ABSTRACT

MORRISON, CLAYTON RUSSELL. Identification of Amino Acid Residues Critical for TLR3 Inhibition by the Multifunctional West Nile Virus NS1 Protein. (Under the direction of Frank Scholle Ph.D.).

The flavivirus West Nile virus (WNV) is maintained in nature through an enzootic transmission cycle between mosquitoes and birds. However, epizootic transmission to humans and other animals can occur when an infected mosquito takes a blood meal. While the levels and duration of viremia in these animals are insufficient to maintain the transmission cycle, the virus is capable of causing febrile and encephalitic diseases in these dead end hosts, which can result in death. WNV was first isolated in 1937 in Uganda, Africa, and has since been found to be endemic to regions of Africa, Europe, and the Middle East. In 1999, WNV was first isolated in New York State in birds and humans and has since spread throughout the contiguous United States. WNV poses a major public health threat as no vaccine or antiviral strategies are currently available for humans.

Flaviviruses have evolved numerous mechanisms to both evade detection by the innate immune system and to actively suppress its activation, thereby promoting virus dissemination and transmission. The innate immune system is an evolutionarily conserved, first-line defense against invading microorganisms, and functions by detecting pathogen-associated molecular patterns (PAMPS) through pattern recognition receptors (PRR). Toll-like Receptor 3 (TLR3) is a PRR found in the endosomes of many cell types and recognizes dsRNA of many viruses. Activation of TLR3 triggers a highly coordinated signaling cascade that results in the production of pro-inflammatory cytokines and type I interferon. These cytokines synergistically induce an antiviral state in responsive cells and prime the adaptive immune response.

Recently, work by our laboratory has described a role for the WNV non-structural protein 1 (NS1) in inhibiting TLR3 signal transduction. TLR3 inhibition occurred in WNV infected cells, WNV replicon-bearing cells, cells ectopically expressing NS1, andnaïve cells pretreated with the purified secreted form of NS1. The current study was designed to identify amino acids of the WNV NS1 protein that are important for TLR3 inhibition. Since the NS1 protein is also absolutely required for virus genome replication, a screening strategy was developed that would allow for the identification of random mutations in NS1 which relieve TLR3 inhibition, while still allowing virus genome replication. Fluorescence activated cell sorting (FACS) was used to isolate cells expressing mutant NS1 proteins of the desired phenotype. These cells were subsequently clonally expanded, and the sequences of their NS1 genes were determined.

Ultimately, three amino acid residues (G295, P320, and M333) located in the C-terminal half of NS1 were identified as being important for TLR3 inhibition. Changes at those positions were further characterized, both ectopically and in recombinant WNV. Each of the amino acid changes allowed genome replication and TLR3 signaling in cells infected with recombinant WNV.

Biochemical studies were designed to identify the defects of the NS1 mutants, which allow TLR3 signaling. The amino acid changes resulted in reduced protein stability, improper glycosylation, and inefficient cell surface expression and secretion. The cumulative result of these defects in NS1 processing prevents antagonism of TLR3 signaling by the mutant proteins.

In summary, this work has confirmed that the WNV NS1 protein does inhibit TLR3 and that specific amino acid residues located within the C-terminal half of NS1 can be

mutated to restore signaling. The identification of these residues may allow for more detailed studies of the role of TLR3 in sensing WNV infection, the pathological contributions of NS1 antagonism of TLR3, as well as serving as a tool for understanding the mechanism by which NS1 inhibits TLR3.

Identification of Amino Acid Residues Critical for TLR3 Inhibition by the Multifunctional West Nile Virus NS1 Protein

by
Clayton Russell Morrison

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APPROVED BY:

_____	_____
Frank Scholle Ph.D. Committee Chair	Scott Laster Ph.D.
_____	_____
Michael Sikes Ph.D.	Jeffrey Yoder Ph.D.

DEDICATION

This work is dedicated to my parents for their endless support and encouragement of all my academic endeavors.

BIOGRAPHY

Clayton is originally a resident of Atlanta, GA and attended the University of Georgia where he completed his Bachelor's degree in Microbiology in 2005. Prior to joining the Department of Microbiology at North Carolina State University in 2008, he completed Master's thesis research in Biology at Georgia College & State University in 2007.

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CHAPTER 1

Literature Review

Flavivirus Phylogeny and West Nile virus Origins

West Nile virus (WNV) is a mosquito-transmitted flavivirus of the *Flaviviridae* family. The *Flaviviridae* comprises more than 70 positive-sense RNA viruses which have been identified in vertebrate hosts and insect vectors [1]. In addition to WNV, other important human pathogens of the *Flaviviridae*, such as dengue virus (DEV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV), reside within the *Flavivirus* genus. *Pestivirus* and *Hepacivirus* represent the other two genera of the *Flaviviridae* [1]. Phylogeny of the *Flavivirus* genus is often organized based upon vector transmission, antigenicity, and sequence homology of the viruses. Flaviviruses can be vectored by mosquito (e.g. DEV) or tick (e.g. Tick-borne encephalitis virus (TBEV)) arthropods. For Apoi virus, Rio Bravo virus, and several other flaviviruses, no known vector (NKV) has been described and these are considered non-vectored flaviviruses [2]. The mosquito-borne flaviviruses are further subdivided based upon the genus of mosquito which preferentially transmits the viruses, and primarily includes mosquitoes of the *Aedes* and *Culex* genera. Mosquito-borne flavivirus vectored by *Aedes* mosquitoes are considered 'Old World' viruses whereas those vectored by *Culex* mosquitoes are considered 'New World' viruses [3]. However, there are

inconsistencies with this classification system as many flaviviruses, including WNV, can be transmitted by mosquitoes of both genera.

Antigenically, 10 flavivirus serocomplexes exist. WNV is grouped within the Japanese Encephalitic Antigenic Complex as antibodies produced against members of this serogroup of viruses are cross-reactive [4]. Of note, the JEV serocomplex of viruses are the leading cause of arbovirus encephalitis in humans and other vertebrates worldwide, and includes important human pathogens such as JEV, Murray Valley encephalitis virus (MVE), and St. Louis encephalitis virus (SLV) [5]. The prototype flavivirus, YFV, can be recognized by all serogroups and thus, is not affiliated with any one single antigenic complex [1]. Following its initial introduction into North America in 1999, WNV was incorrectly identified as SLV based upon clinical findings and preliminary serological evidence [6, 7]. Later, sequencing data confirmed WNV as the causative agent of the diseases, but these findings highlight the ambiguity of using serology to identify sources of flavivirus disease.

Sequence homology among members of the *Flavivirus* genus has been found to be the most accurate method for identifying and describing flaviviruses, as noted above. Complete, whole genome sequences, as well as the nucleotide and amino acid sequences of the non-structural protein 3 (NS3) and NS5 proteins have been utilized in phylogenetic studies [8, 9]. In general, it appears that mosquito-borne flaviviruses and tick-borne flaviviruses diverged from a common ancestor [10, 11]. However, tick-borne viruses show far less sequence diversity compared with mosquito-vectored viruses. It has been proposed that this difference in sequence diversity is the culmination of geographical barriers to tick migration, the limited number of tick blood meals, and the longer vector life span of ticks

than mosquitoes, which have limited tick-borne flavivirus evolution [8, 11]. The placement of NKV flaviviruses is unresolved. Several groups posit that NKV viruses diverged from tick-borne viruses, while others maintain that these viruses have their own independent evolutionary history [9, 12].

WNV was first isolated in 1937 from a woman experiencing febrile illness in the West Nile sub-region of Uganda (formerly West Nile Province and West Nile District) and is believed to have originated in Africa [13]. In Africa, WNV was known to cause severe neurological disease in horses. Febrile, self-limiting disease in children was documented in close proximity to the original outbreak and most healthy adults underwent seroconversion [14]. WNV successfully spread into parts of Europe and Asia where it is now endemic. Of interest, WNV has six distinct molecular phylogenetic lineages that correlate along geographical lines, but may also have been shaped by host genetics and host immunity [15]. WNV has the broadest vector and host range of all the flaviviruses and lineages 1 and 2 of WNV contain the most medically important strains of the virus [16]. Interestingly, lineage 1 WNV are phylogenetically related, but are antigentically diverse. Not surprisingly, these viruses have been isolated in diverse regions of the world including Africa, Australia, Asia, Europe, and the Middle East [17]. Lineage 2 WNV have been isolated in Madagascar and Southern Africa from asymptomatic and mildly-ill patients, and are believed to be less virulent than lineage 1 isolates [16, 18-20].

During August-September of 1999, WNV was first detected in the Western Hemisphere in birds and humans in New York State. Initially, veterinarians at the Bronx Zoo identified dead captive birds which later tested positive for the virus. Several human

encephalitic cases of WNV were subsequently identified at local hospitals, and die off of wild bird populations was attributed to WNV [21-23]. Sequencing data identified the NY99 genotype of WNV as a lineage 1 virus and retrospective analysis indicates that the NY99 genotype is most similar to a neuroinvasive strain of the virus circulating in domestic geese in Israel around the same time [23, 24]. The two viruses share 99.7% nucleotide sequence homology [23]. The homogeneous NY99 genotype circulated the eastern coast of the United States until being replaced by the WN02 genotype in 2002 [25]. WN02 only contains 3 mutations when compared with the parental NY99 strain, but displays increased vector transmission and increased temperature tolerance, thought to have aided in its dissemination [25-27]. It is unclear as to how WNV reached North America, but several scientists postulate that the virus was vectored into New York State by either an infected bird or mosquito via global transportation routes. 62 cases of WNV were reported in the first year of surveillance in North America resulting in 7 deaths (http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount99_detailed.htm).

In the years following the initial outbreak in the Northeastern United States, surveillance data demonstrated that WNV successfully spread along the eastern coast of the United States (2000-2001). This spread was most likely facilitated by vector and host competency in the region and a lack of heard immunity in avian hosts. Prior to the introduction of WNV, SLV was the only recognized flavivirus present in the United States and was known to cause sporadic outbreaks of disease (http://www.cdc.gov/sle/resources/slecasesbystateyear.pdf). SLV is not known to cause massive deaths in local bird populations, and while antibodies to SLV can cross-reactive with

WNV, it was unknown whether avian species would be cross protected [1, 28]. DEV was known to be active in Mexico and Central America, and historically, YFV had caused massive outbreaks of disease in the United States during the 19th century, but was successfully controlled by vaccination and mosquito-spraying campaigns.

By 2004, WNV had reached all 48 states in the contiguous United States, and human disease was associated with infected mosquito vectors circulating in the environment (http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount04_detailed.htm). The transit of WNV infected birds across migration routes in North America coupled with vector competency of mosquitoes in the Western United States most likely extended the virus' distribution and range [29-31]. At the height of the outbreak in 2003, 9,862 human cases resulting in 264 deaths were reported to the CDC through ArboNet (http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount03_detailed.htm). As of 2003 WNV is a reportable clinical disease, but the majority of the surveillance data acquired by the CDC is through voluntary submission of environmental assessments of WNV prevalence. WNV is now endemic in the continental United States, regions of Canada, Mexico, and Central America [32, 33]. A decline in the number of WNV cases was noted between the years 2008-2011, but resurgence in WNV during the second half of 2012 has recently been reported. Preliminary assessments of mosquito populations suggest that consecutive mild winters have allowed increased mosquito populations to fuel the outbreak, as virulence of the virus does not appear to have changed. As of mid-November, 2012, 5,045 cases and 228 deaths have been documented in the United States (http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount12_detailed.htm).

West Nile virus Maintenance Cycle and Transmission

WNV is maintained in nature through an enzootic cycle between mosquitoes and birds. The virus is primarily vectored by ornithophilic *Culex* mosquitoes, but has been detected in over 60 mosquito species from 7 different genera as reported by the CDC (http://www.cdc.gov/ncidod/dvbid/westnile/mosquitospecies.htm). Members of the *Culex* genus are also important for vectoring WNV in Europe, Africa, Asia, and Australia. Additionally, the virus has been consistently isolated from *Aedes* and *Anopheles* mosquitoes in these regions of the world, but their contributions to disease maintenance in nature have not been confirmed. In the Northeastern, Southeastern, and Central United States *C. pipiens*, *C. pipiens quinquefaciatus*, and *C. nigripalpus* mosquitoes serve as the major vectors of transmission, respectively, while *C. tarsalis* mosquitoes mainly vector the virus in the Western United States [34-37]. The virus replicates in the midgut and salivary glands of the mosquitoes which then feed on avian hosts to transmit the virus. Chronically infected female mosquitoes hibernate during the winter months and the virus is able to survive the overwintering period. Additionally, mosquito vectors are known to vertically transmit the virus to their offspring [38].

Avian hosts amplify the virus, maintain sufficiently high viremia, and recapitulate the enzootic cycle in nature. A combination of dead bird surveillance, live bird susceptibility testing, mosquito feeding patterns, mosquito blood meal analyses, and wild-caught bird seroconversion surveys have identified over 300 bird species susceptible to WNV [39-41]. Numerous avian hosts that are important in the WNV enzootic disease cycle in North America include, but are not limited to, crows, passerines, ducks, gulls, doves, finches,

sparrows, and robins [40]. Predicting the most important amplification hosts of WNV is complicated by the geographical distribution of avian species, bird migration patterns, and seasonal shifts in mosquito feeding patterns. Nevertheless, wild bird populations are known to have been affected by WNV disease. Bird counts performed by the American Audubon Society over a period of 25 years clearly demonstrated major declines in crow and robin populations that correlated with the year in which WNV was first detected in the regions of study [42]. Though, the establishment of herd immunity in bird populations is thought to limit disease severity among avian species over time, data are severely lacking to confirm this hypothesis.

Epizootic WNV diseases of humans and equines pose major threats to public and veterinary health. Infected mosquitoes have been documented to transmit the virus to humans and other higher vertebrates during feeding and some mosquito surveillance data demonstrate season shifts in mosquito feeding patterns from birds to mammals [43, 44]. More than 23 mosquito species are suspected of serving as 'bridge vectors' for human disease transmission, and include *C. pipiens*, *C. restuans*, *Aedes vexans*, and *Ochlerotatus canadensis* [45]. Mosquito salvia has been demonstrated to modulate early host immune responses to WNV infection and alters early steps in the infection cycle [46, 47]. Humans and horses are considered incidental or 'dead-end' hosts as the levels and duration of viremia achieved are not sufficient to retransmit the virus to a naïve mosquito. However, these animals are susceptible to the virus and disease severity can range from asymptomatic infection to fulminate neurological disease and death.

Non-vectored human transmission of WNV has been documented. Transmission via blood transfusion, dialysis, and organ transplantation during the early years of the epidemic resulted in blood bank screening for WNV by reverse transcription polymerase chain reaction (RT-PCR) [48, 49]. While RT-PCR to detect WNV is highly sensitive, the virus still poses a major threat to the blood supply following the implementation of screening strategies. Currently, pools of donor blood are screened since individual donor screening in expensive and laborious. This practice is complicated by the low levels of viremia inherently present in positive donors, and by the pooling process which further dilutes out the virus [50]. WNV has also been found to transmit from human to human through breast feeding, and intrauterine-, percutenious- and aerosol-transmission routes [43, 51-53]. However, these incidences of WNV are rare and represent only a minority of all the documented cases of disease.

West Nile virus Diseases

West Nile fever

Statistics collected over the last 13 years of the WNV epidemic suggest that approximately 80% of all WNV infected individuals are asymptomatic. In fact, seroprevalence studies of humans in endemic areas indicate that 9.5-19.7% of the population have detectable antibody titers to the virus [54]. The remaining 20% of infected individuals may develop a flu-like febrile illness referred to as West Nile fever [55]. West Nile virus has an approximate incubation period of 2-14 days before the onset of symptoms and West Nile fever is usually a self-limiting illness. Symptoms include fever, headache, myalgia, ocular

abnormalities, GI disturbance, and maculopapular rash formation [56]. A key demographic feature of the WNV epidemic is that the elderly (potential for senescent immune systems) and individuals with compromised immune systems are at greater risk for disease [57]. This finding contrasts with another related neurotropic flavivirus, JEV, which preferentially causes disease in children and individuals with compromised immune systems [58].

West Nile Neuroinvasive Diseases (WNND)

West Nile fever is a systemic syndrome, however 1 out of every 140 (≤1%) WNV infections can lead to WNND [57, 59]. Several other related flaviviruses are neurotropic including JEV, TBEV, and in rare occurrences, DEV. Key features of all neurotropic viruses are the abilities to invade the central nervous system (CNS) (neuroinvasiveness) and to infect neurons (neurovirulence) [60]. This occurs following viremia, passage of the virus across the blood-brain barrier (BBB), and requires direct infection of CNS neurons. How WNV crosses the BBB is a highly debated subject and several theories have been proposed: direct infection of endothelial cells lining the BBB, direct virus dissemination into the CNS via increased permeability of the BBB, transcellular transport, 'Trojan horse' mechanism – infected immune cells recruited to the CNS, paracellular transport, and retrograde axonal transport though peripheral motor neurons [61, 62]. Regardless of the mechanism employed, WNV has a tropism for gray matter areas of the brain stem, including the medulla and spinal cord. In particular, motor neurons of the anterior horn, pyramidal motor neurons, and cerebellar Purkinje cells become infected [63, 64].

Clinical WNND Syndromes: Aseptic Meningitis, Encephalitis, and Poliomyelitis (Acute Flaccid Paralysis)

The three main clinical syndromes listed above constitute the majority of WNND reported to the CDC, and the overwhelming majority of WNV associated mortality is attributed to WNND [65] (reviewed in [66]). There is great overlap in the symptomatolgy of the syndromes, and more than one syndrome may present during WNV infection [67]. One important distinction between WNND and other arboviral encephalitides is that up to 50% of patients present with neuromuscular weakness [68]. As noted previously, WNND incidence increases with age with the highest incidences among individuals ≥70 years of age [69].

Aseptic Meningitis occurs in 25-40% of WNND patients and is characterized by inflammation of the meninges without the presence of bacteria in the cerebral spinal fluid (CSF), and is associated with a ≤1% mortality rate. WNV Encephalitis, or acute swelling of the brain, is the most common WNND syndrome and accounts for 55-60% of all cases resulting in 20-30% mortality. Patients suffering from WNV Encephalitis present with symptoms similar to West Nile fever, but also have altered mental status but lack sensory abnormalities. Poliomyelitis, or acute flaccid paralysis, is the most distinct and well characterized WNND, but accounts for only 5-10% of neuroinvasive disease [64]. Symptoms include asymmetric upper and lower extremity weakness and cranial nerve involvement often requires intubation of patients [64]. Interestingly, there is no age predilection for WNV Poliomyelitis as all ages can be affected [70]. Poliomyelitis is characterized by rapid flaccid paralysis (24-48h) and high mortalities rates of 10-50%. WNV Poliomyelitis patients who

survive often suffer long-term or lifelong complications from the disease. Unfortunately, there are no treatments for WNND and treatment is only supportive (reviewed in [71]).

Neurological sequelae has been documented in 70-75% of convalescent patients suffering from WNND [59]. In general, sequelae presents as subacute relapse and is often associated with long-term persistent WNV infection. In fact, WNV RNA has been detected in the urine of convalescents several years after primary infection and long-term follow-up of these patients has indicated renal failure as a possible outcome [72, 73].

Histological and immunohistochemical findings of the brain, upon autopsy, show distinct perivascular inflammatory infiltration, infected astrocytes and astrogliosis, infected monocytic cells, necrotic foci, neuronal loss and neuronophagia. Neuronal cell apoptosis and bystander damage of neurons due to CD8+ T cells and monocyte infiltration have been noted [59, 74].

In addition to the clinical presentation, suspected WNND syndromes are confirmed by serology of the CSF. Detection of WNV specific IgM via enzyme-linked immunosorbent assay (ELISA) is the most useful diagnostic tool, but requires 1.5-3 days to generate results [75]. Unlike other viral encephalitides, IgM to WNV can be detected in the CSF for up to 7 months after disease onset [76]. Recently, the FDA has approved a qualitative IgM lateral-flow strip assay which takes minutes to complete, has good sensitivity, and does not require special laboratory equipment [77]. If necessary, a highly specific plaque reduction neutralization test (PRNT) can be performed on patient sera or CSF, but takes approximately 3 days to complete [78]. Other useful indicators of WNND include pleocytosis and elevated protein levels in the CSF [79]. RT-PCR to detect WNV RNA is not as useful for diagnosis

as peak viremia usually occurs 3-4 days before the onset of symptoms [80]. RT-PCR has been successfully employed, post-mortem, to confirm suspected lethal cases of WNV, and is the test of choice for screening blood donors.

West Nile virus Structure, Genome Organization, and Replication Cycle

WNV virions are distinctive enveloped icosahedrons of approximately 50nm [81]. The surface of virions are composed of two structural glycoproteins, M (membrane) and E (envelope), surrounding a lipid bilayer derived from a host cell membrane (most likely from the endoplasmic reticulum (ER)). Beneath the envelope lies the electron-dense nucleocapsid, composed of numerous copies of capsid protein encompassing a positive-sense, single-stranded RNA genome (vRNA). Interestingly, the nucleocapsid has no defined symmetry and appears spherical, measuring approximately 30nm [81]. Mature virions contain 90 E dimers which lie flat on the surface and form a unique herring bone pattern as visualized by cryo-electron microscopy (cryoEM) reconstruction [82, 83]. This configuration positions the E protein to engage a receptor on a host cell, thereby inducing clathrin-dependent, receptor-mediated endocytosis [84]. Many putative flavivirus receptors have been described *in vitro* including the integrin αVβ3, the C-type lectins DC-SIGN and DC-SIGNR (found on the surface of monocytes and dendritic cells), and the glycosaminoglycans (GAG) heparan sulfate and chondroitin sulfate E [85-89]. However, an indispensable *in vivo* receptor remains to be defined. On mature virions, the M protein is positioned between E dimers near their fusogenic domain [83]. The fusogenic domain is important for pH-dependent viral envelope fusion within endosomes, and allows release of nucleocapsids into the cytoplasm following

endosomal maturation [90]. During fusogenesis (low pH-shift), E dimers dissociate into monomers and then irreversibly reassemble into homotrimers [91]. This step involves major conformational changes in both the M and E proteins, requires rotation and refolding of the proteins, and allows the viral envelope to fuse with the endosomal membrane.

Following release of the nucleocapsid from an endosome, uncoating proceeds rapidly to deliver the vRNA into the cytoplasm. The WNV genome is approximately 11kb in length and contains a 5' type I cap structure (m7GpppAmpN2) which stabilizes the RNA against degradation by the host cell and ensures efficient translation [92]. Translation is initiated at the 5' end of the genome by ribosomal scanning and efficient translation is also insured by a 5' RNA secondary structure which most likely pauses the ribosome over the consensus start codon, as most flavivirus genomes lack a consensus Kozak sequence [93]. Translation is thought to proceed rapidly following uncoating and occurs in tight association with cellular membranes, particularly the ER. Notably, the 3' end of the genome lacks a polyA tail that is typically found on eukaryotic mRNA [94]. The genome is translated as one large open reading frame (ORF) into a single polyprotein containing the 10 viral proteins (C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Figure 1) [95]. C, prM, and E constitute the three structural proteins and are important for packaging of the nascent immature virions, whereas the seven non-structural proteins (NS1-NS5) are involved in genome replication, interact with host cell pathways, and are not packaged within virions. Signal and stop-transfer sequences within the polyprotein guide multiple translocations across the ER lumen (Figure 1). Topology of the polyprotein at the ER membrane is predicted such that the C and

NS2A-NS5 proteins reside on the cytoplasmic side of the membrane, while prM, E and NS1 proteins are predicted to be translocated into the lumen of the ER [96, 97].

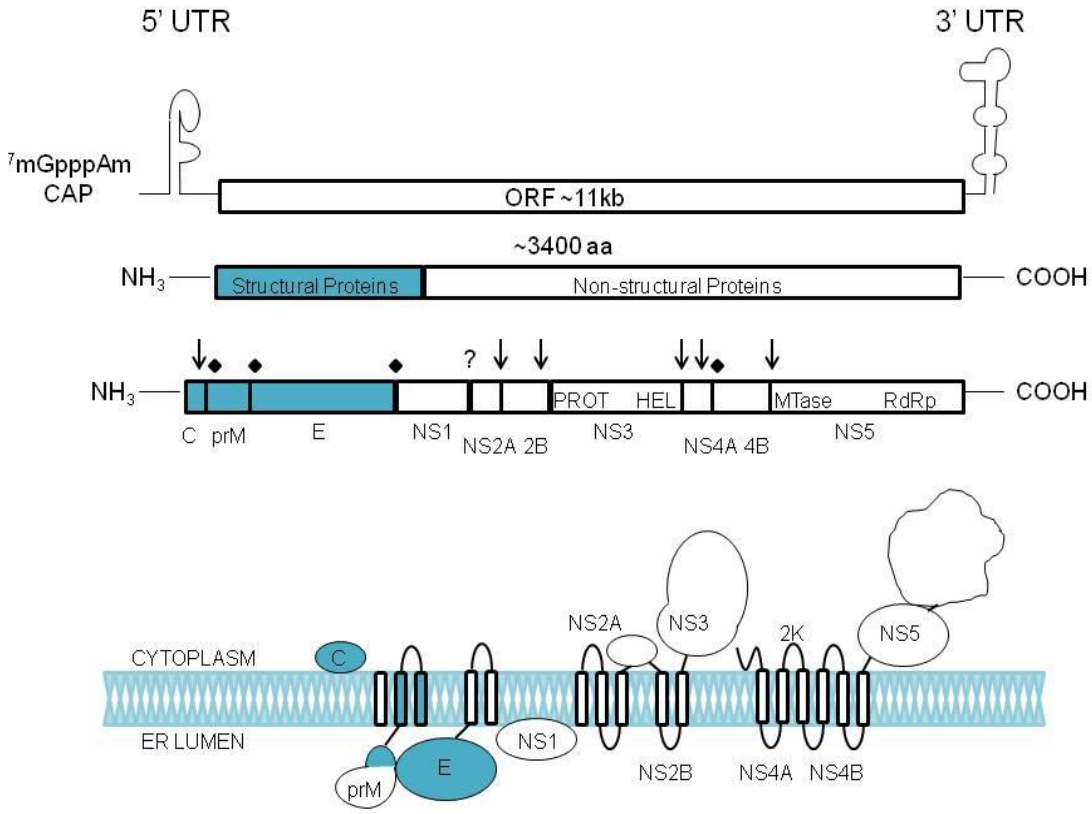

Fig. 1: **Genome organization, polyprotein processing, and predicted topography of the polyprotein at the ER membrane** (adapted from Fields Virology, 5th Edition. D. M. Knipe and P. M. Howley, Eds, 2007). Arrows indicate cleavage sites for the viral NS2B/3 protease, diamonds for cleavage by host cell signal peptidases, and the question mark for cleavage by an undefined ER luminal protease. PROT = serine protease domain, HEL = helicase domain, MTase = methyltransferase domain, and RdRp = RNA-dependent, RNA polymerase domain.

Proper post-translational processing of the polyprotein into the individual viral proteins involves both a virally-encoded serine protease (NS2B/3) and resident cellular signal peptidases [98, 99]. Specifically, the viral protease cleaves proteins on the cytoplasmic side

of the ER membrane while signal peptidases cleave the majority of proteins within the lumen of the ER [98]. The enzyme which cleaves NS1 from NS2A in the ER lumen has not been identified but is thought to be a protease resident to the membrane of the ER lumen [100]. As the majority of hydrophobic non-structural proteins are localized to the cytoplasmic side of the membrane, replication of the vRNA is believed to occur in the cytoplasm. Notably, WNV infected cells display significant ultrastructural morphological changes of cellular membranes. The ER membrane proliferates and smooth membrane structures referred to as vesicle packets and convoluted membranes appear, which are thought to be the sites of vRNA replication [101, 102]. As evidence, several of the non-structural proteins can be identified in these structures along with dsRNA, a replication intermediate of WNV replication [103]. Later in infection, nucleocapsids and immature virions can be seen within higher-ordered, convoluted membrane structures commonly referred to as paracrystalline arrays [104]. These structures serve as defined virus replication platforms (virus factories) and may also protect the nascent RNAs from being degraded or detected by the host innate immune system [105].

Flavivirus replication involves two simple steps, but is a highly coordinated process involving numerous viral and host cell proteins. (1) The positive-sense genome serves as template to make a negative-sense, anti-genome copy. (2) The negative-sense, anti-genome copy is then used as template for making new positive-sense RNA [106, 107]. Importantly, the viral NS5 (RNA-dependent RNA polymerase (RdRp)) and NS3 (helicase activity) proteins catalyze genome replication and are important for 5’ capping. These proteins work in concert with other non-structural proteins which form the replication complex (RC) [108,

109]. The nascent positive-sense RNA can either be translated into more viral protein, replicated again to produce more RNA, or be packaged as vRNA into the nucleocapsids of immature virions [110]. Replication of flavivirus vRNA is asymmetric with approximately ten fold more positive-sense RNA being produced than negative-sense RNA. Additionally, by using a negative-sense RNA intermediate as template, replication of flavivirus genomes is a semiconservative process [106].

For replication to occur, the 5' and 3' ends of the genome contain non-coding regions (NCR), commonly referred to as untranslated regions (UTR), composed of conserved, highly structured RNA elements (hairpins and loops) important for binding by viral replication and host cell proteins [111]. The UTRs also contain complementary sequences/regions which are important for genome cyclization. The 3' UTR of the genome is the initiation site for replication, is directly bound by RC proteins, and may allow these proteins to discriminate between the different replicative forms of RNA [112-115]. The 3' UTR contains three domains (DI-III) of which DI-II sequences are highly variable, dispensable for replication, but are thought to serve as enhancers of replication [116]. DIII is highly conserved, essential for replication, and is involved in long-range RNA:RNA interactions [117]. The 5' UTR is composed of two conserved stem loops (SLA and SLB) and one hairpin (cHP). SLA acts as a promoter for NS5 while SLB is involved in long-range RNA:RNA interactions [116, 118]. cHP is positioned downstream of the translational start codon, within the capsid gene, and is required for translation (ribosomal pausing) and replication [93, 119]. In addition to secondary RNA elements, two pairs of complementary sequences are found at either end of the genome. Regions of complementarity are essential for genome cyclization and

replication, but are not required for translation [111, 117, 120]. Further, mutational studies have found that complementarity of nucleotides is more important than the actual sequence of the nucleotides [121].

Nascent vRNA alongside capsid protein accumulates in the cytoplasm, leading to self-assembly of nucleocapsids. This is most likely achieved by the RNA chaperoning activity of the C protein and the UTRs are predicted to be important for guiding the C protein in properly packaging only positive-sense vRNA into nucleocapsids. [122]. Nucleocapsids then bud into the lumen of the ER, acquiring the viral envelope, and interact with the structural prM and E proteins (present as transmembrane-anchored heterodimers) to produce immature virions [123]. Immature virions contain 60 surface spikes; each spike is composed of a trimer of prM/E heterodimers [124]. Within the lumen of the ER, the prM protein covers/caps the fusion peptide of the E protein (which is at the external tip of the spikes) and plays a critical role in preventing acid-catalyzed rearrangements of the E protein into its fusogenic form. As noted earlier, those rearrangements are critically timed to occur during endocytosis of naïve host cells. The prM and E proteins of immature virions undergo modification steps within the ER and Golgi, including proper *N*-linked glycosylation [125]. Transit of immature virions within the secretory pathway of the ER and Golgi is a rapid process; however a critical prM cleavage step does occur prior to exocytosis [126]. The *trans*-Golgi endoprotease furin cleaves prM to M allowing for structural rearrangements of the E protein (E trimers to E dimers) which produces the mature, infectious virions. Surprisingly, this cleavage step in WNV morphogenesis is actually pH-dependent. The mildly acidic environment of the *tran*s-Golgi (pH 5.8-6) is necessary for exposing the furin

cleavage site on the prM protein. The pr peptide is thought to stay associated with the mature virions, acting as a chaperone, and is secreted within secretory vesicles upon exocytosis of mature WNV virions [126].

West Nile virus Structural Proteins

Capsid (C)

The capsid protein is an approximately 11 kilodalton (kD) basic protein composed of charged amino acid residues located at the termini and a hydrophobic central region important for ER membrane association [127]. Nascent capsid (referred to as anchor C (anchC)) protein contains a C-terminal hydrophobic anchor domain and a signal peptide sequence which are required for proper translocation of the prM protein into the lumen of the ER [128]. The virally-encoded serine protease (NS2B/3) cleaves before the hydrophobic anchor domain releasing monomers of C into the cytoplasm which then rapidly dimerize [128]. The hydrophobic central region of C stabilizes the newly formed dimers at the cytoplasmic side of the ER membrane. The NS2B/3 cleavage step is also critically timed to allow for proper processing of the prM protein within the lumen of the ER (discussed below) [129]. The C dimers then self-associate with vRNA to form nucleocapsids.

Currently, it is unknown how the C dimers organize into nucleocapsids, but *in vitro* addition of RNA or DNA to free C protein is able to induce the formation of nucleocapsid-like structures [130]. It is believed that the nucleic acids are able to interact with the charged termini of the basic C protein to induce oligomerization. The capsid proteins of WNV, JEV, DEV, and HCV have all been shown to localize to the nucleus [131-134]. Capsid protein of

DEV is able to bind four core histones, H2A, H2B, H3, and H4 in the nucleus and it has been proposed that capsid may bind vRNA in a histone-like manner instead of forming an ordered shell [135]. Nuclear localization of the JEV C protein has been demonstrated to be important for neuroinvasiveness, but not neurovirulence [132]. The mechanism for increased neuoroinvasiveness has not been defined. Interestingly, expression of WNV and HCV C proteins can affect the induction of apoptosis. One reported indicated that WNV C protein is capable of activating caspases-9 and -3 to induce apoptosis whereas another report found that WNC C protein blocks apoptosis through a phosphoinositide 3-kinase-dependent (PI3K) mechanism [133, 136]. HCV C protein is able to inhibit caspase-3-induced cellular apoptosis [134]. Thus, it is unclear if C proteins of different flaviviruses are pro- or anti-apoptotic.

The capsid proteins of some flaviviruses have also been implicated in innate immune subversion and will be discussed later.

PreMembrane (prM)

The prM protein is an approximately 26kD precursor glycoprotein (1 *N*-linked glycosylation site, N15) that contains six conserved cysteine residues which are disulfide linked, and two membrane-spanning domains containing a stop transfer sequence and a signal sequence [137]. The signal sequence allows the E protein to be translocated into the lumen of the ER. The prM protein plays a crucial role in virion morphogenesis; a temporally-controlled process that insures production of infectious virions. As noted, prM contains a signal cleavage site at its C-terminus that is positioned before the E protein. Interestingly, studies have indicated that the stretch of residues for peptidase cleavage is shorter than

predicted and the sequence is suboptimal, however optimization of the site increases the number of non-infectious and empty particles (subviral particles) produced by infected cells [129, 138]. It is now appreciated that viral protease cleavage of the C protein is a precursor event for efficient signal peptidase cleavage of native prM. Thus, the delay in prM peptidase cleavage ensures that sufficiently high levels of NS2B/3 protease are available to process C along with the other non-structural proteins. Subsequently, the delay also guarantees that C and vRNA associate into nucleocapsids which are available for packaging into immature virions as they bud into the lumen of the ER. Following this event, prM rapidly folds, chaperones the folding of the E protein, and then heterodimerizes with E [139]. The chaperoning activity of the prM protein is required for proper folding of the E protein and the fates of the two proteins are intimately linked. Notably, prM also contains an ER retention signal that maintains prM/E heterodimers in the lumen of the ER until encapsidation [123]. A furin-mediated cleavage site is also located near the C-terminus of the prM protein and allows for its maturation into M (~8kd C-terminal cleavage product) within the *trans*-Golgi network [140].

Envelope (E)

The approximately 53kD E glycoprotein (1 *N*-linked glycosylation site N154) contains twelve conserved cysteine residues which are disulfide bonded, two transmembrane domains, and is divided into three functional domains (DI-III), [82]. DI is the central structural domain composed of β-barrels that are important for structural rearrangements, and contains the N-terminus of the protein. DI is flanked by DII, which contains a dimerization

peptide in addition to a fusion peptide [90]. Dimerization is predicted to occur via hydrophobic interactions between two E monomers and fusion occurs within low-pH endosomes. DIII contains a characteristic immunoglobulin-like constant domain and includes the putative receptor binding site [141]. DIII of WNV has a consensus arginine-glycine-aspartic acid (RGD) motif which is thought to be important for binding integrins [142]. Additionally, the receptor binding site protrudes from the relatively flat surface of mature virions and is highly immunogenic (discussed later). The E protein also appears important for shaping pathogenesis of WNV. Most virulent, neuroinvasive stains of the virus retain the glycosylation site at position 154, while the majority of attenuated strains lack this modification site [143-145]. Involvement of the E protein in innate immune responses has also been noted and will be discussed in a separate section.

West Nile virus Non-structural Proteins

Non-structural Protein 1 (NS1)

NS1is a non-structural protein of approximately 46kD (352 amino acids), contains 12 invariant cysteine residues that are disulfide bonded, and is important for vRNA replication. Notably, NS1 is the only glycosylated non-structural protein (3 *N*-linked glycosylation sites N130, N175, and N207) expressed by the virus, is localized to the lumen of the ER, and can be secreted from cells as well as associate with the cell membrane [146]. Translocation of NS1 into the ER lumen requires the E protein C-terminal final 24 amino acids, which contain a signal peptidase cleavage site [147]. The C-terminus of NS1 protein lacks a consensus signal peptidase cleavage site before the NS2A protein. Cleavage at this junction is predicted

to involve a resident ER lumen protease and requires expression of the final 8 amino acids of NS1 in addition to the majority of the NS2A protein. The octapeptide sequence of NS1 most likely contains the cleavage site while NS2A may be acting in *cis* to present the cleavage site in its correct conformation [100, 147].

Interestingly, members of the JEV serogroup of flaviviruses, including WNV, are known to express an elongated species of NS1 (52 additional amino acids at the NS1 C-terminus) termed NS1'. NS1' is produced by a -1 ribosomal frame shift that is induced by a conserved slippery heptanucleotide motif in conjunction with an RNA pseudoknot structure present in the NS2A gene [148]. Strains of flaviviruses that express NS1' have increased neurovirulence and neuroinvasiveness in mouse models of infection, suggesting that NS1' may play a role in pathogenesis [148]. As further support for its role in pathogenesis, the live attenuated JEV vaccine strain SA 14-14-2 fails to produce NS1' due to a silent mutation at NS2A position 66 that destabilizes the pseudoknot structure, and contributes to its attenuated phenotype [149]. The mechanism by which NS1' increases viral pathogenesis is not currently known, but is speculated to involve inhibition of type I interferon production in infected cells by either the NS1 or NS2A protein, or by both proteins [148]. Additionally, NS1' is a substrate for cleavage by resident host caspases (producing ΔNS1') and may modulate caspase activity or the induction of apoptosis [150].

NS1 lacks a transmembrane domain and monomers of the protein are largely hydrophilic based on their amino acid content. Following its cleavage, NS1 monomers quickly dimerize in the lumen and associate with the ER membrane [151]. Dimerization requires the final three C-terminal cysteine residues of NS1 as well as proline at position 250

[152, 153]. Dimerization is thought to expose hydrophobic residues on the surface of dimers which could potentiate interactions with the ER membrane [151]. Dimers of NS1 can also enter the secretory pathway where they are glycosylated (*N*-linked high-mannose and complex/hybrid glycosylation), interact with the ER chaperone protein calnexin, and eventually form higher-order soluble structures (hexamers and oligomers) which are secreted into the extracellular milieu [154, 155]. The dimeric form of WNV NS1 has also been demonstrated to be directly targeted to the cell membrane and associates with the membrane through an unknown mechanism not involving GAGs [156]. For DEV, proper glycosylation of the protein has been found to be important for its secretion, cell surface expression, and for stability of hexamers [157]. Additionally, WNV expressing glycosylation-null NS1 displays minimal replication defects *in vitro* but is significantly attenuated *in vivo* for neuroinvasiveness and neurovirulence [158, 159].

The hexameric form of NS1 is composed of three NS1 dimers held together by weak hydrophobic bonds and has been described to organize into a barrel-shaped atypical high-density lipoprotein (HDL) as visualized by cryoEM. The NS1 hexamer is bound by triglycerides, phospholipids, mono- and diacylglycerol, and cholesteryl esters [160]. Recently, a short N-terminal peptide motif has been identified in NS1 that preferentially targets the protein for cell membrane association or for secretion [156]. The secreted form of NS1 (sNS1) is also able to bind back to the cell membrane of infected cells as well as naïve cells through interactions with GAGs (heparan sulfate and chondroitin sulfate E) [161]. Following cell membrane binding, sNS1 can be endocytosed by naïve cells and reside within late endosomes [162].

Studies have determined that flaviviruses with in-frame deletions in the NS1 protein are replication defective, but can be rescued by ectopic expression of NS1 (*trans*-complementation) [163]. Further, mutations in the NS1 protein that attenuate virus replication suggest that the protein is important for negative-sense RNA synthesis early in the replication process [164]. Since vRNA replication occurs in the cytoplasm and NS1 is localized to the ER lumen, it is currently unclear how the protein is directly involved in replication. One possible explanation was provided in a study that found a genetic link between the NS1 and NS4A genes (NS4A protein is a component of the RC) [165]. More recently, the NS1 protein was found to directly interact with the NS4B protein (also part of the RC) of Kunjin virus. It has been proposed that this interaction may allow NS1 to stabilize the RC or to transmit signals to the RC via NS4B [166].

In addition to its role in virion replication, NS1 is highly immunogenic, and is capable of shaping immune responses. Its roles in pathogenesis and immune system subversion will be discussed in detail later.

Non-structural Proteins 2A and 2B (NS2A and NS2B)

NS2A is a small approximately 22kD, hydrophobic, membrane-spanning protein involved in vRNA replication and virion packaging, but lacks identifiable motifs found in enzymatic proteins [115, 167]. NS2A co-localizes with the RC and can directly bind the 3' UTR of vRNA [168]. Cleavage of the N-terminus of NS2A occurs in the ER lumen and has been described above, while the C-terminus is cleaved by the NS2B/3 viral protease in the cytoplasm at a consensus cleavage site [100]. In addition to this consensus cleavage site, an

alternative NS2B/3 cleavage site has been revealed within the NS2A protein of YFV (between amino acids 189-191), the product of which is a truncated form of NS2A, designated NS2Aα [169]. Interestingly, mutagenesis to ablate NS2Aα formation (L190S point mutation in the NS2A gene) is lethal, as no infectious virions were produced following transfection of RNA, however subviral particles consisting of the prM and E proteins were secreted [170]. These findings suggest that the mutation in NS2A does not affect vRNA replication or processing of prM and E, but does play a role in virion packaging. Serial passage of YFV mutants with alternative amino acid changes at position 190 resulted in a compensatory (suppressor) mutation in the helicase domain of the NS3 gene that allowed for production of infectious virions without NS2Aα formation [170]. These findings suggest that NS2Aα formation itself is dispensable for productive infection and that a possible interaction exists between the NS2A and NS3 proteins that requires a basic residue at NS2A position 190 [170]. It is also possible that this interaction is important for the assembly of nucleocapsids or for their budding into the ER lumen.

Efficient flavivirus replication and packaging also appear to require the presence of hydrophobic residues in the NS2A protein, specifically I59 [168]. Notably, position 59 is surrounded by non-polar amino acids. Mutant Kunjin virus expression an I59N point mutation in NS2A is attenuated for plaque formation on BHK cells [168]. Thus, replacement of isoleucine with small polar asparagine may change the hydrophobicity of the protein and in turn affect its ability to support replication and packaging. In addition to its role in flavivirus replication, the NS2A protein of Kunjin virus was shown to induce apoptosis in an interferon-independent manner [171].

The NS2B protein is a very small (~14kD) membrane-spanning protein (3 hydrophobic regions) involved in polyprotein processing [172]. Both the N- and C-termini of the NS2B protein are cleaved by the viral protease. NS2B and NS3 proteins co-localize within convoluted membrane structures and NS2B serves as a cofactor for NS3 serine protease activity. The three hydrophobic regions of NS2B are dispensable for cofactor activity, but are important for localizing the NS3 protein at the ER membrane. The NS2B protein is also important for increasing solubility of the NS3 protein [173]. The hydrophilic region (amino acids 49-95) of NS2B supplies protease cofactor activity by binding a fold within the NS3 protease domain [174].

Both the flavivirus NS2A and NS2B proteins are capable of inhibiting several innate immune signaling pathways which will be discussed in detail in another section.

Non-structural Protein 3 (NS3)

NS3 is a large (approximately 70kD), multifunctional, highly conserved protein essential for replication and processing of the WNV polyprotein. NS3 encodes functions for serine protease, RNA helicase, RNA-stimulated nucleoside triphosphatase (NTPase), and RNA triphosphatase (RTPase) activities [175]. Protease activity is localized to the N-terminus (first 184 amino acids) of the NS3 protein and has sequence similarity to the trypsin superfamily [176, 177]. NS3 cleaves at conserved, adjacent dibasic amino acid residues and requires binding by the NS2B cofactor, which forms a chymotrypsin-like fold in the substrate binding pocket. Autoproteolytic cleavage frees both the N- and C-termini of the NS3 protein, which is subsequently anchored by NS2B to the cytoplasmic side of the ER membrane. The

C-terminus of NS3 possesses the RNA helicase, NTPase, and RTPase domains. The RNA helicase domain shares homology with other DEAD (asparagine-glutamine-alanine-asparagine) box family RNA helicases. Helicase activity utilizes energy generated by nucleoside triphosphate hydrolysis (NTPase) to unwind 3' RNA secondary structures present on the genome and is important for vRNA replication. NS3 can also directly bind the 3' UTR but this is considered a weak interaction. RTPase activity is important for 5' capping of the vRNA and may require a conserved Walker B motif present within the NTPase domain.

Given the multiple functions of the NS3 protein it is not surprising that several of the functional domains overlap. In addition to its interactions with NS2B, the NS3 protein also interacts with the NS5 protein [178]. This interaction involves the C-terminus of NS3 and the N-terminus of NS5, and is important for catalyzing the NTPase activity of NS3 and for 5' capping of the vRNA [112]. Recently, mutagenesis studies of YFV NS3 have revealed that tryptophan at position 349, within the helicase domain, is dispensable for RNA replication, but is absolutely required for packaging of the vRNA into nucleocapsids [179]. This is the first evidence that NS3 may be involved in packaging of the vRNA, a function independent of its other enzymatic activities. In addition to its indispensible roles in replication and virion packaging, the NS3 protein has also been shown to bind to and induce the activation of caspase-8, leading to cellular apoptosis [180]. Further, the NS3 proteins of some flaviviruses can inhibit innate immune responses to infection which will be described in detail later.

Non-structural Proteins 4A and 4B (NS4A and NS4B)

The flavivirus NS4A protein is another small (~16kD) membrane-spanning protein (4 transmembrane domains) important for WNV replication. NS4A can associate with the RC and co-localizes with dsRNA, however its direct role in replication is not currently understood [115]. NS4A has recently been found to induce PI3K-dependent autophagy in epithelial cells which prolongs their survival during flavivirus infection and makes them resistant to apoptosis [181]. The N-terminus of the protein is hydrophilic and associates with the cytoplasmic side of the ER membrane, while the C-terminus contains a signal sequence, named 2K, that translocates the NS4B protein into the lumen of the ER. NS4B is also a relatively small ~27kD hydrophobic, membrane-associated protein involved in flavivirus replication. The protein can be found in membrane complexes with the NS3 protein as well as with dsRNA, and for some flaviviruses, is capable of translocating into the nucleus [182, 183]. Many mutations have been identified within the NS4B protein, which attenuate replication and at least one mutation (P101L) ablates its interaction with the NS3 protein [184, 185]. An amino acid change in the C-terminal end of the NS4B protein, E249G, has also been described that attenuates vRNA replication independent of the effects of interferon [186].

Interestingly, cleavage between the NS4A and NS4B proteins involves both an ER signal peptidase and the viral-encoded serine protease. The final seventeen amino acids of NS4A contain the signal sequence (sig4B), while the upstream NS2B/3 cleavage site (4A/2K) is twenty-three amino acids away in NS4A [97]. Viral protease cleavage at 4A/2K generates the C-terminus of NS4A, and must occur prior to signalase cleavage in order for

sig4B to be properly exposed. Cleavage at sig4B removes the 2K peptide and generates the N-terminus of NS4B, while NS2B/3 cleaves the C-terminus of the protein [97].

Both of these membrane spanning proteins have been implicated in the induction of membrane rearrangements typically observed during flavivirus infection. Co-expression of NS4A/B appears to be a precursor for membrane rearrangement, while cleavage of the two proteins by the viral protease is required for the formation of vesicle packets and membrane convolutions [187]. The contributions of the 2K peptide to membrane rearrangements are not completely resolved. A study by Roosedaal et al found that cleavage of 2K from Kunjin virus NS4A was not necessary for membrane alterations, whereas Miller et al found that cleavage from DEV NS4A was absolutely required [182, 187].

In addition to their roles in WNV replication and membrane rearrangement, both NS4 proteins are capable of inhibiting innate immune signaling pathways. A detailed section on WNV evasion of host innate immune defenses will include a discussion of the NS4 proteins.

Non-structural Protein 5 (NS5)

The NS5 protein is the largest (~103kD) of the non-structural proteins and the sequence is highly conserved among members of the *Flavivirus* genus. NS5 is a basic protein and lacks hydrophobic transmembrane regions. Processing of the N-terminus of NS5 requires the viral protease as described above. NS5 possesses enzymatic activities to function as the viral-encoded RNA-dependent RNA polymerase (RdRp) and as a methyltransferase (MTase). RdRp activity is encoded in the proteins C-terminus which contains a typical glycine-aspartic acid-aspartic acid (GDD) motif found in other viral RdRps [188, 189].

Polymerase activity has been proven to be required for vRNA replication and for virion encapsidation, and the NS5 protein is capable of binding the 3' UTR of vRNA to initiate replication [114]. NS5 RdRp activity in infected cells is also associated with 'heavy' cytoplasmic membrane fractions which are enriched for the RC proteins NS2A, NS2B, NS3, and NS4A.

MTase activity is encoded in the N-terminus of the protein, is involved in 5' capping of the nascent vRNA, and is absolutely required for replication [190]. Notably, the antiviral drug Ribavirin is capable of inhibiting NS5 MTase activity [191]. The NS5 protein is transiently phosphorylated at serine residues by a host cellular serine/threonine kinase (protein kinase G for mosquito-borne flaviviruses) [178, 192, 193]. As noted previously, the NS5 protein interacts with NS3 and can modulate its activity. This interaction is dependent upon the phosphorylation status of NS5 [178]. Specifically, hypophosphorylated (unphosphorylated) NS5 can bind NS3. Additionally, the NS5 protein of some flaviviruses can localize to the nucleus of infected cells [194]. The nuclear localization sequence (NLS) has been identified and is positioned between the MTase and RdRp domains (residues 369-405) [195]. Similar to its interaction with NS3, nuclear localization of NS5 is also phosphorylation-dependent. Hyperphosphorylated NS5 is preferentially localized to the nucleus whereas hypophosphorylated NS5 localizes to the cytoplasm. Cytoplasmic NS5 is able to participate in vRNA replication, however it is currently unknown if nuclear NS5 is important for the viral replication cycle. Nevertheless, nuclear NS5 does interact with a multitude of host proteins, the consequences of which remains to be determined [196].

During vRNA replication the substrate RNA may be damaged at the termini. Uridylyl and adenylyl terminal transferases are able to catalyze the addition of non-template nucleotides to the 3' terminus of RNA. Recently, it has been proposed that WNV NS5 as well as the NS5 proteins of other flaviviruses have intrinsic terminal transferase activity important for 3' UTR repair, but the domain required for this activity has not been identified [197].

The NS5 protein of several flaviviruses has also been found to modulate innate immune responses and will be discussed in detail later.

Adaptive Immune Responses to West Nile virus Infection

Human WNV infection generally results from a mosquito bite of the skin from an infected vector, and involves direct infection of keratinocytes and Langerhans dendritic cells (DCs). Langerhans DCs are thought to migrate to local draining lymphnodes upon activation, where the virus undergoes and an initial round of replication [198, 199]. Viremia ensues and the virus disseminates to visceral organs including the spleen, liver, and kidneys. In a subset of patients the virus can then cross the BBB and proceed to WNND. Similar to another neurotropic flavivirus, SLV, the level and duration of viremia appears to correlate with risk for neuroinvasive disease [200].

Increased risk of severe WNV disease in immunosuppressed and immunosenescent patients suggests that an intact immune system is required for control of the virus. Indeed, both humoral and cell mediated adaptive immune responses are required for virus clearance in mouse models of infection. SCID mice and RAGI deficient mice, which lack functional B

and T cells, uniformly succumb to WNV infection [201, 202]. As further support, young mice (immature immune systems) and cyclophosphamide-treated mice (immunosuppressed) are more susceptible to lethal WNV infection [201, 203, 204].

Humoral Immune Responses

B cells are required for protection against WNV as B cell-deficient mice are completely susceptible to infection, but are protected by passive administration of WNV-specific antisera [203, 205]. In particular, secreted IgM (sIgM) is required for the control of WNV infection as experimental infection of $IgM^{-/-}$ mice is uniformly lethal [206]. These mice are able to produce B cells which only express IgM on the cell membrane. Further, WNV-specific IgM antibody titers in experimentally-infected mice are a strong predictor of disease outcome [206].

Passive transfer of WNV-specific IgG is also protective, but it has been noted that normal IgG responses to WNV do not appear until 6-8 days post-infection [205, 207]. This delay in IgG responses raises questions about the contributions of IgG in clearing WNV infection and about protection of the CNS, as the virus reaches the CNS in this amount of time. The overwhelming majority of neutralizing antibodies to WNV recognize discrete domains on the structural E and PrM (or M) proteins [208-211]. Of these two structural proteins, antibodies to the E protein have the strongest neutralization capacity. The three structural domains of the E protein, DI-III are important for mediating viral attachment to a putative receptor, entry, and virion assembly, and were discussed previously.

Antibodies to the DII epitope are protective when administered prophylactically and following WNV challenge [212]. DIII-specific antibodies recognize a dominant neutralization epitope located on the lateral face of the domain [213, 214]. Work completed with some the most strongly neutralizing DIII antibodies suggests that they may inhibit viral membrane fusion in endosomes at a post-attachment step [215] (and reviewed in [216]). Topologies studies indicate that DIII protrudes from the surface of virions and may explain the predilection of strongly neutralizing antibodies for this region [214]. Surprisingly, administration of DIII-specific antibodies is protective to mice even after WNV has established infection of the CNS, but it is unclear if these antibodies are able to reach the CNS. Importantly, in WNV infected humans, only about 8% of the WNV-specific B-cell clones examined produce antibodies to DIII. In contrast, about 50% of the isolated clones produce antibodies which map to the fusion loop of DII [217]. Further, neutralization capacity of sera taken from convalescent horses suggests that antibodies specific for the DIII region are not required for successful neutralization [218] (reviewed in [219]). Taken together, many scientists have proposed that effective antibody-mediated neutralization of WNV requires the production of multiple neutralizing antibodies, which bind a variety of epitopes and domains of the E protein.

Non-neutralizing, but protective antibodies are also produced against the WNV Non-structural protein 1 (NS1) [220, 221]. NS1 is not packaged in virions, but is a secreted glycoprotein and is found on the surface of infected cells. Mapping studies using yeast and bacterial display of NS1 indicated that the repertoire of NS1-specific antibodies, generated in mice, recognize epitopes on three distinct regions of NS1 [220]. These antibodies protect

against WNV infection through Fc-γ-dependent and -independent opsonization mechanisms. Interestingly, mice are protected against lethal WNV challenge when prophylactically treated with NS1-specic antibodies prior to infection [222].

Cell Mediated Immune Responses

Both CD4+ and CD8+ T cell responses to WNV infection are also required for protection. Mice with genetic deficiencies in MHC class II expression and acquired CD4+ deficiencies (or CD4+ depletion) display protracted WNV viremia, infections of the CNS and increased lethality [223]. CD4+ T cells play a protective role in stimulating antibody responses and are important for priming and activating B cells. In mice lacking functional CD4+ T cell responses, IgM levels decreased 20 fold by 15 days post-infection when compared with wild-type mice and IgG levels were 100-1000 fold lower throughout the course of infection [223]. Taken together, it appears that the population of CD4+ T cells that recognize WNV specific antigens is important for maintaining and maturing B cell responses and can promote IgG class-switching. CD4+ T cells also play synergistic roles in priming CD8+ T cells responses. CD4+ T cells appear important for priming CD8+ T cells and secrete cytokines necessary for their activation and for sustaining their cytotoxic responses. WNV-specific CD8+ T cell responses, as measured by activation and trafficking to the CNS, are severely delayed in mice lacking functional CD4+ T cells [223].

As the data above would suggest, functional WNV-specific cytotoxic CD8+ T cells are also required for clearance of WNV infected cells. Importantly, WNV-infected non-immune cells present WNV peptides via MHC class I [224]. WNV antigen-restricted CD8+

T cells proliferate, release cytokines, and lyse infected cells via perforin and granzymes A and B [224, 225]. CD8+ deficient mice and mice deficient in MHC class I expression generally succumb to WNV infection and the virus can be recovered from the CNS of surviving mice weeks after challenge. Interestingly, the levels of antibody responses and viremia do not appear to be affected in mice lacking CD8+ T cells [226, 227]. These data suggest that CD8+ T cells do not play a critical role in controlling WNV infection in the periphery, but may be more important for controlling infection in the CNS. This is supported by the fact that antibodies to WNV reduce viremia and prevent severe disease, but the virus is still able to enter the brain. This is further supported by experiments in which passive transfer of wild-type CD8+ T cells, but not perforin-deficient CD8+ T cells, increase the survival of perforin-deficient mice infected with the virus [228]. Additionally, adoptive transfer of WNV-specific CD8+ T cells into wild-type mice reduces mortality and prolongs survival [227]. Thus CD8+ T cells clear WNV from infected tissues, including the CNS, and prevent viral persistence.

Innate Immune Responses to West Nile virus Infection

The innate immune system is the first line of defense against invading microorganisms and employees redundant mechanisms to ensure the detection and elimination of pathogens. As outlined above, the adaptive immune response is critical for the control of WNV infection, but is dependent upon an appropriate innate immune response. Further, the adaptive immune responses take a minimum of a week to establish while the innate immune response is initiated upon pathogen detection. The innate immune system is

composed of various cytoplasmic, and cell or endosomal membrane-localized pattern recognition receptors (PRR) which intrinsically recognize pathogen-associated molecular patterns (PAMP) to induce antiviral responses. Mammalian PRRs include the cell and endosomal membrane-associated Toll-like receptors (TLRs), and the cytoplasmic RIG-I-like receptors (RLRs) as well as Nod-like receptors (NLRs).

TLRs

Mammalian TLRs exist as 11 evolutionarily conserved type-I transmembrane glycoproteins which are homologous to the *Drosophila Toll* gene. TLRs may be expressed at the cell membrane or on the endosomal membrane. The generalized TLR structure includes the pathogen-binding ectodomian composed of a leucine-rich repeat (LRR), while the cytoplasmic region is involved in signal transduction and includes a Toll/IL-R homology domain (TIR domain) that transmits signals to a specific adaptor molecule(s) [229]. TIR signaling through the adaptor molecule myeloid differentiation factor 88 (MyD88) and/or TIR-containing adaptor-inducing IFN-β (TRIF) results in the activation of transcription factors such as nuclear factor kappa-B (NFκB), activator protein 1 (AP-1), and interferon regulatory factors 3 and 7 (IRF3 and IRF7). These transcription factors synergistically induce the expression of pro-inflammatory cytokines and type I interferon which induce an antiviral state in responsive cells as well as prime cells of the adaptive immune system (reviewed in [230]).

Several TLRs have been found to be important for recognizing and controlling WNV infection. TLR3 is typically found within endosomes, but may also be localized to the cell

membrane of certain cell types, and recognizes dsRNA of viruses and apoptotic, virus-infected cells [231-233]. TLR3 transmits signals exclusively through the adaptor molecule TRIF [234]. TRIF associates with tumor necrosis factor associated factor 3 (TRAF3) and TRAF6 by binding the N-terminus of TRAF proteins which then activate the transcription factors NFκB, AP-1 or IRF3 (Figure 2) [235]. Induction of type 1 interferon is dependent upon the activation of all three transcription factors while cytokine production requires NFκB and/or AP-1 activation.

Activation of the IRF3 arm of TLR3 signaling involves interactions between TANK-binding kinase 1 (TBK1) and TRIF which is mediated by TRAF3 [235, 236]. TBK1 along with IκB kinase ε (IKKε) phosphorylates IRF3, which then self-dimerizes and translocates to the nucleus to transcribe type 1 interferon in conjunction with several other transcription factors (Figure 2) [237, 238]. The transcription of IFN-β is a tightly regulated event that requires the coordinated assembly of an enhanceosome composed of IRF3, NFκB, AP-1, and co-activator cAMP responsive element binding protein (CBP/p300) [239, 240].

TLR3 is also capable of activating NFκB through two distinct pathways. The canonical TRAF6-dependent pathway involves association of TRAF6 and TRIF, which allows TRAF6 to be polyubiquitinated via its E3 ubiquitin ligase activity alongside the ubiquitinating E2 complex, UBC13, and UEV1A [241]. This facilitates the recruitment of transforming growth factor-beta-activated kinase 1 (TAK1) and TAK-binding protein 2 (TAB2) which form a complex that allows for autophosphorylation of TAK1. TAK1/TAB2 then activate the IκB kinase (IKK) complex consisting of IKKα, IKKβ, and IKKγ (NFκB

essential modulator (NEMO)). This complex phosphorylates and degrades IκBα, releasing NFκB for nuclear translocation and gene transcription (Figure 2) [242].

TLR3 also activates NFκB via a non-canonical pathway involving the receptor-interacting protein kinase 1 (RIP1). The C-terminus of TRIF contains a RIP homotypic interaction domain (RHIM) that allows it to bind RIP1 upon activation [243]. That interaction results in the polyubiquitination of RIP1 leading to the activation of the IκB kinase complex and release of activated NFκB.

In addition to IRF3 and NFκB, TLR3 signaling also activates the transcription factor AP-1. This is achieved by interactions between TAK1 and the mitogen-activated protein kinases (MAPKs), specifically extracellular signal-regulated kinases 1 and 2 (ERK 1 and 2), p38, and c-jun-N-terminal kinase (JNK) [244]. Clearly, there is considerable cross talk within the pathway and many of the downstream molecules involved in TLR3 signaling are also shared with the RLRs (discussed later).

Initial studies using TLR3$^{-/-}$ mice suggested a detrimental role for TLR3 during lethal WNV challenge [245]. Mice showed reduced cytokine responses and increased survival. Most surprisingly, TLR3 deficient mice suffered higher viremia than wt mice but had reduced mortality. The investigators of that study hypothesized that a blunted tumor necrosis factor-alpha (TNF-α) response may have prevented an increase in BBB permeability and thus, transit of WNV into the CNS even though larger viral burdens were detected in the periphery. A more recent study by Daffis et al., using mosquito cell-derived WNV and footpad inoculation clearly demonstrated that TLR3 is protective and plays an critical role in limiting infection in the CNS [246]. WNV replication was enhanced both *in vitro* and *in vivo*

in neurons from TLR3 knock-out mice. Alternatively, Fredericksen and Gale were unable to find a role for TLR3 in controlling WNV infection in mouse embryonic fibroblasts (MEFs). Collectively, the data suggest that TLR3 may have a cell-type-specific role in preventing lethal WNV infection [247].

TLR7 is another Toll-like receptor important for the recognition and control of WNV infection. Similar to TLR3, TLR7 resides within endosomes, but recognizes the PAMP ssRNA and transmits signals through the adaptor molecule MyD88 (reviewed in [248]). MyD88 activation through TLR7 leads to the formation of complex consisting of interleukin-1 receptor associated kinase 1(IRAK-1), IRAK-4, TRAF6, and IRF7 which allows IRF7 to dimerize and translocate to the nucleus to induce the transcription of type 1 interferon, particularly IFN-α (Figure 2) [249-251]. TRAF6 also induces TAK1/TAB2 complex formation resulting in the activation of NFκB and AP-1. Wang et al., previously demonstrated that TLR7 in dendritic cells is capable of recognizing flaviviruses [252]. TLR7$^{-/-}$ mice challenged intraperitoneally with WNV are more susceptible to infection, have larger viral burdens, and increased mortality. TLR7 is also crucial for macrophage immune cell homing following infection. Macrophages from TLR7-deficient mice demonstrate reduced IL-12 and IL-23 responses, which correlated with increased susceptibility to WNV [253]. TLR7 may play an important early role in controlling WNV infection at the site of infection. TLR7-deficient mice show delayed activation and migration of Langerhans DCs following cutaneous infection, but no changes in mortality were observed [254].

With the exceptions of TLR3 (TRIF) and TLR4 (MyD88 and TRIF), all known mammalian TLRs signal solely through the adaptor molecule MyD88 and many are capable

of sensing viral PAMPS to initial antiviral responses. For examples, TLR2 and TLR6 are capable of sensing respiratory syncytial virus (RSV) [255, 256]. WNV pathogenesis studies with MyD88 knockout mice indicated that TLR signaling through MyD88 is important for limiting disease severity and increasing survival. Indeed, MyD88$^{-/-}$ mice exhibited higher viral titers in the brain and reduced survival. *In vitro*, macrophages and subsets of neurons derived from MyD88 deficient mice also produce higher virus titers. This was not observed for myeloid DCs (mDC), suggesting that TLRs which signal through MyD88 do not play a critical role in sensing WNV infection of mDCs [257].

RLRs

The cytoplasmic dsRNA sensors retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) play significant roles in sensing and controlling WNV infection through the induction of type 1 interferon [258, 259]. Both RLRs contain two N-terminal caspase-recruitment domains (CARDs) which transmit signals upon activation. The C-termini of these proteins contain the repressor domain (RD) that is involved in repression as well as PAMP recognition, while the central portion of the proteins include a DExD/H box RNA helicase also important for PAMP recognition (Figure 2) [260, 261]. Interestingly, dsRNA length and phosphorylation status of ssRNA are important in determining RLR recognition. Short sequences of dsRNA (70-300bp) as well as ssRNA containing a 5' triphosphate end are recognized by RIG-I. Alternatively, longer stretches of dsRNA (>2kb) are sensed by MDA5 [262, 263].

RLR signaling involves cytoplasmic recognition of dsRNA by CARDs which become ubiquitinated via the E3 ubiquitinase, tripartite motif 25 (TRIM25) [264]. TRIM25 ubiquitin ligase activity is dependent upon caspase-12, which is thought to recruit RIG-I to TRIM25 [265]. RIG-I binds to IFN-β promoter stimulator-1 (IPS-1) protein located on the surface of the mitochondrial membrane, and allows recruitment of TNFR-associated death domain-containing receptor (TRADD) [266, 267]. TRADD forms a complex with Fas-associated death domain-containing receptor (FAAD), RIP1, and TRAF3 resulting in the activation of IKK, TBK1, and IKKε [268, 269]. This culminates in the phosphorylation and dimerization of IRF3/IRF7 which translocate into the nucleus to transcribe type 1 interferon (Figure 2). IPS-1, TRADD, and FADD signaling also activates NFκB through interactions with caspases 8-10 [270].

RIG-I signaling was found to be critical for interferon responses in MEFs following WNV infection [247]. MEFs deficient for RIG-I showed decreased IFN-β production, reduced activation of IRF3, and higher viral titers compared with wild type MEFs. It was also demonstrated that MDA5 is capable of sensing WNV infection to trigger interferon responses. Interestingly, RIG-I appears to be the dominant RLR in the periphery as interferon responses remained intact in DCs deficient for MDA5 signaling [271]. It is currently believed that MDA5 plays a synergistic role with RIG-I to sense flavivirus infection in the periphery. Studies with IPS-1 deficient DCs, macrophages, and primary cortical neurons indicated reduced interferon responses and increased virus growth. Curiously, IPS-$1^{-/-}$ mice are more susceptible to WNV infection but also produce more interferon and proinflammatory

cytokines. The aberrant cytokine response is thought to prevent the expansion of regulatory T cells (Tregs) which control the immune response [272].

A role for the other known mammalian RLR, laboratory of genetics and physiology 2 (LGP2), in innate responses to WNV has not been described. Unlike RIG-I and MDA5, LGP2 lacks a CARD domain and is thought to actually function as a negative regulator of the other RLRs [273]. Suthar and colleagues recently reported that LGP2 is actually important for WNV antigen-specific CD8+ T cell survival in the CNS and functions by regulating death receptor signaling-induced apoptosis [274].

Interestingly, type I IFN responses in mice appear to largely be elicited independent of IRF3 [275, 276]. IRF3-deficient mice were able to mount significant type I IFN responses dominated by IFN-α. These findings suggest that in the absence of IRF3 cells use alternative PRRs, such as TLR7, to induce IFN-α transcription through IRF7. These findings also highlight the considerable interplay between the PRRs and suggest that multiple PRRs, not a single PRR, are probably responsible for detecting WNV.

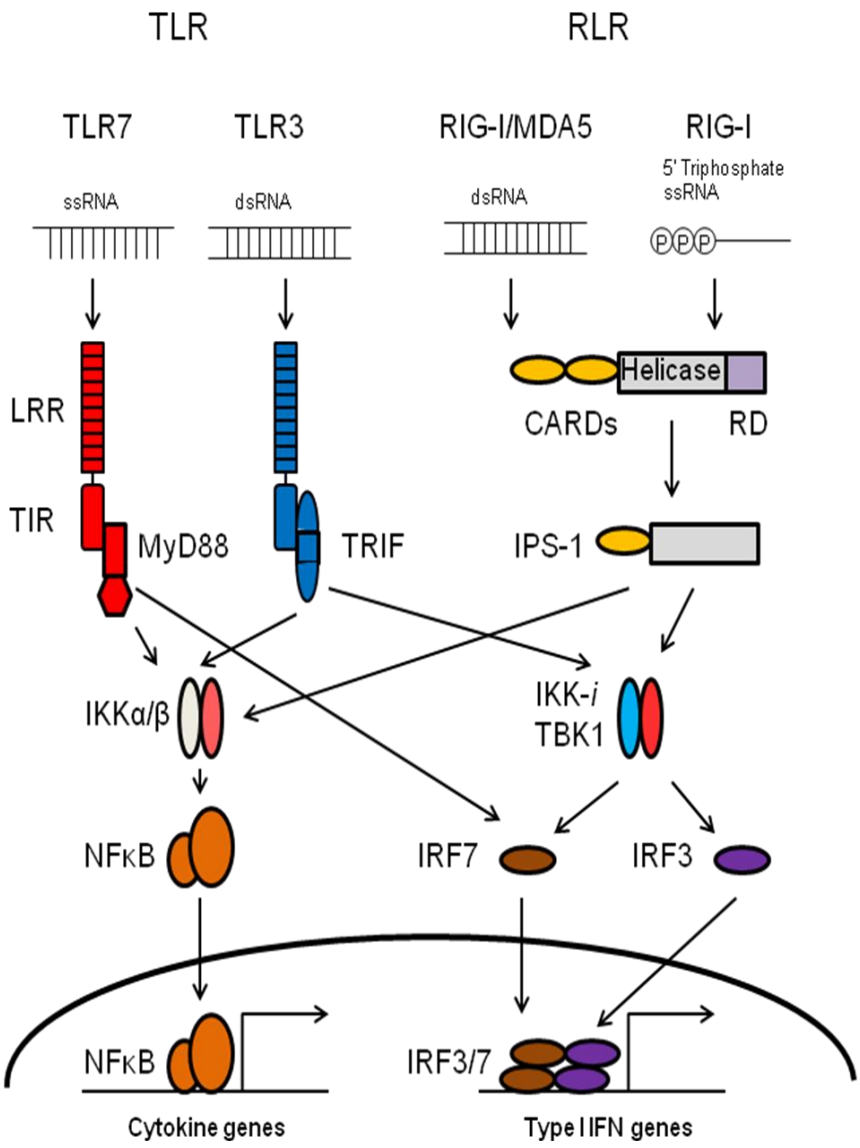

Fig. 2: **Generalized signaling pathways of TLRs and RLRs leading to cytokine and type I interferon production** (adapted from Osamu and Akira, Innate Immunity to Virus Infection, Immunological Reviews, 227: 75-86, 2009). Adaptor molecules = MyD88, TRIF, and IPS-1, kinases = IKKα/β, IKK-*i*, and TBK1, and transcription factors = NFκB, IRF3, and IRF7.

Interferon

A major consequence of WNV detection by PRRs is the production of interferon. Type I interferon (IFN-α and IFN-β) is secreted early in infection from a multitude of cell types. Type I IFN is critical for DC maturation and the activation of B and T cells. Further, type I IFN is able to induce an antiviral state in cells through the activation of interferon-stimulated genes (ISGs). This occurs in an autocrine and paracrine fashion, whereby type I IFN binds the IFNα/β receptor (IFNR), triggering activation of Janus tyrosine kinases (JAKs) which phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. The phosphorylated STAT proteins complex with IRF9 (or IRF3), termed ISGF3, which translocates to the nuclease and binds the IFN-stimulated response element (ISRE) of ISGs to induce their transcription [277, 278].

Mouse pathogenesis studies clearly demonstrate a protective role for type I IFN in WNV infection. IFNR deficient mice uniformly succumb to WNV challenge and display increased viral burdens, particularly in the brain, and altered viral tropism for immune cells in the spleen. Pretreatment of cells with type I IFN prior to WNV challenge completely protects against infection, but these protective effects are lost if type I IFN is used post-infection [279, 280]. More recently, IFN-$\beta^{-/-}$ mice were shown to have increased lethality to WNV infection, but the phenotype was less severe than $IFNR^{-/-}$ mice. Interestingly, while CD8 + T cell responses and B cell antibody responses were preserved, a clear deficit in Treg cells was noted in these mice both before and after WNV infection [281].

Type II interferon (IFN-γ) also contributes to WNV resistance, is primarily produced by CD8+ T cells, natural killer cells (NK), and γδT cells, and functions to induce innate and

adaptive antiviral responses [282]. IFN-γ is important for activating and increasing MHC I expression on antigen presenting cells, such as DCs, and shapes CD4+ T cell TH1/TH2 polarization [283]. Transgenic mice deficient for the IFN-γ receptor (IFNGR), IFN-γ production, or γδT cells are more susceptible to WNV challenge and display higher viral titers in the periphery and the CNS. However, these mice show no changes in their adaptive immune responses indicating that IFN-γ is most important for innate immune control of WNV [284, 285].

ISGs

Signaling through the IFNR triggers the production of ISGs which function as a subset of antiviral effector molecules to directly control WNV infection. While over 100 ISGs have been identified, only a handful have been characterized during WNV infection. RNA-dependent protein kinase R (PKR) is a sensor of dsRNA and is an ISG induced during WNV infection. Binding of flavivirus dsRNA activates PKR to phosphorylate the eukaryotic translation initiation factor 2α (eIF2α), which blocks cellular and viral protein synthesis [286]. PKR is primarily considered an ISG, however Gilfoy and Mason were able to demonstrate a role for PKR as a PRR in several innate immune cell types during WNV infection. Thus, PKR may be an important PRR and an ISG for resistance to WNV infection [287]. 2'-5'-oligodenylate synthase (OAS) proteins are intracellular ISGs which also mediate resistance to WNV. OAS enzymes are activated by binding dsRNA and synthesize 2′,5′-linked oligoadenylates, which subsequently activate RNase L. RNase L is an endoribonuclease that functions to cleave viral RNA as well as cellular mRNA to decrease

protein synthesis and viral replication [288]. Both PKR and OAS have been demonstrated to be important ISGs for resistance to WNV infection. Mice deficient for PKR and OAS are more susceptible to infection, have higher viral titers in the periphery, and show increased mortality following WNV challenge [289]. Inbred mouse susceptibility to WNV infection has also been linked to a non-sense mutation in the *Oasb1* gene which results in a truncated, non-functional form of the protein [290, 291].

Several other ISGs have been reported to be induced during infection and are important in the control of flaviviruses. The cell membrane proteins, IFN-induced transmembrane protein 2 (IFITM2) and IFITM3 are thought to prevent WNV and DEV entry into cells, while viperin and ISG20 block replication of WNV once it has entered cells [292]. Viperin is a putative radical *S*-adeno-syl-L-methionine (SAM) domain containing enzyme and may be involved in controlling the capping of vRNA, while ISG20 is a 3'-5' exonuclease that may cleave flavivirus RNA. WNV-induced expression of viperin within the CNS also suggests that this ISG may be important for controlling infection in the brain [293]. Numerous other ISGs have recently been identified that control flavivirus infection, but their mechanisms of action remain to be elucidated [294].

Complement

The complement cascade consists of a family of proteins produced by the liver, which recognize and bind to pathogens or infected cells leading to their clearance. Three complement pathways have been identified and include the classical, mannose-binding lectin (MBL), and alternative cascades. The protein C1q initiates the classical pathway by binding

antigen-antibody complexes. MBL or ficolin bind lectins present on pathogens, while spontaneous hydrolysis of C3 activates the alternative cascade (C3b deposited on the surface of cells). Each pathway contributes to pathogen elimination through various mechanisms, involving direct pathogen neutralization, pathogen/cellular opsonization and/or cytolysis, or immune cell recruitment/chemotaxis (via the anaphylatoxins C3a and C5a) (reviewed in [295]). The classical and MBL pathways lead to the formation of the C3 convertase composed of C4bC2a. Likewise, the alternative pathway also produces its own C3 convertase composed of C3bBb. Association of C3b with either C3 convertase generates a C5 convertase, ultimately leading to C5b-9 membrane attack complex (MAC) formation. Thus, C3 is a centrally-required element for each of the complement pathways. Notably, the host also expresses complement control proteins (negative regulators), such as complement decay accelerating factor (DAF), C4b-binding protein (C4BP), and factor H (fH), which function to inhibit convertase formation on uninfected cells.

Recent evidence suggests that the complement system is essential for bridging innate and adaptive immunity. Complement receptor 2 (CR2) found on B cells can recognize complement split products, such as C3d, and binding is capable of limiting the threshold required for B cell activation [296]. Similarly, complement mediated-opsonization of microorganisms by professional antigen presenting cells can alter antigen presentation to and activation of T cells [297]. The complement system has been shown to be important for resistance to infection by several viruses and in general is protective in WNV infection. Mice deficient for C3, C4, C1q, factor B, or factor D showed enhanced lethality following WNV infection highlighted by deficiencies in adaptive immunity [298, 299]. Taken together, all

three complement pathways are important for the control of WNV, but clear distinctions do exist in the mechanisms employed by the various pathways to modulate WNV infection. For examples, mice deficient in the alternative pathway display impaired CD8+ T cell responses, while mice deficient in either the classical or MBL pathway are impaired for both B and T cell responses [299].

Flavivirus Tactics to Subvert the Innate Immune System

As discussed in the above sections, the detection of WNV by the innate immune system and subsequent production of interferon, cytokines, and chemokines are critical for limiting infection in cells and for stimulating the adaptive immune response which ultimately clears the virus. On the other hand, flaviviruses have evolved intricate strategies to not only evade innate immune detection, but to also actively suppress its actions. Fredericksen and Gale reported that WNV is able to evade early detection by the innate immune system without directly antagonizing the PRRs, RIG-I and TLR3 [247]. These findings were recently expanded upon by two studies which reported that pathogenic strains of WNV evade innate immune detection and the induction of ISGs through reduced early viral RNA synthesis. The delay was found to be controlled by the non-structural proteins of the pathogenic strains of the virus [105, 300]. Overby et al. also observed a delay in innate immune responses to TBEV infection that was linked to the ability of the virus to conceal dsRNA within intracellular membrane vesicles [301]. In addition to these studies, it was also demonstrated that WNV infected cells are unable to respond to exogenous IFN treatment, suggesting that the virus is able to directly antagonize signaling through the INFR. Currently, all known

vector-transmitted flaviviruses studied have been found to inhibit INFR signaling. Evans et al., observed that WNV infection of HeLa and Vero cells initiates depletion of the IFN-α receptor subunit 1 (IFNAR1) resulting in blunted responses to IFN treatment [302]. The mechanism utilized by WNV to deplete IFNAR1 in these cells remains to be discovered.

Due to the limited coding capacity of the relatively small flavivirus genome, numerous virus proteins have evolved multiple functions and many are capable of inhibiting the innate immune system. These proteins have largely been identified and characterized through over expression studies and will be discussed below.

Capsid protein

The structural capsid protein of WNV has been shown to bind to the host nuclear protein, protein phosphatase 2A (PP2A) inhibitor, I_2^{PP2A} (a potent inhibitor of PP2A) [131]. Binding of C to this phosphatase inhibitor requires the C-terminal half of the C protein and increases PP2A (pro-apoptotic phosphatase) activity while reducing the activation of AP-1. Importantly, knock down of I_2^{PP2A} resulted in decreased WNV production without inducing apoptosis, suggesting that the C protein of WNV has an important role in inhibiting AP-1 activation to increase virus production [131].

Envelope protein

The other major structural protein of WNV, E, is known to affect innate immune signaling. Insect cell-derived recombinant E protein is able to inhibit TLR3 signaling in primary macrophages exogenously treated with the protein. In contrast, recombinant E

protein derived from mammalian cells is unable to inhibit TLR3 signal transduction [303]. Inhibition was mapped to RIP1 in the TLR3 signaling cascade and was dependent upon the presence of high mannose *N*-linked glycans on the surface of the E protein produced in insect cells as opposed to complex glycans produced in mammalian cells. These studies suggest that the WNV E protein is capable of modulating innate immune responses to infection at the very earliest point of infection. However, the ability of the E protein to inhibit TLR3 signaling would be lost as the virus disseminates within the host and its contributions to pathogenesis in this context may be minimal.

NS1 protein

The NS1 proteins of several flaviviruses are capable of inhibiting complement activation. WNV NS1 inhibits the alternative complement pathway by binding to and recruiting factor H to degrade C3b (via factor I) on the cell membrane. Degradation of C3b in turn reduces MAC formation on cells [304]. The NS1 proteins of WNV, DEV, and YFV inhibit the classical and MBL pathways through two mechanisms. The first mechanism involves direct binding of NS1 to C4 and C1s/proC1s in solution. The interaction between NS1 and C1s enhances its serine protease activity leading to the cleavage of C4 to C4b in plasma [305]. The other mechanism requires binding of NS1 to C4BP followed by recruitment to C4b leading to its inactivation in solution and on the cell membrane [306]. The ability of DEV NS1 to bind complement proteins has recently been shown to be dependent upon proper glycosylation and is mediated by the hexameric form of the protein [157].

In addition to its roles in complement inactivation, the NS1 protein of WNV is able to block signal transduction originating from TLR3. Initial studies performed with WNV infected cells demonstrated that the virus was able to inhibit TLR3 signaling in response to pIC, and resulted in reduced IFN-β transcription and failure of IRF3 to translocate to the nucleus. This phenotype was also found for WNV replicon-bearing cells, suggesting that one or more of the non-structural proteins was responsible for antagonizing TLR3 signal transduction [307]. Later, Wilson et al implicated the WNV NS1 protein in preventing IRF3 phosphorylation and NFκB activation, and blocked IFN-β gene transcription in response to pIC treatment [308]. Signal transduction studies performed in multiple cell types indicated that the level of NS1 inhibition is at the adaptor molecule TAK1. NS1-expressing cells fail to phosphorylate TAK1 in response to pIC stimulation, however NS1 was not found to bind TAK1 and the TAK1/TAB2 complex was able to form in cells over expressing these signaling intermediates. Thus, NS1 most likely indirectly modulates TAK1 phosphorylation through an undetermined mechanism (unpublished data and Wilson, JR Dissertation 22, March 2011 http://www.lib.ncsu.edu/resolver/1840.16/6717). Most recently, the secreted form of the NS1 protein was found to inhibit TLR3 signaling in naïve cells treated with the protein (unpublished data and Crook, KR Dissertation: 15, June 2012 http://www.lib.ncsu.edu/resolver/1840.16/7985). TLR3 inhibition in these cells was found to be NS1 dose-dependent and could be abolished by preventing binding of NS1 to the cell membrane. Additionally, mice pretreated with NS1 displayed blunted cytokine induction in local draining lymph nodes in response to pIC treatment or WNV virus replicon particle challenge (WNV VRPs).

NS2A protein

The NS2A proteins of several flaviviruses have been shown to inhibit IFNR signaling in response to exogenous IFN treatment. Initially, Munoz-Jordan and colleagues found that the over expression of the DEV NS2A protein in human A549 cells enhanced the replication of Newcastle disease virus, an interferon-sensitive virus [309]. Work completed by Jones et al., also demonstrated that DEV replicon-bearing cells allowed for the replication of IFN-sensitive encephalomyocarditis virus [310]. Replicon-bearing cells also inhibited IFN-α-stimulated gene expression, but not IFN-γ-stimulated gene expression. STAT2 protein levels, important for IFN-α signaling, were greatly reduced in replicon-bearing cells compared to vector control cells. Liu et al described a role for Kunjin virus NS2A protein in the inhibition of IFNR signaling. An adaptive mutation in the NS2A protein (A30P) of a Kunjin virus replicon, acquired in IFN-deficient BHK cells, allowed the replicon to induce IFN-β gene transcription significantly above the levels observed for wt Kunjin replicon-bearing cells [311]. Ectopic expression of wt Kunjin NS2A, but not A30P NS2A inhibited Semliki Forest virus-induced IFN-β transcription, and A30P NS2A Kunjin virus replicated to wt virus levels in IFN-deficient cells but was abortive in IFN-competent cells [312]. Further, this mutant virus was highly attenuated in a mouse model of infection. Interestingly, the mutant virus was also partially attenuated in IFN-α/β/$\gamma^{-/-}$ mice indicating that NS2A may block other innate immune pathways independent of IFN. Most recently, the JEV NS2A protein was implicated in the antagonism of PKR activation [313]. JEV NS2A inhibits PKR activation and eIF2α phosphorylation to prevent translational inhibition and cell death. A T33I mutation

in the protein abolishes this phenotype and viruses harboring the change are attenuated for growth both *in vitro* and *in vivo*.

NS2B protein

NS2B protein of Kunjin virus was also identified as an antagonist of IFNR signaling. Expression of this protein alone prevented nuclear translocation of STAT2 following IFN-α treatment [314]. In a similar screening approach, the NS2B protein of DEV was not found to antagonize IFNR signaling [309].

NS3 protein and NS2B/3 protease

Similar to the above findings, the NS3 protein of Kunjin virus, not DEV, is capable of blocking STAT2 activation in response to exogenous IFN-α treatment through an unknown mechanism [314]. It is also unclear the differences between the two proteins that define this phenotype. Recently, adaptive mutations in the WNV NS3 (S365G) and 2K (V9M) genes were identified which promoted viral resistance to the antiviral actions of OAS [315]. The mutations were identified in WNV that was serially passage in mouse MEFs expressing the functional *Oas1b* gene, and the NS3 change, which resides in the helicase domain, improves ATPase activity of the protein. Both mutations were important for the resistance phenotype, indicating that NS3 protein can contribute to suppression of innate immune responses originating from the ISG OAS and its functional counterpart, RNase L. Most recently, the NS2B/3 protease of DEV was implicated in attenuating the induction of IFN in infected DCs and A549 cells. The human mitochondrial membrane-associated protein, stimulator of

interferon genes (STING), was shown by two independent groups to be a target for cleavage by the DEV NS2B/3 protease [316, 317]. Importantly, STING is an adaptor molecule that is phosphorylated by TBK1 and allows it to interact with RIG-I and IPS-1 to activate IRF3. Mouse STING lacks the NS2B/3 cleavage site and is resistant to degradation, suggesting a possible mechanism for mouse resistance to DEV infection. This new function ascribed to proteases of the *Flavivirus* genus is similar to that observed for the *Hepacivirus* protease, NS3/4A. NS3/4A of HCV is capable of cleaving both IPS-1 and TRIF to inhibit IFN induction [318, 319]. Similarly, the *Pestivirus* NPro autoprotease is capable of preventing IRF3 from binding DNA in the nucleus to induce IFN transcription [320]. Taken together, the proteases of several flaviviruses prevent IFN induction and signaling through discrete mechanisms.

NS4A protein

The NS4A proteins of Kunjin and DEV virus were both determined to enhance the replication of an interferon sensitive virus through the inhibition of IFN-β-stimulated gene transcription, by preventing STAT2 nuclear translocation [309, 314]. Like NS2A protein, the mechanism of NS4A inhibition is currently unknown. NS4A of JEV was also demonstrated to block ISRE-dependent gene transcription (or ISG transcription) following IFN-α or IFN-β treatment [321]. Cells expressing JEV NS4A consistently blocked the phosphorylation of STAT1 and STAT2 proteins. It was also shown that JEV NS4A interacts with the host cell DEAD-box RNA helicase DDX42 and that over expression of this helicase can prevent NS4A-mediated IFNR signaling inhibition.

NS4B protein

The NS4B proteins of both DEV and Kunjin virus block STAT1 and STAT2 nuclear translocation and prevent transcription of ISRE genes following type I or II IFN treatment [309, 314]. Munoz-Jordan and colleagues expanded these findings to include WNV and YFV NS4B proteins in IFNR inhibition [322]. The N-terminus of the NS4B protein appears to be critical for IFN inhibition and co-expression with NS4A enhances its immunomodulatory function. Further, the inhibitory action of NS4B requires the presence of the 2K peptide, which supplies the signal sequence and is important for proper processing of the protein at the ER membrane. Interestingly, Evans and Seeger reported that mutation of WNV NS4B at amino acid residues E22A and K24A relieved INFR receptor inhibition in replicon-bearing cells but the phenotype was lost in mutant virus expressing the changes [323]. These findings suggest that other non-structural protein may play more dominant roles in INFR inhibition. The NS4B protein of YFV has also been implicated in preventing IFN expression by blocking STING signaling [324]. The sequence of YFV NS4B has strong homology with STING and modulation of its activity is thought to be mediated by a direct protein:protein interaction.

NS5 protein

The NS5 proteins of WNV, JEV, TBEV, and Langat virus have all been demonstrated to suppress IFN-mediated signaling [325-328]. Notably, Kunjin and DEV NS5 do not share this function [314]. Park et al. noted that a single amino acid change, S653F that differs between WNV and Kunjin NS5 is responsible for the two phenotypes. Kunjin virus

expressing S653F NS5 is a potent IFN antagonist and is more resistant to the effects of IFN treatment, while WNV expressing F653S losses its inhibitory function [325]. Langat NS5 inhibits STAT1 phosphorylation and blocks ISRE and IFN-γ-activated gene transcription by directly binding to the IFNAR2 and IFNGR2 subunits [328, 329]. Surprisingly, residues critical for Langat IFNR inhibition are localized to the RdRp domain (residues 332-735), but may be different for other flavivirus NS5 proteins. Indeed, the mechanism employed by the closely related TBEV NS5 protein appears different than Langat NS5. Two regions of residues within the TBEV NS5 protein MTase domain bind to the PDZ protein scribble (hScrib) at the plasma membrane and inhibit STAT1 phosphorylation thereby blocking type I and II IFN responses [327]. Mutation of the PDZ binding regions resulted in attenuation TBEV replication suggestion that they may be important for replication and pathogenesis [330]. The DEV NS5 protein has also been found to induce the transcription and secretion of IL-8, and it has been proposed that this may promote virus dissemination through the recruitment of immune cells to the site of infection [331].

Current Studies

The initial discovery of WNV in the late 1930s prompted research efforts to understand the virus and have lead to significant advances in our knowledge of the disease cycle, viral pathogenesis, and host immune responses. The WNV non-structural proteins are important for viral genome replication and many have also evolved additional functions to inhibit various aspects of the host innate immune response. Research performed in our lab lead to the discovery that the NS1 protein of WNV actively inhibits the induction of antiviral

responses elicited by TLR3, by preventing the activation of the transcription factors NFκB, AP-1, and IRF3. Currently, the mechanism by which NS1 is able to suppress TLR3 signaling is not known, but is thought to involve TAK1. In the present study we aimed to define amino acid residues in the NS1 protein that are important for TLR3 inhibition. Importantly, the NS1 protein also plays an essential role in virus genome replication through an undefined mechanism.

Study 1

Given the multifunctional nature of the NS1 protein, we developed a fluorescent-activated cell sorting (FACS)-based screen to identify randomly generated mutations in the NS1 protein which restore TLR3 signaling, without compromising its function in virus replication. A random mutagenesis strategy was chosen as the three-dimensional structure of the NS1 protein has not been resolved, and our ability to make targeted mutations was significantly limited by the available literature. We are the only group to date that has reported on a role for NS1 in inhibiting TLR3 signal transduction.

The screen was achieved by first assembling a HeLa reporter cell line in which expression of the red florescent protein, DsRed2, is controlled by the transcription factor NFκB. These cells were then stably transduced with lentivirus particles which ectopically express a library of random NS1 mutant proteins. Thus, TLR3 signaling in these cells could be measured following TLR3 stimulation. Replication competency of the mutant NS1 proteins was also measured by infecting cells with WNV GFP virus replicon particles (VRPs), which contained a lethal in-frame deletion in the NS1 gene (WNV GFP ΔNS1

VRPs). Replication and GFP expression by these VRPs is dependent upon the presence of replication competent NS1 protein.

Several NS1 mutants were identified by the screen and the amino acid changes were further characterized through ectopic NS1 protein expression in the lentivirus vector and through genetic incorporation of the changes into a clone of WNV. Ultimately, we identified three amino acid residue changes in the NS1 protein that individually restored TLR3 signaling in response to stimulation, while still allowing the virus to replicate and produce virions. Interestingly, the residues identified as being important for TLR3 inhibition lie within the C-terminal half of the protein and each residue is completely conserved among mosquito-borne and tick-bore flaviviruses.

Study 2

We also undertook molecular and biochemical studies of the mutant NS1 proteins to better understand the defects which allow TLR3 signaling to occur. We noted that several of the amino acids changes affected antibody recognition of the proteins, altered protein stability, *N*-linked glycosylation, cell surface expression/association, and secretion from cells. However, we also determined that TLR3 inhibition by wt NS1 is not dependent upon glycosylation, cell surface expression/association, or secretion of the protein. Taken together, the NS1 amino acid changes identified by the screen resulted in improper processing of the proteins within cells, which culminated in an inability of the proteins to block TLR3 signaling.

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CHAPTER 2

Identification of amino acid residues important for TLR3 inhibition by the multifunctional West Nile virus NS1 protein

Abstract

West Nile virus is a mosquito-transmitted pathogen which causes significant morbidity and mortality in humans. The innate immune system is a first-line defense against invading microorganism and many flaviviruses, including West Nile virus, have evolved multifunctional proteins which actively suppress its induction and block its antiviral actions. The West Nile virus non-structural protein 1 (NS1) is able to inhibit signal transduction originating at Toll-like receptor 3 (TLR3), through an undefined mechanism. In this study we developed a screen to identify amino acid residues in the NS1 protein which are important for TLR3 signaling inhibition. Amino acid changes G295R, P320S, and M333V of NS1 were independently identified by the screen and restored TLR3 signaling in virus-infected cells. These residues are completely conserved among several mosquito- and tick-borne flaviviruses, suggestion that they are of biological importance to the virus.

Introduction

The *Flaviviridae* family includes several arthropod-borne viruses, which are important human pathogens, such as dengue virus, yellow fever virus, and West Nile virus. West Nile virus (WNV) is a mosquito-transmitted virus that can cause febrile illness, severe encephalitic disease, and death in humans and other vertebrates, and is endemic to regions of Africa, Europe, and North America. The positive-sense RNA genome of WNV codes for three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), which are expressed as a single large polyprotein that is co- and post-translationally processed into the ten individual proteins [1]. The structural proteins arrange in icosahedral geometry to form mature virions and package the infectious genome, while the non-structural proteins are required for viral RNA replication and interact with a multitude of cellular pathways. The amount of different functions that can be carried out is limited by the relatively small coding capacity of the approximately 11kb flavivirus genome. One solution many viruses have adapted to overcome this limitation is to evolve multifunctional proteins. Several examples of multifunctional replication proteins that also interfere with the immune response to infection can be found within the *Flaviviridae*.

For example, the Hepatitis C virus NS3 protein exhibits RNA helicase activity and protease activity required for polyprotein processing in conjunction with its cofactor NS4A [2]. In addition, protease activity also functions to cleave the innate immune adaptor proteins TIR-containing, adaptor-inducing IFN-β (TRIF), and IFN-β promoter stimulator-1 (IPS-1), which transmit signals from the pattern-recognition receptors (PRR) Toll-like receptor 3 (TLR3) and TLR4 and retinoic acid-inducible gene I (RIG-I) and melanomadifferentiation-

associated gene 5 (MDA5) [3, 4]. The NS5 protein is the RNA-dependent, RNA polymerase and has been shown for several flaviviruses to also inhibit signal transducer and activator of transcription 1 (STAT1) signaling originating from the type I interferon (IFN) receptor by different mechanisms depending on the virus species [5-11]. The dengue, West Nile, and Kunjin virus NS2A, NS4A, and NS4B proteins, whose function in RNA replication is not well understood, are also involved in interfering with Janus-activated kinases (JAK) JAK-STAT signaling from the type I IFN receptor [12-16]. Inhibition of these innate immune signaling pathways decreases both type I interferon (IFN) and cytokine production and type I IFN responses in infected cells, which normally lead to interferon-stimulated gene (ISG) expression. Cytokines, such as IL-6, are important for the acute phase response to infection, can induce fever, and support the growth and maturation of B cells [17, 18]. The collective function of these non-structural proteins blunts the establishment of an antiviral state in the host cell and allows for increased virus dissemination within the host.

Non-structural protein 1 (NS1) is a glycoprotein that is expressed intracellularly and secreted from infected cells. A hydrophobic signal sequence derived from the C-terminus of the E protein promotes NS1 translocation into the lumen of the endoplasmic reticulum (ER) following polyprotein translation. This signal sequence is subsequently cleaved by cellular signal peptidases [1]. NS1 contains three *N*-linked glycosylation sites and twelve conserved cysteine residues. NS1 forms heat-labile but detergent-resistant dimers and the secreted form has also been reported to bind to and be endocytosed by naive cells. This is achieved through cell surface interactions between NS1 and the endothelial cell glycosaminoglycans, heparan sulfate and chondroitin sulfate E [19, 20]. While its function during virus replication is not

completely understood, it has been established that NS1 is absolutely required for RNA replication and plays a role in early negative-sense RNA synthesis [21-24]. Recent work by Youn et al. has demonstrated a direct protein:protein interaction between the NS1 and NS4B proteins in infected cells, which may facilitate signaling with the replication complex [25]. NS1 has recently been implicated in various aspects of flavivirus pathogenesis such as binding to and promoting the degradation of complement factors [26-28]. However, antibodies to NS1 aid the clearance of infected cells by complement-mediated phagocytosis and NS1-specific antibodies have been shown to confer a protective effect when administered prior to viral challenge [29-31].

TLR3 is an evolutionarily conserved PRR that recognizes dsRNA, a replication intermediate of many RNA viruses and a component of secondary RNA structures found in viral genomes. TLR3 activation in endosomes leads to the induction of pro-inflammatory cytokines and type I IFN expression, and induces an antiviral state in cells [32, 33]. We have previously reported that NS1 expressed by itself in HeLa and HEK293-TLR3 cells, and NS1 expressed during WNV infection is able to inhibit TLR3 [34, 35]. Aside from the HCV protease, Pestivirus N(pro), and dengue virus protease, which target IPS-1, interferon regulatory factor 3 (IRF3), and stimulator of interferon genes (STING), respectively, NS1 is so far the only other flavivirus non-structural protein to interfere with PRR signaling [36-38]. In this study we aimed to identify amino acids of NS1 that are required for TLR3 inhibition. Traditional approaches, such as progressive truncation of the NS1 protein, neglect the indispensible role of NS1 in genome replication. Due to the multifunctional nature of NS1, a screening approach based on *trans*-complementation was developed that allows selection of

randomly generated NS1 mutants that are 1) still able to support viral RNA replication while being 2) defective in TLR3 inhibition. We successfully identified three amino acid changes within NS1 mutants that fulfilled these criteria to varying degrees.

Further characterization of the mutants revealed that amino acids changes at positions G295, P320, and M333 of NS1 restored TLR3 signaling while allowing virus genome replication. These amino acids lie within the C-terminal half of the NS1 protein, which is more conserved than the N-terminal half, and each of these residues is evolutionarily conserved among mosquito-borne and tick-borne flaviviruses. Severe growth defects in infected Vero and HeLa cells were observed with recombinant WNV expressing either the G295R or M333V NS1 mutations, whereas P320S NS1 WNV replication kinetics were only reduced in HeLa cells. Finally, we determined that HeLa cells infected with each WNV mutant fully respond to treatment with pIC.

Materials and Methods

Construction of VSV-G Pseudotyped Lentivirus Particles Expressing NS1 (pLEX-NS1)

Full length NS1, including the last 30 amino acids of the E protein and a C-terminal 6X histidine tag was cloned into the pLEX vector (ThermoScientific /OpenBiosystems) and pLEX-NS1 was packaged into VSV-G pseudotyped, replication-defective, lentivirus particles through co-transfection of HEK 293T cells with vector, packaging (pREV and pMDLg), and envelope (pVSV-G) plasmids. Empty vector (pLEX-MCS) was packaged identically.

Lentivirus-containing supernatants were harvested and clarified 40h post-transfection, and stored at -80°C until further use.

Construction of VSV-G Pseudotyped Lentivirus Particles Expressing Mutant NS1 (pLEX-mNS1)

NS1 mutants (mNS1) were generated by error-prone PCR of the pLEX-NS1 plasmid using a Gene MorphII EZ clone kit (Agilent Technology/Stratagene), according to the manufacturer's instructions, with the following primers; mutFw 5' AAT TCG CCC TTG AAT CGG ATC C 3' and mutRev 5' GGT GAT GGT GAT GAT GAC CGG T 3'. The input amount of template DNA was selected to achieve a mutation frequency between 1nt and 2nt change(s)/kb. The E signal sequence and NS1 are 1146nt in length. Total transformed bacteria were expanded in 250ml of 2XYT broth (Fisher) containing 100µg/ml ampicillin (Sigma), plasmid DNA was purified using a Maxi prep kit (Qiagen), and packaged as lentivirus particles (pLEX-mNS1) as described previously.

Construction of HeLa NFκB-ELAM DsRed2 Reporter Cell Line (HeLa-NFκB-DsRed2)

The CMV promoter of the pDsRed2-C1 plasmid (Clontech) was removed by digestion with *PciI* and *AgeI* restriction endonucleases. The 5X NFκB-ELAM promoter of the pNiFtY-SEAP plasmid (Invivogen) was PCR amplified with the following primers; NFκB5XELAM Fw (***PciI***) 5' ATT AT**A CAT GT**A TCG CTG AAT TCT GGG GAC T 3' and NFκB5XELAM Rev (***AgeI***) 5' ATT AT**A CCG GT**G GCT CTG TCT CAG GTC AGT A 3', subsequently digested with *PciI* and *AgeI* enzymes, and ligated into the pDsRed2-C1

plasmid (NFκB-DsRed2). HeLa cells were plated as semi-confluent monolayers in a 24-well plate and transfected (Mirus IT LT-1) with 500ng of NFκB-DsRed2 plasmid. A polyclonal cell population (HeLa-NFκB-DsRed2) was selected with 500µg/ml G418 (Lonza) and response to treatment with 20µg/ml Polyinosinic:polycytidylic acid (pIC) (Calbiochem) was confirmed by detection of DsRed2 with fluorescence microscopy and flow cytometry 36h later.

Construction of NFκB-ELAM Luciferase Reporter Cell Line (HeLa-NFκB-luc)

The NFκB 5X endothelial-leukocyte adhesion molecule-1 (ELAM) promoter of the pNiFty-SEAP plasmid (Invivogen) was PCR amplified using the following primers: NFκB5XELAM Fw (***KpnI***): 5' ATT AT**G GTA CC**A TCG CTG AAT TCT GGG GAC T 3' and NFκB5XELAM Rev (***HindIII***): 5' ATT AT**A AGC TT**G GCT CTG TCT CAG GTC AGT A 3' The 278bp amplicon was digested with *KpnI* and *HindIII* restriction endonucleases and ligated into the multiple cloning site of the pGL4.15 vector (Promega) using *KpnI* and *HindIII* sites upstream of the luciferase reporter gene. HeLa cells were transfected via lipofection (Mirus IT LT-1) with the reporter construct (500ng) and a stable, polyclonal cell line was selected by 400µg/ml hygromycin (Cellgro). Responsiveness of the HeLa NFκB-luc cell line was confirmed by luciferase assay following 8h treatment with 20µg/ml pIC.

VSV-G Pseudotyped Lentivirus Particle Transduction and Titration

For creation of a random mutant NS1 reporter cell library (HeLa-NFκB-DsRed2-mNS1), HeLa-NFκB-DsRed2 cells were transduced at an MOI of 0.02 with pLEX-mNS1 particles to ensure fewer than 1 integration event per cell. HeLa-NFκB-DsRed2 cells were plated in 6-well plates as semi-confluent monolayers and overlaid with 2ml of 1+1+1 DMEM (1X DMEM (CellGro) with 1% Pen/Strep (CellGro), 1% HEPES buffer (CellGro), 1% FBS (Gemini)), containing 8µg/ml polybrene (Sigma), and lentivirus-containing supernatant. Medium was changed 24h post-transduction to 1X DMEM with 10% FBS and cells were placed under puromycin selection (2µg/ml) (Gemini) 48h later. HeLa-NFκB-DsRed2 cells were also transduced with pLEX-NS1 and pLEX-MCS lentivirus to serve as controls.

Lentivirus titers were determined by counting crystal violet-stained, puromycin-resistant HeLa cell colonies following transduction with serial dilutions of lentivirus-containing supernatants.

Construction, Packaging, and Titration of ΔNS1 GFP VRPs

A GFP-expressing WNV replicon (WNV GFP rep, a gift from P.W. Mason), based on WNR C-NS1-5 with an insertion of GFP and the FMDV 2A autoprotease coding sequences between the capsid and the signal sequence preceding NS1 served as a backbone for introduction of a lethal 138bp (46aa) in-frame deletion in NS1. Initially, the WNV GFP rep plasmid served as template for PCR to amplify the final 60bp of NS1, (incorporating a *BstEII* restriction site at the 5' end the amplicon) along with full-length NS2A (which contains a unique *XmaI* restriction site), using the following primers; delNS1 ***BstEII*** Fw 5'

ACG T**GG TCA CC**A TGG AGA TCA GAC CAC AGA GAC ATG 3' and delNS1 NS2A Rev 5' TCT GGC ACA TCG TAT GGG TAG CGT TTA CGG TTG GGA TCA CAT GC 3'. The 790bp amplicon was subsequently digested with *BstEII* and *XmaI* restriction endonucleases and ligated into the *BstEII* and *XmaI* restriction sites of WNV GFP rep DNA, and transformants (WNV ΔNS1 GFP rep) were confirmed by standard sequencing.

WNV ΔNS1 GFP rep DNA was linearized by *XbaI* digestion followed by phenol:chloroform extraction and RNA transcription using an *in vitro* transcription reaction kit (Ambion) incorporating m7GpppAmpN2 cap analogue (NEB), according to the manufacturer's instructions. BHK packaging cells expressing WNV C, PrM, E, and NS1 (BHK-Pack NS1, a gift from P.W. Mason) from a non-cytopathic Venezuelan equine encephalitis alphavirus (VEE) replicon, were electroporated with the *in vitro* transcribed WNV ΔNS1 GFP rep RNA. WNV ΔNS1 GFP VRP-containing supernatants were collected every 24 in 1+1+1 MEM medium, clarified, and stored at -80°C until further use. For titration, wt NS1 expressing HeLa cells were infected for 24h with WNV ΔNS1 GFP VRPs and GFP positive cells were quantified by flow cytometry.

Screen Conditions

HeLa-NFκB-DsRed2-pLEX-mNS1 cells were plated at 2.5 X 10^6 total cells in a T-75 flask and allowed to grow for 24h, reaching approximately 50% confluency. Medium was removed and cells were infected at an MOI of 1 with WNV ΔNS1 GFP VRPs in 1+1+1 DMEM for 24h. Infected cells were treated with 20μg/ml pIC in 1+1+1 DMEM for an additional 36h. Cells were removed from the flask by trypsinization, washed, and double-

positive cells (GFP and DsRed2) were sorted by Fluorescence-activated Cell Sorting (FACS Cytomation). Sorted cells were washed twice in antibiotic containing medium, counted, and plated at low density in 10cm cell culture dishes. Cells were maintained in medium containing 2µg/ml puromycin and 500µg/ml G418, and surviving colonies were isolated and expanded. The phenotypes of the resulting clonal cell lines were confirmed by infecting with an MOI of 1 with WNV ΔNS1 GFP VRPs or treating with 20ug/ml pIC and monitoring GFP or DsRed2 expression by fluorescence microscopy.

Genomic DNA was purified from the clonal cell lines using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Integrated mNS1 was PCR amplified using the following primers; CMVIEFw 5' ACT TAC GGT AAA TGG CCC GC 3', IRESRev and 5' AAA GGA AAA CCA CGT CCC CG 3' and purified amplicons were sequenced.

Site-Directed Mutagenesis

NS1 point mutations identified in the clonal cell lines were reconstituted individually or as pairs in the pLEX-NS1 vector, using the PCR based Quick Change II XL kit (Agilent Technologies/Stratagene). Site-directed primer pairs were designed using the Quick Change primer design software according to the kit manufacturer. Each primer pair was designed to incorporate a single nucleotide change resulting in a single point mutation. Fidelity of the mutagenesis was confirmed by standard DNA sequencing. Reconstituted mutants were packaged as lentivirus particles and used to produce stable, polyclonal mutant NS1-expressing HeLa NFκB-luc cells as described previously.

NFκB-luciferase Reporter Assays

HeLa-NFκB-luc cells expressing the reconstituted NS1 mutants were plated overnight at 4 X 10^4 cells total per well in a 48-well tissue culture plate. Triplicate wells containing each cell line were either treated with 20µg/ml pIC or left untreated for 8h. Supernatants were removed and kept at -80°C for IL-6 ELISA (described below). Cells were washed once with PBS and lysed for 30min in lysis buffer (0.3M NaCl, 0.05M TrisHCl, 0.1%TritonX-100) at -20°C. Lysates were centrifuged at 14K RPM for 10min to pellet cell debris and 20µl of the supernatant was loaded into the wells of a 96-well luminometer plate, alongside 10µl of each serial diluted luciferase standard. 75µl of luciferase reagent (Promega) were added to each well and light emission was measured on a luminometer (Berthold) as Relative Light Units/ml (RLU/ml). The RLU/ml of each sample was then normalized to total protein in the whole cell lysate (RLU/ml/µg protein), as determined by a commercial protein assay kit (BioRad). Data were calculated as fold NFκB induction over mock.

ELISA

Cell culture supernatants were collected as described and assayed for human IL-6 (Ebioscience) using a commercial ELISA kit according to the manufacturer's instructions. Absorbance at 450nm was determined by a microplate reader (BioTek) and IL-6 concentrations were calculated based on a standard curve.

Immunoblotting

Whole cell lysates were prepared in lysis buffer. Equivalent amounts of protein (5µg) were mixed with LDS buffer (Invitrogen) containing 2.5mM DTT, heated at 90°C for 5min, and loaded into 4-12% Bis-Tris NuPAGE gels (Invitrogen). Proteins were electrophoretically separated at 150V for 3h, transferred to a PVDF membrane (Immobilon Millipore) at 30V for 1.5h, blocked with 5% milk in 1X Tris-buffered saline-0.1% Tween-20 (TBST), and probed with one of the following primary antibodies: 1:4000 dilution of mouse anti-tetra-HIS monoclonal antibody (34679; Qiagen) or 1:10000 dilution of mouse anti-β-actin monoclonal antibody (A1978; SIGMA). Following three 5min washes in TBST, membranes were incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) secondary antibody (474-1806; KPL). Following another round of washes, membranes were incubated with an enhanced chemiluminescence detection system as recommended by the manufacturer (Santa Cruz Biotechnology) and visualized by exposing autoradiographic film (Blue Devil) to the blots.

Flow Cytometry

Detection of DsRed2 and GFP

Following the indicated treatments, HeLa cells were trypsinized, resuspended in 1X DMEM medium with 10% FBS, washed with PBS, and 10,000 events per treatment group were recorded on a BD FACS Calibur flow cytometer using CellQuest Pro (BD) software. Data were analyzed and displayed with WinMDI 2.8 software (The Scripps Institute).

Detection of WNV-infected cells

HeLa cells were infected with WNV or mutant WNV using the indicated conditions, trypsinized, resuspended in 1X DMEM medium with 10% FBS, and washed once with 250µl flow staining buffer (1X PBS, 1% BSA, 1% Sodium Azide). All other steps were performed in the dark on ice. Cells were fixed and permeabilized for 20min with 200µl cytofix/cytoperm solution (BD), washed three times with cytofix/cytoperm wash buffer (BD) and then incubated for 1h with a 1:1000 dilution of mouse anti-dengue virus E protein monoclonal antibody (D14G2) in cytofix/cytoperm wash buffer. Cells were washed in cytofix/cytoperm wash buffer, incubated for 1h with 200µl of a 1:1000 dilution of secondary goat anti-mouse IgG (H+L) AlexaFluor-488-conjugated antibody (Invitrogen) in cytofix/cytoperm wash buffer, followed by additional washes in cytofix/cytoperm wash buffer, then resuspended in flow staining buffer prior to analysis. 5,000 events per treatment group were recorded on an AccuriC6 flow cytometer using CFlow software (BD Accuri).

Reconstitution of NS1 Mutations into a West Nile virus Infectious Clone

PCR-mediated, site-directed mutagenesis, as described earlier, was used to clone the identified idividual changes at NS1 amino acid positions 295, 320, and 333, respectively, into an infectious cDNA clone of West Nile virus of a human 2002 isolate from Texas [39]. Fidelity of the mutagenesis was confirmed by standard DNA sequencing of NS1 in the cDNA clones, using the primer set listed below. Plasmid DNA was linearized by *XbaI* digestion followed by phenol:chloroform extraction and *in vitro* RNA transcription was carried out as mentioned for VRPs. Wild type virus as well as the WNV mutants were

produced in BHK cells following electroporation of *in vitro* transcribed RNAs, and virus was captured in the supernatants of infected cells every 24h for 120h post-infection. Virus-containing supernatants were clarified, aliquoted, and stored at -80°C prior to use (see below).

The coding sequence of NS1 of each WNV mutant was assessed following virus production in BHK cells to determine the stability of the mutations. One hundred microliters of each passage 0 (P0) virus stock was used to extract total RNA using an RNeasy mini kit (Qiagen) as specified by the manufacturer. Total virus RNA was quantified by a Nanodrop ND1000 spectrophotometer, and 20ng of each virus RNA was used in an RT-PCR containing random primers (Promega). Two microliters of each cDNA were used as template in a PCR containing AccuTaq (Sigma) to amplify a 1198bp fragment containing full-length NS1, using the following primers: WNVmNS1SEQFw 5' TGC TCG TGA CAG GTC CAT AGC TCT 3' and WNVmNS1SEQRev 5' CTG GGT GGC CAA GAA CAC GAC C 3'. The NS1 gene from wt WNV was identically amplified and sequenced as a control. A small volume of the PCR was used to visualize amplicons on an agarose gel and the remaining PCR product was purified by standard salt:ethanol precipitation prior to DNA sequencing with the primers listed above. Equivalent volumes of P1 virus containing supernatants, collected 96h post-infection from the Vero and HeLa cell growth curves, were sequenced identically (described below).

Titration of WNV

WNV was titered on monolayers of Vero cells in 48-well plates. Virus was allowed to attach to the cells for 1h with rocking every 15min, and cells were overlayed with 1X MEM/1X Tragacanth Gum and allowed to incubate for 48h. Cells were washed with PBS then fixed with a 1:1 ratio of acetone and methanol. The fixed cells were blocked with 1% Normal Horse Serum (NHS) (Gemini Bioproducts) in PBS (1%NHS/PBS) for 10min. E protein in infected cells was detected by incubating cells at room temperature with a 1:500 dilution of D14G2 monoclonal antibody in 1%NHS/PBS for 1h followed by washing three times in PBS and secondary antibody detection with a 1:1000 dilution of horseradish peroxidase-conjugated (HRP) goat anti-mouse IgG (H+L)in 1%NHS/PBS for 1h. Immunofoci were visualized using a colorimetric HRP detection kit according to the manufacturer's instructions (Vector Labs). Virus titer was expressed as focus forming units/ml (FFU/ml).

WNV Growth Kinetics

Multi-step virus growth curves were performed on Vero and HeLa cell monolayers in 24-well plates by infecting at an MOI of 0.01 with wt WNV or each WNV NS1 mutant. Following 1h of virus attachment, cells were washed with PBS then overlaid with 1ml of 1+1+1 MEM medium. Virus-containing supernatants were collected every 24h for 96h post-infection and stored at -80°C prior to titering on Vero cells. Virus containing supernatants were serially diluted in 1+1+1 MEM medium and titered on Vero cell monolayers using the titration method described above.

TLR3 Signaling in WNV-Infected HeLa cells

TLR3 signaling in HeLa cells infected with WNV was assessed following pIC stimulation. HeLa cells were plated as semi-confluent monolayers in 48-well plates and infected for 1h at an MOI of 5 with wt WNV or each WNV NS1 mutant. Virus inoculum was removed, cells were washed with PBS, and medium was replaced with 1X DMEM medium containing 10% FBS. Twenty hours post-infection medium was removed and replaced with 1X DMEM containing 10% FBS with or without 20µg/ml pIC and allowed to incubate for an additional 8h. IL-6 was quantified by ELISA. Infectivity of the viruses was assessed 28h post-infection by flow cytometry detection of the E protein in identically-infected cells (described above).

Results

Strategy

As outlined above, the overall goal of the mutational screen was to identify amino acid changes in the NS1 protein that allow TLR3 signaling, but do not compromise viral RNA replication. The strategy was based on 1) the ability of ectopically expressed wt NS1 protein to rescue replication of a WNV replicon containing a lethal deletion in NS1 (ΔNS1 GFP rep), and 2) on the ability of wt NS1 to inhibit TLR3 signaling. Figure 1 outlines the overall strategy. To successfully perform the screen, first a GFP-expressing WNV replicon with a lethal deletion in NS1 was created (WNV ΔNS1 GFP rep) and packaged into virus replicon particles (VRPs). Based on previous studies describing *trans*-complementation of NS1, this replicon would only be able to replicate and express GFP in the presence of wt NS1

or an NS1 mutant that is able to complement the defect [22]. A library of NS1 mutants was established in a recombinant lentivirus background to allow expression in a reporter cell line, in which DsRed2 is expressed under control of an NFκB-responsive promoter. DsRed2 is only induced by pIC stimulation in cells that express NS1 proteins defective for TLR3 inhibition. During the actual screen, reporter cells expressing NS1 mutants were infected with WNV ΔNS1 GFP VRPs and treated with pIC, subsequently the presence of GFP and DsRed2 double-positive cells indicates NS1 mutants of the desired phenotype.

Creation of an NFκB-dependent Red Fluorescent Protein Reporter Cell Line (HeLa-NFκB-DsRed2)

A reporter construct using the canonical NFκB response element and a synthetic ELAM promoter, controlling expression of DsRed2 was assembled and a stable HeLa reporter cell pool (HeLa-NFκB-DsRed2) was established. Background fluorescence was very low and inducible expression of DsRed2 was confirmed by fluorescence microscopy 36h post-treatment with pIC (data not shown). To create a system for ectopic expression of NS1, WNV NS1 cDNA was cloned into the pLEX lentivirus vector and recombinant lentiviruses (pLEX-NS1) were produced according to standard procedure. An empty vector control lentivirus was used as a negative control (pLEX-MCS). Stable cell lines were created after transduction of either HeLa-NFkB-DsRed2 cells or standard HeLa cells with either pLEX-NS1 or pLEX-MCS followed by antibiotic selection. HeLa-NFκB-DsRed2-pLEX-MCS and HeLa-NFkB-DsRed2-pLEX-NS1 cells were treated with pIC for 36h and DsReds expression

was analyzed by flow cytometry. Approximately 40% of pIC treated NFκB-DsRed2-pLEX-MCS cells expressed DsRed2 after 36h (Figure 2A). In agreement with our previously-published work, the number of DsRed2 positive cells was decreased by approximately 80% in wild type NS1-expressing cells after pIC stimulation (Figure 2A) [34, 35].

Trans-complementation of an NS1-deleted Replicon

A 138nt lethal in-frame deletion within the NS1 coding sequence of a GFP-expressing WNV replicon was introduced to create a replicon (WNV ΔNS1 GFP rep) whose replication was dependent on *trans*-expressed functional NS1. *In vitro* transcribed RNA of WNV ΔNS1 GFP rep was replication defective when transfected into naïve HeLa cells or HeLa-pLEX-MCS cells. In contrast, GFP expression from transfected WNV ΔNS1 GFP rep RNA was readily observed in HeLa pLEX-NS1 cells. The number of GFP positive cells remained constant over a 24h period, confirming that trans-expressed wt NS1 was able to sustain WNV ΔNS1 GFP rep replication (data not shown).

To facilitate delivery of WNV ΔNS1 GFP rep RNA to reporter cells, virus replicon particles were produced. A BHK packaging cell line (BHK-Pack NS1) was used in which the WNV C, prM, E, and NS1 proteins are expressed from a VEEV replicon. HeLa-NFκB-DsRed2-pLEX-MCS and HeLa-NFkB-DsRed2-pLEX-NS1 cells were infected with WNV ΔNS1 GFP VRPs for 24h. Only NS1-expressing cells, but not vector control cells, supported replication as evidenced by GFP detection by flow cytometry (Figure 2B) and immunofluorescence (data not shown). Taken together these results demonstrate that the individual reagents and cell lines necessary for the screen functioned as designed.

NS1 Mutagenesis

To produce a library of NS1 mutants, the coding sequence of NS1, as well as the upstream E signal sequence, in the pLEX vector, were randomly mutated using an error-prone PCR approach. Mutagenesis of the E signal sequence was included to address the possibility that signal sequence or signalase cleavage site mutants might alter TLR3 inhibition by the NS1 protein. Reaction conditions were chosen with the objective to introduce 1 or 2nt changes per NS1 copy. Ten independent bacterial clones were sequenced to verify the mutation frequency and to assess gene coverage. All clones were found to harbor at least two nucleotide substitutions located throughout the 1146nt NS1 coding sequence (data not shown). As an initial test prior to performing the entire screen, mNS1 cells from the library were plated and either infected with WNV ΔNS1 GFP VRPs or treated with pIC for 36h. Fluorescence microscopy confirmed the presence of cells expressing NS1 that supported *trans*-complementation as well as cells expressing NS1 that allowed TLR3 signaling (data not shown). It is important to note that WNV ΔNS1 GFP VRP infection for up to 72h did not induce NFκB activation by itself (data not shown).

Screen

To perform the screen, 2.5 X 10^6 mNS1 reporter cells were infected with WNV ΔNS1 GFP VRPs at an MOI of 1. GFP fluorescence in some cells was readily observable at 24h post-infection. The cells were subsequently stimulated with pIC for 36h and GFP and DsRed2 double-positive cells were sorted by FACS. The screen was performed twice using independently-established mNS1 reporter cell libraries in order to increase the chance of

selecting unique NS1 mutants. Approximately 3 X 10^4 double-positive cells were isolated and plated into 10cm culture plates at low density. 134 clones survived expansion. Phenotypes of clones were confirmed by independently monitoring DsRed2 expression following pIC stimulation and GFP expression post-infection with WNV ΔNS1 GFP VRPs. Ultimately, 18 clones were considered promising candidates and the NS1 sequence was determined, which result in 4 unique sequences (Table 1). There was redundancy in the sequences of several of the clones. This is most likely attributable to allowing the original mutant cell library to expand prior to the sort. Multiple independent selections of the same sequence confirm the reproducibility of our screen.

All NS1 sequences had at least two nucleotide substitutions resulting in point mutations. Notably, no clones were identified that encoded truncated or elongated forms of NS1, and no mutations were found in the E signal sequence. Identified mutations were reconstituted both individually as well as in combination in the pLEX NS1 sequence, and were packaged into recombinant lentiviruses. These lentivirus particles were used to transduce HeLa cell lines, which contained a luciferase reporter gene driven by the NFκB responsive ELAM promoter (HeLa-NFκB-luc). To verify that the mutations did not adversely affect protein expression, immunoblot analysis of cell lysates was performed (Figure 3A). All NS1 mutants, as well as wt NS1 were expressed to similar levels. The mutation at position 177 eliminates one of the NS1 *N*-linked glycosylation sites at position 175 (see below) explaining the increased mobility of NS1 mutants containing this substitution.

Ability of NS1 Mutants to *Trans*-complement

HeLa cell lines expressing NS1 with the individual and double mutations were infected with WNV ΔNS1 GFP VRPs for 36h and GFP expression was investigated by fluorescence microscopy and by flow cytometry to determine the mean fluorescent intensity of the GFP signal (Figure 3A and B). An arbitrary threshold of 25% of MFI observed with wt NS1 was set as a cutoff.

Trans-complementation by NS1 encoding the individual T177N mutation was similar to wt NS1, while the G295R mutation reduced *trans*-complementation only slightly (Figure 3A). The combined double mutant T177N/G295R showed the same level of *trans*-complementation as the single G295R mutant.

Similarly, replication competence of the N191I mutant remained at near wild type levels. Alternatively, the MFI was significantly reduced for the P320S mutant, but remained above the cutoff. *Trans*-complementation by the N191I/P320S double mutant was reduced even further to a level below the MFI cutoff.

Replication competencies of the E238V and N255D mutants mirrored those of the N191I and P320S mutants, where E238V behaved like wt NS1 and N255D was reduced to just below the threshold level. Similarly, the combination of E238V and N255D mutations further reduced ΔNS1 GFP VRP replication.

WNV ΔNS1 GFP VRPs replicated in HeLa cells expressing M333V NS1, but GFP intensity was diminished by approximately 50% compared with wt NS1 protein. *Trans*-complementation was further reduced in cells expressing the single T317I mutation and the T317I/M333V double mutant.

In summary, NS1 mutants T177N, N191I, and E238V *trans*-complemented at levels comparable to wt NS1. Some mutations, despite their identification in the screen, were severely impaired for *trans*-complementation (191/320,255, 238/255, 317, and 317/333), suggesting that these mutations, while not completely defective, are severely impaired in their support of genome replication. Several mutants *trans*-complemented at intermediate levels above the threshold fluorescence intensity (295, 177/295, 320, and 333) (Figure 3A).

TLR3 Inhibition by NS1 Mutants

TLR3 signaling in HeLa-NFκB-luc cells expressing NS1 mutants was measured by IL-6 ELISA (Figure 4A) and NFκB activation by luciferase assay (Figure 4B) after pIC stimulation. Luciferase units were normalized to protein concentrations in the cellular lysates and expressed as fold NFκB induction over unstimulated cells. IL-6 secretion was calculated as the percentage of IL-6 secreted from mutant NS1-expressing cells compared to vector control (MCS) cells set at 100%, following pIC treatment.

Initially, we dissected the contributions of the T177N/G295R mutations on inhibiting TLR3 signaling. Although glycosylation of NS1 was affected, the T177N change behaved like wt NS1 for both NFκB activation and IL-6 secretion. However, the G295R change was found to partially allow TLR3 signaling as measured by NFκB activation and IL-6 secretion (Figure 4). Interestingly, we did observe a small additive effect on IL-6 secretion when the T177N mutation was combined with G295R, as originally isolated by the screen (Figure 4A).

The effects of the N191I/P320S mutations on TLR3 signaling were also assessed. N191I alone inhibited TLR3 signaling at levels comparable to wt NS1 protein. Like the

G295R mutation, the single P320S mutation was able to partially restore TLR3 signaling. Combining the N191I mutation with the P320S mutation, as it was identified by the screen, had an additive affect on restoring IL-6 secretion, but not on NFκB activation. These data indicate that the P320S mutation partially restores TLR3 signaling while the N191I/P320S double mutation fully restores signaling.

The E238V mutant inhibited TLR3 signaling similar to wt NS1 (Figure 4). Conversely, N255D NS1 behaved similar to vector control cells when TLR3 signaling was assessed, and indicates that the mutation at position 255 fully restores TLR3 signaling. Nearly identical results were found for the E238V/N255D double mutant, suggesting that the N255D change is responsible for the observed phenotype (Figure 4).

The final NS1 double mutant, T317I/M333V, was also dissected for TLR3 inhibition. T317I NS1 fully responded to pIC treatment. TLR3 signaling in HeLa cells expressing the single M333V NS1 mutations revealed that NFκB activation and IL-6 secretion were partially restored. The T317I/M333V NS1 double mutant was found to have the same phenotype as the T317I single mutant. From these data we conclude that the T317I change is the dominant phenotype which fully restores TLR3 signaling, while the M333V single mutant partially restores TLR3 signaling.

Taken together, the mutations at positions 177, 191, and 238 suppressed TLR3-mediated NFκB activation and IL-6 secretion similar to wt NS1, while the mutations at positions 255, 295, 320, 317, and 333 allowed NFκB activation and IL-6 secretion in response to TLR3 engagement above wt NS1 levels. Notably, the T177N/G295R double mutant restored TLR3 signaling in HeLa cells slightly above the single G295R mutant while

the N191I/P320S double mutant significantly increased TLR3 signaling over the P32S single mutant.

Combined with the *trans*-complementation data, we conclude that NS1 mutations T177N, N191I, and E238V behave like wt NS1 for the functions we assessed. Several other mutations identified by the screen completely restored TLR3 signaling, but are severely impaired for *trans*-complementation (191/320, 255, 238/255, 317, and 317/333). The remaining NS1 mutants (295, 177/295, 320, and 333) efficiently *trans*-complemented WNV ΔNS1 GFP VRPs and showed significant loss of TLR3 inhibition (Figures 3 and 4). The individual mutations required for this phenotype were selected for further investigation (G295R, P320S, and M333V).

HighlyConserved NS1 Amino Acid Residues at the C-terminal Half of the Protein are Important for TLR3 Inhibition

The amino acid sequences of 10 mosquito-borne and 6 tick-borne flavivirus NS1 proteins were aligned using ClustalW. All of the mutations indentified by the screen lie at the C-terminal half of the NS1 protein. G295, P320, and M333 are evolutionarily conserved among all flavivirus NS1 proteins that were aligned, suggesting that these positions are of biological importance to the viruses (Figure 5). In contrast, T177N and N191I amino acid residue changes had no effect on the functions we assessed, and were found to have variable residues at those positions (data not shown). E238, while not conserved among all flaviviruses, was completely conserved among mosquito-borne flaviviruses, and a change at that residue behaved like wild type NS1. Residue N255 is completely conserved and a

change to aspartic acid resulted in loss of function, while residue T317 was only partially conserved among mosquito-borne flaviviruses and a change to isoleucine also negatively impacted both NS1 functions we assessed.

Not surprisingly, the NS1 proteins of the examined flaviviruses share significant homology as determined by Shannon entropy calculations (average H value = 1.24). Overall, the C-terminal half of the protein was more conserved than the N-terminal half (average H value N-terminal half = 1.36, C-terminal half = 1.11). Taken together, the observation that residues 295, 320, and 333 are evolutionarily conserved and involved in TLR3 inhibition implies that they are biologically significant.

NS1 Mutations Affect WNV Growth *in vitro*

During the screen, NS1 was ectopically expressed, independent of other viral proteins. During flavivirus replication, non-structural proteins need to interact with each other properly to form a functioning replication complex. To investigate the effects of the identified mutations on genome replication and virus production, the mutations were introduced individually into a WNV infectious clone and recombinant viruses were produced in BHK cells. Initially, immunofocus formation assays were conducted on Vero cells (Figure 6A). Notably, WNV NS1 G295R produces small immunofoci, whereas WNV NS1 M333V virus produces pin-point immunofoci. In contrast, WNV NS1 P320S produces immunofoci that were similar to those observed for the wild type virus. Multistep growth curves were also performed in Vero cells (Figure 6B). WNV NS1 G295R and WNV NS1 M333V were highly attenuated in Vero cells with virus titers being significantly lower than wild type virus at all

time points examined. Wild type WNV reached peak titer by 48h post-infection. Peak virus titers for WNV NS1 G295R and WNV NS1 M333V were delayed until 72h post-infection and were approximately 2 logs lower than wt WNV. WNV NS1 P320S reached a peak titer at 48h, nearly identical to wt WNV, and only a slight reduction in virus production was observed at 24h post-infection.

Multistep growth curves were also performed on HeLa cells (Figure 6C). Growth kinetics for WNV NS1 G295R and WNV NS1 M333V were similar to those observed on Vero cells with significantly reduced virus production at all time points examined and a 2-3 log difference in peak titer at 48h compared with wt WNV. Interestingly, WNV NS1 P320S was also highly attenuated in HeLa cells; titers were significantly lower compared to wt WNV at all time points and peak titer was approximately 2 logs lower than wild type virus.

To assess the stability of the NS1 mutations, the NS1 sequences of Vero and HeLa cell-derived viruses were analyzed at the 96h time point of the growth curves. While WNV encoding G295R NS1 or M333V NS1 were stable out to 96h post-infection in both cell types, P320S NS1 WNV derived from both Vero and HeLa cells partially reverted to wild type sequence. Importantly, the BHK-derived stock viruses used to produce the growth curves showed no evidence of reversion or second-site mutations within NS1.

The cumulative findings from the growth curves suggest that WNV encoding G295R or M333V NS1 mutations are severely attenuated in both Vero and HeLa cells, and produce small immunofoci. Conversely, WNV expressing the P320S NS1 mutation replicates to near-wild type levels in Vero cells, produces normal-sized immunofoci, but is highly attenuated in HeLa cells. Additionally, the G295R and M333V NS1 mutations are stable after one passage

in cell culture, whereas P320S NS1 WNV shows a mixed population of mutant and revertant viruses.

WNV NS1 Mutants Do not Inhibit TLR3 in HeLa Cells

TLR3 signaling was measured in WNV infected cells in response to pIC. Previous studies with HeLa cells demonstrated that IL-6 secretion in response to pIC treatment is TLR3-dependent and can be abolished in cells transfected with TLR3-specific siRNA [34]. HeLa cells were infected for 20h with WNV followed by 8h of pIC treatment. Wild type WNV significantly inhibited IL-6 secretion following pIC treatment (Figure 6D). Cells infected with WNV encoding each of the identified NS1 mutations fully responded to pIC stimulation. Interestingly, the levels of IL-6 secreted by P320S NS1 WNV-infected cells were higher than mock-infected cells treated with pIC (Figure 6D). Given that each virus displayed growth attenuation in HeLa cells, the percentage of infected cells in each case was determined by flow cytometry. All viruses were equally able to infect HeLa cells (Figure 6E).

Discussion

We have previously demonstrated that the WNV non-structural glycoprotein NS1 is able to inhibit TLR3 signaling [34, 35]. In this study, we have developed a FACS-based screen to identify randomly-generated mutations within the NS1 protein that restore TLR3 signaling, while allowing the protein to still function in genome replication. Initially, the components of the screen were successfully validated and mutagenic coverage was assessed.

The mutagenesis was unbiased and nucleotide changes were found throughout the coding sequence of the NS1 transformants we randomly selected for sequencing. Following two screens using independently-constructed mutant NS1 libraries, we identified approximately 3 X 10^4 double-positive cells of which 134 survived clonal expansion. Of the expanded clones, 18 displayed the desired phenotype. While cell viability due to VRP infection alone was not affected as determined by MTT assay (data not shown), the combination of VRP infection, pIC treatment, and the stress of the sorting process likely contributed to a decrease in clonal cell survival. Nevertheless, the identification of several mutants with the correct phenotype confirms that our screening strategy was successful.

Overall, four unique double mutants were identified: T177N/G295R, N191I/P320S, E238V/N255D, and T317I/M333V. Analysis of the effect of individual amino acid changes at each of these eight positions demonstrated that mutations at residues 177, 191, and 238 did not affect replication in our *trans*-complementation assay. These same residues also did not seem to be involved in TLR3 inhibition. The T177N mutation was initially of particular interest because it ablates *N*-linked glycosylation at position 175, and numerous other studies have described a role for NS1 glycosylation in virulence and pathogenesis of several flaviviruses [40-45]. Surprisingly, T177N NS1 behaved like wild type protein for *trans*-complementation and TLR3 inhibition. These findings, however, do not rule out the possibility that the other glycosylation sites in NS1 are important.

The mutations at residues 255 and 317 conferred the opposite phenotype, negatively impacting *trans*-complementation of viral RNA synthesis while allowing TLR signaling to occur. These results could either be interpreted that residues 255 and 317 are involved in

TLR3 inhibition but that simultaneously, the amino acid substitutions have a negative effect on replication, or that mutation of these residues render NS1 simply non-functional.

Ultimately the G295R, P320S, and M333V mutations were shown to allow TLR3 signal transduction, while still permitting the mutant proteins to *trans*-complement and rescue replication of an NS1-deleted VRP. Interestingly, none of the mutations fall in the N-terminal half of the NS1 amino acid sequence. Consistent with these results, a previous clustered charged-amino-acid-to-alanine mutagenesis study of the yellow fever virus NS1 protein identified mutations lethal to virus replication only in the N-terminal half of the protein, while the majority of tolerated mutations mapped to the C-terminal half [46]. That study also noted that paired alanine changes at positions K296/R299 and E334/R336 resulted in a small-plaque phenotype. These residues are in close proximity to WNV residues G295 and M333, which were identified in our screen, and also resulted in small-immunofoci phenotypes. ClustalW alignment of the C-terminal half of 10 mosquito-borne and 6 tick-borne flavivirus NS1 proteins revealed high sequence similarity in this region of the protein, and all 3 of the residues we identified as being important for TLR3 inhibition are fully conserved. Based upon these observations, it will be important to determine if TLR3 inhibition by the flavivirus NS1 protein is restricted to WNV NS1.

In a separate study, Baronti et al, were unable to find a role for the NS1 proteins of several flaviviruses in inhibiting TLR3 signaling [47]. In that study, the investigators relied on the induction of IFN-β transcripts as a primary readout for TLR3 signaling within HEK293-TLR3 cells and in HeLa cells. We have previously demonstrated in another study that HeLa cells fail to produce significant levels of IFN-β transcript and protein following

pIC stimulation alone, which may explain these conflicting results [34]. Further, the authors of that study had to resort to overexpressing IRF3 in the HeLa cell lines in order to induce IFN-β transcription following pIC stimulation. As the mechanism NS1-mediated TLR3 inhibition is currently unknown, IRF3 overexpression in these cells may have subverted the level at which NS1 blocks signal transduction. Finally, in this report we were able to demonstrate that NFκB activation and IL-6 transcription and secretion, in response to pIC stimulation of TLR3, were dependent upon the presence of individual amino acid residue changes in the WNV NS1 protein.

Growth kinetic studies of mutant WNV expressing changes in the NS1 protein were performed in Vero and HeLa cells. HeLa cells are competent for TLR3 signaling while Vero cells are not. We observed that both G295R NS1 WNV and M333V NS1 WNV were attenuated in both cell lines, while P320S NS1 WNV was only attenuated in HeLa cells. It is possible that different innate immune responses in the two cell lines may be important for the growth phenotypes of the different mutant viruses. Further investigation in other cell lines, as well as cells deficient for individual innate immune pathways will be required to address this possibility, but is outside the scope of this report. We also assessed the stability of the mutations during replication and found that G295R and M333V are stable out to 96h post-infection in both Vero and HeLa cell lines after a single passage. However, P320S showed partial reversion in both cell lines. Taken together, it would appear that there is considerable selective pressure on P320S NS1 WNV to revert that was not observed for the other viruses.

TLR3 signaling studies performed using HeLa cells, infected with mutant WNV, confirmed that the amino acid changes G295R, P320S, and M333V restored signaling in

response to pIC treatment. Only wt WNV infected HeLa cells failed to respond to pIC treatment. Interestingly, P320S NS1 WNV-infected HeLa cells secreted more IL-6 than uninfected control cells treated with pIC. Though this may be the result of increased PRR recognition of the virus, further investigations are required. These data indicate that the NS1 mutations identified in the screen with ectopically-expressed protein also behave similarly in the context of the WNV genome with regard to TLR3 inhibition.

As has been previously shown, HeLa cells fail to secrete IFN-β in response to pIC stimulation alone [34, 48]. Nevertheless, we attempted to assess IFN-β secretion in response to pIC treatment following infection with all of the viruses. In agreement, IFN-β production by HeLa cells in response to this stimulus was determined to be at or below the level of detection in all cases.

Our screening process was designed to separate the NS1 function in viral RNA replication from its function to inhibit TLR3. As our results demonstrate, these functions are not easy to separate completely with tradeoffs in either the ability to support RNA replication or partial restoration of TLR signaling. The most promising mutation is the change at residue P320, which in Vero cells allowed replication of the virus to near-wild type levels while being completely deficient in TLR3 inhibition.

Ultimately, interference with TLR3 signaling is expected to alter the host response to benefit to the virus. However, pathogenesis studies, as well as investigation of the exact interplay between various innate immune pathways and our mutant viruses are outside the scope of this study.

We are currently in the process of evaluating other substitutions at position 320 in order to obtain a more stable virus. Successful creation of such a virus would generate an important tool to investigate the role of the TLR3 inhibitory function of NS1 during WNV pathogenesis. Additionally, the identification of P320 as a residue critical for TLR3 inhibition is likely to prove valuable for determining the mechanism by which NS1 antagonizes TLR3 signal transduction.

Table 1: NS1 mutants identified in screen

Clone	*Amino Acid Position	Amino Acid Change
1	177	T : N
	295	G : R
2	191	N : I
	320	P : S
3	238	E : V
	255	N : D
4	317	T : I
	333	M : V

*NS1 is 352 amino acids in length

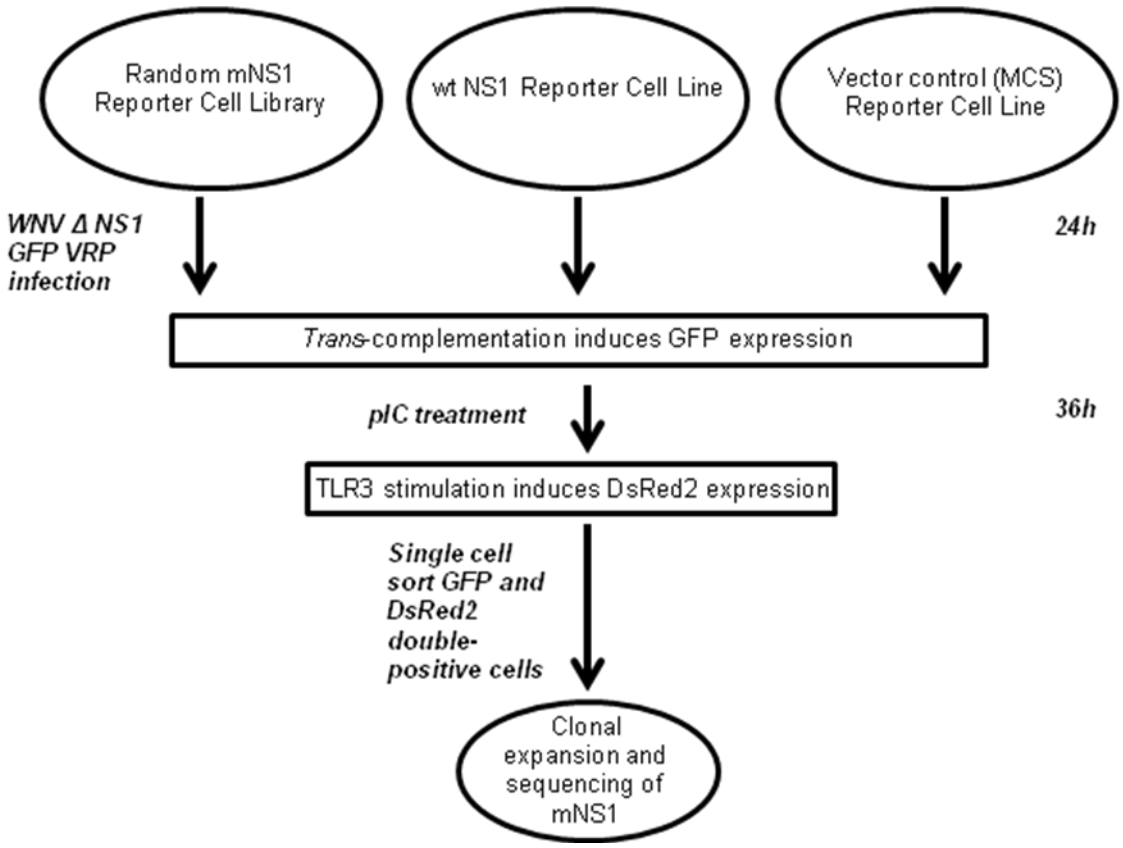


Figure 1: **Screen work-flow for the identification of mutant NS1 proteins that allow TLR3 signaling and support replication of a WNV NS1-deleted GFP replicon particle.** A HeLa reporter cell library expressing random NS1 mutants (HeLa-NFκB-DsRed2-pLEX-mNS1) was infected at an MOI of 1 with a NS1-deleted WNV GFP replicon (WNV ΔNS1 GFP VRP) for 24h followed by pIC treatment for an additional 36h. GFP and RFP double-positive cells were single-cell sorted by Fluorescence-activated cell sorting (FACS), individual clones expanded and the NS1 sequence of each clone was determined after confirmation of the desired phenotype.

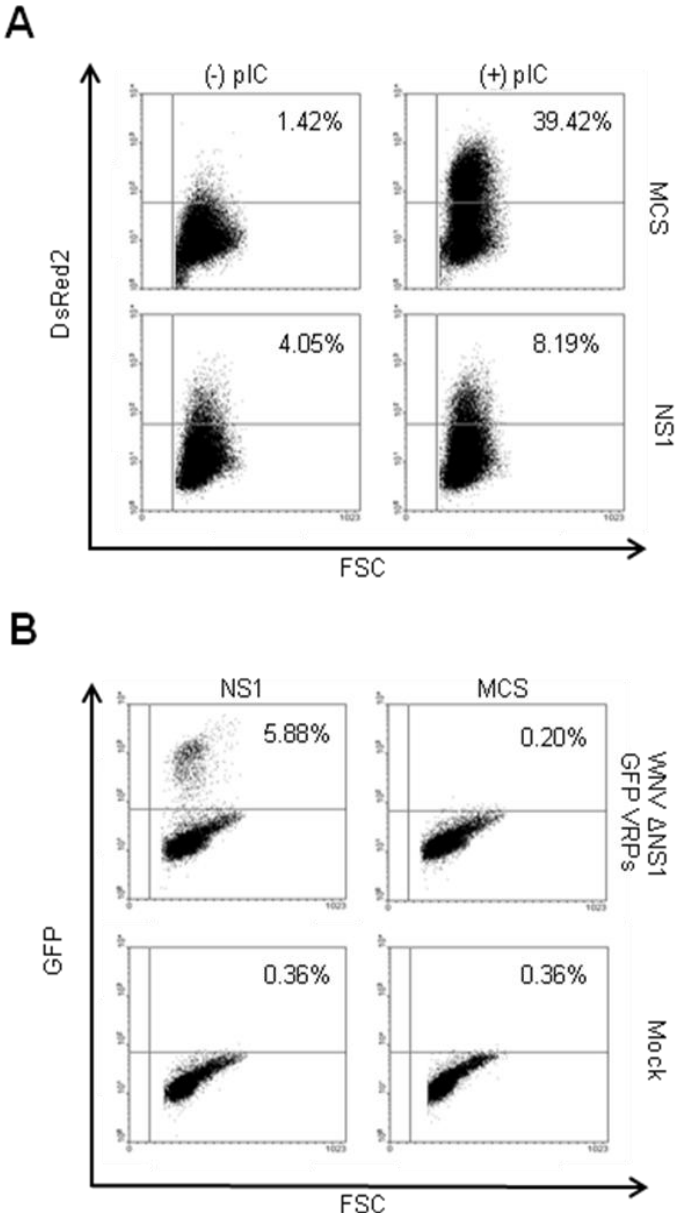


Figure 2: **Validation of the tools employed by the screen.** (A) HeLa NFκB-DsRed2 cells stably transduced with either pLEX-NS1 or pLEX-MCS (vector control) lentivirus were treated with pIC for 36h or left untreated and DsRed2 expression was determined by flow cytometry. (B) HeLA-NFκB-DsRed2 cells stably transduced with either pLEX-NS1 or pLEX-MCS (vector control) lentivirus were either infected for 24h with WNV ΔNS1 GFP VRPs or mock infected. GFP expression was determined by flow cytometry. Data are representative of three independent experiments.

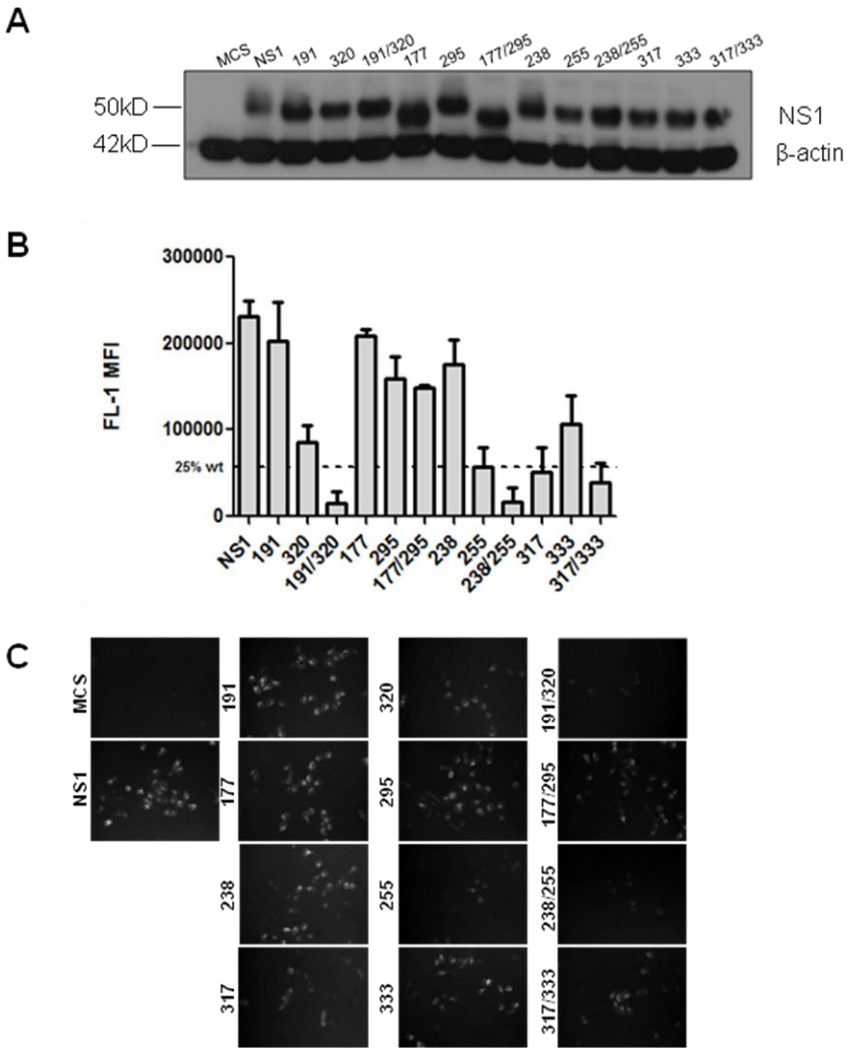


Figure 3: **Efficiency of identified NS1 mutants to *trans*-complement WNV ΔNS1 GFP VRPs.** HeLa-NFκB-luc reporter cells stably transduced with lentivirus expressing either wt NS1, MCS, or single or double NS1 mutations were infected at an MOI of 0.15 for 36h with WNV ΔNS1 GFP VRPs or mock infected. (A) 5μg of whole cell lysate from each cell line was probed for NS1 expression by immunoblot and normalized to β-actin expression. (B) Expression of GFP was quantified by flow cytometry by determining the mean fluorescent intensity (MFI). The background MFI of identically-infected MCS cells was subtracted from the MFI determined for other infected cells. An arbitrary cut off (dashed line) was set at 25% of MFI obtained in wt NS1 expressing cells. (C) GFP expression due to trans-complementation by wild type and NS1 mutants was monitored by fluorescence microscopy. Data are the average of two independent experiments and error bars represent standard deviation from the mean.

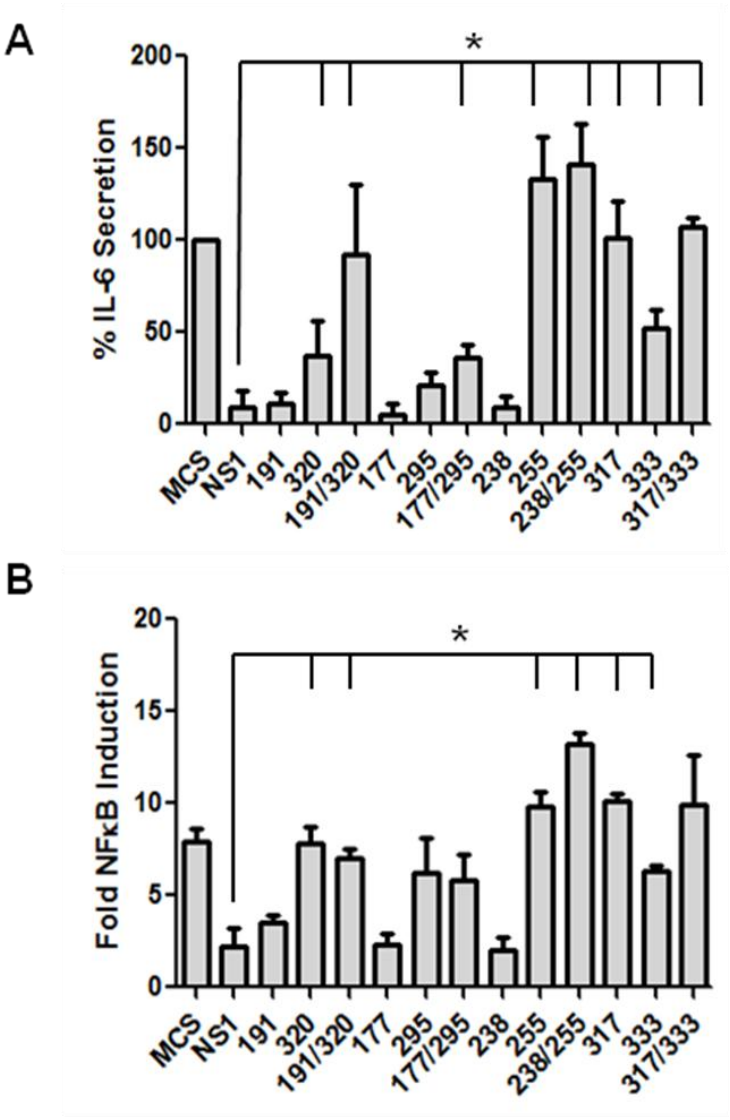


Figure 4: **Efficiency of TLR3 signaling in cells expressing NS1 mutants.** HeLa-NFκB-luc reporter cells stably transduced with lentivirus expressing either wt NS1, MCS, or single or double NS1 mutations were treated with 20μg/ml pIC for 8h or left untreated. (A) IL-6 secretion in the supernatants of cells was determined by enzyme-linked immunosorbent assay (ELISA) and percent secretion was calculated by comparing IL-6 produced by wild type and mutant NS1 expressing cells to that of MCS vector control cells set to 100%. Data are the average of three independent experiments and error bars represent standard deviation from the mean. (B) Fold NFκB induction was determined by luciferase reporter assay. Relative light units were normalized to total protein, and reported as fold induction over untreated cells. Data are the average of two independent experiments and error bars represent standard deviation from the mean. Statistical significance was determined by student's t-test, where *$p \leq 0.05$.

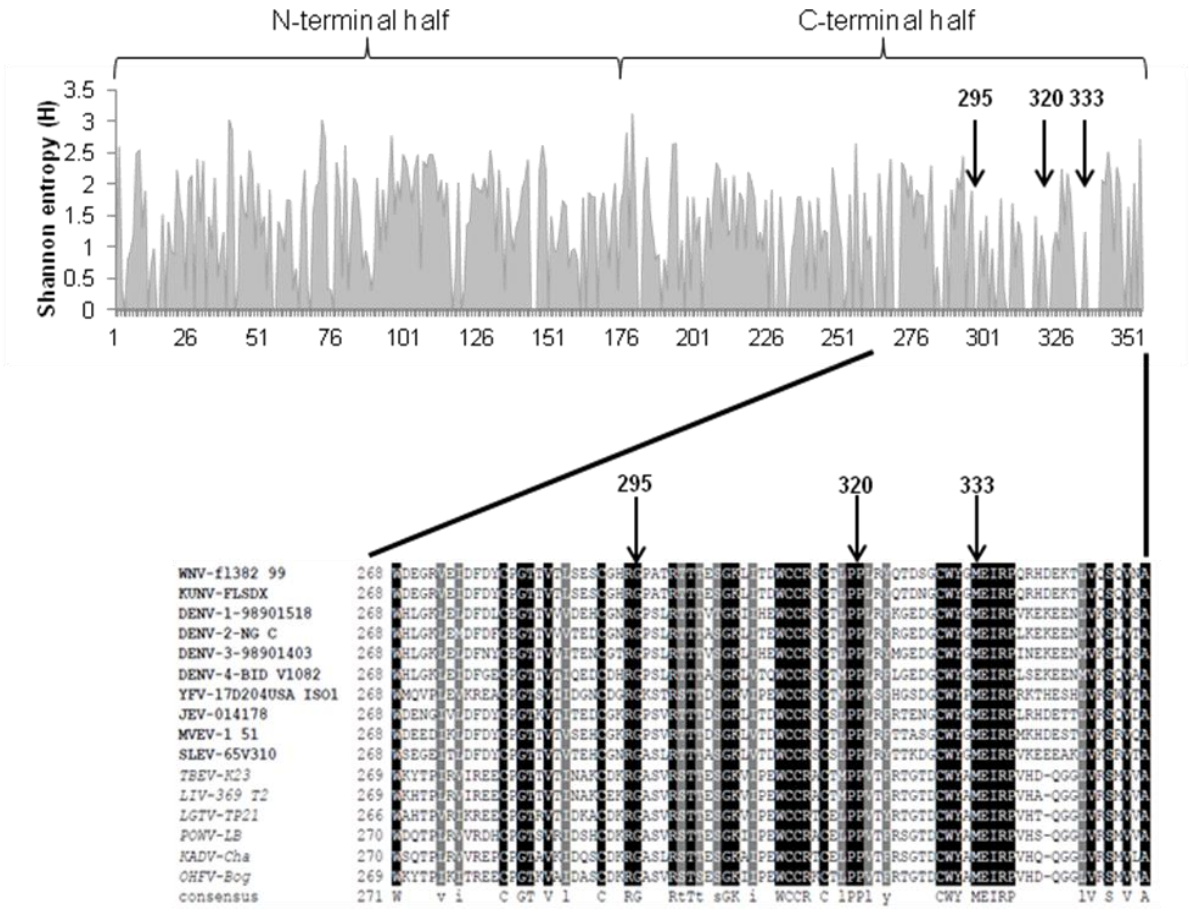
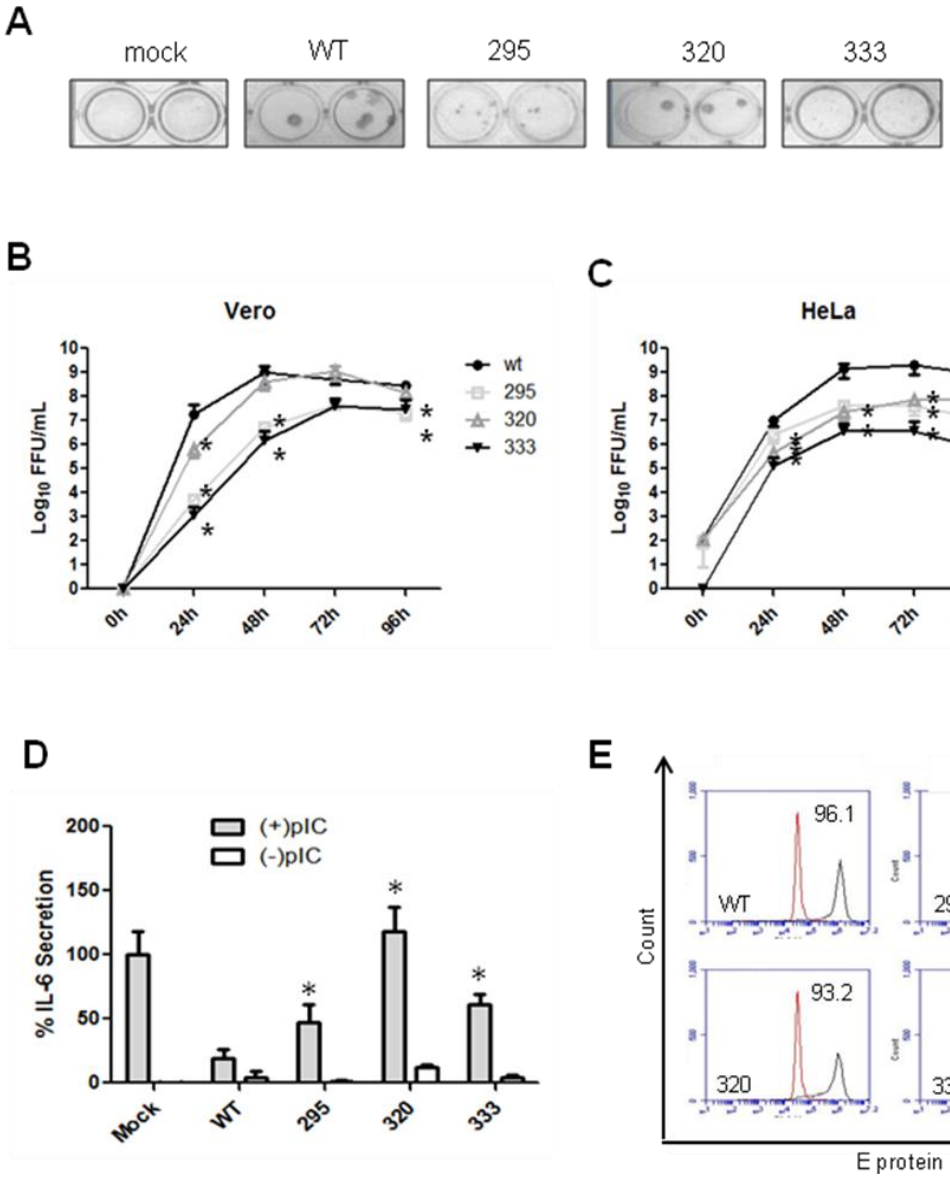


Figure 5: **Shannon entropy (H) and multiple protein alignment of the NS1 proteins of several mosquito- and tick-borne flaviviruses.** The amino acid sequences of 10 mosquito-borne and 6 tick-borne flaviviruses were aligned with ClustalW using the Blosum scoring matrix. The multiple-alignment file was displayed with BOXshade 3.21 where black shading represents identical residues and gray shading represents conserved substitutions. Shannon entropy analysis of the multiple protein alignment was performed by the Protein Variability Server (PVS) at the Complutense University of Madrid, and the data generated was displayed in MS Excel. $H \leq 1$ are considered highly conserved residues. Mosquito-borne flaviviruses are represented in bolded font and tick-borne flaviviruses in italics.

Figure 6: **Replication kinetics and TLR3 inhibition of mutant WNV expressing the identified amino acid residue changes in the NS1 protein.** (A) Immunofocus size of WNV expressing G295R, P320S, or M333V NS1 was determined on Vero cells. Cells were infected for 1h, overlaid with tragacanth gum, and fixed 48h later. Immunofoci were detected with using an anti E protein primary antibody and an HRP-conjugated secondary antibody. Data are representative of three independent experiments (B) Vero cells were infected at an MOI of 0.01 with each WNV NS1 mutant and virus containing supernatants were collected every 24h for 96h. Infectious virus was titered on Vero cells and quantified by immunofocus assay. Growth curves were performed in biological triplicate and data are representative of two independent experiments. Error bars represent standard deviation from the mean. (C) HeLa cells were infected at MOI 0.01 with each WNV NS1 mutant and virus-containing supernatants were collected every 24h for 96h. Infectious virus was titer on Vero cells and quantified by immunofocus assay. Growth curves were performed in biological triplicate and data are representative of two independent experiments. Error bars represent standard deviation from the mean. (D) HeLa cells were infected at MOI 5 with each WNV NS1 mutant for 1h, washed with PBS, replaced with fresh medium, and allowed to incubate for 20h. Medium was removed and replaced with medium with or without pIC (20μg/ml) for 8h. Cell culture supernatants were collected and IL-6 concentrations were determined by ELISA. Data are representative of three independent experiments and error bars represent standard deviation from the mean. (E) Identically infected HeLa cells were fixed and permeabilized 28h post-infection and infected cells were identified by flow cytometry using an E protein-specific monoclonal antibody. Red histograms represent uninfected control cells and black histograms represent E protein in WNV infected cell. Statistical significance was determined when appropriate, by student's t-test, where *$p \leq 0.05$. Data are representative of 3 independent experiments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Log10 FFU/mL
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72h
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(-)pIC
Mock
WT
295
320
333
E
Count
96.1
WT
90.3
295
93.2
320
98.1
333
E protein

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CHAPTER 3

Improper processing and cellular localization of West Nile virus NS1 mutants allow TLR3 signaling

Abstract

Flaviviruses have evolved several functions to either avoid innate immune system detection or to actively suppress its induction. The West Nile virus (WNV) non-structural protein 1 (NS1) actively inhibits Toll-like receptor 3 (TLR3) signal transduction in infected cells through an unknown mechanism. NS1 is a secreted glycoprotein that can also function to inhibit TLR3 signaling in naïve, uninfected cells treated with the purified protein, and can bind to complement factors in serum and on the cell membrane to block their activation. In addition to modulating the innate immune response, NS1 is also required for virus genome replication. Suppression of the innate immune response by the NS1 protein may allow for increased virus dissemination within the host and play an important role in WNV pathogenesis. Recently, we identified three independent amino acid changes in the C-terminal half of the NS1 protein that restored TLR3 signaling in virus-infected cells, while still allowing virus production. In this study, we biochemically characterize these NS1 mutants, in order to better understand NS1 protein function. Several defects in the processing

of the mutant NS1 proteins were identified and included reduced recognition by NS1-specific antibodies, reduced protein stability, improper glycosylation, reduced cell surface expression/association, and reduced secretion. Further experiments proved that glycosylation, cell surface expression/association, and secretion of wt NS1 are not required for inhibition of TLR3 signaling in expressing cells. Taken together, the NS1 mutants are improperly processed within cells leading to a failure to inhibit TLR3.

Introduction

The flavivirus, West Nile virus (WNV), is a causative agent of febrile and encephalitic diseases in humans and other mammals. WNV is endemic in the continental United States and poses a major public health threat as no approved vaccines or treatments are available. Previous work by our group has described a role for the intracellular and secreted forms of the WNV non-structural protein 1 (NS1) in inhibiting innate immune responses originating from Toll-like receptor 3 (TLR3) [1]. In those studies, expression of the NS1 protein, as well as pretreatment of naïve cells with the purified secreted form of NS1, were sufficient to inhibit TLR3 signaling in response to the synthetic TLR3 ligand, polyinosinic:polycytidylic acid (pIC). NS1 inhibits the activation of the transcription factors nuclear factor kappa-B (NFκB), activator protein-1 (AP-1), and interferon regulatory factor 3 (IRF3), and subsequently blunts the expression of cytokines and interferon which prevents establishment of an antiviral state in cells.

TLR3 is a pattern recognition receptor (PRR) that senses the pathogen-associated molecular pattern (PAMP), dsRNA, produced by many viruses [2, 3]. TLR3 is widely expressed by several cell types, including endothelial cells and macrophages, is located throughout the body, and primarily resides within endosomes. TLR3 can also be found on the surface of some cell types and is an interferon-stimulated gene (ISG) [4-7]. Following its activation, TLR3 signals through the adaptor molecule TIR domain-containing adaptor inducing IFN-β (TRIF), this in turn activates the transcription factors NFκB, AP-1, and IRF3, which drive the expression of pro-inflammatory cytokines and type I interferon [8]. The cumulative action of these cytokines induces an antiviral state aimed at limiting virus dissemination, and primes the adaptive immune response. The role of TLR3 in controlling WNV infection in vivo has not been completely resolved. However, some studies suggest a protective role for TLR3 within the CNS and in certain cell types [9].

NS1 is a secreted glycoprotein which can be found within infected cells and on the cell membrane. Following translation of the WNV genome into a large polyprotein, a hydrophobic signal sequence at the C-terminus of the E protein promotes translocation of NS1 into the lumen of the ER. A resident ER signal peptidase cleaves the N-terminus of NS1, while a resident ER protease is predicted to cleave the C-terminus of the protein [10]. Free NS1 rapidly dimerizes within the lumen of the ER and acquires glycosylation at three *N*-linked glycosylation sites. Oligosaccharyl transferase attaches high-mannose oligosaccharides to the three *N*-linked glycosylation sites (N130, N175, and N207) of NS1 within the ER (reviewed in [11]). Dimeric, glycosylated NS1 enters the secretory pathway and is targeted to the Golgi stacks, where the enzyme alpha-mannosidase cleaves and

modifies two terminal mannose residues on at least one of the *N*-linked glycans [12]. NS1 can then be directly target to the cell membrane as a dimer by an unknown mechanism [13]. Alternatively, the protein can be directly secreted from cells as a hexamer which is then able to bind back to the cell membrane through an interaction with glycosaminoglycans (GAG) [14].

The cell surface-associated and secreted forms of the NS1 protein have been found to antagonize complement activation. The complement system is important in controlling WNV infection *in vivo* and all three complement pathways can be inhibited by NS1. Secreted NS1 binds to the C1s and C4 complement proteins, promoting cleavage of C4, and binds C4b binding protein (C4BP) to prevent assembly of C3 and C5 convertases. Additionally, NS1 binds to complement factor H and promotes cleavage of C3b. These inhibitory actions prevent opsonization of the virus and infected cells and block complement-mediated cell lysis [15-17].

In addition to its role in inhibiting innate immune responses, the NS1 protein is also involved in virus genome replication. NS1 has been found to co-localize with other non-structural proteins of the replication complex and with dsRNA, a replication intermediate of flavivirus replication, although the localization of NS1 to the luminal side of the ER membrane would preclude it from being a direct component of the replication complex (RC) [18]. Several studies have demonstrated that truncation of NS1 is lethal to flaviviruses, and numerous mutations of the protein severely attenuate flavivirus replication [19-21]. It has also been demonstrated that NS1 is important for negative-sense RNA synthesis, and a genetic link between NS1 and NS4A has been described [21]. More recently, NS1 has been

found to directly associate with NS4B, another non-structural protein of the replication complex [22]. This interaction is predicted to allow NS1 to functionally interact with the RC. The cumulative data indicate that NS1 is absolutely required for virus replication, however its direct role in replication has not yet been determined.

Recently, we identified three amino acid residue changes in NS1, G295R, P320S, and M333V, which independently restored TLR3 signaling while allowing virus replication (Chapter 2). HeLa cells infected with WNV encoding the identified mutations fully responded to treatment with pIC. While the mechanism of inhibition is not understood, we undertook molecular and biochemical studies aimed at characterizing the defects of the NS1 mutants that allow TLR3 signaling.

In this study, we found that some of the NS1 mutants are inefficiently recognized by NS1-specific antibodies and are less stable over time within cells. Additionally, the NS1 mutants either fail to acquire complex/hybrid glycosylation within the Golgi or show reduced acquisition of complex/hybrid glycosylation when compared with the wt NS1 protein. These mutants also reach the cell surface inefficiently, and fail to be secreted and accumulate in cell supernatants. Importantly, we also demonstrated that intracellular targeting of wt NS1 is sufficient for TLR3 inhibition, as prevention of cell surface-association and secretion did not restore signaling. The cumulative findings of the study indicate that the NS1 mutants are improperly processed within cells leading to aberrant localization and a failure to inhibit TLR3.

Material and Methods

Cell lines

HeLa cells stably expressing NS1 mutants from a lentivirus vector have been described previously (Chapter 2). Cells were maintained in Dulbecco's modification of Minimal Essential Medium (DMEM) (Cellgro) supplemented with 10% FBS (Gemini) and 10µg/ml puromycin (Gemini) at 37°C in a 5% CO_2 atmospheric incubator. HeLa-NFκB-luc cells have also been described in Chapter 2 and were maintained as above with the addition of 400µg/ml of hygromycin (Gemini).

Immunofluorescence Microscopy

HeLa cells stably expressing the NS1 mutants, wt NS1, and empty vector control (MCS) from a lentivirus vector were plated overnight as semiconfluent monolayers in tissue culture-treated chamber slides (Lab-Tek). Cells were washed with PBS and fixed with 4% paraformaldehyde for 20min, washed with PBS, and permeabilized (1X PBS + 0.5% Triton X-100) for 10min. Fixed and permeabilized cells were blocked (1X PBS, 2% BSA (Fisher), 5% normal horse sera (Gemini), 10mM Glycine (Sigma)) for 30min and incubated with a 1:1000 dilution of mouse anti-WNV hyper-immune ascitic fluid (HIAF) in blocking buffer for 1h to detect NS1. The mouse HIAF was previously reported to contain NS1-specific antibodies [1]. Cells were washed in PBS and primary antibody was detected by incubating with a 1:1000 dilution of goat anti-mouse IgG (H+L) conjugated to Alex Fluor 488 in blocking buffer for 1h. Nuclei were counterstained with DAPI in Vectamount (Vector Labs).

Slides were viewed under a Zeiss Axioskop 2 microscope and images were captured with SPOT Basic software (Diagnostic Instruments).

Immunoblotting

Whole cell lysates were prepared by washing cells once with PBS and lysing for 30min in 1X RIPA buffer with leupeptin (Cell Signaling) containing PMSF (1mM) (Sigma) on ice. Lysates were centrifuged at 14K RPM for 10min to pellet cell debris and protein was estimated using a detergent-compatible protein assay kit (BioRad), using BSA standards and read at 650nm on a microplate reader (BioTek).

Equivalent amounts of protein (5µg) were mixed with 1X LDS buffer (Invitrogen), containing 2.5mM DTT, heated for 5min at 90°C, and loaded into 4-12% Bis-Tris NuPAGE gels (Invitrogen). Proteins were electrophoretically separated at 150V for 3h, transferred to a PVDF membrane (Immobilon Millipore) at 30V for 1.5h, blocked with 5% milk in 1X Tris-buffered saline-0.1% Tween-20 (TBST), and probed with one of the following primary antibodies: 1:4000 dilution of mouse anti-tetra-His monoclonal antibody in TBST (34679; Qiagen) or 1:10000 dilution of mouse anti-β-actin monoclonal antibody in TBST (A1978; SIGMA). Following three 5min washes in TBST, membranes were incubated with 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) secondary antibody in TBST (474-1806; KPL). Following another round of washes, membranes were incubated with an enhanced chemiluminescence detection system as recommended by the manufacturer (Santa Cruz Biotechnology) and visualized by exposing autoradiographic film (Blue Devil) to the blots.

To detect NS1 dimer, lysates were prepared as above and mixed with 1X LDS buffer without heating and without reducing agent. Proteins were separated and blotted as described. Secreted NS1 was also detected in cellular supernatants following 48h of capture in serum free, 1X DMEM medium. 16µl of supernatant was mixed with 1X LDS buffer containing 2.5mM DTT, and either heated for 5min at 90°C or left unheated. Proteins were then separated and blotted as described.

Endoglycosidase H Digestions

5µg of whole cell lysate containing each NS1 mutant, wt NS1, or empty vector were mixed with 1X glycoprotein denaturing buffer (NEB) and heated at 100°C for 10min according to the manufacturer (NEB). Denatured protein was then subjected to digestion with 500U of recombinant Endoglycosidase H (Endo H) (NEB) by mixing in 1X G5 buffer (NEB), followed by 5min of incubation at 37°C. Digested proteins were then immunoblotted as described above.

Site-directed Mutagenesis

Each asparagine-linked glycosylation site was mutated to an alanine residue in the NS1 coding sequence of the pLEX-NS1 vector, using the PCR-based Quick Change II XL kit (Agilent Technologies/Stratagene). Site-directed primer pairs were designed using the Quick Change primer design software according to the kit manufacturer. Each primer pair was designed to incorporate a single nucleotide change resulting in a single point mutation to alanine. Fidelity of the mutagenesis was confirmed by standard DNA sequencing. Mutants

were packaged as lentivirus particles and used to produce stable, polyclonal pools in HeLa-NFκB-luc cells as described previously (Chapter 2).

IL-6 ELISA

Following the indicated treatments, cellular supernatants were collected and assayed for human IL-6 using a commercial ELISA kit (Ebioscience), as described in Chapter 2. Concentrations of IL-6 are reported as pg/ml.

NFκB-luciferase reporter assays

Following the indicated treatments, a luciferase assay was performed as described in Chapter 2.

Flow Cytometry

Surface NS1

For surface detection of NS1, HeLa cell monolayers expressing the NS1 mutants were washed with PBS and incubated with 5mM EDTA in PBS for 10min on ice to disrupt the monolayers. Following resuspension in complete medium, cells were washed with staining buffer (1% FBS, 0.09% Sodium Azide in PBS) and incubated for 1h on ice with a 1:500 dilution of mouse anti-WNV NS1 monoclonal antibody 10NS1 (a gift from M.S. Diamond) in staining buffer. Residual antibody was removed by washing twice with staining buffer followed by a 30min incubation with a 1:500 dilution of goat-anti-mouse Alexa Fluor 488-conjugated secondary antibody in staining buffer (Invitrogen). Cells were washed twice

with staining buffer, and analyzed on an Acuri C6 flow cytometer by capturing 20,000 events per treatment/cell line. Where indicated, mouse anti-JE NS1 monoclonal antibody (1:1000 dilution in staining buffer) was used in place of 10NS1 antibody for surface NS1 detection.

Total NS1

For detection of total NS1, cells were PBS washed, detached with EDTA, resuspended in complete medium, washed twice with staining buffer, and fixed and permeabilized for 20 min on ice with CytoFix/CytoPerm buffer (BD). Subsequent antibody treatments were identical to surface detection of NS1 using the 10NS1 antibody, with the following exception; all wash steps, as well as antibody dilutions, were performed in CytoFix/CytoPerm wash buffer (BD).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Following the indicated treatments, total RNA was extracted from cells using an RNeasy mini kit (Qiagen) as specified by the manufacturer. Total RNA was quantified by a Nanodrop ND1000 spectrophotometer, and 500ng total RNA was used in an RT-PCR containing oligo dT as primer (Promega). Transcript copies of GAPDH, IL-6, or NS1 were determined by qPCR of the cDNA using a SensiMix SYBR & Fluorescein kit (BioLine), containing the following primer sets: hGAPDHFw 5’ GGA TTT GGT CGT ATT GGG GC 3’ and hGAPDHRev 5’ TGG AAG ATG GTG ATG GGA TTT C 3’, hIL-6Fw 5’ ACA GCC ACT CAC CTC TTC AGA ACG 3’ and hIL-6Rev 5’ AGT GCC TCT TTG CTG CTT TCA 3’, or NS1Fw 5’ AAA CGC CTC ACC GCC ACC AC 3’ and NS1Rev 5’ GGA CAG GTC

ACT GTG GAT CGC C 3'. Reactions were set up in 96-well PCR plates (Genesee). Amplifications were carried out for 50 cycles, followed by a melt curve analysis of resulting products to confirm the specificity of the reactions. RNA without RT enzyme and dH_20 served as negative controls and transcript copies were determined based upon standard curves.

Cycloheximide and Brefeldin A Treatments

HeLa cells expressing each NS1 mutant, wt NS1, or empty vector MCS from a lentivirus vector were plated overnight at 5 X 10^5 cells per well in a 6 well plate. Medium was removed and replaced with 1X DMEM containing 10% FBS, 1X brefeldin A (Biologend), and 500µg/ml cycloheximide (CalBiochem). At the indicated time points, medium was removed, cells were washed with PBS and lysates were made as described above.

Sodium Chlorate and pIC Treatments

HeLa cells expressing either wt NS1 or empty vector MCS from a lentivirus vector were plated overnight at 4 X 10^4 cells per well in a 48-well plate. Medium was removed and replaced with sulfate-free 1X SMEM medium (Lonza) containing 10% FBS (Gemini) (dialyzed against SMEM) with or without 20mM $NaClO_3$ (Sigma) and allowed to incubate for 20h. Medium was then removed, cells were washed several times in PBS, then replaced with 1X SMEM containing 10% dialyzed FBS with or without 20µg/ml pIC for 8h. Surface

NS1 expression was also determined on identically treated cells as described above using the JE NS1 antibody.

Brefeldin A and pIC Treatments

HeLa cells expressing either wt NS1 or empty vector MCS from a lentivirus vector were plated overnight at 4 X 10^4 cells per well in a 48-well plate. Medium was removed and replaced with 1X DMEM containing 10% FBS with or without 1X brefeldin A for 20h. Surface NS1 expression was also determined on identically-treated cells as described above.

Results

Intracellular Distribution of NS1 mutants

As an initial assessment of mutant NS1 processing within cells, we determined the intracellular distribution of each NS1 mutant as well as wt NS1 by immunofluorescence microscopy (Figure 1). Wild type NS1 protein was found to be distributed throughout the cell, except within the nucleus and localized primarily to perinuclear regions that consistently displayed distinct punctuate accumulation of the protein. This distribution pattern was largely repeated with G295R NS1, however the fluorescent signal was slightly reduced when compared with wild type protein. Staining of both P320S and M333V NS1 was found to considerably weaker than wt NS1 and punctuate accumulation of the proteins in perinuclear regions was absent. Additionally, these NS1 proteins were not detectable in some of the cells (Figure 1). These findings suggest that each of the NS1 mutants is improperly distributed

within the cell and either expressed to low levels or inefficiently recognized by the antibodies.

Intracellular Stability of NS1 Mutants

Based upon the findings from the immunofluorescence microscopy study, we hypothesized that the NS1 mutants are misfolded and therefore not recognized in their native conformations by the antibodies. Misfolded proteins can also induce ER stress and be preferentially targeted for degradation [23]. To address this possibility, we investigated intracellular protein stability of the mutant proteins and wt NS1 protein. HeLa cells expressing each NS1 mutant, or wt NS1, were treated with 1X brefeldin A and 500µg/ml cycloheximide for up to 24h and protein levels were determined by immunoblot at the indicated times. Brefeldin A prevents NS1 secretion and cycloheximide prevents *de novo* protein synthesis, allowing for accurate assessments of the steady-state levels of NS1 protein over time.

Wild type NS1 shows a modest reduction in protein levels after 6h of treatment, but remains constant at approximately 70% after 24h of treatment. The G295R mutant was determined have no difference in stability when compared to wt NS1. Interestingly, two of the NS1 mutants, P320S and M333V, were found to be less stable over time when compared to the wt NS1 protein (Figure 2A and B). P320S NS1 is the least stable of the mutants with protein levels becoming almost undetectable by 24h post-treatment (~6%). M333V NS1 is also less stable than wt NS1 protein after treatment, but levels remained relatively constant (~40%) between 6 and 24h post-treatment. Figure 2C shows that the steady state mRNA

levels for the NS1 mutants are either comparable to those of wt NS1 or higher in the case M333V, suggesting that the observed differences in NS1 protein levels over time are indeed due to differences in protein stability. Figure 3A shows that all of the mutant NS1 proteins are expressed to levels similar to wt NS1. Therefore, the differences in signal level in the immunofluorescence studies are most likely due to inefficient recognition of the mutant NS1 proteins by the antibodies.

We also observed that wt NS1 and G295R NS1 produced two bands by immunoblot, with the top band appearing as a smear. Two bands were not observed for the P320S and M333V NS1 mutants (Figure 2A). Instead, P320S and M333V NS1 proteins migrated as a single, tight band. The top band/smear is predicted to be NS1 modified by complex/hybrid *N*-linked glycosylation and this possibility was addressed by Endo H digestion of the NS1 proteins (see below). In summary, several NS1 mutants which fail to inhibit TLR3 signaling are inefficiently recognized by NS1-specific antibodies and also have reduced protein stability within HeLa cells.

Dimerization of NS1 Mutants

The NS1 protein dimerizes within the lumen of the ER following post-translational processing of the polyprotein. Based upon the previous findings that several of the NS1 mutants are less stable in HeLa cells, we assessed the ability of the proteins to dimerize. As Figure 3A indicates, both monomeric and dimeric forms of all the NS1 mutants were observable in unheated cell lysates, similar to wt NS1. Longer expose of these blots also

revealed higher order oligomerization of all the NS1 mutants (data not shown). Thus, the ability of NS1 mutants to dimerize does not appear to be affected by the amino acid changes.

Glycosylation of NS1 Mutants

Following dimerization, NS1 acquires *N*-linked glycans in the ER and Golgi. We determined whether the NS1 mutants are able to acquire proper glycosylation by digesting proteins, present in cell lysates, with the enzyme Endo H. High-mannose glycans are sensitive to digestion by the enzyme while complex/hybrid glycans are resistant. Wild type NS1 produced two bands by immunoblot following Endo H digestion; a faster migrating band of Endo H sensitive NS1 (~40kd) and a slower migrating band of Endo H resistant NS1 (~50kd) (Figure 3B). Similar to the wild type protein, two bands of NS1 at the correct apparent molecular weights were also observed for G295R NS1 following Endo H digestion. However, the intensity of the G295R resistant band was significantly lower than that of wt NS1, while the intensity of the Endo H sensitive band was increased above that of wt NS1. This suggests that a proportion of G295R NS1 undergoes modification of N-linked glycans within the Golgi but that this fraction of G295R NS1 is significantly less abundant than that of wt NS1. On the other hand, we found that the P320S and M333V NS1 mutants are completely sensitive to digestion with Endo H (Figure 3B). The intensity of the P320S and M333V Endo H sensitive bands were also greater than that of the wt NS1 protein following digestion.

Notably, all of the NS1 mutants migrated at the predicted size of approximately 50kd, without Endo H digestion, suggesting that they were able to acquire high-mannose *N*-linked

glycosylation in the ER (Figure 3C). Further, undigested wt NS1 and G295R NS1 proteins migrated as slightly higher molecular weight smears whereas the other mutants produced tight bands. The smearing pattern observed for wt NS1 and G295R NS1 is most likely due to a proportion of the proteins acquiring complex/hybrid N-linked glycosylation, which retards their migration. Taken together, these data suggest that NS1 mutants acquire high-mannose glycosylation within the ER, but are either improperly processed within the Golgi, fail to reach the Golgi, or that the amino acids changes prevent *N*-linked glycan modification to complex/hybrid in the Golgi.

Role of NS1 Glycosylation in TLR3 Inhibition

Several studies have determined that glycosylation-null NS1 flaviviruses grow well *in vitro,* but are highly attenuated in mouse models of infection [24, 25]. These data combined with our Endo H resistance data for the NS1 mutants suggested that proper glycosylation of NS1 may be required for TLR3 inhibition. To address whether glycosylation of NS1 is a prerequisite for TLR3 inhibition, we mutated the three *N*-linked glycosylation sites (N130, N175, and N207) of NS1 to alanine residues, and performed TLR3 signaling studies. NS1 glycosylation mutants were expressed from the same lentivirus vector, following site-directed mutagenesis, and stable HeLa cell lines, containing an NFκB-dependent luciferase reporter were selected as described in Chapter 2. Immunoblot analysis of the proteins confirmed that each glycosylation mutant migrated faster than wt NS1, and migration was dependent upon the number of *N*-linked sites present on the protein (Figure 4A). As shown in Figure 4B and C, loss of NS1 glycosylation at any and all positions does not affect TLR3

inhibition as measured by IL-6 secretion and NFκB reporter activity. In contrast to another study, we also found that glycosylation-null NS1 is able to be secreted from the cells, albeit to levels slightly below wt NS1 (Figure 4D). We also demonstrated that glycosylation-null NS1 is able to properly dimerize (Figure 4D).

Cell Surface Expression of NS1 Mutants

Improper glycosylation of the NS1 mutants indicated that the proteins were not properly processed within the Golgi or failed to reach the Golgi. Following its transit in the Golgi network, NS1 is either directly targeted to the cell surface as a dimer, or secreted into the extracellular milieu as a multimer, which can then bind back to the cell membrane. We used flow cytometry to assess cell surface expression of the NS1 mutants using a monoclonal antibody that efficiently recognizes cell surface-associated NS1 protein. Initially, we noticed that the monoclonal antibody used for surface NS1 detection did not equally recognize some of the NS1 mutants regardless of cell permeability (Figure 5A). This was similar to the results from our immunofluorescence microscopy experiment, which found inefficient antibody recognition of intracellular NS1 mutants (Figure 1). Therefore, comparative studies of NS1 positive cells were problematic. To address differences in mutant NS1 detection by the antibody, we analyzed the mean fluorescence intensity (MFI) of the NS1 signals on and within NS1 positive cells and then calculated surface and intracellular NS1 MFI as a ratio of the total NS1 MFI (Figure 5B). Intracellular NSI MFI was calculated by subtracting the surface NS1 MFI from the total NS1 MFI acquired by flow cytometry. As illustrated in Figure 5B, the ratio of surface NS1 MFI to intracellular NS1 MFI was lower for all the NS1

mutants than that observed for wt NS1. In fact, the ratio of wt NS1 surface and intracellular MFI were nearly equivalent. These data suggest that for wt NS1 the amount of NS1 on the surface of cells is nearly equivalent to the amount of NS1 within the cells. In contrast, significantly more mutant NS1 is localized within cells than is present on the cell surface, suggesting that the NS1 mutants inefficiently reach the cell surface.

Secretion of NS1 Mutants

NS1 protein can also be secreted as a hexamer from expressing cells and a proportion of this protein can subsequently bind back to the plasma membrane through interactions with GAGs. The reduced amounts of mutant NS1 protein on the surface of cells, observed in Figure 5A and B, may be the result of a failure of the proteins to be directly targeted to the plasma membrane or a failure of the proteins to be secreted and bind back to GAGs present on the cell membrane. To address these possibilities, and to determine the overall amount of mutant NS1 being secreted, we investigated the ability of NS1 mutants to be secreted from expressing cells and to accumulate in cellular supernatants. Serum-free cellular supernatants were captured after 48h of incubation and analyzed for the presence of NS1 by immunoblot using antibody specific to the epitope tag. As expected, wt NS1 was readily detected in the supernatant of expressing cells (Figure 5C). Strikingly, considerably less G295R NS1 was detected in the cell culture supernatants compared to wt NS1, and both P320S and M333V NS1 proteins were undetectable in the cellular supernatants by immunoblot.

These data indicate that the amino acids residue changes in the NS1 protein result in either inefficient secretion or failure to be secreted from expressing cells. These data also

suggest that the lower levels of mutant NS1 present on the surface of expressing cells are most likely due to a failure of the mutants to be secreted and bind back to the membrane.

Roles of NS1 Cell Surface Expression/Association and Secretion in TLR3 Inhibition

As indicated previously, the mechanism of TLR3 inhibition is not understood. Our previous studies demonstrated that NS1 expressed within cells inhibits TLR3 signal transduction and the purified secreted form of wt NS1 is sufficient to inhibit TLR3 signaling in cells pre-treated with the protein. Therefore, the intracellular and secreted forms of the NS1 protein may be inhibiting TLR3 signaling through a common mechanism involving NS1 secretion and subsequent binding of the protein to cells, or by divergent mechanisms. The findings that surface expression and secretion of mutant NS1 proteins are reduced suggested that their inability to block TLR3 signaling may be the result of a failure to be secreted from the cell and/or interact with a cell surface molecule. To address these possibilities, we undertook two experiments to determine if TLR3 signaling could be restored through 1) prevention of secreted wt NS1 binding back to the cell membrane or 2) prevention of wt NS1 reaching the cell membrane and being secreted from cells.

Initially, we used the chemical inhibitor sodium chlorate ($NaClO_3$) to prevent the secreted form of wt NS1 from binding back to GAGs present on the cell surface. $NaClO_3$ blocks the sulfation of GAG linkages, which is required for their proper expression on cell membranes [14]. As Figure 6A indicates, $NaClO_3$ was able to significantly reduce the number of surface NS1 positive cells following 20h of treatment. This data would also suggest that a large proportion of detectable surface NS1 present on cells is represented by

the secreted form of the protein that has bound back to GAGs. Interestingly, despite preventing NS1 from binding back to the cell membrane we were unable to restore TLR3 signaling in response to pIC treatment (Figure 6B). Therefore, in cells expressing wt NS1, secreted NS1 binding back to GAGs is not required for inhibition of TLR3 signal transduction.

In the previous experiment, the binding of secreted NS1 to cell membrane GAGs was prevented by $NaClO_3$ treatment. While the flow cytometry data indicated that a large proportion of surface NS1 was lost on these cells (Figure 6A), dimeric NS1 was still able to reach the surface and NS1 protein was still actively secreted into the extracellular medium. It is possible that this surface targeted and/or secreted NS1 was functioning to inhibit TLR3 signaling. To address this question, we blocked NS1 secretion and cell surface targeting of the protein by treating cells with the chemical inhibitor brefeldin A for 20h. Brefeldin A blocks protein transport in the ER and Golgi and consequently, prevents NS1 from reaching the cell surface and being secreted from cells. Figure 6C demonstrates that 20h of brefeldin A treatment almost entirely eliminated surface NS1 positive cells as detected by flow cytometry. Therefore, intracellular NS1 is presumably retained within the ER and Golgi of these cells. Nevertheless, these cells are still able to completely inhibit TLR3 signal transduction in response to pIC treatment as measured by IL-6 transcript copy number (Figure 6D). These data indicate that intracellularly NS1 is likely targeted to a specific as of yet unidentified location and that this is sufficient to inhibit TLR3 signaling.

Discussion

In this study we have defined several defects in the processing of mutant NS1 proteins that contain individual amino acid changes previously identified to allow TLR3 signaling. Initially, we discovered differential antibody recognition of several of the NS1 mutants using antibodies directed against NS1 epitopes. However, immunoblot to detect the epitope tag present on the NS1 proteins demonstrated that all mutants were expressed to similar levels and we were able to accurately quantify NS1 protein levels throughout the study. Immunoblot was successfully used to quantify NS1 intracellular levels, glycosylation, and secretion. Using an analysis of the NS1 MFI of positive cells as a ratio of surface to intracellular NS1 MFI, we were also able to successfully quantify NS1 surface expression by flow cytometry, despite the differential recognition of the NS1 mutants by NS1-specific antibodies.

The G295R mutant acted most similar to wt NS1, but still showed reduced glycosylation and was only inefficiently secreted from cells (Figure 5C). Two of the NS1 mutants (P320S and M333V) were found to be less stable than the wt NS1 protein, however, we observed equivalent levels of mutant NS1 protein and wt NS1 protein within resting cells, as determined by immunoblot (Figure 2). Thus, differences in secretion between the wt NS1 and mutant NS1 proteins would most likely explain this discrepancy. This hypothesis was confirmed by treating expressing cells with either brefeldin A or cycloheximide. Brefeldin A-treated wt NS1 cells accumulate NS1 protein over time, which was not observed in mutant NS1 expressing cells. Conversely, cycloheximide-treated wt NS1 cells rapidly loose NS1

protein over time, whereas mutant NS1 cells loose NS1 protein at a less rapid rate (data not shown).

The combined results of differential antibody recognition and protein stability data suggest that the NS1 amino acid changes result in misfolding of the proteins. If these NS1 proteins are indeed misfolded, they could possibly induce an unfolded protein response (UPR) in the ER of expressing cells (reviewed in [26]). A key feature of the UPR is reduction in unessential gene translation concomitant with an increase is stress response gene transcription and translation. The untranslated gene transcripts are then degraded within cells undergoing a UPR. However, the induction of UPR is unlikely in our mutant NS1 expressing cells as transcript levels of the mutant NS1 genes appear to be equivalent or slightly elevated compared to those of the wt NS1 protein in resting cells, and these cells respond to treatment with pIC through the secretion of cytokines. A quantitative assessment of UPR induced gene transcripts and proteins, such as BIP and XBP-1, would be necessary however, to fully exclude this possibility [27]. How exactly misfolding of the NS1 proteins could impact TLR3 signaling is not known, since the mechanism of inhibition has not been resolved. Aberrant folding of the mutant proteins could prevent NS1 interactions with a binding partner(s) that is required for TLR3 inhibition, or the proteins may be degraded prior to this interaction. To properly address these questions, mechanistic studies must be performed with NS1, but are beyond the scope of this manuscript.

Additional consequences of improper folding of the mutant NS1 proteins include increased degradation and improper targeting within the cell. This is already demonstrated by the fact that all NS1 mutants were not secreted and inefficiently reach the cell surface. An

additional observation that could illustrate improper intracellular targeting was the finding that *N*-linked glycosylation of all of the NS1 mutants was altered in comparison with the wild type protein. We directly investigated the role of NS1 glycosylation in TLR3 inhibition using glycosylation-null NS1 mutants. The results indicated that glycosylation of the NS1 protein per se is not a requirement for TLR3 inhibition. This suggests that improper glycosylation is the cause of improper transport and processing of the mutants but not the defect responsible for loss of TLR3 inhibition.

Previous work by our group established that the secreted form of wt NS1 is sufficient to inhibit TLR3 signaling in cells pretreated with the protein. These findings combined with the observation that NS1 mutants are inefficiently expressed on the cell surface and fail to be efficiently secreted led us to the hypothesis that lack of secretion and/or cell surface association of the NS1 mutants may explain their TLR3 inhibition phenotype. Surprisingly, neither inhibition of cell surfaceassociation, nor inhibition of secretion of wt NS1, were able to restore TLR3 signaling. These results combined with our other observations described above strongly indicate that correctly-targeted intracellular NS1 is necessary and sufficient for TLR3 inhibition in cells.

These findings also raise important questions in the context of TLR3 inhibition by the secreted form of NS1. One possibility is that secreted and intracellular NS1 proteins function through independent mechanisms to inhibit TLR3. However, given the very limited coding capacity of the WNV genome, we consider it unlikely that two separate mechanisms would have evolved by the same protein to inhibit the same PRR. The other possibility is that intracellular and secreted NS1 inhibit TLR3 through a common mechanism, which would

require that both forms of the protein get correctly targeted to an unidentified subcellular localization. Our group and others have shown that secreted NS1 can be actively endocytosed and resides within late endosomes and potentially other structures [28]. We would then speculate that intracellularly expressed wt NS1 is targeted to the same localization(s) and that this targeting is defective in our NS1 mutants. Additional studies will be required to address these issues.

It is important to also discuss the NS1 mutants in the context of RNA replication. Our previous study (Chapter 2) demonstrated that the NS1 amino acid residue changes specifically affected NS1-mediated inhibition of TLR3 signaling, while replicative function of the proteins was preserved. However, several of the NS1 defects we characterized in this study raise questions about how these proteins are still able to function in replication. During WNV infection, a portion of NS1 is retained in the ER where it is proposed to contribute to virus genome replication. Our data indicate that the mutant forms of NS1 dimerize normally in the ER and acquire proper high mannose glycosylation therein. Thus, it is probable that the mutant NS1 proteins in the ER are folded in a conformation that is replication competent. The cumulative findings of this study indicate that amino acid changes G295R, P320S, and M333V directly affect processing and localization of the NS1 protein that is required for inhibition of TLR3, but not for replication.

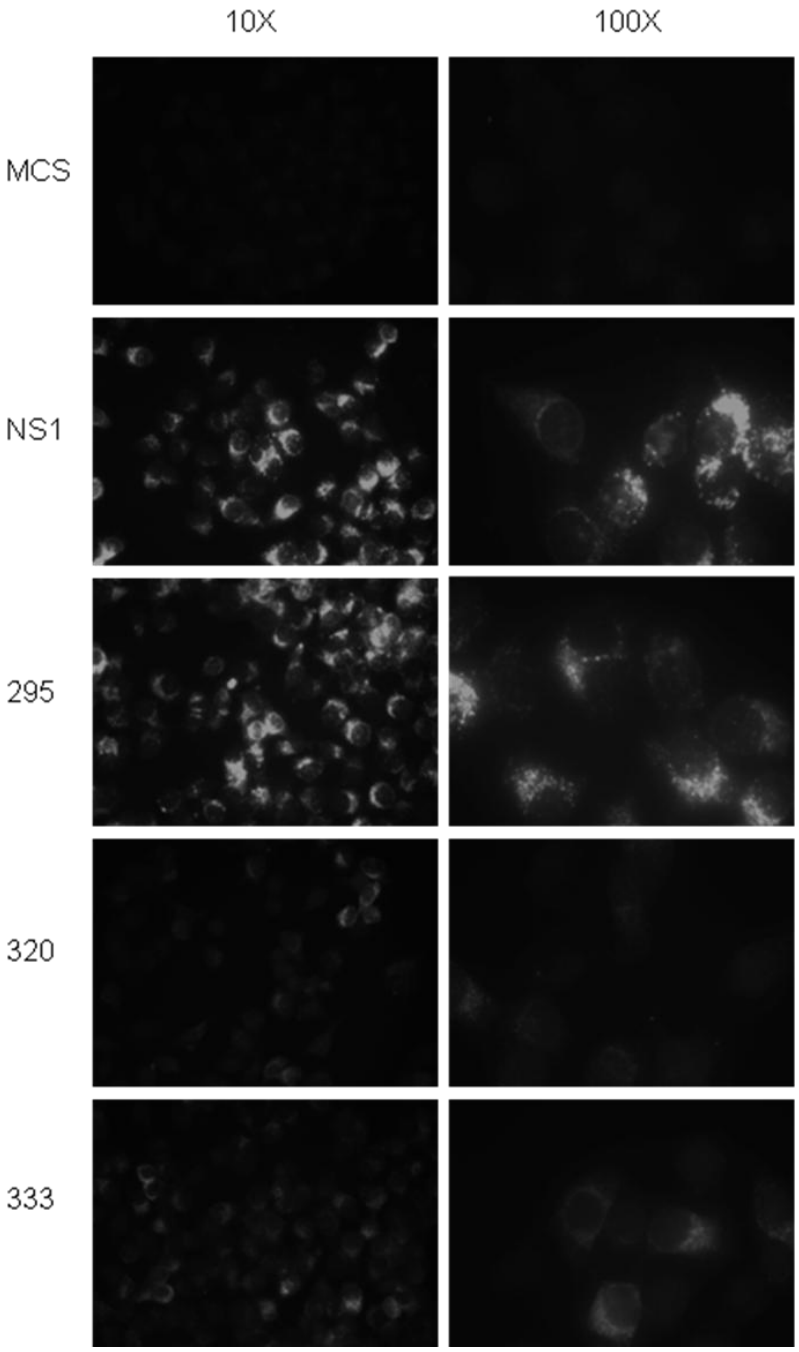


Figure 1: **Intracellular distribution of NS1 proteins.** HeLa cells stably expressing G295R NS1, P320S NS1, M333V NS1, wt NS1, or empty vector (MCS) were plated in chamber slides overnight then fixed and permeabilized. NS1 protein was detected by mouse anti-WNV hyper-immune ascitic fluid (HIAF) and secondary goat-anti mouse Alexa Fluor 488-conjugated antibody by immunofluorescene microscopy. Images are representative of three independent experiments.

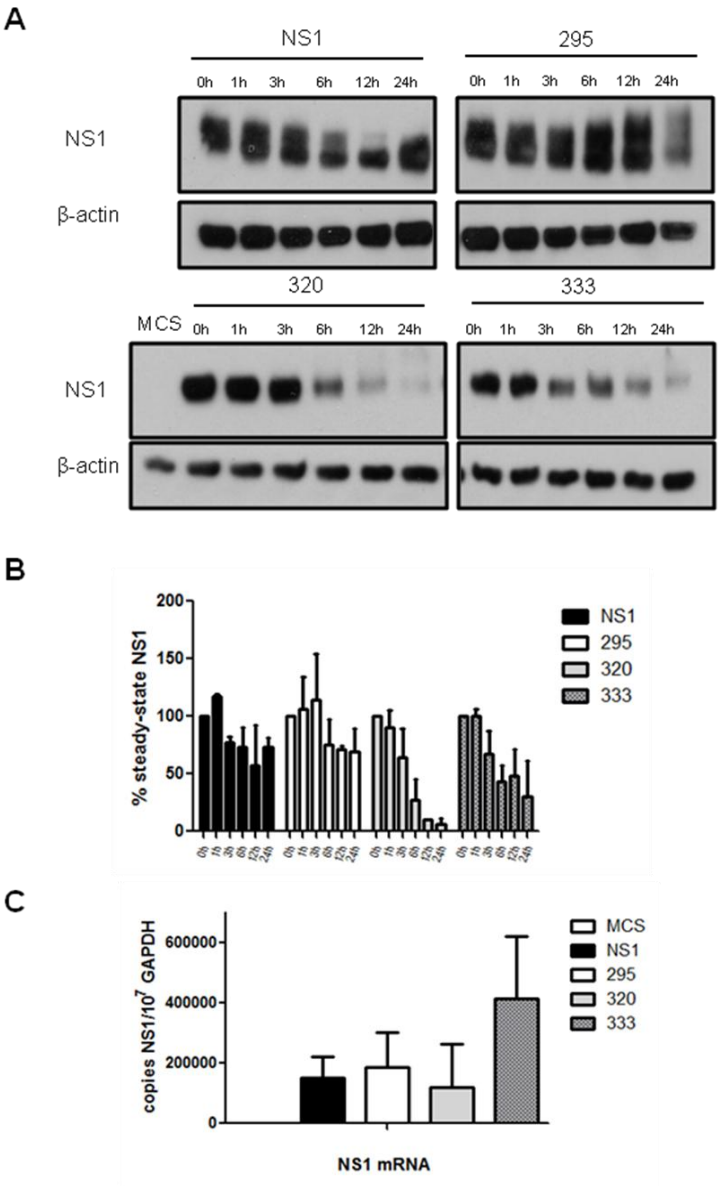


Figure 2: **Intracellular stability of NS1 proteins.** (A) HeLa cells stably expressing G295R NS1, P320S NS1, M333V NS1, wt NS1, or empty vector (MCS) were plated in 6-well tissue culture dishes and were treated for up to 24h with brefeldin A and cycloheximide. Cell lysates were prepared at 0, 1, 3, 6, 12, and 24h post-treatment. NS1 protein in the lysates was detected by immunoblot using an anti-Tetra His antibody specific to the epitope tag and was normalized to β-actin. (B) Densitometry of the blots was performed with ImageJ and percent NS1 steady-state levels were calculated by setting the 0h time-point as 100% NS1 for each mutant. (C) Transcripts of NS1 mRNA in resting cells were determined by qRT-PCR and were normalized to GAPDH. Error bars are representative of standard deviation from the mean. Data are the average of two independent experiments

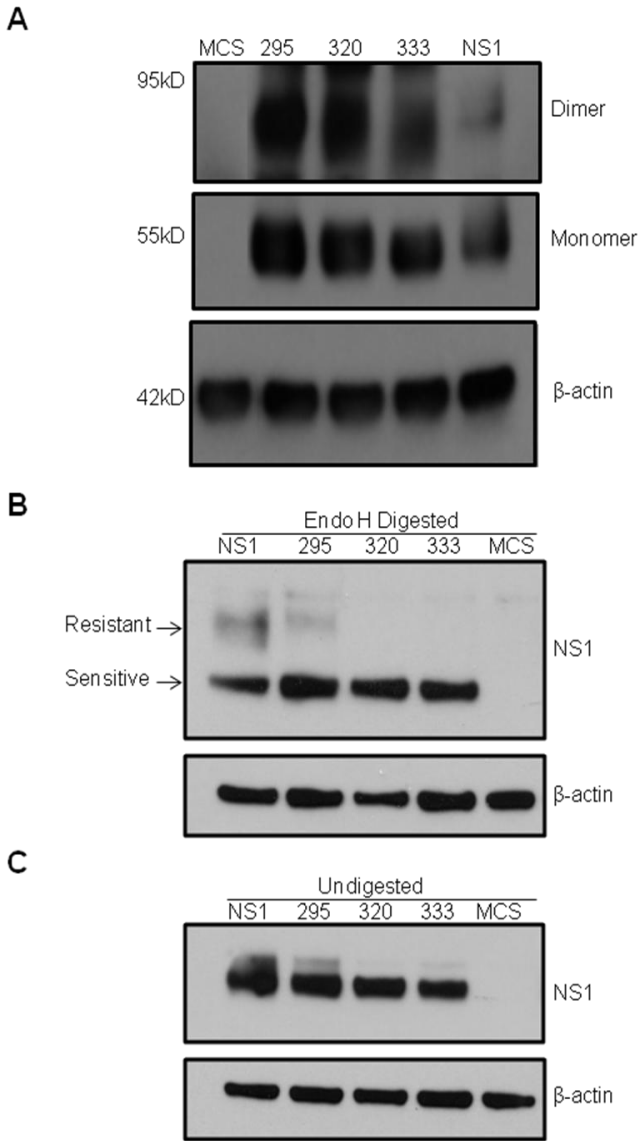


Figure 3: **Dimerization and glycosylation of NS1 proteins.** (A) Unheated cell lysates of each NS1 expressing cell line were prepared and NS1 monomers and dimers were detected by immunoblot using an anti-Tetra His antibody specific to the epitope tag and was normalized to β-actin. Detection of NS1 dimers required longer exposure of the blot. (B) Whole cell lysates of each NS1 expressing cell line were prepared, and glycans were digested with Endo H for 5min. NS1 protein was detected by immunoblot using an anti-Tetra-His antibody specific to the epitope tag and was normalized to β-actin. (C) Heated cell lysates of each NS1 expressing cell line were prepared and NS1 protein was detected by immunoblot using an anti-Tetra His antibody specific to the epitope tag and was normalized to β-actin. Data are representative of three independent experiments.

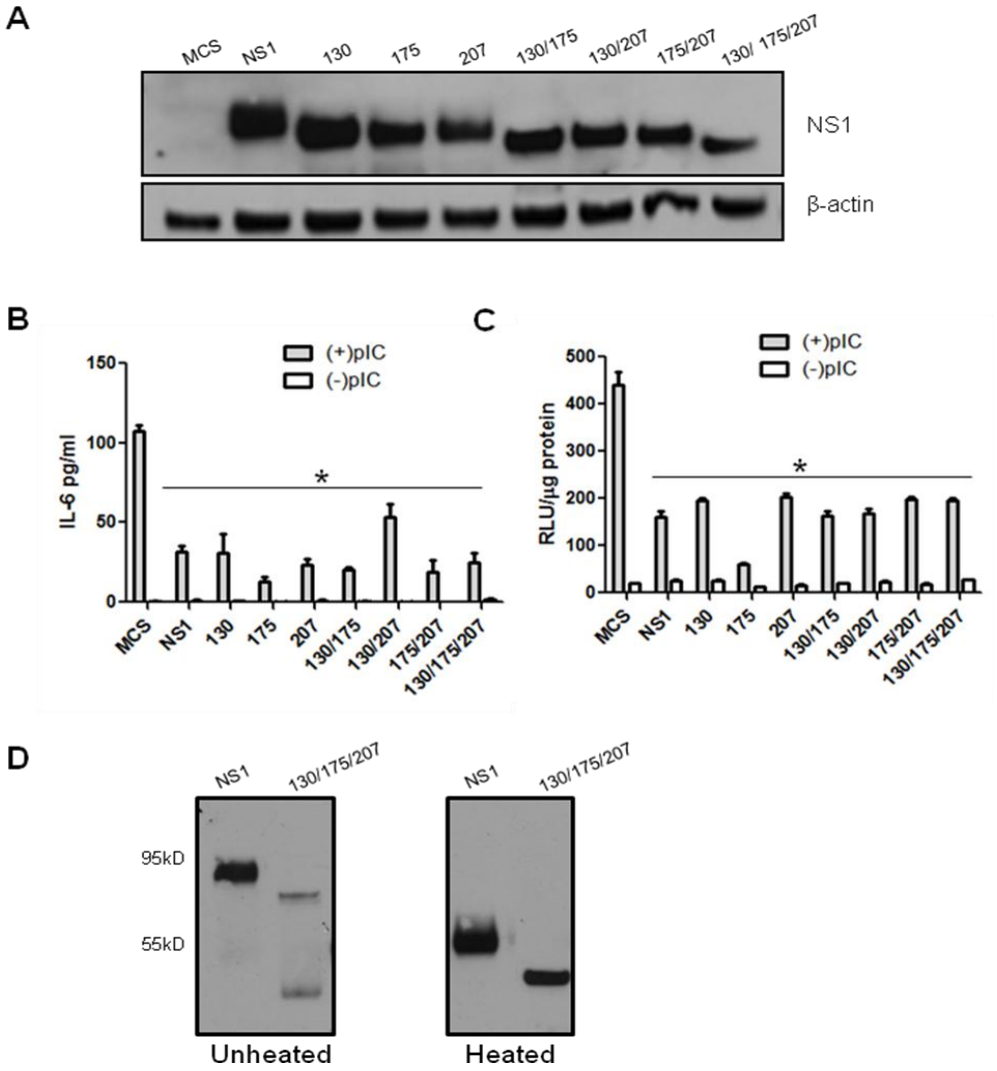


Figure 4: **Role of NS1 glycosylation in TLR3 inhibition and NS1 secretion.** (A) Cell lysates were prepared from HeLa cells stably expressing NS1 glycosylation mutants and NS1 protein was detected by immunoblot using an anti-Tetra His antibody specific to the epitope tag and was normalized to β-actin. (B) IL-6 concentrations in the supernatants of HeLa cells expressing the NS1 glycosylation mutants were determined by ELISA following 8h pIC stimulation. (C) NFκB induction in HeLa cells expressing the NS1 glycosylation mutants was determined by luciferase reporter assay following 8h pIC stimulation. (D) HeLa cells expressing glycosylation-null NS1 were plated overnight and serum-free medium was added to the cells for 48h. NS1 protein in unheated or heated supernatants was detected by immunoblot using an anti-Tetra His antibody specific to the epitope tag. Error bars represent standard deviation from the mean and statistical significance was determined by t-test when appropriate. Data are representative of three independent experiments.

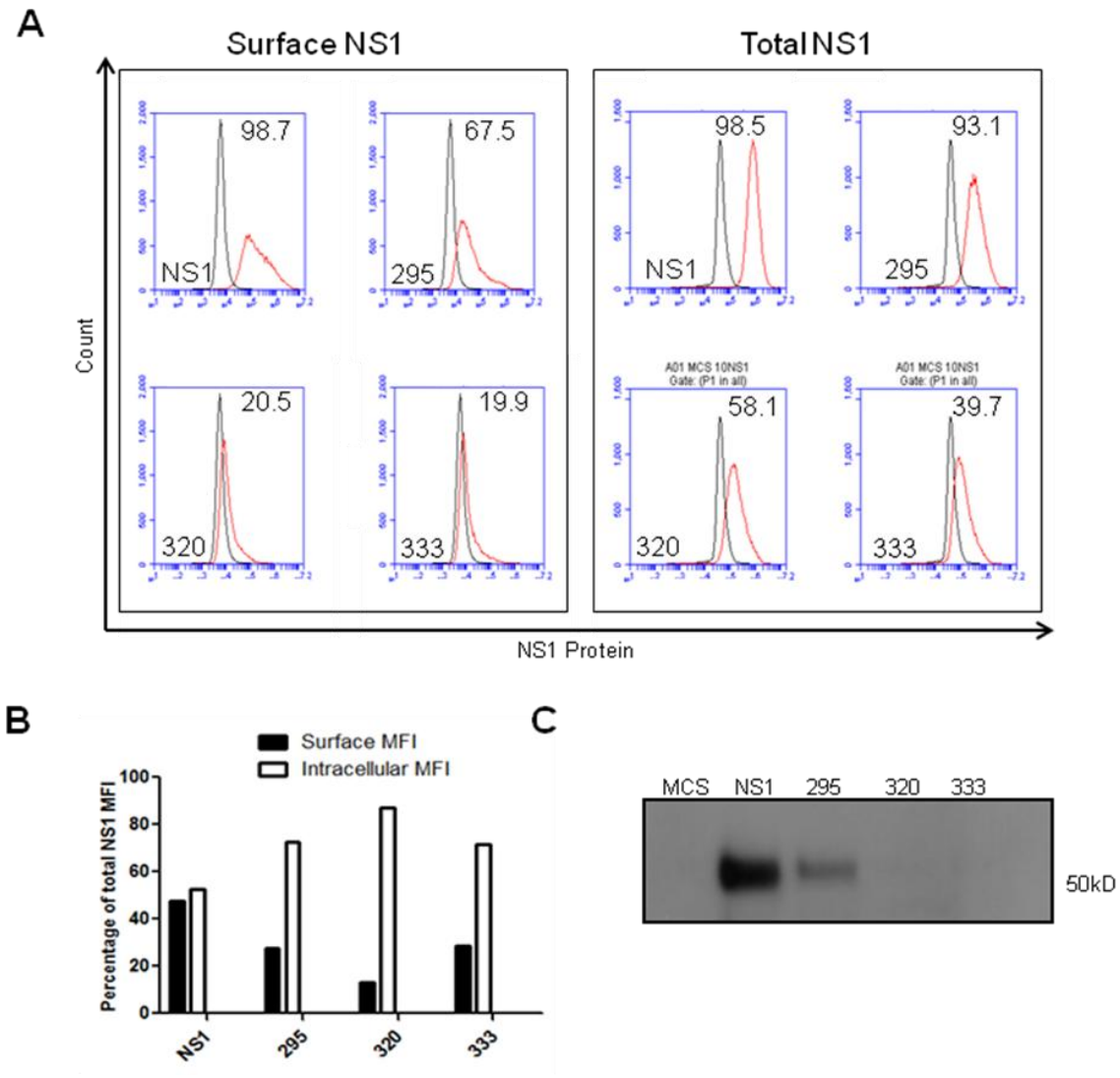
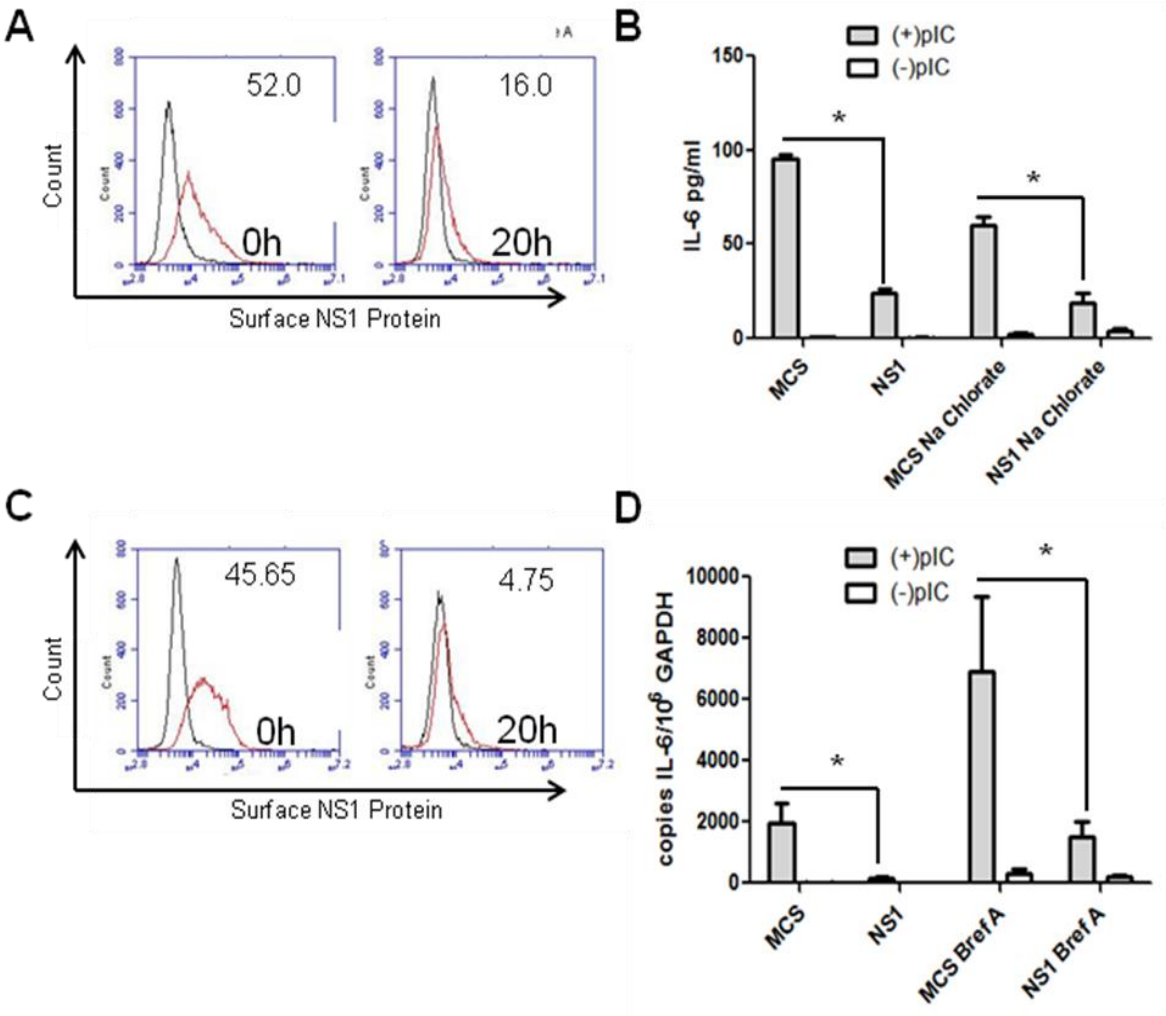


Figure 5: **Cell surface expression and secretion of NS1 proteins.** (A) HeLa cells stably expressing G295R NS1, P320S NS1, M333V NS1, wt NS1, and empty vector (MCS) were detached from tissue culture flasks with EDTA and surface NS1 was detected by mouse anti-WNV monoclonal antibody (10NS1) and secondary goat-anti mouse Alexa Fluor 488-conjugated antibody by flow cytometry. Total NS1 expressed in these cells was also determined by flow cytometry using the same antibodies, following fixing and permeabilization of the cells. (B) The total NS1 MFI of the cells was calculated as a ratio of surface to intracellular NS1. (C) HeLa cells stably expressing G295R NS1, P320S NS1, M333V NS1, wt NS1, and empty vector (MCS) were plated overnight and serum-free medium was add to the cells for 48h. NS1 protein was detected by immunoblot using an anti-Tetra-His antibody specific to the epitope tag. Data are representative of three independent experiments.

Figure 6: **Roles of NS1 cell surface expression/association and secretion in TLR3 inhibition.** (A) HeLa cells stably expressing wt NS1 or empty vector control (MCS) were plated as semiconfluent monolayers in tissue culture plates and were treated for 20h with sodium chlorate or left untreated. Cells were detached with EDTA and surface NS1 was detected by mouse anti-NS1 monoclonal antibody (JE NS1) and secondary goat-anti mouse Alexa Fluor488-conjugated antibody by flow cytometry. (B) Cells were treated as above with or without sodium chlorate and sodium chlorate-containing medium was removed, cells were washed multiple times in PBS, and medium was replaced with or without pIC and/or sodium chlorate for 8h. IL-6 concentrations in the supernatant were determined by ELISA. (C) HeLa cells stably expressing wt NS1 or empty vector control (MCS) were plated as semiconfluent monolayers in tissue culture plates and were treated for 20h with Brefeldin A or left untreated. Cells were detached with EDTA and surface NS1 was detected by mouse anti-NS1 monoclonal antibody (JE NS1) and secondary goat-anti mouse Alexa Fluor 488-conjugated antibody by flow cytometry. (D) Cells were treated as in (C) with or without Brefeldin A, Brefeldin A-containing medium was removed, cells were washed multiple times in PBS, and medium was replaced with or without pIC and/or Brefeldin A for 8h. IL-6 transcripts in the cells were determined by qRT-PCR and were normalized to GAPDH. Error bars represent standard deviation from the mean and statistical significance was determined by t-test when appropriate. Data are representative of two independent experiments.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CHAPTER 4

Summary

Previous investigations by our group and others have described several novel roles for the WNV NS1 protein in modulating innate immune responses both *in vitro* and *in vivo*. In particular, we have defined that both intracellular and secreted NS1 are capable of inhibiting signal transduction originating from TLR3. To expand upon these initial findings, we developed a FACS-based screen aimed at identifying amino residues within NS1 that are involved in the inhibition of TLR3 signaling. Due to the essential role of NS1 in WNV replication, the screen was engineered to only select NS1 mutants that still supported virus replication. The NS1 coding sequence was randomly mutated and expressed in a HeLa reporter cell line via a lentivirus vector. The HeLa reporter cell line was constructed so that expression of the red fluorescent protein DsRed2 was under the control of NFκB, a transcription factor activated by TLR3 signaling. The reporter cell line, expressing different NS1 mutants in *trans*, was infected with GFP expressing single-cycle, WNV replicon particles (VRPs). Replicon replication and thus GFP expression were dependent upon expression of replication-competent NS1. Other similar studies of multifunctional flavivirus replication proteins have largely failed to consider the effects of mutagenesis on replication, and therefore, have considerably less biological significance. By addressing the affects of

mutagenesis on replication competency we have significantly advanced the scope and impact of our screen.

The screen successfully identified three NS1 double mutants, each of which 1) allowed TLR3 signaling to occur above the levels determined for wt NS1 protein, and 2) supported *trans*-complementation of WNV GFP ΔNS1 VRPs. Each double mutant was dissected and the amino acid changes were reconstituted within the lentivirus NS1 vector to determine the contribution of each individual amino acid change to the function of the protein. The findings from this work strongly implicate the contribution of amino acids in the C-terminal half of WNV NS1 to TLR3 inhibition. In particular, TLR3 signaling occurred above the level observed for wt NS1 expressing cells when residues at positions 295, 320, and 333 were mutated. Additionally, the changes at those positions still allowed the NS1 proteins to support vRNA replication as measured by replicon particle *trans*-complementation. Based on ClustalW alignment the amino acid residues identified by our screen were conserved across numerous mosquito- and tick-borne flavivirus NS1 proteins. These findings suggest that the C-terminal half of the NS1 protein, and in particular residues G295, P320, and M333, are evolutionarily conserved and most likely of biological significance.

We also investigated defects in the mutant NS1 proteins which allowed TLR3 signaling. The mutant NS1 proteins had reduced stability, improper glycosylation, were inefficiently expressing on the cell surface, and failed to be secreted from cells. Therefore, it is likely improper processing and localization of the mutants resulted in failure to inhibit TLR3.

Based upon these cumulative findings, other flavivirus NS1 proteins may also be capable of inhibiting TLR3 signaling through a conserved mechanism and it will be important to experimentally test this hypothesis going forward. Other investigators in our lab have begun this work and have generated strong preliminary evidence that suggest the DEV, Kunjin, and YFV NS1 proteins are capable of inhibiting TLR3 signaling, to varying degrees, in HeLa cells (unpublished data). Ideally, this work would expand to include investigations of a panel of NS1 proteins produced by mosquito-borne, tick-borne, and NKV viruses, that cause a variety of clinical diseases including fever, encephalitis, and hemorrhagic fever. It will be interesting to determine if the phenotypes of flavivirus NS1 proteins correlate with certain types of disease.

In the presented studies immortalized HeLa cells were used as a powerful tool to investigate WNV replication kinetics and interactions with the innate immune response. However, HeLa cells are not representative of primary cell types which are normally infected by WNV. Infection of primary innate immune cells, such as dendritic cells and monocytes, with the NS1 mutant viruses would allow for more accurate assessments of the role of NS1 *in vitro*.

Another key finding from our lab is that WNV NS1 protein inhibits signal transduction originating from TLRs other than TLR3 and can potentially inhibit RIG-I signaling (unpublished data). Interestingly, we observed that HeLa cells infected with the P320S NS1 WNV secreted more IL-6 in response to pIC stimulation. We also determined that HeLa cells secreted significantly more IL-6 in response to infection alone with the P320S virus than wt WNV or the other NS1 mutant viruses (Figure 1). These data indirectly

suggest that P320S NS1 WNV may be more efficiently detected by the innate immune system, or that NS1 is functioning to inhibit multiple PRRs and mutation of residue 320 relieves the inhibition. Preliminary studies using HeLa cells knocked-down for TLR3 expression indicated that the IL-6 produced in response to P320S WNV is not TLR3-dependent. To further address these possibilities innate immune cells derived from specific knock-out mice (e.g. TLR3, TRIF, MyD88, or RIG-I) could be infected with the mutant viruses to measure cytokine responses. Alternatively, growth curves in these cells could also be performed to determine if growth kinetics of P320S NS1 WNV can be restored in the absence of specific innate immune signaling receptors/molecules.

Ultimately, *in vivo* pathogenesis studies must be performed with P320S NS1 WNV. Those studies would allow us to gain insights into the role of NS1 in pathogenesis and the role of TLR3 in controlling infection. Given the controversy over the role of TLR3 in pathogenesis, it will be interesting to see how the P320S virus behaves in wild type mice. Its attenuated growth phenotype in HeLa cells would suggest that it will also be attenuated in mice. However, its ability to induce cytokines may lead to increased immunopathology. As detailed in Chapter 2, the stability of the P320S virus is a concern for moving work into the animal model and we are currently in the process generating a more stable virus. Another alternative may be to simply mutate paired, charged amino acids residues between positions 255 and 333 and screen for the P320S phenotype with increased genetic stability.

Addressing the role of NS1 in the natural maintenance cycle of the virus will also be important. WNV is a virus of mosquitoes and birds, with humans and other mammals representing incidental hosts. From an evolutionary standpoint the virus co-evolved within its

two natural hosts and inhibition of TLR signaling in humans may simply be the consequence of selective pressure from the mosquito and/or bird innate immune systems. In the future, it will be interesting to determine if the WNV NS1 protein is able to inhibit Toll signaling in mosquito cells, and to investigate innate immune signaling in mosquito cells in response to infection with wt WNV and P320S WNV.

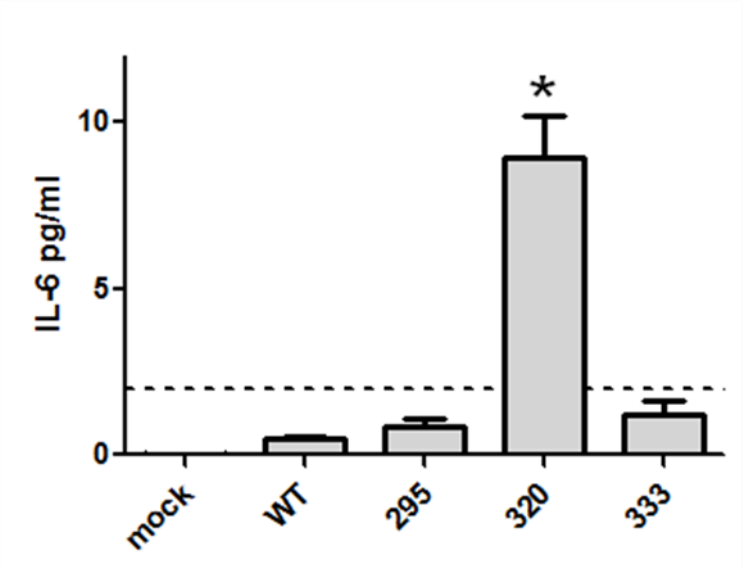

Figure 1: **IL-6 secretion in response to WNV infection.** HeLa cells were infected with wt WNV or each WNV NS1 mutant at MOI 5. IL-6 in supernatants was determined 20h post-infection by ELISA. Data are representative of three independent experiments. Error bars represent standard deviation from the mean. Statistical significance was determined by student's t-test.