PATHWAYS DEPLOYED BY HUMAN PLACENTAL TROPHOBLASTS TO CONFER VIRAL RESISTANCE

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During pregnancy, the placenta plays a vital role in protecting the developing fetus from microbial infections. The multinucleated and terminally differentiated syncytiotrophoblasts actively coordinate host defense through a multitude of mechanisms. In addition to forming a cellular barrier, the syncytiotrophoblasts release extracellular vesicles containing microRNAs from the chromosome 19 microRNA cluster (C19MC). These vesicle-packaged miRNAs, upon uptake by recipient cells, induce autophagy and limit viral infection, acting against a diverse panel of both DNA and RNA viruses. We have shown that medium conditioned by primary human trophoblast (PHT) cells, which contain these vesicles, confers resistance from pathogens associated with congenital infections to recipient non-placental cells. Furthermore, we have demonstrated that primary human trophoblasts constitutively produce interferon lambda 1 (IFNλ1), leading to a robust induction of interferon stimulated genes in an autocrine and paracrine manner. These parallel pathways, actively coordinated by PHT cells, contribute to the barrier function of the placenta, protecting the developing fetus from viral infections.

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PREFACE

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1.0 INTRODUCTION

An infection during pregnancy can have severe consequences to a mother's health and to her developing fetus [1]. Infection and inflammation are the leading causes of preterm births, which account for approximately 13 million pre-term births annually worldwide [2, 3] and are a major cause of infant mortality. Maternal infections, whether resulting from bacteria, viruses, or protozoa, can lead to infection during delivery or pregnancy loss, due to systemic spread [4]. Infections that are transmitted to the fetus can result in developmental abnormalities, fetal, or neonatal disease.

The placenta, a fetal-derived organ that interfaces the mother and the fetus and coordinates the exchange of nutrients, waste, gas, and also the production of essential hormones, is a remarkably effective barrier against viral infections. While historically considered a passive barrier to infection, we now appreciate that the placenta actively coordinates a multitude of immunological responses to protect the fetus from microbial infections as well as preventing immune rejection of the fetus [5]. Once the placenta is formed, the main site of maternal-fetal interaction is the placental villi, tree-like structures that are bathed in maternal blood, and facilitate maternal-fetal communication. This villous tree is comprised of trophoblasts, with a core of fibroblasts, macrophages and fetal endothelial cells. The apical surface of the villous trees is covered by the terminally differentiated, multinucleated trophoblasts, known as syncytiotrophoblasts. These cells are in direct contact with the maternal blood, and are the point

of communication between the mother and the fetus. Our lab has been studying trophoblasts, seeking to understand their role in immunity during pregnancy, and specifically investigating how the syncytiotrophoblasts inhibit viral infection and confer protection to viral infection to neighboring non-placental cells. The cellular composition of the villi, as well as structural differences between the human placenta and other eutherian organisms (organisms having a placenta) are discussed in later sections.

In the following sections of this chapter, we summarize relevant aspects of human pregnancy, with a focus on the role of the placenta as a physical and functional barrier to infection, as well as review information regarding the immune system during pregnancy, and how infection from a diverse range of pathogens can affect the maternal and fetal health. Next, we review data on the expression and function of chromosome 19 miRNA cluster (C19MC), a placenta-specific cluster of small non-coding RNAs shown to confer antiviral effects. Lastly, we provide an overview on interferons, antiviral signaling proteins, focusing on their expression during pregnancy. Subsequent chapters will cover our recent findings regarding the role of the PHT cells in conferring resistance to viral infections associated with congenital diseases, the production of IFNλ1 by the PHT cells, and PHT cell resistance to infection by Zika virus (ZIKV). The final chapter will summarize our findings and the implications of the work, touching on relevance and potential future directions.

1.1 INTRODUCTION TO HUMAN PREGNANCY

Human pregnancy lasts an average of 38-42 weeks, arbitrarily divided into three trimesters, starting from the last menstrual period. Following fertilization of the oocyte by the spermatozoon

in the fallopian tubes, the zygote travels to the uterus, where it implants into the maternal endometrium. The trophectoderm is the first tissue of the blastocyst to differentiate, eventually forming the extra-embryonic tissues, including the placenta. The inner cell mass of the blastocyst develops into the fetus. While these early developmental processes take place, the maternal body undergoes a wide range of physiological changes, including differentiation of the uterine endometrium into the decidua, hormonal changes, and shifts in the maternal immune system. Because the prevention of fetal rejection was considered the main function of the maternal immune system, pregnancy has historically been viewed as in a state of suppression, termed "pregnancy–associated immune deficiency" [6]. It was suggested that in order to prevent rejection of the semi-allograft fetus, the maternal immune system must be globally suppressed. However, a growing body of literature challenges the immune suppression hypothesis, demonstrating a complex regulation of maternal immunity and active coordination of innate immunity by the placenta. Furthermore, the local maternal immune system in the uterus has been demonstrated to remain competent, and play an essential role in implantation, early embryonic development, and intricate immune signaling that coordinates maternal-placental interactions.

The following sections discuss the events underlying placental development, noting important placental structures and functions, the maternal immune system during pregnancy, and characterizing both the innate and adaptive responses. The consequences of microbial infections during pregnancy are also discussed, focusing on their impact on the fetus. These sections provide the necessary context for later chapters, which discuss the role of the placenta in attenuating fetal viral infections, and how that protection can be conferred to non-placental cells, as discussed in the later chapters.

1.1.1 The Human Placenta

The placenta is a fetal-derived organ, resulting from the differentiation of the trophectoderm layer of the developing embryo, early in gestation. At term, the placenta is large discoid organ, with size averaging 22 cm in diameter, 2.5 cm in thickness, and usually weighing half a kilogram [7]. The placenta functions in gas, nutrient, and waste exchange between the mother and the fetus. Additionally, the placenta produces many pregnancy specific hormones, such as human chorionic gonadotropin (hCG), which are essential for maintaining the pregnancy. The role of the placenta in providing the fetus with nutrients has been known since the 5th century BC, though it was initially thought to act as a barrier, preventing the maternal-fetal blood mixing [8]. The term *placenta,* meaning “cake” in Latin, was proposed by Realdo Columbo in the sixteenth century, based on the organ’s discoid shape. In the seventeenth century, William Harvey theorized separate maternal and fetal circulations, which was then proven in the eighteenth century by William and John Hunter, using molten wax to model the utero placental vessels and distinct circulatory systems [7, 9, 10]. Weber and Dalrymple later expanded on the descriptions of the placental anatomy, characterizing the placental villi and the capillary structures. This was then followed by identification of the trophoblast bilayer in 1882 by Langhans. William Carpenter’s experiments in 1889 defined the intervillous space [11]. Ambrosius Hubrecht in 1889 defined and named the tissue barrier between the maternal and fetal circulations trophoblast (nutrition and germ), due to the importance of nutrient exchange between the mother and the fetus [12].

1.1.1.1 Placental Structure

The fetus is connected to the placenta by the umbilical cord, which contains two umbilical arteries and an umbilical vein. The maternal side of the placenta, referred to as the basal plate, is in direct

contact with the decidua, the lining of the uterus. The villus is the main functional unit of the placenta. Each villus is comprised of an outermost layer of cells, the syncytiotrophoblasts. Directly subjacent to the syncytiotrophoblasts are the mononuclear cytotrophoblasts, which become a discontinuous layer after the first trimester. The villous stroma comprises fibroblasts, as well as placental macrophages known as Hofbauer cells. In the stroma are fetal blood vessels lined by endothelial cells. The uterine walls are comprised of a layer of smooth muscle tissue, known as the myometrium, which is responsible for uterine contractions. Internal to the myometrium is the decidua, which forms the anchoring point for the placenta, and is shed after parturition. At the site of implantation, the extravillous trophoblasts invade the decidua and inner third of the myometrium. These structural features of the placenta are depicted in figure 1-1, below.

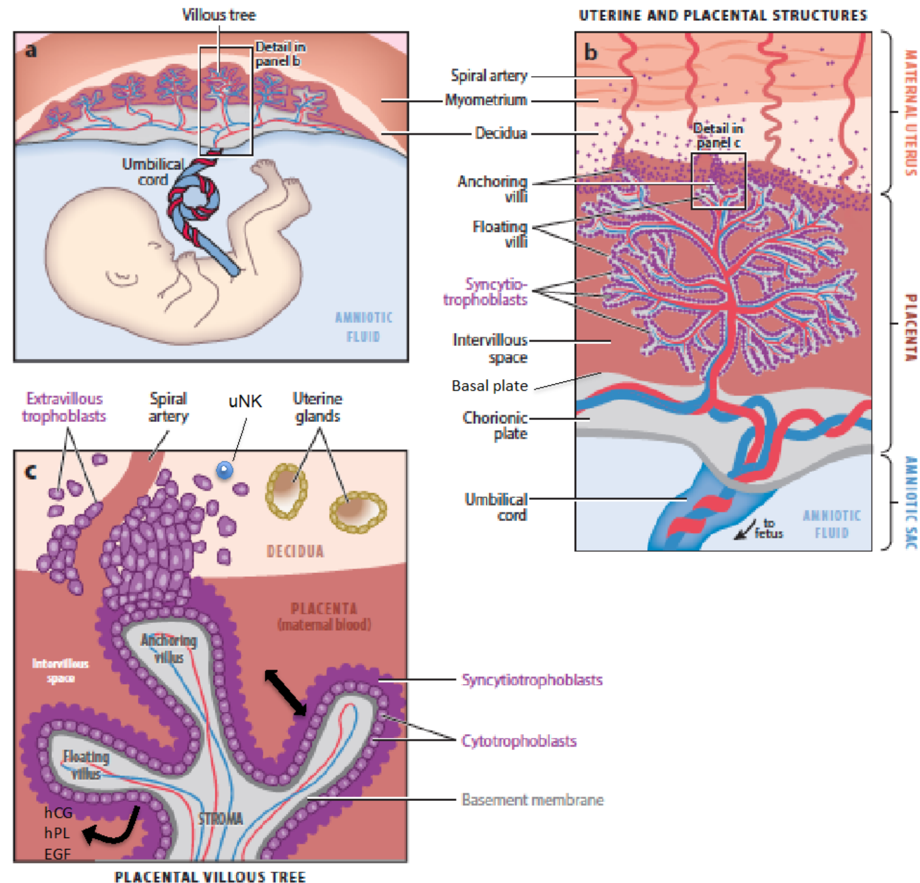

Figure 1-1 - Structure of the Human Placenta **(A) A schematic of a human fetus, surrounded by amniotic fluid, attached to the placenta by the umbilical cord. (B) Enlarged view of one of the villous trees, demonstrating the anchoring villi as well as the floating villi. The placenta attaches to the maternal decidua. Adjacent to the decidua is the myometrium. The villus is surrounded by intervillous space, perfused with maternal blood. The umbilical cord attaches the fetus to the chorionic plate. The basal plate contacts the decidua. (C) An enlarged view of the villus tree. Extravillous (intravascular) trophoblasts (EVT) are depicted invading the maternal spiral artery. Adjacent to the EVTs are uterine natural killer cells (uNK). The distal cells of the villus tree are syncytiotrophoblasts, which produce and secrete hormones, such as human chorionic gonadotropin (hCG). The syncytiotrophoblasts are the site of maternal fetal interaction, with exchange of nutrients, waste, gases, and hormones. Subjacent to the syncytiotrophoblasts are the cytotrophoblasts. (From Delorme-Axford et al., 2014 [13]; modified, with permission, from Annual Reviews 2014.)**

1.1.1.2 Placental Development

During early blastocyst development, the extra-embryonic trophoblast cell lineage is the first to develop. 6-7 days after fertilization, the blastocyst attaches to the uterine endometrium, beginning implantation [14]. At this stage, the blastocoel and the inner cell mass are surrounded by a single layer of mononuclear cells. The cells overlaying the inner cell mass form the contact point with the endometrium. At 7-8 days post fertilization, the trophoblasts that attach to the endometrium fuse laterally, forming the first multinucleated syncytiotrophoblasts. This process is completed by day 11 after implantation, as a fully formed syncytium covers the entire implanted blastocyst [15]. Days 8-12 after fertilization, the lacunas begin formation, which will eventually give rise to the intervillous space.

Concurrent with lacunar formation, the endometrial cells regrow to cover the placenta, and the inner cell mass begins to differentiate into the three germ layers. The lacuna formation partitions the trophoblasts into three major sections. The first is the chorionic trophoblasts near the embryo, which will form the chorionic plate. The second section is the lacunar zone, which will become the intervillous space. The third section is made up of the trophoblastic shell, which interacts with the endometrium to produce the basal plate [7]. Around day 12 after fertilization, the progenitor extravillous trophoblasts begin to invade each of these three layers, extending both laterally and deeply into the endometrium. Some of the invasive trophoblasts give rise to endovascular trophoblasts, remodeling the maternal spiral arteries and replacing the vascular smooth muscle. This permanently dilates the spiral arteries, preventing their constriction, causing reduced perfusion pressure of the placental bed [16, 17].

The proliferating cytotrophoblasts increase the partitions and branching, leading to the development of the primary villi. The surrounding trabeculae will eventually form the intervillous

space. The villous core is formed by the penetration of the extraembryonic mesoderm, with the cytotrophoblasts at the distal end. These columns invade into the maternal uterine capillaries. The proliferation of the columnar cells supplies the extraembryonic trophoblasts. The hemangioblastic progenitors, an undifferentiated and proliferating cell type, become the source for the developing early fetal capillaries endothelial cells [18]. Trophoblastic columns invade maternal vessels, plugging the distal segments, and therefore blocking maternal and fetal circulation, which maintains a state of hypoxia [19, 20]. After 8-9 weeks, the plugs begin to lyse, starting at the periphery and progressing centripetally, beginning the perfusion of the placental intervillous space with maternal blood.

1.1.1.3 Placental functions

The placenta's predominant function is to regulate the maternal-fetal exchange of gases, supply of nutrients, and removal of waste, to produce hormones, and to provide immunological defense [21, 22]. The syncytiotrophoblasts are key players in these processes, and facilitate the transfer of molecules across two plasma membranes. The transcellular transport across the syncytium occurs via non-mediated transport, mediated transport, or endocytosis. Non-mediated transport relies on the physical and chemical properties of the molecules, rather than a specific transport mechanism. Mediated transport is more common, relying on transporter proteins present at the plasma membrane, such as transport of glucose via the glucose transporters (GLUT family) [23], present at the microvillous membrane and basement membrane. The process of endocytosis involves the formation of vesicles through invagination at the plasma membrane. When transporting cargo to the fetal blood, these vesicles are transported to the basal side of the cell, where they fuse and release their cargo. Endocytosis transport is important for the transfer of maternal IgG to the fetus [24].

A key function of the placenta is the production of critical hormones that are needed for proper maintenance of the pregnancy. The syncytiotrophoblasts produce large quantities of hormones including hCG, human placental lactogen (hPL), and many other growth factors, such as insulin like growth factor (IGF-1), transforming growth factor beta (TGFβ), and epidermal growth factor (EGF) [25].

It is now appreciated that the placenta also functions as a physical and functional barrier to infection. Although previously suggested to be a passive barrier, lacking many of the receptors necessary for pathogen entry [5], we now know that the placenta actively coordinates an immune response. Furthermore, recent studies have demonstrated that the placenta is able to detect and respond to microbial infections, mounting a coordinated immune response. This is discussed in more detail in the following sections.

1.1.1.4 Modeling the placenta

Certain aspects of human pregnancy and placental function can be determined by observations from non-invasive procedures and post-pregnancy examination. For example, ultrasound is used to image placental structure during routine pre-natal care, and can also be used to measure placental perfusion with maternal blood [7, 26]. Other biomarkers of pregnancy can be found in maternal blood, and can be used for indicators of potential disease states [27-29]. With regards to studying infections during pregnancy, many retrospective and case studies have been used to assess the health of the mother and fetus in the presence of infections, such as with HIV or Zika [27, 30-33]. These studies can include placental biopsies, where histological examination of the placenta provides information about infection or immune cell infiltration.

In order to study the mechanisms underlying vertical transmission of infections, a number of different animal models can be used, with advantages and disadvantages to each. Mouse models

of infection during pregnancy allow researchers to understand the mechanisms of trans-placental infections as well as fetal pathologies. However, the mouse placenta is structurally distinct from the human placenta, with differences in the vascularity, maternal-fetal inter-digitation, barrier interface, and blood flow [7, 34]. As previously stated, the human placenta is a discoid shape, contrasting the diffuse placenta of the pig or the zonary placenta of many carnivores. Unlike the human villous trees, which are bathed in maternal blood, the mouse placenta has a complex inter-digitation, referred to as a labyrinth, where maternal and fetal vessels are placed in close proximity [7]. Additionally, while both are hemochorial, the human placenta is dichorial early during pregnancy, becoming monochorial later in gestation as the cytotrophoblast layer becomes sparse. In contrast, the mouse placenta is trichorial. Additional differences include the number of offspring per pregnancy. While humans and other primates tend to be singleton, many other animals such as mice, have litters of varying size. Along with the structural differences, the mouse placenta differs immunologically from the human placenta. To recapitulate the disease states observed in humans, high titers of virus are required, or ablation of the maternal IFN system using IFNAR knockout mice or IRF knockout mice [35, 36].

Lastly, there are in vitro models, which can be utilized to study the placenta and infection during pregnancy, including explant studies, cell culture models, and "placenta-on-a-chip" systems. Villous trees collected from first, second, and third trimester can be dissected out and grown in extracellular matrix like substitutes, such as Matrigel [37]. These explant models have the benefit of maintaining the cellular composition and morphology of the villous trees, and can be used to determine specific cells susceptible to infection, as shown with *T. gondii* [38]. A range of human choriocarcinoma cell lines have been developed to study the placenta *in vitro*, such as BeWo, JEG-3, and JAR cells [39, 40]. These cells can be easily propagated, and are amenable to

genetic manipulations. However, some of the disadvantages to using these lines in that they reflect the carcinogenic cell origins, and are distinct from trophoblasts in a number of different ways. For example, they are polyploidy, and some of these lines do not produce many of the trophoblastic hormones mentioned above, and are morphologically distinct from trophoblasts, as the cell lines do not spontaneously form syncytium in culture [41-43]. The BeWo cells are able to form syncytium and produce hormones after exposure to forskolin or 8-bromo-cAMP, but pharmacological treatment can also affect other cellular processes [43-45]. Our lab has recently developed a system to model the syncytiotrophoblasts, utilizing a three dimensional co-culture system of JEG-3 cells, grown together with human brain microvascular endothelial cells (HBMEC), and recapitulate some of the morphology and secretory phenotype of the syncytiotrophoblasts, as well as recapitulating microbial resistance [46].

In addition to immortalized cell lines, primary cells can be used for studying the placenta. Our lab and others have demonstrated that primary human trophoblasts, grown in culture for longer than 24 h differentiate, produce relevant hormones and form syncytium [47]. Our studies have focused on singleton third trimester placentas for culturing of trophoblasts. However, other groups have used first and second trimester trophoblasts, either cultured, or used in explant studies [38, 48-50]. Lastly, placenta-on-a-chip models have recently been described as a system to study placenta barrier function and simulate transport across cellular layers. Some of the chips are made of Poly-dimethylsiloxane (PDMS) plates with microchannels that are seeded with choriocarcinoma cell lines, either JEG-3 or BeWo on the “maternal’ side, and endothelial cells on the “fetal” side, both attached to fibronectin-based ECM “basement-membrane” [51, 52]. However, these systems still have the disadvantages described for the trophoblast cell lines for infection studies.

1.1.2 Immunity during pregnancy

Viviparity, growth and development of the fertilized egg inside the maternal body, creates the situation where the embryo and its related extraembryonic tissues, all expressing paternal or "foreign" antigens, are in contact with the maternal immune system. Hence, pregnancy is referred to as a semi-allograft. The maternal immune system is regulated throughout pregnancy to ensure that the adaptive immune response is not activated, ensuring tolerance of the fetus, and allowing for the remodeling of the maternal endometrium to facilitate placental development and function. The section below discusses key changes that occur in the maternal immune system, noting both the role of the maternal immune system to facilitate proper placental development and implantation, as well as the placental innate immune responses to infections.

1.1.2.1 Adaptive immunity during pregnancy

In response to the cycling estrous hormones, the uterine endometrial cells recruit leukocytes, through the production and secretion of different cytokines [53]. These leukocytes, including uterine natural killer (uNK) cells, macrophage, dendritic cells, and regulatory T cells, are important for ensuring proper implantation and placentation [54-56]. The majority of the decidua leukocytes are comprised of uNK cells [55, 57-59]. During the first trimester the uNK cells, dendritic cells, and macrophage begin to invade the decidua, interacting with extravillous trophoblasts [60, 61]. Furthermore, these immune cells are critical for proper invasion of the trophoblast cells, and deletion of these leukocytes has negative effects on implantation and fetal development [62-67].

For instance, in the absence of uNK cells, decidual vascular remodeling and placental implantation are impaired [63, 68].

An important component of maternal immune regulation and prevention of fetal rejection is the restricted expression of Human Leukocyte Antigens (HLA) by trophoblasts. The villous trophoblasts do not express any HLA-class I or class II antigens *in vivo*, though expression can be induced *in vitro* with inflammatory cytokines [69, 70]. As the extravillous trophoblasts differentiate, they begin to express HLA-E and HLA-G, which are important for suppressing the killing action of uNK cells, binding to the CD94/NKG2 receptor and immunoglobulin like receptor B1, respectively [71]. Importantly, while the class Ia HLA genes produce membrane bound proteins, the class Ib genes, such as HLA-G, produce membrane bound as well as soluble forms. This is the result of alternative splice variants [72]. While most class I HLA genes are regulated by enhancer sequences (enhancer A, interferon stimulated response element (ISRE), and class II major histocompatibility complex trans-activator (CIITA), these sequences are divergent in HLA-G, rendering the protein unresponsive to NFκB, IRF-1, and CIITA [73-75].

Another attribute of the maternal immune system during pregnancy is the ability of the maternal immunoglobulin to be transmitted passively to the fetus. Among the different immunoglobulins, only IgG is transferred across the placenta in significant quantities, with low level transfer during the first half of pregnancy, and a gradual increase until term [76]. The process is mediated by the neonatal FC receptor [77, 78]. Normally, the transfer of IgG is important for conferring immunity to the fetus, which has not yet fully developed its own adaptive immune response.

1.1.2.2 Innate immune responses of the placenta

The trophoblasts are capable of recognizing a broad range of pathogens, expressing many pattern recognition receptors (PRR). These include Toll like receptors (TLR) 3, 7, 8, and 9 [79]. In addition to recognition, the trophoblasts produce many different anti-microbial peptides, such as beta defensins 1 and 3 [80, 81]. The cells can also produce secretory leukocyte protease inhibitor (SLPI), which has been shown to potently inhibit HIV infection [80, 82, 83]. Stimulation of trophoblasts choriocarcinoma cell lines, including JAR, BeWo, and JEG-3 with polyinosinic:polycytidylic (PIC) or infection with Sendai virus (SeV) has been shown to induce the production and release of type I interferons [79, 84]. Furthermore, we have demonstrated that trophoblast cell lines also potently induce type III interferons upon infection with SeV, or transfection with PIC (Figure 3-3D) [85].

1.2 INTERFERONS

Interferons (IFNs) were discovered in the 1950s [86]. These proteins induce a signaling cascade that results in the up-regulation of a broad range of antiviral effector proteins, referred to as interferon stimulated genes (ISGs) [87]. Almost every mammalian cell type produces IFN in response to viral infection, in contrast to plants, insects, and lower chordates, which do not produce IFNs [88]. Different cell types produce specific IFNs, such as the production of IFNα by dendritic cells [89], or IFNκ by keratinocytes [90]. Since their initial discovery, three different types of interferons have been characterized. Type I and type III interferon will be discussed in detail below. IFNγ is the sole type II IFN. Activated T cells and NK cells produce IFNγ, and it signals through a distinct heterodimeric receptor. In most instances, IFNγ does not result in the induction

of a potently antiviral state, and is therefore outside the scope of this work, but has been reviewed by others [91]. These different types of IFN, along with their respective receptors and signaling pathways, are depicted in figure 1-2, below. In the following sections, we discuss the sensing of viral pathogens and induction of IFNs in cells, and review the IFN signaling pathway, leading to the induction of the antiviral effector proteins. We focus the last section on IFNλ, and how the type III interferons are distinct from the type I.

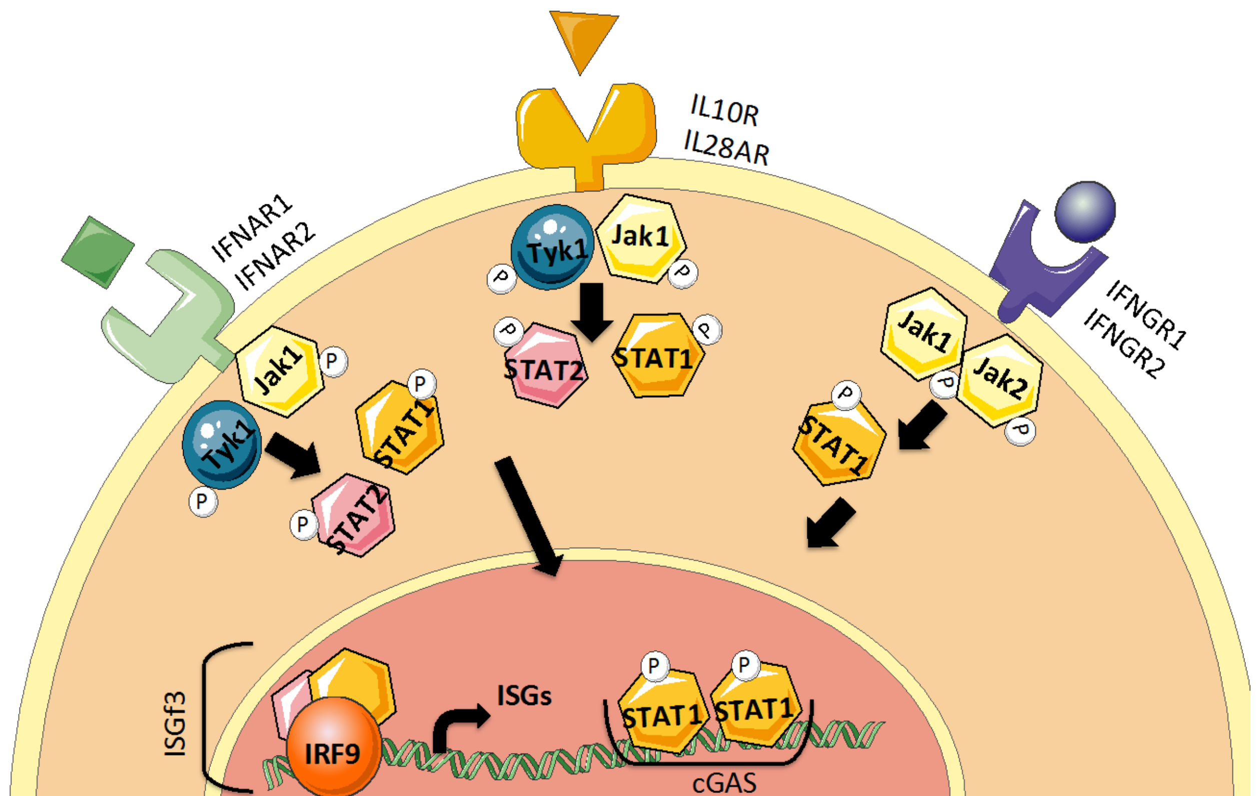

Figure 1-2 - Different types of IFN. Depicted are the three classes of interferon molecules, their respective receptors, and signaling pathways. Type I interferon (green), predominantly made up of IFNα and IFNβ, signals through the IFNAR receptor heterodimer. This results in subsequent signaling through the JAK/STAT pathway, leading to the nuclear translocation of ISGf3 complex, comprised of STAT1, STAT2 and IRF9. The result of translocation of ISGf3 to the nucleus is the upregulation of hundreds of different ISGs. Type II

interferons (purple) are comprised of IFNγ, which is predominantly expressed by T cells in response to antigen presentation. Signaling through the IFNγ receptor, this pathway is distinct from the Type I and Type III IFN, in that it relies on cGAS nuclear translocation, and upregulates a subset of ISGs. Type III interferons (yellow), or IFNλ, signal through the IL-10R/IL-28AR heterodimer. The downstream signaling of IFNλ largely overlaps with the type I.

1.2.1 Pattern Recognition/Interferon induction

Upon microbial infections, the innate immune system detects pathogens using pathogen sensing molecules referred to as pathogen recognition receptors (PRR). These receptors recognize specific pathogen associated molecular patterns (PAMPS) that are well conserved amongst classes of pathogens [92]. Among the PRRs are the Toll-like receptors (TLRs), which are able to recognize a broad range of pathogens, including bacteria, viruses, fungi, and parasites, and also some host molecules that are indicators of pathogen caused damage to the cell, referred to as DAMPs (danger associated molecular patterns) [93]. Another group of cytosolic PRRs are the retinoic acid-induced gene like receptors (RLRs).

The predominant TLRs involved in RNA virus sensing are TLR3 [94] and TLR7, which recognize dsRNA and ssRNA respectively. The cytosolic RNA-helicase domain containing protein RIG-I recognizes the distinctly negative ssRNA viral 5'-triphosphate containing RNA or short dsRNA. Melanoma differentiation-associated gene 5 (MDA5) recognizes longer dsRNA molecules. Binding of viral RNA to either MDA5 or RIG-I leads to association with mitochondrial antiviral signaling protein (MAVS). MAVS activation and dimerization results in the recruitment of adapter proteins that activate NFkB, IRF3 and IRF7. Upon dsRNA binding to TLR3, the receptors dimerize, and bring the Toll/Interleukin-1 receptor domain (TIR) in close proximity. This allows for the subsequent recruitment of adapter proteins, such as myeloid differentiation factor

88 (MYD88), TIR-domain-containing adaptor-inducing interferon B (TRIF), and TRIF-related adaptor molecule [95]. TRIF binding to TLR3 leads to the activation of IRF3, as well as NFkB and AP-1. TRIF recruits a number of different proteins including TNF receptor-associated factor 3 (TRAF3) and TRAF6 [96, 97]. TRAF3 interacts with TRAF family member associated NFkB activator (TANK), which complexes with the kinases TANK-binding kinase 1 (TBK1) and IKKe. These two proteins phosphorylate IRF3, which then dimerizes, translocating to the nucleus to bind IFNβ enhancer sequences [98, 99]. Whereas the TLR3 receptor recognizes dsRNA, TLR7, upon recognition of ssRNA, dimerize and recruits MYD88, which then complexes with IL-1 receptor associated kinase 1 and 4 (IRAK1 and IRAK4), as well as with TRAF6. TRAF6 phosphorylation and activation leads to activation of NFkB and IRF7, which is bound to IRAK1 [100]. The above-mentioned signaling cascades are depicted below, in figure 1-3.

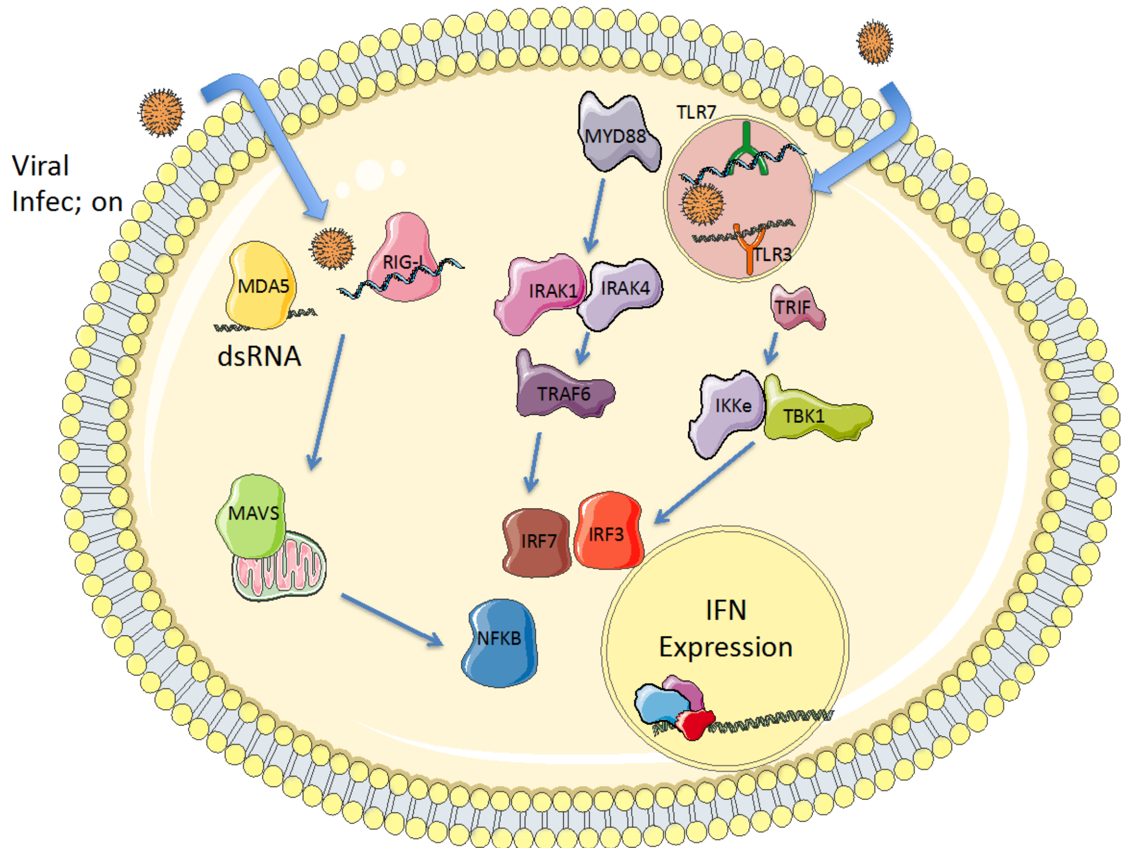

Figure 1-3 **- Pathogen recognition and IFN induction. RNA virus recognition by pattern recognition receptors (PRR) involved in the induction of IFN α and β. TLR7, present in the endosomal compartment of trophoblast cells, encounters ssRNA from viral infection or uptake of viral components from phagocytosis. Signaling from TLR7 through Myd88, complexes with IRAK1, IRAK4 and TRAF6 to activate IRF7. TLR3 is expressed in a wide range of cells types, and recognizes dsRNA, leading to the activation of TRIF, and then IRF3 through the IKK kinases and TBK1. Nuclear translocation of the interferon regulatory factors (IRF) leads to the induction of IFN.**

1.2.2 Type I interferons

Type 1 Interferons, IFNβ and IFNα, are the most well studied interferons. Humans express 1 IFNβ, and 13 functional IFNα. These are defined based on their structural similarities. IFN ω, IFNε, and

IFNκ are more distantly related. IFNω is produced by leukocytes, and has properties that are very similar to IFNα. IFNκ expression is unique, in that it is only expressed in keratinocytes, and seems to be cell associated, only affecting the immediate surrounding cells [90, 101]. Studies using a murine model demonstrated that IFNε plays a specific role in the female reproductive tract, and is coordinated by the estrous cycle [102].

1.2.2.1 IFNAR - Type I IFN Receptor

The receptor for the Type I interferons belongs to the class II cytokine family receptors, and consists of relatively long intracytoplasmic domain (ICD), and relatively short intracytoplasmic domain [103]. These two subunits are termed IFNAR1 and IFNAR2. The ICD serves as a docking site for the tyrosine kinase, Janus kinase 1 (Jak1). Jak1 binds to sites on IFNAR2, while tyrosine kinase 1 (Tyk1) binds to sites on IFNAR1, in both instances, binding to the ICD adjacent to the transmembrane domain [104, 105]. As the ligand binds the receptor subunits, the proximity of each receptor and subsequently, the associated kinases, becomes closer, allowing for the auto and cross phosphorylation.

1.2.2.2 JAK/STAT Pathway

Phosphorylation of the IFNAR receptor, by the tyrosine kinases JAK and Tyk, produces multiple docking sites within the ICD, which can then be docked by the signal transducer and activator of transcription (STAT). Three different STAT proteins, STAT1, 2, and 3, have been demonstrated to bind the IFNAR complexes. STAT1 and STAT2 form a complex with IRF9, referred to as the ISGf3 complex. The ISGf3 complex, upon entering the nucleus, associates with the histone acetyltransferase CBP/p300, GCN5, and with the chromatin remodeling factor BRG1 [106-109].

This complex enters the nucleus, binding to the DNA motif, interferon stimulated response element (ISRE), and enhancing the transcription of interferon stimulated genes (ISGs).

1.2.2.3 Interferon stimulated genes (ISGs)

Interferon stimulated genes are known for their role in inhibiting viral infection. To date, there are hundreds of ISGs that have been identified, though many have not yet been characterized for their direct role in inhibiting viral infection [87]. Additionally, ISGs have a diverse range of functions to inhibit viral infection, including interference with translation (PKR), degradation of RNA (OAS), or interference with viral assembly and release (Vipirin) [110]. Some viruses are sensitive to specific ISGs. For example, parainfluenza virus type 5 (PIV5) is sensitive to the ISG IFN-induced protein with tetratricopeptide repeats 1 (IFIT1), but not to MxA, OAS, or PKR [111]. ISG15 functions to modify over a hundred different host proteins through ubiquitin like conjugation, referred to as ISGylation. Additionally, many of the genes involved in the IFN pathway, such as STAT1 and IRF9, are also upregulated, contributing to the positive feedback [112].

1.2.2.4 Type I IFN in the placenta

As was mentioned, choriocarcinoma trophoblast cell lines can respond to PRR agonists or viral infection by the induction of Type I interferons. Studies in this area, carried out in the 1980s and 1990s, utilized infectivity assays and neutralization experiments to identify the presence of IFN [113, 114]. In some of these early studies, amniotic fluid or placental biopsies were collected, and tested for their ability to inhibit viral infections *in vitro* [115-118]. However, there were conflicting reports about the presence of IFNs in placentas and amniotic fluid in the absence of viral infection [119, 120]. Some studies suggesting the presence of IFN in amniotic fluid was derived from

amniotic cells were dismissed by other groups, contending that the IFN was actually derived from the placenta [121]. Other hurdles related to studying placental IFN included contamination of maternal blood, potentially masking the contribution of the placenta. Later studies addressed these concerns by using perfused term placentas from healthy mothers [122]. Interestingly, this group and others noted only moderate effects of anti-sera against IFNα and IFNβ [123], suggesting the presence of a then uncharacterized antiviral protein. Other reports supporting the production of IFN from the placenta, and possibly systemic effects during pregnancy, demonstrated that peripheral blood mononuclear cells (PBMCs) from healthy pregnant women showed an upregulation of many ISGs (ex. IFI44L, IFIT3, OAS1) [124], compared to the PBMCs from healthy non-pregnant women.

1.2.3 Type III interferons

In contrast to the type I interferons, which have been studied for over 60 years, the type III interferons, IFNλ1-4, were identified in 2003 [125, 126]. IFNλ4, a frameshift variant of IFNλ3 and thus considered a pseudogene, was reported in the last 5 years, identified largely based on genetic data [127]. Also belonging to the class II cytokine family, the type III IFNs are functionally similar to type I, resulting in the upregulation of ISGs. Structurally, they resemble the IL-10 family of cytokines [128], and there is no cross reactivity with the type I IFNs. The type III IFNs signal through a distinct heterodimeric receptor, IFNLR. The function and expression of IFNλ and IFNLR appear to be restricted to certain cell types, activating predominantly in epithelial and endothelial cells. This targeted signaling is in contrast to the ubiquitous nature of IFNβ. In this section we highlight the role of IFNλ in infections, and discuss the attributes that make the type III

distinct from the type I IFNs, particularly with regards to the expression in mucosal tissues and epithelial cells, as well as discussing differences in IFNλ4 expression in certain populations.

1.2.3.1 Induction of IFNλ

Type III IFNs are induced by the same mechanisms that lead to the induction of type I IFNs, as viral infections trigger PRR activation and signaling. One exception to this is the expression of IFNλ following activation of the cytosolic DNA sensor Ku70, which was demonstrated to be active in a broad range of cell types, which does not lead to induction of type I IFN [129]. The genetic locus of IFNλ is also distinct from IFNα/β. The genes encoding IFNα/β are located on chromosome 9 in humans, whereas IFNλ1-4 are located on chromosome 19 [130]. The IFNλ promoter regions share similarities with the type I IFNs, containing binding elements for IRF-1, IRF-3, IRF-7, and NFκB [131, 132]. However, while IRF3, NFkB and AP-1 can lead to induction of IFNβ, IFNλ1 can be activated by NFkB alone [133]. Additionally, IFNλ expression is regulated by the transcriptional repressor ZEB1 in epithelial cells [134].

1.2.3.2 IFNλ signaling

The type III IFNs are structurally more aligned with IL-10 cytokines based on crystal structure analysis, though the primary amino acid sequence closely resembles the type I IFNs. IFNλs signal through a distinct heterodimeric receptor, shown in figure 1-3. This structural and functional similarity with type I IFNs and IL-10 cytokines, such as IL-22, likely reflects a common evolutionary origin, protecting the epithelium from bacteria and viruses respectively [135]. The heterodimeric receptor contains one subunit is made up of the IL-10 family cytokine receptor IL-10R. The other subunit is specific to IFNλ, referred to as IFNLR1. Despite similarities with other IL-10 cytokine family members, the receptor binding is unique to IFNλ [136].

Downstream from receptor binding, the signaling of IFNλ overlaps with the type I IFN signaling, with activation of the JAK/STAT pathway, and subsequent ISGf3 nuclear translocation [125, 126, 137-139] and induction of hundreds of ISGs. However, despite the overlapping pathways, there appear to be differences in antiviral activity and in ISG induction [137, 140, 141]. These differences have been attributed to the magnitude of induction and the differences in the duration of the induction [142]. IFNα induction of an antiviral state is transient, lasting for approximately 6 hours. In contrast, IFNβ and IFNλ1-3 have longer lasting effects, with ISG expression sustained for 12-24 hours. The mechanism of for this difference in expression is not known, though it has been suggested that IFNα uniquely induces negative regulators [142]. This suggests that there are no uniquely expressed ISGs induced by IFNλ. Rather, the magnitude of expression is different, with IFNβ causing the most robust ISG induction, followed by IFNα, and then IFNλ 3, 1, and 2 (in that order). However, there may be specific promoter elements that distinguish the different IFNs and more a thorough comparison is required to further delineate them.

1.2.3.3 Tissue expression of IFNλ

Other than differences in the signaling and expression of ISGs, the effect of the type III IFNs is the most apparent in mucosal tissues and epithelial and endothelial cells, which are constantly exposed to commensal and pathogenic microbes [139, 143, 144]. Numerous studies have demonstrated the role of IFNλ in the respiratory tract, gastrointestinal tract, the liver, and the blood brain barrier. The IFNLR is expressed at high levels in human hepatocytes, and when infected with HCV or HBV, these cells produce IFNλ [145]. Other studies have demonstrated high levels of expression of IFNLR in gastrointestinal epithelial cells, with robust responsiveness to IFNλ [146]. In models where IFNLR was knocked out, such as in mouse airway or the intestinal epithelial

cells, viral pathogenesis was increased [134, 147]. IFNLR knockout mice also demonstrated increased sensitivity to West Nile virus (WNV), which might be related to the recently demonstrated role of IFNλ in maintaining the blood brain barrier integrity (BBB) [148].

1.2.3.4 IFNλ polymorphisms

Many humans have a functional IFNλ4. However, a single nucleotide polymorphism (SNPs), which causes a frameshift mutation, ablates production in populations encoding this mutation [127, 149]. Interestingly, loss of IFNλ4 confers enhanced clearance of HCV infection, although the cause is unclear. IFNλ4 may potentially downregulate the expression of other IFNλ members. Another possibility is that high expression of IFNλ4 results in expression of negative regulators of the IFN pathway, rendering the cells less sensitive to future IFN stimulation [150]. Genome wide association studies revealed multiple IFNλ polymorphisms that are linked to improved clearance of viral infections, such as HCV, HBV, hCMV, and HSV-1 [151-155]. Other SNPs in the promoter region of IFNλ3 lead to higher expression, and better control of infections [156]. The mechanisms by which these polymorphisms affect IFNλ production and clinical responses remain unclear.

1.3 MICROBIAL INFECTIONS DURING PREGNANCY

Relatively few pathogens are capable of crossing the placental barrier, resulting in fetal infections. However, there are a number of viral, bacterial, or protozoan pathogens linked to pregnancy complications, resulting in severe maternal and/or fetal disease. Infection by these pathogens can lead to severe developmental abnormalities, morbidity and mortality [157] with some morbidity related to maternal immunological responses. In response to PIC, murine models as well as

trophoblast cell lines (HTR8) and primary trophoblasts produce proinflammatory cytokines, including TNFα, IFNγ, IL-12, and IL-6 [158]. These cytokines may activate the maternal immune response, which can lead to placental damage, miscarriage, fetal developmental or growth abnormalities, abortion, and spontaneous preterm birth [158]. There are also a number of different routes of infections, allowing the pathogens access to the fetus. In terms of viral infections, the mode of transmission is most frequently hematogenous. In this section, we highlight some of the different types of pathogens that have been linked to perinatal infections, discussing the basic biology of these pathogens, as well as pathways of maternal-fetal transmission. A more comprehensive list of pathogens associated with pregnancy complications and placental infections are presented in table 1.

Table 1-1. Pathogens linked to placental/fetal infections

Pathogens	Type	Tropisms
Brucella spp.	Bacteria	Leukocytes, Epithelial cells[159]
Coxiella burnetii	Bacteria	Leukocytes[160, 161]
Listeria monocytogenes	Bacteria	Epithelial, Phagocytes[162]
Mycobacterium tuberculosis	Bacteria	Leukocytes[163]
Treponema pallidum	Bacteria	Unknown[164, 165]
Leishmania spp.	Bacteria	Leukocytes[166, 167]
Plasmodium falciparum	Parasite	Erythrocytes, hepatocytes[168]
Toxoplasma gondii	Parasite	All nucleated cells[169]
Trypanosma spp.	Parasite	Epithelial, Endothelial[170]
Cytomegalovirus	Virus	Leukocytes, trophoblasts[171, 172]

Lymphocytic choriomenigitis virus	Virus	Leukocytes[173, 174]
Parvovirus B19	Virus	Hematopoietic, endothelial[175, 176]
Rubella virus	Virus	Broad[177]
Varicella zoster virus	Virus	Leukocytes, neurons, epithelial[178]
Herpes simplex virus	Virus	Leukocytes, neurons, epithelial [179, 180]

1.3.1 Viral infections during pregnancy

Viruses can gain access to the maternal-fetal compartment by a diverse range of routes, including infected sperm during fertilization, intrauterine infections occurring before or during the implantation process, hematogenous spread of the virus causing direct placental infection or transmission across the placenta, infected macrophages from the maternal blood supply crossing the placenta, infection resulting from a medical procedure such as amniocentesis, or infection of the fetus during the delivery process.

1.3.1.1 Herpesviridae

A number of members of the herpesviridae are connected with congenital infections and pregnancy complications, especially during primary infection. Broadly, the herpesviruses contain large linear dsDNA genomes packaged into a nucleocapsid core. Here we discuss three different herpesviruses: herpes simplex virus, cytomegalovirus, and Varicella Zoster virus, and the contribution they play towards perinatal infections.

Herpes simplex virus 1 and 2 (HSV) can lead to pregnancy related disease either *in utero*, during delivery, or postnatally, resulting from breastfeeding. These viruses are members of the α-

herpesvirus family, and are enveloped viruses with linear ds DNA genomes of approximately 152 and 155 kb, respectively. There are approximately 1500 cases of neonatal infection from Herpes simplex virus each year in the United States [180]. Most of the infections occur perinatally from an infected maternal lower genital tract [179, 181]. Transplacental transmission is rare, and has been linked to fetal anomalies, including microcephaly, intracranial calcifications, and chorioretinitis. Using explant models, other groups have demonstrated that the syncytiotrophoblasts are resistant to HSV, but the underlying mesenchymal cells are permissive. However, these subjacent cells were only accessible following exposure of the explants to trypsin, which damages the trophoblast layer [182]. In contrast, extravillous trophoblasts may be more permissive to infection, and could explain poor placental attachment to the uterine wall during infection and subsequent miscarriage [182].

Human Cytomegalovirus (hCMV) is one of the leading causes of severe prenatal viral infection. A member of the β-herpesvirus family, it is an enveloped dsDNA virus, with a genome of approximately 240 kb. For immune-competent individuals, hCMV usually manifests as a relatively benign disease. However, CMV can have severe consequences during pregnancy. Congenital CMV infection is not rare, although the risk associated with recurrent viral infection during pregnancy is low [183]. However, primary infection during pregnancy has a 30-40% risk of intrauterine transmission and clinical disease [184, 185]. Approximately 15% of women who acquire a primary infection early in their pregnancy have a spontaneous abortion [186]. During these instances, the placenta, but not the fetus, demonstrates signs of infection. Later during pregnancy, CMV infection can cause premature delivery or intrauterine growth restriction [187]. In instances where the virus is transmitted to the fetus, severe congenital defects can occur, such

as fetal growth restriction, cerebral ventriculomagly, microcephaly, intracranial calcifications, and chorioretinitis [188].

Varicella zoster virus (VZV), the causative agent of chicken pox, is a highly contagious infectious agent, and a common childhood illness. However, infection of varicella during pregnancy is rare, since mothers are often immune to the virus by the time of childbearing age. VZV, like other herpes viruses, is a DNA virus, in which humans are the only host, and transmission occurs via the conjunctive and mucous membranes of the nasopharynx [189]. Generally, primary infection provides life long immunity. Herpes zoster infection during pregnancy, resulting from reactivation of the virus, has not been associated with congenital varicella syndrome, and incidence of vertical transmission is rare [190]. While not associated with first-trimester spontaneous abortion [191-193], vertical transmission may result in congenital varicella syndrome, where primary infection during the first two trimesters can result in range of devastating development abnormalities, including microcephaly, eye disorders like microphthalmia [178, 190, 194-196], or gastrointestinal abnormalities. Additionally, fetuses born with congenital varicella syndrome often present with hypertrophic skin scarring [178].

1.3.1.2 Togaviridae

The togaviridae are a family of enveloped simple single stranded RNA viruses. Originally named based on resemblance to roman cloaks in electron microscope micrographs, the togaviridae have a spherical virion structure [197]. This family of viruses includes many pathogens associated with human disease, and in particular a number of different viruses linked with perinatal infection. This section focuses on members of the two genera, rubivirus, and alphaviruses. In the genus rubivirus there is only a single virus, rubella, also known as German measles. In contrast to rubivirus, the alphavirus genus contains over 30 different viruses. These two are distinguished by genome

organization and nucleotide sequence. The togaviruses have non-structural replication proteins encoded by the 5' end of the genome, and the structural proteins, making up the viral particles, encoded by the 3' end. These structural proteins are encoded by a subgenomic mRNA.

Rubella virus, the only member of the rubivirus genus, is a single stranded positive sense RNA virus, of approximately 9.7 kb. Currently, with the widespread vaccination against rubella, infection during pregnancy is rare. However, prior to vaccination, fetal infection during the first trimester had infection rates as high as 50%, with severe congenital defects associated with infection, including congenital rubella syndrome, characterized by deafness, CNS damage such as microcephaly, cardiac defects, and cataracts [198].

The old world alphaviruses, which include Sindbis virus and Chikungunya virus, can cause acute febrile illness as well as more lasting arthrogenic diseases in humans, including myalgia and rash. The new world alphaviruses, including eastern equine encephalitis and Venezuelan equine encephalitis, are known to cause encephalitic disease. Among the alphaviruses, there is a link between gestational infection with Venezuelan equine encephalitis virus and spontaneous abortion [199].

1.3.1.3 Human immunodeficiency virus

Human immunodeficiency virus -1 is a member of the retroviridae. These viruses are characterized by the virion particles containing an RNA genome, and after entry into the host cell, the genome is reverse-transcribed into DNA. The transcribed viral genomic DNA is then integrated into the host chromosomal DNA. Through direct interaction between the viral envelope protein and the receptor CD4 and the chemokine receptor CCR5 or CXCR4, the virus targets cells of the immune system, causing suppressed immune systems [197]. HIV is the causative agent of AIDS, which currently affects 36.7 million people worldwide living with HIV. The World Health Organization

has estimated that there have been approximately 10 million babies born with Human immunodeficiency virus (HIV). While the rates of vertical transmission in developed nations have dropped significantly with the use of advanced anti-retroviral therapies, there is still a major burden in the most afflicted areas, such as sub-Saharan Africa. Interestingly, while most viruses linked to congenital pathologies infect the fetus predominantly in the first or second trimester, vertical transmission by HIV results mainly from invasive procedures during the pregnancy, or upon delivery [31]. In rare cases of fetal infection during pregnancy, it has been hypothesized that maternal immune cells, crossing into fetal circulation, transmit infection [200].

1.3.1.4 Flaviridae

The flaviridae comprises a family of single stranded positive sense RNA viruses. The genus contains over 50 viruses, most of which are arthropod-borne. In particular, two of the flaviviruses have been implicated in pregnancy complications, Dengue virus (DENV) and Zika virus (ZIKV). DENV, which is a major global health problem in tropical and subtropical areas, infects over 100 million people each year, resulting in 25,000 deaths worldwide [201]. Dengue fever is caused by four different dengue virus serotypes, and is transmitted primarily by the mosquito *Aedes aegypti,* with *Aedes albopictus* as a secondary vector [202]. A retrospective study in West Guinea, where dengue is not endemic, found that infection during pregnancy resulted in an increase in premature births and preterm labor [203]. While there have been no reports about congenital infections and fetal malformations resulting from dengue infection during pregnancy, there is an increased risk of hemorrhage during delivery, with possible perinatal transmission at delivery [203-206].

Unlike DENV, the recent ZIKV pandemic has demonstrated that ZIKV is strongly linked to congenital defects and developmental abnormalities. Zika virus was initially discovered in Uganda in 1947 [207]. For most infected individuals, the symptoms of ZIKV infection resemble

dengue fever, with acute febrile illness, headache, arthralgia, and rash. ZIKV infections tend to be less severe than DENV, with many individuals never developing symptoms. However, the recent pandemic in Brazil has demonstrated that infection with ZIKV during pregnancy is strongly linked with congenital malformations, most prominently microcephaly and fetal growth restriction. The Brazilian strain of ZIKV has been detected in the placenta, as well as in amniotic fluid and fetal brain, indicating the virus is capable of crossing the placental barrier [208, 209]. However, it remains unclear how this virus gains access to the fetus. Recent studies have demonstrated that ZIKV infects primary placental cells, including cytotrophoblasts, endothelial cells, fibroblasts, Hofbauer cells from chorionic villi, and amniotic epithelial cells [48]. However, that same study also demonstrated that the syncytiotrophoblasts from first trimester placental explants were not infected. We have recently published work (see below) further supporting these findings, as syncytiotrophoblasts from term placentas were refractory to ZIKV [85].

1.3.2 Protozoan infections during pregnancy

Perinatal parasite infection can also lead to severe consequences for the developing fetus. Of particular relevance is the protozoan parasite *Toxoplasma gondii*. This intracellular parasite is able to infect almost all nucleated cell types [169]. Contraction of Toxoplasma can occur from ingestion of uncooked meats, contaminated foods, or transmission of the oocysts from infected cat feces. Infection with *T. gondii* during pregnancy can result in congenital toxoplasmosis, leading to neurological disorders, blindness, or even miscarriage or stillbirth [210]. Severity of the infection is dependent on the stage of pregnancy. Maternal infection during the first trimester often results in miscarriages, while infection during the second trimester results in blindness, microcephaly, or other cognitive disabilities. The risk of vertical transmission usually occurs during a primary

infection [211]. Using first trimester villous explants, it has previously demonstrated that EVTs are preferentially colonized by the parasite, relative to the more resistant syncytiotrophoblasts [38].

Malaria, endemic in 103 countries, is estimated to affect between 300-500 million people each year [212]. Caused by plasmodium falciparum (*P. vivax, P. ovale and P. malariae* cause less severe disease) studies have suggested that infants surviving a placental malaria infection can suffer adverse neurodevelopment sequelae and have abnormal responses to parasite infections later in life [213]. In these instances, the adverse effect on fetal development is attributed to the inflammatory response in the placenta, and the parasite itself [214].

1.3.3 Bacterial infections during pregnancy

A comprehensive list of bacterial pathogens linked to congenital defects is presented in Table 1. Here, we highlight one of those bacteria, *Listeria monocytogenes*, which is a ubiquitous bacterial pathogen that often causes food borne illness in humans and many other animals. Healthy adults frequently ingest *L. monocytogenes.* In pregnant women, *L. monocytogenes* can spread to the feto-placental unit, resulting in spontaneous abortion, stillbirth or preterm labor, depending on the stage of pregnancy [215-217]. Other groups have demonstrated that the syncytiotrophoblasts are resistant to infection by *L. monocytogenes*, and that the extravillous trophoblasts are the predominant site of infection [218]. They suggest that this resistance is the result of the syncytiotrophoblasts lack of E-cadherin, limiting the entry of the bacterium.

1.4 MICRORNAS

In recent years the appreciation for ncRNA has grown tremendously, as we now understand that many of these non-protein coding RNAs are crucial for maintaining proper balance and homeostasis. A major class of these ncRNAs is the small regulatory RNAs known as microRNAs (miRNA). These miRNAs were first identified in the *Caenorhabditis elegans*, by Lee et al [219] and by Wightman et al., [220]. These initial studies were of the gene lin-4, which does not produce a functional gene product, but rather a small 22 nt transcript which targets the 3' untranslated region (UTR) of lin-14. Within these 3' UTRs are conserved elements that are complimentary to the lin-4 transcript. Since their initial characterization, miRNAs have been identified in a broad range of genomes, including protists, plants, animals and viruses. These small ncRNAs are approximately 20-24 base pairs in length. Functionally, miRNAs act to prevent translation of mRNA transcripts through a block in translation or by degradation of the transcript. This process is completed through the RNA-induced silencing complex (RISC), a complex of proteins including Argonaute (Ago), which guides the miRNA to the corresponding mRNA, binding through an imperfect match in the base pairs, discussed further below. The degree to which miRNAs inhibit protein expression is often modest [221, 222]. However, disruption of miRNA expression levels can have profound biological consequences. There is a growing list of miRNAs implicated in human disease, including cancer, heart disease, kidney disease, and psychiatric disorders [223-227].

The expression profile for miRNAs in different tissues is highly variable, and dependent upon developmental and pathological states. Within the human placenta there is a distinct repertoire of miRNAs, with the majority belonging to the two largest miRNA clusters in the human genome. The first is the chromosome 14 miRNA cluster, and the second is the chromosome 19

miRNA cluster. In this section, we focus on the biogenesis of miRNAs, the machinery involved in regulation of gene expression by miRNAs, and the specific attributes and relevance of the C19MC miRNAs as they relate to pregnancy, focusing on their role in the placenta and contributions to protecting the fetus from viral infections.

1.4.1 MiRNA biogenesis

Encoded by the genome, miRNAs are initially transcribed by RNA-Pol II as long primary miRNA transcripts, termed pri-miRNAs [228]. This primary transcript, derived from either mRNA or intronic sequences, then undergoes a number of processing steps before becoming a mature and functional miRNA. The primary structure contains self-complementary regions, allowing for folding back on itself to become a hairpin, ranging between 65-80 nucleotides in length. This primary sequence then undergoes endonucleolytic cleavage by the microprocessor, a complex consisting of the RNAse III endonuclease Drosha, guided by the RNA binding protein (RBP) DGCR [229, 230]. Following this processing step, the hairpin stem loop precursor, now referred to as a pre-miRNA, is exported out of the nucleus by the RanGTPase-dependent exportin 5. Once in the cytoplasm, the pre-miRNA is further cleaved by another RNAse III endonuclease, known as Dicer, in conjunction with the RBP TPBP [231-234]. Following this cleavage step, the mature 20-24 nt duplex is loaded onto the RISC effector protein complex [235-237]. The process of miRNA biogenesis is depicted in figure 1-4, below.

Once the mature duplex is loaded onto the RISC complex, it is unwound by the helicase activity, and the guide strand (the strand with the lowest internal stability at the 5’ end) is retained to form the active RISC complex, while the other passenger strand is discarded [238, 239]. These

canonical pathways for miRNA biogenesis and RISC complex formation and function are the most common in mammalian cells, though other pathways have recently been described.

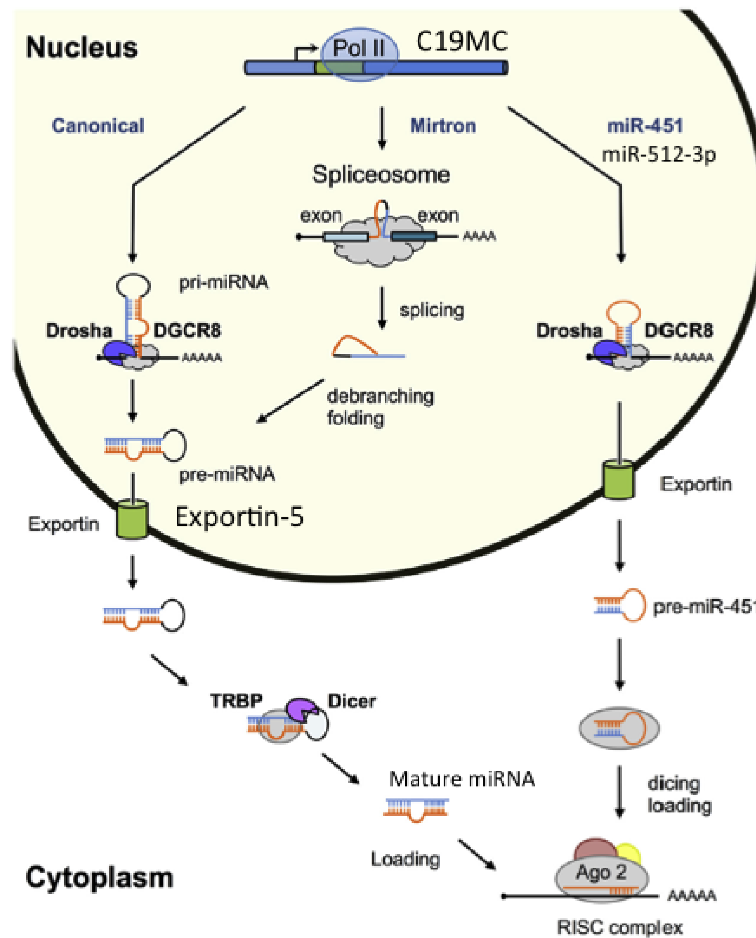

Figure 1-4 - miRNA biogenesis. In the canonical miRNA biogenesis pathway, miRNAs are initially transcribed from intronic, exonic, or intergenic sequences. Following transcription by Pol II, the primary miRNA is then bound by the RNA binding protein DGCR8 and cleaved by the RNAse III Drosha. Following this, the pre-miRNA, at this point ranging from 55-70nt, is then exported from the nucleus by exportin-5, where Dicer processes it. The mature miRNAs are then loaded onto the RNA induced silencing complex, where they bind the seed sequence of the complementary 3'UTR of the target mRNA. (From Mouillet et al., 2015 [240]; modified, with permission, from Elsevier 2015.)

1.4.2 Placental specific miRNAs

With its inherent transcriptional complexity, it is not surprising that the placenta expresses a broad range of miRNAs, with a subset of these being specific to the trophoblasts [241-245]. Thus, understanding the role of these placental specific miRNAs has been challenging. Murine models that lack either Dicer or Ago2 are embryonic lethal [246-248]. Mir-675, expressed exclusively from the placentally-abundant H19 transcript [249], demonstrated elevated expression levels during the second half of gestation. This expression has been correlated with reduced growth, as mir-675 appears to inhibit placental cell proliferation, likely due to the silencing of insulin like growth factor receptor 1 [249].

To date, our understanding of the functional significance of many of placental miRNAs is severely limited. For instance, murine models that lack the C14MC cluster (containing 77 mature miRNAs), which is abundant in the placenta, do not exhibit any phenotypic differences. Some of the best studied miRNAs in the placenta, mir-17~92 cluster, have been shown to play a role in tumorigenesis [250] and specific members from this cluster have been shown to regulate the differentiation of trophoblast cells, targeting specific proteins such as CYP19A1 (aromatase) and transcription factor GCM1 which play a pivotal role in syncytium formation [251]. However, a targeted deletion of these miRNAs in the murine model did not generate an obvious phenotype [252]. Computational analysis has indicated a role for a few miRNAs within the placenta, and these have subsequently been validated experimentally. For instance, mir-378-5p and mir-376c were demonstrated to enhance trophoblast proliferation and invasion, through the targeting of the nodal pathway [253, 254]. In contrast, mir-155 inhibits trophoblast invasion, targeting CYR61 and Cyclin D1, potentially contributing to the development of preeclampsia [255, 256]. Other miRNA

species such as mir-210 and mir125b-1-3p, were implicated in the impairment of trophoblast proliferation and invasion, thereby contributing to placental disorders [257-260].

1.4.2.1 C19MC

Our lab has a focused on the chromosome 19-miRNA cluster (C19MC), which is the largest miRNA cluster in the human genome, and is nearly exclusively expressed in the placenta of humans and primates, with no murine ortholog [261]. This is the result of primate specific evolutionary expansion, potentially due to Alu-mediated rearrangements of the C19MC [262]. Spanning 100kb, this cluster of miRNAs contains 46 intronic miRNA genes, and produces 58 mature miRNAs [261]. The C19MCs are extraordinarily enriched for repetitive Alu sequences, a class of short interspersed nuclear elements (SINE), each approximately 300 nt long of highly repetitive DNA [263]. This cluster is highly expressed in the syncytiotrophoblasts, as well as other trophoblast cell lines.

The C19MC miRNAs are also expressed in human embryonic stem cells, and that expression declines upon differentiation [264-266]. The expression of C19MC miRNA in the extravillous trophoblasts is markedly lower in comparison to the villous trophoblasts [267]. Further, overexpression of the C19MC in extravillous trophoblasts reduces cell migration *in vitro.* While expression of the C19MCs in healthy individuals is limited to expression in the trophoblasts, aberrant expression of these miRNAs has been demonstrated in a number of different malignancies [268-274].

1.4.2.2 C19MCs: A role in autophagy

An unexpected role of the C19MCs is their involvement in viral resistance. The C19MCs, which are expressed in particularly high levels in primary human trophoblasts, are capable of conferring

resistance to a diverse panel of DNA and RNA viruses [275]. Through exosome-mediated delivery, the C19MC miRNAs were determined to induce autophagy in recipient non-placental cells, thereby conferring viral resistance.

Autophagy is an evolutionarily conserved catabolic process, in which cells maintain homeostasis by sequestering aged, damaged, or ubiquitylated proteins into double membraned vesicles, known as autophagosomes. The autophagosome cargo is then digested upon fusion of the vesicle with the lysosome. Autophagy is initiated during periods of cellular stress, such as starvation and nutrient deprivation, or in response to the cellular stress of viral infection. There are a number of viruses, which can lead to congenital infections, including CMV [276], VZV [277], B19V [278], and ZIKV [279], and are known to induce autophagy. Autophagy has been shown to be both antiviral and proviral in a number of different studies [280]. In cases where autophagy is antiviral, cytoplasmic viruses can be sequestered by the autophagosome and degraded upon fusion with the lysosome, potentially leading to the viral components activating innate immune pattern recognition receptors. This is the case with sindbis virus and VSV [281, 282]. However, certain viruses have evolved mechanisms to antagonize the autophagic machinery, such as HSV and hCMV [283, 284]. For other viruses, autophagy is beneficial, as is the case with VZV and dengue [285].

1.4.2.3 Extracellular vesicles released by trophoblasts

It has recently become clear that miRNAs not only function in the cells producing them, but are capable of being released by the cells and taken up by distant recipient cells, broadening their role to cell-cell communications [286, 287]. This miRNA based cell-cell communication is important for maintaining homeostasis, tissue physiology and certain disease states [288-290]. This can be

observed in the release of individual protein bound miRNAs, but also in the packaging of miRNAs into extracellular vesicles such as exosomes [291-294].

The syncytiotrophoblasts, like other epithelial cells, release a variety of extracellular vesicles into the maternal circulation. Among these are the apoptotic blebs, microvesicles, and exosomes [295-298]. The largest of these vesicles, the apoptotic bodies (AB), range from 1-5 μm, and are produced by shedding of trophoblast at baseline with an increase of fragments during apoptosis [299]. The microvesicles are smaller than the AB, ranging between 200 nm and 1 μm. These vesicles are the result of budding from the plasma membrane [298]. The exosomes are produced from invagination of the microvesicular body (MVB), which then fuses with the plasma membrane releasing its cargo of exosomes into the circulation [290, 300]. The exosomes range in size between 40-150 nm, and as our group has previously reported, contain miRNAs from the C19MC family [301]. Exosomes, known to be involved in cell-cell communication [292], are involved in immune signals [302, 303]. These trophoblast exosomes may be involved in immune tolerance, potentially through the reduction in NK cell receptor NKG2D expression, Fas ligand mediated apoptosis, or tumor necrosis factor (TNF)-related ligand inducing apoptosis [245, 302, 304]. In particular, our lab has delineated the role of exosomes in conferring viral resistance to non-placental recipient cells [275].

1.5 SPECIFIC AIMS

Given the severe consequences of primary infection during pregnancy and risk of subsequent fetal infection and disease, understanding the mechanism by which the placenta limits fetal infection by viruses is crucial for designing treatment strategies and therapeutics. Many groups

have demonstrated, using explant models, that the placenta can actively limit infection by a number of different pathogens such as protozoan parasites, bacterial infections, and viral infections. In many of these studies, it was demonstrated that the syncytiotrophoblasts were highly resistant to infection, but that the underlying cytotrophoblasts and adjacent extravillous trophoblasts were permissive [38, 48, 305]. However, the mechanisms of syncytiotrophoblasts' resistance to infection are not fully understood. Additionally, while previous groups demonstrated that in response to PRR agonists or in response to infection trophoblast explants could produce type I interferon, the protective function of syncytiotrophoblasts in the absence of infection was not known [80, 117]. Therefore, we set out to elucidate the underlying mechanisms by which syncytiotrophoblasts were resistant to viral infection, as well as the mechanisms by which the trophoblasts were able to confer resistance to non-trophoblast recipient cells. Previous work from out lab, using primary human trophoblasts, identified high levels of basal autophagy in the PHT cells as one such mechanism of viral-resistance. This work also demonstrated that this resistance could be conferred to other cells through exosomal mediated delivery of the C19MC miRNAs, and subsequent induction of autophagy. **We hypothesize that PHT cells utilized additional mechanisms of viral resistance, which could also be conferred to non-placental cells, and that this resistance was active against a number of pathogens associated with perinatal infections**. Identifying the different antiviral pathways deployed by the primary human trophoblasts and how they can inhibit microbial infection in the placenta and surrounding tissues may lead to the development of new treatment and prevention strategies for perinatal infections.

Aim 1) Characterize the effects of the C19MC microRNAs on pathogens associated with intrauterine infections Rationale: Our lab previously found that non-placental cells exposed to CM were resistant to the majority of viruses screened. Here we address a broader panel that includes microbial pathogens linked to congenital syndromes and perinatal infections.

Aim 2) Identify additional antiviral pathways utilized by primary human trophoblasts Rationale: We observed differences in the degree of antiviral activity between conditioned medium from PHT cells and the C19MC miRNA mimics.

Aim 3) Elucidate the differences among PHT derived extracellular vesicles, focusing on antiviral effects Rationale: We previously reported that exosomes derived from PHT cells are able to confer viral resistance to non-placental recipient cells. Here we expand these finding to examine additional extracellular vesicles derived from PHT cells and their functional significance.

Understanding how the placenta is able to limit fetal infection and how this process is regulated is important for developing treatments and therapies for a broad range of highly relevant diseases. This research will elucidate some of the mechanisms involved in defense at the maternal-fetal interface.

2.0 HUMAN TROPHOBLASTS CONFER RESISTANCE TO VIRUSES IMPLICATED IN PERINATAL INFECTIONS[1]

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Data for figure 2-1A was contributed by Teryl Frey and Christie Sleigher. Data for figure 2-1B and 2-1C was partially contributed by Derek Trobaugh and William Klimstra. Data for figure 2-5 was contributed by Stephanie Seveau.

2.1 INTRODUCTION

Beyond providing a physical barrier between the maternal and fetal vasculature, the placenta governs the exchange of gases, nutrients, and waste products between these two compartments. In the hemochorial placenta, this exchange is regulated primarily by the syncytiotrophoblasts, a layer of multinucleated, terminally differentiated cells that are bathed in the maternal blood and play a critical role in protecting the developing fetus from invading pathogens [5]. Despite this defensive barrier, some pathogens are able to invade the fetal environment.

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[1] Reprinted with permission from the American Journal of Obstetrics and Gynecology

Viral infection of the intrauterine compartment can spread to the fetus and/or the mother. Active maternal viral infections can lead to infection during delivery or to pregnancy loss (either early or late) resulting from systemic spread of the infection [4]. Viruses that are transmitted directly to the fetus can result in developmental abnormalities or fetal or neonatal disease. For example, prior to the widespread use of vaccination, fetal infection rates by the rubella virus were nearly 50% during maternal rubella infection in the first trimester of pregnancy and were associated with congenital rubella syndrome, characterized by deafness, cataracts, damage to the central nervous system, and cardiac defects [198]. Congenital infection with varicella during pregnancy can lead to spontaneous abortion or neonatal varicella infection, which may result in devastating birth defects known as congenital varicella syndrome [178, 190]. Venezuelan equine encephalitis virus has been linked to pregnancy complications such as spontaneous abortion [199]. HIV tends to be transmitted during vaginal delivery or invasive procedures [31].

Beyond the formation of a syncytial physical barrier, mechanisms by which placental trophoblasts influence viral infections are insufficiently understood. We recently demonstrated that primary human trophoblasts (PHT) are resistant to infection by an unrelated panel of viruses [275]. Furthermore, viral resistance was conferred to non-trophoblast cells when incubated with conditioned medium from PHT cells. This resistance was mediated, at least in part, by exosomal delivery of miRNAs from the chromosome 19 microRNA cluster (C19MC), which is the largest miRNA cluster in humans unique to primates and almost exclusively expressed in the placenta [263]. C19MC miRNAs are highly expressed in exosomes released from PHT cells, and can be found circulating in the plasma of pregnant women [245, 301]. In cells exposed to PHT-conditioned medium we also observed a strong induction of autophagy, a pro-survival catabolic process where cellular organelles are partly or fully enclosed in cytoplasmic phagosomes, and

degraded upon fusion with the lysosomes. Autophagy was also observed in cells transfected with selected miRNA members of the C19MC, and attenuation of autophagy mitigated this antiviral effect [275]. Here we expand upon our previous observations and focus on viruses that are pertinent to fetal infection during pregnancy and/or delivery, including rubella virus and other togaviruses, HIV, and varicella zoster virus (VZV). Additionally, we compared these effects to infection by two clinically relevant non-viral perinatal pathogens, *Listeria monocytogenes*, and *Toxoplasma gondii*.

2.2 RESULTS

2.2.1 PHT conditioned medium or miRNA mimics from the C19MC attenuate infection of select members of the *Togaviridae* family

We found that, when compared to non-conditioned medium, pre-exposure to PHT conditioned medium markedly reduced the infection of Vero cells by rubella virus (Fig. 2.1A)[2]. We expanded our studies to other members of the *Togaviridae* family and measured the activity of luciferase from reporter viruses to represent the relative infection of a panel of viruses from the alphavirus genus [306]. We found that infection by EEEV, VEEV, CHIKV, and SINV was attenuated in cells exposed to conditioned medium (Fig. 2.1B)[3]. Because we had previously shown that PHT-conditioned medium contained relatively high levels of antiviral C19MC miRNAs [275], we tested the antiviral activity of one of these miRNAs, miR-517-3p, shown previously to confer resistance to VSV, VV, and herpes simplex virus-1 (HSV-1). Indeed, we found that transfection of a miR-517-3p mimic significantly reduced infection of only CHIKV and SINV when compared to cells transfected with a scramble control miRNA (Fig. 2.1C).

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[2] Data related to rubella plaque assays was provided by Dr. Teryl Frey (Georgia State University, Atlanta GA)

[3] Data related to alphavirus infectivity assays was contributed to by Dr. Derek Trobaugh (University of Pittsburgh, Pittsburgh PA)

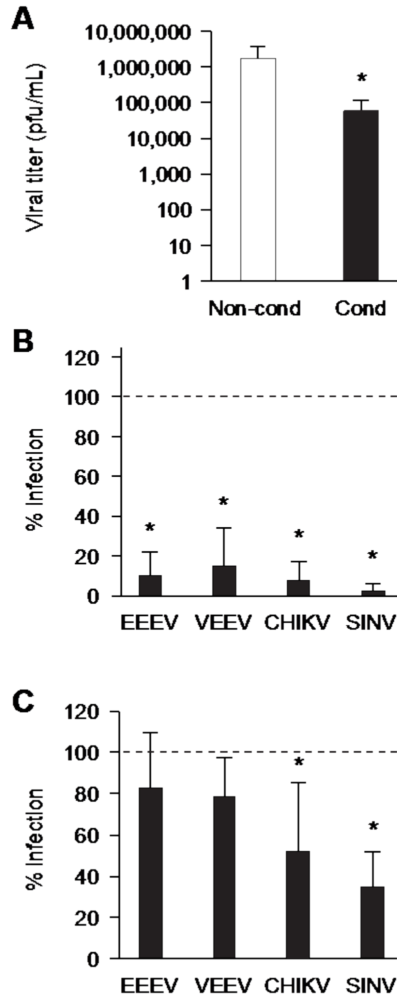

Figure 2-1 - PHT-conditioned medium or miRNA mimics from Chromosome 19 miRNA cluster attenuate infection of select togaviruses. (A) Log scale of Rubella virus titers from cells exposed to non-conditioned or conditioned PHT medium. Data are mean of three independent plaque assays, each run in duplicate. p <0.05 (Student's t test). Data related to figure 2-1A provided by Dr. Teryl Frey (Georgia State University, Atlanta GA) (B) Activity of alphavirus luciferase reporter constructs. Eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), Chikungunya virus (CHIKV), and Sindbis virus (SINV), expressing luciferase in Vero cells, were exposed to conditioned PHT or control non-conditioned medium. Data are presented as percent infection relative to control and represent a mean of three independent infections, each run in triplicate. p <0.0001 (ANOVA with Bonferroni correction). (C) EEEV, VEEV, CHIKV, and SINV luciferase expression in cells transfected with miR-517-3p or control scrambled mimic. Data are presented as percent infection relative to control and represent a mean of three independent infections, each run in triplicate.

p <0.0001 (ANOVA with Bonferroni correction). Data related to figure 2-1A and 2-1B partially contributed by Dr. Derek Trobaugh (University of Pittsburgh, Pittsburgh PA)

2.2.2 PHT-conditioned medium attenuates HIV-1 infection

To determine whether conditioned PHT medium could inhibit HIV-1 infection in non-trophoblastic cells, we used an HIV-1 infectivity assay, described in the materials and methods. We observed a significant reduction in Tat-induced luciferase expression in cells that were exposed to conditioned medium compared to cells exposed to non-conditioned medium (Fig. 2.2A). To investigate whether or not miR-517-3p was capable of conferring resistance to HIV-1, we transfected TZM-bl cells with scramble control miRNA, miR-517-3p, or miR-720, a non-C19MC miRNA expressed in trophoblasts. We observed a non-significant trend in reduction of HIV-1 reporter activity in cells transfected with miR-517-3p, but not with miR-720 (Fig. 2.2B).

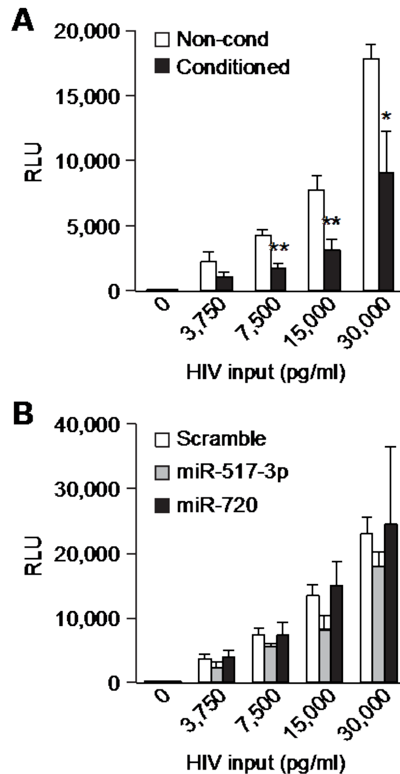

Figure 2-2 - PHT - conditioned medium inhibits HIV infection. (A) Relative luciferase activity (RLU) of an HIV Tat-inducible reporter in TZM-bl cells exposed to either conditioned PHT or control non-conditioned medium and infected with increasing concentrations of HIV, as described in Methods. Data are representative of three experiments, each performed in triplicate. * denotes p <0.05, and ** denotes p <0.001 (Student's t test). (B) Relative luciferase expression in TZM-bl cells transfected with miR-517-3p, miR-720, or control scrambled mimic and infected with increasing concentrations of HIV. Data are representative of three experiments, each performed in triplicate. None of the differences were statistically significant.

2.2.3 PHT conditioned medium or C19MC miRNA mimics attenuate VZV replication

Using either a VZV reporter virus that expresses luciferase after an immediate early varicella gene (ORF63) or a reporter virus expressing luciferase after a late VZV gene (ORF9), we found that pre-exposure to PHT conditioned medium only impacted the late viral gene reporter, but did not

significantly impact expression of the immediate early viral reporter (Fig. 4A). We next assessed the effect of miR-517-3p on VZV infection. Because of inefficient transfection of HFF cells with miRNA mimics, we transfected VZV permissive MeWo cells [307] with miR-517-3p, miR-720 (non-C19MC control), or scrambled miRNA mimics. We found that transfection of miR-517-3p, but not the control miRNAs, significantly reduced luciferase activity due to infection with VZVORF9 (Fig. 4B), albeit to a lesser extent than PHT conditioned medium.

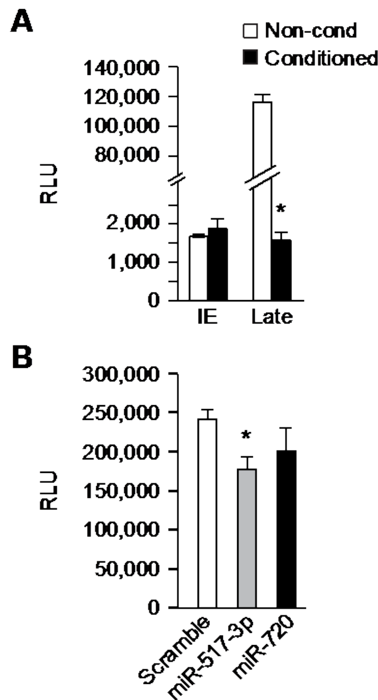

Figure 2-3 - PHT-conditioned medium and miRNA mimics from chromosome 19 miRNA cluster attenuate VZV. (A) Relative luciferase activity (RLU) from cells infected with either immediate early (IE) reporter virus, or "late" ORF9 reporter virus, exposed to either non-conditioned or conditioned PHT medium. Data are representative of three independent experiments, each performed in triplicate. p <0.001 (Student's t test. (B) Relative luciferase activity (RLU) in MeWo cells transfected with either miR-517-3p, miR-720, or scrambled mimic and infected with VZV_ORF9_Luc. Data are representative of three independent experiments, each performed in triplicate. p <0.05 (ANOVA with Bonferroni correction).

2.2.4 PHT conditioned medium has no effect on infection by the non-viral perinatal pathogens *Toxoplasma gondii* or *Listeria monocytogenes*

To determine whether conditioned PHT medium could inhibit *T. gondii* infection, we exposed HFF cells to either PHT conditioned or non-conditioned medium for 24 h and determined infection by YFP-expressing *T. gondii* [308]. We found that PHT conditioned medium had no effect on *T. gondii* infection (Fig. 5A) quantified by vacuole number and size (Fig. 5B-C). We next tested whether PHT conditioned medium could confer resistance to *L. monocytogenes* in Caco-2 cells. Bacterial burden was measured after 1.5, 5, and 10 h time points. Our results showed a lack of consistent reduction in *L. monocytogenes* burden (Fig. 6)[4].

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[4] Data related to *L. monocytogenes* assays was provided by Dr. Stephanie Seveau (Ohio State University, Columbus OH)

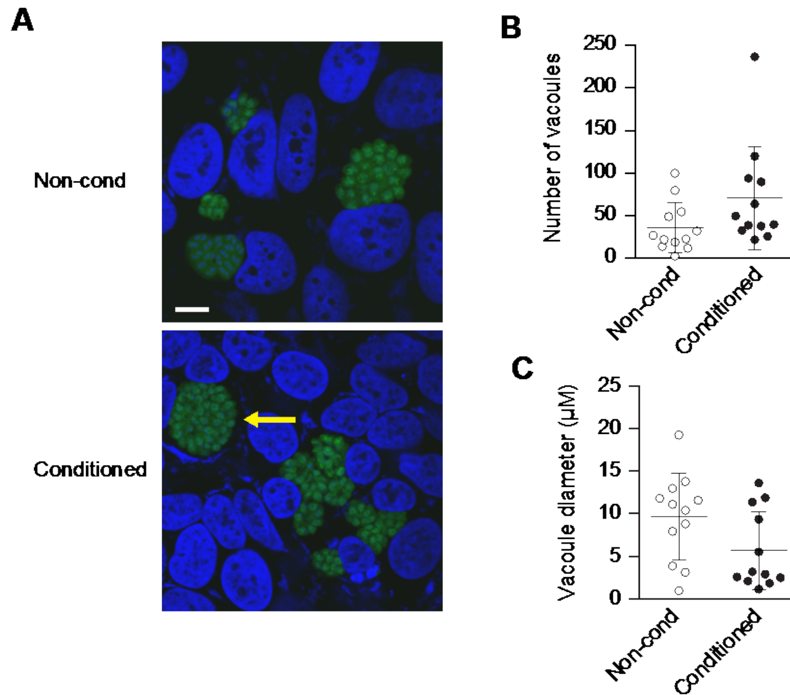

Figure 2-4 - PHT-conditioned medium has no effect on Toxoplasma gondii infection. (A) Representative micrographs showing fluorescent parasites within vacuoles in U2OS cells exposed to non-conditioned or conditioned PHT media. Arrow denotes parasitophorous vacuole. Scale bar =10 µM. (B) Vacuole number, or (C) vacuole size in cells exposed to conditioned or non-conditioned media. Quantities are the average of four fields per sample and were assessed via three independent experiments. None of the differences were statistically significant.

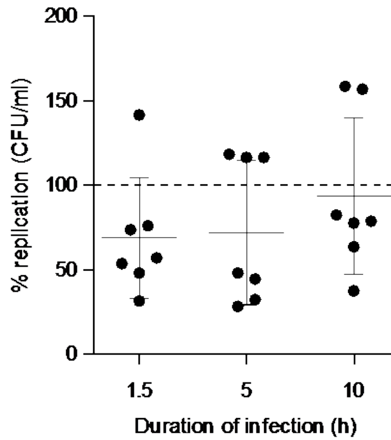

Figure 2-5 -Primary human trophoblast conditioned medium has no effect on Listeria Monocytogenes replication. Colony forming units for *L. monocytogenes* after infection of 1.5, 5, and 10 h, as detailed in Methods. Data are presented as percent replication of bacteria in cells exposed to conditioned medium compared to that of bacteria in cells exposed to non-conditioned medium. Data presented are the mean of three independent experiments. None of the differences were statistically significant. Data provided by Dr. Stephanie Seveau (Ohio State University, Columbus OH)

2.3 DISCUSSION/COMMENT

2.3.1 Study Design

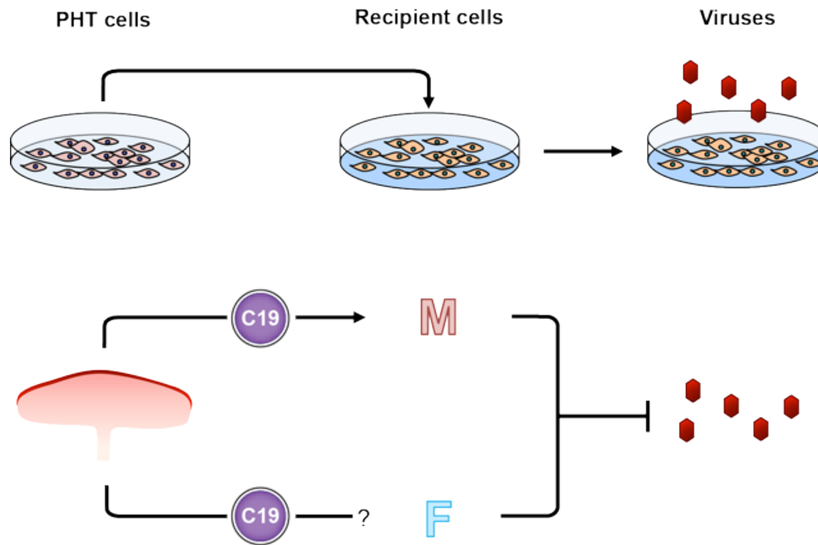

Figure 2-6 - Experimental design and Model. (A) Primary human trophoblasts (PHT cells) were collected from healthy singleton term placentas, and cultured for 48-72 hours. The conditioned medium from these cells was then transferred to non-trophoblast recipient cells and incubated for 24 h prior to infection. Infectivity assays were then performed to quantify relative pathogen infection between control non-conditioned and conditioned medium. (B) Proposed model of transmission for C19MC miRNAs packaged into exosomes. Exosomes are transferred from the placenta into maternal circulation (M), and can lead to subsequent inhibition of viral infection. It is unclear exosomes are transferred to the fetus in biologically meaningful amounts (F).

2.3.2 Principle findings and their meaning

We recently demonstrated that PHT cells are resistant to a diverse panel of viruses, including coxsackie virus B, poliovirus, VSV, vaccinia virus, HSV-1, and cytomegalovirus. Importantly, we found that this resistance could be conferred to non-trophoblast cells by exposing them to PHT conditioned medium and, to a lesser degree, to C19MC miRNAs [275]. Here we expanded our experiments to include viruses that are associated with human perinatal infection and to assess the effect of PHT conditioned medium on non-viral pathogens [5, 309]. We first tested rubella, a togavirus that crosses the placenta by hematogenous dissemination and causes congenital rubella syndrome [198]. We then broadened our studies to include other members of the togavirus family. The Old world alphaviruses, including CHIKV and SINV, cause febrile and arthritogenic disease in humans, while the New world alphaviruses, including EEEV and VEEV, can cause acute encephalitis in humans [310-312]. Infection by all four alphaviruses was significantly attenuated in cells pre-exposed to PHT conditioned medium. Interestingly, transfecting cells with miR-517-3p attenuated infection by the Old world, but not the New world, alphaviruses.

We also found that PHT conditioned medium attenuates infection by HIV-1, which is transmitted to the fetus predominantly during delivery [30], with less frequent antenatal transmission during invasive procedures such as amniocentesis [313]. In addition, we found that pre-exposure to PHT conditioned medium significantly reduced cell infection by VZV, the causative agent of chickenpox and a significant perinatal pathogen which causes the rare but devastating congenital varicella syndrome [314]. VZV gene expression occurs in a cascade, with immediate-early, early, and late viral genes being expressed in sequence. Interestingly, our data suggest that the effect of PHT conditioned medium is observed only on late viral genes during VZV infection. We previously observed inhibition of early gene expression in other large DNA

genome viruses, vaccinia virus and HSV-1, suggesting that the underlying mechanism of inhibition may depend on the viral life cycle and on the timing of infection during pregnancy.

We found no effect of PHT conditioned medium on infection by the protozoan *Toxoplasma gondii*, which is an important perinatal pathogen, that causes direct fetal organ damage.[315] Similarly, PHT conditioned medium had no consistent effect on infection by *L. monocytogenes*, a Gram-positive facultative intracellular pathogen that can cause miscarriage, stillbirth, or neonatal meningioencephalitis [316]. As syncytiotrophoblasts were previously shown to be resistant to colonization by *T. gondii* or by *L. monocytogenes,*[38, 218] our findings may indicate that PHT-conditioned medium induces a primarily antiviral state, while other mechanisms may drive resistance to bacterial or protozoan intracellular pathogens.

2.3.3 Strengths and weaknesses, and implications of findings

Our results, presented here and in our previous work [275], indicate that the conditioned medium from PHT cells is broadly antiviral, inhibiting infection by viruses known to cause perinatal infection in humans. This inhibition was observed for RNA and DNA viruses. Previously we showed that viral inhibition was, in part, due to induction of autophagy, as recipient cells exposed to PHT medium demonstrated increased autophagy with internalizing virions localizing to autophagosomes. Further, the antiviral effect was mitigated when autophagy was blocked [275, 317]. We previously showed that PHT conditioned medium contains high levels of miRNAs from the C19MC family that correspond to the expression level of these miRNAs in PHT cells [301]. Interestingly, our data show that the effect of C19MC miRNAs is markedly weaker than that of PHT conditioned medium [275], and not all viruses tested were sensitive to C19MC-associated

miRNAs previously shown to exert antiviral effects on VSV and HSV1. It is possible that expression of additional members of the C19MC miRNAs, or the entire miRNA cluster, might be needed for a more potent antiviral effect. In addition, a more effective miRNA delivery system (e.g., via exosomes) might potentiate the antiviral effect [275, 290]. This might be particularly relevant to VZV, in which infection might be enhanced by the initial stages of autophagy, but impaired by the complete, more effective autophagy process, which includes protein degradation [318].

Our data do not rule out additional mechanisms for the antiviral effect of trophoblastic conditioned medium. We previously reported [275] that cells rendered incapable of responding to type I interferon retain their antiviral activity upon exposure to conditioned medium. Nonetheless, PHT conditioned medium may contain non-classical interferons or additional factors (see below) that contribute to the antiviral state and exhibit an additive or synergistic effect with the miRNAs. Importantly, the lack of effect on infection with either *Listeria monocytogenes* or *Toxoplasma gondii* suggests that the PHT conditioned medium and, to an extent, the C19MC miRNAs, stimulate a selective viral-specific response in non-trophoblast cells. Given the diversity of the viruses tested, it is possible that this response may inhibit viral infection at different points of the viral life cycle. Future studies have been designed to address the mechanisms underlying the antiviral effect, and elucidate the targets of the C19MC and other antiviral factors potentially present in PHT conditioned media.

2.4 MATERIALS AND METHODS

2.4.1 Cells

Human osteosarcoma U2OS, human foreskin fibroblast (HFF), melanoma-derived cells (MeWo), and TZM-bl cells [319] were cultured in DMEM (Corning, USA) supplemented with 10% FBS (Sigma, USA) and antibiotics. HFF cells were provided by Jon Boyle, Department of Biological Sciences, University of Pittsburgh. Human epithelial cells (Caco-2 cells, ATCC HTB-37) were cultured in Eagle's MEM containing 20% heat-inactivated FBS and 10 U penicillin and streptomycin. Vero African green monkey kidney cells were maintained in DMEM supplemented with 5% FBS and antibiotics.

PHT cells were acquired from healthy singleton term placentas, using the procedure previously described [47], with modifications [320, 321]. Cells were maintained in DMEM containing 10% bovine growth serum (HyClone, USA), 20 mM HEPES, and antibiotics at 37°C. Cells were maintained 72 h after plating, with cell quality monitored morphologically and by human chorionic gonadotropin (hCG) levels (ELISA, DRG International, USA) in the medium, which show a characteristic increase as cytotrophoblasts differentiate into syncytiotrophoblasts [322].

2.4.2 Conditioned medium preparation

Conditioned medium samples were collected from PHT cultures as previously described, and only medium that demonstrated at least 70% reduction in vesicular stomatitis virus (VSV) infection was used for subsequent infectivity assays [275]. Briefly, U2OS cells were exposed to conditioned or

non-conditioned PHT medium for 24 h, and infected with VSV at a multiplicity of infection (MOI) of 1 for 6 h or until cytopathic effect was evident. Cells were then lysed with 1mL Qiazol lysis reagent, and infection was quantified by RT-qPCR, as previously described [275].

2.4.3 Rubella plaque assays

Vero cells were pre-exposed to either conditioned or non-conditioned medium and infected with rubella virus at an MOI of 10. Plaque assays were performed with serial dilution of the virus. One milliliter of each dilution and a control (PBS/1% FBS) were plated on 30 mm plates confluent with Vero cells, and the plates were incubated for 1 h at 37°C. Cells were overlaid with a liquid agar solution (60 mL 0.4% liquid agar, 34 mL 3X MEM, 1 mL FBS, 3 mL 5% $NaHCO_3$, 0.1 mL penicillin/streptomycin and 0.1 mL diethylaminoethanol). The plates were incubated for 7 days at 37°C. After incubation, the agar was removed, and the plates were stained with crystal violet solution to reveal plaques. Duplicate plaque assays were performed for each infection, and the final titer of each infection was the average of the two plaque assays.

2.4.4 Alphavirus Luciferase assays

Vero cells were pre-exposed to either conditioned or non-conditioned media for 24 h prior to infection. Reporter alphaviruses that expressed a cleavable firefly luciferase in between the capsid and PE2 proteins (as described in [306] were added to the cells at an MOI of 0.1 for 8 h and lysed with 1X Passive lysis buffer (Promega). Infection was quantified by measuring luciferase expression and normalized to protein level. Alphavirus constructs included eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV), chikungunya virus

(CHIKV), and sindbis virus (SINV). These virus constructs were also used to infect U2OS cells transfected with miRNA mimics from the C19MC, as described below. These experiments were conducted with an MOI of 1 for EEEV, VEEV, and CHIKV while SINV was used at an MOI of 0.1.

2.4.5 HIV infectivity assay

Utilizing the TZM-bl cell line, a HeLa cell derivative that are CD4+ and expresses CCR5 and CXCR4, as well as a Tat-inducible luciferase reporter, HIV-1 infectivity was quantified on the basis of relative luciferase expression [319, 323]. These cells, which are permissive to wild type HIV, were pre-exposed to PHT-conditioned media or non-conditioned media for 24 h prior to infection. HIV-1 NL4-3 was added at increasing concentrations, indicated in the figure legend. After 48 h, infected cells were washed with PBS and lysed in luciferase lysis buffer (Promega, USA) by rocking for 15 min. Lysates (40 µl) were transferred to white 96-well plates, and 50 µl luciferase reagent (Promega) was injected into each well. Luciferase activity was determined by detection of luminescence recorded by Synergy 2 SL luminescence microplate reader (BioTek, VT, USA).

2.4.6 VZV Luciferase assays

Varicella zoster virus (VZV) infectivity was measured using reporter viruses expressing firefly or renilla luciferase reporter enzymes respectively. Recombinant viruses were developed from a VZV BAC using methods detailed previously [324]. The luciferase gene was placed directly downstream of either the immediate early gene encoding the latency associated regulatory protein

IE63 or the late VZV ORF9 gene encoding the abundant VZV tegument protein at the native locus, and was expressed as bi-cistronic mRNAs with a T2A ribosome skipping motif (MB Yee and PR Kinchington, manuscript in preparation.). VZV permissive HFF cells were pre-exposed to conditioned or non-conditioned media for 24 h. Cells were then infected with 1000 pfu/mL of either VZVORF9Luc or VZVORF63Luc vector for 48 h. Cells were lysed, and luciferase expression was quantified as described above.

2.4.7 Fluorescence microscopy

Toxoplasma gondii infection was quantified using a yellow fluorescent protein (YFP)-tagged RH strain provided by David Roos, University of Pennsylvania, as previously described [308]. HFF cell monolayers were cultured in eight-well chamber slides (Nunc Lab-Tek, Thermo-Fisher, USA) at 37°C, 5% CO_2. Either conditioned or non-conditioned medium was added to the cells 24 h prior to infection. *Toxoplasma gondii* RH-YFP was added at an MOI of 0.5. Forty-eight h post-infection, cells were washed and fixed with 4% paraformaldehyde in PBS and permeabilized with 0.25% Triton X-100 in PBS. Fixed monolayers were then mounted with Vectashield (Vector Laboratories, USA) containing DAPI. Images were captured with an Olympus FluoView 1000 laser scanning confocal microscope. Parasitophorous vacuole number and size, measured by region of interest length (uM), were quantified using Image J software (NIH, USA).

2.4.8 Listeria infectivity assay

Wild type *Listeria monocytogenes* (DP10403S) was cultured overnight at 37°C in brain and heart infusion (BHI). The following day, bacteria were diluted (1/20) in BHI and grown at 37°C until

OD 600 nm reached 0.7–0.8. Bacteria were washed three times with PBS and suspended in MEM at the indicated MOI. Caco-2 cells were plated in 24-well cell culture plates at a density of 40,000 cells/well on glass coverslips coated with rat tail collagen. After 24 h, the cell culture medium was replaced with 500 μL conditioned or control medium, in triplicate, for 24 h. Caco-2 cells were washed with MEM and infected with *L. monocytogenes* at the indicated MOI. The bacterial suspension (500 μL MEM) was added to each well, and the cell culture plate was centrifuged at 1,500 rpm for 2 min at room temperature. Caco-2 cells and bacteria were then co-incubated for 30 min at 37°C, followed by two washes with warm MEM. Caco-2 cells were further incubated for the indicated times in the presence of conditioned or control medium supplemented with 15 μg/mL gentamicin [325, 326]. Cells were washed three times with warm PBS. A volume of 300 μL PBS containing 0.2% Triton X-100 was added to each well to lyse the Caco-2 cells. Cell lysates were diluted and plated on BHI agar plates to enumerate bacterial colony forming units.

2.4.9 Mimic transfections

Mimics for C19MC miRNAs (miRIDIAN) as well as a non-targeting control miRNA mimic were obtained from Thermo-Fisher USA as previously described [275]. U2OS cells were reverse transfected with miRNA mimics or miRNA mimic control (final concentration, 25 nM for each miRNA mimic), using DharmaFECT-1 transfection reagent (Thermo-Fisher) according to the manufacturers' instructions. Cells were assayed 48 h post-transfection.

2.4.10 Statistics

Experiments were performed at least three times as indicated in the legend of each figure. Data are presented as mean ± SD. Except where specified, Student's t-test was used to determine statistical significance for virus infections when two sets were compared, and one-way analysis of variance (ANOVA), with Bonferroni's correction for post hoc analysis of multiple comparisons, was used to determine statistical significance for reporter gene assays; $p < 0.05$ was considered significant.

2.4.11 Ethical approval

PHT cells were cultured from term or near-term placentas collected by the Obstetrical Specimen Procurement Unit at Magee-Womens Hospital of the University of Pittsburgh Medical Center. Sample collection was conducted according to a protocol approved by the Institutional Review Board of the University of Pittsburgh. All specimens were de-identified.

3.0 TYPE III INTERFERONS PRODUCED BY HUMAN PLACENTAL TROPHOBLASTS CONFER PROTECTION AGAINST ZIKA VIRUS INFECTION[5]

Avraham Bayer[6], Nicholas J. Lennemann[6], Yingshi Ouyang, John C Bramley, Stephanie Morosky, Ernesto Torres De Azeved Marques, Jr., Sarah Cherry, Yoel Sadovsky, and Carolyn B. Coyne.

Data for figure 3-3D was provided by Stephanie Morosky. Data for figure 3-3E, F, G and figure 3-6B was provided by Nicholas Lennemann. Data for figure 3-5A and C was provided by Carolyn Coyne. Data for figure 3-6C was provided by John Bramley.

3.1 INTRODUCTION

In eutherian organisms, the placenta acts as a physical and immunological barrier between the maternal and fetal compartments, and protects the developing fetus from the vertical transmission of viruses. In the human hemochorial placenta, the frontline of fetal protection are the syncytiotrophoblasts, which cover the surfaces of the human placental villous tree and are directly bathed in maternal blood following the establishment of the maternal circulatory system during the later stages of the first trimester.

The mechanisms by which viruses can be transmitted vertically are multifaceted, and can involve entry into the gestational sac via direct hematogenous spread, trophoblastic transcellular

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[6] Co-first author

or paracellular pathways, transport within immune cells or infected sperm, pre-pregnancy uterine colonization, introduction during invasive procedures during pregnancy, and/or transvaginal ascending infection. The emerging ZIKV pandemic poses a new threat to the developing fetus. While usually causing relatively mild symptoms in non-pregnant individuals, ZIKV infection in Brazil has been associated with increased incidence of microcephaly [33, 327-329]. In addition, ZIKV infections have also been associated with other disorders such as placental insufficiency and fetal growth restriction, ocular disorders, other CNS anomalies, and even fetal death [32, 209].

While direct evidence for a causal relationship between ZIKV infections and the development of abnormal pregnancy outcomes is still emerging, recent reports have directly identified the presence of viral RNA and infectious virus in the placentas, amniotic cavity and brains of fetuses that had developed fetal anomalies [208, 330, 331]. Interestingly, other flaviviruses, such as dengue virus (DENV), which is endemic in the regions of Brazil most impacted by the recent ZIKV outbreak, have not been associated with microcephaly or other congenital disorders, suggesting that ZIKV may exhibit unique mechanism(s) to directly infect and/or bypass the placental barrier, and access the fetal compartment and cause organ-specific damage.

The innate immune system is a primary host defense strategy to suppress viral infections and converges on the induction of interferons (IFNs), which function in autocrine and paracrine manners to upregulate a cadre of other genes, known as interferon stimulated genes (ISGs). The effects of IFNs and ISGs are potent and wide-ranging; they are pro-inflammatory, enhance adaptive immunity, and are directly antiviral [332]. In most cell types, type I IFNs, which include IFNα and IFNβ, are the primary IFNs that are generated in response to viral infections. In contrast, cells of epithelial origin mount antiviral responses primarily mediated by type III IFNs, which

include IFNλ1-4 (also known as IL-29, IL-28A-C) [150]. The role of IFN signaling in the protection of placental trophoblasts from viral infections is unclear. Previous work has pointed to unidentified IFN(s) present in first trimester human placentas [333]. Ruminants express IFNτ at various stages of gestation [334], and the mouse placenta can produce IFNλs in response to *Listeria monocytogenes* infection [335].

Here we show that primary human trophoblast (PHT) cells, isolated from full-term placentas, are refractory to infection by two strains of ZIKV, one derived from an African lineage, and one derived from an Asian lineage that exhibits >99% amino acid sequence similarity to strains currently circulating in Brazil [336]. We also found that conditioned medium isolated from PHT cells protected non-trophoblast cells from ZIKV infection through the constitutive release of the type III IFN IFNλ1. Our findings thus suggest that for ZIKV to infect syncytiotrophoblasts, it must overcome the restriction imparted by IFNλ1 and other syncytiotrophoblast-specific antiviral factors, and/or gain access to the fetal compartment by a mechanism that does not involve syncytiotrophoblast infection, at least in the later stages of pregnancy.

3.2 RESULTS

3.2.1 PHT cells produce an ISG inducing factor

Using microarrays, we found that exposure of human fibrosarcoma HT1080 (2fTGH) cells to PHT CM induced a subset of previously characterized ISGs [87], which did not occur in HT1080 cells with defective signal transducer and activator of transcription 1 (STAT1; 2fTGH-U3A cells) signaling [337] (Figure 3-2A and Table 2-1). We obtained similar results when cells were treated

with IFN□ (Figure 3-2A), as previously described [338]. We confirmed these results by RT-qPCR in human osteosarcoma U2OS cells that were exposed to PHT CM, which led to the robust induction of two known ISGs, interferon induced protein 44 like (IFI44L) and interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) (Figure 3-2B) and in human monocyte THP-1 cells as determined by an interferon regulatory factor (IRF)-inducible SEAP reporter assay (Figure 3-5A)[7]. In addition, using datasets from previously published RNASeq studies from our laboratory [46], we found that PHT cells express high levels of ISGs (Figure 3-2C). In contrast, the trophoblast cell line JEG-3 did not endogenously express ISGs (Figure 3-2C) and CM isolated from these cells did not induce ISGs in non-placental recipient cells (Figure 3-5B).

In order to identify the ISG inducing factor present in our conditioned medium, we utilized an unbiased biochemical approach. Using gel filtration chromatography, we fractionated medium conditioned on PHT cells, which was confirmed to be potently antiviral, as well as robustly induce ISGs. We observed a single absorbance peak, which corresponded with elution fractions containing ISG inducing activity, measured by IFI44L induction using qPCR (Figures 3-1A and 3-1C). These fractions were then further resolved using anionic exchange chromatography (MonoQ column), and we observed multiple peaks, with only one corresponding to ISG induction (Figure 3-1B and 3-1C). We next applied conditioned medium, pooled fractions from the gel filtration column, and two fractions from the anionic exchange column, corresponding to no ISG inducing activity and ISG inducing activity (MonoQ5 and MonoQ7, respectively) to an SDS-PAGE gel, and silver-stained for proteins (Figure 3-1D). We observed a band unique to fractions that contained ISG inducing activity, which ran at 25 kd, consistent with the size of IFNλ.

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[7] Data provided by Dr. Carolyn Coyne (University of Pittsburgh, Pittsburgh PA)

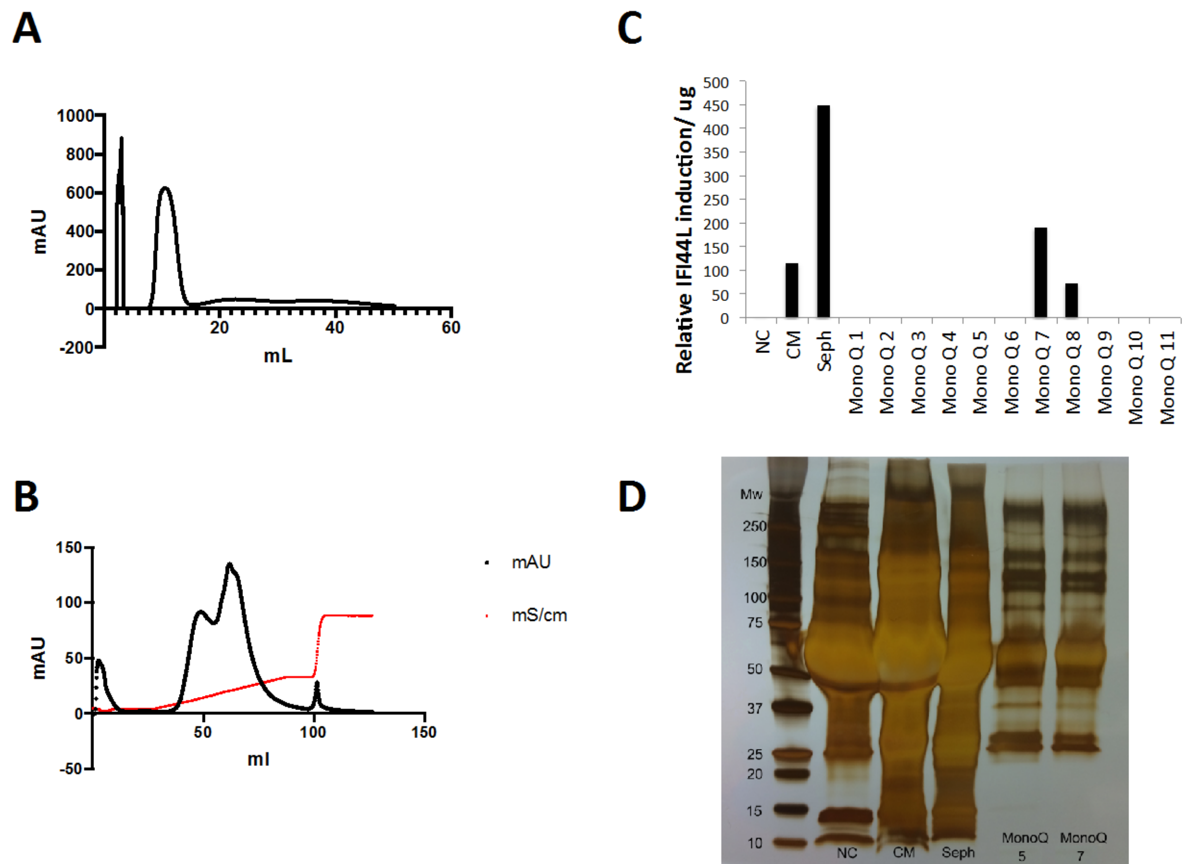

Figure 3-1 – Biochemical isolation of ISG inducing ligand in conditioned medium. (A) PHT conditioned medium was loaded onto an HPLC S300 sepharose column equilibrated with PBS, and eluted into 5mL fractions. Protein absorbance was measured at 280nm. The peak at 0-10 mL corresponds with the void fraction (proteins too large to enter into the dextran beads. >300kd). (B) Pooled sepharose fractions were loaded onto a MonoQ anionic exchange column, and eluted into 2mL fractions, with increasing concentrations of NaCl (Up to 1M). Protein absorbance was measured at 280nm. Conductivity was measured in mS/cm. (C) IFI44L induction, normalized the amount of protein present in each fraction. Conditioned medium and pooled S300 fractions exhibited robust induction. Only fractions 7 and 8 from the MonoQ column demonstrated ISG induction. (D) Non-conditioned medium, conditioned medium, elutions from the S300 sepharose column, and two fractions from the MonoQ anionic column were run on an SDS-PAGE and then silver stained. MonoQ fractions were less complex in composition, with fraction 7 having a distinct band at approximately 25 kd.

During culturing *in vitro*, PHT cells undergo fusion to form syncytiotrophoblasts (Figure 3-5C)[6] similar to their natural differentiation process *in vivo*, which can be inhibited by exposing the cultures to DMSO [339]. We found that attenuation of PHT differentiation by DMSO reduced the ability of PHT CM to induce IFI44L in recipient cells (Figure 3-2D). Consistent with a role for syncytiotrophoblast fusion in the induction of ISGs, we found that exposure of PHT cells to epidermal growth factor (EGF), which promotes cell-cell fusion of trophoblasts [340], enhanced the ISG inducing properties of PHT CM (Figure 3-2E). Importantly, ISG induction in recipient cells was specific for PHT CM and did not occur when cells were exposed to CM from the trophoblast-derived cell lines BeWo, JEG-3, JAR, or HTR8 cells, suggesting that this induction is specific for CM derived from primary trophoblasts (Figure 3-2E and Figure 3-6B). Furthermore, although BeWo cell fusion can be stimulated by forskolin treatment [41], this treatment did not confer ISG-inducing properties to BeWo CM (Figure 3-2F), suggesting that cell-cell fusion alone is not sufficient to confer ISG inducing properties to trophoblasts. Lastly, we previously showed that PHT-derived exosomes released into PHT CM mediated some of the antiviral properties of PHT CM [275]. We found that CM depleted of vesicles was still capable of inducing ISGs in recipient cells (Figure 3-6D), indicating that an ISG-inducing pathway is present in PHT CM and bestows antiviral properties independently from PHT-derived exosomes.

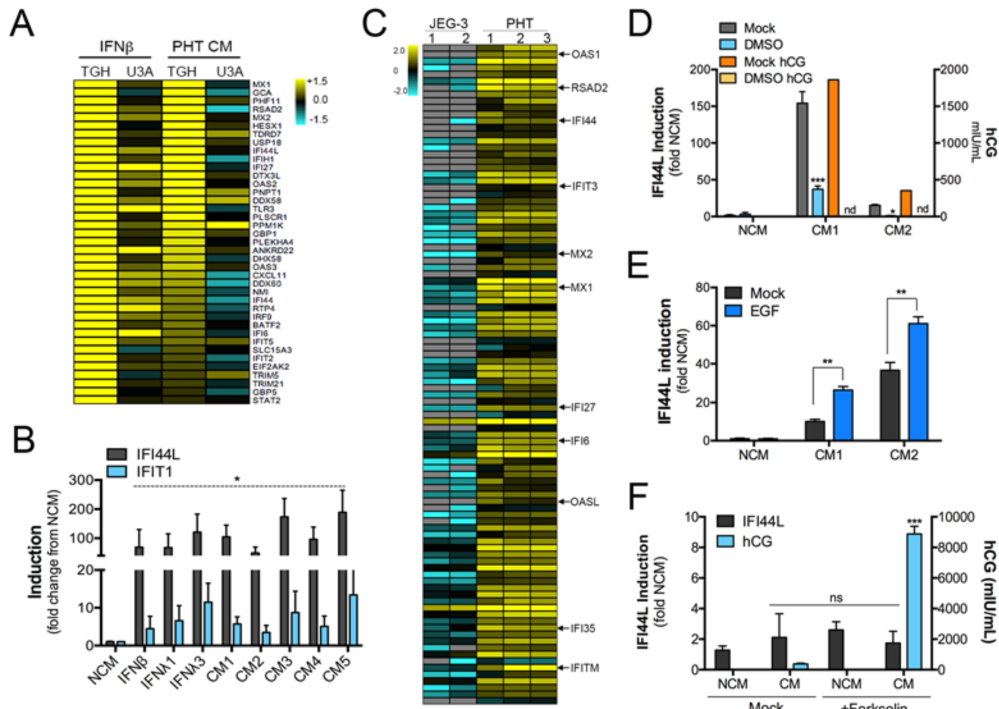

Figure 3-2 - conditioned medium from PHT cells induces ISGs. (A), A heat map of interferon stimulated genes (ISGs) differentially expressed between control (TGH) or STAT1 signaling deficient (U3A) HT1080 cells exposed to purified IFNλ or PHT CM for 24hrs. (B), RT-qPCR analysis for IFI44L or IFIT1 in U2OS cells exposed to control PHT non-conditioned medium (NCM) or five independent preparations of PHT CM. Data are shown as a fold change from NCM. (C), Heat map of differentially expressed interferon stimulated genes (ISGs) between two cultures of JEG-3 cells and a single preparation of PHT cells as assessed by RNASeq (p<0.05). Data related to 3-2C was provided by Dr. Carolyn Coyne (University of Pittsburgh, Pittsburgh PA) (D), Two preparations of PHT cells were exposed to DMSO to inhibit cell fusion, CM collected, and then IFI44L induction assessed by RT-qPCR (left y axis). In parallel, the levels of human chorionic gonadotropin (hCG) were determined by ELISA (right y axis). (E), BeWo cells were exposed to forskolin to induce fusion, CM collected, and ISG induction in CM-exposed cells assessed by RT-qPCR (for IFI44L, left y axis). In parallel, the levels of hCG were assessed by ELISA (right y axis). (F), In (B), (D-F), data are shown as mean ± standard deviation (*p<0.05, **p<0.01, ***p<0.001, ns not significant). The color intensity in (A) and (C) indicates the level of gene expression (yellow for up-regulation and blue for down-regulation), and grey indicates that no transcripts were detected in that sample.

3.2.2 PHT cells release the type III IFN IFN□1

We found by ELISAs that PHT CM contained negligible levels of IFNβ that were comparable to those in control non-CM, but contained IFNλ1, and to a lesser extent, IFNλ2, which was detected in one PHT preparation (Figure 3-3A). In addition, PHT cells expressed high levels of IFNλ1 mRNA (Figure 3-3B)[8], which were consistent with the levels induced in non-PHT cells (HBMEC) transfected with the synthetic ligand polyinosinic-polycytidylic acid (poly I:C) to induce IFN production (Figure 3-3B)[9]. In addition, we found that anti-IFNλ1/2 neutralizing antibodies partially inhibited the induction of the ISG IFI44L by PHT CM (Figure 3C). Furthermore, although CM isolated from uninfected trophoblast-derived cell lines did not contain detectable levels of IFNλ1 (Figure 3-6A), we found that these cells potently induced type III IFNs, primarily IFNλ1, in response to infection by Sendai Virus (SeV, Figure 3-3D)[8] and by both DENV and ZIKV (Figure 3E)[10]. In contrast, PHT cells did not induce IFNλ1 or the ISG 2'-5'-Oligoadenylate Synthetase 1 (OAS1) in response to ZIKV, or DENV, infection, yet were highly resistant to infection when compared to JEG-3 cells (Figure 3-3F and Figure 3-6B)[9]. However, PHT cells do induce both IFNλ1 and ISGs in response to toll like receptor 3 (TLR3) stimulation by poly I:C (Figure 3-7C). Finally, we found that RNAi-mediated silencing of a subunit of the type III IFN receptor (IL28RA) partially restored ZIKV infection in recipient cells exposed to PHT CM depleted of vesicles (Figure 3-3G)[9]. Collectively, these data point to a direct role for type III IFNs, particularly IFNλ1, in the antiviral signaling of placental syncytiotrophoblasts to viral infections, including ZIKV.

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[8] Data provided by John C Bramley (University of Pittsburgh, Pittsburgh PA)
[9] Data provided by Stephanie Morosky (University of Pittsburgh, Pittsburgh PA)
[10] Data provided by Dr. Nicholas Lennemann (University of Pittsburgh, Pittsburgh PA)

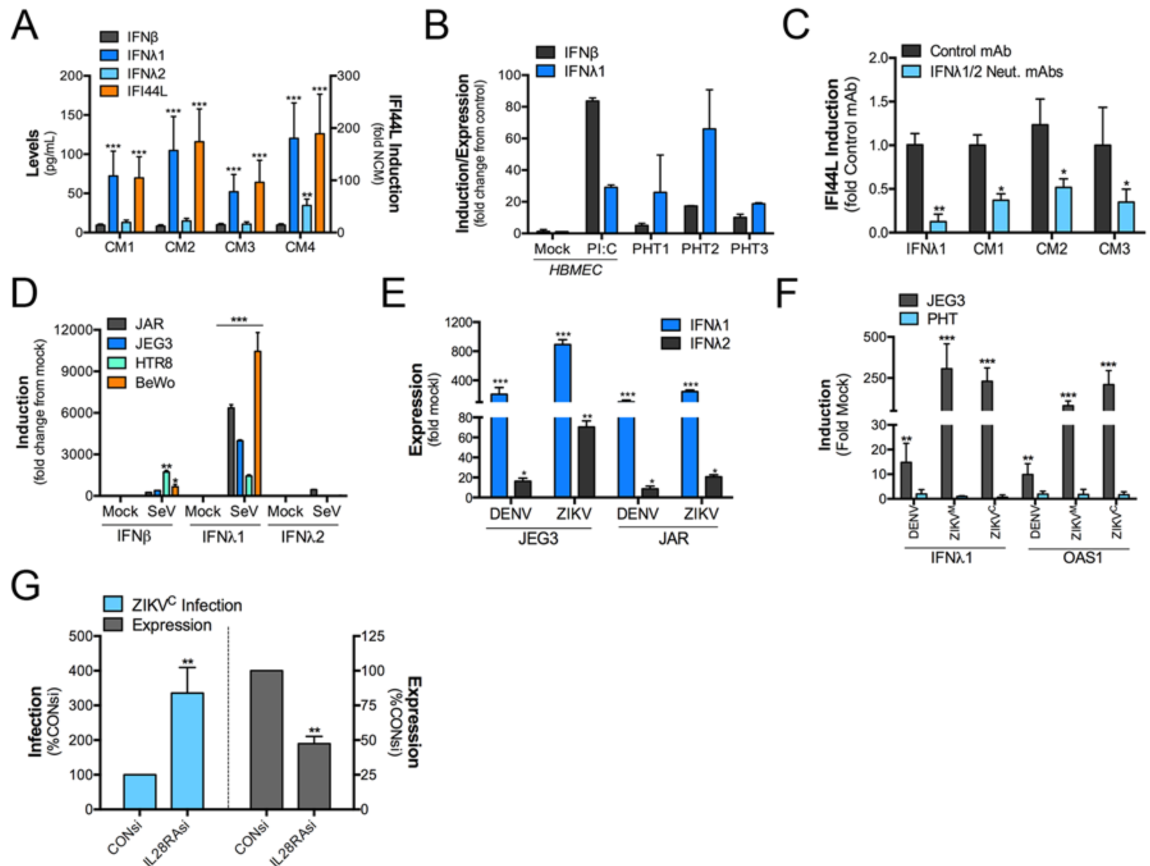


Figure 3-3 - Conditioned medium from PHT cells contains IFNλ1, which is required for ISG induction. (A), ELISA for IFNβ, IFNλ1, and IFNλ2 in four independent PHT CM preparations (left y axis). In parallel, the extent of ISG induction in each sample was determined by RT-qPCR for the levels of IFI44L induced in U2OS cells exposed to the sample (right y axis). (B), The levels of IFNβ and IFNλ1 mRNA in three preparations of PHT cells was assessed by RT-qPCR. In parallel, IFNβ and IFNλ1 mRNA levels were determined in mock-treated HBMEC, or in HBMEC exposed to 10µg poly I:C ('floated' in the medium) for ~24hrs. Data are shown as a fold change from mock-treated HBMEC cells. Data related to 3-3B was provided by Stephanie Morosky (University of Pittsburgh, Pittsburgh PA) (C), Level of ISG induction (as assessed by IFI44L RT-qPCR) in U2OS cells exposed to purified IFNλ1, or to three preparations of PHT CM incubated with a non-neutralizing monoclonal antibody (MOPC21) or anti-IFNλ-1-3 neutralizing antibodies. (D), RT-qPCR for IFNβ, IFNλ1, or IFNλ2 in indicated trophoblast cell lines infected with Sendai virus (SeV) for ~24hrs. Data related to figure 3-3D provided by Stephanie Morosky (University of Pittsburgh, Pittsburgh PA) (E), RT-qPCR for IFNλ1 or IFNλ2 in the indicated trophoblast cell lines infected with DENV or ZIKV^{M} for ~24hrs. (F), RT-qPCR for

IFN□1 or OAS1 in JEG-3 or PHT cells infected with DENV, ZIKV^{M}, or ZIKV^{C} for ~24hrs. (G), ZIKV^{C} infection in HBMEC transfected with control siRNA (CONsi) or IL28RA siRNAs and exposed to PHT conditioned medium depleted of vesicles for ~24hrs prior to infection. In all panels, data are shown as mean ± standard deviation (*p<0.05, **p<0.01, ***p<0.001, ns not significant). Data related to figure 3-3(E-G) provided by Dr. Nicholas Lennemann (University of Pittsburgh, Pittsburgh PA)

3.3 DISCUSSION

The strong association between ZIKV infection in pregnant women and the development of fetal growth restriction and/or CNS and other fetal congenital abnormalities, in addition to the positive culture of ZIKV from feto-placental tissues of affected pregnancies, suggest that ZIKV is capable of gaining access into the intrauterine cavity to directly affect fetal development. Our work presented here suggests that ZIKV is unlikely to access the fetal compartment by direct replication in placental syncytiotrophoblasts, as least in the later stages of pregnancy, unless ZIKV can bypass the antiviral properties of type III IFNs and other syncytiotrophoblast-derived antiviral pathways. Because we observed potent protection from ZIKV infection by type III IFNs, specifically IFNλ1, which is constitutively produced by syncytiotrophoblasts, it likely functions in an autocrine manner to protect these cells from viral infections. In addition, we show that trophoblast-derived IFNλ1 protects non-placental cells from ZIKV infection in a paracrine manner. A schematic of the human placenta and the mechanisms by which IFN□λ1 protects syncytiotrophoblasts from ZIKV infection is shown in Figure 3-4. Our work thus provides evidence that ZIKV may not directly infect placental villous syncytiotrophoblasts during the later stages of pregnancy, suggesting instead that the virus must either evade the potent type III IFN antiviral signaling pathways

generated by these cells and/or bypass these cells through an as-yet-unknown pathway to gain access to the fetal compartment.

Our previous studies implicated a role for trophoblast-specific miRNAs associated with the placental-specific chromosome 19 miRNA cluster (C19MC), contained within PHT-derived exosomes, as part of the antiviral arsenal secreted by PHT cells [275, 341]. Indeed, our work presented here demonstrates another facet of the antiviral mechanisms utilized by PHTs to protect the developing fetus. These potent antiviral pathways likely function in parallel to provide multiple mechanisms to protect syncytiotrophoblasts, and other cell types at the maternal-fetal interface, from ZIKV, and other viral infections. It is also possible that other as-yet-undiscovered pathways intrinsic to placental trophoblasts provide additional pathways to protect these cells from viral infections. While we have not been able to reliably measure IFNλ in the plasma of pregnant women, this may be because IFNλ is below the limits of detection in the expanded plasma volume of pregnant women and/or that the effects of IFNλ are local, affecting trophoblastic and non-trophoblastic placental cells (such as villous fibroblasts) in the immediate vicinity of the feto-placental unit.

Type III IFNs share significant structural homology with members of the IL-10 cytokine family [135], but induce ISGs similar to type I IFNs [125] through a distinct receptor [126]. We found that PHT cells expressed high levels of IFNλ1. Remarkably, IFNλ1 was constitutively released from PHT cells and did not require the activation of antiviral innate immune signaling pathways to become induced. Thus, in addition to studies that implicate an important role for type III IFNs in antiviral signaling in the respiratory and gastrointestinal tracts and the blood-brain barrier [150], our work directly points to a role for type III IFNs, specifically IFNλ1, in antiviral signaling at the maternal-fetal interface. Although type I IFNs are conserved between mice and

humans, there is significant divergence in the type III IFN pathway, where humans express IFNλ1-4 and mice express only IFNλ2 and IFNλ3. PHT cells expressed IFNλ2 at significantly lower levels than IFNλ1 and did not express mRNA for either IFNλ3 or IFNλ4. Thus, in addition to the morphological differences between the human and mouse placentas [342], these data suggest that the IFNλ1-mediated antiviral properties of placental syncytiotrophoblasts may be distinct between humans and mice, which may complicate the use of the mouse placenta as a model for viral infections of the placenta during human pregnancy.

Another important implication of our work is that cells that do not express the type IIII IFN receptor, or do not respond robustly to type III IFNs, may be more susceptible to ZIKV infection, particularly at the maternal-fetal interface. In mice, the expression of the alpha subunit of the IFN-lambda receptor (IL-28RA) is restricted to epithelial-derived cells, which respond most robustly to type III IFNs [343]. Recent evidence also supports a role for type III IFNs in the microvascular endothelium comprising the blood brain barrier [148]. Because syncytiotrophoblasts and other trophoblasts are epithelial cells, and are likely protected by the potent stimulation of ISGs in response to their constitutive production of IFNλ1, our data suggest that ZIKV may invade the intrauterine cavity by mechanisms that are distinct from direct infection of villous trophoblast. In addition to the trophoblast cell layers, the human placenta is also composed of mesenchymal cells, placental-specific macrophages (termed Hofbauer cells), and fibroblasts, located within the villous core between trophoblasts and fetal vessels. These cell types may exhibit differences in their responsiveness to IFNλs. It addition, it is also possible that the less differentiated, first trimester trophoblasts as well as extravillous trophoblasts are more permissive than late pregnancy villous trophoblasts to ZIKV infection and/or the antiviral effects of IFNλs. Finally, it is possible that the levels of IFNλ1 vary throughout pregnancy, or between individuals, which could markedly impact

the ability of the virus to infect the syncytiotrophoblast cell layer at specific times during pregnancy, or in specific individuals

The rapidly emerging human health crisis associated with the ZIKV epidemic highlights the growing need to identify mechanisms by which ZIKV accesses the fetal compartment. These data will be instrumental in order to design therapeutic measures to limit ZIKV replication and/or spread. Our experimental cell system is directly relevant to the study of congenital ZIKV infections, defining unique antiviral mechanisms at play in this specialized environment. We provide evidence that ZIKV is unlikely to access the fetal compartment by its direction infection of late pregnancy villous syncytiotrophoblasts, and potentially neighboring cells that express INFLR, due to the role of type III IFNs in the antiviral defense produced by villous trophoblasts, and suggests that the virus may circumnavigate these cells or overcome this restriction *in vivo* in order to bypass the placental barrier.

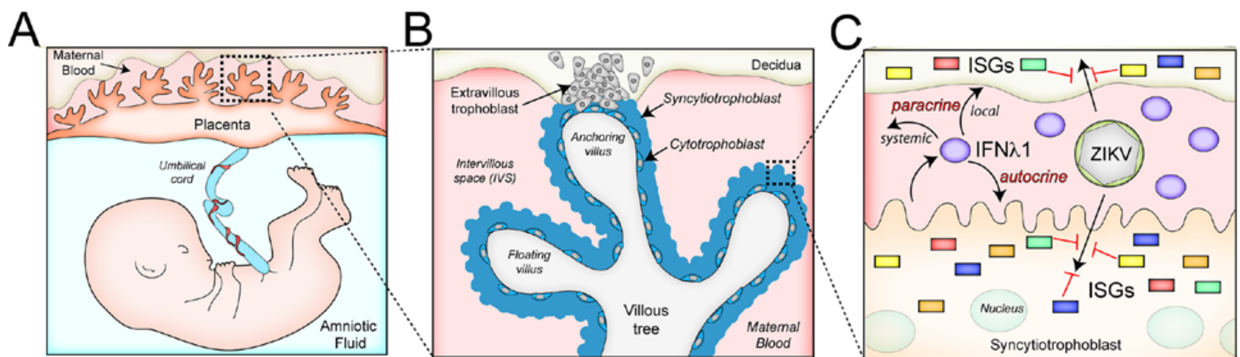


Figure 3-4 - Schematic depicting the structure of the human placenta and the role of IFNλ1 in protecting against ZIKV infection. (A), The intrauterine environment during human pregnancy. Embryonic structures include the villous tree of the human hemochorial placenta and the umbilical cord, which transfers blood between the placenta and the fetus. (B), An overview of a single placental villus. Extravillous trophoblasts invade and anchor the placenta to the maternal decidua and to the inner third of the myometrium. The villous tree consists of both floating and anchoring villi. Multinucleated syncytiotrophoblasts overlie the surfaces of the villous tree and are in direct contact with maternal blood (which fills the intervillous space (IVS) once the placenta is fully formed.

Mononuclear cytotrophoblasts are subjacent to the syncytiotrophoblasts and the basement membrane of the villous tree, and serve to replenish the syncytiotrophoblast layer throughout pregnancy. (C), In the work presented here, we show that syncytiotrophoblasts release IFN□1 that can act in both autocrine and paracrine manners to induce ISGs, which protect against ZIKV, and other virus, infections. The paracrine function of IFN□ could work locally within the direct maternal-fetal compartment, or might circulate more systemically to act on other maternal target cells. Schematic created by Dr. Carolyn Coyne (University of Pittsburgh, Pittsburgh PA)

3.4 MATERIALS AND METHODS

3.4.1 Culture of primary human trophoblasts

PHT cells were isolated from healthy singleton term placentas using the trypsin-DNAse-dispase/Percoll method as described [47], with previously published modifications under an exempt protocol approved by the institutional review board at the University of Pittsburgh. Patients provided written consent for the use of de-identified and discarded tissues for research purposes upon admission to the hospital. Cells were maintained in DMEM (Sigma) containing 10% FBS (HyClone) and antibiotics at 37°C in a 5% CO_2 air atmosphere. Cells were then maintained for 72 h after plating, with cell quality ensured by microscopy and production of human chorionic gonadotropin (hCG), determined by ELISA (DRG international). The cells exhibited a characteristic increase in medium hCG levels as the cytotrophoblasts differentiated into syncytiotrophoblasts.

3.4.2 Cells and viruses

Human osteosarcoma U2OS cells, Vero cells, 2fTGH (STAT1 wild-type) and U3A (STAT1 mutant) fibrosarcoma cells (previously described [337]) were cultured in DMEM supplemented with 10% FBS and antibiotics. BeWo cells were maintained in F12K Kaighn's modified medium supplemented with 10% FBS and antibiotics. JAR cells and immortalized, human, first-trimester, extravillous trophoblast cells (HTR8/SVneo) were maintained in RPMI 1640 medium supplemented with 10% FBS with antibiotics. Human choriocarcinoma JEG-3 cells were maintained in EMEM medium, supplemented with 10% FBS with antibiotics. Human brain microvascular endothelial cells (HBMECs) were maintained in RPMI 1640 medium supplemented with 10% FBS, 10% NuSerum, MEM vitamins, non-essential amino acids, sodium pyruvate, and antibiotics. THP-1 cells stably expressing an interferon regulatory factor (IRF)-inducible SEAP reporter construct were obtained from Invivogen and were cultured in RPMI medium, 2mM L-glutamine, 10% FBS supplemented with 100 µg/ml Zeocin. SEAP levels in tissue culture medium were assessed according by QUANTI-Blue to the manufacturer's instructions. HeLa CCL-2 cells were maintained in MEM supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, and antibiotics. Development of HeLa cells stably propagating a DENV subgenomic replicon has been previously described [344]. Plasmids used to generate stable replicon cells were provided by Theodore Pierson (NIAID). *Aedes albopictus* midgut C6/36 cells were maintained in DMEM supplemented with 10% FBS and antibiotics at 28°C in a 5% CO_2 air atmosphere.

DENV2 16681 and ZIKV FSS13025 (Cambodian origin) were propagated in C6/36 cells, as previously described [345]. ZIKV MR766 (Ugandan origin) was propagated in Vero cells. Viral titers were determined by fluorescent focus assay, as previously described [346], using anti-DENV

envelope protein monoclonal antibody 4G2 (provided by Margaret Kielian, Albert Einstein College of Medicine) for DENV and anti-double stranded RNA monoclonal antibody J2 (provided by Saumendra Sarkar, University of Pittsburgh) for ZIKV. SeV was purchased from Charles River Laboratories. Experiments measuring productive DENV and ZIKV infection were performed with 1 to 3 focus forming units/cell for 24 h, unless otherwise stated, and SeV was used at 100 hemagglutination units/cell for 24 h. Infection was determined by either RT-qPCR or immunofluorescence microscopy, as stated in the figure legends.

3.4.3 Preparation and characterization of conditioned medium

CM samples from PHT cells or other cells were harvested at 72h after plating followed by centrifugation at 800 xg for 5 min. Non-conditioned medium (NCM) was complete PHT medium (described above) that had not been exposed to PHT cells. Recipient cells were exposed to conditioned medium for ~24 h before assays. Vesicle depleted conditioned medium was generated by three centrifugation steps: 2,500 xg for 5 min at room temperature, followed by 12,000 xg for 20 min at room temperature, and 100,000 xg for 2 h at 4°C.

Levels of IFNλ1, IFNλ2 (R&D Systems), and IFNβ (PBL Source) present in CM produced from individual PHT cell preparations were analyzed by ELISA, according to manufacturer protocol. Antiviral activity of CM preparations was determined in HBMEC exposed to CM for 24 h prior to infection with DENV, $ZIKV^{M}$, or $ZIKV^{C}$.

3.4.4 Neutralization assay

Conditioned medium diluted 4-fold, recombinant IFNβ (100 U/mL; PBL Source), or recombinant IFNλ1 and IFNλ2 (5ng/mL each; R&D Systems) was incubated with neutralizing antibodies against IFNβ (200ng/mL; R&D Systems), IFNλ1 and IFNλ2 (1µg/mL each; R&D Systems), or mouse IgG1 (1µg/mL; Sigma) for 1 h at room temperature then added to cells. After 24 h of exposure, the RNA was harvested and analyzed for induction of ISGs.

3.4.5 Differentiation assay

PHT differentiation was blocked by growing the cells in the presence of 1.5% dimethyl sulfoxide (DMSO) [339]. The levels of hCG in DMSO-exposed cells confirmed the attenuation of differentiation by DMSO. For BeWo cell differentiation, BeWo cells were exposed to 25 nM of Forskolin for 24 h, and differentiation was confirmed by increased medium level of hCG. For EGF exposure, PHT medium was replaced 4 h post-plating and with medium containing 10□g/mL EGF (BD Biosciences), which was replenished with fresh medium containing EGF before medium was harvested at 48 h.

3.4.6 RNA extraction, cDNA synthesis, and Real-Time quantitative PCR

For cellular mRNA analysis, total RNA was extracted using TRI reagent (MRC) or GenElute total RNA miniprep kit (Sigma) according to the manufacturer protocol. RNA samples were treated

with RNase-free DNAse (Qiagen or Sigma). Total RNA was reverse transcribed using HiCapacity cDNA synthesis kit (Applied Biosystems) or iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer protocol. Strand specific cDNA was produced with primers targeting the negative RNA strand DENV or ZIKV using iScript Select cDNA Synthesis kit (Bio-Rad). RT-qPCR was performed using SYBR select or iQ SYBR green supermix (BioRad) in a StepOnePlus real-time PCR system (Applied Biosystems), ViiA 7 systems (Applied Biosystems), or CFX96 Real-Time system (Bio-Rad). Gene expression was calculated using the 2-delta delta CT method normalized to GAPDH or actin. Primer sequences were as follows: GAPDH (5'-GAAGGTCGGAGTCAACGGATTT -3' and 5'- GAATTTGCCATGGGTGGAAT -3'); Actin (5'-ACTGGGACGACATGGAGAAAA-3' and 5'-GCCACACGCAGCTC-3'); IFI44L (5'-TGCAGAGAGGATGAGAATATC-3' and 5'-ACTAAAGTGGATGATTGCAG-3'); IFIT1 (5'-CAACCAAGCAAATGTGAGGA-3' and 5'-GGAGACTTGCCTGGTGAAAA-3'); IFNβ (5'-GAGCTACAACTTGCTTGGATTC-3' and 5'-CAAGCCTCCCATTCAATTGC-3'); IFNλ1 (5'-CGCCTTGGAAGAGTCACTCA-3' and 5'-GAAGCCTCAGGTCCCAATTC-3'); IFNλ2 (5'-ACATAGCCCAGTTCAAGTC-3' and 5'-GACTCTTCTAAGGCATCTTTG-3'); IFNAR1 (5'-CAGTTGAAAATGAACTACCTCC-3' and 5'-ACTTGAAAGGTCATGTTTGC-3'); IL28RA (5'-ATCCTCAGTTAACCTACACC-3' and 5'-CAGATACTCCACCACAAAAC-3'); OAS1 5'-ATAAAAGCAAACAGGTCTGG-3' and 5'- TCTGGCAAGAGATAGTCTTC-3'); ZIKV (5'-AGATGACTGCGTTGTGAAGC-3' and 5'-GAGCAGAACGGGACTTCTTC-3'); and DENV (5'-AGTTGTTAGTCTACGTGGACCGA-3' and 5'-CGCGTTTCAGCATATTGAAAG-3'). The specificity of ZIKV and DENV primers were confirmed by RT-qPCR analysis (Figure 3-5F).

3.4.7 RNASeq and microarray analyses

RNASeq from JEG3 and PHT cells was performed as previously described [46]. Briefly, libraries were prepared with the NEB Ultra Library Preparation kit and library quality was determined using the Qubit assay and the Agilent 2100 Bioanalyzer. Sequencing was performed with the Illumina HiSeq2500 rapid-run mode on one flow cell (two lanes). CLC Genomics Workbench 8 (Qiagen) was used to process, normalize, and map sequence data to the human reference genome (hg19). Differentially expressed genes were identified using DESeq2 [347] with a significance cut-off of 0.05, and heat maps were generated using MeViewer software.

We used high-throughput microarray analysis as previously described [338], to screen for transcriptional changes in control (2fTGH) *vs*. STAT1 signaling deficient (U3A) HT1080 cells, both exposed to 100U of purified IFNβ (PBL) or PHT CM for 24 h. In parallel, mock-treated 2fTGH and U3A were also included and were used to identify differentially expressed genes in IFNβ- and CM-treated cells.

3.4.8 Immunofluorescence and transmission electron microscopy

Cells cultured in chamber slides (LabTek, Nunc) were fixed in ice cold methanol, washed, incubated with primary antibody for 1 h, followed by Alexa Fluor conjugated secondary antibody for 30 min (Figure S1G). Slides were mounted with VectaShield (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI). Images were captured on an Olympus IX83 inverted fluorescent microscope and analyzed using ImageJ.

For transmission electron microscopy, cells were fixed in 2.5% glutaraldehyde as previously described [275]. Micrographs were captured using a JEOL 1011 transmission electron microscope.

3.4.9 RNAi mediated silencing

HBMECs were reverse transfected with 25nM Mission siRNA Universal Negative Control (Sigma) or two pooled Silencer Select siRNAs targeting IL28RA (Ambion). At ~48 h post transfection, cells were exposed to PHT CM depleted of vesicles for 24 h followed by infection with ZIKVC. At 24 h post-infection, RNA was isolated and infection analyzed by RT-qPCR.

3.4.10 Protein fractionation

Gel filtration chromatography was accomplished using an AKTA FPLC and Superdex S75 300GL size exclusion column (GE Healthcare) equilibrated in PBS at 4° C. Protein elution was measured at 280 nm. Elutions from the S75 300GL SEC were pooled, and filter sterilized using 0.20 µm sterile syringe filters (Corning). These samples were loaded onto an anion exchange column (MonoQ, GE Healthcare) and eluted with a salt gradient (NaCl up to 1M). Salt concentration was measured by conductivity.

3.4.11 Micro BCA protein assay

Protein concentration from the non-conditioned medium, conditioned medium, and fractions was measured using a Micro BCA kit (Thermo Scientific) according to the manufacturers protocol.

3.4.12 Statistics

Experiments were performed at least three times as indicated in the figure legends or as detailed. Data are presented as mean ± standard deviation. Except were specified, a Student's t- test was used to determine statistical significance for virus infection assays when two sets were compared, and one-way ANOVA with Bonferroni's correction used for *post hoc* multiple comparisons was used to determine statistical significance. Specific p-values are detailed in the figure legends.

3.5 SUPPLEMENTAL INFORMATION

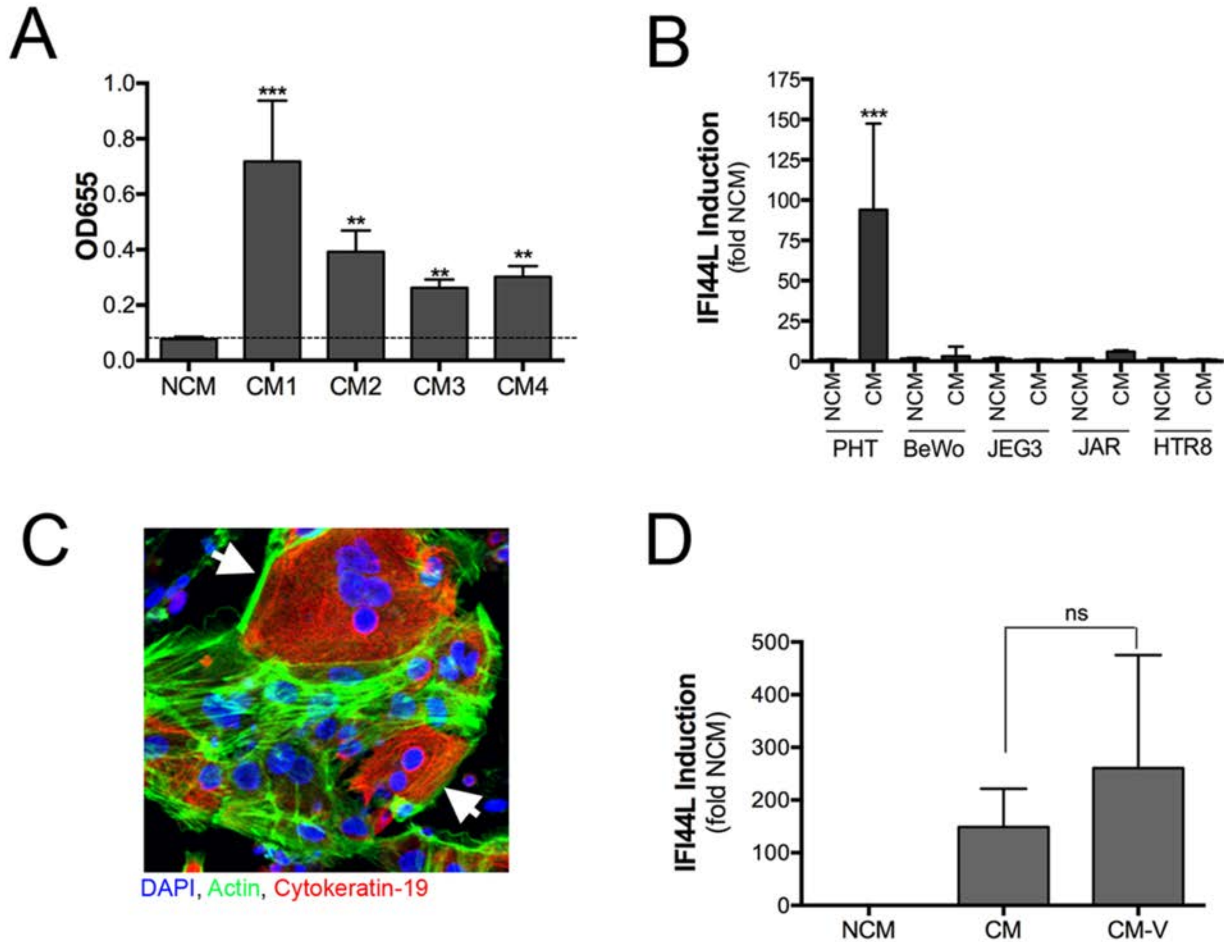
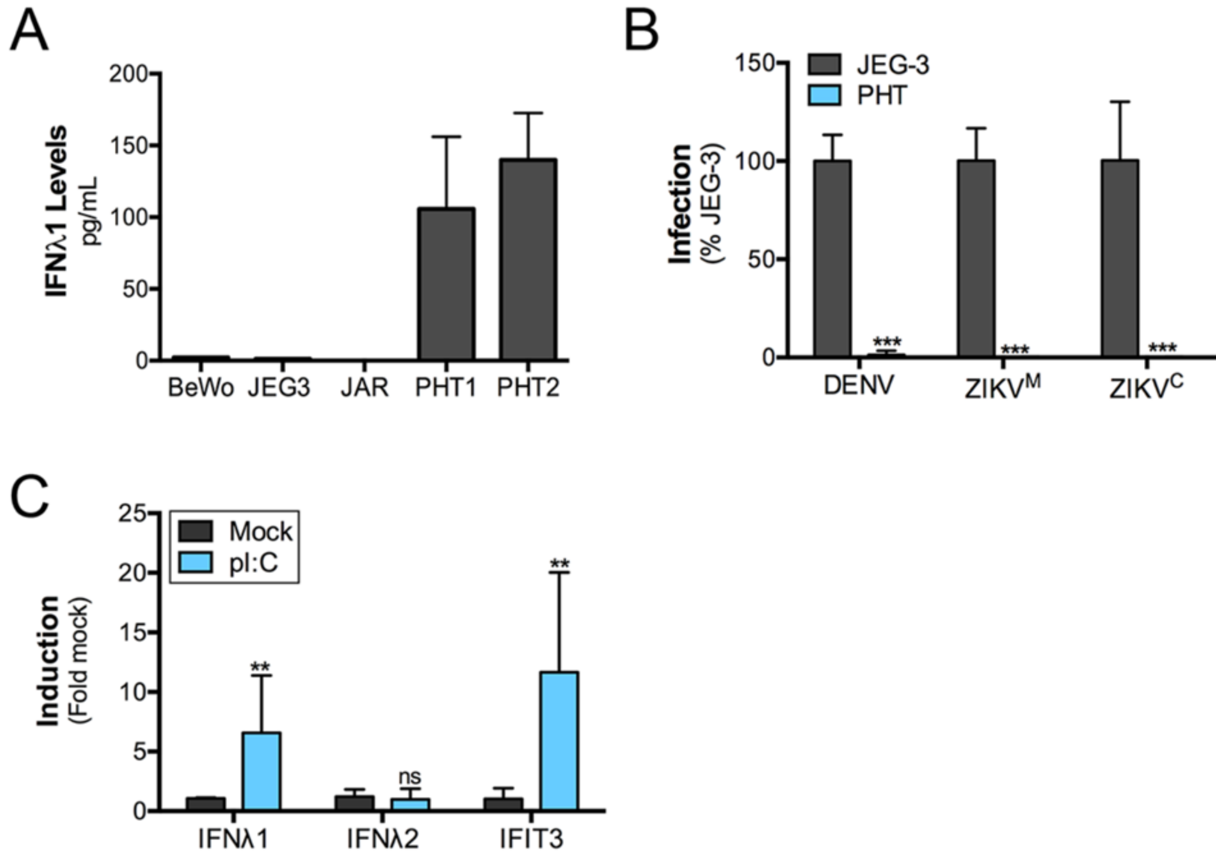


Figure 3-5 Supplemental figure related to figure 3.2. (A), SEAP levels in the cell culture medium of THP-1 ISG-Blue cells treated with NCM or four preparations of PHT CM using the QUANTI-Blue assay. Data are shown as OD655. (B), RT-qPCR for IFI44L in U2OS cells exposed to non-conditioned medium (NCM) or the CM from the indicated trophoblast cell lines, or from PHT cells. Data are shown as a fold change from NCM matched control. Data are shown as mean ± standard deviation,* $p<0.001$. (C), Confocal micrograph of PHT cells. DAPI-stained nuclei are shown in blue, actin is shown in green, and cytokeratin-19 is shown in red. White arrows denote multinucleated syncytiotrophoblasts. (D), The level of IFI44L induction from PHT CM depleted of exosomes as determined by RT-qPCR. Data related to figure 3-6A and 3-6C provided by Dr. Carolyn Coyne (University of Pittsburgh, Pittsburgh PA)**



Supplemental Figure 3-6 (A), ELISA for IFNλ1 in the medium from the indicated trophoblast-derived cell lines and from two independent preparations of PHT cells. (B), RT-qPCR for total vRNA in DENV, ZIKV$_M$, or ZIKV$_C$ infected PHT cells, or in JEG-3 cells, which are matched to the data shown in Figure 3F. (C), RT-qPCR for IFNλ1, IFNλ2, and IFIT3 in mock-treated PHT cells or in cells incubated with 10µM pI:C ('floating'). Data are shown as mean ± standard deviation. Data related to figure 3-7B provided by Dr. Nicholas Lennemann (University of Pittsburgh, Pittsburgh PA)

Table 3-1 - Related to Figure 2

Gene	TGH.(IFNvCON)	U3A.(IFNvCON)	TGH.(CMvCON)	U3A.(CMvCON)
MX1	4.896358663	0.388294157	3.263410147	-0.271622325
GCA	4.517715329	-0.395744301	3.044818852	-0.833194482
PHF11	4.485801453	0.10987201	2.825646995	0.383666568
RSAD2	4.804955007	0.615113013	2.730072248	-1.189510583
MX2	4.77559591	0.782529649	2.640376575	0.124378476
HESX1	2.395931562	-0.047611623	2.490728829	0.279331787
TDRD7	2.586238529	0.321782908	2.310714294	0.921060686

USP18	5.730437534	0.115370405	2.284359399	0.329455223
IFI44L	8.913031589	0.924534909	2.164678967	0.063553645
IFIH1	5.001070005	0.395044768	2.112201	-0.911184453
IFI27	5.745581718	2.492179908	1.95267877	-0.240694057
DTX3L	2.689138978	0.689019188	1.796073483	0.290002187
OAS2	5.145123424	0.88088955	1.745493029	0.018104964
PNPT1	2.829664487	0.172126122	1.668212042	0.890894168
DDX58	4.206286299	0.741261345	1.615580997	1.099575989
TLR3	2.45286905	2.253218622	1.519366804	-0.403987308
PLSCR1	2.883172842	0.150574065	1.503924418	0.004749209
PPM1K	2.277909683	0.564597375	1.379270543	1.575616035
GBP1	4.91359994	0.298253437	1.365440211	0.381459194
PLEKHA4	2.912923786	0.159430372	1.326477622	-0.034119997
ANKRD22	6.079696462	1.555961834	1.27404633	0.29687178
DHX58	5.511157214	0.283502849	1.223487303	-0.358185471
OAS3	3.402291872	0.556019625	1.204068818	0.223923078
CXCL11	3.472110173	1.051661136	1.055936092	-0.94359852
DDX60	5.043230844	0.95734797	0.959847314	-1.017719499
NMI	3.188299747	0.487135496	0.802370817	-0.45530595
IFI44	4.73855272	1.023600087	0.782485247	-0.872754664
RTP4	5.280825809	1.38514923	0.723133744	-0.525119712
IRF9	3.433175564	0.712113478	0.722101364	-0.301602672
BATF2	3.02129754	0.339364537	0.710870521	0.022435634
IFI6	4.645271804	1.862423076	0.692992535	-0.276437265
IFIT5	2.934888011	0.518821813	0.688222994	0.572404181
SLC15A3	5.760020987	-0.502168785	0.521449159	-0.009730073
IFIT2	4.608131777	0.227396865	0.487998324	-0.656114793
EIF2AK2	2.904046139	0.411798817	0.445902929	-0.295878668
TRIM5	2.049548111	-0.276493838	0.416625857	0.691593987
TRIM21	2.244186934	0.219315257	0.378410223	-0.230646938
GBP5	3.930868705	-0.03250477	0.28785213	-0.547688963
STAT2	2.093108083	0.169721686	0.261996504	0.050812407

Table 3.1 - Expression of ISGs as determined by microarray analyses in 2fTGH (TGH) or U3A HT1080 cells treated with 100U of purified IFNβ (in grey) or PHT CM (in purple). Values shown are log2 fold changes from untreated control cells.

4.0 CONCLUSIONS AND FUTURE CHALLENGES

During pregnancy, microbial infections can compromise the health of the mother, as well as the health of her developing fetus. There are many pathogens that have been linked to severe congenital complications, infecting the fetus and resulting in miscarriages, fetal death, and developmental abnormalities such as growth restriction, microcephaly, and cardiac abnormalities. Even if an infection does not directly cross the placenta, severe maternal infection may compromise maternal homeostatic adaptation to pregnancy, with a secondary, adverse effect on pregnancy outcome. Understanding the mechanism deployed by the placenta to prevent fetal infections, and the mechanism pathogens use to subvert the placental barrier, will help establish strategies to prevent adverse pregnancy outcomes.

The work presented in this thesis identifies potent and broadly acting pathways by which the human placenta defends against viral infections. Our cultures of PHT cells, which model the trophoblasts during the third trimester (including the differentiation of cytotrophoblasts to syncytiotrophoblasts), are resistant to a diverse panel of viruses, including ZIKV. We find that this viral resistance can be conferred to non-placental cells through exposure to conditioned medium. Furthermore, individual miRNA mimics, representative of some of the C19MC miRNAs, are able to confer resistance to pathogens associated with perinatal infections. We demonstrate that PHT cells constitutively produce the type III IFN, IFNλ1, which elicits a robust induction of interferon-stimulated genes. Taken together, our results suggest that the term placenta, through at least two separate pathways, is able to confer resistance to non-placental cells. Considering that the miRNAs packaged in exosomes, as well as high levels of IFNλ1, are released by the syncytiotrophoblasts into maternal circulation, it is possible that the placenta can confer resistance to viral infection

broadly to the mother, and potentially also to the fetus. While we were unable to detect IFNλ in plasma samples from pregnant women, detection could be made more difficult as a result of dilution in the expanded maternal blood volume during pregnancy. This may suggest that IFNλ is acting locally, effecting tissues in the uterine environment that express the IFNLR. Future experiments testing cord blood may provide evidence for transport of IFNλ to fetal tissue.

In the concluding sections, we address areas of continuing research in the lab, focusing on understanding the regulation and function of the chromosome 19 microRNA cluster (including the induction of autophagy), regulation of IFNλ, and characterization of the trophoblast derived extracellular vesicles. Lastly, we discuss remaining questions in the field, regarding how viruses and other pathogens circumvent the placental barrier, and what mechanisms of entry are being employed, leading to congenital disease.

4.1 CONDITIONED MEDIUM AND C19MCS

In chapter 2, we presented data demonstrating that conditioned medium from PHT cells and the individual miRNA mimic miR-517-3p from the chromosome 19 miRNA cluster were able to confer resistance to non-placental cells infected with viruses associated with perinatal infections. Building on our previous studies, in which we demonstrated that PHT cells were resistant to a diverse panel of RNA and DNA viruses [275], we have broadened our studies to include rubella virus and other members of the togaviridae, varicella zoster virus, HIV, as well as the protozoan parasite *Toxoplasma gondii*, and the bacterium *Listeria monocytogenes*. We demonstrate that PHT conditioned medium has broad antiviral capacity, significantly reducing infection in all the viruses we had tested. This breadth of antiviral activity, capable of inhibiting viruses with distinct viral

lifecycles, reflects the multifaceted functions of the different ISGs that are induced upon IFN stimulation. Furthermore, we note that conditioned medium exposed to cells prior to the establishment of infection was able to confer resistance, however we observe no reduction in infection in cells that are exposed to conditioned medium from an already established infection (Appendix Figure 4-2D).

4.1.1 The differences between PHT CM and C19MC miRNA mimics

We observed disparate results when comparing the antiviral effects of PHT conditioned medium to the introduction of individual miRNA mimics from the C19MC family. While the conditioned medium consistently conferred significant protection to non-placental recipient cells (HeLa, U2OS, HBMEC, HUVEC, HFF, Vero, HT1080) from viral infection, the miRNA mimic miR-517-3p only limited the replication of select viruses tested (Figure 2-1C and Figure 2-3B), indicating that the conditioned medium contained additional factors contributing to the antiviral effects. As we demonstrated in chapter 3, the conditioned medium, in addition to the exosome packaged miRNAs, contains significant levels of IFNλ1, a potent inhibitor of viral infection. This partially explains differences observed between the CM and miRNA mimic experiments presented in chapter 2. However, it does not address why individual miRNAs, which were previously demonstrated to be antiviral against VSV infection, demonstrated no significant effect on HIV, EEEV, or VEEV (Figure 2-1C). These pathogens may have mechanisms to subvert miRNA mediated antiviral activity, likely downstream of the mRNA targets. Alternatively, it is possible that combinations of miRNAs from the C19MC are required for optimal effects. Past experiments using clusters of different miRNAs or cells stably expressing the entire C19MC were consistently antiviral.

4.1.2 Autophagy and viral infections

In our previous studies, we demonstrated that the C19MC miRNAs confer viral resistance through the induction of autophagy. Furthermore, RNAi mediated knockdown of Beclin 1, a known component of the autophagic machinery, or pharmacological inhibition of autophagy using 3-MA, which inhibits phosphatidylinositol 3 kinases (PI-3K), restored viral infection in cells stably expressing the C19MCs [275]. Additionally, we observed increased infection of VSV in PHT cells exposed to 3-MA. Interestingly, the HIV protein NEF is known to inhibit autophagy, by binding to Beclin 1 [348]. This subversion of the autophagic pathway enhances virion production [349]. This could explain the observed difference between the significant reduction in HIV infection with exposure to conditioned medium, but not by the introduction of miR-517-3p.

While HIV has been shown to antagonize the autophagy pathway, VZV is known to induce and benefit from autophagy [318, 350]. Therefore it was surprising that transfection of miR-517-3p into cells exposed to VZV was able to confer resistance (Figure 2-3B). It is possible that the pathway being induced by the C19MCs may differ from the canonical autophagy pathway, and could be unique and placenta-specific. VZV infection stimulates autophagy through ER stress and the unfolded protein response resulting from production of the VZV glycoprotein [350]. Interestingly, VZV also lacks ICP34.5 and US11, two HSV viral proteins known to antagonize the autophagic pathway [350], again suggesting a non-canonical autophagy pathway is being induced by the C19MC miRNAs.

4.1.3 C19MC miRNA target identification

The functions of the vast majority of miRNAs are largely unknown, due to the poorly established links between miRNA and their target mRNAs. Some individual miRNAs have been connected to certain regulatory pathways, involved in differentiation, development, organ function, and disease [226, 351, 352]. However, these represent the exception, rather than the rule. Sequence complementarity is restricted to the seed sequence, 6 base pairs at the 5' region [353], resulting in multiple potential target mRNAs and limiting the accuracy of target predictions algorithms [354-358].

Recently, attempts have been made to identify direct targets through the use of cross linked immune-precipitation, using the Ago2 protein attached to both the miRNA and the target mRNA sequence [359-362]. In addition to these pull-down methods, overexpression or deletion of specific miRNAs and the subsequent effect on transcripts can be used to identify potential targets. However, these latter types of studies do not necessarily demonstrate direct miRNA targeting to mRNAs. The characterizations by transcript could reflect indirect interactions, with miRNAs targeting upstream regulators. Additionally, experiments employing specific knockout of individual miRNAs commonly do not seem to produce a phenotype, presumably due to the redundancy among miRNA species.

Ongoing research in our lab focuses on identifying potential targets, utilizing cross-linked immune precipitation techniques to isolate and identify Ago2 bound miRNA complexes in association with their target transcripts [360]. These studies have the benefit of identifying direct miRNA:mRNA target interactions, and should provide clues to the role the C19MCs play in autophagy induction, and whether this pathway is distinct from canonical autophagy pathways. With the identification of the target transcripts, we can then address the possibility that certain

pathogens linked to perinatal infections have developed mechanisms to attenuate this pathway. Furthermore, these studies may elucidate other regulatory functions of C19MCs, including a potential role in placental development and regulation of extravillous trophoblasts invasion [267].

4.1.4 Exosomes and communication of antiviral signals

Based on our previous findings, we know that exosomes derived from the PHT conditioned medium are able to confer viral resistance to recipient non-placental cells. This function is specific to the exosomes, with apoptotic bodies and microvesicles derived from PHT cells exhibiting a much weaker effect (Appendix B, Figure 4-6). As discussed in section 1.4.3.3, extracellular vesicles are known to be involved in cell-cell communication. However, we currently do not know which tissues are targeted by these vesicles. Furthermore, the critical mass required for exosomes and their miRNA cargo to induce antiviral effects remains unclear. MiRNA copy number per exosome is variable, and the concentration of exosomes being taken up per recipient cell is unknown [363]. Other areas of ongoing research in the lab are focused on deciphering the exosome-mediated viral resistance. As demonstrated in appendix B, we have observed a significant inhibition of viral infection in cells exposed to exosomes. Comparative analysis of the different extracellular vesicles has revealed unique proteins that are present in the exosomes, which may affect cellular uptake by the recipient cells, such as the endogenous retroviral encoded protein Syncytin-1 [364]. Enhanced cellular uptake of exosomes may explain the unique antiviral effects, potentially due to more efficient loading of the miRNAs into the right cellular compartment. Recent studies have demonstrated concentrated uptake of exosomes through the certain cellular pathways, such as uptake via filopodia of certain cells [365]. Furthermore, what cells are being targeted by the exosomes, and what those targeting elements are remain to be determined. It is

possible that proteins exclusively expressed by the exosomes, unlike the other extracellular vesicles derived from the PHTs is mediating the uptake, but could also be due to unique lipid signatures present in the exosomes.

So far we have been unable to determine if exosomes have effects that are independent of miRNAs. Generating miRNA-free exosomes derived from PHT cells or trophoblast cell lines has remained a challenge, as we still do not fully understand the regulatory mechanisms of the C19MCs. Ongoing work in the lab is focused on understanding what these regulatory factors are, particularly examining the function of C19MC enhancer elements. Interestingly, transgenic mice that express a BAC containing the entire 100kb C19MC cluster also demonstrate restricted expression in the placenta (unpublished work). Identification either enhancer or repressor sequences regulating the expression of the C19MC will enable us to silence the entire cluster. Furthermore, ongoing experiments in the lab will uncouple the C19MCs and the exosomes, generating type III endonuclease Drosha knockout cells, and isolating vesicles from these cells for comparison in functional assays.

Our data also demonstrated that the conditioned medium from PHT cells had no significant impact on the infection of either *T. gondii* or *L. Monocytogenes*. This suggests that the conditioned medium and the miRNAs are specifically antiviral. Other groups have used a combination of in vitro studies and explant models to demonstrate the role of syncytiotrophoblasts in protecting the fetus from infection by these pathogens [38, 218]. Given that the CM and C19MCs do not confer resistance to these pathogens, it suggests that the trophoblasts have additional, as yet undefined mechanisms to resist bacterial and parasitic infections. While some proposed mechanisms of resistance have included lack of required pathogen receptors, such as E-cadherin, it is likely that there are additional mechanisms involved [5]. We initially utilized unbiased biochemical

approaches in the identification of factors present in our conditioned medium that were conferring viral resistance as well as ISG induction (figure 3-1). While these studies strongly indicated the presence of a protein band corresponding to IFNλ (~25kd), they were discontinued due to alternative methods available at the time. However, it is likely that the PHT cells release many other factors into the conditioned medium, some of which may be antimicrobial. Future studies to continue the biochemical identification of these factors will couple different methods of protein biochemistry and gel filtration with functional assays and mass spectrometry to identify potential anti-microbial factors.

4.2 PLACENTAL PRODUCTION OF IFNΛ1

In appendix A we present data demonstrating that the primary human trophoblasts are resistant to ZIKV infection, and that this resistance can be conferred to non-placental recipient cells (figure 4-2). PHT cells constitutively produce the type III IFN, IFNλ, which robustly induces antiviral effector proteins, leading to a cellular state refractory to viral infection. As was mentioned in section 1.2.2.4, a few groups have previously noted that the placenta produced an IFN that was not neutralized by anti-sera for type I IFNs [117]. Here, we demonstrate that PHT cells, and trophoblast cell lines produce IFNλ constitutively and in response to PRR agonists, respectively. These findings further support the role of the syncytiotrophoblasts as a crucial barrier to viral infection during pregnancy.

Animal models of pregnancy have indicated the role of IFN in maintaining the conceptus. Ruminant and ungulate species, such as bovine, produce a unique pregnancy related IFN, known as IFNτ, which has also been demonstrated to be constitutively expressed during pregnancy [366].

However, humans do not have an IFNτ ortholog. In the porcine conceptus, early during gestation IFNδ is produced, functioning similarly to other type I interferons [366]. Like IFNτ, IFNδ is also not expressed in humans, thought to have been lost over evolution, potentially due to redundancy. In the mice, at the site of implantation, some ISGs have been demonstrably up-regulated, including ISG15, indicating the role of IFNs during murine pregnancy as well [367].

While these examples from other species highlight a functional role for IFN in maintaining pregnancy, it is difficult to draw comparisons to human pregnancy. As was discussed in the introduction, there are a number of differences between human pregnancy and pregnancy in other mammals, including placentation. Furthermore, humans do not encode the above mentioned IFNs, while mice lack the gene for IFNλ1, which we have shown to be highly expressed in term human trophoblasts.

4.2.1 IFNλ regulation in the placenta

The type III IFNs, discussed in section 1.3, are an important component of the innate immune system in epithelial cells and endothelial cells. Syncytiotrophoblasts, like other epithelial barriers, are polarized cells constantly exposed to pathogenic microbes. The trophoblasts are unique in that they constitutively produce IFNλ, in the absence of viral infection. We have previously demonstrated that neither NFκB nor IRF3 translocate to the nucleus in PHT cells [275]. It is possible that expression of the IFNλs results from global transcription of chromosome 19, which contains many pregnancy related genes, including the α subunit of hCG [368], and pregnancy specific glycoproteins (PSGs) [369]. Both the chromosome 19 miRNA cluster (C19MC), located at chromosome 19q13.41, and the IFNλ genes, located at chromosome 19q13.13, are highly expressed in primary human trophoblasts. Alternatively, high expression of IFNλ could be related

to the fusion of the cytotrophoblasts as they differentiate to syncytiotrophoblasts. This hypothesis is attractive, since we have demonstrated that differentiation of the trophoblasts was necessary for the ability of CM to induce ISGs in recipient cells, presumably through the expression of IFNλ.

4.2.2 Viral entry into the fetal compartment

Our group and others have shown that the placenta is a remarkably efficient barrier to infection, conferring protection against most pathogens. In particular we have demonstrated that the syncytiotrophoblasts, through high levels of basal autophagy and autocrine signaling of IFNλ, create a formidable barrier to pathogens. However, despite these barrier functions, certain viral pathogens can lead to severe disease in the fetus. Here, we will discuss the potential routes of viral access to the fetus.

4.2.2.1 First and second trimester trophoblasts

We have shown that primary human trophoblasts from term placentas are remarkably resistant to viral infection. Recent studies from other groups have begun to address whether or not trophoblasts from earlier periods during gestation are as efficient at preventing infection from viruses, such as ZIKV [48]. Considering that many perinatal infections early during pregnancy manifest in severe congenital defects, such as microcephaly and other developmental abnormalities, it is possible the placenta during early gestational periods in gestation is not as effective as a fully developed placenta at term. Alternatively, these early gestational periods are the most critical for fetal development, and it is possible that similar magnitudes of fetal infection may lead to more dramatic consequences for the fetus during these initial stages. As was discussed in section 1.3, primary infection during the first trimester of pregnancy by viruses including CMV, rubella, and VZV, may

cause congenital syndromes, while infections during later periods of pregnancy is less frequent, or cause less severe disease. Recent reports on ZIKV suggest that infection early in gestation leads to intrauterine growth restriction (IUGR), microcephaly, and malformation of the fetal brain [209, 330]. Using first trimester placental explants, the Pereira group demonstrated that ZIKV infects cytotrophoblasts, endothelial cells, fibroblasts, Hofbauer cells from chorionic villi, and amniotic epithelial cells [48], but that the syncytiotrophoblasts had no detectable virus. They also demonstrated that ZIKV titers were significantly higher in amniotic cells isolated from mid-gestation, 16-23 weeks of pregnancy, compared to those isolated from later gestational periods, at term [48]. At these later periods, the fetus may be less susceptible to developmental injuries, as many of the tissues are already formed.

It is important to note that the placenta does not become perfused with maternal blood until 12 weeks of pregnancy, when the placental plugs of the maternal spiral arteries begin to degrade. These plugs are comprised of extravillous columnar cells, and may be susceptible to viral infection, similar to what has been demonstrated for the EVTs, mentioned above. Future studies to address the susceptibility of these cells can utilize induced pluripotent stem cells to recapitulate the in vivo phenotype of these columnar cells, and provide a model for primary infection early in gestation.

4.2.2.2 Routes of virus infection

While the syncytiotrophoblasts are refractory to viral infection, it has been previously reported that viruses bound by maternal IgG can be transported across the placental barrier via neonatal FC receptor. This was shown for hCMV, as IgG-virion complexes crossed via transcytosis in explant models [370]. This mechanism may be utilized by ZIKV to gain access to the fetus. Dengue virus is endemic in Brazil, and large portions of the population are sero-positive, including women of childbearing age. Recent reports have indicated that the four different sero-types for DENV are

cross reactive to ZIKV [371, 372], however cross reactivity is not required for ZIKV infection, as mothers sero-negative for DENV were infected with ZIKV, and passed the virus to the fetus [209]. Exposure to DENV leads generation of antibodies specific for one of the four serotypes. Upon exposure to a different serotype the antibodies, which recognize the viral glycoprotein or fusion loop epitope (FLE), do not provide protection [371], but instead facilitate entry into cell types that would not typically be infected, via the FC receptor. This was recently demonstrated *in vitro*, as DENV plasma potently induced ADE, enhancing ZIKV infection [371]. Therefore, prior immunity to DENV may drive greater replication of ZIKV.

Other groups have demonstrated that cytotrophoblasts, extravillous trophoblasts and Hofbauer cells, from first trimester explants are susceptible to a variety of viruses, including CMV and ZIKV [35, 48, 373, 374]. We currently don't know the targets cells that uptake the trophoblast exosomes, nor do we know the extent to which IFNλ confers resistance to neighboring cells. Future studies will need to explore the expression of IFNLR in the uterine environment.

4.2.3 IFNλ polymorphisms

In our studies we have observed variability amongst the placental preparations, regarding the derived cells ability to confer viral resistance. Conditioned medium preparations also contained variable quantities of IFNλ. These differences in the potency of the antiviral effects could potentially reflect differences in the feto-placental genome. Genome wide association studies (GWAS) examining differences in response to HCV and HBV infection, indicated that populations demonstrating improved outcomes in terms of spontaneous clearance of viral infection as well as response to antiviral therapy, contained polymorphisms in the promoter of IFNλ. One allele identified corresponded with increased production of IFNλ3, potentially leading to better control

of infection [156]. A separate allele demonstrated an altered 3'UTR sequence, rendering the IFNλ3 less susceptible to miRNA degradation following HCV infection [375]. It is possible that the differences we observe amongst the placental cell isolates, and potentially differences in the population among women who are more susceptible to viral infection during pregnancy, are due to single nucleotide polymorphisms in IFNλ genes. We have observed variability in the potency of conditioned medium from different placental preparations. Given that we have noted variability in, the antiviral potency of conditioned media, it may be relevant to test our primary human trophoblasts for SNPs, and compare the efficacy of the conditioned medium to determine if genetic differences are a factor in contributing to susceptibility to fetal infection.

4.3 CONCLUDING REMARKS

We have shown that the primary human trophoblasts deploy at least two distinct antiviral mechanisms, which can be communicated to non-placental cells. These two pathways are depicted in figure 4-1. The PHT derived exosomes, which contain miRNAs from the C19MC, have been shown to be antiviral through the induction of autophagy. This induction is presumably through classical miRNA shutoff of translation or by transcript degradation. We have previously demonstrated that the exosomes are capable of conferring viral resistance in cells deficient in the JAK/STAT pathway, and we have demonstrated here (Figure 4-6B) that the exosomes or other extracellular vesicles do not lead to an ISG induction. The second pathway is the constitutive production of IFNλ and subsequent ISG induction in non-placental recipient cells.

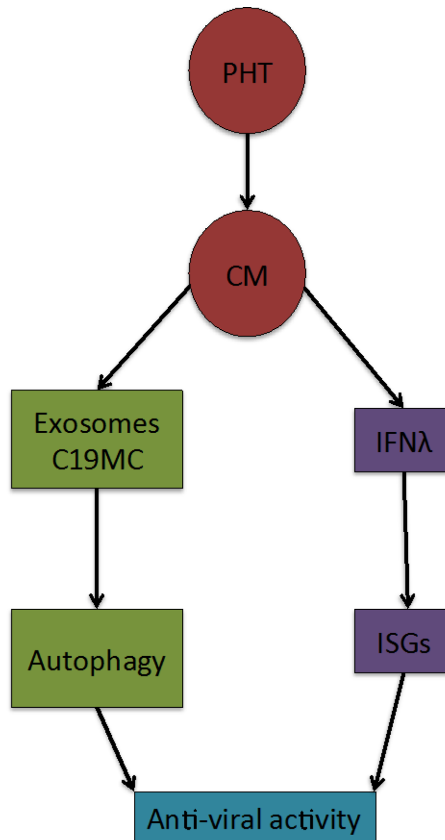


Figure 4-1 Parallel paracrine pathways deployed by PHT cells

The multiple mechanisms work in parallel, and it is unclear what, if any, interactions exist these two pathways. Other groups have suggested that RNAi and IFN pathways are mutually exclusive [376, 377]. Studies have demonstrated that in the presence of type I IFN induction by Poly I:C, Ago2 is inhibited, blocking the function of the RISC complex [378].

The regulation of IFNλ1 expression in the PHT cells is currently unknown, and it is possible that there are differences in IFNλ1expression in early and late pregnancy. The gene locus for IFNλ 1-4 is adjacent to the locus for the C19MC [150, 301]. It is therefore possible that constitutive expression of IFNλ is linked to expression of the C19MC miRNAs, or that they are

both regulated by distant enhancer sequences. While the mechanisms underlying the placenta-specific regulation of the C19MC is not yet known, it is possible that the two pathways share a placental-specific genomic activation or repression.

Understanding how viruses gain access to the fetal compartment is a crucial yet understudied area of research. Some of the reasons for our lack of knowledge regarding how viruses can circumvent the placental barrier include a lack of adequate models that fully recapitulate the events in vivo. Animal models have advantages of being able to model vertical transmission of infection, but have the disadvantage of being morphologically and genetically different from human placenta. Placental cell lines allow for genetic manipulation and characterization of the molecular events of trophoblast infection, such as the production of IFNλ in response to ZIKV infection (figure 3-3E). However, these choriocarcinoma cell lines often are more characteristic of their carcinoma origins, and do not fully represent the trophoblasts in vivo. Explant models of first and second trimester villi have facilitated the study of early events of infection during pregnancy. However, these tissue samples are difficult to attain. Future advances in the available models will provide better understanding of the role of the placenta in protecting the fetus against infection, and mechanisms by which the pathogens are able to access the fetus.

In the remaining sections of the dissertation, we provide evidence for PHT resistance to ZIKV infection (appendix A), and the ability of the PHT conditioned medium to confer resistance to non-placental cells. This work directly ties in with the findings presented in chapter 3, as IFNLR knockdown by RNAi restored ZIKV infection in cells exposed to conditioned medium (figure 3-3G). In appendix B, we provide preliminary evidence in support of the model presented in figure 4-1, demonstrating that the exosome mediated C19MC pathway and the IFNλ pathway presented in this work are independent of one another.

APPENDIX A

PHT CELLS ARE REFRACTORY TO ZIKA VIRUS[11]

Data for Appendix A was generated by Nicholas Lennemann and Carolyn Coyne

To assess the ability of ZIKV to replicate in human placental trophoblasts, we measured the replication of two strains of ZIKV, one of African lineage [336] (MR766, termed $ZIKV^M$ hereafter) and one of Asian lineage [336] (FSS13025, termed $ZIKV^C$ hereafter) in PHT cells and a panel of trophoblast-derived cell lines including BeWo, JEG-3, and JAR choriocarcinoma cells, and the extravillous trophoblast cell line HTR8/SVneo [50]. In addition, we compared the level of infection of these cell types by DENV. We also compared the infectivity of these cell types with that of human brain microvascular endothelial cells (HBMEC), which is a cell-based model of the blood-brain barrier [379] and is permissive to DENV and both strains of ZIKV (Figure 1A, Figure S1A)[12]. We found that BeWo, JEG-3, JAR, and HTR8/SVneo cells supported infection by both $ZIKV^M$ and $ZIKV^C$, although BeWo cells were less susceptible to infection by both DENV and ZIKV than the other trophoblast-derived cells lines (Figure 3-1A and Figure 3-5A).

In contrast, we were unable to detect any evidence of ZIKV or DENV replication in PHT cells by immunofluorescence microscopy (not shown). Consistent with this, we found that PHT

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[12] Data for ZIKV infection assays was provided by Dr. Carolyn Coyne and Dr. Nicholas Lennemann (University of Pittsburgh, Pittsburgh PA)

cells resisted infection by ZIKV^{M}, ZIKV^{C}, and by DENV, as evidenced by very low levels of total viral RNA (vRNA) (Figure 3-5B and 3-5C)[4] and the lack of production of the negative strand of vRNA, which is only produced during viral replication (Figure 3-1B)[4]. These results are consistent with our previous observations that PHT cells resist infection by diverse RNA and DNA viruses [275] and show that ZIKV is unable to replicate efficiently in primary trophoblasts.

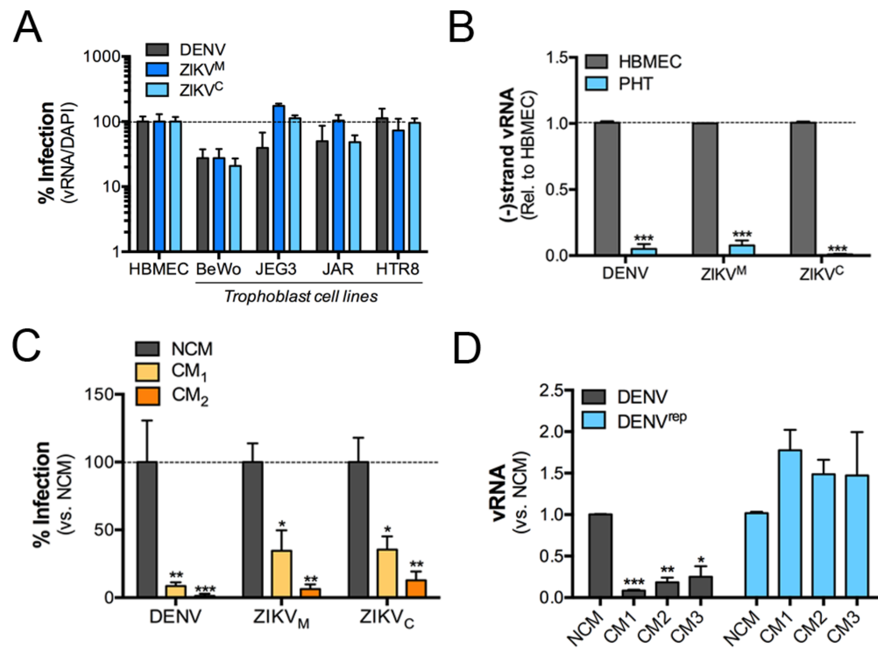

Figure 4-2 **PHT cells are resistant to ZIKV (A), The indicated cell lines were infected with DENV, ZIKV^{M}, or ZIKV^{C} for ~24hrs, fixed, and then stained with anti-dsRNA (J2) antibody. Data are shown as the percent of vRNA positive cells relative to the total number of nuclei (as assessed by DAPI). (B), Levels of DENV, ZIKV^{M}, or ZIKV^{C} negative strand vRNA were assessed by RT-qPCR in non-PHT (HBMEC) or PHT cells infected for ~48hrs. (C), HBMEC were exposed to non-conditioned (NCM) PHT medium or conditioned PHT medium (CM, two independent preparations) for ~24hrs and then infected with DENV, DENV, ZIKV^{M}, or ZIKV^{C}. The level of infection was assessed for fluorescence microscopy for dsRNA. Data are shown as the percent of vRNA positive cells relative to the total number of nuclei (as assessed by DAPI). (D), Control HeLa cells, or HeLa cells constitutively expressing a DENV replicon were exposed to NCM or three independent preparations of PHT**

CM and then the levels of DENV vRNA assessed by RT-qPCR ~24hrs after exposure. In all, data are shown as mean ± standard deviation (*p<0.05, **p<0.01, ***p<0.001). Data related to figure 3-1(A-D) was provided by Dr. Nicholas Lennemann and Dr. Carolyn Coyne (University of Pittsburgh, Pittsburgh PA)

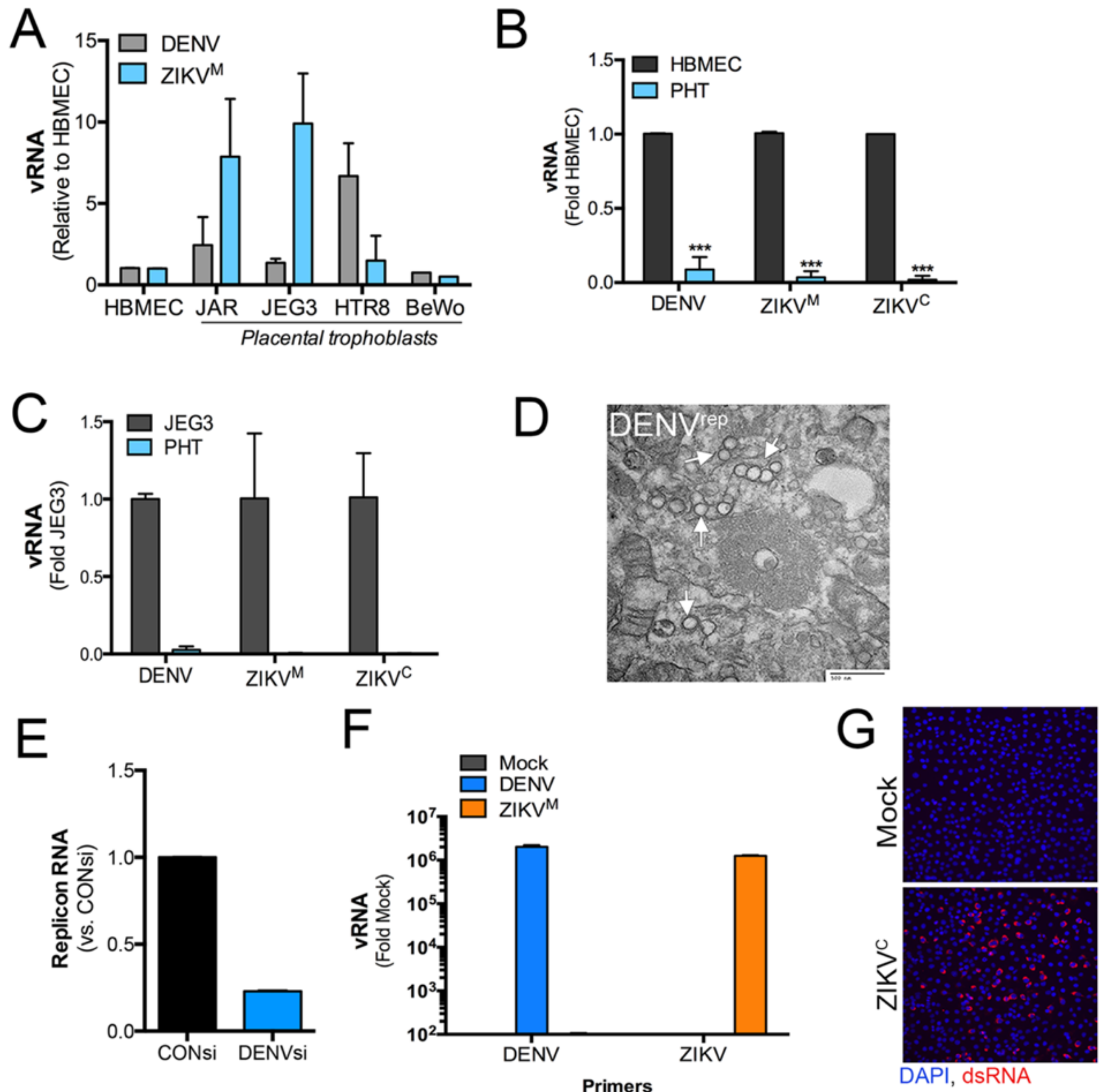

Figure 4-3 Supplemental data related to Figure 4-2. (A), RT-qPCR for total vRNA in the indicated DENV or ZIKV$_M$ infected trophoblast cells lines, or in HBMEC. Data are shown as a fold from HBMEC. (B,C), RT-qPCR for total vRNA in DENV, ZIKV$_M$, or ZIKV$_C$ infected PHT cells, or in HBMEC (B) or JEG-3 (C) cells.

Data are shown as a fold from HBMEC (B) or JEG-3 (C). (D), Transmission electron micrograph (TEM) from HeLa cells stably expressing a DENV replicon. White arrows denote DENV-induced replication organelles. Scale, 500 nm, at bottom right. (E), RT-qPCR for DENV vRNA in HeLa cells stably expressing a DENV replicon and transfected with a control

APPENDIX B

CHARACTERIZATION OF TROPHOBLAST EXTRACELLULAR VESICLES ON ISG INDUCTION AND ANTIVIRAL EFFECTS

B.1 INTRODUCTION

Primary human trophoblasts have previously been shown to confer viral resistance to non-placental cells, through the release and subsequent uptake of extracellular vesicles, that contain miRNAs from the C19MC family of miRNAs [275]. Based on our results from chapter 3, we know that the PHT cells, in addition to the release of the extracellular vesicles, constitutively produce type III interferon, IFNλ1. In this section, we assessed the potential synergy between miRNA pathways to observed ISG induction in recipient cells, as well as further characterizing the PHT derived extracellular vesicles. While previous studies examined the role of the exosomes from PHT cells, here we expand the panel of extracellular vesicles to include apoptotic bodies as well as microvesicles, exploring their potential contribution to the observed antiviral phenotype.

Additionally, we address the questions about potential interaction between the two pathways, combining both extracellular vesicles with recombinant IFNλ1 in recipient cells. We found no significant interaction between these two pathways.

B.2 RESULTS

B.2.1 Extracellular vesicles from PHT cells inhibit viral infection but do not induce ISGs

We added individually isolated extracellular vesicles, including apoptotic bodies, microvesicles, and exosomes, to U2OS cells for approximately 18 h prior to infection with VSV. Figure 4.5A demonstrates that isolated conditioned medium and exosomes showed significant reduction in VSV infection, while apoptotic bodies and microvesicles reduced infection, but not significantly. The antiviral activity of the vesicles was independent of the IFNλ activity reported in chapter 3, as isolated vesicles added to recipient U2OS inhibited viral infection but did not induce IFI44L, while conditioned medium used as the source for those vesicles did significantly induce IFI44L (Figure 4.6B).

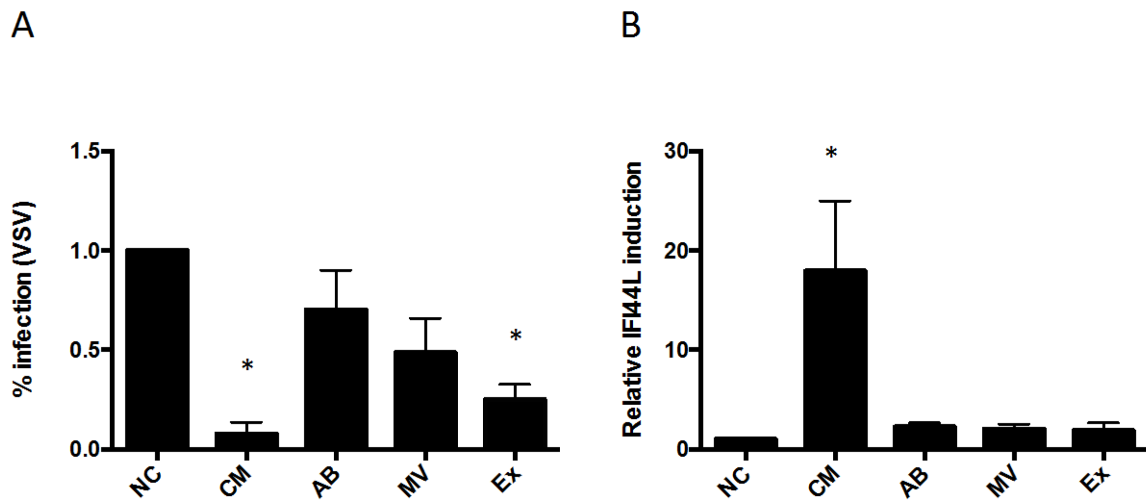

Figure 4-4 - Extracellular vesicles from PHT cells and their effects on VSV and ISG induction. Extracellular vesicles from primary trophoblast cells were tested for their effects on VSV replication and ISG induction in U2OS cells. Viral replication and ISG induction were measured using qPCR, as described in the methods. Vesicles were prepared from PHT cells from three different placental preparations. Analysis was performed using one-way ANOVA (Prism 6.0 software). The asterisks indicate statistical significance. * denotes $p<0.05$.

B.2.2 Extracellular vesicles and IFNλ are independent pathways

To address whether there is any interaction between the two pathways reported in chapters 2 and 3, we combined isolated extracellular vesicles from PHT cells, which did not have any ISG induction on their own, with recombinant IFNλ1. Conditioned medium, isolated vesicles, and IFNλ

independently inhibited VSV infection to a variable degree. When IFNλ was combined with isolated vesicles there was no significant change in VSV infection, suggesting no interaction between the two pathways (4.7A). Furthermore, ISGs measured in cells exposed to CM, isolated vesicles, IFNλ, or a combination of the vesicles with IFNλ, again did not demonstrate any influence between the two pathways. ISG induction, measured for IFI44L (Figure 4.7B), IFIT1 (Figure 4.7C), or OAS1 (Figure 4.7D), do not indicate any change in expression from IFNλ with the addition of either microvesicles or exosomes.

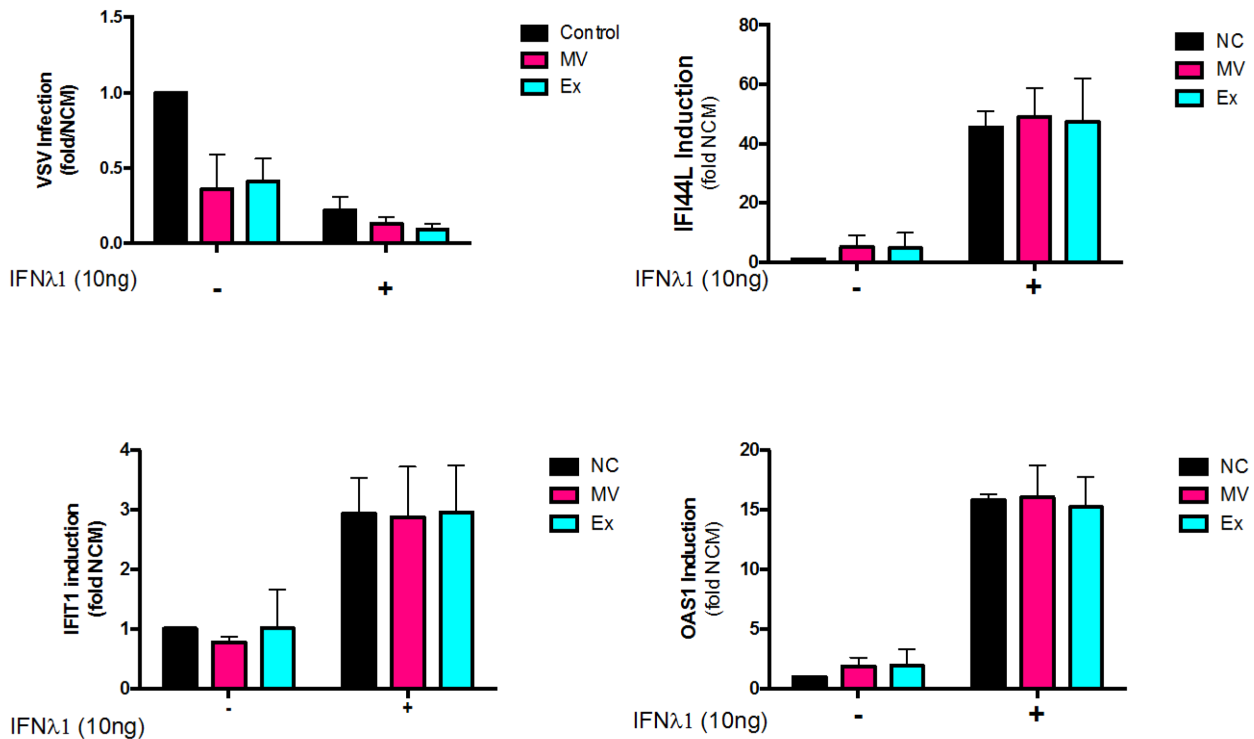

Figure 4-5 - Interaction between trophoblast vesicles and IFNλ1. (A) VSV infection was measured in U2OS cells exposed to Mock (untreated cells), PHT CM, or IFNλ1 with or without the addition of PHT derived microvesicles or exosomes. Cells were exposed for 24 h prior to infection, and infection was measured by RT-

qPCR. (B-D) ISG induction was measured in the cells exposed to IFNλ1 (10 ng/ml) with the addition of either isolated microvesicles or exosomes derived from PHT cells. ISG induction was quantified by RT-qPCR.

B.3 MATERIALS AND METHODS

B.3.1 Cells and Viruses

Human osteosarcoma U2OS cells were cultured in DMEM supplemented with 10% FBS and antibiotics.

For viral infection assays, U2OS cells were seeded into 24 well plates. 24 h following plating, the cells were infected with vesicular stomatitis virus (VSV) at the multiplicity of infection (MOI) equivalent to 0.5-1, for 5 h, and then washed three times with PBS, to remove residual non-infected virus.

B.3.2 RNA extraction, cDNA synthesis, and Real-Time quantitative PCR

For cellular mRNA analysis, total RNA was extracted using TRI reagent (MRC) RNA samples were processed with RNase-free DNAse (Qiagen or Sigma). Total RNA was reverse transcribed using HiCapacity cDNA synthesis kit (Applied Biosystems) RT-qPCR was performed using SYBR select in a ViiA7 system (Applied Biosystems). Gene expression was calculated using the 2-delta delta CT method normalized to GAPDH. Primer sequences were GAPDH (5’-GAAGGTCGGAGTCAACGGATTT -3' and 5’- GAATTTGCCATGGGTGGAAT -3') and IFI44L (5’-TGCAGAGAGGATGAGAATATC-3' and 5’-ACTAAAGTGGATGATTGCAG-3');

IFIT1 (5'-CAACCAAGCAAATGTGAGGA-3' and 5'-GGAGACTTGCCTGGTGAAAA-3'); OAS1 5'-ATAAAAGCAAACAGGTCTGG-3' and 5'- TCTGGCAAGAGATAGTCTTC-3').

B.3.3 PHT conditioned medium and isolated vesicle preparations

CM samples from PHT cells were harvested at 72h after plating followed by centrifugation at 800 xg for 5 min. Non-conditioned medium (NCM) was complete PHT medium (described above) that had not been exposed to PHT cells. Recipient cells were exposed to either conditioned medium or filtered fractions for ~24 h before assays.

PHT cells were cultured in 7x15-cm plates at 37° for 72 h in complete DMEM containing 1% antibiotics and 10% fetal bovine serum depleted of exosomes by overnight ultracentrifugation. Approximately 400 mL of conditioned medium was collected per vesicles purification. Serial centrifugation steps, including an initial spin at 500g for 10 min to remove cell debris, followed by 2,500g for 20 min to pellet and isolated the apoptotic bodies. These were washed three times with PBS, and resuspended in 50-100 µL of PBS (Sigma). Supernatant from the preceding step was then spun at 12,000g for 30 min to pellet and isolate the microvesicles. These were washed three times in PBS and resuspended. The supernatant from the preceding step was then used for exosome isolation. The supernatant was spun at 100,000 xg overnight. This supernatant was removed, and he pellet was resuspended in 0.5 mL PBS mixed with 1.5 mL OptiPrep 60% (Sigma Aldrich). This mixture was placed at the bottom of a tube, and overlayed with 6-40% OptiPrep gradient, using a gradient formation chamber and peristaltic pump. The mixture was spun at

100,000 xg for 22h, and fractions containing exosomes were isolated and confirmed by western blot.

B.4 CONCLUSIONS

We have previously demonstrated that exosomes isolated from medium that was conditioned by primary human trophoblast are capable of conferring viral resistance to non-placental recipient cells. Here we have shown that the PHT-exosomes significantly reduced viral infection in U2OS cells, compared to the other extracellular vesicles (EV) derived from the PHT cells, which reduced infection to a lesser extent (Figure 4.6A). Furthermore, based upon the results presented in chapter 3, we know that conditioned medium from PHT cells can induce ISGs robustly in recipient cells. Here we have confirmed that this ISG induction was independent of the extracellular vesicles, and likely exclusively mediated by IFNλ1 (Figure 4.6B). This strongly suggests that the two antiviral pathways, discussed in chapter 2 and chapter 3, are independent of one another.

To further explore the relationship between the antiviral activity of EVs and IFNλ, we incubated recipient cells for 24h with EVs, IFNλ, or the two combined, and measured the effects on VSV infection and ISG induction. In support of the findings from figure 4.6, the EVs did not induce ISGs, but were antiviral. IFNλ was both antiviral and strongly induced ISGs, as expected. Interestingly, the combination of EVs with IFNλ did not cause any noticeable synergistic effect. This strongly suggests that the two pathways act independently, with no evidence for a synergistic relationship or cross talk between them. As was mention in chapter 4, other groups have suggested that the IFN pathway blocks RNAi, and the two pathways are mutually exclusive. Future studies

will explore the relationship between the C19MC miRNAs and IFNλ1, including any potentially shared regulation, and also for any potential negative feedback between the two pathways.

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