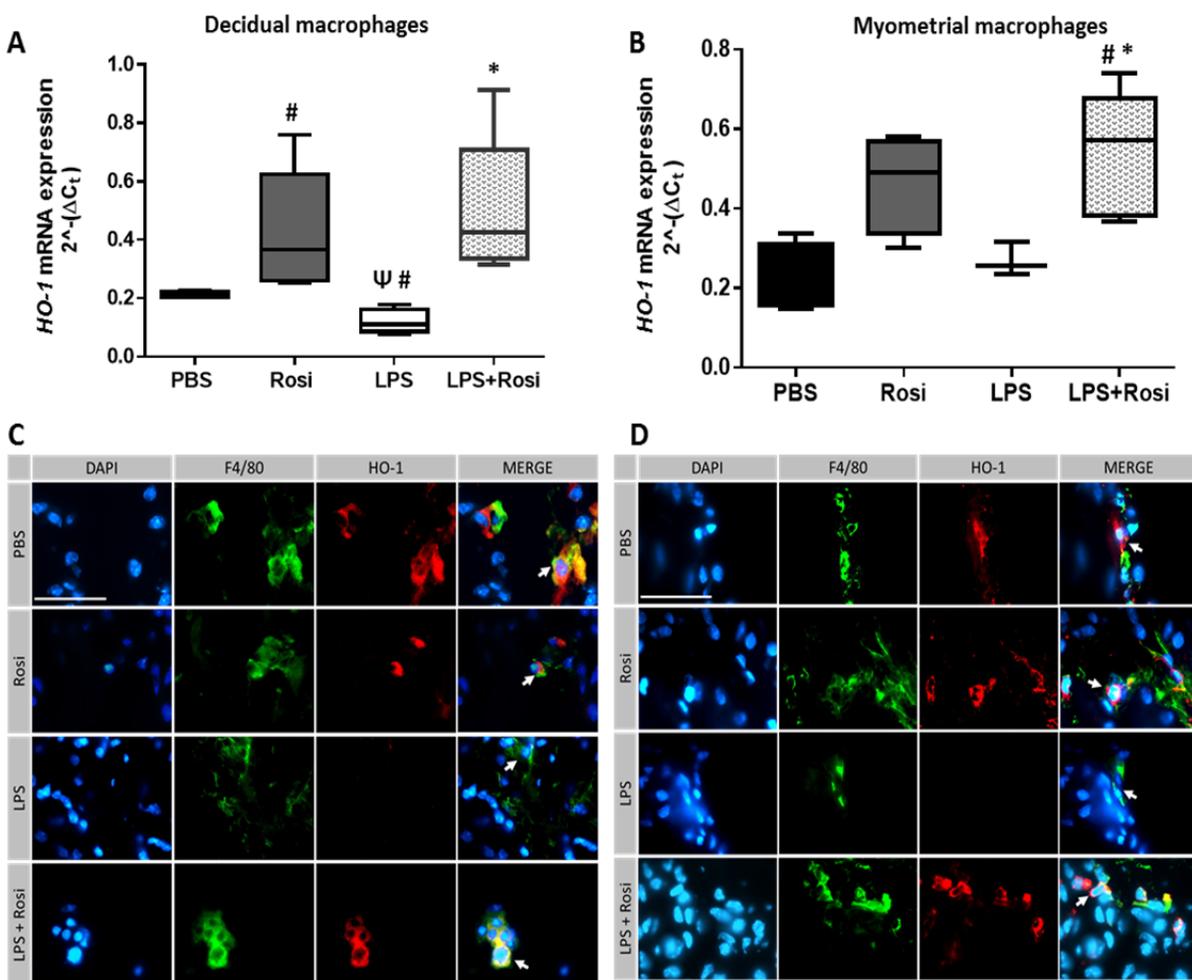


Figure 11: Rosiglitazone rescued LPS mediated decrease in HO-1 expression. Mean relative expression of Ho-1 mRNA in decidual (A) and myometrial (B) macrophages isolated from animals (n=4-7) in the 4 treatment groups. Macrophages from the LPS treated group showed a lower expression of Ho-1, which was significantly upregulated when Rosiglitazone was administered. Representative images show immune-staining for HO-1 protein expression (red) in (C) decidual and (D) myometrial tissues macrophages (green, arrows). The nuclei were stained with DAPI (blue). Data are shown as box plots (median). ‘*’, ‘#’, ‘ψ’ indicates significance at p<0.05, when compared to the LPS group, PBS group and ROSI group respectively. Scale bar: 50μM, Magnification is 1000X, n=3

The relative protein levels of HO-1 observed in myometrial tissues also supported the mRNA expression data. HO-1 was expressed in myometrial and decidual macrophages isolated from LPS injected mice treated with Rosiglitazone as well as those isolated from mice injected with PBS and Rosiglitazone alone. A reduced expression of HO-1 was observed in myometrial

and decidual tissues from mice injected with endotoxin compared to those injected with LPS+ Rosiglitazone and PBS and Rosiglitazone alone (Figures 11C and D).

Discussion

In the current study, we expanded the involvement of molecular pathways in the response to Rosiglitazone treatment in a murine model of LPS induced PTB. Our previous study showed that administration of Rosiglitazone reduced the rate of LPS induced preterm birth by 30%, increased the pup viability by 41% and lowered systemic & local inflammation (235). We also showed a downregulation of the NF- κ B pathway mediators – TNF- α and NF- κ B1- in decidual and myometrial macrophages. These findings demonstrated that treatment with Rosiglitazone contributes to reduced inflammation via reducing Nf- κ B activity in local macrophages. Herein, we investigated whether treatment with Rosiglitazone also regulated TLR4 expression and induced the expression of the anti-oxidants HO-1 & NRF2, which could further support the reduction of PTB and pup mortality.

Depletion of macrophages in pregnant mice abrogates the effects of LPS and prevents PTB (155), suggesting that these innate immune cells are involved in mediating the effects of LPS. Therefore, we focused on the role of macrophages in our LPS induced model of preterm birth. Decidual and myometrial macrophages from mice injected with LPS only, LPS + Rosiglitazone, Rosiglitazone only, and PBS only were isolated and their gene expression was analyzed by qPCR. The protein expression was assessed by staining decidual and myometrial tissues with antibodies against F4/80⁺ and the proteins of interest. We observed a significant increase in *Tlr4* expression in both decidual and myometrial macrophages from mice injected with endotoxin compared to those isolated from mice injected with PBS and Rosiglitazone alone. Administration of Rosiglitazone post LPS prevented this upregulation and macrophages from the

LPS + Rosi group had TLR4 levels (protein and mRNA) comparable to those seen in control groups, suggesting an active regulation of TLR4 in our model.

Induction of *Tlr4* expression by LPS has been observed in smooth muscle cells and microglia; however, information on the expression of this protein in mouse decidual and myometrial tissues and tissue macrophages is inconclusive (216, 254, 255). Salminen *et al* reported TLR4 mRNA expression in the uteri of pregnant mice; however, its expression significantly declined post LPS treatment (256). *In-vitro* studies in mouse macrophages showed no alteration in TLR4 expression after LPS treatment whereas human mononuclear cells showed an upregulation at the mRNA level with no alteration in protein expression (257)(258). In contrast, we observed that the LPS upregulated TLR4 mRNA expression and protein in macrophages from both tissues. These results suggest that LPS regulates the expression of its own receptor in these cells. Increased expression of TLR4 is associated with increased sensitivity to LPS and increased activation of inflammatory pathways e.g. NF- κ B (254). In return, we suggest that Rosiglitazone mediated the downregulation of TLR4 expression (as observed in the LPS + Rosi group) and impaired the activation of pro-inflammatory pathways, which could ultimately contribute to a reduction in PTB (259). Further, in both decidual and myometrial tissues, the TLR4 protein was exclusively localized in the macrophages (F4/80⁺ cells) (Figure 9C, D). While it is known that human decidual cells express the TLR4 protein, its expression in mouse decidual tissue remains unknown (260). We report that in murine decidual and myometrial tissues, only the macrophages express the TLR4 receptor, suggesting an active role in pathogen recognition and clearance during pregnancy.

In addition to activation of NF- κ B, TLR4 activation has been shown to induce production of reactive oxygen species (ROS), which leads to oxidative stress (261). Oxidative stress in an

inevitable part of pregnancy and in normal circumstances, a balance between the production of ROS and the anti-oxidants that scavenge them is maintained. However, excess ROS – induced by inflammation or lowered anti-oxidant capacity- leads to oxidative stress damage which has been associated with pathologies like PE, IUGR and PTB [detailed review in (84, 262)]. Further, elevated ROS has been shown to augment the expression of TLR4 in mice which, in turn, has been shown to downregulate the expression of anti-oxidant enzyme HO-1. These data suggest that there is a mutual regulation between inflammatory and oxidative stress pathways (263-265).

The transcription factor NRF2 is the master regulator of anti-oxidant enzyme expression and is induced under normal cellular stress conditions by various ROS (252) (252, 253). Activation of NRF2 leads to its release from the cytoplasmic inhibitor Kelch Like ECH Associated Protein 1 (Keap-1) and translocation to the nucleus where it induces expression of anti-oxidant enzymes like HO-1, which then help in scavenging ROS and other oxidants. NRF2 has been reported to play a role in murine placental development (266). Its activation was shown to reduce thrombin induced PTB, suggesting its active role in inflammation induced PTB. HO-1 has also been implicated in playing crucial roles during pregnancy (267, 268). It was reported to regulate recruitment and maintenance of myeloid cells in pregnant uteri and placental vasculature development (269, 270). Additionally, HO-1 activation via statins was shown to delay myometrial contractions, cervical ripening and inhibit pathological complement activation in the LPS induced PTB model (271). However, expression of both HO-1 and NRF2 in myometrial and decidual tissues and tissue macrophages has not been described.

We observed that LPS treatment downregulated the expression of both NRF2 and its downstream target HO-1. These results demonstrate, for the first time, that LPS actively inhibits the anti-oxidant response in the pregnant mice by downregulating NRF2. Thus, LPS mediated

inflammation contributes to the elevated oxidative stress in this model. As reported in other cell systems, Rosiglitazone treatment induced the expression of both NRF2 and HO-1. In addition, the expression of these proteins in the LPS + Rosiglitazone group was comparable to that in the PBS and Rosiglitazone groups (249). A cross talk between the NRF2-HO-1 signaling and TLR4-NF- κ B signaling pathway has been suggested and activation of NRF2 has been shown to rescue the effects of TLR4 mediated pro-inflammatory pathways in mouse liver and adipose tissue cells (272-274). We suggest activation of a similar pathway due to Rosiglitazone administration in our model.

Furthermore, HO-1 expression was significantly upregulated in decidual macrophages isolated from mice injected with Rosiglitazone only, suggesting its regulation by a NRF2 independent mechanism. Similar to TLR4, the NRF2 and HO-1 proteins were predominantly localized to macrophages from decidual and myometrial tissues, suggesting their role in mediating the oxidative and inflammatory stress cross talk.

Taken together, our results demonstrate that LPS might promote inflammation and oxidative stress by upregulating TLR4 expression and downregulating the anti-oxidants NRF2 and HO-1 in the LPS induced model of PTB. In addition, Rosiglitazone prevents PTB by downregulating TLR4 mediated pro-inflammatory signaling and upregulating the anti-oxidant response via NRF2 and HO-1.

Limitations of Aim 1

The current study evaluated the effects of Rosiglitazone intervention in preventing endotoxin induced PTB in mice via its effects on inflammatory and anti-oxidative pathways. However, Rosiglitazone has been known to contribute to PPAR γ activity in several metabolic and cell differentiation pathways. Evaluating the effects of Rosiglitazone intervention on these

pathways would provide further insights into the efficacy of intervention. Further, the current study focused on macrophages as they are the critical cell type involved in LPS mediated PTB. Assessing the effects of PPAR γ activation in other immune cell types – NK cells, neutrophils – would help in better understanding of the intervention model.

CHAPTER 4 - PPAR γ ACTIVATION RESCUES ENDOTOXIN MEDIATED EFFECTS ON TROPHOBLAST INFLAMMATORY RESPONSE AND DIFFERENTIATION IN 1ST TRIMESTER HUMAN PLACENTA

Introduction

Crosstalk between inflammatory and trophoblast differentiation pathways at the maternal fetal interface is crucial for successful establishment and maintenance of pregnancy. The trophoblast cells of the placenta play a critical role in maintaining allogenic tolerance by selective expression of cell surface receptors such as HLA-E, HLA-G and HLA-C (275, 276). They prevent fetal rejection by suppressing local inflammatory responses via regulation of T cell populations and impairing responses to immune-activating cytokines present at the maternal fetal interface (19, 277, 278). Additionally, trophoblast cells express pattern recognition receptors like Toll like receptors (TLR's) and Nod like receptors (NLR's) which are known to be involved in pathogen recognition (26, 279). Upon ligation, these receptors initiate an inflammatory cascade via transcription factors like NF- κ B, AP-1 and STAT3 to eliminate pathogens (26, 280, 281). Furthermore, the uterine NK cells and macrophages at the site of implantation direct trophoblast migration and invasion. They secrete several cytokines and chemokines that contribute to a local environment of growth factors at the maternal fetal interface (282, 283). These cytokines play a role in trophoblast differentiation (invasive extravillous trophoblast phenotype), inducing secretion of matrix degrading enzymes and selective remodeling of uterine spiral arteries (283-285). The proinflammatory transcription factor NF- κ B was recently shown to regulate expression of Placental growth factor (PlGF) – a protein known for its role placental angiogenesis, trophoblast proliferation. PlGF as well initiates differentiation towards the invasive phenotype further highlighting the role of inflammatory mediators in trophoblast function (286, 287). Dysregulation in these complementary processes could affect pregnancy success. Elevated inflammation at the maternal–fetal interface and abnormal placental function are both

common features in several pregnancy pathologies (288). Localized inflammation (Chorioamnionitis) has been reported to be present in >85% spontaneous preterm births even in the absence of systemic inflammation (289). Elevated placental cytokine release has been observed in cases of both preterm birth (PTB) and PE (290-294). Furthermore, proinflammatory cytokines like TNF- α and IL-6 released as a part of inflammatory cascade have been shown to induce trophoblast cell apoptosis as well commonly found in placentas from IUGR and PE cases (293, 295-297). Understanding the mutual molecular mechanism between inflammation and trophoblast differentiation could provide further insights into the etiologies of above mentioned syndromes. It could as well potentially highlight molecular targets common to both pathways which can then be evaluated for development of therapeutic strategies.

In vitro studies have shown that exposure to inflammatory stimuli induces proinflammatory cytokine secretion from trophoblast cells (298, 299). Additionally, exposure to proinflammatory cytokines such as TNF- α , IL-6 induced trophoblast cell apoptosis and affected trophoblast invasion, although contradictory results were reported by various groups (300, 301). Jovanovic, M *et al.* reported that proinflammatory cytokines increased invasion in both isolated human primary trophoblast cells as well as HTR cells, whereas other groups reported an overall decrease in invasion (300-302). To date, the effects of inflammation on trophoblast differentiation and related proteins including transcription factors has not been explored.

Trophoblast differentiation is a tightly regulated process. The transcription factor PPAR γ recently emerged as an important protein in early trophoblast lineage differentiation as well as placental function. Initially known for its role in energy metabolism and anti-inflammatory processes, PPAR γ knockouts were shown to die in-utero due to placental abnormalities (178, 212). It was reported to regulate the transcription factor Glial cell missing-1 (GCM1) and the

glycoprotein hormone human chorionic gonadotropin (CG- β); both known to be crucial players in trophoblast differentiation (69, 78, 303).

Our group recently showed that activation of PPAR γ in a mouse model of inflammation induced preterm birth prevented premature delivery, reduced systemic and local inflammation by repressing NF- κ B activity and improved placental and fetal weights, further emphasizing its role in placental function and as an anti-inflammatory agent (235, 304). Surprisingly, the role of PPAR γ in inflammation mediated effects on trophoblast differentiation in 1st trimester human placenta so far has not been evaluated.

In the current study, we therefore aimed to measure the effects of inflammation on trophoblast differentiation and the potential role of PPAR γ in this process. ***We hypothesized that activation of PPAR γ would reverse the inflammation mediated effects on trophoblast differentiation.***

We applied the commonly used bacterial endotoxin lipopolysaccharide (LPS) to induce the inflammatory cascade and the drug Rosiglitazone (specific PPAR γ agonist) to induce PPAR γ activity in a 1st trimester placental explant culture model. We assessed the effects of LPS mediated inflammation and PPAR γ activity by (i) measuring the rate of apoptosis and proliferation in trophoblast cells (ii) evaluating expression of differentiation related markers (GCM1 and CG- β), and (iii) analyzing trophoblast function by studying invasion capacity.

Materials and Methods

Tissue collection

Human first trimester placental tissues (5-12 weeks) were obtained with written informed consent from healthy pregnant women following elective termination of pregnancy at the Michigan Family planning facility, Michigan, US and Morgentaler Clinic, Toronto, Canada. The

Institutional Review Board (IRB) of Wayne State University and Mount Sinai Hospital (MSH) Research Ethics Board approved all consent forms and protocols used in this study.

First trimester explant culture

The chorionic villi from 1st trimester human placenta were micro-dissected under a microscope into small pieces (20-30 mg wet weight) as previously described in (79) and cultured in 1ml of Dulbecco's Modified Eagle Medium – F12 (DMEM/F12) media, without phenol red (Life Technologies, CA) containing 10% Fetal bovine serum (FBS, Atlanta Biologicals, GA) and 1% Antibiotic-Antimycotic (Life Technologies, CA). The explants were treated in triplicates, by adding the respective treatments to the culture medium and then incubating at 8% O₂, 37°C for 24 hrs. After 24 hrs, the explants were weighed and snap frozen to be used later for either RNA extraction or protein analysis. One replicate was fixed in 4% paraformaldehyde (PFA, Fischer Scientific, MA) for 60 minutes and paraffin embedded to be used for immunohistochemistry. The media was frozen and used for ELISA.

Cell culture

The HTR-8/SVneo cytotrophoblast cell line (passage number 25-35) was cultured on plastic in T-75 tissue culture flasks (Corning) in DMEM/F12 media (Life Technologies, CA) containing 10% Fetal bovine serum (Atlanta Biologicals, GA) and 1% Antibiotic-Antimycotic (Life Technologies, CA) in a humidified incubator at 5% CO₂. Culture medium was replaced with serum-free medium prior to all treatments (305).

Explants and cell culture treatments

LPS (*Escherichia coli* 055: B5; Sigma-Aldrich, MO) was reconstituted in sterile 1X Phosphate buffered saline (PBS) (Life technologies, CA) and stored at -20°C. Cell or villous explant cultures were treated by supplementing culture medium with 1µg/ml LPS in presence or

absence of 10 μ M Rosiglitazone (Sellekchem, MA). Cells/explants treated with 1X PBS and 10 μ M Rosiglitazone were used as controls. The treatment groups will be identified as outlined in Table 3 in the rest of the chapter. In addition to these, HTR-8/SV Neo cells were also treated with 100nM 2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1) (Tocris, UK), a specific inhibitor for NF- κ B activity.

Table 3: Trophoblast explants/cell treatment groups and names.

Treatment	Group Name
LPS - 1 μ g/ml	LPS/Endotoxin
LPS - 1 μ g/ml + Rosiglitazone- 10 μ M	LPS + Rosi
1X PBS	Control
Rosiglitazone- 10 μ M	Rosi

RNA extraction and real time PCR

The explants/cells were lysed in 0.9 ml Qiazol (Qiagen, germany). Total RNA was extracted (RNAeasy Plus Universal Mini kit, Qiagen, Germany) and all samples were simultaneously reverse transcribed using the RT synthesis kit from Bio-Rad per the manufacturer's protocol (iScript Reverse Transcription Supermix, Bio-Rad Laboratories, CA). Real-time PCR was performed on the Bio-Rad CFX384 real time system in triplicates in 10 μ L total reaction volume containing 10 ng of template cDNA, 5 μ L of SYBR-green master mix (LuminoCT, Sigma-Aldrich, MO) and 500nM of primers. The primers used for assessing the expression levels of target and housekeeping genes are outlined in Table 4. Data was analyzed using the delta delta CT method as described in (251).

Table 4: List of primer sequences used for studying expression levels of trophoblast differentiation markers

Gene Name	Gene Symbol	Sequence
Cytochrome - C 1	<i>Cycl</i>	5'-CAT CAT CAA CAT CTT GAG CC-3'
		5'-CAG ATA GCC AAG GAT GTG TG-3'
Tyrosine 3-monooxygenase	<i>Ywh</i>	5'- CCG CCA GGA CAA ACC AGT AT -3'
		5'- ACT TTT GGT ACA TTG TGG CTT CAA -3'
TATA Box Binding Protein	<i>Tbp</i>	5'-CAC ATC ACA GCT CCC CAC CA-3'
		5'-TGC ACA GGA GCC AAG AGT GAA-3'
Glial cell missing 1	<i>Gcm1</i>	5'-TGA ACA CAG CAC CTT CCT C-3'
		5'-CCA CTG TAA CTA CCA GGC AAT-3'
Human chorionic gonadotropin - beta	<i>CG-β</i>	5'-GGT TGA GGC TTC AGT CCA G-3'
		5'-AGG GAG TAG GGT GTA GGA AG-3'

Protein extraction and western blotting

Total proteins were extracted from the explants by homogenizing the explants in lysis buffer containing: 1% (w/v) SDS, 50mM Tris, pH 6.8 and 10mM NEM followed by heating the lysate at 100⁰C for 10 mins. The lysates were then spun at 10,000 rpm for 15 mins and the supernatant was collected. Protein concentration was then determined using Pierce[®] 660 nm protein assay reagent (Thermo Scientific, MA), per manufacturer's instructions. Purified protein (30mg) was separated on 12% SDS-polyacrylamide gel (TGX Stain Free Fastcast Acrylamide kit, Bio-Rad Laboratories, CA) and transferred on PVDF membrane (Bio-Rad Laboratories, CA). Membranes were blocked with 5% skimmed milk in Tris-buffer saline (TBST) containing 0.1% (v/v) Tween for 1hr at room temperature (RT) and incubated over night at 4⁰C with the anti-GCM1 antibody (1:3000) (Aviva System Biology, UK). The membranes were washed with TBST and incubated with a HRP-conjugated secondary antibody (Cell signaling) for 1 hr. at RT. The antibody binding was detected using the Western Lightning[®] ECL Pro detection kit (Perkin Elmer, MA). Signals were visualized using a ChemiDoc Imaging System (Bio-Rad Laboratories, CA) and Image Lab V.5.1 software (Bio-Rad Laboratories, CA). Densities of immunoreactive bands were measured as arbitrary units by ImageJ software. Protein levels were normalized to a housekeeping protein β-actin (1: 20,000; Abcam, UK).

Enzyme Linked Immunosorbent Assay

The media collected from placental explant cultures was assayed for levels of secreted inflammatory cytokines – IL-6, IL-8, IL-1 β , TNF- α , CCL5 using the Duo Set ELISA development kits (R & D Systems) as per the manufacturers protocol. The levels of CG- β were assayed using the Beta-Human Chorionic Gonadotrophin (β -hCG), free (Human) - ELISA Kit (Phoenix Pharmaceuticals, CA) again as per the manufacturer's protocol. The optical density of the final colored reaction product was measured at 450nm using multispectral UV/VIS plate reader (Bio-Tek, VT). Standard curves were used to calculate protein in content in the samples. The level of proteins detected was divided by the weight of the explant to obtain the amount of protein secreted per milligram of explant tissue. The data was then normalized to control treatment to take sample to sample variations into account. The data was analyzed using the Graph pad prism 7.0 software.

Immunohistochemistry

Immunostainings of placental villi were performed as described before (306). Briefly, the sections were deparaffinized and rehydrated, followed by antigen retrieval using Dako Target retrieval solution (Agilent-DAKO, CA). The intrinsic peroxidase activity was then quenched by incubating the sections with 3% Hydrogen peroxide (Fisher Scientific, MA) for 30 mins at RT, followed by a wash with 1X PBS. The sections were then incubated overnight at 4°C with anti-PCNA (Santa Cruz, TX), or 10 μ g/ml nonimmune Rabbit IgG (Jackson Immunoresearch, PA) (used as a negative control). On the following day, the slides were washed 3 times (5 minutes/wash) with 1X PBS containing 0.1% Tween 20. The samples were then incubated for 30 min with a peroxidase-conjugated polymer coupled to anti-rabbit IgG (EnVision Systems Peroxidase, Agilent-DAKO, CA). The peroxidase was visualized with 3,3-diaminobenzidine

(DAB, Agilent-DAKO, CA) and hydrogen peroxide for 5 min. Tissues were counterstained with hematoxylin, dehydrated and were cover slipped. The staining was visualized using Nikon Eclipse 90i epifluorescence microscope (Nikon Inc., Japan) and the images were analyzed using ImageJ software.

HTR-8/SV neo cells were fixed with 4% PFA in 1X PBS for 10 mins at RT, washed 3 times with 1X PBS (5 mins/wash) and permeabilized with PBS containing 0.1% Triton-X100 for 10 mins. Cells were then incubated overnight at 4⁰C with various primary antibodies against: anti-Integrin alpha 1 (EMD Millipore, MA), and anti-Integrin alpha 6 (Cell Signaling Technologies, MA) or 10 µg/ml of nonimmune mouse serum (Jackson Immunoresearch, PA), the next day cells were washed 3 times with 1X PBS (5 mins/wash) and incubated with peroxidase-conjugated polymer coupled to anti-mouse IgG (EnVision Systems Peroxidase, Agilent - DAKO, CA). The peroxidase was visualized with 3,3-diaminobenzidine (DAB, Agilent - DAKO, CA) and hydrogen peroxide for 5 min. The cells were then imaged using the Leica DM IRB epifluorescence microscope (Leica, Germany), and images were captured using a Hamamatsu Orca digital camera (Hamamatsu Corp, Japan). All samples were stained similarly to avoid staining bias.

To quantify the staining intensity, monochromatic bright field images of the antibody/DAB stained cells were obtained at ×400. Before imaging the brightness was adjusted in a region of each slide devoid of tissue/cells by setting the gray level to 255. Using Simple PCI imaging software (Hamamatsu Corp. Japan), 5 random fields were imaged per well and the mean gray level was determined. The intensity from 5 images fields was averaged to obtain the mean intensity per well and the intensity from 3 wells was combined to get the average intensity per treatment.

Matrigel invasion assay

For placental explants, individual clusters of 6–8 week villi were dissected under a stereomicroscope and verified for the presence of extravillous trophoblasts (EVT's) on the villous tips. These clusters were cultured on Millicell-CM inserts (12-mm diameter, 0.4- μ m pores; EMD Millipore, MA) precoated with 0.2 mL of undiluted Matrigel (Corning, MI) in a 24-well culture plate for a total of 72 hours. The bottom chamber contained 300 μ L DMEM/F12 (Life Technologies, CA) without serum, medium supplemented with 10% Fetal bovine serum (Atlanta Biologicals, GA) and 1% Antibiotic-Antimycotic (Life Technologies, CA). The upper chamber contained approximately 200 μ L of the same medium. The explants were treated by supplementing the media with respective drugs. The explants were imaged every 24 hrs. for 72 hrs using Hamamatsu Orca digital camera (Hamamatsu Corp, Japan) and the outgrowths were measured using the ImageJ software. Each treatment was performed in duplicates for every tissue, and the experiment was repeated 3 times.

For assessing invasion of cell line, HTR-8/SV Neo cells (100,000) were cultured with treatments outlined in Table 3 on matrigel in 6.5mm Transwell inserts as previously reported in (305). Briefly, 15 μ L of undiluted growth factor reduced matrigel was added onto the membrane and the inserts were placed into 24-well culture plates and incubated at 37°C for 1 hr. for polymerization. After gel formation, the lower chamber was filled with 500 ml of serum-free DMEM/F12 medium and approximately 100,000 cells were cultured at 20% O₂ & 37°C for 24hrs on the matrigel in 100 μ L of medium. Cells that penetrated the matrigel and populated the lower chamber were detached using 500 μ L Trypsin-EDTA solution (Life technologies, CA). The number of invading cells in response to treatment was quantified by microscopic imaging of 5 random fields per well and averaging the number of cells counted per field. Each treatment was

performed in duplicates and the entire experiment was repeated 3 times. The calculation was performed by combining the average number of cells for each treatment across all experiments.

TUNEL assay

For histological evaluation and quantification of apoptosis in paraffin embedded sections DNA strand breaks were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL), using a fluorescein-based in situ cell death detection kit (Roche Applied Science, IN), per the manufacturer's instructions. Sections were imaged with a Nikon Eclipse 90i epifluorescence microscope (Nikon Inc., NY). The apoptotic trophoblast cells (TUNEL-positive nuclei) were counted at 200 \times from 5 random fields on each section from three samples for each treatment, along with the total number of nuclei (DAPI-labeled) to calculate the percentage of TUNEL/DAPI-labeled nuclei (TUNEL index). Sections subjected to treatment without TdT were assessed as negative controls. The calculation was performed by averaging counts for four fields of each specimen from duplicate samples of at least three independent experiments.

Results

Rosiglitazone reduced endotoxin induced cytokine secretion

The changes in inflammatory cytokines expression due to endotoxin exposure were quantified by ELISA. Exposure to endotoxin upregulated the secretion of inflammatory cytokines. The explants from the LPS group had significantly elevated levels of TNF- α (≥ 3.0 fold, $p=0.034$), RANTES (≥ 2.7 fold $p=0.013$), IL-8 (≥ 1.4 fold, $p=0.034$) and IL-1 β (≥ 2.0 fold, $p=0.042$) when compared to the PBS treated control explants (Figure 12 A-D). Rosiglitazone treatment reduced this secretion and explants from the LPS+Rosi group had significantly reduced secretion of TNF- α ($p=0.009$), RANTES ($p=0.0134$), IL-8 ($p=0.039$) and IL-1 β ($p=0.016$) when

compared to the LPS treated explants. LPS treatment significantly upregulated the expression of anti-inflammatory cytokine IL-10 (≥ 1.8 fold, $p=0.0005$) which was reduced by Rosiglitazone presence (LPS + Rosi group, $p=0.0007$) (Figure 12-E).

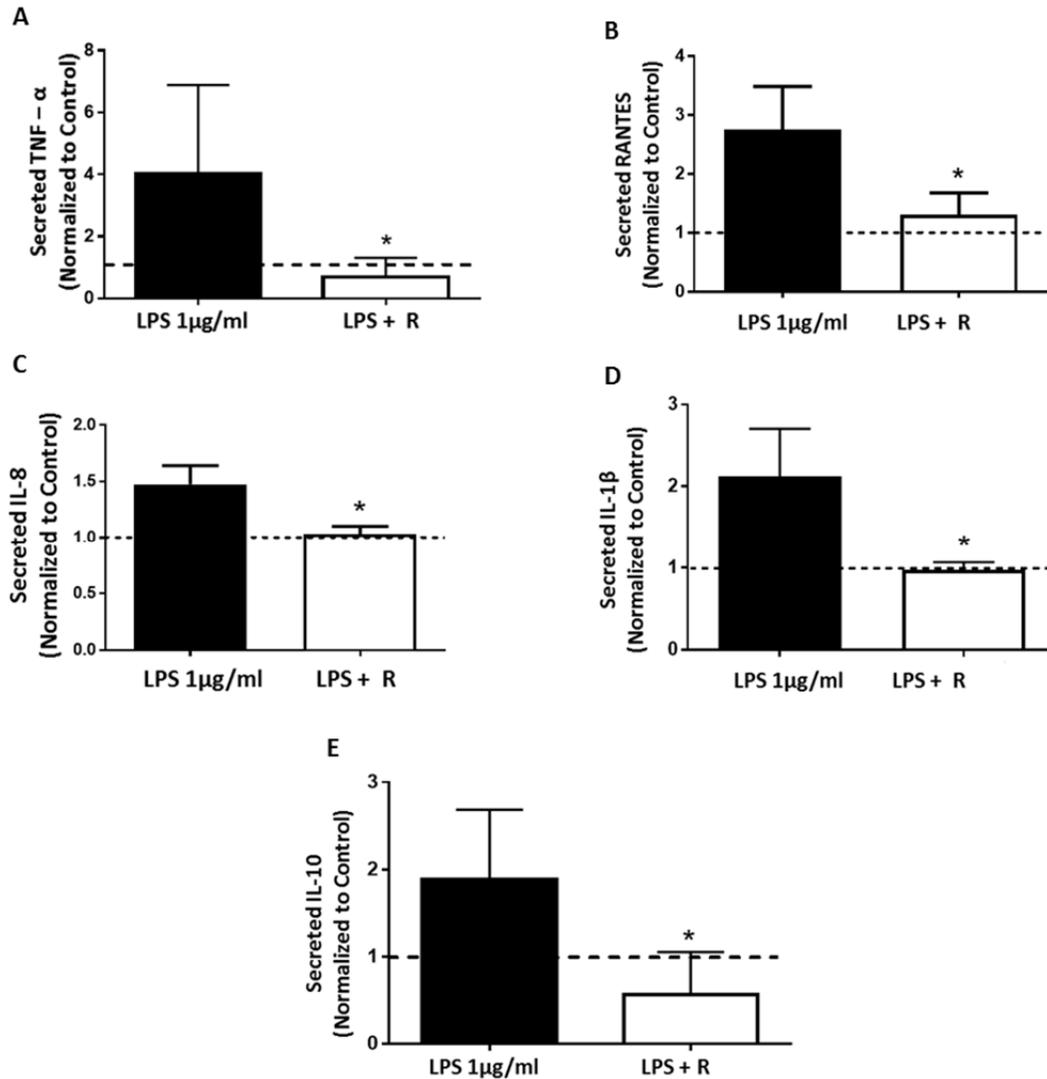


Figure 12: Rosiglitazone treatment reduced the inflammatory cytokine secretion in first trimester human placental explants. Cytokines secretion by first trimester human placental explants post 24hr LPS+/- Rosiglitazone treatment was assessed by ELISA. Rosiglitazone treatment significantly downregulated the secretion of inflammatory cytokines (A) TNF- α , (B) RANTES, (C) IL-8, (D) IL-1 β and (E) IL-10. $n \geq 12$, '*' indicates significance at $p < 0.05$ when compared to the LPS treated group.

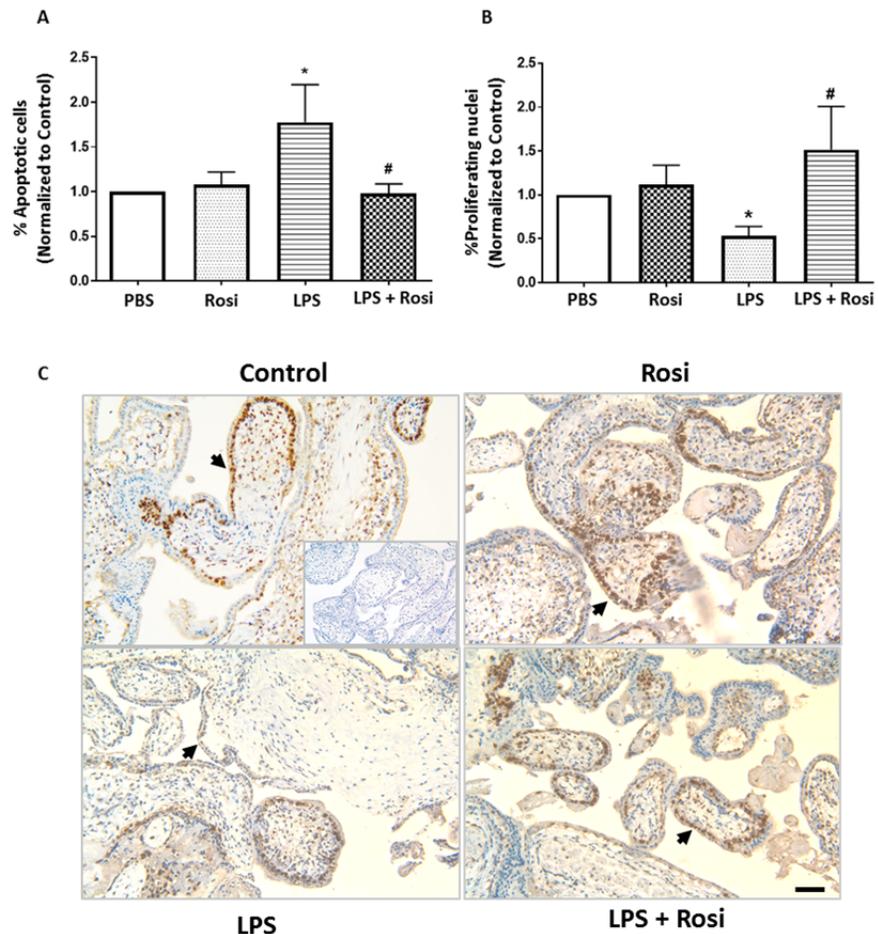
Rosiglitazone reduced endotoxin induced apoptosis and restored proliferation

Pro-inflammatory cytokines have been shown to have effects on proliferation and cell

apoptosis in hematopoietic and mesangial cells (307, 308). To evaluate if endotoxin induced inflammatory cytokines had similar effects in trophoblast cells / placental tissue we assessed the rate of apoptosis – using a TUNEL assay. The endotoxin treated explants (LPS group) showed a significantly higher percentage of apoptotic trophoblast nuclei (>1.7 fold, $p=0.02$) when compared to the PBS treated control explants. The percentage of apoptotic nuclei in explants treated with LPS + Rosi was significantly lower when compared to the only endotoxin/LPS group ($p=0.03$) and comparable to the PBS control group (Figure 13A). The percentage of apoptotic nuclei between the Rosi and PBS control groups was not significantly different (Figure 13A).

Figure 13: Rosiglitazone treatment reduced endotoxin induced apoptosis and increased proliferation.

(A) Percentage of apoptotic trophoblast cells identified by TUNEL staining and (B) Percentage of proliferating trophoblast nuclei after 24 hrs of treatment. (C) Representative images for positive PCNA staining across the 4 treatment groups, positive nuclei identified by brown color due to oxidation of DAB (black arrowheads). Inset shows the IgG control $n=4-8$, ‘*’ indicates significance at $p<0.05$ when compared to the PBS control group, ‘#’ indicates significance at $p<0.05$ when compared to the LPS treated group. Scale bar: $50\mu\text{m}$.



The rate of proliferation in treated tissues was assessed by counting the trophoblast nuclei

stained positive for PCNA antigen over the total number of nuclei. PCNA is an auxiliary protein involved in DNA replication and used as a marker for cell proliferation (309). The explants treated with endotoxin (LPS group) had significantly lower number of positively stained trophoblast nuclei (lowered to $\leq 50\%$, $p=0.01$) when compared to the PBS control group. Treatment with Rosiglitazone increased the rate of proliferation when compared to both the endotoxin treated explants and PBS control explants. The values reached statistical significance for the comparison between LPS and LPS + Rosi groups ($p=0.02$) (Figure 13B and C). The rate of proliferation between the Rosi and PBS control groups was comparable (Figure 13B and C).

Rosiglitazone reversed endotoxin mediated reduction in trophoblast differentiation marker expression

To assess the effects of endotoxin on trophoblast differentiation, we assessed the expression of GCM1 and CG- β – both have been shown to be crucial for trophoblast differentiation (78, 310). Exposure to endotoxin significantly reduced the expression of both *Gcm1* mRNA (~40% reduction, $p=0.04$) as assessed by qPCR and protein (~30% reduction, $p=0.01$) as assessed by western blotting (Figure 14A, B). Activation of PPAR γ via Rosiglitazone reversed this reduction and significantly induced expression of GCM1 at both mRNA ($p=0.01$) and protein levels ($p=0.05$) (Figure 12A, B).

For CG- β , the endotoxin mediated downregulation was not significant on the mRNA level (~40% reduction, $p=0.06$), but achieved significance at the protein levels secreted in the media (~40% reduction, $p=0.009$) as quantified by ELISA (Figure 14C, D). PPAR γ activation did not significantly upregulate mRNA expression of CG- β , however, secretion of CG- β (≥ 2.5 fold over LPS group, $p=0.002$) in the media was significantly upregulated (Figure 14C, D). Interestingly, treatment with Rosiglitazone (Rosi group) also significantly induced protein expression of both GCM1 (1.3 fold over PBS controls, $p=0.03$) and CG- β (1.6 fold over PBS

controls, $p=0.002$) (Figure 14A - D).

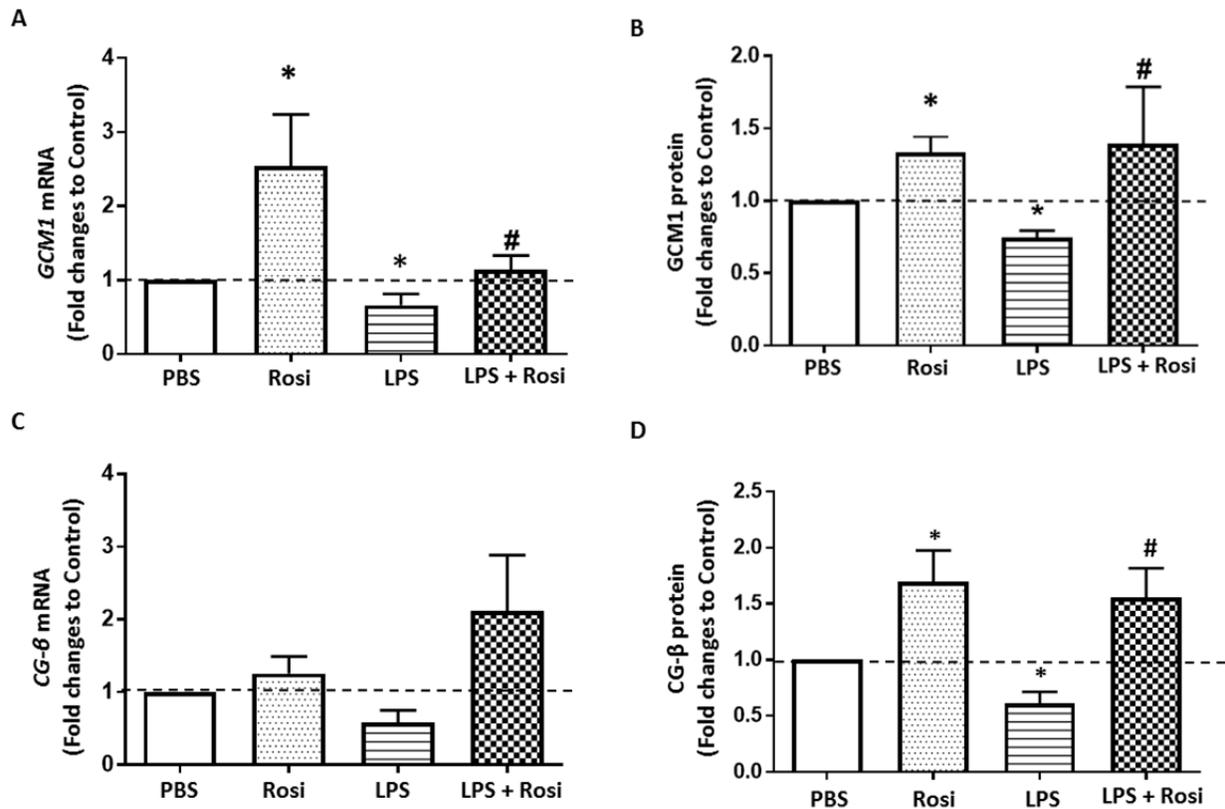


Figure 14: Rosiglitazone reversed endotoxin mediated reduction in trophoblast differentiation marker expression. Expression of trophoblast differentiation markers GCM1 and CG-β was assessed at mRNA level by qPCR (A, C) and protein level using ELISA and western blotting (B, D). Endotoxin exposure downregulated expression of both mRNA and protein levels of GCM1 and CG-β which were induced by Rosiglitazone treatment. $n=4-8$, '*' indicates significance at $p<0.05$ when compared to the PBS control group, '#' indicates significance at $p<0.05$ when compared to the LPS treated group.

Rosiglitazone reduced endotoxin induced trophoblast invasion to control levels

To determine the effects on trophoblast function, we assessed the effects of endotoxin exposure on trophoblast invasion in 1st trimester placental explants. Villous clusters with EVT tips were cultured on matrigel at 3% O₂, treated and the length of outgrowth monitored and quantified as described in the Methods section.

Endotoxin exposure induced invasion and the length of outgrowth into the matrigel 2.0 ± 0.4 (Mean \pm SD) was significantly higher when compared to the control group: 0.9 ± 0.2 ($p=0.0007$) (Figure 15A, B). In the presence of Rosiglitazone (LPS + Rosi group), the outgrowth

length in was maintained at a mean length: 1.0 ± 0.4 , comparable to that of the control group.

The mean outgrowth length in Rosi group was similar to the control group: 0.99 ± 0.2 .

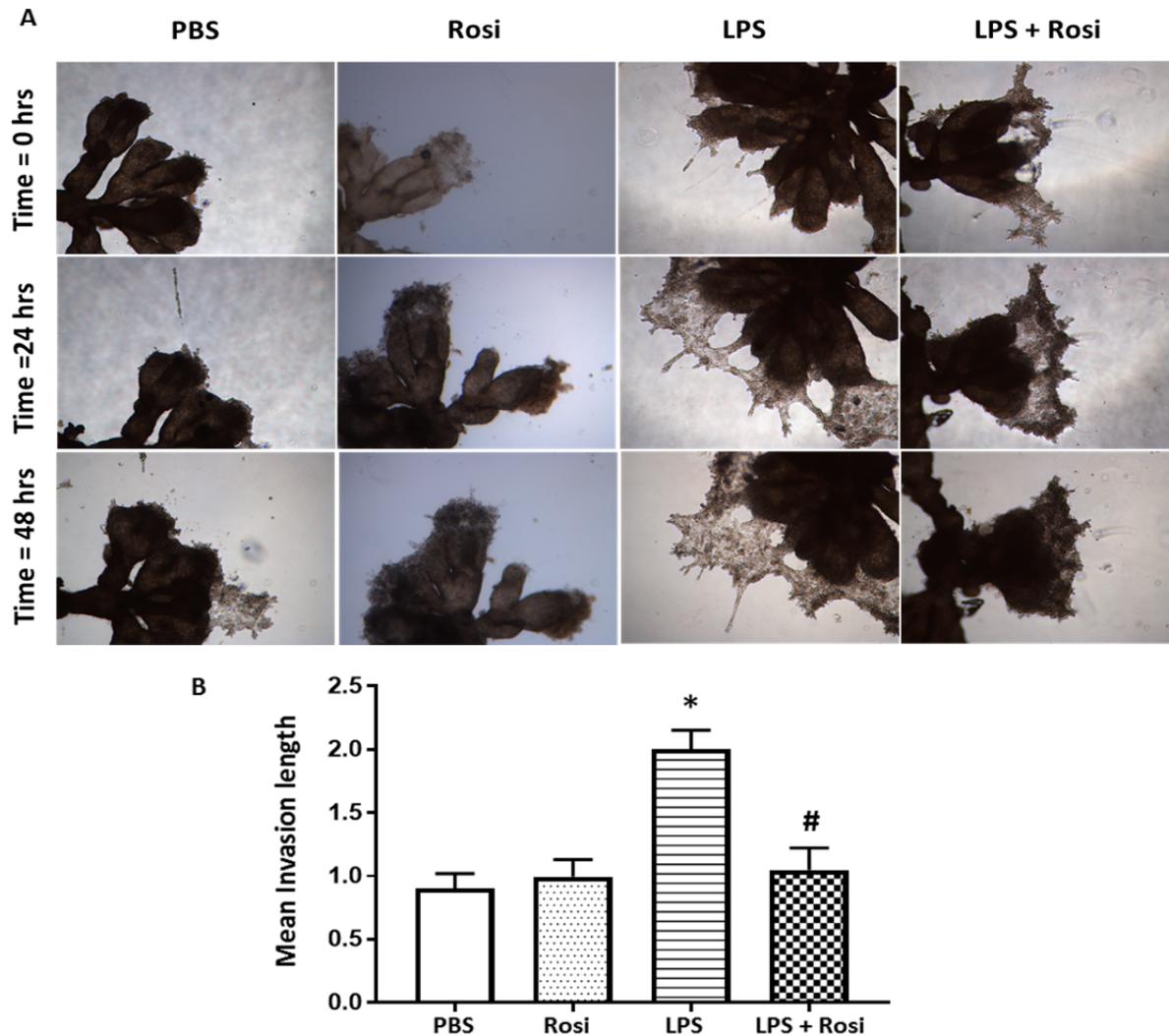


Figure 15: Endotoxin increased invasion in 1st trimester human placenta after 24 hrs of treatment. Villous clusters with EVT tips were cultured on matrigel and treated for a period of 48 hrs. (A) Representative images for mean length of invasion over the culture period. (B) Graph shows mean length of invasion in different groups measured after 24 hrs of treatment. $n = 3$, '*' indicates significance at $p < 0.05$ when compared to the PBS control group, '#' indicates significance at $p < 0.05$ when compared to the LPS treated group. Magnification: $\times 100$

Endotoxin exposure increased invasion in the trophoblast cell line HTR-8/SV neo

Endotoxin mediated increase in the length of trophoblast outgrowths observed in the treated explants was counterintuitive due to the decrease in expression of trophoblast

differentiation markers. Gestational age and oxygen concentration have been shown to play a critical role in trophoblast invasion (311) in 1st trimester placenta. To validate our observations on endotoxin mediated effects on invasion are independent of O₂ levels and gestational age, we used the trophoblast cell line HTR-8/SV neo.

Similar to the observations in explants, exposure to endotoxin significantly increased the number of HTR-8/SV Neo cells (12,974± 3128 (Mean± SD), p=0.01) invading into the matrigel, when compared to the PBS control group (5560 ± 3221) (Figure 14A, B). Presence of Rosiglitazone in the culture significantly lowered these numbers (L+R group: 7819 ± 1607, p=0.05) when compared to the LPS group (Figure 16A, B). The number of invading cells was comparable in the PBS, Rosi only and LPS+ Rosi groups.

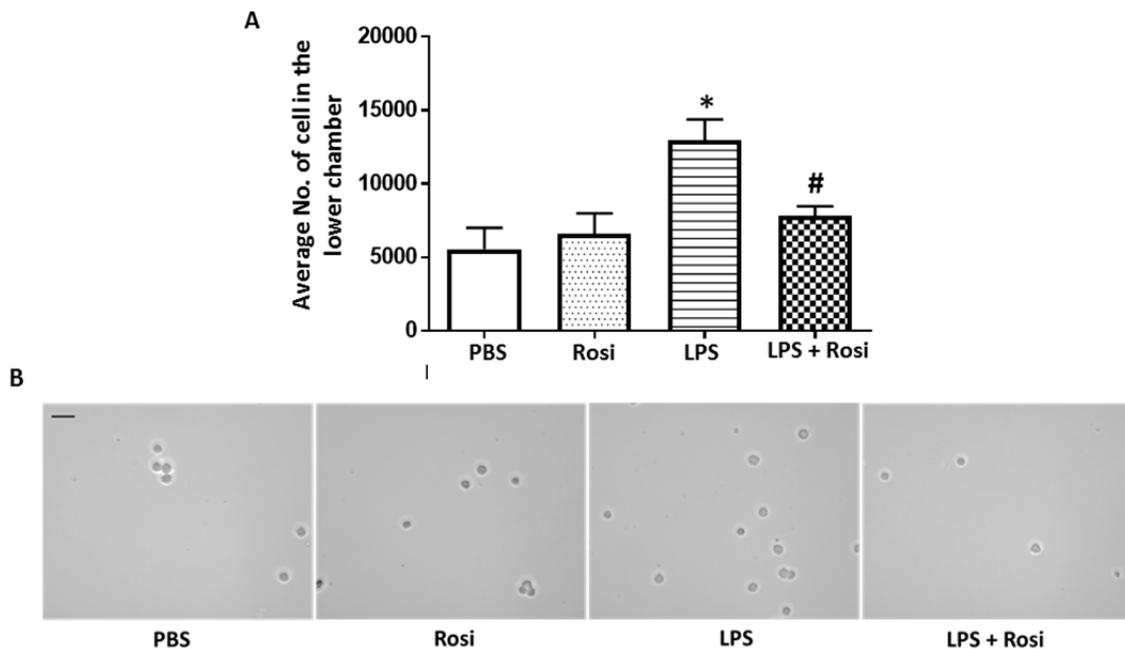


Figure 16: Rosiglitazone reduced endotoxin induced invasion in trophoblast cell line HTR- 8/SV Neo. Graph shows the average number of cells counted in the lower chamber in the matrigel invasion assay with HTR-8/SV Neo cells. Treatment with LPS significantly increased number of cells in the lower chamber which reduced when treated with Rosiglitazone. (B) Representative images shows cells in the lower chamber in wells with respectively labelled treatments. n=6, *' indicates significance at p<0.05 when compared to the PBS control group, '#' indicates significance at p<0.05 when compared to the LPS treated group. Scale bar: 50µm

Endotoxin exposure induced integrins switching in HTR-8/SV neo cells

Integrin switching is a physiological event during trophoblast invasion (312). The expression of integrin $\alpha 1$ expression is upregulated in differentiating and invasive trophoblast cells whereas, integrin $\alpha 6$ expression is restricted to noninvasive cytotrophoblasts (312). To further validate the effects on trophoblast invasion, the HTR-8/SV neo cells were stained with antibodies against integrins $\alpha 1$ and $\alpha 6$ (Figure 16C) and the staining intensity quantified.

HTR-8/SV neo cells treated with endotoxin had a significantly higher expression of integrin $\alpha 1$ (LPS v/s PBS: 29.4 ± 1.6 v/s 8.3 ± 1.4 arbitrary units (AU), (Mean \pm SD), $p < 0.0001$) and reduced expression of integrin $\alpha 6$ (LPS v/s PBS: 10.4 ± 2.0 v/s 38.3 ± 1.9 AU, $p < 0.0001$) when compared to the PBS controls (Figure 16D). Treatment with Rosiglitazone, prevented this switch and the LPS + Rosi group had significantly lower expression of integrin $\alpha 1$ (LPS + R v/s LPS: 8.6 ± 1.9 v/s 29.4 ± 1.6 AU, $p < 0.0001$) and higher expression of $\alpha 6$ (LPS + R v/s LPS: 33.1 ± 1.5 v/s 10.4 ± 2.0 AU, $p < 0.0001$) when compared to the LPS treated group. The expression of both integrin $\alpha 1$ and $\alpha 6$ in the LPS + Rosi, Rosi and PBS groups were comparable (Figure 16C and D). Additionally, we also assessed $\alpha 1$ and $\alpha 6$ staining intensity in cells treated with endotoxin and NF- κ B inhibitor. Treatment with NF- κ B inhibitor prevented the integrin switching in response to LPS exposure. The staining intensity of $\alpha 1$ in the LPS + NF- κ B inhibitor group was significantly lower (LPS + NF- κ B inhibitor v/s LPS: 10.3 ± 2.4 v/s 29.4 ± 1.6 AU, $p < 0.0001$), and $\alpha 6$ was significantly higher as compared to the LPS group (LPS + NF- κ B inhibitor v/s LPS: 48.7 ± 3.4 v/s 10.4 ± 2.0 AU, $p < 0.0001$)

Discussion

Inflammation and trophoblast differentiation are complementary processes that ensure successful placentation and pregnancy maintenance. The cytokines and growth factors secreted

by both the immune cells and trophoblast cells at the maternal fetal interface control the proliferative and invasive capacity of trophoblasts (19, 277). The trophoblast cells in turn engage in processes like selective expression of cell surface receptors and regulation of local immune cell activation that protect the fetus from rejection (130, 275, 276). Failure to do so has been associated with a wide spectrum of pregnancy related disorders (289, 290, 296).

Several studies have evaluated the molecular pathways involved in the inflammatory response of the trophoblast cells in response to infection and/or inflammatory stimuli (288, 302). However, the effects of inflammation on trophoblast differentiation have not been assessed in great detail. Studying the effects of inflammation on trophoblast differentiation will help to better understand disorders such as PE, IUGR and PTB. All of the latter are known to be associated with increased inflammation and abnormal trophoblast differentiation (293, 294, 313).

A protein involved in trophoblast differentiation is the transcription factor PPAR γ (150, 185). Interestingly, PPAR γ also has a known anti-inflammatory action and its activation was recently shown prevents PTB and reduces inflammation in the mouse model of endotoxin induced PTB. However its anti-inflammatory role in 1st trimester human placenta has not been explored. In the current study we assessed the effects of LPS on trophoblast inflammatory response and differentiation and the potential role of PPAR γ activation in reversing these effects.

Here, exposure of 1st trimester placental explants to LPS (1 μ g/ml) for 24 hrs significantly induced the secretion of inflammatory cytokines - TNF- α , IL-1 β , IL-8, and RANTES - similar to the observations made in other cell culture models (288, 302). Additionally, we observed a significant increase in secretion of the anti-inflammatory cytokine IL-10 post endotoxin exposure. LPS mediated induction in IL-10 expression was shown in human alveolar macrophages, kupffer cells and blood monocytes (314, 315). Gniesinger *et al* reported a similar

induction of IL-10 in primary trophoblasts isolated from term placentas (316). Upregulation of IL-10 secretion in response to endotoxin is interpreted as a defense mechanism against the ensuing inflammatory damage (317-321). We suggest a similar mechanism for IL-10 upregulation in our study. Activation of PPAR γ via Rosiglitazone significantly reduced the secretion of TNF- α , IL-1 β , IL-8, and RANTES - cytokines (Figure 12 A-D) including IL-10, confirming its anti-inflammatory action in 1st trimester human placenta.

PPAR γ activation by Rosiglitazone has been reported to exert its anti-inflammatory action via transrepression NF- κ B activity in several non-pregnancy related animal and human models of diseases (146, 199, 212). Our group recently validated the anti-inflammatory action of PPAR γ at systemic and local levels by repressing NF- κ B activity in a mouse model of PTB (235, 304). In the current study, we observed a significant reduction in TNF- α and RANTES - specific targets of the NF- κ B pathway (Figure 12 A, D). Based on the evidence present in literature and our previous observations, we speculate a similar mechanism to be present in the human first trimester placenta.

We further studied the effects of endotoxin exposure on apoptosis and proliferation specifically on the trophoblast cells in cultured explants. LPS exposure has been shown to induce apoptosis and reduce proliferation in immune cells (322). Studies with LPS and isolated primary trophoblast cells reported increase in apoptosis post three days of exposure and no effects on proliferation (288, 323). Exposure to high concentrations of recombinant inflammatory proteins - TNF- α and IFN- γ - was however reported to increase apoptosis and block proliferation in trophoblast cell line JEG-3, whereas IL-8 exposure was shown to increase proliferation in HTR-8SV/Neo cells (297, 324, 325). In our studies, we observed a significant increase in apoptosis and reduction in proliferation in trophoblast cells post 24 hrs of endotoxin

exposure suggesting a negative effect on cell viability (Figure 13). In the explants treated with LPS + Rosiglitazone, both apoptosis and proliferation rates were restored to control levels (Figure 13 A-D). Further, the levels of both apoptosis and proliferation in the explants from the Rosi only group were comparable to the PBS treated controls (Figure 13 A, C). These results suggest that in the placenta, PPAR γ mediates the effects on trophoblast apoptosis and proliferation indirectly, possibly via a downregulation of the inflammation cascade.

The proliferative capacity in the trophoblast cells is coupled to differentiation with reduction in proliferation as cells differentiate (326). To determine if the reduction in proliferation observed in our study affected trophoblast differentiation, we assayed the expression of Glial cell missing 1 (GCM1) and chorionic gonadotropin – beta (CG- β) as proxies for this process. GCM1 is a transcription factor reported to be crucial for terminal differentiation of trophoblast cells and consequently for maintaining a balance between trophoblast proliferation and differentiation (78). CG- β is a glycoprotein hormone secreted by and involved in differentiation of trophoblast cells (310, 327). Contrary to our expectations, exposure to endotoxin significantly downregulated expression of both GCM1 and CG- β (Figure 14A-D). Treatment with Rosiglitazone stimulated expression of both proteins and expression in LPS + Rosi group was significantly higher over the endotoxin treated group. The expression of GCM1 and CG- β in the Rosi only group was also significantly higher over the PBS treated control. PPAR γ mediated upregulation in GCM1 was previously reported by our group in the BeWo trophoblast cell line, whereas upregulation in CG- β was reported by us as well as other groups (69, 150). Endotoxin mediated downregulation in GCM1 protein has never been reported and expression of CG- β was shown to be unaffected (328). Further, since PPAR γ activation upregulated the expression of both targets irrespective of the endotoxin mediated

downregulation, we suggest that LPS and PPAR γ regulate expression of GCM1 and CG- β by independent mechanisms. Interestingly, decreased placental expression of GCM1 and lower CG- β levels were reported in pre-eclamptic women (77, 329). Endotoxin mediated decrease in GCM1 and CG- β observed in our study might explain the relationship between increased inflammation and lowered differentiation marker expression observed in PE.

Both GCM1 and CG- β have been shown to affect trophoblast function - specifically trophoblast invasion capacity (78, 327). GCM1 knockdown in 1st trimester human explants significantly inhibited invasion (78). Whereas contradictory results have been reported for the effect of CG- β on trophoblast invasion by other groups. It was reported to increase invasion in the trophoblast cell line JEG-3 whereas higher concentrations were shown to reduce invasion in isolated primary trophoblast cells (327, 330). More recently, Prast *et al* reported that CG- β stimulated trophoblast invasion in a culture system similar to ours (331). As endotoxin exposure affected both GCM1 and CG- β expression in our study, we evaluated if this reduction resulted in any perturbations in trophoblast invasion. Surprisingly, we observed that endotoxin treated explants displayed significantly higher invasion capacity (longer mean outgrowth length) when compared to the PBS treated controls. This was restricted to control levels in explants treated with Rosiglitazone (LPS + Rosi group). Mean invasion length in explants from the Rosi only group was also comparable to the PBS control group. Previous studies evaluating effects of endotoxin on invasion have reported a decrease in invasion in isolated primary trophoblast cell model (288, 302). While the contrasting results may be attributed to differences in cell models used for the studies, we asked if these discrepancies were due to differences in gestational age of the tissue and the O₂ tension used for the matrigel invasion experiments. The experimental setup for the invasion assay was different from the one used for previously discussed experiments in

two ways: (i) it used early first trimester tissue (5-7 weeks v/s the 10-12 weeks of gestation age), as at an older gestation age the (invading EVT cells are rare and) invasion capacity is potentially reduced (ii) it used a lower O₂ tension (3% v/s the 8%) as it is considered to be more reflective of the physiological O₂ concentration at 5-7 weeks of gestation (332). Both these factors have been shown to impact trophoblast cell invasion (311, 333, 334). To confirm our observations on endotoxin mediated effects on trophoblast invasion are independent of age and O₂ tension, we repeated the matrigel invasion assay using the HTR-8/SV neo trophoblast cell line. Similar to the effects observed in placental explants, exposure to endotoxin significantly induced invasion in HTR-8/SV neo cells, which was reduced to control levels when treated with Rosiglitazone (LPS+ Rosi, Rosi group) (Figure 16). To further validate our results and confirm induction of an invasive phenotype, we performed immunostaining for the integrins $\alpha 1$, $\alpha 6$ and quantified their expression in HTR-8/SV neo cells. Trophoblast cells undergoing differentiation towards the invasive phenotype have been shown to temporally and spatially switch their cell surface integrin expression from integrin $\alpha 6$ to integrin $\alpha 1$ (312). We observed that the changes in $\alpha 1$ and $\alpha 6$ expression were in accordance with our invasion results. Endotoxin exposure caused the HTR-8/SV neo cells to significantly upregulate $\alpha 1$ expression while simultaneously downregulating $\alpha 6$ compared to the controls (Figure 17). Rosiglitazone treatment prevented this switch and expression levels in the LPS + Rosi and Rosi groups were comparable to the ones in PBS treated control groups i.e. significantly low $\alpha 1$ and high $\alpha 6$ expression. Previous reports assessing the effects of PPAR γ activation on trophoblast cells invasion have reported negative effects of the transcription factor (335-337). However, these studies were performed in isolated primary trophoblast cells cultured at 5% O₂ tension. We suggest that differences in the cell model and culture conditions might be the confounding factors for the contrasting observations made in the

current study. Also, since Rosiglitazone treatment alone did not inhibit/reduce invasion, we concluded that the inhibitory activity of PPAR γ does not directly affect trophoblast invasion and acts via regulation of inflammatory pathways triggered by LPS.

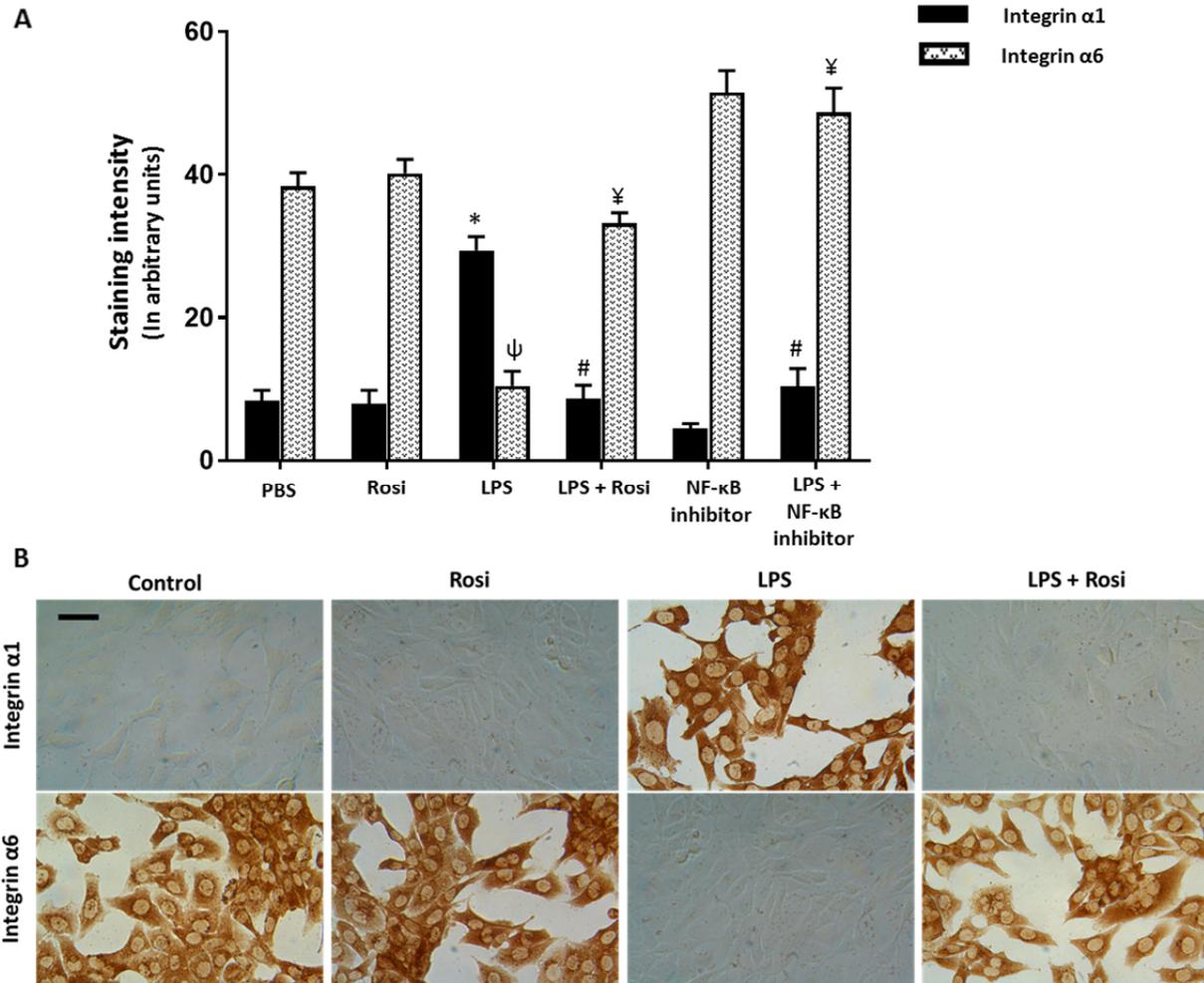


Figure 17: Rosiglitazone prevented endotoxin mediated integrin switching in trophoblast cell line HTR- 8/SV Neo. HTR-8/SV Neo cells were stained for assessing the expression of integrins $\alpha 1$ and $\alpha 6$. (A) Graph shows mean staining intensity for $\alpha 1$ and $\alpha 6$ across groups. (B) Representative images showing $\alpha 1$ and $\alpha 6$ integrin staining across different treatments, showing the integrin switch in the LPS group which was prevented in the LPS + Rosi and LPS+ NF- κ B inhibitor group. n=6, ‘*’, ‘ ψ ’ indicates significance at p<0.05 when compared to the PBS control group for $\alpha 1$ and $\alpha 6$ respectively, ‘#’, ‘ \yen ’ indicates significance at p<0.05 when compared to the LPS treated group for $\alpha 1$ and $\alpha 6$ respectively. Scale bar: 50 μ m.

The inflammatory action of LPS is triggered by binding to its specific receptor Toll like receptor – 4 (TLR4) and downstream activation of the the inflammatory transcription factor NF- κ B (338, 339). To determine if the effects on trophoblast invasion were mediated via this

pathway, we treated HTR cells with LPS +/- TPCA, a specific inhibitor of NF- κ B activity and assessed for induction of invasive phenotype. We observed that in presence of NF- κ B inhibitor, the trophoblast cells failed to switch integrin subtype (Figure 17) suggesting that the LPS driven differentiation towards the invasive phenotype is mediated by activation of NF- κ B. We also qualitatively confirmed this pathway in our explant culture model by matrigel invasion assay. Additionally, Rosiglitazone via PPAR γ activation has been shown to repress the NF- κ B activity, which would further explain Rosiglitazone mediated reduction in trophoblast invasion post endotoxin treatment (100).

To summarize, in the current study we report that endotoxin exposure in 1st trimester human placenta (i) causes inflammatory cytokine expression, (ii) induces trophoblast cell apoptosis, (iii) reduces trophoblast cell proliferation, (iv) downregulates expression of trophoblast differentiation related proteins and (v) increases trophoblast invasion via activation of NF- κ B. Activation of PPAR γ via Rosiglitazone reduces the endotoxin mediated effects on inflammatory cytokine production, apoptosis, proliferation and invasion and further stimulates expression of trophoblast differentiation markers.

Limitations of Aim 2

The current study focusses on the inflammation mediated via activation of NF- κ B and its effects on trophoblast function. LPS treatment has been shown to induce inflammation by activation of transcription factors such as STAT and AP-1 in addition to NF- κ B (340, 341). Effects of inflammation due to activation of STAT and AP1 on trophoblast differentiation would further help in understanding the link between inflammation and placental function. The current study also outlines the anti-inflammatory action of PPAR γ in the placenta and its role in reversing inflammation mediated effects on trophoblast differentiation. However, PPAR γ has

been suggested to play a role in trophoblast differentiation beyond inflammation. Evaluating the molecular pathways governed by PPAR γ in trophoblast lineages will further help in understanding its role in placental physiology and disease.

CHAPTER 5 - PPAR γ REGULATES TROPHOBLAST DIFFERENTIATION VIA GLIAL CELL MISSING -1

Introduction

Peroxisome proliferator-activated receptor-gamma (PPAR γ) belongs to the nuclear hormone receptor superfamily. Apart from being involved in lipid metabolism, like its other two subtypes PPAR α and β , it was shown to be crucial for successful placentation. Barak *et al.* reported that PPAR γ knockouts die *in utero* due to gross placental and cardiovascular abnormalities (178). Replenishing PPAR γ expression in the extra-embryonic lineages of PPAR γ null mutants resulted in viable pups suggesting a critical role of PPAR γ in placental development (178). Further analysis of the PPAR γ null embryos revealed that these placentas had a significantly thicker layer of undifferentiated trophoblast cells and an under - developed placental vasculature. These findings suggested a dysregulation of the trophoblast differentiation pathway mediated by PPAR γ . Parast *et al.* using PPAR γ ^{-/-} murine trophoblast stem cells that re-introducing PPAR γ in these cells rescued differentiation of the syncytiotrophoblast and labyrinthine trophoblast – crucial components of mouse placental structure and vasculature - by upregulating the expression of Glial cell missing 1 (GCM1) (303). GCM1 is a transcription factor regulating differentiation of mouse and human trophoblast lineages (74). Functionally, the labyrinth trophoblast in the mouse placenta resembles the villous trophoblast in human placenta which also expresses GCM1 (76). Baczyk *et al.* reported that knockdown of GCM1 expression in 1st trimester human placenta altered trophoblast proliferation, syncytial fusion in villous trophoblast and inhibited invasion in extra-villous trophoblast cells – supporting its critical role in human trophoblast differentiation (78). Recently, Levytska *et al.* showed that activation of PPAR γ in the human choriocarcinoma trophoblast cell line – BeWo significantly upregulated GCM1 expression (150). Further, our studies in 1st trimester human placenta also showed

upregulation in GCM1 and its downstream target CG- β on treatment with Rosiglitazone at both transcript and protein levels (Figure 14, Chapter 4). *In-silico* analysis of GCM1 promoter identified 2 putative PPAR γ target sites 20bp and 211bp upstream of the transcription start site (Figure 19). Unpublished work from our laboratory using the electrophoretic mobility shift assay (EMSA) showed that PPAR γ can bind to both these sites in the GCM1 promoter. For the current study, *we hypothesized that in human trophoblast cells PPAR γ regulates GCM1 expression by binding to its specific target sites in the GCM1 promoter.*

Materials and Methods

Cell culture and treatments

The human choriocarcinoma cell line JEG-3 was cultured in Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 (1:1; DMEM/F12) media (Invitrogen, CA) containing 10% FBS and 1% Antibiotic-Antimycotic (Life Technologies, CA) in a humidified incubator at 5% CO₂. For the manipulation of PPAR γ activity, the cells were treated in duplicates with a specific PPAR γ antagonist SR-202 (TOCRIS, Bioscienc, UK) at 400 μ M concentration or the agonist Rosiglitazone at 50 μ M for 3hrs. At the end of 3hrs of incubation the cells from one replicate were lysed in Qiazol (Qiagen, Germany) for RNA extraction. The second replicate was used for chromatin immunoprecipitation to investigate the physical locations of the transcription factor. The experiment was repeated 4 times.

Chromatin Immunoprecipitation

After the respective treatments, the media was aspirated from the wells and cells were gently washed with 1ml ice cold PBS (Life Technologies, CA). The cells were then cross-linked by adding 4% formaldehyde solution (Thermo Scientific, MA) and placing on a shaker at RT for 6 mins. The crosslinking was stopped by adding 125mM glycine solution (Fisher

Scientific, MA) and cells were washed with 1ml cold PBS for 5 mins to remove the residual formaldehyde. The cells were then lysed in cell lysis buffer (10mM HEPES, 60mM KCl, 1mM EDTA, 1mM 1,4-dithiothreitol, 1mM PMSF, 0.075% NP-40, pH 7.6) for 3 mins and spun at 1500 rpm for 4 mins. The pellet was re-suspended in nuclei lysis buffer (10mM EDTA, 50mM Tris HCl, 1% SDS, pH 8.1) and incubated on ice for 30 mins to obtain chromatin. The resulting chromatin lysate was then sonicated on a focused ultra-sonicator (S220, Covaris, MA) to obtain fragments between 200-500bp. Post-sonication, the amount of DNA was directly quantified in the sonicated chromatin using a spectrophotometer (NanoDrop). For each treatment immunoprecipitation reactions were set up using anti- RNA Pol II CTD and anti- PPAR γ antibodies (Thermo Scientific, MA) and an aliquot was saved as input fraction. Each immunoprecipitation reaction contained ~5 μ g chromatin, 4 μ g respective antibody and 9 μ L Dynabeads Protein G magnetic beads (Invitrogen, MA) and was rotated overnight at 4 $^{\circ}$ C. On the second day, the beads were washed by rotating for 5mins at 4 $^{\circ}$ C. The bound DNA was eluted by adding 40 μ L Chelex beads (Bio-Rad Laboratories, CA) and heating at 100 $^{\circ}$ C for 10 mins followed by reverse crosslinking by adding 2 μ L Proteinase K and incubating at 55 $^{\circ}$ C for 60 mins and RNase treatment for 30 mins at 37 $^{\circ}$ C. 3 μ L of the eluted DNA was used for qPCR analysis using the primer pairs: 5' -- CCAGGC TGCTCTTACCTATCC --- 3' & 5'-- TCTTACTGCTGGTTCAAGTCCC -- 3' for site I and 5'-- CTA CTGTGAATCGTCTGCCT -- 3' and 5'--TCTTCCCAGAATGCCAGCAA -- 3' for site 2 spanning the two PPAR γ target sites in the GCM1 promoter. The enrichment of the GCM1 promoter was calculated using the percent input method outlined in (342).

RNA extraction and qPCR analysis

The cells were lysed in Qiazol and RNA was extracted using RNeasy Plus Universal

Mini kit (Qiagen, Germany) as per the manufacturer's protocol. The extracted RNA was quantified using Nanodrop and 1µg was reverse transcribed using iScript RT synthesis kit (Bio-Rad Laboratories, CA). Real-time PCR was performed on the Bio-Rad CFX384 real time system in triplicates in 10uL total reaction volume containing 10 ng of template cDNA, 5µL of SYBR-green master mix (LuminoCT, Sigma-Aldrich, MO) and 500nM of primers. The primers used for assessing the expression levels of target and housekeeping genes are outlined in Table 5. Data was analyzed using the delta delta CT method as described in (251).

Table 5: Primer sequences used for gene expression analysis in JEG-3 cells.

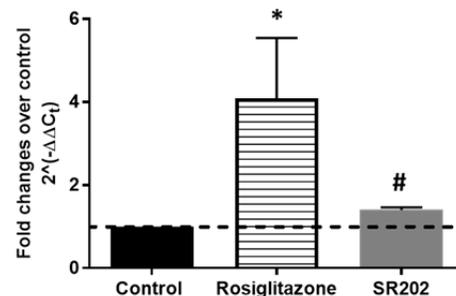
<u>Gene Name</u>	<u>Gene Symbol</u>	<u>Sequence</u>
Cytochrome - C 1	<i>Cycl</i>	5'-CAT CAT CAA CAT CTT GAG CC-3'
		5'-CAG ATA GCC AAG GAT GTG TG-3'
Tyrosine 3-monooxygenase	<i>Ywh</i>	5'- CCG CCA GGA CAA ACC AGT AT -3'
		5'- ACT TTT GGT ACA TTG TGG CTT CAA -3'
TATA Box Binding Protein	<i>Tbp</i>	5'-CAC ATC ACA GCT CCC CAC CA-3'
		5'-TGC ACA GGA GCC AAG AGT GAA-3'
Glial cell missing 1	<i>Gcm1</i>	5'-TGA ACA CAG CAC CTT CCT C-3'
		5'-CCA CTG TAA CTA CCA GGC AAT-3'

Results

Gcm1 expression is differentially regulated by PPAR γ agonist and antagonist

Similar to the observation reported by Levytska *et al.* Rosiglitazone treatment significantly induced *Gcm1* mRNA expression in JEG-3 cells (3.7 fold, p=0.01) when compared to the untreated controls (150). Cells treated with the PPAR γ antagonist SR202, had significantly lower *Gcm1* expression (1.4 fold, p= 0.05) as compared to the Rosiglitazone treatment. The expression of *Gcm1* was comparable between the untreated control cells and SR202 treated cells (Figure 18).

Figure 18: *Gcm1* expression correlates to PPAR γ activation status. Treatment with PPAR γ agonist Rosiglitazone significantly upregulated the expression of *Gcm1*, whereas treatment with the antagonist significantly reduced *Gcm1* expression. N=4, *: Significant V/s control at p<0.05, #: Significant v/s Rosiglitazone at p<0.05



PPAR γ and active RNA polymerase II preferentially bind to Site I in the GCM1 promoter to regulates its expression

The binding of transcription factors PPAR γ and active RNA Pol II to the GCM1 promoter was evaluated by performing chromatin immunoprecipitation assay. We observed that both RNA Pol II and PPAR γ precipitates were positively enriched for both target sites, I and II. Treatment with Rosiglitazone resulted in higher enrichment of the site I (2.7 ± 1.1 (Mean fold change \pm SD), $p=0.02$) when compared to its enrichment in untreated controls. The enrichment for target site II located -211bp for immunoprecipitation with PPAR γ antibody was $1.5 + 0.62$, but did not reach statistical significance (Figure 19 & 20A). For immunoprecipitation with RNA Pol II, the enrichment for site I was $2.2 + 1.2$ ($p=0.028$) and site II was $1.5 + 0.3$ when compared to the enrichment of respective sites in untreated controls.

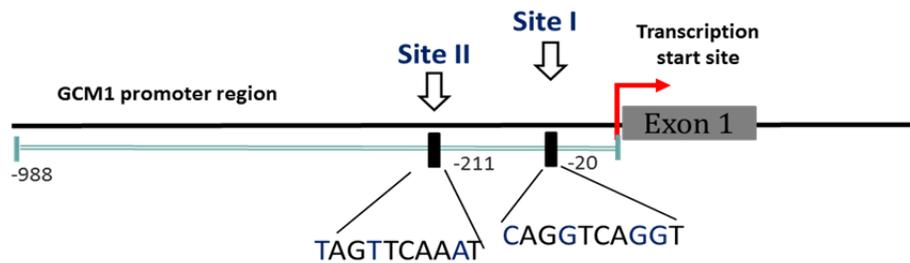


Figure 19: Illustration for putative PPAR γ binding sites in GCM1 promoter.

Further, we also observed that PPAR γ binding to the site I correlated to its activation status. Treatment with Rosiglitazone significantly increased the enrichment of the binding site I ($2.71 + 1.1$, $p=0.02$) when compared to the untreated controls. The enrichment of site I in the antagonist treated cells was lower ($0.39 + 0.2$, $p=0.026$) when compared to the untreated controls, however the values did not reach statistical significance (Figure 20B).

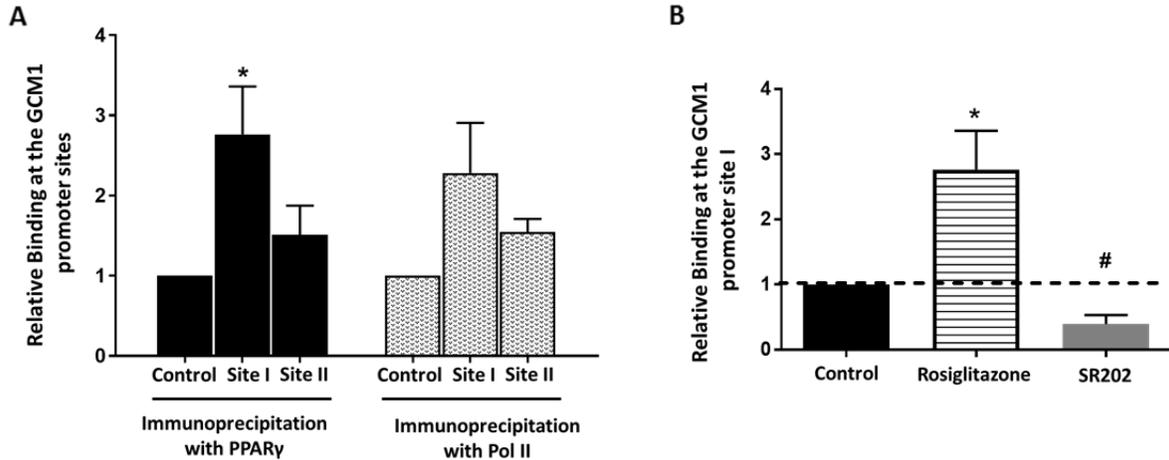


Figure 20: PPAR γ activation led to preferential binding and enrichment of the binding site I in GCM1 promoter. (A) PCR analysis of PPAR γ and Pol II immunoprecipitants showing significantly higher enrichment of the binding site I when compared to the untreated controls. (B) Treatment with Rosiglitazone significantly increased the enrichment of binding site I, which reduced significantly after treatment with the antagonist SR202. N=4, *: Significant V/s control at $p < 0.05$, #: Significant v/s Rosiglitazone at $p < 0.05$

Discussion

PPAR γ mediated upregulation of GCM1 expression was previously reported by Levytska *et al.* in the BeWo choriocarcinoma cell line (150). Our studies in 1st trimester villous explants showed a similar upregulation (Chapter 4, Figure 14A, B). *In silico* analysis revealed two putative PPAR γ binding sites in the GCM1 promoter. In this study, we aimed to extend our previous observations by showing that PPAR γ increases *Gcm1* expression by direct binding to its target sites in the GCM1 promoter region. We performed chromatin immunoprecipitation with PPAR γ and active RNA Pol II antibodies to evaluate if (i) PPAR γ physically binds to these target sites and (ii) PPAR γ binding recruit's RNA Pol II to the target sites for transcription.

The JEG-3 cells were treated with PPAR γ agonist – Rosiglitazone and antagonist – SR202 for 3 hrs. Successful modulation of PPAR γ activity post agonist/antagonist treatment was validated by assessing the expression of known PPAR γ target gene *Fabp4*. Like the observations made by Levytska *et al.*, treatment with Rosiglitazone significantly upregulated *Gcm1* expression confirming changes in PPAR γ activity due to agonist treatments in our

experimental set up (Figure 18). The chromatin immunoprecipitation assay further showed that PPAR γ and RNA Pol II bound to both target sites I and II in the GCM1 promoter however, post Rosiglitazone treatment the enrichment for site I was significantly higher compared to site II. This suggested that PPAR γ activation causes preferential binding of PPAR γ and recruitment of RNA Pol II to target site I. This also correlated with the increase in *Gcm1* expression observed in Figure 18. We also observed that treatment with PPAR γ antagonist SR202 significantly reduced enrichment of binding site I. However, we observed that the decreased in PPAR γ activity did not reduce the expression of *Gcm1* suggesting that in JEG-3 cells, the basal expression of *Gcm1* may be regulated by factors other than PPAR γ . Further, these observations were also contrary to the observations made by Levytska *et al.* in BeWo choriocarcinoma cell line further suggesting that trophoblast cell lines might differ in regulation of *Gcm1* expression and that results obtained should be interpreted with caution.

Based on previous data and the current study we report that upon activation, PPAR γ regulates GCM1 expression by preferentially binding to the specific target site I (-21bp upstream of transcription start site) in the GCM1 promoter in JEG-3 cells. Also, in combination with the data obtained by Levytska *et al* our data highlights the need for caution while interpreting molecular data from cancerous cell lines and calls for development of better models to study trophoblast differentiation.

CHAPTER 6 - DEVELOPMENT OF A CULTURE MODEL FOR SIMULTANEOUSLY STUDYING PPAR γ MEDIATED REGULATION IN EXTRA-VILLOUS AND VILLOUS TROPHOBLAST LINEAGE DIFFERENTIATION

Introduction

Abnormal trophoblast differentiation has been associated with pathologies like PE – affecting 5-8% pregnancies, IUGR – affecting 4-7% pregnancies, GDM – affecting 2-5% pregnancies and PTB – that accounted for 9.5% live births in 2015 (61, 62, 343, 344). Understanding the molecular basics of placental development and function could provide insights into the etiologies of these disorders. Interestingly, abnormal expression of PPAR γ has also been reported in cases of IUGR with PE and IUGR (103, 104). PPAR γ expression was reported to be unaltered in women with PE however significantly lower levels of PPAR γ activators were reported in their serum (103, 105). These studies highlight a crucial role for PPAR γ activity in placental pathologies. However, we still lack an understanding of the molecular pathways regulated by PPAR γ in human placenta.

In-vitro studies focusing on PPAR γ activity in isolated 1st trimester human EVT's showed that treatment with PPAR γ agonists reduced invasion, suggesting its negative involvement in differentiation towards the EVT lineage (335, 337). However, a similar study with isolated villous trophoblast showed induction of differentiation on treatment with agonists (66). Handschuh *et al.* showed that PPAR γ activation had opposite effects on human chorionic gonadotropin (hCG) subunit expression patterns in the villous and extra-villous trophoblast (336). hCG is a critical hormone shown to be involved in proliferation and differentiation processes in both trophoblast lineages (310, 330). Differential regulation of hCG by PPAR γ suggests specialized role for the receptor in each lineage. The current study aimed to explore the differential role of PPAR γ in VT and EVT lineages. A critical limitation for this aim was availability of a suitable culture model. Most studies evaluating the role of PPAR γ in trophoblast

cells, rely on the use isolated primary trophoblast cells. Primary cell models, although generally helpful, have the common obstacle that they lack tissue integrity and tend to spontaneously differentiate (345, 346). We therefore aimed to develop a culture model using 1st trimester placental explants that would enable simultaneous assessment of both lineages. The data shown here outlines our preliminary work in which we (i) established the culture and treatment conditions to ensure maintenance of tissue viability (ii) confirmed the purity of isolated EVT fractions by assessing expression of specific markers - Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM 1) and Human Leukocyte Antigen-G (HLA-G).

Materials and Methods

Tissue collection

Human first trimester placental tissues (5-7 weeks) were obtained with written informed consent from healthy pregnant women following elective termination of pregnancy at the Michigan Family planning facility, Michigan, US and Morgentaler Clinic, Toronto, Canada. The Institutional Review Board (IRB) of Wayne State University and Mount Sinai Hospital (MSH) Research Ethics Board approved all consent forms and protocols used in this study.

Placental explant culture

Individual clusters of 5-7 week villi were dissected under a stereomicroscope and verified for the presence of extra-villous trophoblasts (EVT's) on the villous tips. These clusters were cultured on Millicell-CM inserts (12-mm diameter, 0.4- μ m pores; EMD Millipore, MA) pre-coated with 0.2 mL of undiluted growth factor phenol red -free Matrigel (Corning, MI). The matrigel containing inserts were placed in a 24-well culture plate. The bottom chamber was filled with 300 μ L DMEM/F12 (Life Technologies, CA) medium supplemented with 10% Fetal bovine serum (Atlanta Biologicals) and 1% Anti-Anti (Life Technologies, CA). The upper

chamber contained 200 μ L of the same medium. The explants were treated in duplicates by supplementing the media with either 10 μ M Rosiglitazone (Selleckchem, MA) or 1 μ M of T0070907. The explants were cultured at 3% O₂, 37°C for a total of 72 hrs. Explants that did not show visible outgrowth in the first 24 hrs were discarded. The culture media was replaced with fresh media (with respective treatments) every 24 hrs. The explants were imaged every 24 hrs to record the outgrowth using Hamamatsu Digital camera and the outgrowths were measured using the ImageJ software. Each treatment was performed in duplicates for every tissue and the entire experiment was conducted thrice. At the end of 72 hrs, one replicate was processed for RNA extraction and the other for immunohistochemistry.

Tissue dissection, RNA extraction and qPCR analysis

To dissect the explants for RNA extraction, media from both the lower chamber and Millicell-CM inserts was aspirated. The inserts were taken and the lower membrane holding the matrigel was carefully separated. The matrigel with the explants was then taken out and dissected under a stereomicroscope as outlined in Figure 21. The non-invaded part of the explant was considered as enriched VT fraction whereas the invaded part was collected as enriched EVT fraction. Both the VT and EVT fractions were lysed in Qiazol and processed for RNA extraction.

The explants/cells were lysed in 0.9 ml Qiazol (Qiagen, Germany). Total RNA was extracted (RNAeasy Plus Universal Mini kit, Qiagen, Germany) and all samples were reverse transcribed simultaneously using the RT synthesis kit from Bio-Rad per the manufacturer's protocol (iScript Reverse Transcription Supermix, Bio-Rad Laboratories, CA). Real-time PCR was performed on the Bio-Rad CFX384 real time system in triplicates in 10 μ L total reaction volume containing 5 ng of template cDNA, 5 μ L of SYBR-green master mix (LuminoCT, Sigma-

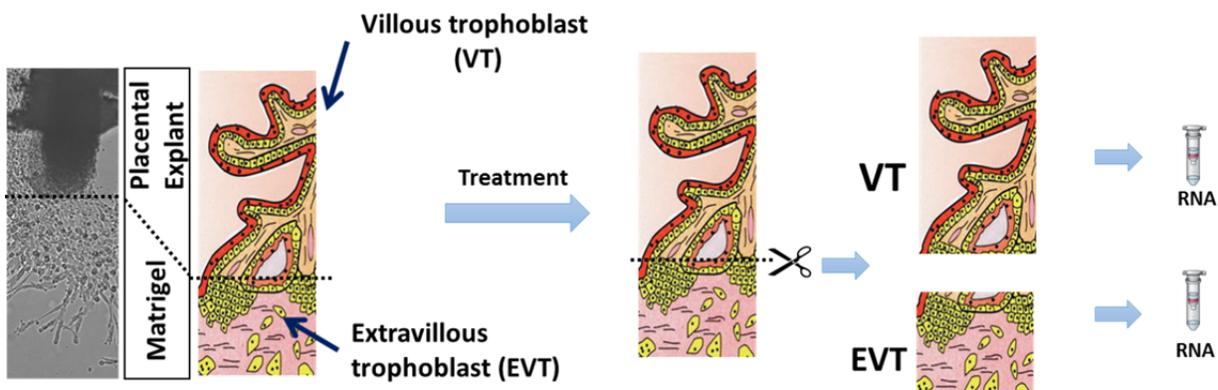


Figure 21: Illustration showing the separation of VT and EVT parts from cultured explants.

Aldrich, MO) and 500nM of primers. The primers used for assessing the expression levels of target and housekeeping genes are outlined in Table 6. Data was analyzed using the delta CT method as described in Drewlo 2011.

Table 6: List of primer sequences for gene expression analysis in invaded and floating villous trophoblast

Gene Name	Gene Symbol	Sequence
Cytochrome - C 1	<i>Cyc1</i>	5'-CAT CAT CAA CAT CTT GAG CC-3'
		5'-CAG ATA GCC AAG GAT GTG TG-3'
Tyrosine 3-monooxygenase	<i>Ywh</i>	5'- CCG CCA GGA CAA ACC AGT AT -3'
		5'- ACT TTT GGT ACA TTG TGG CTT CAA -3'
TATA Box Binding Protein	<i>Tbp</i>	5'-CAC ATC ACA GCT CCC CAC CA-3'
		5'-TGC ACA GGA GCC AAG AGT GAA-3'
Human Leukocyte Antigen-G	<i>Hla-G</i>	5'-GTG TGG TAC TTT GTC TTG AGG A-3
		5'-AGA GTA GCA GGA AGA GGG TT-3
Platelet And Endothelial Cell Adhesion Molecule 1	<i>Pecam 1</i>	5'-CAG GCC CCA TTG TTC CC-3
		5'-ATT GCT CTG GTC ACT TCT CC-3'

Immunohistochemistry

The explants were fixed (with the matrigel) by adding 4% paraformaldehyde for 10 mins. The explants were then dehydrated and paraffin embedded, sectioned at 5µm thickness and mounted onto glass slides. For immunostaining, the sections were rehydrated and deparaffinized, followed by antigen retrieval using Dako Target retrieval solution (Agilent, Dako, CA). The intrinsic peroxidase activity was then quenched by incubating the sections with 3% peroxide (Fischer Scientific, MA) for 30 mins at RT, followed by 1 wash with 1X PBS. The

sections were then incubated overnight at 4°C with anti-Ki67 antibody (Santa Cruz TX), and 10µg/ml nonimmune Rabbit IgG (Jackson ImmunoResearch, PA) (used as a negative control). On the next day, the slides were washed 3 times with 1X PBS containing 0.1% Tween 20 (5 minutes/wash). Then the slides were incubated with a peroxidase-conjugated polymer coupled to anti-rabbit IgG (EnVision Systems Peroxidase, Agilent, DAKO, CA) for 30 min. The peroxidase was visualized with a 1:1000 dilution of 3,3-diaminobenzidine (DAB, Agilent, DAKO, CA) in hydrogen peroxide for 3 min. The tissue was counterstained with hematoxylin, dehydrated and cover slipped. The staining was visualized using Nikon Eclipse 90i epifluorescence microscope (Nikon Inc, Japan).

Results

Cultured explants contained proliferating cells and were viable at 72 hrs of culture

We assessed the viability in the cultured explants by immunostaining for Ki67. The cells from both compartments, villous trophoblast (outside the matrigel) and extravillous trophoblast (invaded into the matrigel) stained positive for Ki67 indicating that the tissue still maintained proliferating cells (Figure 22).

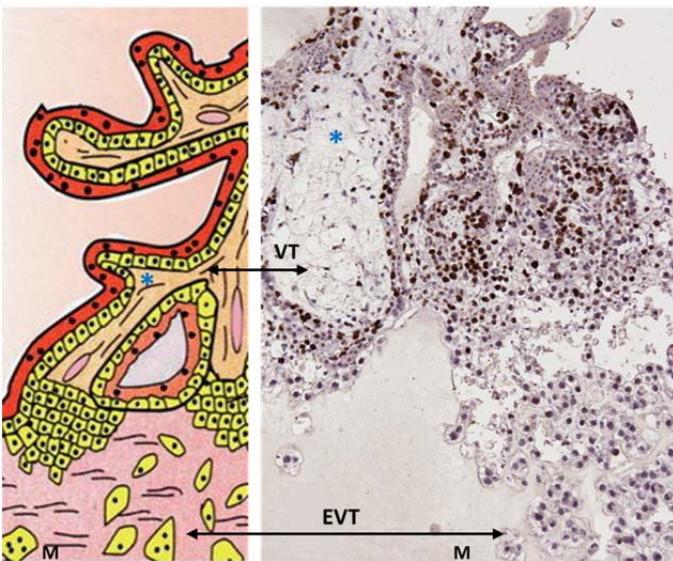


Figure 22: Immunohistochemistry for Ki-67 showed presence of proliferating cells in cultured explants. Representative image shows the VT and EVT structures in cultured explants. The explants stained positive for the proliferation marker Ki-67. ‘*’ stroma, ‘M’ matrigel.

The isolated EVT fractions enriched for EVT-specific markers

We assessed the expression of EVT markers, *Pecam1* and *Hla-G* in both our EVT and VT fractions. We observed that the EVT/matrigel fraction had significantly higher expression of both *Pecam1* and *Hla-g* when compared to the villous/floating fraction (Figure 23A & B).

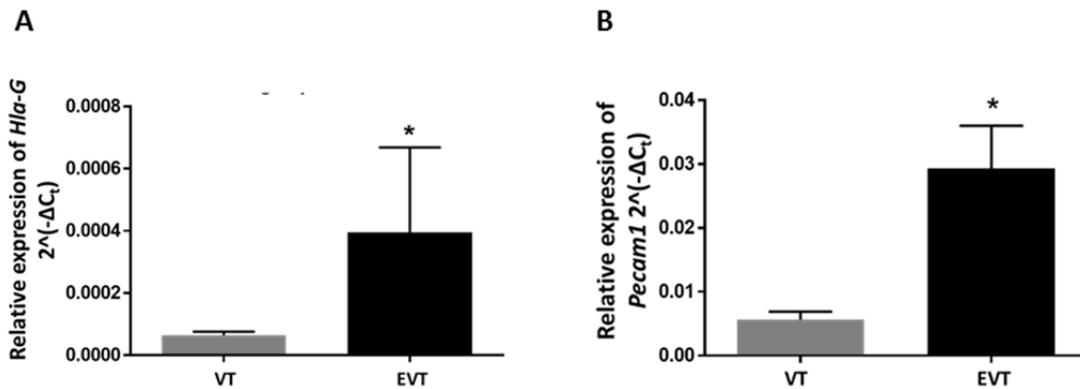


Figure 23: The extra-villous tissue fractions dissected out from matrigel inserts enriched for EVT markers. The graphs show expression of EVT markers (A) *Pecam 1* and (B) *Hla-g* in villous (VT) and extra-villous fraction isolated from matrigel cultures. The expression of both markers is significantly higher in EVT fractions. N=, *: Significant V/s VT at $p < 0.05$.

PPAR γ antagonist increased invasion

In preliminary experiments, we assessed the mean outgrowth length in the explants treated with PPAR γ agonist (Rosiglitazone) and antagonist (T0070907). The outgrowth index was calculated by taking a ratio of the length of outgrowth after 24hr of treatment over the length before treatment for each explant. We observed that after 24hrs of treatment, the outgrowth index in Rosiglitazone treated explants was 1.1 ± 0.31 (Mean \pm SD), similar to the 0.9 ± 0.3 in the control explants (Figure 24A and B). The outgrowth index in the T0070907 treated explants was higher (2.1 ± 1.4), however the values did not reach statistical significance.

Discussion

In the current study, we aimed to develop a culture model for simultaneous assessment of EVT and VT lineages. Currently used primary culture models suffer from the lack of tissue

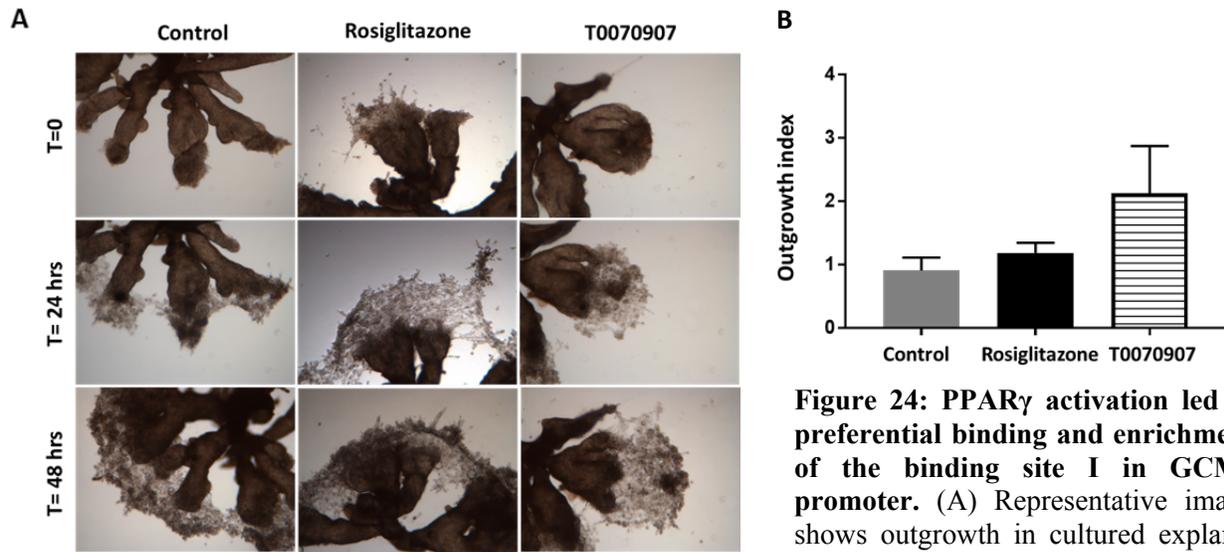


Figure 24: PPAR γ activation led to preferential binding and enrichment of the binding site I in GCM1 promoter. (A) Representative image shows outgrowth in cultured explants treated with Rosiglitazone and T0070907. (B) graph shows the mean outgrowth index in explants after Rosiglitazone and T0070907 treatments. N=3. Magnification X20

integrity and spontaneous differentiation in culture conditions (346). Here we cultured early first trimester (5-7 weeks of gestational age) villous clusters with visible EVT's at the tips on matrigel for 3 days. We observed that at the end of the culture period, the tissue still maintained its integrity and stained positive for proliferating cells marker Ki-67 (Figure 22). This suggests that the explants were still viable. We suggest a more comprehensive assessment such as analysis of apoptotic rate and estimating tissue necrosis would provide further insights into tissue viability. A crucial step in the current study was to ensure the precise dissection of explant structures to obtain the VT and EVT's with minimum cross-contamination. We assessed this by analyzing the mRNA expression of two EVT specific markers in both fractions – *Pecam1* and *Hla-g*. We observed that both *Pecam1* and *Hla-g* had significantly higher expression in the isolated EVT fraction when compared to the VT fraction. We suggest a similar analysis of the VT specific markers would further help ascertain the purity of isolated lineages. We also conducted preliminary experiments assessing the effects of PPAR γ agonist and antagonist on outgrowth in

cultured explants. We observed that while PPAR γ agonist did not significantly alter outgrowth, treatment with antagonist T0070907 increased it. Similar analysis in more explants would provide a concrete conclusion and more definitive answer for the effect of PPAR γ on EVT outgrowth. Performing gene expression analysis of EVT and VT fractions isolated after agonist/antagonist treatments would further help in understanding role of PPAR γ in differentiation of trophoblast lineages.

The current study thus provides the preliminary data towards establishment of a culture model for simultaneous assessment of EVT and VT lineages. Once completely established, the model can be used for assessment of molecular cross-talk and pathways involved in EVT/VT differentiation as well as placental development. Further, the model will also help in developing and screening therapeutics for intervention in placental dysfunction disorders.

Limitations of Aim 3

The current study provides proof for transcriptional regulation of GCM1 expression by PPAR γ and suggests that PPAR γ regulates trophoblast differentiation via GCM1. However further studies are needed to validate both these observations. Experiments involving site specific mutagenesis in GCM1 promoter would further verify the specificity of the PPAR γ binding at the GCM1 promoter sites. And GCM1 knockdown experiments would reveal if the effects of PPAR γ activation on trophoblast differentiation are GCM1 dependent. The current study also explored the role of PPAR γ in trophoblast differentiation beyond regulation of GCM1 expression. It provided preliminary data for development of a unique culture model that would enable simultaneous assessment of PPAR γ mediated regulation in EVT and VT lineages. Further development and analysis of the culture model is needed to validate its use for studying differentiation in trophoblast cell lineages.

APPENDIX A

IACUC Approval Letter



INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A3310-01

PROTOCOL # A 07-03-15

Protocol Effective Period: 8/26/2015 – 7/31/2018

AMENDMENT # 3

TO: Dr. Nardhy Gomez-Lopez
OB/GYN
275 E. Hancock

FROM: Lisa Anne Polin, Ph.D. 
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Amendment # 3 to Protocol # A 07-03-15
"Immunological mechanisms during term and preterm labor/birth"

DATE: September 1, 2015

The following requested changes to the above protocol have been reviewed and approved for immediate effect:

Request dated August 24, 2015:

To do further studies on preventative measures for preterm birth, since rosiglitazone, a PPAR γ agonist, suppresses the inflammatory signal transduction pathway initiated during preterm birth as described in the amendment request.

Mice, C57BL/6J, Females, 96, "D"

**To be purchased*

Mice, C57BL/6J, Male and Female Offspring, 576, "D"

**Bred in house*

This protocol, as amended, will be subject to annual review on the anniversary date of the initial IACUC review. This protocol was last reviewed on August 26, 2015.

APPENDIX B

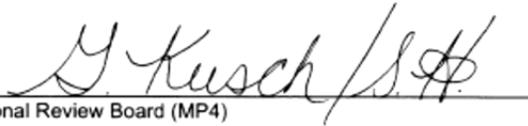
IRB Approval Letter

**WAYNE STATE
UNIVERSITY**

IRB Administration Office
87 East Canfield, Second Floor
Detroit, Michigan 48201
Phone: (313) 577-1628
FAX: (313) 993-7122
<http://irb.wayne.edu>

NOTICE OF EXPEDITED APPROVAL

To: David Armant
Obstetrics/Gynecology
339 Mott Center

From: Sabrina Heidemann, M.D. or designee 
To: Chairperson, Medical/Pediatric Institutional Review Board (MP4)

Date: August 03, 2015

RE: IRB #: 013515MP4E
Protocol Title: First Trimester Trophoblast Investigations
Funding Source: Sponsor: NATIONAL INSTITUTES OF HEALTH
Sponsor: KELLOGG FOUNDATION
Protocol #: 1501013712

Expiration Date: August 02, 2016

Risk Level / Category: Research not involving greater than minimal risk

The above-referenced protocol and items listed below (if applicable) were **APPROVED** following *Expedited Review* (Category (5)*) by the Chairperson/designee for the Wayne State University Institutional Review Board (MP4) for the period of 08/03/2015 through 08/02/2016. This approval does not replace any departmental or other approvals that may be required.

- Revised Protocol Summary Form (revision received in the IRB Office 7/24/2015)
- Protocol (received in the IRB Office 1/9/2015)
- Medical records are not being accessed for this study. Therefore, HIPAA does not apply.
- Research Informed Consent (revision dated 6/18/2015)

-
- Federal regulations require that all research be reviewed at least annually. You may receive a "Continuation Renewal Reminder" approximately two months prior to the expiration date; however, it is the Principal Investigator's responsibility to obtain review and continued approval **before** the expiration date. Data collected during a period of lapsed approval is unapproved research and can never be reported or published as research data.
 - All changes or amendments to the above-referenced protocol require review and approval by the IRB **BEFORE** implementation.
 - Adverse Reactions/Unexpected Events (AR/UE) must be submitted on the appropriate form within the timeframe specified in the IRB Administration Office Policy (<http://www.irb.wayne.edu/policies-human-research.php>).

NOTE:

1. Upon notification of an impending regulatory site visit, hold notification, and/or external audit the IRB Administration Office must be contacted immediately.
2. Forms should be downloaded from the IRB website at **each** use.

*Based on the Expedited Review List, revised November 1998

APPENDIX C

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Title: The balancing act – PPAR- γ 's roles at the maternal-fetal interface
Author: Leena Kadam, Hamid Reza Kohan-Ghadr, Sascha Drewlo
Publication: SYSTEMS BIOLOGY IN REPRODUCTIVE MEDICINE
Publisher: Taylor & Francis
Date: Mar 4, 2015
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APPENDIX D

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April 7, 2017

Leena Kadam
Wayne State University
CS Mott Center for Human Growth and Development 275 E. Hancock
Detroit, MI 48201
Email: lkadam@med.wayne.edu

Dear Dr. Kadam,

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APPENDIX E

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Title: Rosiglitazone Regulates TLR4 and Rescues HO-1 and NRF2 Expression in Myometrial and Decidual Macrophages in Inflammation-Induced Preterm Birth

Author: Leena Kadam, Nardhy Gomez-Lopez, Tara Mial, et al

Publication: REPRODUCTIVE SCIENCES

Publisher: SAGE Publications

Date: 03/21/2017

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ABSTRACT**THE ROLE OF PPAR γ IN PLACENTAL DEVELOPMENT AND DISEASE**

by

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The placenta in mammals forms the maternal fetal interface serving as the source of nutrition for the fetus throughout gestation. It comprises of two major trophoblast lineages: (i) the decidua invading extra-villous trophoblast (EVT) and (ii) the placenta residing villous trophoblast (VT). The EVT's invade the maternal endometrium to establish pregnancy and secure blood-flow to the implantation site. The VT villous trophoblast forms the main maternal-fetal exchange surface and ensures nutrient and gas exchange to facilitate growth throughout pregnancy. Additionally, both lineages are involved in immunological functions such as maintaining allogenic tolerance and regulation of immune cell activation at the maternal fetal interface. They also express pattern recognition receptors like TLR (toll-like receptors) and NLR (Nod-like receptors) involved in pathogen recognition. Activation of these receptors leads to an inflammatory cascade aimed at elimination of the pathogen which in severe conditions can lead to preterm birth (PTB) and even fetal death. Proper development of trophoblast cells is thus crucial for placental function and hence for a successful pregnancy. Indeed, pregnancy disorders like pre-eclampsia (PE), intra – uterine growth disorders (IUGR) and preterm birth (PTB) have all been associated with abnormal trophoblast differentiation. Interestingly, elevated levels of

systemic as well as placental inflammation is another feature commonly associated with these disorders. Localized inflammation (Chorioamnionitis) has been reported to be present in >85% spontaneous preterm births even in the absence of systemic inflammation. However, the potential effects of inflammatory pathways on placental function (and trophoblast differentiation) remain largely unexplored. Consequently, there is also less information available on molecular targets common to both these processes that can be used for development of therapeutic interventions.

The ligand activated transcription factor PPAR γ has a known anti-inflammatory role and plays a crucial role in placental development. Abnormal levels of the receptor were also associated with disorders IUGR associated PE, GDM and even PTB. However, the potential role of PPAR γ in regulation of the placental and systemic immune responses remains unexplored. Our preliminary studies in the mouse model of inflammation induced PTB showed that activation of PPAR γ significantly reduced PTB and improved both placental and fetal weights. The current dissertation therefore aimed to evaluate PPAR γ as the potential common link between inflammation and placental function. Based on our preliminary results, *we hypothesized that PPAR γ is an important modulator of placental immune responses and is as well involved in trophoblast function.*

AIM 1: To determine the molecular mechanism of PPAR γ mediated prevention of PTB in endotoxin induced PTB mouse model

In our preliminary studies, we observed that treatment with Rosiglitazone (specific agonist for PPAR γ) significantly reduced pre-term birth in the mouse model for endotoxin (bacterial lipopolysaccharide) mediated inflammatory preterm birth. The current aim was designed to evaluate the mechanism involved. Our results revealed that activation of PPAR γ via Rosiglitazone had anti-inflammatory effects at both systemic and local levels. We also reported that the endotoxin (LPS) increases inflammation by upregulating its receptor TLR4 and

contributes to oxidative stress by downregulating the anti-oxidant pathway. Rosiglitazone via PPAR γ activation decreased the inflammatory cytokine levels in serum and downregulated activity of NF- κ B pathway in macrophages at the maternal-fetal interface. It also upregulated expression of anti-oxidant pathway mediators NRF2 and HO-1 and reduced the expression of TLR4. We thus report for the first time, that PPAR γ activation via Rosiglitazone prevents LPS induced preterm birth in mice by acting on inflammatory as well as anti-oxidative pathway.

AIM 2: To determine the effects of LPS exposure and PPAR γ induction on human trophoblast physiology

In our mice studies, we observed that endotoxin treatment caused reduction in placental weights suggesting inflammation mediated effects on mouse placenta. Since elevated inflammation is associated with approximately 30% of preterm deliveries in humans, we asked if inflammation had any effects on human placental function. Previous studies have focused on evaluating the inflammatory response of human placental trophoblast cells. However, the effects of inflammation on trophoblast cell differentiation and function remain comparatively unevaluated. Additionally, the potential anti-inflammatory effect of PPAR γ in human placenta also remains unexplored. The current aim was designed to evaluate the effects of endotoxin exposure on the human trophoblast cell differentiation and function and the potential role of PPAR γ in reversing these effects. Our results showed for the first time that inflammation alters trophoblast cell differentiation and function -- by downregulating expression of trophoblast differentiation proteins GCM1 and CG- β and reducing invasion -- which can be reversed by PPAR γ activation.

AIM 3: To determine the role of PPAR γ in differentiation of EVT and VT trophoblast lineages.

The data obtained in Aim 1 and Aim 2 highlighted the key role for anti-inflammatory activity of PPAR γ in placental function and pregnancy in general. However, PPAR γ is

implicated to be involved in placental development via pathways beyond inflammation. Previous studies and results presented in chapter 4 suggested a key role for PPAR γ in trophoblast differentiation via regulation of GCM1 – transcription factor crucial for differentiation towards both trophoblast lineages. The first part of Aim 3 therefore focused on validating the PPAR γ – GCM1 molecular axis. Our results showed that PPAR γ transcriptionally regulated GCM1 expression by binding to its specific binding site in the GCM1 promoter. The second part of Aim 3 was designed to further delineate the role of PPAR γ in differentiation towards the VT and EVT lineages. A crucial initial step was to develop a model to simultaneously study both lineages. In the current study, we established the model and conducted preliminary experiments for determining the specific role of PPAR γ in trophoblast lineage differentiation.

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Publications (selected):

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- Kohan-Ghadr HR, Kilburn BA, **Kadam L**, Armant DRA, Drewlo S. Rosiglitazone attenuates Redox-associated damage and improves survival rate in human trophoblasts under oxidative stress. (*Under review*)
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Picture abhi baaki hai mere dost....