Type III Interferon Control of Rift Valley Fever Virus at Epithelial Cell Barriers

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Abstract

Rift Valley fever virus (RVFV) is an emerging infectious disease of domesticated livestock that is endemic to many regions of Africa and is spread by multiple mosquito species. RVFV epizootic outbreaks commonly affect sheep and cattle, resulting in hepatic disease and hemorrhagic fever. One of the most devastating characteristics seen in previous outbreaks are among pregnant livestock which experience "abortion storms" wherein the virus induces abortogenic rates of 90-100%. While human RVF carries a small risk of mortality, RVFV is of great public health significance to women during pregnancy. An association has been demonstrated between women who are infected with RVFV and increased likelihood of late term miscarriage. Thus, RVFV infection in pregnant women carries the potential for vertical transmission to the child. Vertical transmission cases have been documented in naturally occurring outbreaks. Our lab has also demonstrated vertical transmission in a pregnant rat model of RVFV congenital infection. Pregnant rats were inoculated with wildtype RVFV subcutaneously, mimicking the mosquito bite transmission seen in natural infections. Type III interferons, the IFN-λ family of proteins, are a group of innate, antiviral molecules that function in modulating host immune responses to viral infection at epithelial barriers, including the placenta. Unlike type I interferons which elicit antiviral responses in all nucleated cells, which express the IFNAR1 receptor, type III interferons elicit a more localized innate antiviral response, with receptors for

IFN-λ proteins only found on barrier epithelial cells. Previous work has examined a type III interferon, IFN-λ1, and its ability to confer protection at the placental barrier during Zika virus infection. Primary human trophoblasts, the barrier cells of the placenta, constitutively release IFN-λ1 which protects trophoblast and non-trophoblast cells from Zika virus infection, suggesting the virus must use alternative pathways to cross the placental barrier. The research presented herein represents the first investigationsperformed to define the possible modulatory effects of type III IFNs on RVFV pathogenesis.

We performed experiments *in vitro* in cell lines mimicking the hepatic and placental tropism for RVFV infections. Exogenous treatment with interferon revealed that type III IFN can reduce viral titers in infected cells in a time- and cell-dependent manner. Further, cells infected with wildtype RVFV or RVFV-ΔNSs/GFP exhibited modulated IFN stimulated gene (ISG) expression. The research presented herein represents the first investigations performed to define the possible modulatory effects of type III IFNs on RVFV pathogenesis.

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1.0 Introduction

Rift Valley fever virus (RVFV) is an emerging infectious disease endemic to many regions of Africa. It is primarily a veterinary disease with occasional zoonotic events and is spread by multiple mosquito species (1). It is the causative agent of Rift Valley fever (RVF). Formerly a member of the *Bunyaviridae* family of viruses, RVFV has recently been reclassified within the *Phenuiviridae* family of viruses, genus *Phlebovirus*, within the now expanded order of *Bunyavirales (*2*)*. It is a single-stranded, enveloped RNA virus with a tripartite segmented genome encoding two negative sense segments large (L) and medium (M), as well as a small (S) ambisense segment (3). RVFV encodes for 7 proteins, 4 structural and 3 nonstructural (3). The structural proteins are RNA polymerase (L), glycoprotein Gn (M), glycoprotein Gc (M), and nucleocapsid protein (S) (3). The nonstructural proteins are NSm1 (M), NSm2 (M) and NSs (S) (3). NSs is the major virulence factor for the virus.

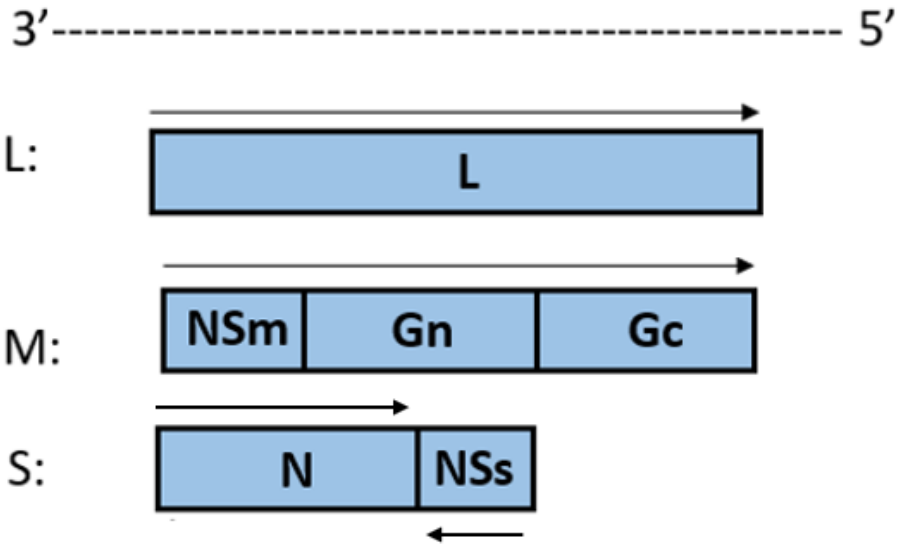

Figure 1: Schematic of RVFV genome structure and coding regions, with coding strategy indicated by arrows.

Rift Valley fever virus was first identified in 1930, during an outbreak in sheep and cattle in Kenya's Rift Valley (4). The virus is primarily a veterinary disease, causing a mortality rate of 10-20% among domesticated ruminants (5). Clinical manifestations in humans include hemorrhagic fever, hepatic disease and encephalitis. The main disease manifestation in ruminants is hepatic disease. One of the most devastating characteristics of RVF are among pregnant livestock which experience "abortion storms" wherein the virus can induce abortogenic rates of 90-100% (6, 7). An association has recently been demonstrated between women who are infected with RVFV and an increased likelihood of late term miscarriage (8). RVFV infection in pregnant women may also carry the potential for vertical transmission to the child. There are a few documented cases of vertical transmission in naturally occurring outbreaks (9). Previous data in our lab has also demonstrated vertical transmission using a rat model (10).

1.1 Rift Valley Fever Virus

1.1.1 Epidemiology

RVFV was first isolated during an epizootic outbreak in the Great Rift Valley of Kenya (11). Major rainfall followed by largescale fatal illness in lambs and febrile illness among the surrounding human population precipitated the discovery of RVFV by local veterinarians (11). Since its initial outbreak, the geographic range of confirmed RVFV outbreaks has steadily increased, growing to include South Africa, Madagascar, Eastern Africa and into Egypt, most recently expanding into the Arabian Peninsula with outbreaks in Saudi Arabia and Yemen (11).

RVFV is an arbovirus, spread primarily by mosquito-bite (12). RVFV replicates in a life cycle common to viruses in the *Phlebovirus* genus. As an arbovirus, RVFV utilizes mosquito species to mature and spread to other host species. The virus can lie dormant in desiccated mosquito eggs during dry periods. In subsequent periods of heavy rainfall, blooms in the mosquito population, already infected with RVFV, can contribute to enzootic and epizootic outbreaks, as was the case in the 1930 outbreak during which RVFV was first identified (11). RVFV has been found in numerous mosquito species including widespread genera such as *Aedes* and *Culex* mosquitoes (13).

RVFV infection in humans is possible from working with infected animals or tissues as well as infection via aerosol exposure (14-16). To date, no human-to-human transmission has been documented.

The history of RVFV human epidemiology includes a series of outbreaks with disparate scales, both in terms of the size of the outbreaks and the range of human outcomes. The 1977 Egyptian outbreak caused an estimated 18,000-200,000 human infections, and 598 disease-attributable deaths (11, 15). Infected individuals in this outbreak predominantly presented with undifferentiated febrile illness (17).

In 2000, Saudi Arabia experienced a smaller but more clinically severe outbreak, with 245 human deaths out of 834 confirmed cases and thousands of livestock succumbing to disease (11, 18). Human clinical and laboratory features from the Saudi Arabian outbreak included fever, jaundice, and neurological symptoms as well as 98% of tested cases characterized by elevated liver enzymes, highlighting RVFV hepatic tropism. Hemorrhage, neurological symptoms and jaundice were all independently associated with increased mortality rate.

Among livestock in the same Saudi Arabian outbreak, an estimated 40,000 animals, primarily sheep and goats succumbed to RVFV infection themselves or experienced spontaneous abortion. Mass ruminant abortion storms are a hallmark of epizootic events and often precede RVFV human cases (11, 18).

Over time, RVFV has spread steadily throughout Africa, with major outbreaks in both humans and livestock being recorded in Sudan, Somalia, Kenya, Tanzania, Zambia, Zimbabwe, South Africa, Senegal, Mauritania, Yemen, and even Madagascar (19). Expanded geographic distribution in Africa and beyond is expected to continue growing concomitant with increasing global temperatures due to climate change.

1.1.2 Clinical RVF Disease in Humans

The most common clinical manifestation of RVFV infection in humans is a self-limiting febrile illness (20, 21). The virus has a 4-6-day incubation period after which begin with chills, dizziness, malaise, headaches, and nausea. After these initial symptoms, fever typically occurs for between 4 and 10 days (11). The vast majority of infections are limited to this level of severity, however 1-2% of human infections present with more complicated and severe outcomes (20). The most common of the more severe outcomes can include ocular RVFV infection, hemorrhagic fever, and encephalitis.

Ocular RVFV infection can cause blindness, either concurrent with the presentation of initial febrile symptoms, or even up to months after infection, a phenomenon in common with other viral infections occurring in immune-privileged sites where viral flareups long after the primary infection can accompany symptoms and new complications (22).

Hemorrhagic fever occurs in the most fatal cases, often with little warning. In addition to the symptoms common in early RVFV infection, infected individuals can also experience bleeding in the gums and gastrointestinal tract, jaundice, and a reduction in hemoglobin and platelet counts. Direct hemorrhage is not necessary for a hemorrhagic fever distinction and the most common cause of death in these cases is liver failure, typically within 3-6 days (20).

Encephalitis in RVFV infections is accompanied by direct viral infection of the brain. The path the virus takes as well as the dynamics controlling this course of disease are unclear. Individuals progressing to encephalitic RVFV infection show symptoms common to neurological disorders and other encephalitic viruses including headaches, nausea, confusion and seizures (20).

1.1.3 Prevention and Treatment

No vaccines or therapeutic drugs are FDA-approved for Rift Valley fever virus (14). A few experimental therapeutics exist, albeit with significant side effects and questionable efficacy. The vaccines that exist for RVFV include attenuated live vaccines, some of which are licensed for use in livestock, but which carry with them documented teratogenic effects.

The first live-attenuated vaccine was derived from the Smithburn strain of RVFV in 1949 (23). The virus was attenuated by passaging in mouse brains 102 times, and was administered to livestock as a vaccine (24). The vaccine was refined in 1971 and sold as a “modified live virus vaccine” (MLVV), but despite its heavy attenuation, the virus had a high potential for reversion to wildtype virus in livestock (24). To use this vaccine in regions of Africa in which RVFV was not already endemic could essentially result in increasing the pace of the virus’ geographic spread in the likely event that the vaccine would revert to wildtype virus after administration into the livestock population.

The United States government began research on another RVFV vaccine candidate, labeled MP-12, after the 1977 outbreak in Egypt (24). Two clinical isolates from the Egyptian outbreak, ZH501 and ZH548, were serial passaged in the presence of 5-flourouracil to attenuate the virus via mutagenesis (23). The live attenuated mutant that resulted from ZH548 became the MP-12 vaccine, which contained a myriad of deletions in all three segments of the virus (23). The MP-12 strain was again teratogenic, although to a lesser degree than the Smithburn vaccine (25). Despite showing efficacy in preventing abortions in ewes during later pregnancy, a low rate of abortions occurred when the vaccine was administered early in pregnancy, as well as other adverse effects in calves born from vaccinated ewes (24, 25). Nevertheless, the US government needed a vaccine for their military personnel and did conduct a small experimental vaccination of 100 soldiers. MP-12 proved to be safe and efficacious, though the FDA has never licensed the vaccine (24).

Another vaccine candidate is Clone 13, isolated from a plaque from an infected individual in Central Africa (24). This vaccine strain has the majority of the NSs gene deleted, resulting in functional loss of the NSs protein, the major virulence factor in RVFV infections. Reversion is significantly hampered by the extent of the NSs deletion and Clone 13 remains a viable candidate for a veterinary vaccine, demonstrating efficacy and safety in trials (24).

Finally, DDVax, a recombinant RVFV vaccine lacking both NSs and NSm virulence factors, has demonstrated protection against RVFV challenge in sheep, including during pregnancy (26). As of yet, the FDA has not approved it for use in humans.

1.2 Public Health Significance

1.2.1 Endemic RVFV in Africa

Outbreaks in endemic areas of Africa are sporadic but generally follow periods of heavy rainfall which result in blooms in the mosquito population. RVFV infections exhibit some seasonality but it remains difficult to predict outbreaks even in endemic areas.

In addition to human morbidity and mortality due to RVFV infection, veterinary infections can drastically impact the livelihoods of those with livestock who have been affected. In Kenya, where the virus was originally discovered, livestock account for almost 90% of the pastoral economy (27). RVFV can effectively obliterate farmers' livelihoods through a combination of directly killing off their livestock, inducing massive abortogenic rates destroying the next generation of livestock for which farmers could make a profit, and even through stigma attached to those animals that do survive the outbreak, essentially making whatever animals survive also unprofitable (27).

This effect on the supply side of the economy in Kenya naturally has deleterious effects on other institutions, including butchers, meat markets, and slaughterhouses. Further, epidemics tend to inspire surrounding markets to refuse any products coming out of the affected area. During outbreaks in Africa, including the Arabian Peninsula, surrounding countries banned the import of meat from affected areas (27).

1.2.2 RVFV in the United States

Closer to home, the United States is not immune to the potential for RVFV enzootic and epizootic transmission. *Aedes* and *Culex* mosquitos are the most common vectors for RVFV in Africa, but over 50 mosquito species have been shown to be capable of transmitting the virus, including in experiments with species found in North America (28). In addition to US livestock, white tailed deer have also been shown to be a competent host for the virus (29). With the continuing march of climate change and subsequent increases in habitable ranges for mosquito populations, RVFV stands to establish prolonged transmission and endemic status in much of the US, with a $50 billion dollar price tag to attempt to control the virus once it is established here (30).

The spread of Zika virus in the Americas during 2015-2016 is evidence of the potential harm of mosquito-borne viruses increasing range. For a virus with similar congenital and neurological characteristics as RVFV, there were 1,000 confirmed cases of Zika infection in pregnant women (31).

1.3 Animal Models

1.3.1 Mice

Mice are very susceptible to RVFV infection. The most common mouse used in animal research is the BALB/c strain. Mice infected with wildtype Rift Valley fever virus strains show symptoms in 2-3 days and typically die within 24 hours of symptom onset. Death from wildtype

RVFV in mice occurs within 3-5 days, with infected animals succumbing to fatal hepatitis regardless of exposure route or dose (32). Mice given attenuated RVFV with a deletion in the NSs gene (ΔNSs) via footpad injection results in subclinical disease, characterized by rapid antibody responses and robust T cell involvement. When given intranasally, RVFV-ΔNSs-infected mice develop lethal neurological disease in 7-9 days, providing a model of *in vivo* encephalitis (32). $IFNAR^{-/-}$ mice develop severe disease when exposed to attenuated RVFV strains due to their inability to mount interferon-driven antiviral responses (33).

RVFV pathogenesis in mice mimics that of lambs, where the disease manifests as acute hepatitis with capacity to progress to encephalitis. Hemorrhagic fever does not occur in mouse RVFV infections (34).

In mice infected with recombinant ΔNSs RVFV expressing luciferase, showed that luminescence signal in reproductive tissues, including the ovaries and uterus in female mice, was similar to levels seen in the spleen and liver, tissues of high tropism for RVFV infections (35).

While mouse models are valuable for their ability to be manipulated and for studying differences between lethal and subclinical RVFV infection, the use of wildtype strains causes such rapid disease progression that long term immune response studies are difficult, which is a major limitation.

1.3.2 Rats and the Rat Congenital Model

Disease manifestations in rats infected with RVFV are dependent on rat strain, dose, and exposure route (36). Wistar-Furth rats succumb to hepatic disease and viremia within days of either subcutaneous or aerosol infection with RVFV (37). Lewis rats do not develop disease after subcutaneous exposure but become viremic. However, by the end of infection there is no detectable

viremia or virus in other common RVFV target tissues (37). In addition to these models, the Hartman lab has developed both a reproducible immunocompetent rat model of encephalitic disease in Lewis rats, and a congenital model of RVFV vertical transmission in pregnant Sprague-Dawley rats (10, 38).

McMillen et al. 2018 developed the first reproducible rodent model of congenital Rift Valley fever virus in Sprague-Dawley rats. Pregnant rats subcutaneously infected with ZH501 strain RVFV exhibited vertical transmission and intrauterine fetal demise similar to what has been seen in livestock "abortion storms" during natural outbreaks. Vertical transmission occurred regardless of disease outcome in the dams. Sublethal dams gave birth to still-born pups with gross pathological changes and stunted development (10). Interestingly, despite RVFV generally displaying a hepatic tropism, viral titers were higher in the placenta than in the livers of these infected rats (10). Rat placentas have significant morphological and structural differences from the human placenta, but they are similar enough that rats are common in research on placental development and vaccine teratogenicity during pregnancy (39). This model of disease is understudied and interactions between pathogens and hosts during pregnancy remain poorly defined, particularly in the case of Rift Valley fever virus congenital disease. Studying the effects of RVFV infection in reproductive and fetal tissues is crucial to the continuing development of non-teratogenic vaccines and therapeutics.

1.3.3 Non-Human Primates

NHPs have long been used in RVFV studies, particularly in studies on the innate antiviral IFN response. Rhesus macaques are the most used non-human primate (NHP) model in RVF studies, with lethal to severe disease being seen in 20% of infections. Rhesus macaque studies have

been used to define pathogenesis of RVFV infection, investigate the dynamics of IFN responses and viral IFN antagonistic proteins, and for a number of vaccine studies (40-44). In a rhesus macaque model, IFN-α administration initiated 24 hours before or 6 hours post-infection was effective in protecting infected rhesus macaques from viremia, hepatocellular damage, and death (45).

Across infection routes (aerosol, subcutaneous, intravenous) RVFV infection in rhesus macaques results in non-lethal fever, viremia, and in rare cases lethal hemorrhagic disease. Hartman et al. 2014 characterized RVFV aerosol infection in NHP models using three additional species: cynomolgus macaques, African green monkeys, and marmosets to evaluate them for their potential in developing an encephalitic model more similar to human disease progression (14). Cynomolgus macaques developed fever similar to rhesus macaques but did not develop encephalitis (14). Marmosets succumbed to severe neurological disease in low dose aerosol exposures, and encephalitic disease at high dose aerosol exposures. African green monkeys succumbed to similar neurologic disease after aerosol exposure but did not progress to symptoms or disease when exposed to RVFV subcutaneously (14).

1.4 Rift Valley Fever Virus and Interferons

1.4.1 Type I Interferons

Interferons constitute the first line of immunological defense against viral infections. They are broken down into three classes: type I, II, and III IFNs. Each of these types of IFN function by activating intracellular antimicrobial responses through changes in gene expression and

subsequent protein production. Type I IFNs, IFN-α and IFN-β, are the first innate immunological response to viral infection, signaling through the receptor IFNAR which is expressed on all nucleated cells (46). Secretion of type I IFN in response to immune recognition of pathogen and damage-associated molecular patterns occurs through a well-defined system that was first described over 50 years ago (47). Type I IFNs are the cytokines most predominantly produced in response to immunological pattern recognition receptor (PRR) binding to microbial peptides (48). The initial round of IFN-α/β signaling is currently understood to be induced through IRF3 and NF-κB stimulation. Subsequent rounds of IFN signaling and induction are mainly the result of type I IFN endocytosis by IFNAR, followed by a signal cascade and the phosphorylation of STAT1 and STAT2, leading to altered gene expression and upregulated IFN production (49). Thus, type I IFNs can signal in a positive feedback loop where pro-inflammatory signaling feeds into itself further stimulating the immune response. Type I IFNs are potent proinflammatory interferons capable of inducing widespread antiviral immunity. Type I IFNs contribute to a general antiviral state through a variety of inducible changes including upregulated expression of MHC class I molecules and antigen presentation on all cells, activation of macrophages and dendritic cells, activation of Natural Killer (NK) cells, recruitment and activation of lymphocytes and enhanced effector function in CD4+ and CD8+ T cells (50-52). Type I IFNs also contribute to antiviral immunity through protein kinase R (PKR) which inhibits protein translation and synthesis through phosphorylation of eIF-2 protein synthesis initiation factor (46, 53). Type I IFN production and signaling results in a positive feedback loop of increasing type I IFN gene expression and protein release in an autocrine, paracrine, and endocrine manner. IFN-α/β have also been shown to induce over 300 interferon-stimulated genes (ISGs), 51 of which had host response specific functions, contributing to inflammation, immunomodulation and changes in host cell transcription to create

a more hostile microenvironment for the invading pathogen (54). These IFNs are the quintessential antiviral innate signaling molecules and play an indispensable role in the very early stages of infection.

1.4.2 Type II Interferons

Type II IFNs also play an important role in viral infections and are predominantly secreted by NK cells (55). In surrounding cells, NK secretion of type II IFN, IFN-γ, and subsequent binding to its receptor IFNGR, resulted in increased reactive oxygen species production particularly with nitric oxide (NO) production which is a potent inhibitor of viral replication (56). IFN-γ also stimulated phagocytotic function in macrophages as well as gearing immune cells, including NK cells, macrophages, and T cells to skew them towards a more proinflammatory profile (57). IFN-γ plays a heavier role in dedicated myeloid and lymphoid immune cell signaling than it does in epithelial barrier cells where viral infection first occurs and functions more-so to coordinate the immune response after infection than controlling the very early stages of viral infections as type I IFNs do.

1.4.3 Type III IFNs

Type III interferons, the IFN-λ family of proteins, are a recently characterized group of innate antiviral molecules that function in modulating host immune responses to viral infection at epithelial barriers, including at the placenta. Unlike type I interferons which elicit antiviral responses in all nucleated cells, expressing the IFNAR1 receptor, type III interferons elicit a more localized innate antiviral response, with the heterodimeric receptor for IFN-λ proteins,

IL28Rα/IL10Rβ, only found on barrier epithelial cells (58). The type III interferon family includes IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4 in humans; with IFN-λ1-3 proteins exhibiting localized pro-inflammatory and antiviral functions, and IFN-λ4 demonstrating immunoregulatory function in attenuating the antiviral response (59). Interestingly, type III IFNs have been demonstrated to be induced by type I IFN stimulation, making IFN-λs an ISG of type I IFNs (60). In mice, only IFN-λ2/3 are functional (61).

1.4.4 The role of IFNs in Congenital Viral Infection

Type I IFNs contribute extensively to the immune environment during pregnancy and are necessary for protection of the pregnancy through their antiviral properties (62). Type I IFN dysregulation can also contribute to immunopathology during pregnancy (62). Elevated levels of type I IFN are associated with antiphospholipid syndrome events and earlier onset for preeclampsia in human pregnancies (63). Additionally, type I IFN responses induced by viral infection during pregnancy can block fusion of the syncytiotrophoblast layer of cells in the placenta, which is necessary for viral defense, and can contribute to immunopathology and loss of barrier integrity at the placenta (64). Zika virus (ZIKV), a flavivirus with similar transmission dynamics to RVFV including vertical transmission, has been implicated in this phenomenon. In a mouse model of ZIKV vertical transmission, researchers found that only infected mice with competent IFNAR1 receptors exhibited resorption of the fetus after ZIKV infection. IFNAR signaling as a result of ZIKV infection inhibited placental labyrinth development resulting in abnormal architecture of the maternal-fetal environment and further work in human chorionic villous explants suggested that type I IFNs, but not type III IFNs, altered placental morphology implicating type I IFNs as potential

mediators of adverse pregnancy complications, including spontaneous abortion in the context of viral infections (65).

Previous work has examined a type III interferon, IFN-λ1, and its ability to confer protection at the placental barrier during Zika virus (ZIKV) infection (66). ZIKV is an emerging flavivirus and the latest in a group of pathogens known as TORCH pathogens (*Toxoplasma gondii*, other, rubella virus, cytomegalovirus and herpes simplex virus) which cause congenital infections and can gain access to the fetal compartment in infections during pregnancy. IFN-λ administration during pregnancy reduced ZIKV burden in maternal and fetal tissues and reduced placental injury and fetal demise (67). Further, gestational stage has been shown to impact ZIKV pathogenesis, with IFN-λ mediated immunity at the maternal-fetal interface. This phenomenon may be due to changes in IFN-λ signaling throughout gestational stage or possibly changes in expression of potential entry receptors for the virus (66, 68). Mouse models lacking IFN-λ receptors had increased ZIKV replication in the placenta and fetus, while IFN-λ treatment reduced ZIKV infection in pregnant mice (69). Primary human trophoblasts, the barrier cells of the placenta, constitutively release IFN-λs which protects trophoblast and non-trophoblast cells from Zika virus infection, suggesting the virus must use alternative pathways to cross the placental barrier (66, 70).

Human Cytomegalovirus (HCMV), another congenital viral infection, is a significant cause of congenital morbidity and mortality with roughly 5 out of every 1,000 infants born every year infected, 10% of whom will experience neurological disease (71). IFN downregulation by HCMV is well-documented (72). To date, work done to characterize the relationship between type III IFNs and HCMV has focused on IFN-λ signaling during CMV infection post-organ transplant and with HIV co-infection, immunocompromised at-risk populations for CMV infection and reactivation. In this field, IFN-λ has been shown to be a potent antiviral ISG activator, and IFN-λ induced

signaling resulted in significant reduction of cells positive for CMV immediate-early protein (73, 74). However, in the case of HIV-coinfected individuals, IFN-λ genotype did not have an effect on susceptibility to CMV reactivation (75). To date, no work has been published characterizing congenital CMV infection and its relationship to IFN-λs.

Herpesviruses also have congenital properties when infections occur during pregnancy. Both HSV-1 and HSV-2 have been shown to be sensitive to the antiviral properties of IFN-λs, where signaling has resulted in reduced viral titers, however these effects were cell type dependent. IFN-λ treatment was effective in reducing titers in the case of hepatic and neuronal cell infection (60, 76). Additionally, type III IFNs are capable of and necessary in inducing NF-κB, ICP27, and IRF-3 in HSV-1 infections (77). To date, no work has been published on HSV congenital infection and its relationship to type III IFNs.

1.4.5 Rift Valley fever virus and type I IFNs

In RVFV infection, IFN-α has been shown to be capable of preventing clinical disease in RVFV infected rhesus macaques and can suppress RVFV viremia (45). However, RVFV non-structural protein NSs has also been demonstrated to block type I IFN production by targeting the transcription factor TFIIH (78). NSs inhibits IFN-β production by interacting with the transcription factor YY1 that regulates IFN-β gene expression through a SAP30 complex which acts as a repressor on the IFN-β promoter (79). The presence of NSs in infected cell nuclei also induces DNA damage responses and causes cell cycle arrest and apoptosis. NSs also promotes the degradation of TFIIH p62 and PKR proteins (80).

While an extensive body of research has been done investigating the relationship between Rift Valley fever virus and IFN-α/β, thus far no work has been done to investigate interactions

between type III IFNs and RVFV. This work addresses that gap in the field by determining the effect of type III IFNs on RVFV with a direct comparison to type I IFNs.

1.5 Placenta Structure and Function

1.5.1 Placenta Structure

The human placenta is organized in a villous structure, resembling branches of a tree, to maximize surface area for the exchange of nutrients in the intervillous space and the maternal-fetal interface (81). Syncytiotrophoblasts form the outermost cell layer of the placenta and this layer is a long, continuous multinucleated unit of cytoplasm. Directly beneath this layer are the cytotrophoblasts which function in anchoring the developing fetus and placenta to the uterine wall. Cytotrophoblasts expand and proliferate into the maternal decidua where they are termed extravillous trophoblasts (EVTs). EVTs may represent a potential entry point in for other TORCH pathogens like Zika and CMV, as well as the possibility that these infections are mediated by trafficking of infected maternal immune cells across the placenta, through breaks in the syncitiotrophoblast layer, or through ascending infection (81). In the case of RVFV vertical transmission, RVFV has been shown to directly infect syncytiotrophoblasts, a phenomenon unique to RVFV (10).

1.5.2 Function of the Placenta as an Immune Barrier

The placenta represents the physical barrier layer between the maternal blood supply and the developing fetus. Of importance are placental syncytiotrophoblasts which develop into a complete layer enveloping the implanted embryo by day 7 post-fertilization and are largely responsible for nutrient and waste exchange in the maternal-fetal interface (82). Once the uteroplacental circulatory system is established at about 12 weeks of gestation, these fetal-origin syncytiotrophoblasts are the only cells that come into direct contact with maternal blood (83, 84). This makes this cell layer indispensable in microbial defense and these cells are traditionally considered to be entirely microbially resistant. The source of this resistance is due largely to their morphological novelty, lacking intercellular junctions and receptors, as well as the elasticity conferred by having one multi-nucleated cytoplasm (85). In addition to these properties, syncytiotrophoblasts constitutively secrete type III IFNs, establishing an immune microenvironment at the placental barrier that primes trophoblasts in a paracrine and autocrine fashion for resistance to microbial infection (86). Little is known about specific mechanisms by which viruses capable of congenital infection breach or bypass the placental barrier to access the developing fetus. This work represents the first characterization of how RVFV may circumvent the type III IFN-mediated immune response in this critical syncytiotrophoblast layer.

2.0 Statement of Project and Specific Aims

Rift Valley fever virus (RVFV) is an emerging virus of domesticated livestock endemic to many regions of Africa that is spread by multiple mosquito species (1). RVFV outbreaks commonly affect sheep and cattle, resulting in a wide range of clinical manifestations including hepatic and neurological disease. Human clinical manifestations have included hepatic disease and hemorrhagic fever as well as encephalitic disease. One of the most devastating characteristics seen in previous outbreaks is the phenomenon of widespread spontaneous virally-induced abortion among pregnant livestock (6). An association has also recently been demonstrated between women who are infected with the virus and increased likelihood of late term miscarriage (8). RVFV infection in pregnant women may carry the potential for vertical transmission to the child. There are a few documented cases of vertical transmission in naturally occurring outbreaks (9). Vertical transmission has also been demonstrated in recent laboratory experiments using a rat model in the Hartman lab (10). Pregnant rats were inoculated with wildtype RVFV subcutaneously, mimicking the mosquito bite transmission seen in natural infections. Pregnant rats more susceptible to death as compared to their nonpregnant counterparts. In addition, reproductive tissues were shown to be targets of RVFV infection including the placenta, uterus, ovary, and amniotic sac and direct vertical transmission of the virus to pups during late gestational stage resulted in pup deformity and death.

All these data and findings taken together indicate that RVFV may have significant potential for vertical transmission and serious adverse consequences in pregnant women. What the above evidence lacks is a clear understanding of how RVFV accomplishes such a prolific and

detrimental infection in the maternal-fetal environment. The work performed here represents a start at understanding this critical phenomenon by examining the effect of type III IFNs on RVFV.

IFNs are the main arm of innate antiviral immunity. They function in the earliest host response to infection. IFN responses utilize autocrine and paracrine signaling to induce a hostile microenvironment for the invading pathogen, alter host-cell transcription to induce an antiviral state, and function in activating and recruiting immune cells to sites of infection. IFNs are separated into three types: I, II, and III.

The relationship between RVFV and IFNs is complex. RVFV nonstructural protein NSs has been previously demonstrated to downregulate type I IFN (IFN-β) expression in infected cells (45, 87). Conversely, IFN-α treatment has been shown to be effective in preventing or greatly suppressing viremia (58).

Type III interferons, the IFN-λ family of proteins, are a recently characterized group of innate antiviral molecules that function in modulating host immune responses to viral infection at epithelial barriers, including at the placenta. To date, no work has been performed to study the possible effects of type III IFNs on RVFV infection or pathogenesis in any context, let alone pregnancy and vertical transmission.

We will address this gap in the field by examining the relationship between RVFV and IFN-λ in vitro, using relevant epithelial cell lines. Placental tissues from livestock, rodents and humans have been demonstrated to be particularly permissive to RVFV infection (10, 88). *Therefore, we hypothesize that* RVFV may be resistant to the effects of type III IFNs at epithelial cell barriers such as the placenta, contributing to the frequency of vertical transmission of the virus. *We further hypothesize that* resistance of RVFV to type III IFNs is due to the action of NSs, a nonstructural protein that is known to inhibit other interferon type pathways (23,24). The

sensitivity of RVFV to type III interferons will be determined, and we will also determine the mechanisms by which RVFV may avoid or downregulate type III IFN responses during infection. In these studies, we hope to gain more information on how RVFV avoids the specific barrier-layer immune defense of IFN-λ at the placenta. We will address our hypothesis using the following specific aims:

AIM 1: Determine RVFV sensitivity to exogenous IFN-λ1. Type I IFNs (i.e. IFN-β) can inhibit infection and replication of RVFV despite the fact that the viral NSs protein acts as an interferon antagonist. It is currently not known whether RVFV is sensitive to type III IFNs. To address the ability of type III IFNs to inhibit RVFV infection, the permissivity of relevant cell lines to RVFV infection will be determined in the presence or absence of the addition of IFN-λ or IFN-β (as a control) at different times before or after infection.

AIM 2: Determine the role of RVFV non-structural protein NSs in modulating expression of IFN-λ1 stimulated genes (ISGs). We will determine whether the viral NSs protein can inhibit ISG promoter activation and gene expression after stimulation of cells with either IFN-λ or IFN-β. Three ISGs of interest were selected: PKR, CXCL3, and CXCL8.

AIM 3: Determine the role of RVFV non-structural protein NSs in modulating IFN-λ1 expression. The goal of this aim is to determine whether NSs can prevent the induction of IFN-λ protein expression after treatment of relevant cell lines with a potent IFN inducer polyI:C. Presence of interferons in cell culture supernatant will be measured by ELISA for differing doses of NSs and poly (I:C) concentrations.

At the conclusion of this study, we will have characterized the sensitivity of RVFV to IFN-λ1 at epithelial cell barriers and compared that to IFN-β, in which the effects of RVFV are more

well characterized. In addition, we hope to describe the effect of NSs protein on IFN-λ stimulated gene expression, and on expression of IFN-λ protein in epithelial cells.

3.0 Materials and Methods

3.1 Biosafety

All work with RVFV was performed in the biosafety level 3+ Regional Biocontainment Laboratory (RBL) at the University of Pittsburgh Center for Vaccine Research. Personnel wore powered air-purifying respirators (PAPRs), waterproof apron, and facility designed shoes. The RBL is a shower-out facility that requires a full clothing change into scrubs before entry and a personal shower and change into new scrubs to exit. Work was conducted in a class III biosafety cabinet (BSC) using Vesphene IIse (Steris Corporation, cat. #646101) at a 1:128 working dilution as a disinfectant. All samples removed from the BSL-3 were inactivated using methods described below which have been verified and approved by a University of Pittsburgh biosafety oversight committee.

3.2 Viruses and Cells

The ZH501 strain of RVFV was provided by Barry Miller (CDC, Fort Collins, CO) and Stuart Nichol (CDC, Atlanta, GA). The recombinant GFP-RVFV virus, containing a deletion for the NSs gene, was obtained courtesy of Jeroen Kortekaas (Wageningen University, Wageningen, Netherlands). Virus was propagated on VeroE6 (CRL-1586, ATCC) cells in standard culture conditions using Dulbecco's modified Eagle's medium (DMEM) containing 2% (D2) or 10% (D10) fetal bovine serum (FBS), 1% penicillin-streptomycin (pen/strep), and 1% L-glutamine.

Two immortalized cell lines HepG2 (HB-8065, ATCC) and BeWo (CCL-98, ATCC) cells, were used to model hepatocyte and placental trophoblast cell types, respectively. HepG2 cells are an immortalized hepatocellular carcinoma cell line chosen to mimic the hepatic tropism of RVF disease. BeWo cells are a choriocarcinoma cell line chosen for their ability to form syncytia and for closely mimicking placental syncitiotrophoblasts previously described as a unique cell type infected by RVFV. HepG2 cells were maintained in standard culture conditions using D10 or D2. BeWo cells were maintained in standard cell culture conditions using Kaighn's Modification of Ham's F12 medium (F-12K) with 20% FBS during growth or 2% FBS during infection (30-2004, ATCC).

3.3 Viral Plaque Assay

To determine viral titers via plaque assay, VeroE6 cells were seeded in a 6-well plate with D10 media at high density one day before to ensure confluency on the day of the assay. The following day the virus to be titered was serially diluted in D2 media at 1:10 dilutions, from 10^{-1} to 10^{-6}. For each dilution, 200 ul were added to each well in duplicate and each 6-well plate was incubated at 37 C, 5% CO_2 for 1 hour, rocking every 15 minutes. After an hour, the inoculum was removed and replaced with 3 ml of a 1:1 mixture of nutrient media and agar. Once the mixture solidified to create a gel plug, plates were placed back in the 37 C incubator for 3 days. After 3 days, 2 ml of 37% formaldehyde was added to each well and left overnight to inactivate virus and fix the wells. The following day, formaldehyde and gels were disposed of as chemical waste. The cells were then rinsed with distilled water and stained with 0.1% crystal violet (CV) working

solution for 10 minutes. After CV solution was rinsed off, the resulting plaques were counted from each duplicate, averaged, and used to calculate viral titer.

3.4 Quantitative Real-Time Reverse Transcriptase PCR

Infectious materials to be analyzed by PCR were first inactivated using an approved protocol by the University of Pittsburgh RBL Biosafety Officer. To do this, 100 ul of infectious sample was added to 900 ul of TRIzol reagent (Invitrogen, cat. #15596026) in a 1.7 mL Eppendorf tube, capped and vortexed. The tube was kept capped and allowed 10 minutes of contact time at room temperature for full inactivation. Samples were then transferred to a new Eppendorf tube, removed from the BSL-3 to BSL-2 lab space, and stored at -80 C until ready for RNA extraction.

RNA extraction was performed using a modified version of the PureLink RNA Mini Kits protocol (Invitrogen, cat. #12183018A). Final samples were run on a 96-well plate in duplicate against a known standard curve and quantified based on the standard curve generated on each plate. PCR results were reported in relative PFU/ml equivalence based on prior calculations comparing standard curves to plaque assay results.

3.5 PKR, CXCL3, and CXCL8 Quantitative Real-Time PCR

Samples to be analyzed for interferon stimulated gene (ISG) expression came from isolated RNA as previously described. RNA samples underwent cDNA synthesis to generate 1X stocks of cDNA for qPCR. PCR analysis was conducted in multiplex using AB TaqMan Multiplex Master

Mix 2X (Applied Biosystems, cat. #4484263), and a primer probe specific for each ISG: PKR (ThermoFisher Scientific, assay ID: Hs00169345_m1 (FAM)); CXCL3 (ThermoFisher Scientific, assay ID: Hs00174103_m1 (FAM)); and CXCL8 (ThermoFisher Scientific, assay ID: Hs00174103_m1 (FAM)) normalized to an endogenous β-actin control (ThermoFisher Scientific, assay ID: Hs01060665_g1 (VIC)). mRNA expression levels were calculated using the ΔΔCT method and expressed as fold-change compared to untreated controls.

3.6 Lipofectamine Transfection

To analyze potentials effects of RVFV protein NSs on IFN protein production, cells were plated to 70-90% confluency in Opti-MEM reduced serum medium (ThermoFisher, cat. #31985062) and incubated overnight at 37 C, 5% CO_2. Transfections were performed using Lipofectamine 3000 Transfection Reagent (ThermoFisher Scientific, cat. #L3000001) following manufacturer instructions. Cells were transfected with either an NSs-GFP expression plasmid or an empty backbone control and transfection efficiency was verified by fluorescence microscopy. The following day, transfection media was replaced with fresh growth media with 2% FBS. Wells were then treated with poly(I:C) dsRNA mimic (R&D Systems, cat. #4287/10) at a range of concentrations and supernatants were collected at 4, 12, and 24 hours post-stimulation.

3.7 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs to detect presence of secreted IFN proteins were performed on samples of interest using Human IL-29/IL-28B (IFN-λ1/3) DuoSet ELISA kits (R&D Systems, cat. #DY1598B-05), Human IFN-β DuoSet ELISA kits (R&D Systems, cat. #DY814-05) along with DuoSet ELISA Ancillary Reagent Kit 2 (R&D Systems, cat. #DY008). Supernatants were analyzed in duplicate in 96-well plates and compared to a known standard for each plate per manufacturer's instructions. Results were reported in pg/ml.

4.0 Results

4.1 AIM 1: Determine RVFV sensitivity to exogenous IFN-λ1.

HepG2 and BeWo cells were the cell lines chosen for these experiments. HepG2 cells are a hepatocellular carcinoma epithelial cell line used in IFN-λ research, particularly in the field of hepatitis (89). Their ability to secrete and respond to type III IFNs has been well-documented in the literature (90). Due to this and their extensive use in hepatitis virology, they were chosen as an appropriate cell line to mimic the hepatic form of RVF disease. BeWo cells are a choriocarcinoma cell line widely used to model placenta trophoblast cells *in vitro* (91). BeWo cells are an immortalized trophoblast cell line and have been used in viral research, importantly being demonstrated to form syncytia, which set these cells apart from other common placental cell lines (92). Due to their trophoblast origin and record of viral research in the literature, BeWo cells were selected as the best placenta cell line to investigate RVFV infection *in vitro*.

To determine the optimal timepoint to use for viral infections in HepG2 and BeWo cell lines, a time-course experiment was conducted. BeWo or HepG2 cells were plated on 6-well plates at a density of 1.5×10^5 cells/well. The following day, each cell line was infected at 0.01 MOI with the pathogenic, wildtype ZH501 strain of RVFV (wt RVFV). This MOI was chosen as a moderate MOI that would result in productive infection over a short timeframe but avoiding killing the cells too rapidly. Viral production in each cell line peaked at 48 hours-post-infection (hpi), and this timepoint was chosen as the point of analysis for subsequent experiments (Figure 2).

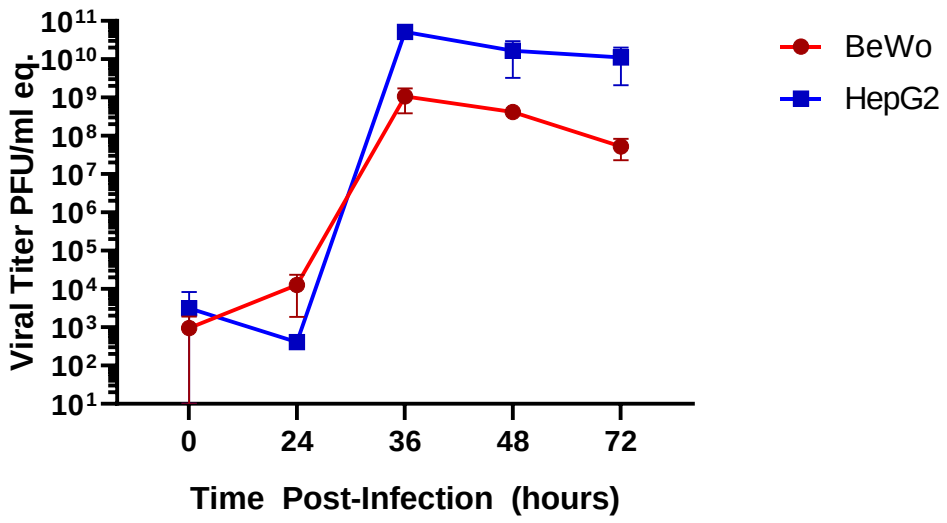

Figure 2: ZH501 RVFV viral titers over time in HepG2 and BeWo cells.

Mean viral titers in cell supernatants by qRT-PCR for HepG2 and BEWo cells infected at 0.01 MOI. (n=3, bars indicate SD)

To assess whether RVFV is sensitive to exogenous type III IFN treatment, HepG2 and BeWo cells were plated at a density of 1.5 x10^5 cells/well on 6-well plates. The following day, cells were infected at 0.01 MOI wt RVFV and treated with increasing doses of exogenous IFN-λ1 (0, 10, 100, or 1,000 ng/mL) at the time of infection (0 hours post-infection; hpi) or 24 hpi. These treatment times were chosen based off of *in vivo* RVFV infection data demonstrating that pre-treatment or immediate post-infection treatment with IFN-α was effective in reducing viral titers in animals compared to delayed treatment (45). Cells were also infected similarly and treated with IFN-β at 0 or 24 hpi as a control based on previously published data, with the expectation that immediate treatment of cells with type I IFN would result in reduced viral titers. Supernatants and cell lysates were taken at 48 hpi and analyzed by viral plaque assay and qRT-PCR to determine viral titers (Figure 3).

Exogenous IFN-β and IFN-λ1 treatment inhibited RVFV production in HepG2 cells when cells were treated immediately after infection (0 hpi) with 10, 100, or 1,000 ng/ml, as compared to untreated controls; conversely, delayed treatment with either IFN at 24 hpi had no effect on virus production in HepG2 cells (Figure 3). We hypothesize, and will test later in this thesis, that the resistance of HepG2 cells to delayed IFN treatment may be due to the action of the RVFV IFN antagonist NSs protein. Notably, IFN-λ1 treatment did not diminish RVFV production in BeWo cells regardless of time of addition (Figure 3). This may help to explain the underlying mechanism of why RVFV replicates well in the placenta despite constitutive IFN-λ secretion under normal conditions (10, 66). These experiments indicate that RVFV is sensitive to and can be inhibited by type III IFNs, although this is cell-type dependent as treatment of BeWo cells did not affect RVFV viral production.

As previously discussed, Rift Valley fever virus non-structural protein NSs is a known type I IFN antagonist (78). To assess whether NSs may be mediating RVFV resistance to the antiviral

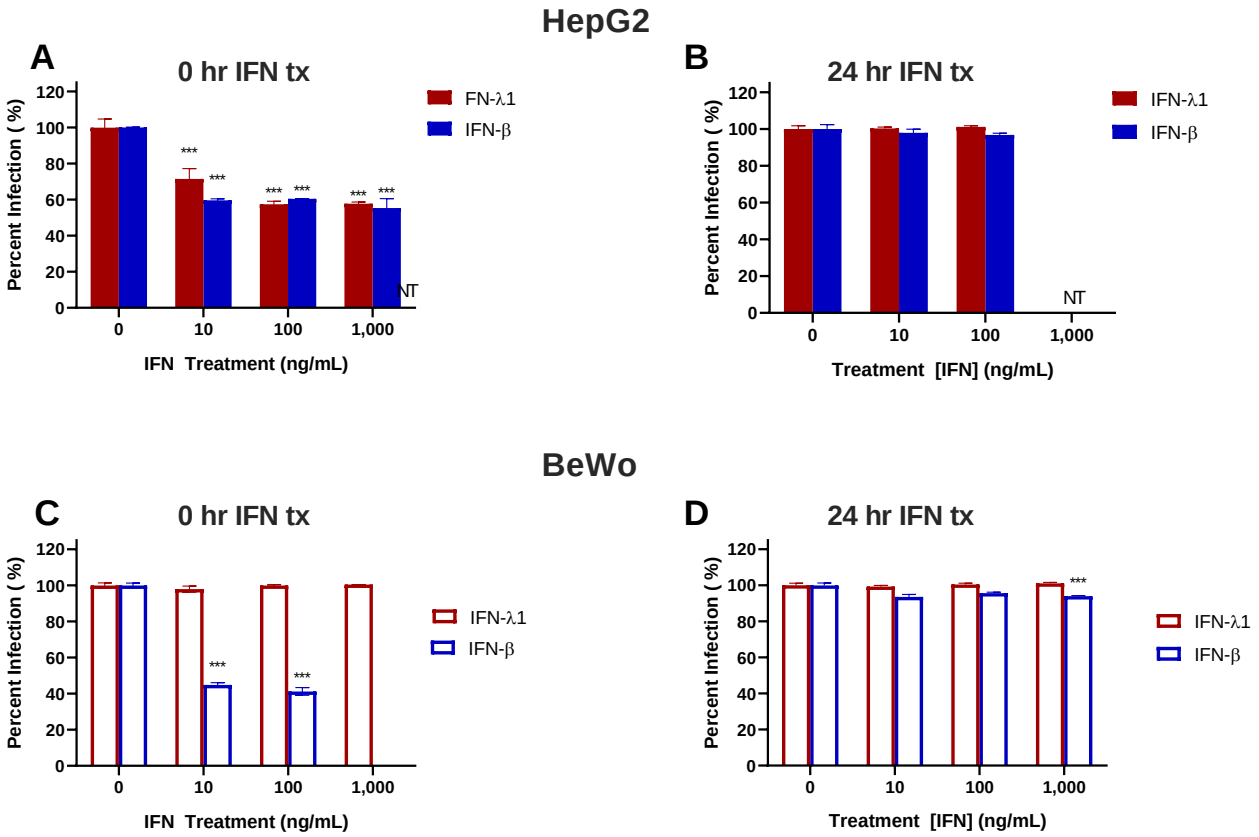

Figure 3: The effect of IFN treatment on ZH501 RVFV viral titers in HepG2 and BeWo cells.

Mean viral titers at 48 hpi measured by viral plaque assay (VPA) for HepG2 and BeWo cells infected at 0.01 MOI with ZH501 RVFV and treated with exogenous IFN-λ1 or IFN-β at 0 hpi (A, C) or 24 hpi (B, D). (n=3, bars indicate SD). Data are expressed as percentage of untreated controls from log-transformed viral titers. Asterisks indicate significance (*, P <0.05, ***, P < 0.001) by Two-Way ANOVA, Tukey's multiple comparison test. NT = not tested.

effects of type III IFNs, the same infection protocol was repeated with RVFV-ΔNSs/GFP, containing a deletion for the NSs protein and an insertion of eGFP in its place. HepG2 and BeWo cells were infected with RVFV-ΔNSs/GFP at 0.01 MOI and treated with IFN-λ1 or IFN-β at 0 and 24 hpi, and virus production was measured by qRT-PCR at 72 hpi. This later time point was chosen to account for the slower growth kinetics of ΔNSs-RVFV.RVFV-ΔNSs/GFP infection of HepG2 cells was dramatically inhibited by IFN-β but not IFN-λ1 regardless of time of treatment (Figure

4A). These resulted indicated that sensitivity of RVFV to IFN-β but not IFN-λ1 was enhanced in the absence of the NSs protein.

Contrary to what we expected, RVFV-ΔNSs/GFP was not more sensitive to IFN-λ1 in BeWo cells when added at either treatment time (Figure 4), indicating that RVFV infection and antiviral resistance to type III IFNs in this trophoblast cell line may not be mediated by the action of NSs. RVFV-ΔNSs/GFP was also less sensitive to IFN-β in BeWo cells compared to wt RVFV, which was contrary to our hypothesis.

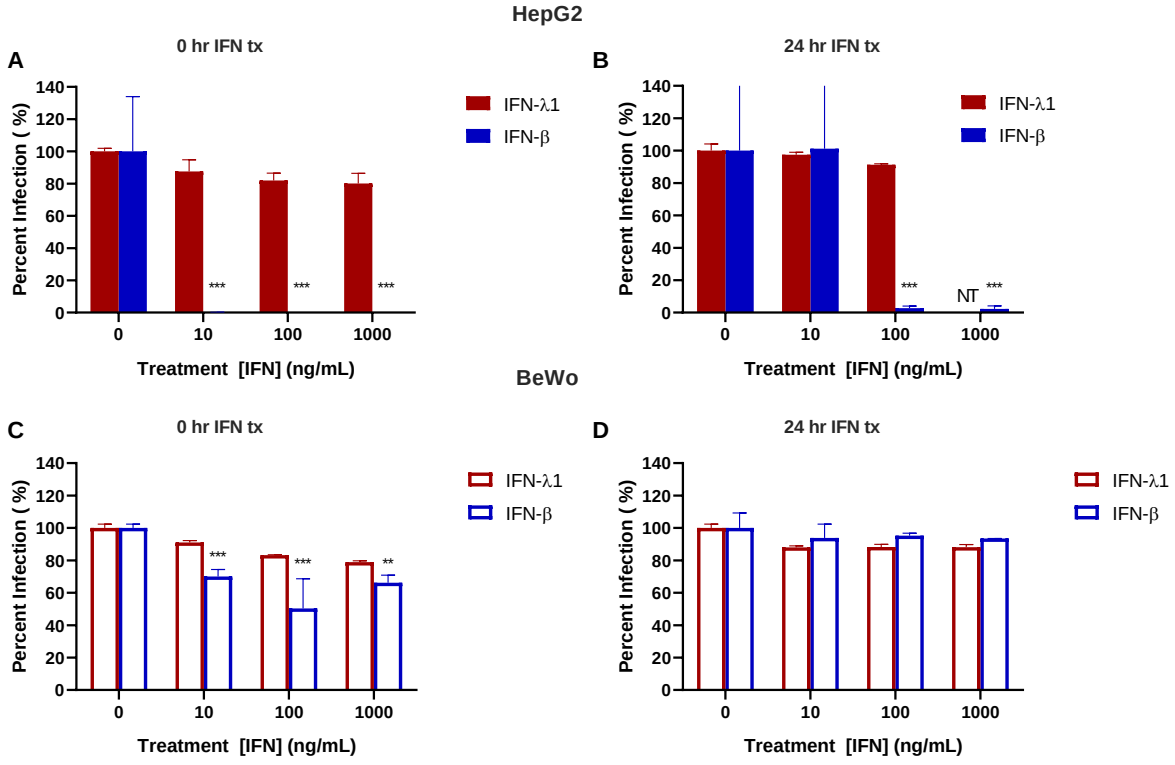

Figure 4: RVFV-ΔNSs/GFP viral titers at 72 hpi in HepG2 and BeWo cells.

Mean viral titers by qRT-PCR for HepG2 and BeWo cells infected at 0.01 MOI and treated with exogenous IFN-λ1 or IFN-β at 0 hpi (A) or 24 hpi (B). Data are expressed as percentage of untreated controls from log-transformed viral titers. Asterisks indicate significance (***, $P < 0.001$) by Two-way ANOVA, Tukey's multiple comparison test. NT = not tested.

To confirm these results and expand the range of timepoints to analyze for effects of IFN-λ1 treatment on RVFV infection in BeWo cells, a time course experiment was conducted. BeWo cells were plated as in Aim 1 and treated with 0, or 1,000 ng/mL of IFN-λ1 or IFN-β at 6 hours pre-infection, 0, 24, or 48 hours-post-infection. Cell supernatants were taken every 24 hours from 0 to 72 hpi and analyzed by qRT-PCR to quantify viral titers (Figure 5).

In support of data from Figure 3, IFN-λ1 treatment did not result in a significant reduction in viral titers with any treatment time. Conversely, IFN-β treatment 6-hour pre-infection and 0 hpi did reduce viral titers as compared to controls.

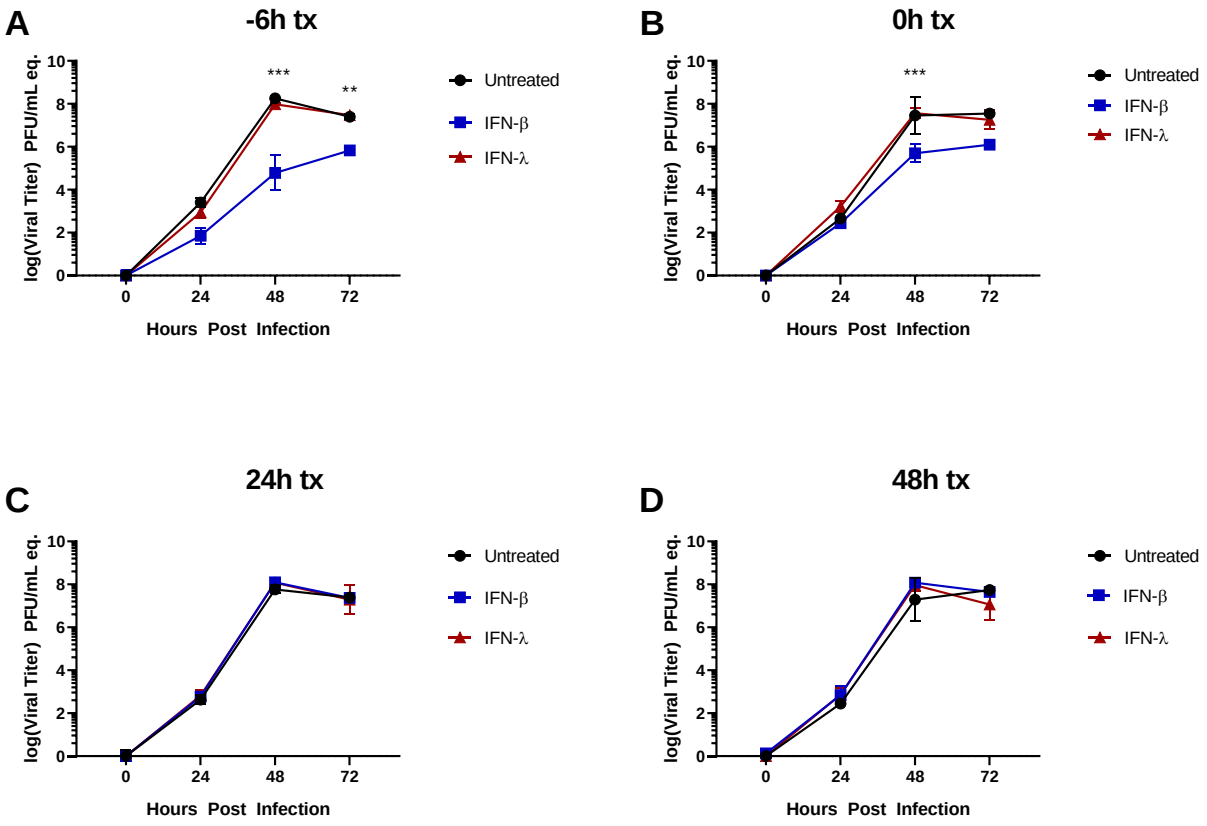

Figure 5: ZH501 viral titers at 0, 24, 48, and 72 hpi in BeWo cells.

Mean viral titers by qRT-PCR in BeWo cells infected with 0.01 MOI ZH501 RVFV and treated at -6 (A), 0 (B), 24 (C) or 48 (D) hpi, with 1,000 ng/mL IFN-λ1 or IFN-β, compared to an untreated control. Asterisks indicate significance (**, $P < 0.01$; ***, $P < 0.001$) by Two-way ANOVA, Tukey's multiple comparison test.

4.2 AIM 2: Determine the role of RVFV nonstructural protein NSs in inhibiting expression of IFN-λ1 stimulated genes (ISGs).

Results from Aim 1 indicate that treatment of HepG2 cells with IFN-λ1 can limit the production of infectious RVFV virus. Interestingly, this was not the case with the trophoblast cell line BeWo, and the resistance of BeWo cells could be only partially attributed to the presence of NSs. In Aim 2, we seek to specifically determine the effect of the viral NSs protein on activation of ISG promoter and several ISGs in response to IFN-λ1 treatment.

In order to determine whether the viral NSs protein can affect ISG promoter activation and gene expression after stimulation with either IFN-λ1 or IFN-β, HEK-293T cells were transfected with a luciferase reporter plasmid under the control of an interferon-stimulated response element (ISRE) promoter along with an NSs expression plasmid or an empty vector control. Transfected cells were treated with IFN-λ1 or IFN-β, and 24 hours after IFN treatment, ISRE activation was quantified by dual luciferase assay.

The ISRE promoter was activated in a dose-dependent manner by treatment with either IFN-λ1 or IFN-β. This activation was inhibited by co-transfection with NSs (Figure 6). These results indicate that NSs can prevent activation of the ISRE promoter after both IFN-λ1 and IFN-β treatment, suggesting that in theory NSs can mediate resistance to IFN-λ treatment similarly to

IFN-β. However, clearly other factors are at play given that RVFV titers were unaffected by IFN-λ treatment in BeWo but not HepG2 cells.

Knowing that NSs can function to inhibit IFN-λ mediated ISRE activation despite the observation that there was a discrepancy in titer reduction between BeWo and HepG2 cells, we next measured ISG expression in cell lysates from the infection experiments described in Aim 1. We measured three ISGs that have a known or suspected role in the pathogenesis of RVFV: protein kinase R (PKR), CXCL3(also known as GRO3 and MIP2B), and CXCL8 (also known as IL-8). PKR functions early in the antiviral response pathway, primarily by inhibiting protein synthesis (53). PKR inhibits viral protein synthesis by targeted protein ubiquitination and proteosomal degradation through E3 ligases. RVFV NSs protein is known to degrade protein kinase R, an early E3 ligase in the IFN-response pathway, as well as promoting post-transcriptional downregulation and inhibiting necessary phosphorylation for PKR to function (87, 93). CXCL3 is the cytokine ligand of CXCR2 (also a receptor for CXCL8) and mainly functions as a chemoattractant

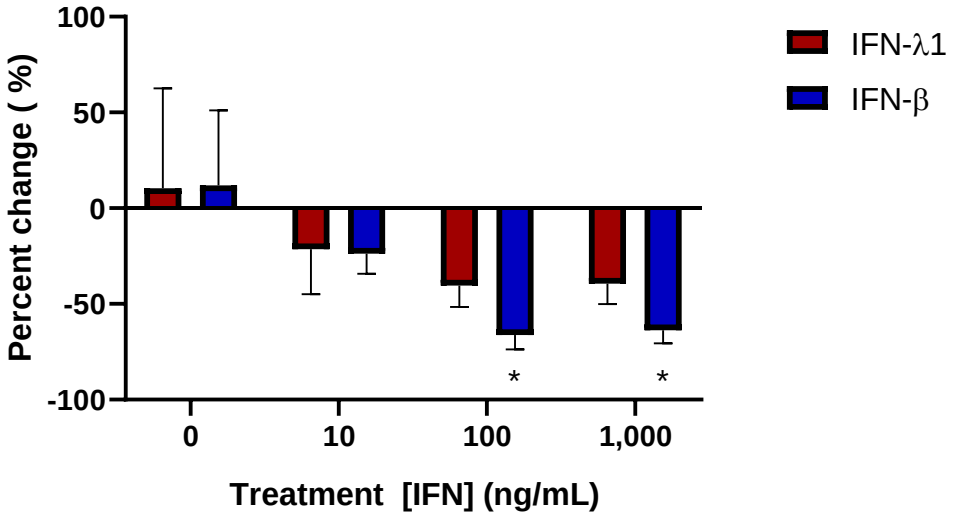

Figure 6: NSs inhibits ISRE activation in response to IFN-λ1 and IFN-β treatment.

ISRE-luciferase expression in HEK-293T cells transfected with NSs expression plasmid at 24 hours post-treatment with IFN-λ1 or IFN-β. Luciferase expression displayed in percent change (%) normalized to constituitively

expressed renilla plasmid luminescence. Cells were transfected with 1 ug/well each of NSs expression plasmid or empty backbone control plasmid, ISRE-luciferase reporter plasmid, and renilla expression plasmid. (n=3, bars indicate SD) Asterisks indicate significance compared to untreated controls (*, $P < 0.05$) by Two-way ANOVA, Tukey's multiple comparison test.

controlling the migration and adhesion of monocytes and neutrophils in sites of infection, particularly the cerebellum (94). Recent work on neutrophil and macrophage influx during encephalitic RVFV infection has highlighted the inflammatory components of lethal RVFV in a rodent model (95). Additionally, immunostaining has shown localization of CXCL3 in trophoblasts of human placenta explants (96). CXCL3 has also been shown to play a role in the migration, invasion, and proliferation of placental trophoblast cells (97). CXCL8, also known as IL-8, is a chemokine produced by macrophages, epithelial cells, and airway smooth muscle cells. It is a proinflammatory chemokine that attracts macrophages and neutrophils as well as stimulating phagocytosis. In RVFV infections in mice, increases in CXCL8 signaling are associated with pathogenesis in the brain and liver (98). In rats, high levels of the IL-8 homolog GRO/KC were associated with lethal infection (38). Similarly in NHPs, higher IL-8 levels late in infection, along with other proinflammatory markers, were found in animals with severe disease, however early expression of those same markers was seen in survivors (99). In human cases, elevated CXCL8 level in serum are indicative of more sever disease (100).

In IFN-β treated HepG2 and BeWo cells, expression of each of these ISGs was downregulated in ZH501 RVFV infection compared to uninfected IFN treated cells, regardless of whether those cells were treated at 0 or 24 hpi. In IFN-λ1 treated HepG2 cells, PKR and CXCL8 expression was downregulated in ZH501 infected cells compared to uninfected controls. In BeWo cells however, IFN-λ1 treatment was only significantly downregulated in ZH501 infected cells for

CXCL3. BeWo cell ISG expression was relatively unresponsive overall, even in uninfected cells, in response to IFN-λ1 treatment.

Overall, these data indicate that the NSs protein is not necessary in RVFV inhibition of IFN-λ1 stimulated genes for both HepG2 and BeWo cells; but, RVFV-ΔNSs/GFP is unable to inhibit IFN-β stimulation of CXCL3 and CXCL8 in BeWo cells.

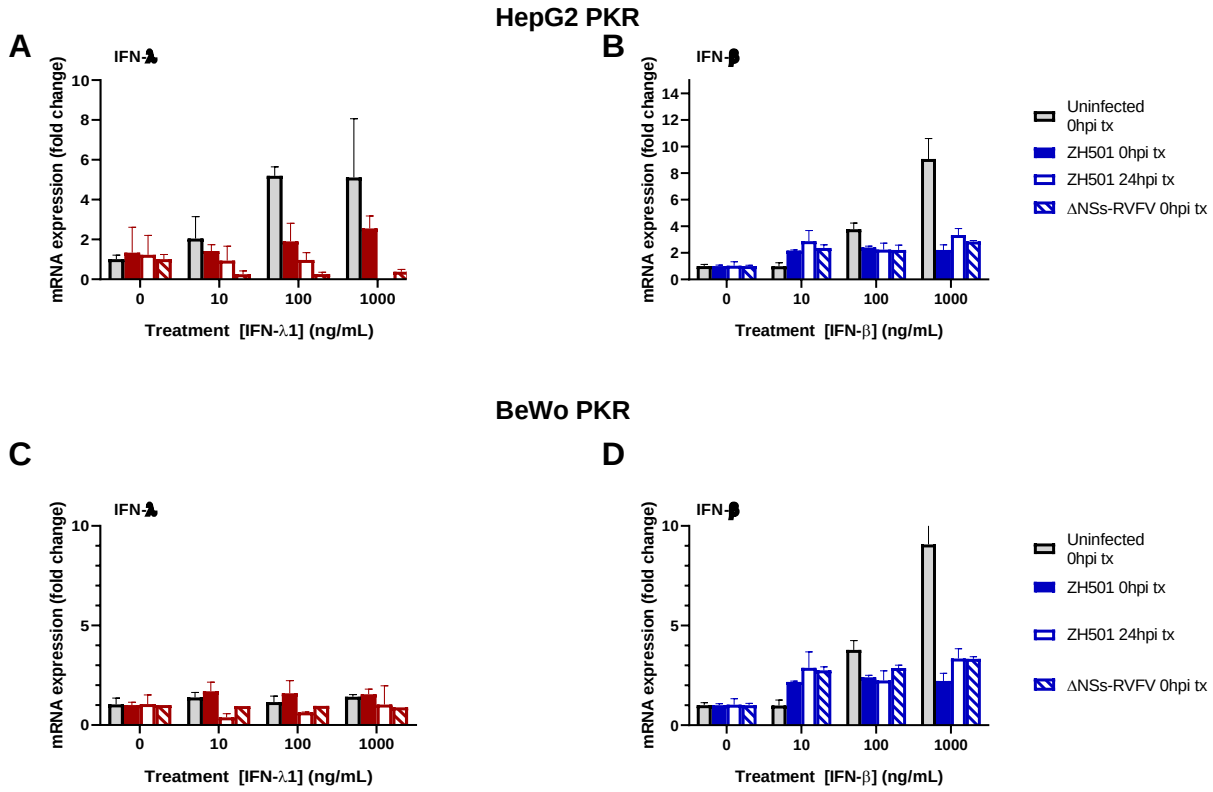

Figure 7: RVFV infection inhibits PKR mRNA expression in HepG2 and BeWo cells, independent of the presence of NSs.

Mean mRNA expression levels by qPCR for 0.01 MOI infected HepG2 (A, B) and BeWo (C, D) cells treated with IFN-λ1 at 0 or 24 hpi. Expression of each ISG normalized to an endogenous β-actin control.

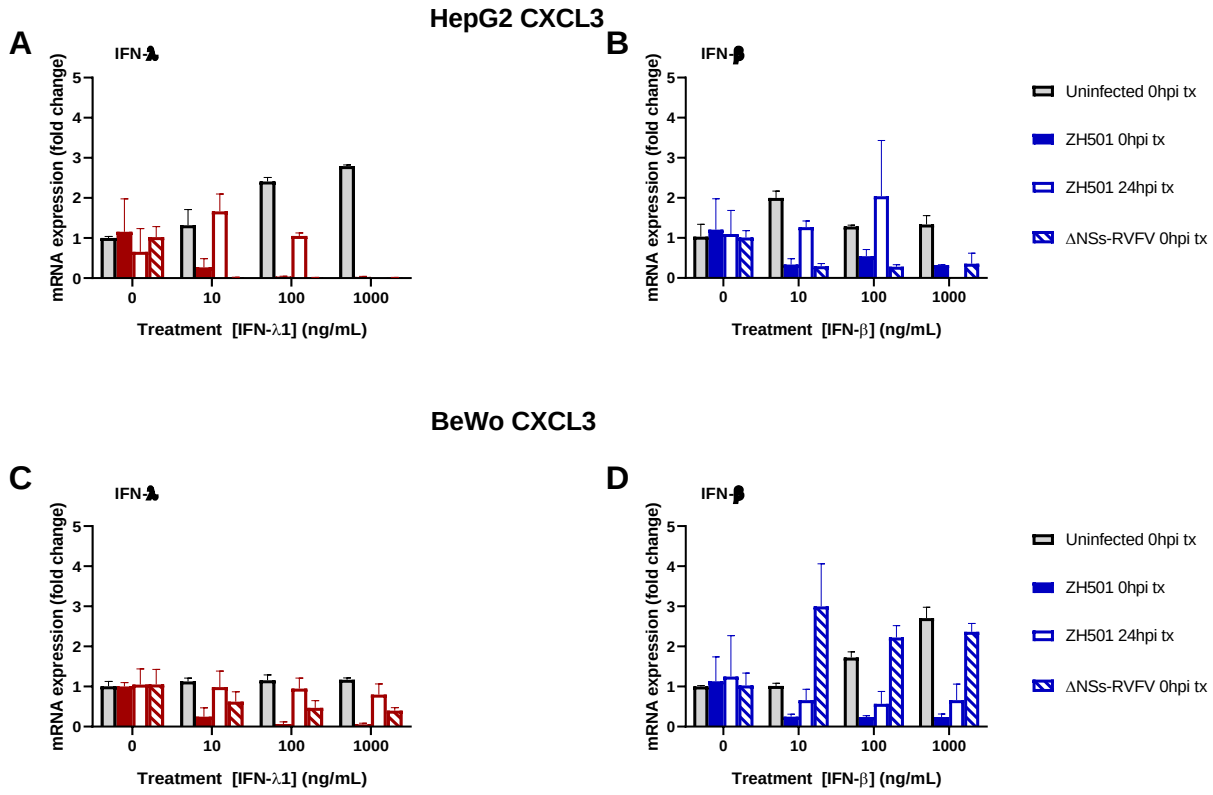

Figure 8: RVF infection inhibits CXCL3 mRNA expression in HepG2 cells independent of the presence of NSs, but requires NSs to inhibit expression in IFN-β treated BeWo cells.

Mean mRNA expression levels by qPCR for ZH501 0.01 MOI infected HepG2 (A, B) and BeWo (C, D) cells treated with IFN-β at 0 or 24 hpi. Expression of each ISG normalized to an endogenous β-actin control.

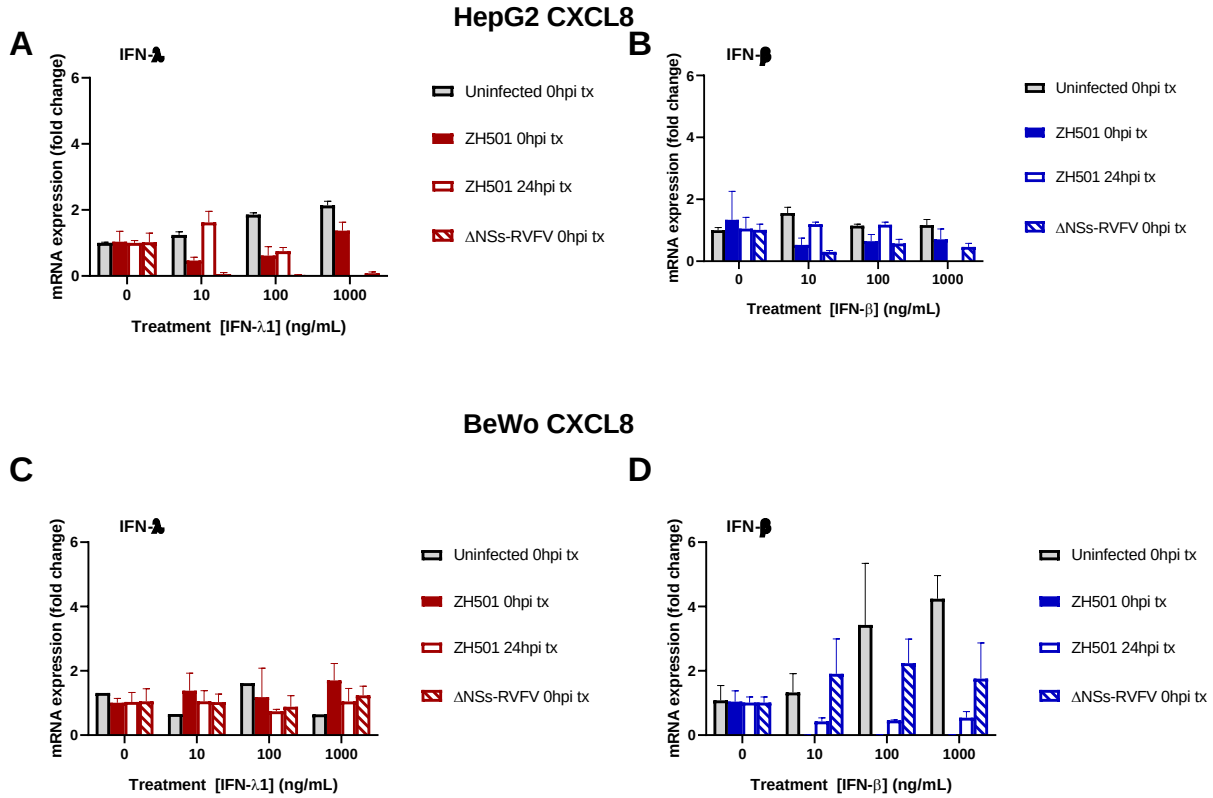

Figure 9: RVFV infection inhibits CXCL8 mRNA expression in HepG2 cells, independent of NSs, but requires NSs to inhibit expression in IFN-β treated BeWo cells.

Mean mRNA expression levels by qPCR for ZH501 0.01 MOI infected HepG2 (A, B) and BeWo (C, D) cells treated with IFN-β at 0 or 24 hpi. Expression of each ISG normalized to an endogenous β-actin control.

4.3 AIM 3: Determine the role of RVFV non-structural protein NSs in reducing IFN-λ1 protein production.

Aim 2 determined that NSs can inhibit IFN-λ1-mediated ISG production under certain circumstances. Aim 3 is designed to determine whether NSs can inhibit the induction of IFN-λ protein after treatment of cells with the potent IFN-inducer poly(I:C). Poly(I:C) is a dsRNA mimic used to induce TLR3 signaling and subsequent IFN-λ1 or IFN-β protein production (90). IFN production from NSs transfected cells was measured at 0, 4, 12, and 24 hours post-poly(I:C)-stimulation and compared to cells transfected with an empty backbone control. Presence of IFNs in cell supernatant was measured by enzyme-linked immunosorbent assay (ELISA). HepG2 or BeWo cells were plated in 24-well plates and transfected with 0, 0.5, 1, or 2 ug NSs expression plasmid as in Aim 2. 24 hours post-transfection, cells were treated exogenously with 0, 5, 10, or 15 ug/mL of poly(I:C) dsRNA mimic to induce TLR3 stimulation and subsequent IFN protein production. Supernatants were taken at 4, 12, and 24 hours post-stimulation and analyzed by ELISA for the presence of IFN-λ or IFN-β (Figure 10).

In BeWo cells, both IFN-β and IFN-λ protein production was below the limit of detection under all treatment conditions. In HepG2 cells, IFN-β production was below the limit of detection (data not shown). In contrast, IFN-λ production was detectable, but protein levels in cell supernatants were not reduced in cells transfected with NSs expression plasmid, indicating that while NSs may have an effect on IFN-stimulated genes, it does not appear to have an effect on IFN-λ protein secretion in HepG2 cells (Figure 10).

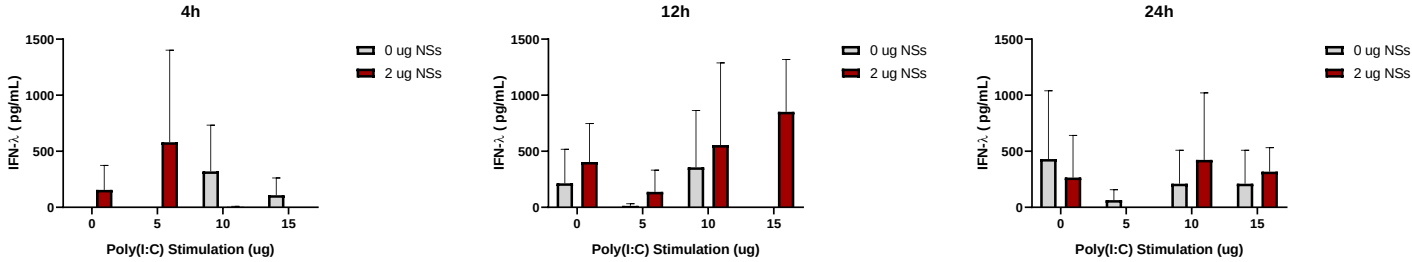

Figure 10: NSs transfection has no effect on IFN-λ protein production in HepG2 cells in response to poly (I:C) stimulation at 4, 12, and 24 hours post-stimulation.

IFN-λ protein levels measured by ELISA, calculated based on a standard curve of known concentrations of IFN-λ in pg/mL.

5.0 Discussion

Since its discovery in 1931, RVFV has caused outbreaks throughout Africa, the Saudi Peninsula, and Madagascar. Mosquito-facilitated RVFV spread has resulted in the establishment of endemic RVFV in Africa where frequent enzootic and epizootic transmission has been documented. In livestock, RVF primarily presents as a fulminant hepatic disease, which can occur in a variety of domestic ruminants (101-104). Vertical transmission and mass-abortogenic infections in livestock has been a consistent phenomenon in enzootic outbreaks.

In humans, the most common form of RVF disease is a self-limiting febrile illness, however more serious clinical manifestations can occur (20). In a large case study of individuals with laboratory confirmed cases of RVF during the Saudi Arabian outbreak in 2000, 93% of cases presented with fever, followed by 17% with neurological disease, and 7% including hemorrhagic manifestations (18). Despite well-documented epidemiological and clinical data from a myriad of human outbreaks, confirmed cases of human vertical transmission are limited to only two cases. Two pregnant women gave birth to an infant with jaundice, and an enlarged spleen and liver, and a serologically positive infant that later succumbed to infection (9, 105). More broadly, a cross-sectional study of pregnant women with concomitant RVFV infection established an association between infection and late-term miscarriage in 54% of the women with an odds ratio of 7.4 (8). Despite the lack of data on this phenomenon in humans, abortion storms in livestock infected with RVFV are a hallmark of zoonotic outbreaks and highlight the ability of the virus to cause congenital infection. Laboratory work exploring RVFV vertical transmission has been understudied. To this end, McMillen et al. (2018) developed a rodent model for congenital RVFV infection. This model has the benefit of the rat and human placenta structures sharing key

characteristics, both being hemochorial and discoid-shaped (106). While there remain differences in structure and function between rat and human placentas, this model represents a significant step in the development of a tractable and reproducible laboratory model of RVFV vertical transmission from which findings can merit further investigation in humans. Further, RVFV can infect similar placental structures as other arboviruses including ZIKV, West Nile virus and Chikungunya virus (107). One of the more surprising findings from McMillen et al. (2018) was the ability of RVFV to infect syncytiotrophoblasts in placental explants (10).

Building off of the foundation of the work done developing the rodent model of RVFV congenital infection, the work presented in this thesis represents a continuation of the project, with an emphasis on examining the relationship between type III IFNs, known to be constitutively expressed at the placenta and RVFV infection *in vitro*. RVFV and type I IFNs have a dynamic relationship, each with the capacity to inhibit the other. Type I IFNs have been known to reduce viral titers and even prevent clinical disease in RVFV infection, however, they are also known to be detrimental to pregnancy (62, 78).

The work described in this thesis has been the first to determine the possible effects of type III IFNs on RVFV infection. Type III IFNs are known to be constitutively expressed at the placenta and can be effective in controlling other congenital arboviral infections like Zika virus both in mice and *in vitro* (66, 67, 86).

Using HepG2 and BeWo cell lines to mimic target tissues of RVFV infection, *in vitro* data supports the hypothesis that RVFV is sensitive to the effects of type III IFNs. Viral titers were reduced in HepG2 cells treated with IFN-λ pre-infection or immediately post-infection, as was also the case in IFN-β treated HepG2 cells. These data synergize well with existing literature describing HepG2 cells as supportive of viral replication as well as mounting effective type III IFN responses

to reduce that viral replication (89, 90). Notably in opposition to the HepG2 data, BeWo cells did not experience a reduction in viral titers when treated with IFN-λ regardless of the time of addition. This finding speaks to key differences in the cell lines and may also offer clues as to why RVFV replication occurs to such a high degree in the maternal-fetal interface, despite the constitutive release of IFN-λs during pregnancy.

Neither cell line experienced significant reduction in viral titers when treated with IFN-λ or IFN-β 24 hpi. We hypothesized that this could be due to the action of NSs, a known viral IFN antagonist. NSs expression plasmid was able to reduce induction of an IFN-stimulated promoter ISRE in HEK-293T cells. Strengthening this finding, mRNA expression levels of key IFN-stimulated genes (ISGs), previously associated with RVFV infection (PKR, CXCL3 and CXCL8) were downregulated in infected HepG2 and BeWo cells as compared to uninfected controls. Interestingly, RVFV lacking NSs was also able to downregulate all three ISGs tested, though this was partially dependent on cell type and the type of IFN the cells were treated with. PKR and CXCL3 were both downregulated in HepG2 and BeWo cells treated with IFN-λ in an NSs-independent manner. This may point to a role for NSm in inhibiting the ISG response to RVFV, as CXCL3 is heavily involved in immune migration which has been shown to be critical in RVFV pathogenesis and disease outcome (95).

CXCL8 expression in both cell lines was reduced in ZH501 infected cells, but was NSs-dependent, with a partial or full rescue of CXCL8 expression in cell infected with RVFV lacking NSs, compared to uninfected controls. This effect was also specific to IFN-β treated cells.

Further clarifying the role of NSs in inhibiting the innate antiviral response, IFN protein production in NSs transfected cells compared to controls was only measurable in HepG2 cells, and only IFN-λ production was above the limit of detection. This result points to the fact that BeWo

cells do not secrete either IFN after stimulation with poly(I:C) and its effect on IFN production in those cells. Lacking better information to inform these findings, two obvious conclusions include BeWo cells lacking in stimulation from poly(I:C) despite having a functional TLR3 receptor, or that the IFN production response in BeWo cells in dysfunctional in some way. In HepG2 cells, IFN-λ production was not affected by the presence of NSs protein or by the concentration of poly(I:C) added, however this may be due to the concentrations of poly(I:C) added. A better treatment design for the Aim 3 poly(I:C) stimulation experiments would have been a logarithmic increase in dose concentration as was the case for the Aim 1 exogenous IFN treatments. Dose dependence may have been easier to show using a scale of treatment groups increasing successively 0, 10, 100, 1,000 ug/ml poly(I:C) stimulation. As designed, it is difficult to say whether a significant increase should be expected in IFN production following a linear increase in poly(I:C) stimulation from 0 to 15 ug/ml.

The investigations described in this thesis highlight the intricate relationship between IFNs and RVFV in general, as well as the emerging interactions between RVFV and type III IFNs. In sum, these experiments have been able to show that the ability of type III IFNs to inhibit RVFV infection and replication is cell type dependent as well as dependent on the time of IFN treatment. The role of NSs in mediating differences in type III IFN efficacy against RVFV infection and replication remains not well-understood, but these findings suggest NSs or even NSm may be playing a role in viral resistance to the effects of IFN-λ in BeWo cells. Indeed, RVFV lacking NSs is still able to inhibit ISG expression of PKR, CXCL3 and CXCL8, all ISGs associated with RVFV infections, in a cell type dependent and IFN type dependent manner. Finally, there is not enough evidence to conclude much about the effect that NSs may have in inhibiting the production of type I or III IFN protein from these cell types and more work is needed to identify greater context for

the interplay between innate antiviral interferons and Rift Valley fever virus particularly in the case of infections at the maternal-fetal interface, which have been shown to be crucial for so many viruses causing congenital infections including in the case of Rift Valley fever virus.

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