

ABSTRACT

Title of Document: **INTERFERON-DEPENDENT AND
-INDEPENDENT MODULATION OF WEST
NILE VIRUS INFECTIONS OF HUMAN
DERMAL FIBROBLASTS.**

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Although dermal fibroblasts are one of the first cell types exposed to West Nile virus (WNV) during a blood meal by an infected mosquito, little is known about WNV replication within this cell type. Here, I demonstrate that pathogenic, WNV-New York (WNV-NY), and nonpathogenic, WNV-Australia (WNV-AUS60) strains are able to infect and replicate in primary human dermal fibroblasts (HFFs). However, WNV-AUS60 replication and spread within HFFs was reduced compared to that of WNV-NY due to an interferon-independent reduction in viral infectivity early in infection. Additionally, replication of both strains was constrained late in infection by an IFN- β -dependent reduction in particle infectivity. Overall, our data indicate that dermal fibroblasts are capable of supporting WNV replication; however, the low infectivity of particles produced from HFFs late in infection suggests that this cell type likely plays a limited role as a viral reservoir *in vivo*.

INTERFERON-DEPENDENT AND -INDEPENDENT MODULATION OF
WEST NILE VIRUS INFECTIONS OF HUMAN DERMAL FIBROBLASTS.

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Dedication

I would like to dedicate this dissertation to my loving and supportive husband, Jarrod Hoover. Without his encouragement and help at home, I would not have been able to put in as many hours researching and writing.

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I'd like to start by thanking my adviser, Dr. Brenda Fredericksen. She taught me many different scientific methods while pipetting alongside me and forced me to really think about the words I was using to describe my work. After each time I believed I had failed, she stayed positive and encouraged me to think of my next steps. She also encouraged me not to be a "wussy girl" and said that I could wear whatever I wanted to lab, as long I was willing to climb up on a lab bench in it. Next, I'd like to thank my committee members, Drs. James Culver, Alison McBride, David Mosser, and Xiaoping Zhu. They have taken the time to review my ideas and advise me on how to proceed with my experiments and research career.

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List of Abbreviations

C	Capsid
DENV	Dengue virus
E	Envelope
EM	Electron Microscopy
ER	Endoplasmic reticulum
HFF	Human dermal foreskin fibroblast
IFN	Interferon
IRF	Interferon regulatory factor
ISG	IFN-stimulated gene
ISRE	IFN-stimulated response element
JAK	Janus kinase
KUNV	Kunjin virus
LCMV	Lymphocytic choriomeningitis virus
LGP2	Laboratory of genetics and physiology 2
M	Membrane
MCMV	Murine cytomegalovirus
MDA5	Melanoma differentiation associated gene 5
MEF	Mouse embryonic fibroblast
MOI	Multiplicity of infection
NF- κ B	Nuclear factor-kappa B
NLS	Nuclear localization signal
NS	Nonstructural
NTPase	Nucleoside Triphosphatase
NTR	Nontranslated region
OAS	2',5'-oligoadenylate synthetase
PAMP	Pathogen associated molecular pattern
PrM	Premembrane
PRR	Pattern recognition receptor
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene 1
RLR	RIG-I-like receptor
RTPase	RNA triphosphatase
STAT	Signal transducers and activators of transcription
TGN	<i>Trans</i> -Golgi network
TLR	Toll-like receptor
VSV	Vesicular stomatitis virus
WNV	West Nile virus

WNV-AUS60 West Nile virus-Australia 1960
WNV-NY West Nile virus-New York 2000
YRA Yield reduction assay

Chapter 1: Introduction

1. WNV History

West Nile virus (WNV) is a positive-sense single-stranded RNA virus in the family *Flaviviridae*. This family is made up of three genera: *Hepacivirus*, *Pestivirus*, and *Flavivirus*. WNV belongs to the genus *Flavivirus*; other members include yellow fever virus, dengue virus, and tick-borne encephalitis virus. WNV was first isolated from a febrile woman in Uganda in 1937 [1]. By the 1960s, WNV spread to humans and horses in the Middle East, Europe, Australia and Asia where it caused a mild fever known as WN fever with few cases of encephalitis [2-7]. Further spread in the 1990s led to outbreaks in Algeria, Morocco, Tunisia, Italy, and Israel, contributing to encephalitis and death in humans and horses [8-15]. By studying WNV seroprevalence in birds, correlations were drawn between the movement of migratory birds and spread of the virus [16-17].

In 1999 in New York, veterinarians noted unusually high numbers of dead crows with detectable virus in their brains. Virus was isolated from the dead birds and later identified as WNV [18]. Between 1999 and 2000, 83 human cases were reported in New York, Connecticut, and New Jersey. The virus quickly spread and in 2002, there were reports of 4,156 cases in 38 states [19]. Since emerging in the United States, WNV infections have been confirmed in every state of the continental United States. Compared to previous outbreaks in African countries, recent outbreaks in the Americas, Europe, and the Middle East have a marked

increase in both the number of reported cases and the severity of disease [20]. WNV is now the leading cause of mosquito-borne neuroinvasive disease in the United States. Between 1999 and 2013, over 17,000 cases with neurological complications, such as meningitis, encephalitis, and acute flaccid paralysis and over 1,600 deaths due to WNV, were reported [19]. While there are approved vaccines for horses, there is still no approved vaccine or treatment for WNV in humans.

2. WNV transmission

The transmission cycle of WNV was first reported by a study conducted on the ecology of WNV in Egypt and southern Sudan published in 1956 [6]. Surveying human blood from approximately 1,500 people in Egypt and southern Sudan, they determined that over 50% of the samples were seropositive for WNV. The widespread prevalence of WNV in humans in the area led to the study of various birds and arthropods to discover the mode of transmission. Many species of birds tested positive for WNV, but mosquitoes were the only WNV-positive arthropods out of the 26,000 sampled [6]. This study demonstrated that the transmission cycle of WNV utilizes mosquitos as vectors and birds as hosts (Figure 1). Several genuses of mosquitoes can transmit WNV and many avian species can serve as reservoirs, but in the United States *Culex* mosquitoes are known to be the primary vector and robins have been identified as the most prevalent reservoir [21].

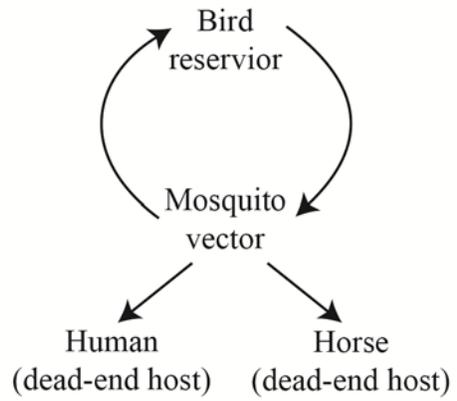


Figure 1. WNV transmission cycle

WNV is transmitted by a mosquito vector and birds serve as the reservoir of the virus. Humans and other mammals, such as horses can be infected, but are dead-end hosts.

Culex mosquitoes are present in all of the Americas and have the highest populations in seasons of high temperature and heavy rain. Viral transmission is also high when water is scarce because mosquitoes and birds drink from the same pools of water [22]. Straying from the transmission cycle, mosquitoes can also infect dead-end hosts such as humans and horses. It is thought that levels of viremia in these hosts are very low, which does not allow for transmission back to the mosquito [23].

3. WNV genome, structure, and proteins

West Nile virus (WNV) is a positive-sense single-stranded RNA virus with a 5' cap. Its genome is approximately 11 kb in length and consists of a single open reading frame encoding three structural proteins (Capsid, Premembrane/membrane, and Envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The genome is flanked by 5' and 3' nontranslated regions (NTRs) (Figure 2).

WNV virions are small, spherical and enveloped. Early cryo-electron microscopy (cryo-EM) images suggested that both the nucleocapsid and the envelope had icosahedral symmetry [24]. However, later studies using improved cryo-EM technology show that the nucleocapsid has no discernable symmetry [25]. The nucleocapsid is composed of capsid (C) proteins, which coat the viral RNA. The viral envelope includes envelope (E) and premembrane/membrane (prM/M) proteins.

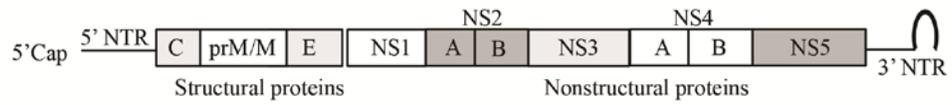


Figure 2. WNV genome

The 11 kb genome encodes 3 structural proteins and 7 nonstructural (NS) proteins that are flanked by 5' and 3' nontranslated regions (NTRs).

While the WNV structural proteins are integral parts of the envelope and capsid, the nonstructural proteins serve functions related to viral RNA synthesis. Studies focusing on NS1 suggest it has a role in viral RNA replication since it has been shown to colocalize with viral replication complexes [26, 27]. Further supporting this work, neither (+) nor (-) strand viral RNA could accumulate in the absence of NS1 [28]. The secreted form of NS1 can be detected in the blood of viremic hosts and has been linked to disease severity in a hamster model [29]. In addition to being secreted, NS1 associated with cell surfaces has been shown to protect the cell from complement-mediated lysis by binding to glycoprotein factor H, which is known to regulate the alternative complement pathway [30].

Specific functions of NS2A, NS2B, NS4A, and NS4B have not been fully characterized. Studies focusing on NS2A suggest that this protein participates in viral assembly [31, 32]. In complex with NS3, NS2B is a required cofactor for serine protease activity of NS3 [33]. The serine protease activity domain of NS3 exists at the N-terminus of the protein and functions to cleave the polyprotein into individual proteins [34-37]. The C-terminus of NS3 contains RNA helicase, nucleoside triphosphatase (NTPase), and RNA triphosphatase (RTPase) motifs, which are important for RNA replication [38-40].

WNV and dengue virus (DENV) NS4A contain amino acid residues that act as a signal sequence for the translocation of NS4B into the lumen of the ER (named the C-terminal transmembrane domain 2K), were both shown to induce cytoplasmic membrane rearrangement [41-43]. This rearrangement resembled the convoluted membranes found to house viral replication complexes in infected

cells. Molecular dynamics computer simulations of DENV NS4A have shown that this protein has membrane-altering properties [44]. Both WNV NS4A and NS4B have been reported to block innate immune signaling [45-47]. Like NS3, NS5 is also known to have several functions. In addition to its role of partially blocking the innate immune cascade, the N-terminal region of the protein encodes a methyl transferase, which is required for viral RNA capping [48]. The C-terminal portion of NS5 contains the viral RNA-dependent RNA polymerase [49-53].

4. WNV classification

Based on the sequence of the structural proteins, WNV has been classified into five lineages and two clades that differ from each other by 20-27% (Figure 3) [54, 55]. Lineages one and two are the two main lineages; they include both highly pathogenic and less pathogenic strains of the virus [56-60]. Lineage one strains have been isolated from the United States, Europe, Israel, Africa, Russia, and Australia. While lineage two strains were initially identified in sub-Saharan Africa and Madagascar, they have recently also been detected in Eastern and Southern Europe as well as South Africa [62-70].

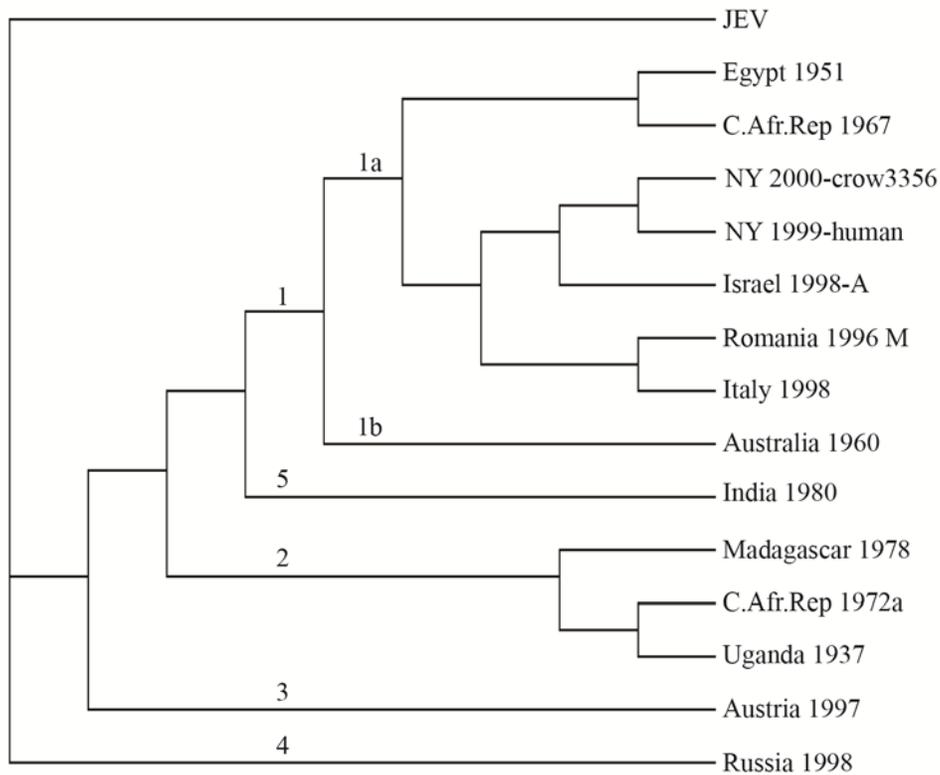


Figure 3. WNV classification

WNV is grouped into five lineages, labeled 1-5 on the left, and 2 clades (1a and 1b). Phylogenetic analysis of selected WNV strains compared sequences of the E region of the WNV genome in a nearest neighbor joining tree. Strains listed are representative of each lineage and include isolates from around the globe. JEV serves as an outgroup. Figure adapted from Lanciotti 2002, Bondre 2007, and Aliota 2012 [54, 55, 61].

5. WNV life cycle

WNV can replicate in many different avian, mammalian, amphibian, and insect cell types. Known receptors that can mediate WNV attachment include DC-SIGN, DC-SIGNR, and $\alpha_v\beta_3$ integrins [71-73]. Unknown receptors also exist because WNV can infect cell types lacking these receptors. Following attachment, WNV is internalized via receptor-mediated endocytosis of a clathrin-coated pit [71, 74] (Figure 4). As the endosome acidifies, the virus membrane fuses with the endosomal membrane and the nucleocapsid enters the cell [75, 76]. The genome is uncoated and translated by the host cell ribosome as a polyprotein. The polyprotein is co- and post-translationally cleaved into ten separate proteins by viral and cellular proteases. Transcription of minus-strand RNA from positive-strand RNA utilizes the RNA-dependent RNA polymerase (RdRp), NS5. While the minus strand is sequestered in replication complexes [49, 77-79], it serves as the template for genomic RNA synthesis [80]. Nonstructural proteins induce the formation of the endoplasmic reticulum (ER) membrane invaginations that house the replication complexes [41-44].

In association with ER membranes, viral genomes can be translated, replicated, or assembled into a virion. E and prM have transmembrane domains that insert into the ER membrane such that their exodomains are located in the ER lumen. When membrane-associated capsid dimers interact with viral genomic RNA in areas where E and prM are inserted, immature virions bud into the ER lumen [81]. To keep immature particles from fusing, the pr portion of prM covers

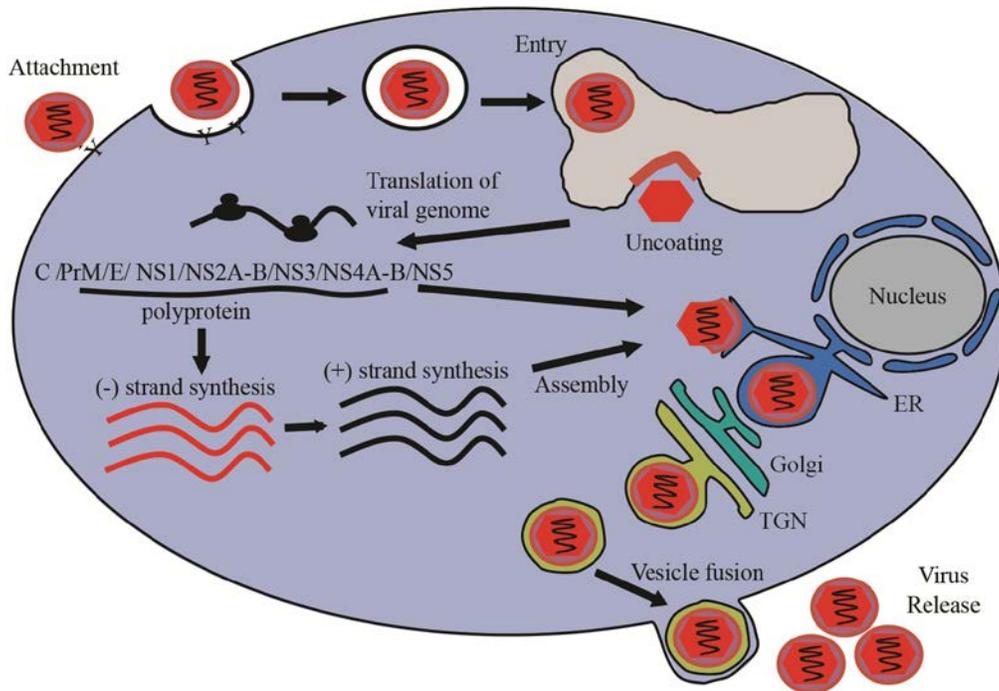


Figure 4. WNV life cycle

The virion attaches and enters the target cell through receptor-mediated endocytosis. Uncoating from an endosomal vesicle occurs in a pH-dependent manner. This is followed by polyprotein translation, polyprotein cleavage into individual proteins, and replication of the viral genome. The virus particle is assembled, buds through the ER membrane, moves through the Golgi and *trans*-Golgi network (TGN), and is released from the cell.

the fusion peptide on E [24, 82]. In order for the particle to mature, the pr portion of prM must be cleaved by a cellular furin-like protease in the *trans*-Golgi compartment [83]. In the mature conformation, the E trimers rearrange into antiparallel dimers, which makes them fusion competent [84-86]. After 10 to 12 hours in a mammalian cell, virions are finally exocytosed and mature or partially mature particles are capable of infecting other cells [22, 87].

6. WNV dissemination pathway

Infection of human hosts occurs during a WNV-infected mosquito's blood meal, where it inserts its proboscis through the epidermis and into the dermis. Probing for a blood vessel, the mosquito salivates and deposits high levels of WNV along with factors that can enhance infection [88-91]. While keratinocytes, the major cell type comprising the epidermal layer of the skin, have been shown to support high levels of WNV replication [90], primary human dermal fibroblasts are also capable of sustaining the replication of WNV and several other *Flaviviruses* [92]. Langerhans dendritic cells and neutrophils traffic to the site of infection, take up the pathogen, and transport it to the draining lymph node and other secondary lymphoid organs [93-96]. The virus then spreads through the circulatory system and subsequent viremia leads to infection of other organs such as the kidneys, liver, lungs, heart, and brain.

7. Innate immune recognition of WNV

When a host encounters a viral pathogen, the first line of defense is the innate intracellular immune system. The cell's pattern recognition receptors (PRRs) can detect pathogen associated molecular patterns (PAMPs) and induce the expression of antiviral genes. One PAMP is double-stranded (ds)RNA; initial reports demonstrated that Toll-like receptor 3 (TLR3) recognized this PAMP when it was extracellular or within an endosome [97]. Studies using TLR3-deficient mice showed that the mice succumbed to infection by murine cytomegalovirus (MCMV), but they remained resistant to infections by some other viruses such as lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV) and reovirus [98]. In addition, the introduction of (ds)RNA in the cytoplasm of TLR3-deficient cells has also been shown to stimulate the host antiviral response [99, 100]. Combined, these studies showed that TLR3 was not required for an effective host antiviral response to some pathogens and suggested that other PRRs existed.

A more recently discovered PRR is retinoic acid-inducible gene 1 (RIG-I), which is a DexD/H RNA helicase that was identified as an essential regulator for poly(rI):poly(rC)-induced signaling in a functional screen [101]. Later *in vitro* studies showed that RIG-I bound to intracellular poly(rI):poly(rC) [51, 102]. These studies also identified other genes that encode RIG-I related proteins: melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [51, 102]. This class of intracellular PRRs is now termed the RIG-I like receptors (RLRs).

TLR3, RIG-I, and MDA5 pathways activate various signaling cascades to lead to the activation and nuclear translocation of nuclear factor-kappa B (NF- κ B) and interferon regulatory factor-3 (IRF-3) (Figure 5) [103-116]. IRF-3, NF- κ B, and c-jun/ATF-2 assemble on the IFN- β promoter to form the enhanceosome and induce IFN- β production [117].

IFN- β is secreted from cells and binds to the IFN- α/β receptor in either an autocrine or paracrine manner, to activate the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) (JAK/STAT) signaling pathway (Figure 5) [118]. The IFN- α/β receptor is made up of IFNAR-1 and IFNAR-2, which are associated with Tyk2 and JAK1, respectively. Tyk2 and JAK1 become phosphorylated following IFN- β binding to the receptor. This triggers downstream formation and phosphorylation of the stable STAT1-STAT2 heterodimer. The dimerization and phosphorylation of this complex leads to a conformational change, attributing to the exposure of nuclear localization signal (NLS) [119]. This STAT1-STAT2 heterodimer complexes with IRF-9 to form the ISGF3 heterotrimer and translocates to the nucleus [120]. ISGF3 then binds to the IFN-stimulated responsive element (ISRE), which is present on the promoter of most IFN-stimulated genes (ISGs) [121]. ISGs include proteins with direct antiviral effects such as ISG15, ISG56 and 2',5'-oligoadenylate synthetase (OAS); components involved in antigen presentation; RLRs and TLRs; transcription factors such as IRF-1, IRF-7 and IRF-9; and proinflammatory cytokines and chemokines [122-128]. The expression of IRF-7 in the presence of

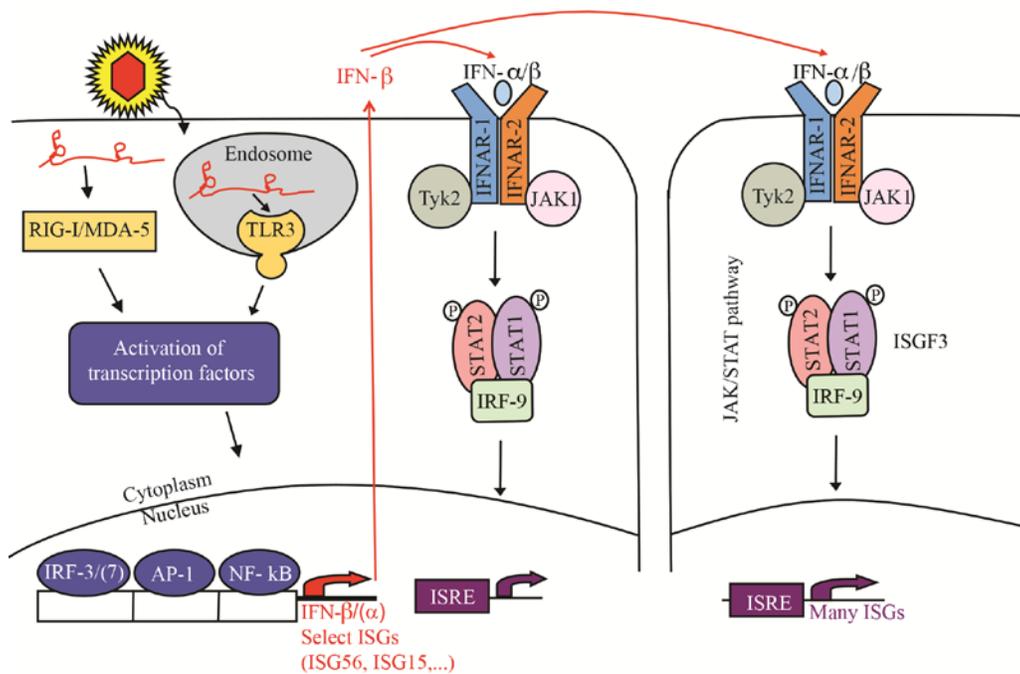


Figure 5. Innate immune signaling

WNV PAMPs are recognized by PRRs such as RIG-I, MDA-5, or TLR3. This triggers the activation of transcription factors that translocate to the nucleus and promote the transcription of IFN- β , IFN- α , or a subset of ISGs. Secreted IFN- β and IFN- α can act in an autocrine or paracrine manner by binding to the IFN- α/β receptor and triggering the JAK/STAT pathway. This leads to the activation of the transcription factor ISGF3, which translocates to the nucleus and induces the transcription of many ISGs.

continued infection leads to the production of IFN- α [127, 129, 130]. Like IFN- β , IFN- α is secreted from cells and binds to the IFN- α/β receptor to activate the JAK/STAT signaling cascade [118]. Ultimately, ISG expression controls infections and triggers an antiviral state within the cell.

While the production of ISGs typically follows IFN induction, ISGs can also be produced directly following viral infection of cells (Figure 5) [129, 131-136]. A study by Grandvaux et al. identified that a subset of ISGs, including ISG15, ISG54, ISG56, and ISG60, could be transcribed following IRF-3 activation [137]. This subset of ISGs is involved in the establishment of an antiviral state prior to and following IFN signaling.

8. WNV evasion and inhibition of the innate immune response

In response to the host cell developing mechanisms to detect and block WNV infection, WNV has evolved means to evade innate intracellular antiviral responses and block JAK/STAT signaling. While WNV can be recognized by TLR3, RIG-I, and MDA-5, the first PRR to detect WNV in most mammalian cell types is RIG-I [138, 139]. However, WNV evades detection by RIG-I by masking viral PAMPs until later times post infection. Consequently, downstream IRF-3 activation is delayed until approximately 12 to 16 h post-infection [140]. Evading detection by a cell's defenses allows WNV to replicate before the cell can suppress translation or block viral replication [139]. RNA segments in both the WNV genome and antigenome have been found to trigger a RIG-I-specific response. When these segments are incorporated into a larger segment, they lose

their stimulatory capacity. This suggests that the ability of WNV to evade the host antiviral response lies in its ability to sequester these stimulatory segments of viral RNA [141].

In addition to evading innate antiviral responses, many structural and nonstructural WNV proteins have been found to act in different ways to prevent or inhibit JAK/STAT signaling (Figure 6). Measuring the induction of ISGs following infection with WNV, it was noted that the host antiviral response was attenuated [140]. ISGs are typically induced following JAK/STAT signaling, which requires the type-I IFNs, IFN- α - β , to be secreted from infected cells. If signaling through PRRs is inhibited, IFN- β will not be produced and the JAK/STAT pathway will not be initiated. TLR-3 signaling may be inhibited by the viral NS1 protein or by specific E protein glycosylation profiles [142, 143]. However, a separate study showed that neither WNV nor DENV NS1 was able to disrupt TLR3 signaling [144]. In the less pathogenic strain of WNV, Kunjin virus (KUNV), NS2A has been found to specifically inhibit IFN- β transcription while the introduction of the NS2A A30P mutation leads to rapid IFN- β production [45, 47].

Comparing highly pathogenic to less pathogenic strains of WNV, the ability of a strain to control IFN and JAK/STAT signaling proved to be a distinct characteristic of highly virulent strains [145]. Some studies have found that WNV NS5 can inhibit phosphorylation of Tyk2 and JAK1, which will prevent the downstream transcription of ISGs [146, 147]. Alternatively, another study

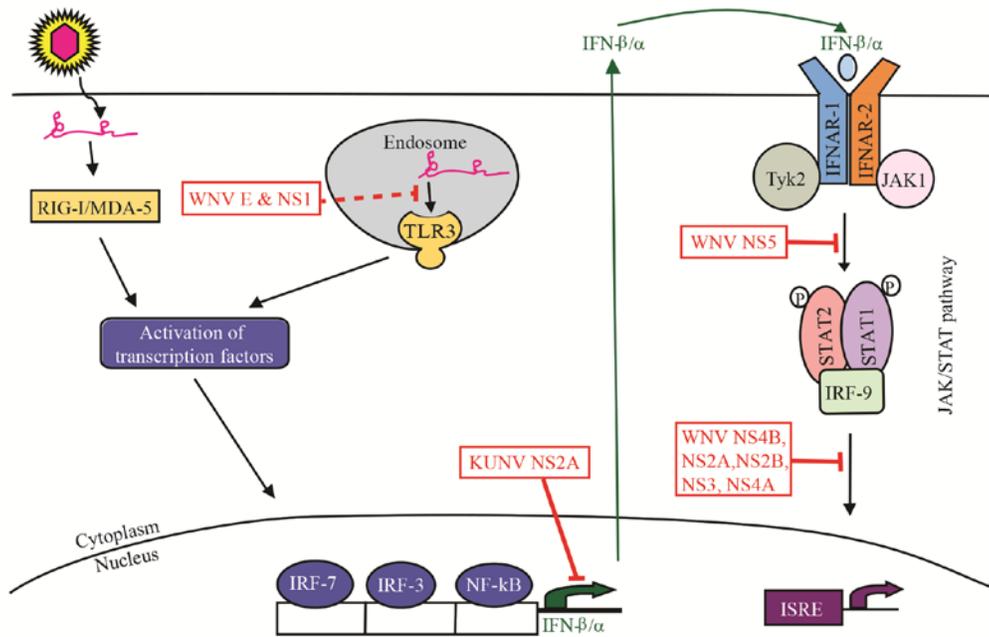


Figure 6. Blocking innate immune signaling

WNV has evolved several different ways to block the innate intracellular immune response. Both WNV E and NS1 may be able to block TLR3 signaling, KUNV NS2A can inhibit IFN- β transcription, WNV NS5 was reported to inhibit Tyk2 and JAK1 phosphorylation, and WNV NS4B can partially block STAT1 signaling while NS2A, NS2B, NS3, NS4A, and NS4B have been shown to inhibit STAT2 nuclear translocation [45-47, 142-144, 146-149].

showed that IFN- α signaling and STAT2 nuclear translocation was inhibited by WNV NS2A, NS2B, NS3, NS4A, and NS4B, but not NS1 and NS5 [46]. WNV NS4B was found to have the ability to partially block STAT1 signaling, which induced fewer ISGs [148]. By mutating certain residues within WNV NS4B in a subgenomic replicon, the protein's ability to block IFN signaling was abolished. However, WNV generated from infectious clones containing these same mutations retained their ability to block the IFN response, suggesting a role for structural genes in the inhibition of IFN signaling [150].

9. Significance of dissertation research

Very few studies have focused on the initial infection of dermal fibroblasts with WNV. Since these cells are part of the initial infection, they may play an important role in the early propagation and dissemination of the virus. Here, I examine the host-pathogen interactions between WNV and human dermal fibroblasts (HFFs) with both a pathogenic strain (WNV-NY) and a nonpathogenic strain (WNV-AUS60). More specifically, I study the HFF innate intracellular immune response to both WNV strains. I also examine the factors that control WNV replication and spread. While both strains are capable of replicating within dermal fibroblasts, WNV-AUS60 replicates to lower levels and spreads less from cell to cell in HFFs than WNV-NY. At the very low multiplicity of infection (MOI) of 0.005 and the low MOI of 0.05, both viruses stimulate similar IFN responses. IFN- β can be detected at 24 h post-infection, but does not limit WNV

infection of HFFs until later times post infection. Specifically, IFN- β plays a role in modulating WNV particle infectivity at late, but not early times post-infection in HFFs. In addition, peak viral titers are lower for WNV-AUS60 compared to WNV-NY due to an IFN-independent reduction in viral infectivity and an inability to spread beyond the initially infected cells. These studies indicate that dermal fibroblasts are capable of supporting WNV replication; however, the low infectivity of particles produced from HFFs late in infection suggests that this cell type likely plays a limited role as a viral reservoir *in vivo*. The work in this dissertation highlights both IFN-dependent and IFN-independent mechanisms that can reduce WNV infectivity in dermal fibroblasts, which comprise an initial site of infection, the dermis.

Chapter 2: Characterization of Factors Reducing West Nile virus Infectivity in Human Dermal Fibroblasts

1. Introduction

West Nile virus (WNV) is a neurotropic *Flavivirus* that has recently emerged as a significant threat to human health. Prior to the 1990s, most WNV infections were asymptomatic or associated with a mild febrile illness known as West Nile fever. However, the recent introduction of WNV into naïve populations in Europe, Israel, and the Americas has resulted in a marked increase in both the number of reported cases and the severity of disease when compared to previous outbreaks.

Based on the phylogenetic analysis of partial genomic sequences of structural genes, WNV has been grouped into five lineages and two clades, which differ from each other by 20-27% [54, 55]. Most strains reside within the two main lineages, designated Lineage 1 and Lineage 2. Lineage 1 strains have been isolated from North America, Europe, the Middle East, Africa, Asia and Australia. While Lineage 2 strains were initially confined to sub-Saharan Africa, they have recently been detected in eastern and southern Europe as well as South Africa [62-64, 66-70]. Experimental infections in rodents and birds demonstrated that the virulence and neuroinvasiveness of strains from both Lineage 1 and 2 are highly variable, ranging from nonpathogenic to highly neuroinvasive [56-58, 60].

WNV is primarily maintained in nature in an enzootic transmission cycle between avian hosts and mosquito vectors. Though mosquitos can transmit WNV to humans and other mammals, this normally results in a dead-end infection since levels of viremia are not sufficient for transmission back to the mosquito vector [23]. Transmission of WNV to avian and mammalian hosts occurs when an infected mosquito deposits saliva containing high doses of virus into the dermal layer of the skin while probing for a blood vessel [89, 91,151-153]. The deposited virus is thought to infect local skin cells as well as immune cells that are recruited to the inoculation site, such as neutrophils and Langerhans dendritic cells [154]. Studies with WNV and dengue virus (DENV) suggest that the immune cells promote viral dissemination by transporting the virus to draining lymph nodes, where a second round of replication occurs in Langerhans cells [93, 95, 96, 155, 156]. The amplified virus then enters the circulatory system via the efferent lymphatic system and the thoracic duct. The subsequent viremia allows WNV to access distal organs, including the spleen, heart, liver, kidneys, and brain.

As one of the first cell types exposed to WNV during a mosquito's blood meal, the nonmigrating cells within the skin may function as an early reservoir for WNV infection. While keratinocytes, the major cell type comprising the epidermal layer of the skin, have been shown to support high levels of WNV replication [90], primary human dermal fibroblasts are also capable of sustaining the replication of WNV and several other *Flaviviruses* [92]. Because little else is known about WNV replication within cells comprising the dermal layer of the

skin, we further assessed the ability of WNV to propagate in primary dermal fibroblasts. Specifically, we compared the ability of a pathogenic, WNV-New York (WNV-NY) [157], and a nonpathogenic, WNV-Australia (WNV-AUS60) [59], Lineage I strain to replicate in these cells. While both strains of WNV replicated in dermal fibroblasts, WNV-AUS60 achieved lower overall peak viral titers compared to WNV-NY. By treating HFFs with supernatants from previous infections, we determined that a factor secreted from WNV-infected HFFs was limiting subsequent WNV infections. Although IFN was not detected, IFN-dependent ISGs were induced in WNV-infected HFFs. Together, this work suggests that WNV-infected HFFs secrete very low levels of type-I IFN and these cells are highly sensitive to the inhibitory effects of small amounts of IFN.

2. Results and Discussion

2.1 WNV replication in human dermal fibroblasts

The replication kinetics of pathogenic (WNV-NY) and nonpathogenic (WNV-AUS60) strains of WNV in human dermal foreskin fibroblast cells (HFFs) were assessed using a multistep growth curve at an MOI of 0.005. Both viruses replicated within HFFs without obvious induction of cytopathic effects (CPE) (data not shown). WNV-AUS60 reached peak infectious particle production by 24 h post-infection, while the viral set point of WNV-NY was obtained at 40 h post-infection (Figure 7A). Examination of viral protein expression indicated that

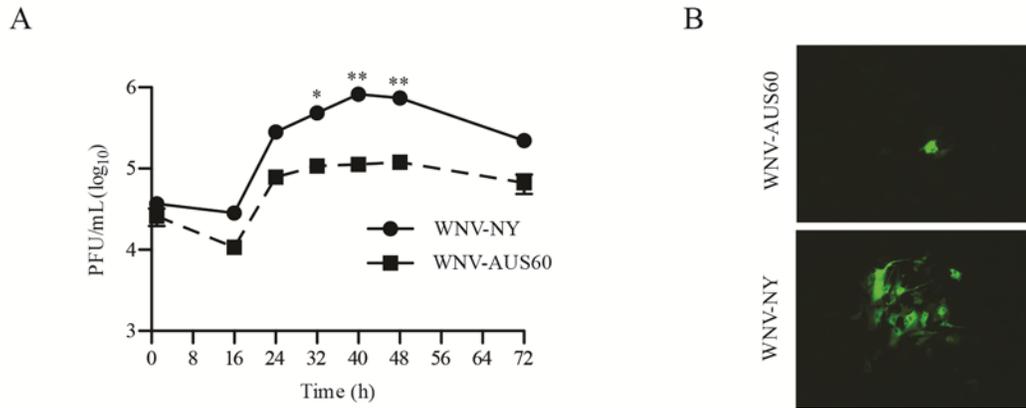


Figure 7. WNV replication and spread in HFFs

HFF cells were infected at an MOI of 0.005 with either WNV-AUS60 or WNV-NY. **(A)** Levels of infectious virus in supernatants collected at 1, 16, 24, 32, 40, 48, and 72 h post-infection were measured by plaque assay on Vero cells. Each point represents duplicate titers of at least three independent experiments.

Statistical significance relative to WNV-AUS60 levels at each time point were determined by an unpaired t-test where * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$. **(B)** Cells were fixed with 3% PFA 24 h post-infection, permeabilized and labeled with WNV antisera followed by FITC conjugated-Goat anti-Mouse IgG. Images are representative of three independent experiments.

WNV-AUS60 was restricted in its ability to spread from cell to cell within the HFF monolayer compared to WNV-NY at 24 h post-infection (Figure 7B). Therefore, reduced cell-to-cell spread may contribute to lower peak titers of WNV-AUS60 compared to WNV-NY.

2.2 Sequential infection in HFFs limits WNV-NY replication

We hypothesized that WNV-AUS60-infected HFFs secrete a factor that limits the ability of this strain to spread from cell-to-cell. Since this factor may also be able to limit the spread of WNV-NY, we performed sequential infections in HFFs (Figure 8A). Cultures were mock- or WNV-AUS60-infected for 24 h. Following these initial infections, the HFFs were mock- or WNV-NY-infected for an additional 24 h and analyzed using quantitative reverse transcriptase-PCR (qRT-PCR) to determine viral levels (Figure 8 B,C). WNV-AUS60 levels were not affected by subsequent WNV-NY infection. However, WNV-NY replication was significantly decreased when HFFs were initially infected with WNV-AUS60 compared to mock (Figure 8C). Because the cells were infected at an MOI of 0.005 with each virus, it is not likely that any cells were co-infected. Therefore, WNV-AUS60-infected cells likely secrete a factor to limit further WNV infection.

2.3 WNV infections of HFFs are limited by a secreted factor

To confirm the presence of a secreted factor, supernatants from previous infections were used to treat HFFs prior to and throughout WNV infections.

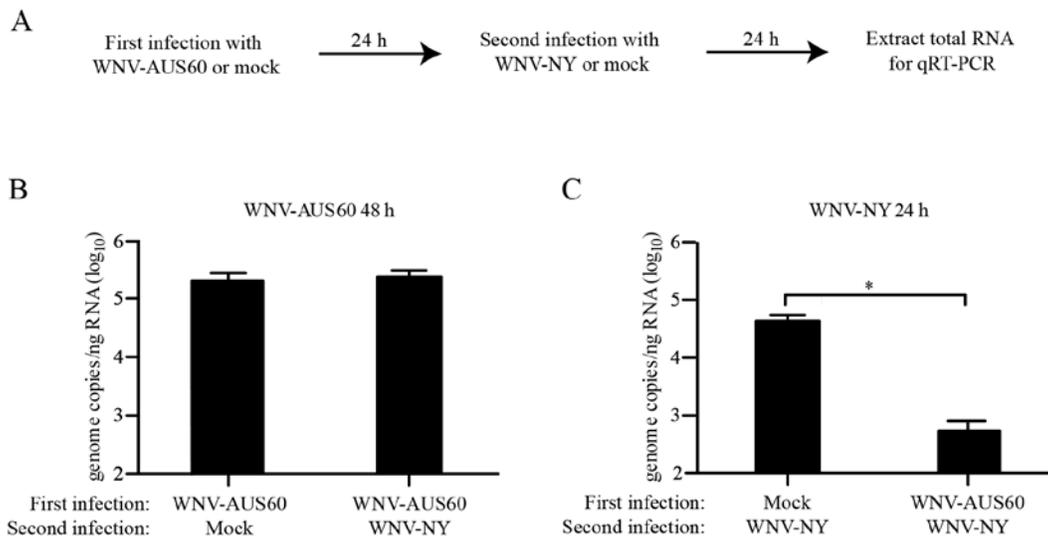


Figure 8. WNV infection is limited after sequential infection in HFFs

(A) A schematic of the sequential infection. (B, C) HFF cells were mock- or WNV-AUS60-infected for 24 h at an MOI of 0.005. After, cells were mock- or WNV-NY-infected at an MOI of 0.005 for 24 h. Total RNA was extracted and analyzed by qRT-PCR with primers specific to a portion of the genome encoding the envelope of either (B) WNV-AUS60 or (C) WNV-NY. Genome copies per ng RNA for each sample were determined by comparing genome copy levels to GAPDH levels. Each point represents triplicate analysis of at least three independent experiments.

Supernatants from 24 h mock- or WNV-AUS60-infections of HFFs were UV-treated and virus was inactivated to levels undetectable by plaque assay. HFFs were treated with inactivated supernatants prior to infection with WNV-NY (Figure 9A). WNV-NY infectious particle production was lower when HFFs were treated with supernatants from WNV-AUS60 infections compared to supernatants from mock infections (Figure 9B). WNV-NY infectious particle production was similarly inhibited in HFFs treated with UV-inactivated supernatants recovered from WNV-NY-infected cultures (data not shown). The inhibitory effect of treatment with supernatants from WNV-AUS60 infections suggests that WNV-infected HFFs secrete a factor that limits subsequent WNV infections.

2.4 Characterization of the inhibiting factor(s) secreted by WNV-AUS60-infected HFFs

To further characterize the factor(s) secreted by WNV-AUS60-infected HFFs, we assessed its effect on other cell lines. Initial characterization involved verifying if the secretion of and response to this factor(s) was cell-type specific. To make this determination, A549 (Figure 10A,B,C) and Vero (Figure 10A,D,E) cells were first mock- or WNV-AUS60-infected for 24 h. Subsequently, the HFFs were mock-or WNV-NY-infected for 24 h, and then total RNA was extracted from the cells using Trizol. The RNA was analyzed with qRT-PCR analysis. Similar to the HFF infections, WNV-AUS60 replication remained the same after 48 h in the presence of a secondary 24 h infection with either mock or WNV-NY (Figure 10A,C). This demonstrates that WNV-AUS60 replication in

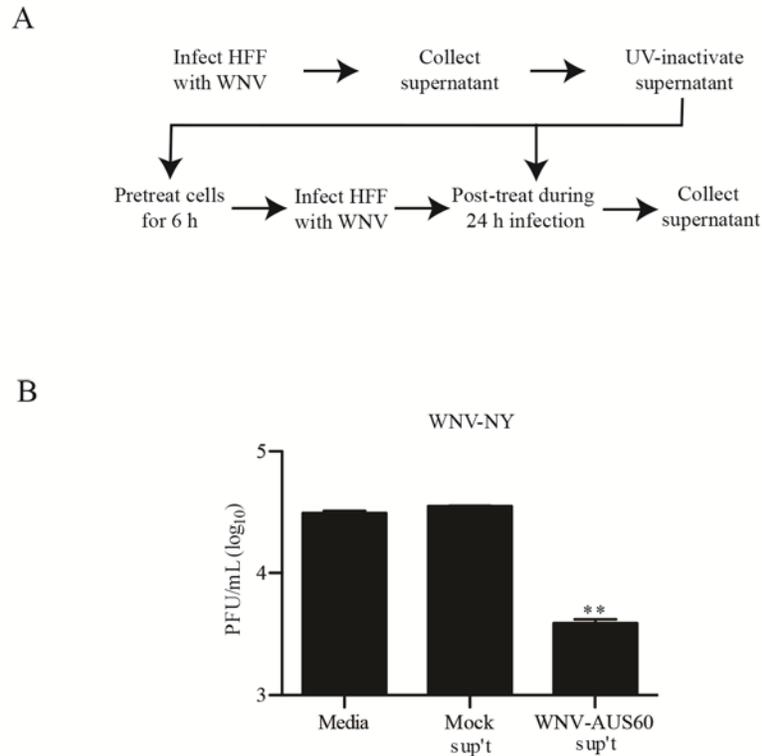
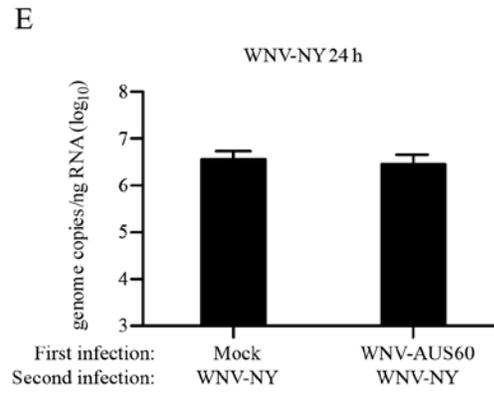
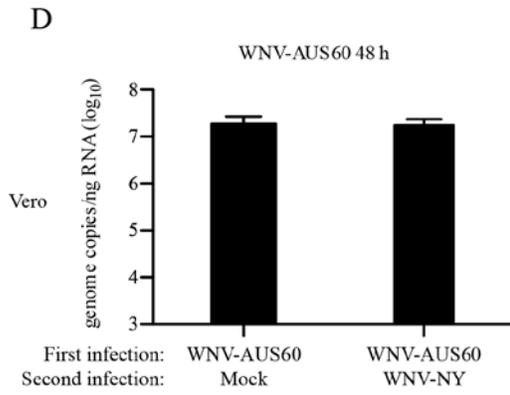
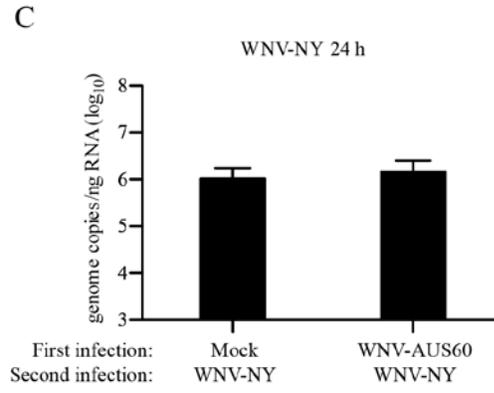
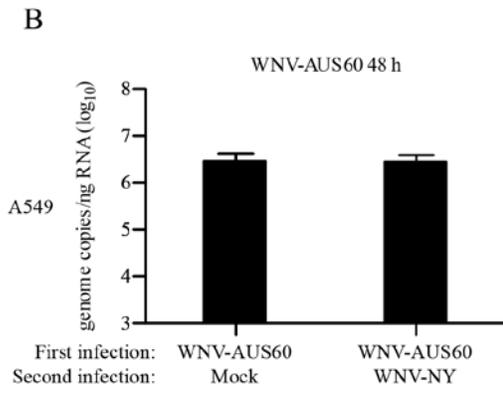
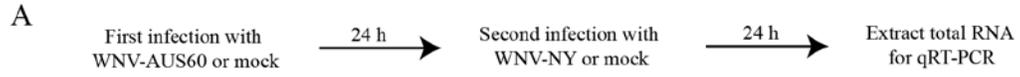


Figure 9. Treatment of HFFs with supernatants from previous WNV HFF infections

(A) Schematic of infection with supernatant treatment. **(B)** HFF cells were treated with media or supernatants from previous mock- or WNV-AUS60-infections for 6 h prior to and throughout infections with WNV-NY (MOI of 0.005) for 24 h. Levels of WNV-NY infectious particle production were determined by plaque assay on Vero cells. Each point represents duplicate titers of at least three independent experiments. Statistical significance was determined by an unpaired t-test where **represents $p < 0.01$.

Figure 10. WNV infection is not limited after sequential infection in A549 and Vero cells

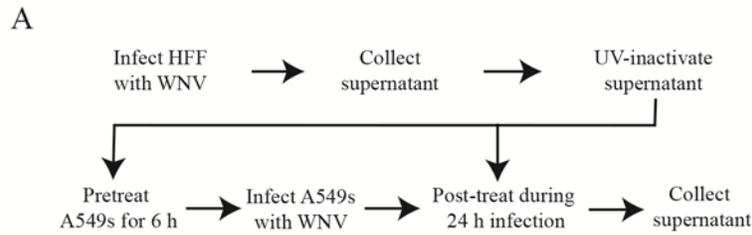
(A) A schematic of the sequential infection. (B, C) A549 or (D, E) Vero cells were mock- or WNV-AUS60-infected at an MOI of 0.005 for 24 h. After, cells were mock- or WNV-NY-infected at an MOI of 0.005 for 24 h and total RNA was extracted for qRT-PCR analysis with primers specific to a portion of the genome encoding the envelope of either (B, D) WNV-AUS60 or (C, E) WNV-NY. Comparing genome copy levels to GAPDH levels, genome copies per ng RNA for each sample was determined. Each point represents triplicate analysis of at least three independent experiments.



A549 and Vero cells is not altered by a subsequent WNV-NY infection. Next, we focused on the effect of the initial WNV-AUS60 infection on the subsequent WNV-NY infection. Unlike sequential infections in HFFs, WNV-NY replication in A549 and Vero cells remained unchanged when HFFs were initially infected with WNV-AUS60 compared to mock (Figure 10B,D). Together, these data suggest that WNV-AUS60-infected A549 and Vero cells do not produce and/or respond to an inhibitory secreted factor comparable to the factor secreted by WNV-AUS60-infected HFFs.

Further characterization involved determining the effect of supernatants from WNV-infected HFFs on WNV infection of A549 cells. Supernatants from 24 h WNV-AUS60 infections of HFFs were used to treat A549 cells prior to and throughout a 24 h WNV-NY infection (Figure 11A). Unlike HFF infections, treatment with supernatants from WNV-AUS60 infections of HFFs had no effect on WNV-NY infections of A549 cells (Figure 11B), suggesting that A549 cells do not respond to the inhibitory factor present in supernatants recovered from WNV-AUS60 infected HFFs.

To assess some basic biochemical properties of the inhibitory factors within the supernatants recovered from WNV infections of HFFs, we evaluated their thermal stability. While incubation at 56°C for 30 min is sufficient to inactivate some viruses, WNV supernatants required additional treatment with UV-light to decrease levels of virus below detectable limits. HFFs were exposed to UV- and heat-treated supernatants prior to and throughout infection with



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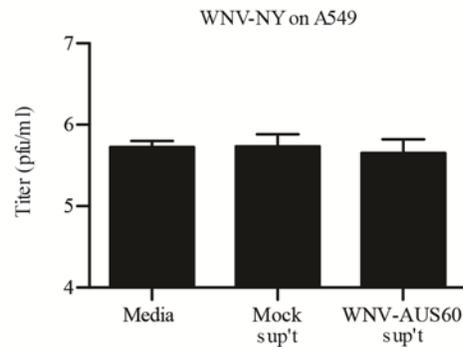


Figure 11. Treatment of A549 cells with supernatants from previous WNV HFF infections

(A) Schematic of A549 infection with HFF supernatant treatment. (B) A549 cells were exposed to UV-treated media or supernatants recovered from mock or WNV-AUS60 HFF infections for 6 h prior to and throughout infections with WNV-NY at an MOI of 0.005 for 24 h. Each point represents duplicate titers of at least three independent experiments.

WNV-NY (Figure 12A). Exposure of WNV-AUS60 supernatants to heat abolished their inhibitory effects (Figure 12B) compared to supernatants that were only exposed to UV-light treatment in Figure 9B. Therefore, the secreted factor produced by WNV-AUS60-infected HFFs is heat-labile.

An additional biochemical property that we tested was pH stability of the factors in the supernatants from WNV-infected HFFs. Like heat-treatment, acid treatment was not sufficient to inactivate WNV. WNV supernatants required additional treatment with UV-light to fully inactivate virus. HFFs were incubated with UV- and acid-treated WNV-AUS60 supernatants prior to and throughout infection with WNV-NY (Figure 12C). Low pH and UV light also abolished the inhibitory effects of WNV-AUS60 supernatants (Figure 12C) when compared to supernatants that were only exposed to UV light treatment in Figure 9B.

Therefore, the secreted factor produced by WNV-AUS60-infected HFFs is both heat- and acid-labile. Work by Rentsch and Zimmer showed that IFN- β retained its antiviral activity, as measured by bioassay in normal dermal human fibroblasts, when heated up to 60°C for 30 min or treated with 0.1 M HCl for 30 min [159]. This suggested that the factor secreted by WNV-AUS60-infected HFFs was not IFN- β .

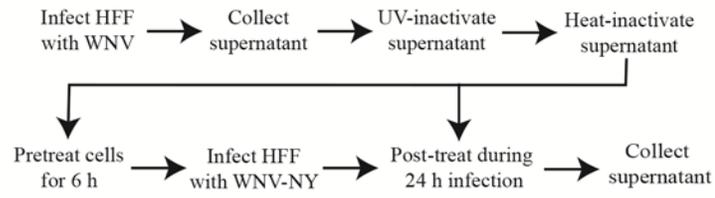
2.5 IFN α/β was not detected in supernatants from WNV HFF infections

To directly assess whether IFN was induced during WNV-infection in HFFs, we measured type-I IFN levels with an IFN bioassay. The type-I IFN

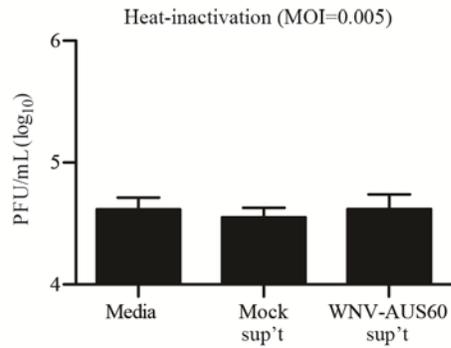
Figure 12. Treatment of HFFs with heat- and acid-treated supernatants from previous WNV HFF infections

(A) Schematic of infection with heat-inactivated supernatant treatment. (B) HFF cells were treated with supernatants from previous WNV-AUS60 infections, mock infections or media before and after infection with WNV-NY. Specifically, supernatants from 24 h mock- or WNV-AUS60-infections that were UV-inactivated then incubated at 56°C for 30 min, were used to treat HFF cells for 6 hours prior to and throughout infection with WNV-NY at an MOI of 0.005 for 24 h. (C) Schematic of infection with acid-treated supernatant treatment. (D) HFF cells were treated with supernatants from previous WNV-AUS60 infections, mock infections or media before and after infection with WNV-NY. Specifically, supernatants from 24 h mock- or WNV-AUS60-infections that were UV-inactivated then incubated with 0.1 M HCl for 30 min then neutralized, were used to treat HFF cells for 6 hours prior to and throughout infection with WNV-NY at an MOI of 0.005 for 24 h. Each point represents duplicate titers of at least three independent experiments.

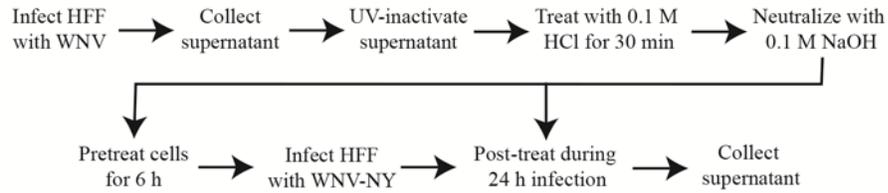
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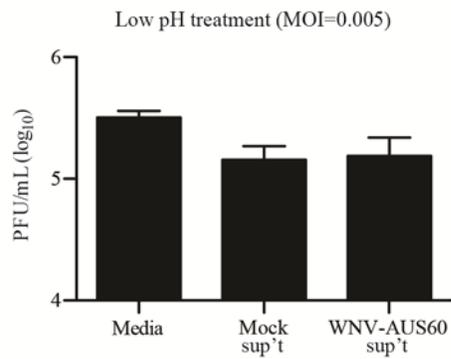
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bioassay quantified protection from infection with a virus that is highly susceptible to type-I IFN treatment, vesicular stomatitis virus (VSV) [160]. For the bioassay, A549 cells were pretreated with supernatants from mock, WNV-AUS60, and WNV-NY HFF infections alongside an IFN standard for 24 h or cells were untreated. After a 24 h VSV infection, levels of VSV in supernatants were assessed by plaque assay (Figure 13A). Decreased VSV levels were observed when cells were pretreated with an IFN standard as compared to untreated cells. Conversely, treatment with supernatants from mock-, WNV-AUS60-, and WNV-NY-infections of HFFs had no effect on VSV titers (Figure 13B). This suggests that IFN is not present or is below the limit of detection for this assay.

To assess IFN induction with a more sensitive assay, we analyzed total RNA isolated from WNV-infected HFFs using qRT-PCR analysis. While IFN- β transcription was induced in control A549 cells infected with WNV-NY, it was not induced in WNV-AUS60- or WNV-NY-infected HFFs (Figure 13C). Combined, these data suggest that IFN- β is not present or below detectable limits in cells and supernatants from WNV infections of HFFs.

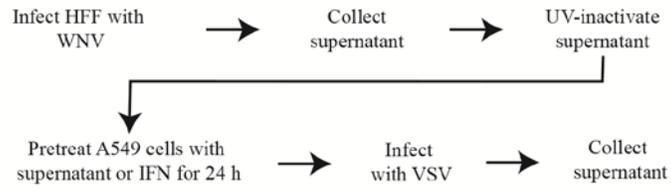
While it is known that IFN- β signals pathways that lead to the inhibition of WNV infection, other cytokines may also play a role in the inhibition of HFF infection. In addition to testing for the presence of type-I IFN, levels of type-III IFN, IFN- λ , were also assessed. IFN- λ is an interferon stimulated gene (ISG) that is induced following IFN- α or IFN- β expression and has been shown to have

Figure 13. Detection of IFN in supernatants from WNV HFF infections

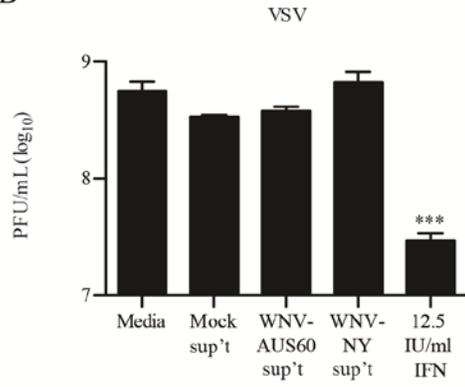
(A) Schematic of IFN bioassay on A549 cells. **(B)** IFN bioassay. Prior to a 24 h infection with VSV at an MOI of 1, A549 cells were treated for 24 h with media, 12.5 IU/ml IFN- β , or UV-inactivated supernatants from 24 h-WNV-AUS60 or WNV-NY infections. VSV titers were determined by plaque assay on Vero cells. Each point represents duplicate titers of three independent experiments. Statistical significance was determined by an unpaired t-test where *** represents $p < 0.001$.

(C,D) One-step qRT-PCR analysis. Total RNA was isolated from HFF cells infected at an MOI of 0.005 with mock, WNV-AUS60, WNV-NY or A549 control cells infected at an MOI of 1 with WNV-NY. RNA from WNV-infected cells was analyzed in the presence of SYBR Green with primers specific to human **(C)** IFN- β and GAPDH or **(D)** IFN- λ and GAPDH then compared to values from mock-infected cells. **(C,D)** Each point represents triplicate analysis of at least two independent experiments. Statistical significance was determined by an unpaired t-test where * represents $p < 0.05$.

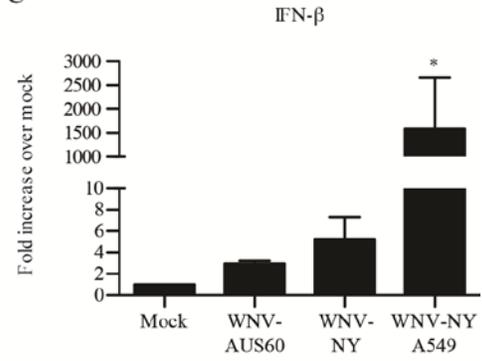
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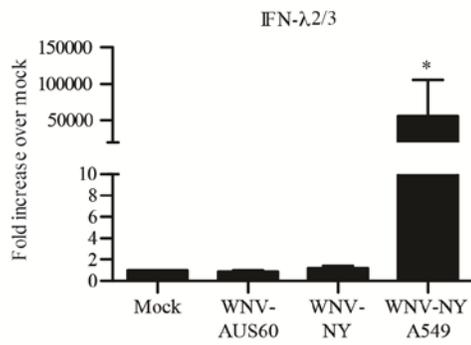
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antiviral activity against positive-sense ssRNA viruses, like encephalomyocarditis virus (EMCV) [161]. A study examining the effects of IFN- λ on WNV infection showed that IFN- λ could slightly inhibit the infection of epithelial cells with WN virus-like particles, but not virus replication [162]. To assess levels of IFN- λ 2/3 transcription, total RNA from mock-, WNV-AUS60-, and WNV-NY-infected cells was analyzed using qRT-PCR. Like IFN- β levels, IFN- λ 2/3 transcription could be detected in control A549 cells infected with WNV-NY, but not in WNV-AUS60- or WNV-NY-infected HFFs (Figure 13D). This suggests that IFN- λ does not contribute to the inhibition of WNV infection in HFFs.

2.6 Characterization of the innate intracellular antiviral effector proteins produced during WNV infection

While IFN was not detected by type-I IFN bioassay in cells and supernatants from WNV infection of HFFs at an MOI of 0.005, western blot analysis of the intracellular proteins suggests that IFN is secreted by HFFs infected with WNV. By 24 h post-infection with either WNV-AUS60 or WNV-NY, ISGs, such as Interferon Regulatory Factor-9 (IRF-9), Interferon Stimulated Gene 15 (ISG15), Interferon Stimulated Gene 56 (ISG56) and phosphorylated Signal Transducer and Activator of Transcription-2 (phospho-STAT-2) can be detected by western blot (Figure 14). Production of ISG15 and ISG56 can be a result of either IFN-independent IRF-3 activation or downstream IFN production [137]. However, phosphorylation of STAT-2 is specific to type-I IFN signaling

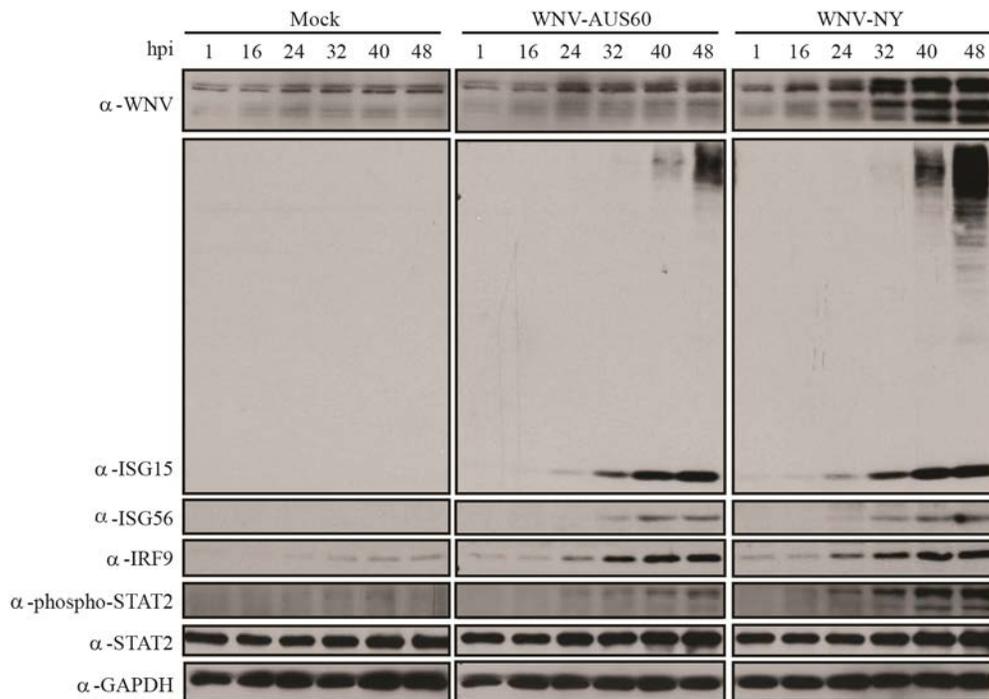


Figure 14. Induction kinetics of antiviral effector proteins resulting from WNV-AUS60 and WNV-NY infection of HFFs

HFF cells were infected at an MOI of 0.005 with either mock, WNV-AUS60, or WNV-NY. Whole-cell lysates were collected at indicated times post infection. Levels of WNV, ISG56, ISG15, IRF-9, Phospho-STAT-2, STAT-2, and GAPDH were examined by immunoblot. Images are representative of three independent experiments.

[118,163-167]. Phospho-STAT2 was detected in WNV-infected HFFs, suggesting that IFN is being produced at levels that are below the limit of detection of several standard IFN quantification assays.

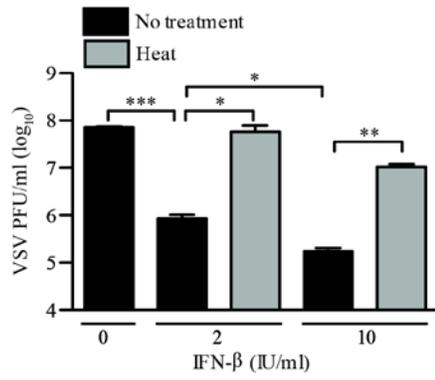
However, these results do not correspond with the biochemical profile previously observed by Rentsch and Zimmer, showing that IFN- α , IFN- β , and IFN- λ retained their antiviral activity, as measured by bioassay in normal dermal human fibroblasts, when heated up to 60°C for 30 min [158]. They also showed that IFN- β and IFN- λ retained their antiviral activity when treated with 0.1 M HCl for 30 min [158]. In these experiments, the normal human dermal fibroblasts were treated with approximately 10 IU/ml IFN- β . However, we have demonstrated that 0.625 IU/ml IFN- β is sufficient to limit VSV infection of human dermal fibroblasts [159]. Therefore, low levels of residual activity in the heat- and acid-treated IFN may be sufficient to inhibit viral infection in dermal fibroblasts.

We hypothesized that heat and acid treatment may abolish the antiviral activity of samples containing IFN at the threshold level of biological activity. Treatment of HFFs with 2 and 10 IU/ml IFN- β reduced VSV titers by 2 and 2.5 logs, respectively, when compared to an untreated control (Figure 15A, B). When testing the effects of heat and low pH on WNV supernatants, additional treatment with UV-light was required to completely inactivate all virus present (Figure 12). Following exposure to UV light, treatment with 2 IU/ml IFN- β decreased VSV titers by 1 log and 10 IU/ml IFN- β decreased VSV titers by 3 logs, as compared to treatment with media exposed to UV-light (Figure 15C, D).

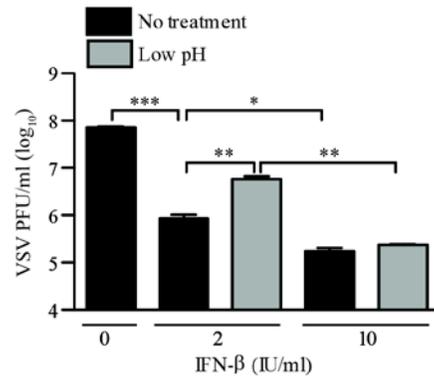
Figure 15. Effect of heat and acid treatment on low levels of IFN- β

(A) HFF cells were incubated with untreated or heat-treated IFN- β before and after infection with VSV. Specifically, 2 or 10 IU/ml IFN- β was incubated at 56°C for 30 min, then used to treat HFF cells for 6 h prior to and throughout the 24 h infection with VSV at an MOI of 1. (B) HFF cells were exposed to untreated or acid-treated IFN- β before and after infection with VSV. Specifically, 2 or 10 IU/ml IFN- β was incubated with 0.1 M HCl for 30 min, neutralized, and then used to treat HFF cells for 6 hours prior to and throughout the 24 h infection with VSV at an MOI of 1. (C) HFF cells were treated with UV-treated or heat- and UV-treated IFN- β before and after infection with VSV. Specifically, 2 or 10 IU/ml IFN- β was UV-treated then incubated at 56°C for 30 min and used to treat HFF cells for 6 hours prior to and throughout the 24 h infection with VSV at an MOI of 1. (D) HFF cells were exposed to UV-treated or acid- and UV-treated IFN- β before and after infection with VSV. Specifically, 2 or 10 IU/ml IFN- β was UV-treated prior to incubating with 0.1 M HCl for 30 min, neutralized, and then used to treat HFF cells for 6 hours prior to and throughout the 24 h infection with VSV at an MOI of 1. Each point represents duplicate titers of at least three independent experiments. Statistical significance was determined by an unpaired t-test. Asterisks indicate differences that are statistically significant (* p<0.05, ** p<0.01, *** p<0.005).

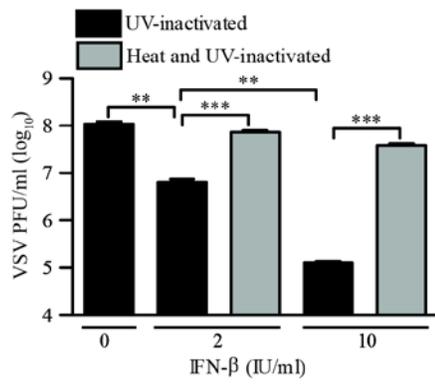
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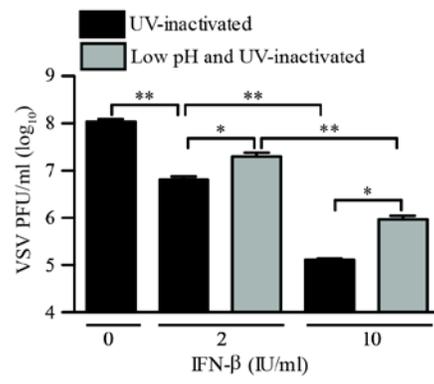
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To compare the effects of heat and acid treatment on low levels of IFN to the previous biochemical treatments of supernatants from WNV-infected HFFs, IFN- β controls were also treated with heat or acid. These heat- and acid-treated IFN controls were used to treat cells pre- and post-infection with VSV. Upon heat-treatment, the inhibitory effect of 2 IU/ml IFN- β was completely abrogated while 10 IU/ml retained a slight effect (Figure 15A). With heat- and UV-treatment, IFN at either concentration had virtually no effect on VSV titers (Figure 15C). Therefore, with and without UV-treatment, heat-treatment of low levels of IFN- β abolishes its biological activity.

When 2 IU/ml IFN- β was exposed to low pH, its effect on VSV infection was slightly inhibited, but 10 IU/ml IFN- β remained unchanged (Figure 15B). With both UV- and acid- treatment, the inhibitory effect of IFN- β on VSV titers only decreased slightly (Figure 15D). Therefore, UV-treatment inhibits the effects of low levels of IFN- β , but higher levels retain their inhibitory effect on VSV infection. However, acid-treatment of IFN- β in the presence and absence of UV-light treatment slightly dampens its ability to inhibit VSV infections.

Based on a previously published analysis of the biochemical properties of type-I IFN, IFN in supernatants from WNV infections of HFF did not appear to be responsible for controlling the inhibition of subsequent WNV infection. However, our analysis indicated that low levels of IFN might be present. These low levels may have been sufficient to inhibit WNV infection, but the biological activity was abolished upon heat and acid treatment.

3. Conclusions

Since little is known about WNV infection of dermal cells and mosquito-borne viruses are known to infect both epidermal and dermal cells upon mammalian infection [90, 168], we further assessed the ability of WNV to propagate in primary dermal fibroblasts. Specifically, we compared the ability of a pathogenic, WNV-New York (WNV-NY) [157], and a nonpathogenic, WNV-Australia (WNV-AUS60) [59], Lineage I strain to replicate in these cells. Peak titers of WNV-AUS60 were lower than those of WNV-NY, which may be due to a lack of cell-to-cell spread. Since treatment with UV-inactivated supernatants from WNV infections of HFFs limits subsequent infections, a secreted factor is likely responsible for this inhibition.

To quantify levels of type-I IFN in supernatants from WNV infections of HFFs, we initially utilized an IFN bioassay on A549 cells. At the MOI of 0.005, the levels of IFN were below the limit of detection of the IFN bioassay on A549 cells, 2 IU/ml. For a more sensitive assay, we assessed levels of IFN- β transcripts in WNV-infected HFFs via qRT-PCR. Further analysis of WNV-infected cells by qRT-PCR analysis demonstrated that IFN- β , as well as IFN- λ mRNA levels were below the limit of detection of this assay.

To further characterize the factor, we assessed its effect on other cell types, its thermal stability and its acid tolerance. When supernatants containing the factor were used to treat WNV-infected A549 cells, the treatment had no effect on the infection. This suggested that the concentrations of the factor in the supernatants could inhibit WNV-infection on HFFs, but not A549 cells.

Following incubation at a high temperature or a low pH, the inhibitory effect of the supernatant from WNV infections was abolished. Although the heat- and acid-sensitivity profile suggested that the factor was not IFN, the detection of phospho-STAT2 by immunoblot suggests that IFN is being produced. However, the levels are below the limit of detection of standard assays. Previous studies focusing on 24 to 48 h infections of human foreskin fibroblasts with dengue virus at an MOI of 10 led to the production of IFN- β , which protected uninfected cells from dengue virus infection [92]. These observations suggest that infection at a higher MOI will lead to the secretion of IFN at detectable levels. The next chapter highlights the detection of IFN at a higher MOI.

Chapter 3: IFN-Dependent and -Independent Reduction in West Nile virus Infectivity in Human Dermal Fibroblasts

1. Introduction

Following the work in the previous chapter, a new stock of WNV-AUS60 was generated that contained ten times more infectious particles. The highest possible multiplicity of infection (MOI) for WNV-AUS60 infection of HFFs with the previous stock was 0.005, but the new stock allowed for infection at a higher MOI of 0.05. Even with the higher multiplicity of infection, WNV-AUS60 achieved lower overall peak viral titers compared to WNV-NY. At this higher MOI, IFN- β could be detected in supernatants from WNV-AUS60 and WNV-NY infections by type-I IFN bioassay. While IFN- β affected replication and particle infectivity of both strains late in infection, it had no effect at early times post-infection. Moreover, IFN- β neutralization increased particle infectivity for both strains late in infection; however a significant difference in WNV-AUS60 and WNV-NY particle infectivity remained.

2. Results and Discussion

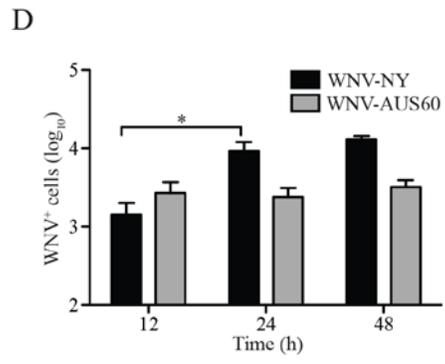
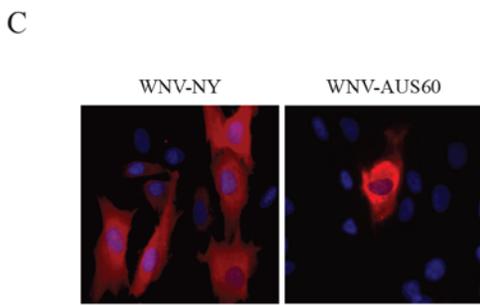
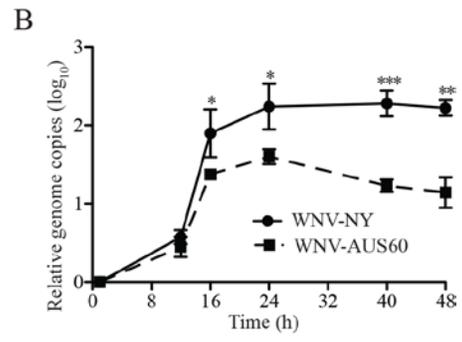
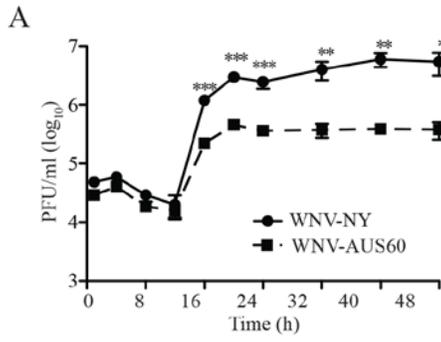
2.1 WNV replication in human dermal fibroblasts

To expand upon the work in the previous chapter, we compared the replication kinetics of insect cell-passaged WNV-NY and WNV-AUS60 in human dermal foreskin fibroblasts (HFFs) at a higher MOI of 0.05 PFU/cell (Figure 16A). Like the very low MOI of 0.005, pathogenic, WNV-NY, and the nonpathogenic, WNV-AUS60, strains replicated within HFFs without obvious induction of cytopathic effects (CPE) (data not shown). At this higher MOI, both strains reached peak infectious particle production between 20 and 24 h post-infection (Figure 16A). However, peak viral titers of WNV-AUS60 were approximately one log lower than WNV-NY. Additionally, quantitative RT-PCR (qRT-PCR) analysis revealed that the kinetics of WNV-NY and WNV-AUS60 genome replication occurred at similar rates (Figure 16B). Peak levels of viral genome accumulation were detected at 24 h post-infection and lower levels of viral genomic RNA were detected at all times in WNV-AUS60-infected cells compared to WNV-NY-infected cells. Therefore, although both strains were capable of establishing an infection within HFFs, the nonpathogenic strain, WNV-AUS60, never reached levels of replication as high as the pathogenic strain, WNV-NY.

We hypothesized that WNV-AUS60 multiplication in HFFs is reduced compared to that of WNV-NY due to a defect in cell-to-cell spread. Therefore, we examined viral protein expression in infected cells by an immunofluorescence assay (IFA). While multi-cell foci of infected cells were detected in WNV-NY-

Figure 16. WNV replication in HFFs

HFF cells were infected (MOI=0.05) with WNV-NY or WNV-AUS60. **(A)** WNV infectious particles production in HFFs. Culture supernatants were recovered at the indicated times and titered by plaque assay on Vero cells. Values represent the average number of plaque forming units (PFU) per mL (+/- standard error) from three independent experiments. **(B)** RNA synthesis of WNV-NY and WNV-AUS60 in HFFs. Total RNA was extracted from cells at the indicated times and viral RNA levels were assessed by qRT-PCR. Relative WNV genome copies were calculated as a change in WNV genome copies per ng of RNA from 1 h post-infection. Values represent the average (+/- standard error) of at least three independent experiments. **(C)** Examination of viral protein expression by IFA. HFFs were fixed with 3% PFA at 24 h post-infection, permeabilized, dyed with Hoescht stain (blue), and probed with WNV hyperimmune ascitic fluid and goat anti-mouse IgG 549 nm-Dylight conjugated secondary antibody (red). Images are representative of at least three independent experiments. **(D)** WNV spread in HFFs. The number of infected cells within the monolayer was determined by flow cytometry. Monolayers were trypsinized at the indicated times, fixed with 3% PFA and probed with WNV hyperimmune serum. Values represent the average number (+/- standard error) of WNV-positive cells per 10^5 cells from three independent experiments. Statistical significance was determined by an unpaired t-test. Asterisks indicate differences that are statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



infected cultures after 24 h, WNV protein expression was primarily restricted to single cells within WNV-AUS60-infected cultures (Figure 16C). Like infection at the very low MOI of 0.005, the detection of unicellular foci with infection at the low MOI of 0.05 suggests that WNV-AUS60 is restricted in its ability to spread from cell to cell within the HFF monolayer compared to WNV-NY. To confirm this result, we quantitated the number of infected HFFs over the course of infection using flow cytometry (Figure 16D). Similar levels of WNV-positive cells were detected at 12 h post-infection in WNV-NY- and WNV-AUS60-infected cultures, indicating that both viruses initially established comparable levels of infection within the HFF monolayer. However, the number of WNV-NY-positive cells increased between 12 and 24 h post-infection, whereas the number of WNV-AUS60-positive cells remained unchanged. Thus, WNV-AUS60 is impaired in its ability to spread beyond the initially infected cells.

2.2 IFN response to WNV in HFFs

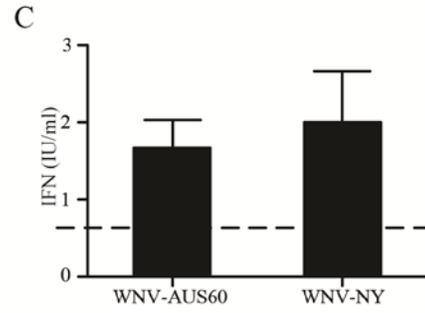
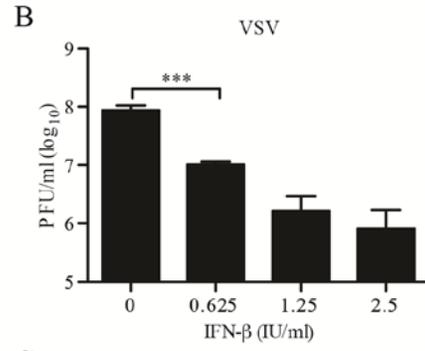
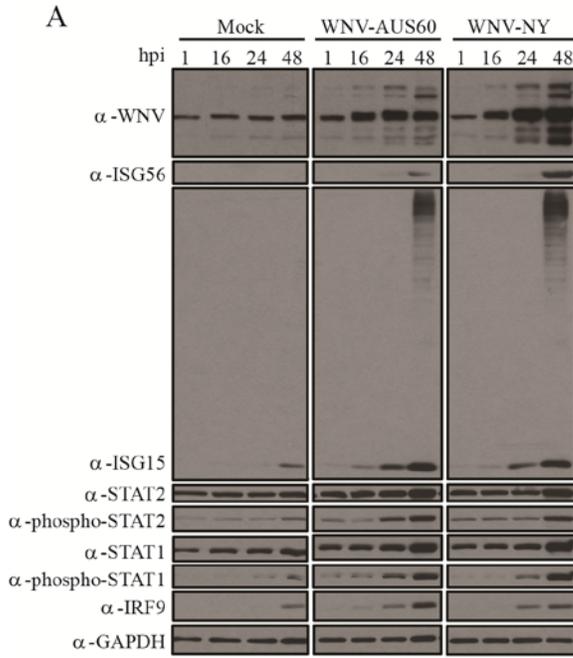
The restriction of viral spread within HFF cultures was suggestive of paracrine protection by type-I IFN since previous work has shown that WNV infection is controlled by IFN- α/β in other *in vivo* and *in vitro* models [169-171]. Therefore, we measured the levels of type-I IFNs in supernatants recovered from WNV-infected HFFs using a bioassay on A549 cells. While approximately 160 IU/ml of IFN was detected in supernatants recovered from WNV-NY-infected cells at 48 h post-infection, IFN was not detected at 24 h post-infection (data not shown). Like supernatants from infections at the very low MOI of 0.005, IFN

was not detected in supernatants recovered from mock- or WNV-AUS60-infected cells at either 24 or 48 h post-infection (data not shown). Consistent with infections at the very low MOI of 0.005, ISGs, such as Interferon Regulatory Factor-9 (IRF-9), Interferon Stimulated Gene 15 (ISG15), Interferon Stimulated Gene 56 (ISG56), phosphorylated Signal Transducer and Activator of Transcription-1 (phospho-STAT-1), and phospho-STAT2 were detected by western blot from cultures infected with WNV-NY- and WNV-AUS60 at the MOI of 0.05 (Figure 17A). Comparable to western blot analysis of lysates from cells infected at the MOI of 0.005, both WNV-NY and WNV-AUS60 infections at the MOI of 0.05 induced the expression of a panel of interferon-stimulated genes (ISGs) with similar amplitude and kinetics. Combined, these data suggest that both strains induced IFN responses in HFFs as early as 24 h post-infection, though the level of IFN expression was below the detection limit of 2 IU/ml of a standard bioassay using A549 cells. Therefore, we assessed whether HFFs were sensitive to low levels of IFN. HFFs were treated with 0.625 to 2.5 IU/ml of IFN- β and infected with vesicular stomatitis virus (VSV), a virus that is highly sensitive to the antiviral effects of IFN (Figure 17B). VSV replication in HFFs was suppressed in a dose-dependent manner, indicating that levels as low as 0.625 IU/ml of IFN- β are capable of inducing an antiviral state within this cell line.

Based on these results, we reassessed IFN levels in supernatants from WNV-NY and WNV-AUS60 infections using a bioassay on HFFs. Using this

Figure 17. Antiviral response to WNV-AUS60 and WNV-NY in HFFs

(A) Steady state protein levels of WNV, ISG56, ISG15, Phospho-STAT-2, STAT-2, Phospho-STAT-1, STAT-1, IRF-9, and GAPDH in mock-, WNV-AUS60- or WNV-NY-infected (MOI=0.05) HFF cells. Extracts prepared at the indicated times post-infection were examined by immunoblot. A representative example from three independent experiments is shown. **(B)** Sensitivity of HFFs to IFN. HFF cells were treated with 0, 0.625, 1.25, or 2.5 IU/ml IFN- β for 24 h prior to infection with VSV (MOI=1). Supernatants were collected at 24 h post-infection and VSV titers were determined by plaque assay on Vero cells. Values represent the average number of plaque forming units (PFU) per mL (+/- standard error) from at least three independent experiments. Statistical significance was determined by an unpaired t-test. Asterisks indicate differences that are statistically significant (***) $p < 0.001$. **(C)** Determination of the WNV-induced IFN levels using a VSV-based bioassay on HFFs. HFF cells were treated with specified supernatants for 24 h prior to infection with VSV (MOI=1). Supernatants were collected at 24 h post-infection and VSV titers were determined by plaque assay on Vero cells. Values represent the level of type-I IFN (IU/mL) (+/- standard error) from three independent experiments. The dashed line represents the limit of detection for the assay.



more sensitive assay, approximately 2 IU/ml of IFN were detected in supernatants recovered from WNV-NY- and WNV-AUS60-infected cells at 24 h post-infection (Figure 17C). Thus, both viruses induce the expression of low levels of IFN at early times post-infection. While higher levels of secreted IFN were detected in supernatants of WNV-NY-infected HFFs at 48 h post-infection, this was likely due to a greater number of infected cells secreting IFN and not higher levels of IFN secreted from each infected cell.

While it is known that IFN- β signals pathways that lead to the inhibition of WNV infection, other cytokines may also play a role in the inhibition of HFF infections. In addition to testing for the presence of type-I IFN, levels of type-III IFN, IFN- λ , were also assessed. IFN- λ is an interferon stimulated gene (ISG) that has been shown to have antiviral activity during infection with positive-sense ssRNA viruses, like encephalomyocarditis virus (EMCV) [161]. A cytokine that has been shown to work in concert with IFN- β is TNF. Treatment of fibroblasts with TNF in addition to IFN- β has shown to induce a distinct antiviral state[172].

Another important aspect of the antiviral response is the attraction of leukocytes. CCL2 (monocyte chemoattractant protein-1 or MCP-1), CCL5 (regulated on activation, normal T cell expressed and secreted or RANTES), and CXCL12 (stromal cell derived factor or SDF-1 α) are chemokines that are known to recruit leukocytes. Previous work with human cytomegalovirus (hCMV) has shown that CCL2 expression is modulated during virus replication [173]. Studies focusing on respiratory syncytial virus (RSV) have shown that the chemokine CCL5 (RANTES) can control RSV infection in a dose-dependent manner [174,

175]. Additionally, upon infection with herpes simplex virus (HSV), fibroblasts were shown to secrete CCL5 [176]. CXCL12 is a chemokine secreted from stromal cells, like fibroblasts, which has been shown to activate the JAK/STAT pathway [177].

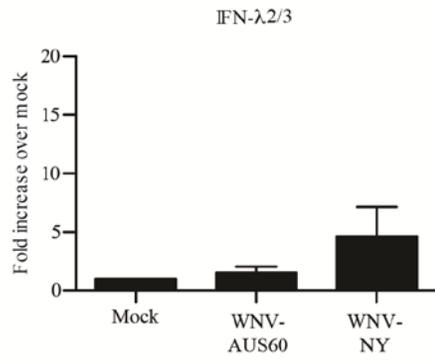
Since other cytokines and chemokines such as IFN- λ , CXCL12, TNF- α , and CCL5 have been previously shown to activate antiviral pathways or have a direct inhibitory effect on viral infection [161, 162, 172-176], we measured their levels in supernatants and cells by qRT-PCR and ELISA (Figure 18). Production of IFN- λ 2/3, CXCL12, TNF- α , and CCL2 were not upregulated with WNV infection. Like IFN, CCL5 levels increased with WNV-AUS60 infection and further increased with WNV-NY infection, which correspond with increased cell-to-cell spread.

To confirm that the low levels of IFN produced in response to WNV infection were sufficient to suppress viral replication, we measured WNV-NY infectious particle production in HFFs treated with UV-inactivated supernatants recovered from mock- or WNV-AUS60-infected cultures. Compared to cultures treated with mock supernatants, WNV-NY titers were only reduced by one log when treated with supernatants from WNV-AUS60 infections at the MOI of 0.005 (Figure 9) and titers were reduced by approximately two logs in cultures treated with WNV-AUS60 supernatants from the MOI of 0.05 (Figure 19A). In control cells treated with 25 IU/ml of IFN- β , WNV-NY titers were reduced by 3 logs. To confirm that the inhibitory effect of WNV supernatant treatment was due to IFN,

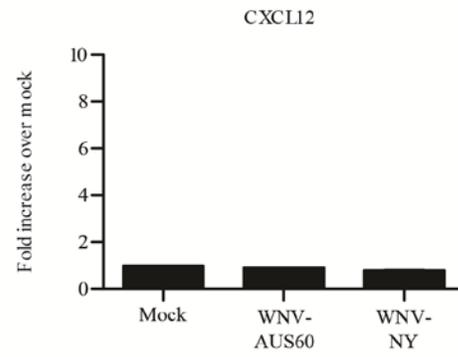
Figure 18. Detection of cytokines in WNV-infected HFFs

One-step qRT-PCR analysis of total RNA isolated from mock-, WNV-AUS60-, or WNV-NY-infected HFF cells (MOI of 0.05). RNA from mock- and WNV-infected cells was analyzed in the presence of SYBR Green with primers specific to **(A)** human IFN- λ 2/3 and GAPDH or **(B)** CXCL12 and GAPDH then compared to values from mock-infected cells. **(C,D,E)** HFFs were infected at an MOI of 0.05 with mock, WNV-AUS60 or WNV-NY and supernatants were removed and UV-treated at 24 or 48 h post-infection. Levels of **(C)** TNF- α , **(D)** CCL2, and **(E)** CCL5 in collected supernatants were measured by ELISA. Dashed line represents the limit of detection of each assay.

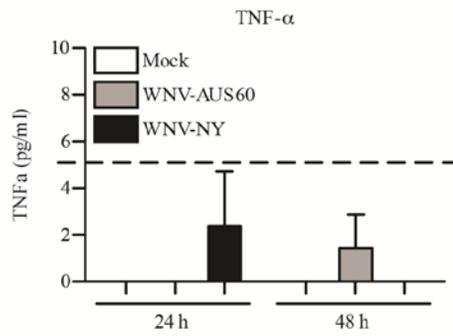
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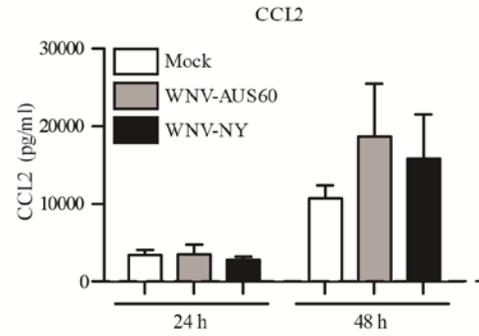
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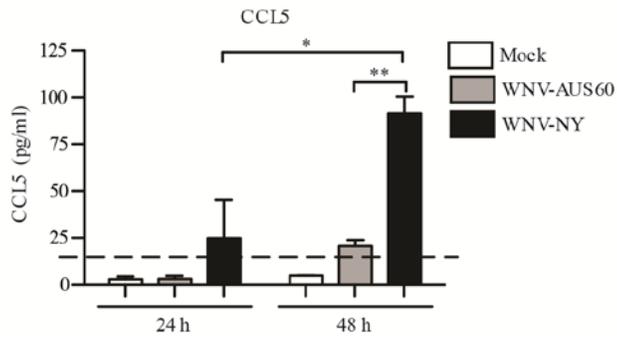
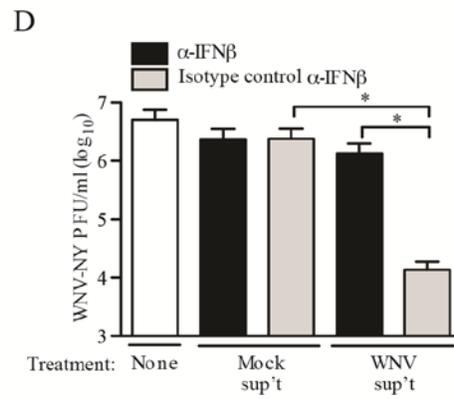
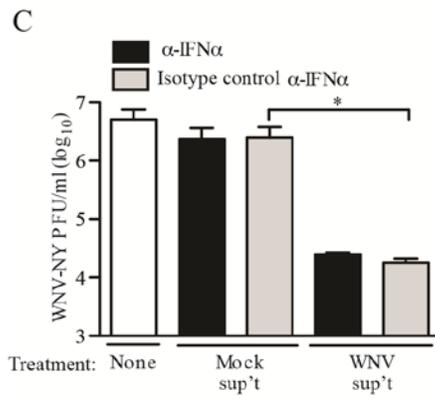
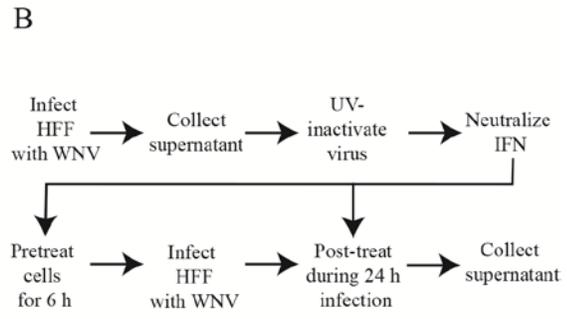
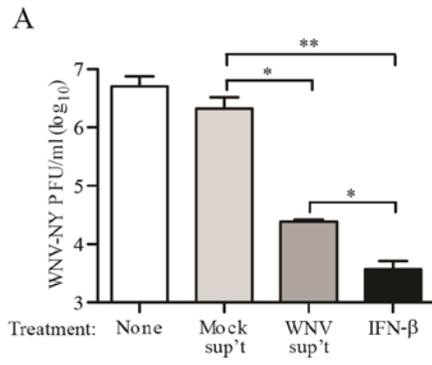


Figure 19. Inhibitory capacity of UV-inactivated supernatants recovered from WNV-AUS60-infected HFFs

(A) HFFs were pretreated with UV-inactivated supernatants recovered from mock- or WNV-AUS60-infected cells or 25 IU/ml IFN- β for 6 h prior to and throughout the 24 h infection with WNV-NY (MOI=0.05). Viral titers were determined by plaque assay on Vero cells. Values represent the average number of plaque forming units (PFU) per mL (+/- standard error) from three independent experiments. **(B)** Schematic of pre- and post-treatment of cells with UV-inactivated supernatants in the presence or absence of neutralizing antibodies to IFN- α or IFN- β . **(C and D)** Effects of neutralizing antibodies or specified isotype controls to **(C)** IFN- α or **(D)** IFN- β on the inhibitory capacity of UV-inactivated supernatants recovered from WNV-AUS60-infected HFFs. Values represent the average number of plaque forming units (PFU) per mL (+/- standard error) from three independent experiments. Statistical significance was determined by an unpaired t-test. Asterisks indicate differences that are statistically significant (* $p < 0.05$ and ** $p < 0.01$).



the UV-inactivated supernatants were incubated with neutralizing antibodies or isotype controls to IFN- α or IFN- β prior to being used to treat HFFs (Figure 19B). While neutralization of IFN- α had no effect (Figure 19C), addition of IFN- β neutralizing antibodies abrogated the inhibitory effect of the WNV-AUS60 supernatants and restored WNV-NY titers to levels equivalent to those observed in control cells treated with mock supernatants (Figure 19D). Thus, the inhibitory effect of the culture supernatant was due to low levels of secreted IFN- β .

2.3 IFN suppresses WNV infectious particle production at late times post-infection

To directly assess the effect of IFN on WNV replication, we examined infectious virus particle production in the presence and absence of neutralizing antibodies to IFN- α or IFN- β . As expected, neutralization of IFN- α had no effect on infectious virus production, indicating that IFN- α does not play a role in controlling WNV replication in fibroblasts (Figure 20A). Likewise, infectious particle production was unchanged for both WNV-NY and WNV-AUS60 at 24 h post-infection in the presence of neutralizing antibodies to IFN- β and their isotype controls. However, neutralization of IFN- β significantly increased WNV-AUS60 and WNV-NY titers at 48 h post-infection, though WNV-AUS60 titers remained approximately one log lower than WNV-NY (Figure 20B). Thus, IFN- β appears to play a role in controlling WNV infectious particle production at late, but not early, times post-infection. Moreover, this demonstrates that the disparity

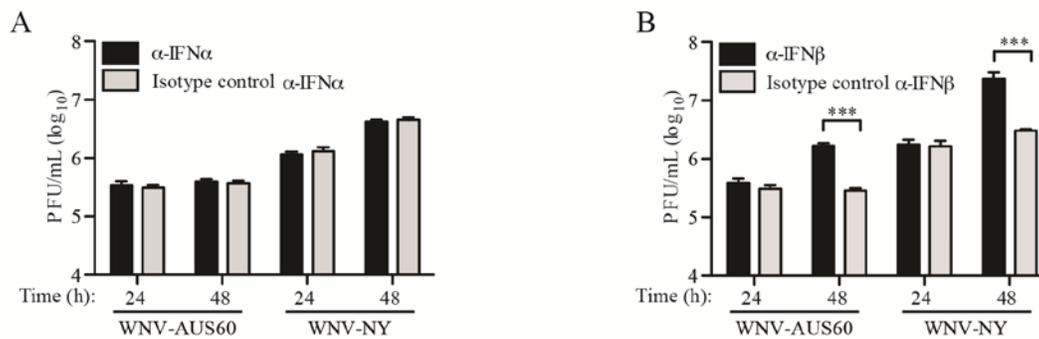


Figure 20. WNV replication in the presence and absence of neutralizing antibodies to IFN- α or IFN- β

HFF cells were infected with WNV-NY or WNV-AUS60 (MOI=0.05) and the inoculum was removed after 1 h and replaced with complete DMEM containing specified isotype control antisera or neutralizing antibodies to (A) IFN- α or (B) IFN- β . Culture supernatants were recovered at the indicated times post-infection and viral titers were determined by plaque assay on Vero cells. Values represent the average number of PFU per mL (+/- standard error) from three independent experiments. An unpaired t-test was performed to determine significance. Asterisks indicate differences that are statistically significant (***) p<0.001).

between WNV-AUS60 and WNV-NY viral set points is regulated by an IFN-independent mechanism.

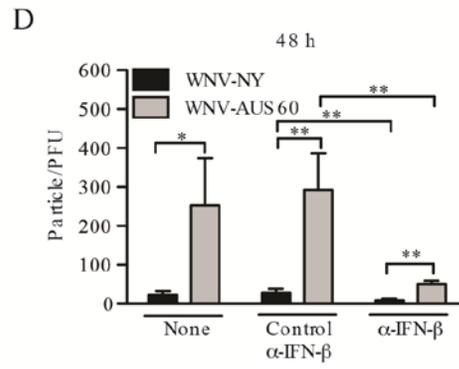
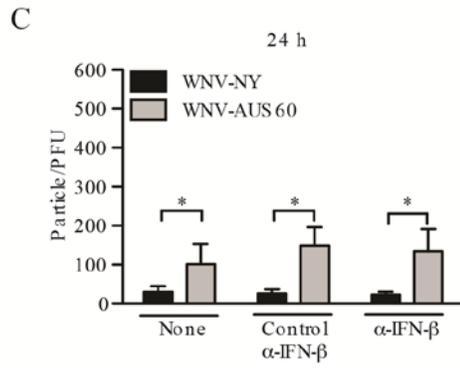
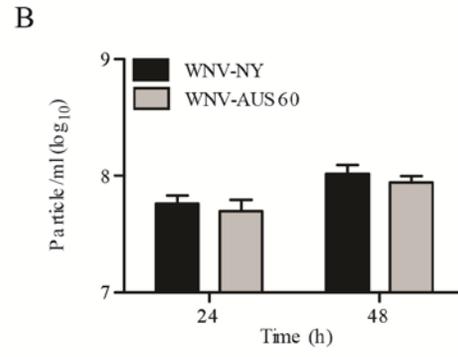
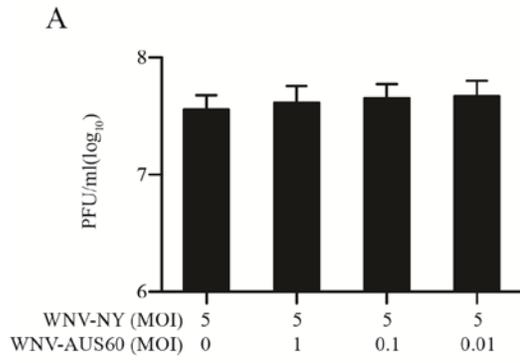
2.4 WNV strain variation in particle infectivity during HFF infection

Key steps in the viral life cycle leading up to infectious particle production include translation and replication of the viral genome, assembly of the virus particle at the ER membrane, transport of virus particles through the secretory pathway, and finally virus release. To assess whether the lower viral set point for WNV-AUS60 was due to impairment at or before the stage of viral assembly, we first assessed the WNV-AUS60 stock for the presence of defective interfering (DI) particles. DI particles have been previously shown to inhibit infection with WNV [178, 179]. Using a yield reduction assay (YRA) as previously described by Barrett et al., we were able to measure levels of DI virus [180]. Briefly, the assay measures the ability of a virus to cause a reduction in the yield of infectious (or standard) virus when cells are co-infected with both standard and DI viruses. With WNV-NY as the standard virus and WNV-AUS60 as the presumed DI virus, WNV-NY titers were not reduced in the presence and absence of various concentrations of the WNV-AUS60 viral stock (Figure 21A). This suggests that the stock of WNV-AUS60 does not contain inhibitory DI particles, so the reduced infectivity of WNV-AUS60 was not due to the generation of defective interfering (DI) particles.

While DI particles were not detected in the WNV-AUS60 stock, we hypothesized that WNV-AUS60-infected HFFs may have a defect in particle

Figure 21. Infectivity of WNV particles produced in HFFs

(A) YRA to detect DI particles in WNV-AUS60 viral stock. A549 cells were infected with standard virus, WNV-NY, at an MOI of 5 and WNV-AUS60 at increasing MOIs. Culture supernatants were recovered at 48 h post-infection and titered by plaque assay on Vero cells. Values represent the average number of plaque forming units (PFU) per ml (+/- standard error) from two independent experiments. (B,C,D) HFF cells were infected at an MOI of 0.05 with WNV-NY or WNV-AUS60. (B) Total viral particle production at 24 and 48 h post-infection was determined using a flow cytometry-based virus counter. Values represent the average number of particles per mL (+/- standard error) from three independent experiments. (C, D) WNV infectivity in the presence and absence of specified isotype control or neutralizing antibodies to IFN- β at (C) 24 and (D) 48 h post-infection. The concentration of total virus particles and infectious particles was determined using a flow cytometry-based virus counter and plaque assays on Vero cells, respectively. (B,C,D) Values represent the average Particle to PFU ratio of three independent experiments. Statistical significance was determined by an unpaired t-test. Asterisks indicate differences that are statistically significant (*p<0.05 and ** p<0.01).



production. To compare WNV-NY and WNV-AUS60 total virus particle production, we used a virus counter. The virus counter utilizes two dyes in a flow cytometry-based system that simultaneously detects nucleic acid and protein, thereby excluding empty particles and cellular debris from the analysis. Similar levels of total viral particles were detected for WNV-NY and WNV-AUS60 at 24 and 48 h post-infection (Figure 21B), suggesting that WNV-AUS60 and WNV-NY replicate and assemble at similar rates and to equivalent levels within HFFs. Using the physical counts of total virus particles obtained from the virus counter and the biological counts determined by plaque assay, we determined the particle to PFU ratio for WNV-NY and WNV-AUS60 at 24 and 48 h post-infection (Figure 21C, D). Based on these calculations, the infectivity of WNV-AUS60 particles was significantly reduced at both time points compared to WNV-NY. These data suggest that the lower viral set point for WNV-AUS60 was due, in part, to WNV-AUS60 particles being less infectious than WNV-NY particles.

In studies utilizing several different viruses, infected cells treated with IFN produced viral particles with lower infectivity compared to untreated control cells [181-186]. To assess whether IFN plays a similar role in reducing WNV infectivity, we examined the effect of IFN- β neutralizing antibodies on the particle to PFU ratios of WNV-NY and WNV-AUS60. Although neutralization of IFN- β had no effect on total particle production (data not shown), the particle to PFU ratios for both WNV-NY and WNV-AUS60 were substantially reduced at 48 h post-infection (Figure 21D). Thus, this demonstrates that IFN- β plays a role

in modulating infectivity of WNV particles late in the course of infection. In contrast, neutralization of IFN- β had no effect on WNV particle to PFU ratios at 24 h post-infection (Figure 21C), which is consistent with our previous data demonstrating that IFN does not regulate viral titers at this time point. Therefore, the higher level of defective particle production observed for WNV-AUS60 at 24 h post-infection was independent of IFN- β and therefore, due to a strain-specific defect in infectious particle production.

3. Conclusions

Here we demonstrate that both nonpathogenic and pathogenic strains of WNV are capable of establishing an infection within dermal fibroblasts. However, a strain-specific defect in infectious particle production limits the ability of WNV-AUS60 to disseminate from the initially infected cells, resulting in a lower viral set point compared to WNV-NY. The defect in WNV-AUS60 infectious particle production was not specific to human dermal fibroblasts. WNV-AUS60 exhibited both a smaller plaque phenotype and reduced titers compared to WNV-NY on Vero cells (data not shown). It is unclear why WNV-AUS60 particles are less infectious than those of WNV-NY.

One possible explanation for limited spread is decreased WNV-AUS60 replication. If its RNA dependent RNA polymerase (RdRp) has an increased error rate, this could push this strain over the error threshold, thus, resulting in an increased production of defective particles. A recent study demonstrated that mutant alphaviruses with higher error rates are reduced in their infectivity

compared to wild-type [187]. Alternatively, the lower infectivity of WNV-AUS60 particles may be due to a reduced capacity to undergo maturation. *Flavivirus* particles secreted from the infected cell are a combination of mature, partially mature and immature virions. Recent reports have demonstrated that partially mature viral particles are still capable of infecting cells, however, immature *Flavivirus* particles are noninfectious due to an inability to efficiently bind to the target cell [24, 188, 189]. Therefore, an increase in the percentage of immature particles released from the infected cell would result in decreased infectivity of WNV-AUS60 particles.

We also demonstrated that WNV replication is restricted late in infection through an IFN- β -mediated reduction in viral infectivity. Conceivably, this reduction in viral infectivity may limit the ability of the dermal layer to serve as a productive reservoir for WNV infection. Moreover, the higher level of defective particles that are produced late in infection may help to stimulate the adaptive immune response to WNV. Since the infectivity of WNV-AUS60 is inherently lower than WNV-NY, the IFN-dependent decrease in infectious particle production may have a greater impact on the ability of this strain to disseminate from the site of infection and ultimately cause disease.

Chapter 4: Discussion

The public health threat of WNV stems from its recent introduction into the Americas, which has resulted in an increase of infections associated with neurological disease [19]. Arthropod-borne transmission of the virus occurs when an infected mosquito is in search of a blood meal and deposits saliva containing high levels of virus into the dermal layer of skin. Upon initial infection, viral replication occurs in cells within the dermal and epidermal layers of skin. Utilizing both a pathogenic strain (WNV-NY) and a nonpathogenic strain (WNV-AUS60), I was able to examine the replication and spread of WNV as well the IFN response to WNV infection in human dermal fibroblasts (HFFs).

1. WNV replication and spread in HFFs

While both strains are capable of replicating within dermal fibroblasts, WNV-AUS60 replicates to lower levels and spreads less from cell to cell in HFFs than WNV-NY. At the very low MOI of 0.005, WNV-AUS60 reached peak infectious particle production by 24 h post-infection, while the viral set point of WNV-NY was obtained at 40 h post-infection (Figure 7A). WNV-NY viral spread was evident at 24 h post-infection by IFA (Figure 7B) and the continued increase in viral titers suggests that the virus underwent a second round of replication and spread between 24 and 40 h post-infection. When ten times more virus was used to infect cells at the higher MOI (0.05), both strains reached peak

infectious particle production between 20 and 24 h post-infection (Figure 16A). Increasing the concentration of virus within the inoculum eliminated the second round of replication and spread after 24 h post-infection (Figure 16A,D).

Examining viral set points of each strain at the two MOIs, WNV-NY reaches 1×10^6 PFU/ml at the MOI of 0.005 and 1×10^7 PFU/ml at MOI of 0.05. WNV-AUS60 peaks at 1×10^5 PFU/ml at the MOI of 0.005 and 5×10^5 PFU/ml at the MOI of 0.05. With both strains, peak viral levels were higher when cultures were inoculated at the slightly higher MOI. This suggests that reaching a viral set point is dependent on the number of initially infected cells. Since WNV-NY is known to initially evade the host antiviral response [138-140], there is only a window of approximately 12 to 16 h for the virus to replicate without cellular detection. By inoculating cells with more virus, higher titers can be observed since an antiviral state will be triggered at the same time post-infection regardless of the initial MOI.

At the MOI of 0.005 or 0.05, WNV-AUS60 and WNV-NY infections both induced the production of innate antiviral effector proteins with similar kinetics and amplitude. Between 16 and 24 h post-infection, ISG15, ISG56, IRF-9, and phospho-STAT2 were detected in WNV-infected HFFs (Figure 14,16). Production of ISG15 and ISG56 can be a result of either IRF-3 activation or downstream IFN signaling [137]. IRF-9 (ISGF3 gamma) can be induced following viral infection [126] and also complexes with the STAT1-STAT2 heterodimer to initiate transcription of various IFN-stimulated genes [163]. When type-I IFN binds to the IFN- α/β receptor, it leads to the phosphorylation of Tyk2

and JAK, which are associated with the receptors. Downstream signaling leads to the dimerization and phosphorylation of STAT1 and STAT2 [118]. Since the IFN-dependent production of phospho-STAT2 [163-167] is detected in WNV-AUS60- and WNV-NY-infected cells at similar times post-infection, this suggests that WNV-AUS60 and WNV-NY-infected HFFs secrete IFN with similar kinetics.

2. Detection of IFN in WNV-infected HFFs

Using a standard type-I IFN bioassay on A549 cells, IFN was not detected in supernatants from 24 h mock, WNV-AUS60, or WNV-NY infections (MOI=0.005) of HFFs (Figure 13B). At the MOI of 0.05, IFN was not detected in 24 h mock, WNV-AUS60, or WNV-NY supernatants or 48 h mock or WNV-AUS60 infections (data not shown). IFN was only detected in HFFs infected with WNV-NY for 48 h. Because immunoblot analysis indicated that IFN was signaling activation of the JAK/STAT pathway by 24 h post-infection in WNV-AUS60- and WNV-NY-infected (MOI of 0.005 or 0.05) cultures, we assessed the sensitivity of a HFF bioassay. HFFs were sensitive to levels as low as 0.625 IU/ml of IFN- β , while inhibition of infection in A549 cells required a minimum of 2 IU/ml IFN. The high sensitivity of HFFs to IFN allowed for the detection of IFN in 24 and 48 h supernatants from WNV-AUS60- and WNV-NY-infected cells (MOI=0.05). Additionally, the increased sensitivity of HFFs to IFN explains why WNV-infection supernatants (MOI=0.005) inhibited subsequent HFF, but not A549 cell, infections (Figure 9,11).

While the secretion of IFN- β can be detected following WNV infection of cultured human dermal fibroblasts after 24 h, other types of cells may affect the local IFN response within the dermis of a mammal. In addition to fibroblasts, the dermal layer of skin also contains mast cells, adipocytes, and immune cells [190]. These other cells in the dermis have their own distinct sets of PRRs that can recognize a variety of PAMPS to trigger host defense mechanisms. Future work could assess local IFN levels within the dermis of a mouse following intradermal WNV inoculation.

Since *in vivo* IFN levels would likely be low in dermal cells, it would be necessary to assess the sensitivity of dermal cells to treatment with IFN. Treatment of cultured human dermal fibroblasts with IFN inhibits WNV infection, but this effect may be altered in the dermis of a mammal. IFN could be injected at the site of inoculation before and after intradermal inoculation of WNV. If IFN treatment at the *in vivo* site of inoculation inhibits WNV infection, further studies focusing on local IFN treatment could be pursued.

There is currently no approved method of treatment or prevention of *Flaviviruses*, including WNV, infection in humans. Standard treatment for persistent *Flaviviral* infection, such as chronic hepatitis C virus, utilizes pegylated IFN- α and ribavirin, but this is often associated with adverse side effects and a slow virologic response [191, 192]. IFN- α stimulates the JAK/STAT pathway to induce ISGs that can have direct antiviral effects or induce an immune response [193, 194]. Ribavirin is a nucleotide analog that incorporates ribavirin triphosphate into viral genomes during viral RNA synthesis [195, 196]. It can

increase the error frequency of genomic RNA in viruses such as HCV and poliovirus because it can bind to both uracil and cytosine [195-197]. While ribavirin decreased levels of some viral infections, WNV-infected patients treated with ribavirin during an outbreak in Israel exhibited increased disease [198]. A later study of WNV-infected hamsters demonstrated that treatment with ribavirin increased their death rate [199]. Utilizing a similar antiviral mechanism as ribavirin, an adenosine nucleoside inhibitor of DENV was developed more recently that reduces infection levels, but has significant adverse side effects [200, 201]. By studying WNV replication and the effects of type-I IFN on infection, we can more thoroughly understand the effects of IFN treatment and improve antiviral therapies.

3. IFN-dependent and -independent modulation of WNV particle infectivity

At the higher MOI of 0.05, IFN- β can be detected at 24 h post-infection, but does not limit WNV infection of HFFs until later times post infection. If the reduction in WNV levels at early times post infection is not due to a secreted factor, it may be tied to the replication of each virus. While qRT-PCR analysis showed that WNV-AUS60 and WNV-NY had similar rates of replication, it only measured the transcription of a small amplicon near the 5' end of the genome. The transcription of this portion of the envelope gene may be replicated at similar rates, but the WNV-AUS60 RdRp may have low processivity. Reports of other *Flaviviruses* show various levels of processivity [52, 202, 203]. Purified NS5, isolated from either DENV or WNV [202, 203], was shown to have an RdRp with

low processivity, while a KUNV NS5 RdRp lacked premature termination [52]. To assess processivity, amplification of a segment in the 3' NTR could be compared to an envelope amplicon of each strain.

Similar replication rates of WNV-AUS60 and WNV-NY as measured by qRT-PCR analysis do not detect mutations within the genome. The lower infectivity of the WNV-AUS60 particles may be due to the decreased fidelity and therefore higher mutation rate of the WNV-AUS60 RdRp. Our data suggest that both WNV-NY and WNV-AUS60 produce similar numbers of viral particles, while the WNV-AUS60 particles have significantly lower infectivity than the WNV-NY particles.

The fidelity of WNV-AUS60 and WNV-NY RdRps could be measured as outlined by Pugachev et al. [204]. They measured *Flavivirus* RdRp fidelity with the use of infectious clones. Most RNA viruses exist as a quasispecies since they replicate with high error rates and display genetic diversity [205]. Transfecting cells with an infectious clone can control the natural genetic diversity. After the transfected virus establishes an infection within cultured cells, it can be serially passaged and plaque purified. Finally, virus isolated from various passages and plaque purifications can be sequenced to compare mutations in the genome from one passage to the next. While Pugachev et al. determined that the yellow fever virus RdRp has high fidelity [204], the RdRps of other RNA viruses, such as poliovirus have demonstrated low fidelity [206, 207]. If the fidelity of the WNV-AUS60 RdRp is lower than the WNV-NY RdRp, this could explain why so many WNV-AUS60 particles are noninfectious. Alternatively, if the fidelity of WNV-

AUS60 RdRp is higher than that of WNV-NY, it could prove to be a potential vaccine candidate. A study using poliovirus by Vignuzzi et al. demonstrated that viruses with higher fidelity RNA polymerases are restrained in their ability to mutate toward a higher-fitness genotype [208]. By enhancing replication fidelity, the strains are less likely to mutate and revert to wild type. They are also restricted in their ability to spread to various tissues throughout the body.

Since the virulence determinant separating WNV-NY from WNV-AUS60 has not yet been identified, it is relevant to continue to compare these two strains. Because they are both within lineage one of the five phylogenetic lineages and display significantly different levels of pathogenicity in mice [59], their differences could be useful in the development of a WNV vaccine. As many different steps within their viral life cycle could control their pathogenicity, it is important to consider the viral particle's ability to bind to cells in addition to their ability to replicate.

The particles produced by WNV-AUS60 infections could also be less effective at binding and entering cells. If particles do not efficiently bind and enter cells, they cannot be infectious. In order to attach to a cell, the E proteins on the WNV envelope must bind to a receptor on the target cell. If the particle is immature or partially mature, the pr portion of prM covers the binding portion of E and it cannot bind cellular receptors.

The production of an increased number of immature particles is another potential mechanism for controlling particle infectivity. WNV-AUS60 infections of HFFs produce the same number of viral particles as WNV-NY infections, but

WNV-AUS60 infections produce fewer infectious viral particles. A possible explanation for the lower infectivity is that WNV-AUS60-infected cells may produce a greater number of immature particles. At both 24 and 48 h post-infection, levels of immature particle production could be assessed for both WNV-AUS60 and WNV-NY infections.

In order for the particle to mature, the pr portion of prM is cleaved by a cellular furin-like protease in a low pH environment, within the TGN [83]. This cleavage exposes the fusion peptide on E and the E trimers rearrange into antiparallel dimers, which makes them fusion competent [84-86]. After 10 to 12 hours in a mammalian cell, virions are finally exocytosed and mature particles or partially mature particles are capable of infecting other cells [22, 87].

To assess the effects of particle maturation, all viral particles could be induced *in vitro* to possess characteristics of mature virions as described by Stadler et al. [83]. Briefly, by exposing immature virions to recombinant bovine/human furin and low pH, prM can be cleaved to induce the characteristic structural rearrangement of mature virions. Exposing WNV to these conditions will drive the particles toward maturation. By inducing the mature conformation on both WNV-AUS60 and WNV-NY particles, their infectivity should be equal, if the decrease in WNV-AUS60 particle infectivity was due to higher levels of immature particle production.

4. Final thoughts

The work in this dissertation demonstrates that both IFN-dependent and IFN-independent mechanisms limit WNV infection in dermal fibroblasts, which are main components of an initial site of infection, the dermis. This work determined that IFN- β is an integral part of the mechanism that controls WNV infection in dermal fibroblasts at late times post-infection. This restriction at late times post-infection was a result of reduced particle infectivity. The inhibitory effect of IFN on WNV infection of dermal fibroblasts could play a key role in the development of future antiviral therapies. The disparity between WNV-AUS60 and WNV-NY particle infectivity remains unclear. Once the mechanisms that inhibit WNV-AUS60 particle infectivity are determined, they could be used toward the development of a WNV vaccine.

Chapter 5: Materials and Methods

1. Cells and viruses

Vero, A549, and human foreskin fibroblast (HFF) (kindly provided by Alison McBride, NIH) cell lines were propagated at 37°C in 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker), antibiotic/antimycotic solution, and nonessential amino acids (complete DMEM). C6/36 cells were propagated at 28°C in 5% CO₂ in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum and antibiotic-antimycotic solution (complete MEM). A WNV-NY strain 3356 stock was generated by passaging the infectious clone pFL-WNV [157] once on HEK293 cells and twice on C6/36 cells. The WNV-AUS60 stock was generated by plaque purifying clinical isolate MRM16, obtained from the World Reference Center of Emerging Viruses and Arboviruses (Galveston, TX), passaging it once on HEK293 cells and twice on C6/36 cells. Titers for each stock were determined for each of the listed cell lines. All WNV infections and handling of WNV-infected samples were carried out in Biosafety Level 3 (BSL3) biocontainment. Vesicular stomatitis virus encoding green fluorescent protein (VSV-GFP) (a gift from Michael A. Whitt) was amplified in BHK-J cells.

2. Focus-forming assays

HFFs were grown in 12-well tissue culture plates and infected with serially-diluted WNV-NY or WNV-AUS60. After 1 h, inoculum was removed and replaced with complete DMEM in 10% methylcellulose. At 24 h post-infection, monolayers were washed three times with phosphate buffered saline (PBS) (Hyclone) and fixed with 3% paraformaldehyde for 30 min at room temperature. Cell monolayers were permeabilized with a solution of PBS/0.2% Triton X-100, blocked with PBS containing 1% normal goat serum, and incubated with WNV hyperimmune ascitic fluid (1:1000, World Reference Center of Emerging Viruses and Arboviruses) followed by Dylight 549nm-conjugated goat anti-mouse IgG (1:800, Jackson ImmunoLaboratories). Foci were visualized with an Olympus IX51 microscope.

3. Virus growth curves

Cell cultures were infected with WNV-NY or WNV-AUS60 (MOI=0.005 or 0.05). The amount of virus added to cultures to achieve the indicated MOI was calculated using the titer of the viral stock as determined on HFFs. Cultures were incubated for 1 hour at 37°C with rocking, the inoculum was removed and complete DMEM was added. Culture supernatants were recovered at the indicated times, clarified by low speed centrifugation for 5 minutes, transferred to new tubes, and stored at -80°C. Viral titers were determined by plaque assay on Vero cells.

4. Plaque assays

Monolayers of Vero cells in six-well plates were inoculated with serial dilutions of viral samples. The cells were incubated in a 5% CO₂ incubator at 37°C with rocking for 30 min (VSV) or 1 h (WNV). The inoculum was removed and a 0.9% agarose-complete DMEM overlay added. VSV plaques were counted 24 hours post-infection. For WNV titration, cell monolayers were incubated for 48 h and a second overlay of agarose-containing complete DMEM supplemented with 0.003% neutral red (ICN Biomedical) was added. The plates were incubated for an additional 48 (WNV-NY) to 72 hours (WNV-AUS60) prior to counting plaques. All titers were performed in duplicate.

5. Quantitative reverse transcriptase-PCR

Total RNA was extracted from HFFs infected with WNV using TRIzol reagent (Invitrogen Life Technologies, Inc) and treated with Turbo DNA-free (Invitrogen). RNA levels were determined by quantitative real-time PCR (qRT-PCR) analysis on a Roche LC480 using Veriquest One-Step SYBR green MasterMix (Affymetrix Biosystems) with 25 ng of RNA. The following primers were used: WNV-NY(s): 5' GGACCTTGTAAGTTCCTATCTCG 3'; WNV-NY(as): 5' AGGGTTGACAGTGACCAATC 3'; WNV-AUS60(s): 5' GGACCCTGCAAGATACCCATTTC 3'; WNV-AUS60(as): 5' GGGGTTGACGGTAACTAACC 3'; WNV lineage 1(s): 5' TGGAACCACCCTTTGGAG 3'; WNV lineage 1(as): 5' GTCCCAAGCTGTGTCTCC 3';

human GAPDH(s): 5' CCACTCCTCCACCTTTGAC 3';
human GAPDH(as): 5' ACCCTGTTGCTGTAGCCA 3';
human IFN- β (s): 5' ATGACCAACAAGTGTCTCCTCC 3';
human IFN- β (as): 5' GCTCATGGAAAGAGCTGTAGTG 3';
human IFN- λ (s): 5' GCCAAAGATGCCTTAGAAGAG 3';
human IFN- λ (as): 5' CAGAACCTTCAGCGTCAGG 3';
human CXCL12(s): 5' TGAGCTACAGATGCCCATGC 3';
human CXCL12(as): 5' TTCTCCAGGTACTCCTGAATCC 3'.

6. Immunofluorescence assay (IFA)

HFFs were grown on coverslips and infected with WNV-AUS60 or WNV-NY at an MOI of 0.005 or 0.05. After 1 h, inoculum was removed and replaced with complete DMEM. At 24 h post-infection, monolayers were washed with PBS (Hyclone) and fixed with 3% paraformaldehyde for 30 min at room temperature. Cell monolayers were permeabilized with a solution of PBS/0.2% Triton X-100, blocked with PBS containing 1% normal goat serum, and incubated with WNV hyperimmune ascitic fluid (1:1000, World Reference Center of Emerging Viruses and Arboviruses) followed by Dylight 549nm-conjugated goat anti-mouse IgG (1:800, Jackson ImmunoLaboratories) and Hoescht stain (0.1 μ g/mL). Foci were visualized with an Olympus IX51 microscope equipped with a digital camera.

7. Flow cytometry

Cultures of HFFs grown on 6-well plates were infected with WNV at an MOI of 0.05. At the indicated times post-infection, cells were removed from plates by trypsinization, washed twice with PBS and fixed in 3% paraformaldehyde. Cells were permeabilized with PBS/0.2% Triton X-100, blocked in PBS containing 0.5% heat-inactivated FBS and probed with WNV hyperimmune ascitic fluid (1:1000, World Reference Center of Emerging Viruses and Arboviruses) followed by DyLight 549 nm conjugated goat anti-mouse IgG (1:2000). For flow cytometry analysis, 100,000 single cell events were collected using a FACS Canto (BD Biosciences).

8. Immunoblot analysis

Cells were washed twice with PBS and lysed in RIPA buffer (10 mM Tris, 150 mM NaCl, 0.02% Na-deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitors (Sigma). Proteins (20 µg) were resolved on 10-12% polyacrylamide gels containing SDS and transferred to NitroPure nitrocellulose membranes (Micron Separations Inc.). Blots were blocked overnight at 4°C and probed with the following monoclonal or polyclonal antibodies: polyclonal rabbit anti-GAPDH (1:4000; Abcam), polyclonal mouse anti-WNV hyperimmune ascetic fluid (1:1000; World Reference Center of Emerging Viruses and Arboviruses), polyclonal rabbit anti-ISG56 (1:2000; kindly provided by Dr. Ganes Sen), polyclonal rabbit anti-ISG15 (1:2500; kindly provided by Dr. Arthur Haas), polyclonal rabbit anti-phospho-STAT-2 (1:1000;

Millipore), polyclonal rabbit anti-STAT-2 (1:1000; Santa Cruz), monoclonal mouse anti-phospho-STAT-1 (1:100; Santa Cruz), polyclonal mouse-anti-STAT-1 (1:1000; Cell signaling), and polyclonal rabbit anti-IRF-9 (1:200; Santa Cruz). Following a secondary incubation with peroxidase-conjugated goat anti-rabbit or goat anti-mouse (Millipore) and treatment with ECL+ Western Blotting detection reagents (Amersham Biosciences), the protein bands were visualized by exposure of the membrane to film.

9. UV-inactivation

UV-inactivation was carried out by exposing a 1 ml aliquot of supernatant recovered from mock- or WNV-infected HFFs to UV (254 nm) for 2 min at room temperature in a Statalinker Model XL-1000 (Spectroincs Corp.). Titers of UV-treated supernatants were below detectible levels by plaque assay on Vero cells, confirming complete inactivation of the WNV-infected supernatants.

10. Interferon bioassay

A549 or HFF cells in 24-well plates were treated with two-fold serial dilutions of human IFN- β (BEI Resources) or cell-free, UV-inactivated supernatants recovered from mock or WNV-infected HFFs. Cultures were incubated for 24 h at 37°C, infected with VSV (MOI=1) and supernatants were collected at 24 h post-infection. VSV titers were determined by plaque assay on Vero cells as described above. IFN concentrations were determined based on a

standard curve generated from the titers of VSV recovered from samples treated with serial dilutions of IFN- β .

11. Neutralization of type-I IFN

The antibody concentration necessary to neutralize the IFN present in supernatants recovered from WNV-infected HFFs was determined by pretreating A549 cells for 24 hours with 25 IU/ml of IFN- α or IFN- β in the presence of 2-fold serial dilutions of the antibodies to IFN- α (NR-3089; BEI resources) or IFN- β (NR-3091; BEI resources). Control wells consisted of cells treated with IFN only, no IFN or isotype matched antisera to IFN- α or IFN- β . Pretreated cells were infected with VSV (MOI=1) and supernatants were collected at 24 h post-infection. Viral titers were determined by plaque assay on Vero cells. Culture supernatants were neutralized with twice the amount of antibody necessary to neutralize 25 IU/ml IFN- α or IFN- β or the appropriate control antisera (NR-3089 or NR-3090; BEI resources) for 1 h at 37°C. For neutralization during WNV-infection, HFFs were inoculated with WNV (MOI=0.05) for 1 hour at 37°C and the inoculum was replaced with complete DMEM containing neutralizing antibodies to IFN- α /IFN- β or the appropriate control antisera. In wells that contained supernatants to be collected at 48 h post-infection, supplemental antisera were added at 24 h post-infection.

12. Detection and enumeration of total virus particles

Culture supernatants were cleared by low speed centrifugation for 5 minutes and analyzed using the Virus Counter 2100 (ViroCyt LLC, Denver, CO) as per the manufacturer's instructions. Briefly, samples were diluted 1:10 or 1:30 to a total volume of 100 μ l and incubated in the dark for 30 minutes with 50 μ l of Combo dye, which stains nucleic acid and protein. Two-channel fluorescence was used to detect co-localization of nucleic acid and protein. Events with simultaneous detection within both channels were defined as virus particles by Virocyt software.

13. Enzyme-linked immunosorbent assay (ELISA)

TNF- α , CCL2, and CCL5 concentrations in culture supernatants from mock- and WNV-infected HFFs were determined by TNF- α (BD Biosciences), CCL2 (R&D systems), and CCL5 (R&D systems) ELISA kits as per manufacturer's instructions. Culture supernatants were UV-inactivated for 2 min then incubated on antibody-coated 96-well plates alongside serial dilutions of standards using the human TNF- α , CCL2, and CCL5 ELISA kits as per manufacturer's instructions (BD Biosciences and R&D systems).

14. Yield reduction assay (YRA)

A549 cells in 12-well plates were infected with standard virus, WNV-NY, at an MOI of 5 and WNV-AUS60 at MOIs of 0, 0.01, 0.1, and 1 PFU/cell. The

amount of virus added to cultures to achieve the indicated MOI was calculated using the titer of the viral stock as determined on A549s. Cultures were incubated for 1 hour at 37°C with rocking, the inoculum was removed and complete DMEM was added. Culture supernatants were recovered at 48 h post-infection, clarified by low speed centrifugation for 5 minutes, transferred to new tubes, and stored at -80°C. Viral titers were determined by plaque assay on Vero cells. Plaques were counted at 72 h post-infection, which is when WNV-NY plaques are visible and WNV-AUS60 plaques are not.

15. Statistical analysis

Graphpad Prism 5 was used for all statistical analyses. Comparative significance was determined with unpaired Student's t-tests.

16. Phylogenetic analysis

The nucleic acid sequences for the E region of the genome of each WNV strain were obtained from GenBank with the following accession numbers: JEV: U0422; Egypt 1951: AF260968; C.Afr.Rep 1967: AF001566; NY 2000-crow3356: AF404756; NY 1999-human: AF202541; Israel 1998-A: AY033389; Romania 1996 M: AF260969; Italy 1998: AF404757; Australia 1960: GQ851602; India 1980: DQ256376; Madagascar 1978: AF001559; C. Afr.Rep 1972a: AF001563; Uganda 1937: M12294; Austria 1997: AY765264; Russia 1998: AY277251. Alignments were performed with the ClustalW run in MacVector

version 10.0.2 (MacVector, Inc., Cambridge, United Kingdom). The nearest neighbor joining tree was developed within MacVector using the Tamura-Nei distance calculation method with gaps distributed proportionally.

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