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Virulence determinants of West Nile virus: how can these be used for vaccine design?

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West Nile virus (WNV), a neurotropic mosquito-borne flavivirus, has become endemic in the USA and parts of Europe since 1999. There is no licensed WNV vaccine for humans. Considering the robust immunity from immunization with live, attenuated vaccines, a live WNV vaccine is an ideal platform for disease control. Animal and mosquito studies have identified a number of candidate attenuating mutations, including the structural proteins premembrane/membrane and envelope, and the nonstructural proteins NS1, NS2A, NS3, NS4A, NS4B and NS5, and the 3' UTR. Many of the mutations that have been examined attenuate WNV using different mechanisms, thus providing a greater understanding of WNV virulence while also identifying specific mutations as candidates to include in a WNV live vaccine.

First draft submitted: 15 December 2016; Accepted for publication: 14 February 2017; Published online: 28 April 2017

West Nile virus (WNV) is a mosquito-borne neurotropic flavivirus of worldwide public health importance [1]. The genus *Flavivirus* includes many pathogenic viruses including mosquito-borne pathogens yellow fever virus (YFV), Japanese encephalitis virus (JEV), dengue virus (DENV) and Zika virus, plus tick-borne encephalitis virus (TBEV) [2]. WNV was first isolated in Uganda in 1937 and has since been identified throughout Africa, Asia, Europe and the Americas [3]. WNV was not detected in the western hemisphere until its isolation in New York in 1999, and the outbreaks observed thereafter in North, Central and South America have been associated with a high incidence of disease among avian, equine and human populations [3]. WNV has a primary transmission cycle between *Culex* sp. mosquitoes and birds as amplifying hosts, however, other vertebrates including equines and humans can be infected with WNV and potentially have clinical disease if a mosquito infects them during a blood meal [1]. Most vertebrates other than birds have low viremias and are unable to be transmitted to mosquitoes during feeding and thus are deemed 'dead-end' hosts [1]. These incidental hosts can still contract WNV disease. Overall, 80% of WNV infections are asymptomatic, 20% get WN fever and 1% of cases progress to severe neurological symptoms including acute flaccid paralysis, meningitis and encephalitis, of which 10% will die [4]. Between 1999 and 2016 there have been approximately 1995 deaths in the USA alone [5,6]. Although there are veterinary vaccines that are effective at controlling the disease, annual WNV outbreaks are observed and anticipated in many countries including the USA, making development of a human vaccine of the utmost importance for WNV control [5–7].

WNV encodes a positive-sense single-stranded RNA genome that is approximately 11 kilobases in length [3]. This encodes a single open-reading frame with terminal 5' and 3' UTRs that have a 5' cap and no poly-A tail, respectively [2]. The viral polyprotein is co- and post-translationally cleaved into ten unique proteins: the structural proteins capsid (C), premembrane (prM) and envelope

KEYWORDS

- attenuation • flavivirus
- live attenuated vaccine
- virulence • West Nile virus

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Figure 1. Attenuating mutations in the West Nile virus genome. A representation of the different genes in the West Nile virus genome with arrows indicating attenuating mutations. The black arrows above the genome denote mutations engineered using reverse genetics, and the gray arrows below the genome denote attenuating mutations identified in natural West Nile virus isolates.

(E) and the seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Figure 1) [3]. While the three structural proteins compose the WNV virion, the NS proteins are important for intracellular multiplication, including proper virion assembly, viral replication and evasion of host immunity [2].

Currently, there are no antiviral drugs that effectively target any members of the flavivirus genus, however, the flaviviruses YFV, JEV and TBEV each have safe and effective licensed vaccines [8,9]. The TBEV vaccine is formalin-inactivated, while there is an empirically derived live attenuated YFV vaccine and recently a chimeric recombinant live attenuated vaccine has been licensed for DENV in approximately 13 countries [9,10]. Additionally, there are three types of JEV vaccines: formalin-inactivated, empirically derived live attenuated and a recombinant live attenuated vaccine [9]. There are ongoing studies investigating candidate WNV vaccines including inactivated, recombinant and chimeric versions, however, none of these vaccines have achieved licensure [7].

Naturally attenuated WNV isolates

The genotype of WNV that entered the USA in 1999 (NY99) has undergone few nucleotide changes that have become fixed in isolates during the past 17 years in the USA [11]. In 2002, a new dominant genotype was identified in North America (NA/WN02) that had replaced the NY99 genotype [12,13] and this is still the

dominant circulating genotype today [11]. It has 13 conserved nucleotide changes encoding only a single amino acid substitution at the E protein residue 159 (V159A) [11]. Hundreds of natural isolates of WNV have been analyzed using genomic sequencing, and although the dominant NA/WN02 genotype has remained relatively stable, a number of WNV isolates have been identified with mutations that alter their specific virulence phenotype but these have only been seen transiently during a specific WN season [11,14,15]. These mutations are most likely selected based on various host pressures exerted upon circulating WNV as it replicates in drastically different host environments of mosquitoes and avians. While the effects of these naturally acquired mutations can be difficult to interpret, some WNV isolates from birds and mosquitoes have a naturally attenuated phenotype [14]. For example, in 2003 natural isolates were identified in Houston, Texas that produce a small plaque (sp), temperature-sensitive (ts), mouse-attenuated and avian-attenuated phenotype [14,16]. In comparison, virulent WNV typically produces large plaques that are indicative of its rapid multiplication in cell culture. Although pathogenic flaviviruses often have a large plaque phenotype, sps are not always indicative of attenuation. Specifically, the vaccine strain YFV 17D has a large plaque morphology while most wild-type (WT) strains have a sp morphology. Besides plaque size, an additional virulence determinant of highly pathogenic strains of WNV

is that they replicate to comparable titers whether grown at 37°C (humans) or >41°C (birds). Alternatively, a ts phenotype at 41°C is associated with mouse attenuation and indicates that the virus will be attenuated and have reduced replication in the avian host, which has a higher average body temperature, and this was observed in American crows [16]. The sp, ts strains isolated in 2003 also were highly attenuated for murine neuroinvasion following intraperitoneal (ip.) inoculation with LD₅₀ values ranging from 2000 to 645,000 PFU, whereas virulent natural isolates of WNV have ip. LD₅₀ values less than 1 PFU and thus cause rapid mortality in mice [14]. Although attenuated isolates of WNV circulate in nature from time-to-time, genomic sequencing has not so far indicated that one specific set of mutations is responsible for the observed attenuated phenotype. Three of the six isolates discussed above each have a mutation at NS4B E249G, however, each of the three isolates also has several unique mutations that are not shared among all other attenuated isolates [14]. Several additional mutations were observed in some but not all of the six naturally attenuated isolates investigated, including the substitutions prM-N4D and NS5-A804V, as well as several sites in the 3' UTR [14]. Reverse genetics studies showed that these mutations alone are not sufficient to generate the sp, ts phenotype, rather attenuation was observed following various combinations of the natural mutations found in NS4B, prM, NS5 and/or the 3' UTR, in other words, attenuation was due to a combination of multigenic mutations [17]. Investigation of natural WNV isolates provides important information for vaccine development by demonstrating that attenuated WNV isolates typically accumulate unique sets of mutations and the attenuated phenotype relies upon multigenic mutations.

An effective live attenuated human vaccine for WNV must be attenuated in both mosquito and vertebrate hosts, in other words, the virus should have limited replication in mosquito, avian and human hosts. Traditionally, attenuation is examined in animal models such that the candidate vaccine must have high LD₅₀ values following both peripheral (e.g., ip.) and intracranial (ic.) routes of inoculation to ensure attenuation of neuroinvasion and neurovirulence, respectively. Thus far most studies have been undertaken in mouse models and in nonhuman primates, but before vaccine licensure it would also be important to assess the virulence in other relevant models, especially birds as they are the

amplifying hosts for WNV. However, the vaccine virus must not be overattenuated as it has to be effective in inducing a long-term protective immune response.

To achieve a safe level of attenuation without concern for reversion to virulence, it is likely that a live WNV vaccine strain must have multiple independent mutations in different viral genes that contribute to the attenuated phenotype.

Mutations in the 3' UTR

In recent years there have been studies to elucidate the role of the 3' noncoding region (3' UTR) in attenuation as this region is involved in regulation of viral RNA transcription/replication. Interestingly, a cellular 5'-3' exoribonuclease, XRN1, stalls at highly organized secondary structures present at the beginning of the 3' UTR, and this prevents complete degradation of some segments of viral RNA resulting in formation of subgenomic flaviviral RNA (sfRNA) [18]. sfRNA has been specifically attributed to the 3' UTR, and deletion mutants of WNV incapable of producing sfRNA were attenuated both in cell culture and a murine infection model [18]. sfRNA quickly accumulates in virus-infected cells and likely plays many roles in virulence that are still not yet understood, but it is already apparent that these viral RNA species are important for both viral replication and immune evasion [18]. Specifically, the 3' UTR aids genomic RNA cyclization and efficient replication, therefore sfRNA may compete with the 3' UTR during RNA cyclization and thus promote a switch to virion packaging instead of genome amplification [18]. Additionally, sfRNA binds to many viral and host proteins, likely impacting the viral replication complex as well as host transcription and translation [18]. Although the exact impact of various sfRNA binding partner interactions are not yet known, several effects on host immunity have been identified [18]. For instance, studies have shown that the presence of sfRNA inhibits components of the antiviral response by suppressing IFN- α/β signaling and the effects of cellular RNA inhibition pathways (RNAi) [18]. Although there are many questions about the specific roles of sfRNA, it clearly has a crucial role in flavivirus pathogenesis and thus mutation of the 3' UTR may have application to live WNV vaccine development.

Utilization of infectious clone technology to investigate attenuation of virulence

While natural WNV isolates have provided insight into viral genetic diversity and its impact upon the virulent versus attenuated phenotypes of WNV, several studies have investigated the effects when specific mutations are engineered in the WNV genome using site-directed mutagenesis (SDM). Much of the SDM research into WNV utilizes an infectious clone platform based on the highly virulent NY99 genotype (NY99ic). So far there has been investigation into specific mutations in the virus structural prM/M and E genes as well as the NS genes NS1, NS2A, NS3, NS4A, NS4B and NS5.

Attenuating mutations in the prM/M protein

The flavivirus prM protein is a precursor for the viral membrane (M) protein that is expressed on the surface of budding virions along with the E protein [2]. Inside the host cell, prM undergoes furin-mediated cleavage into M and the resulting virion is mature and prepared for exocytosis [2]. While the E protein covers the vast majority of the flavivirus virion when compared with the amount of M present on the viral surface, the prM protein has a vital role in stabilizing the conformation of E and in prevention of premature budding from the host cell [2]. WNV prM protein has a single N-linked glycosylation site present at amino acids 15–17 (i.e., not present in the mature M protein), and ablation of this glycosylation motif alters virus particle release and infectivity [19]. Using WNV reporter virus particles capable of a single round of infection, a decrease in the quantity of viral RNA by real-time PCR was observed when cells were infected with a prM-N15Q mutant and compared with WT WNV [19]. While the differences observed between the mutant and WT viruses in this study were not dramatic, similar studies with the related JEV support the hypothesis that the prM glycosylation site is an important component of viral infectivity [19,20]. Specifically, the JEV mutants prM-N15A, prM-T17A and prM-N15A/T17A had a decreased amount of viral particle release when compared with WT JEV in cell culture [20]. Importantly, these JEV mutants were also tested in a murine infection model and each had a mouse-attenuated phenotype [20]. While WT JEV is highly virulent (ip. LD₅₀ ≈ 1.4 PFU), the mutants had decreased neuroinvasive properties

(ip. LD₅₀ ranging from 29.4 to 497.6 PFU) [20]. Although the mutants were not attenuated for neurovirulence (ic. LD₅₀ = 1.5 PFU), the attenuation of neuroinvasion and the subtle decrease in viral particle release are properties that could be beneficial to include in a live WNV vaccine strain that may increase attenuation without being overattenuated [19,20]. Other studies with JEV have identified a mutation in the mature M protein (M-I36F) that reduces virus-induced apoptosis [21]. While 10³ focus forming units of WT JEV resulted in >80% mortality following ip. inoculation into mice, the M-I36F mutant caused no deaths when administered at doses up to 10⁶ focus forming units, demonstrating a high level of attenuation of neuroinvasion [21]. Importantly, the same residue (M-L36F) is mutated in the attenuation of WT yellow fever strain Asibi virus to generate the live attenuated 17D vaccine [22]. While this mutation has not yet been studied in WNV, the similarity between neurotropic JEV and WNV indicates that M-I36F may also be attenuating to WN.

Role of the E protein in attenuation

The flavivirus E protein is responsible for virus binding and fusion with the host cell [2]. Additionally, the E protein consists of the dominant epitopes that will be recognized by the immune system, making it an important component of both flavivirus pathogenesis and vaccine development [23]. Genome analysis and virulence studies of the live attenuated JEV vaccine strain SA14–14–2 indicated that there are ten E protein substitutions in the vaccine strain that are potentially involved in the attenuated phenotype: L107F, E138K, I176V, T177A, E244G, Q264H, K279M, A315V, S366A and K439R [24]. While the SA14–14–2 vaccine strain has mutations in most viral genes, it has been shown that JEV with only the vaccine-specific E mutations is as attenuated for neuroinvasion and neurovirulence as the fully attenuated vaccine strain [24]. One of these mutations, L107F, is present in the highly conserved flavivirus fusion loop and shared with WNV, among other flaviviruses [25]. Zhang and colleagues examined the impact of the L107F mutation on attenuation of NY99ic [25]. While it was determined that the L107F mutation attenuates neuroinvasion in mice (ip. LD₅₀ >1000 PFU), it did not attenuate neurovirulence significantly (ic. LD₅₀ = 3.1 PFU) [25]. Additionally, there was some evidence of reversion to

WT F107L when virus was harvested from the brains of mice that succumbed to infection [25]. This makes the important point that while identification of attenuating mutations is important, stability of the mutations is critical when developing live attenuated vaccines. Considering that there are nine additional E mutations in the JEV SA14–14–2 vaccine, it is probable that addition of other mutations into the WNV E protein could more strongly attenuate neurovirulence as well as increase the stability of the L107F mutation [24]. The strong attenuating effects of the E mutations in JEV SA14–14–2 indicate that mutations in the viral E may be critical to achieving a safe, effective vaccine [24]. In terms of WNV, further studies are still needed to identify stable E mutations for a candidate vaccine.

Clinical studies and previous veterinary licensure of chimeric WNV vaccine strains have demonstrated the importance of the viral E protein in immunogenicity and safety [7]. In 2006, ChimeriVax-WN01, a veterinary vaccine for WNV, was licensed for use in horses [7]. This chimeric virus utilizes the genome of the licensed YFV vaccine strain 17D with the genes for the structural proteins prM and E removed and replaced by those of WNV [7]. While this chimeric vaccine required annual booster doses to sustain protective immunogenicity, it did provide widespread protection from WNV disease in horses [7]. By 2010 ChimeriVax-WN01 was removed from the vaccine market due to several recorded serious adverse events [7]. ChimeriVax-WN01 has been modified to a new strain, ChimeriVax-WN02, by adding three E substitutions found in the live JE SA14–14–2 vaccine (L107F, A316V and K440R) [7]. ChimeriVax-WN02 has been studied in Phase I and Phase II clinical trials [7]. Results from Phase II clinical studies undertaken in 2007 and 2008 demonstrate 96% seroconversion in the study groups and high antibody titers through 28 days post vaccination as well as no vaccine associated neurological disease [9]. While ChimeriVax-WN02 is a promising candidate for a human WNV vaccine, the durability of elicited protection and evaluation of safety of the candidate vaccine in larger cohorts is still needed.

In addition to evaluation of the JE SA14–14–2 vaccine specific E mutations in WNV, studies have also investigated the effects on WNV attenuation when the single E glycosylation site

(N154) is ablated using SDM. This glycosylation site is not present in all strains of WNV, but most of the highly pathogenic strains responsible for human outbreaks (including outbreaks in the USA) do include the E glycosylation motif [19]. Several studies have demonstrated that the presence/absence of WNV E glycosylation impacts viral infectivity and production [19,23,26]. One study demonstrated that E glycosylated WNV replicated to approximately tenfold higher titer than the nonglycosylated strains in Vero and baby hamster kidney (BHK) cells when using WNV reporter virus particles capable of a single round of infection [19]. Other studies investigated the effects of a WNV E-N154S mutation on mouse virulence, and both demonstrated that this mutation caused moderate attenuation of neuroinvasion (ip. LD₅₀ = 125–200 PFU) and mild attenuation of neurovirulence (ic. LD₅₀ = 1.1–125 PFU) [23,26]. Considering that some natural, attenuated isolates of WNV are already lacking the E glycosylation site, ablation of this site in a vaccine strain could help to maintain attenuation.

Attenuating mutations in the NS genes

The viral E is a critical location for attenuating mutations due to its importance in virus-cell binding, entry and immune recognition, but other studies provide powerful evidence that mutations in the NS protein genes are also vital to virus attenuation. The importance of NS gene mutations to attenuation can be supported by the previously mentioned sequencing studies of natural isolates that found that combinations of mutations in NS4B, NS5 and/or the 3' UTR were sufficient to produce a significantly attenuated phenotype [14,15].

Attenuating mutations in the NS1 protein

Studies by Whiteman *et al.* on mutation of the WNV NS1 have provided additional evidence of the attenuating effects of NS gene mutations [23]. NS1 is not only important to the flavivirus replication complex, but it also is secreted as a hexamer into the extracellular space and expressed on the surface of infected cells [2]. Secreted NS1 protein has been shown to be an important protein in DENV infection [2]. The immune system is able to recognize NS1 and induces non-neutralizing antibodies; however, these antibodies mediate antibody-dependent cellular cytotoxicity and complement fixation [2]. The flavivirus NS1 can antagonize

innate immunity, including components of complement and Toll-like receptor 3 signaling, and thus has a complex role in flavivirus pathogenesis [2]. The NS1 protein is unusual as it is an NS protein that is glycosylated [2]. White-man *et al.* ablated the three N-linked glycosylation sites present in WNV NS1 at amino acid residues 130, 175 and 207 [23]. Initially, attenuation was investigated when the asparagine (N) in each motif was changed to alanine (A) (N130A/N175A/N207A) [23]. This NS1 glycosylation mutant was attenuated for murine neuroinvasion and neurovirulence, however, there was evidence of reversion at residue 130 (A130N) that led to further studies to generate a stable WNV NS1 mutant [23,27]. Follow-up studies altered all three amino acids in the first glycosylation motif (including glutamine rather than alanine substitutions) and left the remaining two sites with single amino acid mutations (NNT130QQA/N175A/N207A) [27]. The resulting virus proved to be attenuated for neuroinvasion (ip. LD₅₀ >10⁶ PFU) and neurovirulence (ic. LD₅₀ = 800 PFU) with no evidence of reversion to virulence [27]. Additionally, infection with the NS1 glycosylation mutant induced protective immunity with only 50 PFU of the mutant protecting 50% of mice from lethal infection with WNV NY99 (PD₅₀ = 50 PFU) [27]. Subsequent studies of the NS1 glycosylation mutant found that deglycosylation caused the NS1 protein to accumulate near the endoplasmic reticulum and thus have reduced secretion compared with WT WNV NS1, indicating a possible mechanism for attenuation [28]. The NS1 glycosylation mutant exhibits many of the properties desirable in a live WNV vaccine strain, however, it is not sufficiently attenuated for neurovirulence and for full confidence in the safety of a candidate vaccine it is still desirable to have additional mutations present in other viral genes that independently attenuate the virus.

Attenuating mutation in the NS2A protein

While the flavivirus NS proteins each seem to have multifaceted effects on virus replication and pathogenicity, one role of the NS2A protein is that it is a potent inhibitor of IFN- β transcription [29]. IFN- α/β are effective antiviral signaling molecules produced by the innate immune response, therefore, suppression of their effects by NS2A is a key immune evasion technique used by flaviviruses. Using the Kunjin subtype of WNV as

a model, WNV_{KUN} Liu and colleagues have identified a single point mutation that can ablate the anti-IFN- β effects of NS2A [29]. WNV_{KUN} with the mutation NS2A-A30P allowed for accumulation of approximately seven- to eightfold more IFN- β mRNA in the A549 cell line [29]. Comparison of the mutant in IFN-competent A549 cells versus IFN-deficient BHK cells demonstrated that the A30P mutation caused abortive replication in the presence of IFN (A549) but the mutated virus was able to replicate as normal in the absence of IFN (BHK) [29]. In addition to the observed attenuation of the NS2A-A30P mutant in cell culture, attenuation was also observed when the mutant was tested in a WNV mouse model [29]. NS2A-A30P caused significant attenuation of neuroinvasion (ip. LD₅₀ >10⁵ PFU), and immunization with 10⁴ PFU of the mutant was completely protective against a challenge of 100 PFU of the highly virulent strain WNV_{NY99} [29]. However, the mutant was only weakly attenuated for neurovirulence (ic. LD₅₀ = 30 PFU) [29].

Attenuating mutation in the NS3 protein

The flavivirus NS3 protein functions as an NTPase, a serine protease and an RNA helicase, and thus it is a key component of viral replication [30]. Work by Ebel and colleagues utilized a WNV infectious clone platform passaged 20-times in mosquitoes to identify attenuating mutations by selecting for small plaques produced by the viral population [30]. One mutant that was isolated with a sp phenotype contained an RNA helicase deletion of an aspartic acid residue at position 483 in NS3 (NS3 Δ 483) [30]. While a subcutaneous dose of 10 PFU of parental virus caused approximately 87.5% mortality in mice, the NS3 Δ 483 mutant administered at 10⁵ PFU by the subcutaneous route reduced mortality to approximately 14%, demonstrating that the sp phenotype was associated with murine attenuation [30]. Specifically, this NS3 mutant mimicked WT infection at early stages of infection but had reduced viremia on days 2–4 resulting in a decrease in CNS invasion as measured by viral titers in CNS tissues [30]. Importantly, coinfection of NS3 Δ 483 mutant and WT WNV in to chickens demonstrated avian attenuation as NS3 Δ 483 was outcompeted by WT WNV by 2 dpi [30]. Considering that the NS3 deletion mutant has exhibited attenuation in both mice and chickens, it should be considered in future vaccine development.

Role of the NS4A protein in attenuation

Investigation of mutations in the flavivirus NS4A protein has provided insight into the functional importance of this NS protein. Thus far, it is known that NS4A has roles in viral replication, including a signal sequence for proper translocation of the NS4B protein through the endoplasmic reticulum membrane, and also functions as a cofactor for the RNA helicase activity of the NS3 protein [2,31]. Studies by Shiryayev and colleagues have identified several amino acids in the WNV NS4A protein that are essential for its role as a cofactor of NS3 helicase activity [31]. Briefly, the group generated protein constructs of the soluble fragments of the NS3_{HEL} domain as well as the NS3_{HEL} domain connected to the soluble portion of the NS4A protein (NS3_{HEL}-NS4A) [31]. Using *in vitro* measurements of ATPase activity, the experiments demonstrated that NS3_{HEL} alone had approximately fivefold more ATPase activity when compared with the NS3_{HEL}-NS4A construct [31]. Since it has been previously shown that *in vitro* ATP is required in mM concentrations for NS3 RNA helicase activity, it was hypothesized that the ATP-saving function of the soluble portion of NS4A acts as a cofactor to sustain NS3 helicase activity *in vivo* under lower concentrations of ATP [31]. When they changed three negatively charged NS4A amino acids (Glu, Glu, Asp) at residues 46, 47 and 50 to positively charged lysine residues, the NS3_{HEL}-NS4A construct no longer exhibited low ATPase activity and instead it behaved as the NS3_{HEL} fragment and exhibited a high rate of ATP degradation [31]. Although these mutations have not yet been assessed in a full-length WNV genome, the identified role of NS4A as a cofactor to sustain NS3 RNA helicase activity could serve as a good target for mutagenesis to attenuate WNV by altering its replication efficiency.

Attenuating mutations in the NS4B protein

Other studies have investigated the impact of mutations present in the NS protein NS4B. Like the other NS proteins, NS4B has an essential function in the flavivirus replication complex. There is evidence that NS4B accumulates in the perinuclear region and it may also translocate into the nucleus [32]. NS4B promotes immune evasion by inhibiting STAT phosphorylation and thus halting the interferon-signaling cascade [33]. Detailed nuclear magnetic resonance analysis of DENV NS4B indicates that this

highly hydrophobic protein consists of three helical transmembrane domains and two helical membrane-associated domains [34]. WNV with an NS4B-E249G mutation has been engineered using SDM [35]. When administered at 10³ PFU by the subcutaneous route, the E249G mutant was attenuated in mice, reducing the mortality from 100% following WT infection to 50% following infection with the mutant. While a greater level of attenuation is desired for a vaccine strain, the remaining mice all survived a fully lethal challenge dose of WNV, indicating that the E249G mutation may contribute to decreased virulence and promote immunogenicity. Studies from Wicker and colleagues screened at least 15 different NS4B mutations in the N-terminal and central domains of NS4B and identified two mutations that are strongly attenuating: P38G and C102S [32,36]. The mutation P38G is located in a flavivirus conserved region of NS4B near the N-terminal domain, while C102S is located in the central hydrophobic transmembrane domain [32,36]. Both of the attenuating mutations were highly attenuated for neuroinvasion (ip. LD₅₀ >10,000 PFU) [32,36]. Less than 1 PFU of each virus was highly protective against a lethal challenge of WNV NY99, indicating that these mutants induce strong protective immunity to future infection [32,36]. Unfortunately, the P38G mutation was not attenuated for neurovirulence; however, the C102S mutation provided significant attenuation of both neuroinvasion (ip. LD₅₀ >10,000 PFU) and neurovirulence (ic. LD₅₀ >1000 PFU) [32,36]. The NS4B mutants also have sp and ts phenotypes indicating, much like the naturally attenuated isolates discussed previously, that these characteristics are indicative of attenuation [32,36]. Interestingly, the P38G mutation caused compensating mutations at NS4B-T116I and NS3-N480H or NS4B-T116I only, but when tested independently these compensating mutations were not attenuating but instead provide stability of the P38G mutation, as virus could not be recovered with the P38G mutation alone [32].

Attenuating mutations in the NS5 protein

Another NS gene that has been investigated using SDM is NS5. Flavivirus NS5 contains N-terminal methyltransferase (MTase) activity and C-terminal RNA-dependent RNA-polymerase activity making it a vital component of flavivirus replication [37]. As previously stated, the flavivirus genome has a 5' cap that

mimics eukaryotic RNA and allows for efficient viral gene expression and NS5 MTase activity is responsible for the observed capping mechanism [2]. Specifically, NS5 has been shown to sequentially catalyze methylation of a guanine N-7 residue followed by a ribose 2'O site on the 5' end of viral RNA [37]. Zhou and colleagues have identified three stable, attenuating mutations in NS5 that abolish 2'O methylation activity (K61A, K182A and E218A) in both JEV and WNV [37]. While 2'O MTase activity is abolished by these mutants, they retain varying degrees of N-7 MTase activity, which seems to be essential for virus viability [37]. Continued work on the mutation E218A using JEV as a model to further characterize this mutation has shown substantial evidence of its attenuating properties [38]. In these studies, $>10^7$ PFU of virus by the ip. route was not lethal, indicating that the mutant strain has a greatly decreased neuroinvasive phenotype [38]. Administration of 10^4 PFU by the subcutaneous route was protective against lethal ip. challenge of 10^8 PFU of WT JEV indicating that the mutant virus induces protective immunity [38]. Significantly, the E218A JEV mutant was weakly attenuated for neurovirulence in this study, and ideally neurovirulence would be reduced if combined with additional independent mutations [38].

Vector competence of attenuated WNV

While many of the studies discussed above focus on mouse virulence as a primary determinant of attenuation, another important consideration for vaccine development is whether or not the attenuated mutants can replicate, disseminate and transmit efficiently from their mosquito vectors. Ideally, a live vaccine strain will have little to no capability for mosquito competence and thus will limit spread of the virus. It has been previously shown that the WN02 genotype that displaced the initial WNV NY99 genotype was more efficient at replication, dissemination and transmission in the mosquito vectors *Culex pipiens* and *Culex tarsalis*, indicating an increased fitness profile of the WN02 genotype that allows for more rapid and frequent viral transmission [12,39]. A study by Van Slyke and colleagues tested several attenuated mutants for their vector competence in *Cx. tarsalis* mosquitoes and showed that different mutations cause varying effects [40]. This group compared the mosquito competence of WNVic, NS4B-P38G/T116A, NS4B-C102S,

NS4B-P38A and a naturally attenuated isolate of WNV with the mutations prM-V156I, NS4B-E249G, NS5-A804V and 3' UTR-A10799G [40]. Although each of these mutants was attenuated in mice, the mutations did not cause decreased vector competence [40]. Interestingly, each of these attenuated mutants containing mutations in NS4B had similar or increased rates of mosquito infection, dissemination and transmission when compared with WNVic with no mutations [40]. However, the same study also assessed the vector competence of two WNV glycosylation mutants compared with WNVic: NS1- NNT130QQA/N175A/N207A with the three NS1 glycosylation sites ablated and E-N145S/NS1-NNT130QQA/N175A/N207A with the three NS1 glycosylation sites and the single E glycosylation site ablated [40]. Unlike the WNV mutants with NS4B mutations, both of the glycosylation mutants had decreased rates of mosquito dissemination and transmission compared with WNVic, and the NS1 glycosylation mutant also had significantly decreased ability to infect mosquitoes when compared with WNVic [40]. Another study has demonstrated that soluble, nonmutated DENV2 NS1 downregulates ROS and JAK-STAT immune signaling in the mosquito midgut, thus aiding virus uptake in the mosquito; in addition this study also showed that several deletions in DENV2 NS1 reversed the observed immune suppression and thus decreased virus uptake [41]. Evidence that soluble DENV2 NS1 increases virus uptake by mosquitoes provides a possible mechanism to explain how WNV with glycosylation-deficient NS1 is less capable of infecting mosquitoes. The varying degrees of mosquito competence of the mutants tested demonstrates that attenuated WNV glycosylation mutants may be important for decreasing vector competence, whereas WNV NS4B mutants may be primarily important for attenuation of the virus in vertebrate hosts. Importantly, another study found that WNV with a mutation ablating only the E glycosylation site (E-N154I) had decreased competence in both *Cx. tarsalis* and *Cx. pipiens* mosquitoes, supporting the hypothesis that virus glycosylation is important to WNV multiplication in mosquito vectors [42]. Vector competence was also assessed for the previously described NS3Δ483 mutant in *Culex quinquefasciatus* mosquitoes [30]. Although differences between mosquito

infection, dissemination and transmission between WT WNV and the NS3Δ483 mutant were not statistically significant, there was a trend of decrease in viral fitness of the mutant compared with the parental virus [30]. Specifically, when mosquitoes were fed on equal quantities of the mutant and WT viruses, each virus replicated similarly up to day 7, but by day 14 the NS3Δ483 mutant was replicating to a lower titer, indicating that the mutation caused a decrease in fitness of the NS3Δ483 virus in the mosquito host [30]. Overall, studies of the vector competence of the above-mentioned attenuated mutants have provided further support that a live vaccine strain of WNV should have multigenic mutations to provide an attenuated phenotype in both vertebrate and insect hosts in which the virus replicates.

The balance between attenuation & immunogenicity

Studies in animal models and specimens from humans suggest that B cells and specific antibodies are critical in the control of viral dissemination during WNV infection [43–46]; whereas cellular immunity is more important in controlling and eliminating the virus from host and contributes to long-lasting protective immunity [47–49]. Live-attenuated vaccines offer superior, rapid and long-lived immunogenicity, and typically single-dose protection. Compared with inactivated viruses, they are able to actively replicate and thus mimic natural infection and induce stronger adaptive immune responses without disease development. The primary target for WNV neutralizing antibodies is the E protein [50]. Immune epitopes for T cells are more widely distributed in C, prM, E, NS3, NS4B and NS5 proteins [51–54]. Due to the wide distribution of T- and B-cell epitopes among viral proteins, the complete loss of immunogenicity is less likely to be observed in attenuated viruses generated by either point mutations in viral genes or by constructs of chimeric viruses, that will affect virus replication and virulence. Nevertheless, a recent study with YFV-17D vaccine showed that initial viral load determines the magnitude of human CD8⁺ T-cell responses following immunization [55]. Thus, the attenuation of viruses also needs to be monitored to allow delivery of sufficient antigen during the initial period of immune response in order to elicit robust protective T-cell responses. Mutations in NS

proteins are often correlated with additional changes in virulence and immunogenicity. The NS proteins of flaviviruses, in particular NS1, NS4B and NS5, are reported to be associated with evasion of interferons and other antiviral cytokines, and are important determinants in viral pathogenesis [33,56–62]. Given the importance of type I interferons and other antiviral cytokines in limiting virus dissemination and in efficient induction of memory B- and T-cell antiviral immunity, the co-expression of these antiviral cytokines with the live vaccine will likely lead to further increase in attenuation of virulence and immunogenicity. For example, the highly conserved N-terminal domain (aa35–aa60) of NS4B protein bears a resemblance to an immunomodulatory tyrosine inhibitory motif found in various components of mammalian cell-signaling cascades [63], which is thought to contribute to its antagonist activities for IFN and inflammatory cytokine signaling [59–61]. As a result, the WNV NS4B-P38G mutant is highly attenuated for mouse neuro-invasiveness, but induces stronger innate cytokine and memory T-cell responses than the WT parent WNV strain [32,64]. Overall, mutagenesis studies targeting NS proteins can be utilized as a paradigm to enhance the balance between immunogenicity and attenuation as a rational approach to development of live attenuated vaccines for flaviviruses.

Conclusion

There is a strong body of work on WNV virulence determinants that has provided a foundation for live attenuated vaccine development. Studies of natural isolates have provided insight into the profiles of attenuated, circulating isolates of WNV, while SDM research has expanded our understanding of specific virulence determinants in different viral genes. Genomic sequencing of both natural WNV isolates and live attenuated vaccines such as JEV SA14-14-2 indicate that a stable, attenuated flavivirus has coding and noncoding changes across multiple genes that are cumulatively responsible for the observed phenotype. Independent studies have identified attenuating mutations present in the viral genes prM/M, E, NS1, NS2A, NS3, NS4A, NS4B, NS5 and the 3' UTR. Due to the high level of attenuation observed from some of the mutants, future studies should begin to investigate the impact of combined mutations in multiple genes.

EXECUTIVE SUMMARY

Background

- West Nile virus (WNV) is a neurotropic, mosquito-transmitted flavivirus.
- Since the 1990s, there has been an increase in highly neurotropic strains of WNV.
- There are no licensed antiviral drugs or vaccines for human treatment.

Naturally attenuated WNV isolates

- Attenuated WNV isolates have been collected from birds and mosquitoes in nature.
- While phenotypically similar, naturally attenuated isolates do not indicate that a single viral mutation is responsible for attenuation, rather it is multigenic.
- Attenuation is often measured by a decrease in viral replication and by increased quantities of virus needed for neuroinvasion and neurovirulence in vertebrate hosts following intraperitoneal and intracerebral route of inoculation, respectively, and inability of virus to cross the midgut barrier in mosquitoes.

Attenuating mutations identified to date

- The 3' UTR generates noncoding viral RNA (sfRNA) that impacts viral replication and interferes with host proteins.
- WNV incapable of producing sfRNA are attenuated.
- prM-N15Q mutation decreases viral replication.
- E-L107F attenuates WNV in mice.
- Loss of the E protein glycosylation site (E-N154S) attenuates WNV in mice.
- NS1- NNT130QQA/N175A/N207A mutant ablates three glycosylation sites in WN NS1 and attenuates the virus in mice.
- NS2A-A30P mutation increases virus sensitivity to interferon and attenuates WNV in mice.
- A NS3 helicase deletion mutant, NS3 Δ 483 attenuates WNV in mice and chickens.
- NS4A-E46K/E47K/D50K mutations cause increased ATP degradation by NS3.
- NS4B inhibits STAT phosphorylation during interferon signaling.
- NS4B-E249G (also observed in natural WNV isolates) attenuates WNV in mice.
- WNV NS4B-P38G and NS4B-C102S are attenuated in mice.
- NS5-K61A, NS5-K182A and NS5-E218A deplete methyltransferase activity and attenuate WNV in mice.

Vector competence of attenuated WNV

- A live WNV vaccine strain should have a decreased ability to replicate, disseminate and transmit from mosquitoes.
- Several mouse attenuated WNV isolates with NS4B mutations appear to have an increase in vector competence.
- WNV isolates with NS1 and E glycosylation site mutations have decreased rates of mosquito dissemination and transmission.
- NS3 Δ 483 mutant WNV has no decrease in vector competence but can be outcompeted by co-infection with wild-type.

The balance between attenuation & immunogenicity

- Antibodies target both virus structural and nonstructural genes.
- NS proteins are important for viral evasion of interferon and cytokines, especially NS1, NS4B and NS5, such that mutations in these proteins inhibits viral evasion of the host innate immune response.
- NS4B-P38G induces stronger cytokine and T-cell responses than wild-type WNV.
- High immunogenicity is crucial for a candidate vaccine.

Conclusion

- Phenotypic and genotypic studies have identified attenuating mutations in the WNV genes prM/M, E, NS1, NS2A, NS3, NS4A, NS4B, NS5 and the 3' UTR.
- Combining some of these mutations will aid progress toward vaccine development.

Future perspective

Since there has been an increase in neuro-invasive strains of WNV over the past 20 years, development of a WNV vaccine remains a priority for public health. Genotypic and phenotypic WN studies have indicated that the degree of virulence observed of WN isolates is dependent upon multiple sites in the viral genome indicating that virulence is multigenic. Although several single amino acid substitutions have been identified that strongly attenuate WNV, there is evidence from natural WN isolates as well as from genetically engineered WN isolates that combining independent substitutions in different viral proteins can confer a highly attenuated phenotype in both vertebrate and mosquito hosts, and decrease the likelihood of reversion to virulence. Thus far, many studies in this field have been focused on the impact of one specific mutation in one specific viral gene/protein. There is now a robust portfolio of specific virulence determinants across most of the WN genome, and future work must investigate combinations of these multigenic mutations for vaccine development. Much like the live, attenuated flavivirus vaccines YFV 17D and JEV SA14-

14-2, we anticipate that a safe and immunogenic live attenuated WNV vaccine will have mutations in multiple viral genes. In contrast with the YF and JE live vaccines, which were empirically derived, a live, attenuated WN vaccine can be rationally designed using reverse genetics to include the mutations of interest. Ultimately, vaccine design in this manner will be accompanied by a more in depth understanding of the mechanism of structure–function behind each specific mutation. Moreover, reverse genetics will also ensure that long-term, consistent production of the vaccine strain is possible because it will not rely on limited seed stocks of the virus.

Financial & competing interests disclosure

This work was supported in part by NIH grant R01 AI099123 (T Wang) and a grant from the Gillson Longenbaugh Foundation (ADT Barrett). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

- Petersen LR, Brault AC, Nasci RS. West Nile virus: review of the literature. *JAMA* 310(3), 308–315 (2013).
- A comprehensive overview of West Nile virus (WNV) human disease.
- Lindenbach BD, Thiel H-J, Rice CM. *Flaviviridae: The Viruses and their Replication (5th Edition)*. Lippincott-Raven Publishers, PA, USA (2007).
- Donadieu E, Bahuon C, Lowenski S, Zientara S, Couplier M, Lecollinet S. Differential virulence and pathogenesis of West Nile viruses. *Viruses* 5(11), 2856–2880 (2013).
- West Nile virus.
www.who.int/mediacentre/
- West Nile virus disease cases and deaths reported to CDC by year and clinical presentation, 1999–2015.
www.cdc.gov/westnile/
- CDC. West Nile virus disease cases and presumptive viremic blood donors by state – United States, 2016 (as of October 25, 2016).
www.cdc.gov/westnile/
- Brandler S, Tangy F. Vaccines in development against West Nile virus. *Viruses* 5(10), 2384–2409 (2013).
- A good overview of progress toward West Nile vaccine design.
- Lim SP, Shi PY. West Nile virus drug discovery. *Viruses* 5(12), 2977–3006 (2013).
- Ishikawa T, Yamanaka A, Konishi E. A review of successful flavivirus vaccines and the problems with those flaviviruses for which vaccines are not yet available. *Vaccine* 32(12), 1326–1337 (2014).
- First Dengue Vaccine Approved In More Than 10 Countries.
www.sanofipasteur.com/en/
- Mann BR, McMullen AR, Swetnam DM, Barrett AD. Molecular epidemiology and evolution of West Nile virus in North America. *Int. J. Environ. Res. Public Health* 10(10), 5111–5129 (2013).
- Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD. Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. *Am. J. Trop. Med. Hyg.* 71(4), 493–500 (2004).
- Davis CT, Ebel GD, Lancio I, Robert S *et al.* Phylogenetic analysis of North American West Nile Virus isolates 2001–2004: evidence for the emergence of a dominant genotype. *Virology* 342(2), 252–265 (2005).
- Davis CT, Beasley DW, Guzman H *et al.* Emergence of attenuated West Nile virus variants in Texas, 2003. *Virology* 330(1), 342–350 (2004).
- May FJ, Li L, Davis CT, Galbraith SE, Barrett AD. Multiple pathways to the attenuation of West Nile virus in south-east Texas in 2003. *Virology* 405(1), 8–14 (2010).
- Shows that naturally attenuated variants of WNV are due to multigenic mutations.
- Brault AC, Langevin SA, Ramey WN *et al.* Reduced avian virulence and viremia of West Nile virus isolates from Mexico and Texas. *Am. J. Trop. Med. Hyg.* 85(4), 758–767 (2011).
- Davis CT, Galbraith SE, Zhang S *et al.* A combination of naturally occurring mutations in North American West Nile virus nonstructural protein genes and in the 3′ untranslated region alters virus phenotype. *J. Virol.* 81(11), 6111–6116 (2007).
- Roby JA, Pijlman GP, Wilusz J, Khromykh AA. Noncoding subgenomic flavivirus RNA:

- multiple functions in West Nile virus pathogenesis and modulation of host responses. *Viruses* 6(2), 404–427 (2014).
- 19 Hanna SL, Pierson TC, Sanchez MD, Ahmed AA, Murtadha MM, Doms RW. N-linked glycosylation of west nile virus envelope proteins influences particle assembly and infectivity. *J. Virol.* 79(21), 13262–13274 (2005).
- 20 Kim JM, Yun SI, Song BH *et al.* A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. *J. Virol.* 82(16), 7846–7862 (2008).
- 21 De Wispelaere M, Khou C, Frenkiel M-P, Despres P, Pardigon N. A single amino acid substitution in the M protein attenuates Japanese Encephalitis virus in mammalian hosts. *J. Virol.* 90(5), 2676–2689 (2016).
- 22 Hahn CS, Dalrymple JM, Strauss JH, Rice CM. Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. *Proc. Natl Acad. Sci. USA* 84, 2019–2023 (1987).
- 23 Whiteman MC, Li L, Wicker JA *et al.* Development and characterization of non-glycosylated E and NS1 mutant viruses as a potential candidate vaccine for West Nile virus. *Vaccine* 28(4), 1075–1083 (2010).
- 24 Gromowski GD, Firestone CY, Whitehead SS. Genetic determinants of Japanese encephalitis virus vaccine strain SA14–14–2 that govern attenuation of virulence in mice. *J. Virol.* 89(12), 6328–6337 (2015).
- **Characterization of specific subsets of JE SA14–14–2 vaccine mutations.**
- 25 Zhang S, Li L, Woodson SE *et al.* A mutation in the envelope protein fusion loop attenuates mouse neuroinvasiveness of the NY99 strain of West Nile virus. *Virology* 353(1), 35–40 (2006).
- 26 Beasley DW, Whiteman MC, Zhang S *et al.* Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J. Virol.* 79(13), 8339–8347 (2005).
- 27 Whiteman MC, Wicker JA, Kinney RM, Huang CY, Solomon T, Barrett AD. Multiple amino acid changes at the first glycosylation motif in NS1 protein of West Nile virus are necessary for complete attenuation for mouse neuroinvasiveness. *Vaccine* 29(52), 9702–9710 (2011).
- 28 Whiteman MC, Popov V, Sherman MB, Wen J, Barrett AD. Attenuated West Nile virus mutant NS1130–132QQA/175A/207A exhibits virus-induced ultrastructural changes and accumulation of protein in the endoplasmic reticulum. *J. Virol.* 89(2), 1474–1478 (2015).
- **A mechanistic study to identify intracellular differences between attenuated and virulent WNV.**
- 29 Liu WJ, Wang XJ, Clark DC, Lobigs M, Hall RA, Khromykh AA. A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. *J. Virol.* 80(5), 2396–2404 (2006).
- 30 Ebel GD, Fitzpatrick KA, Lim P-Y, Bennett CJ. Nonconsensus West Nile virus genomes arising during mosquito infection suppress pathogenesis and modulate virus fitness *in vivo*. *J. Virol.* 85(23), 12605–12613 (2011).
- 31 Shiryayev SA, Chernov AV, Aleshin AE, Shiryayeva TN, Strongin AY. NS4A regulates the ATPase activity of the NS3 helicase: a novel cofactor role of the non-structural protein NS4A from West Nile virus. *J. Gen. Virol.* 90(Pt 9), 2081–2085 (2009).
- 32 Wicker JA, Whiteman MC, Beasley DW *et al.* Mutational analysis of the West Nile virus NS4B protein. *Virology* 426(1), 22–33 (2012).
- **A screen and characterization of attenuation mutations by site-directed mutagenesis of NS4B.**
- 33 Munoz-Jordan JL, Laurent-Rolle M, Ashour J *et al.* Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *J. Virol.* 79(13), 8004–8013 (2005).
- 34 Li Y, Kim YM, Zou J *et al.* Secondary structure and membrane topology of dengue virus NS4B N-terminal 125 amino acids. *Biochim. Biophys Acta* 1848(12), 3150–3157 (2015).
- **Determines the structure of the N-terminus of flavivirus NS4B based on nuclear magnetic resonance.**
- 35 Puig-Basagoiti F, Tilgner M, Bennett C, Zhou Y. A mouse cell-adapted NS4B mutation attenuates West Nile virus RNA synthesis. *Virology* 361(1), 229–241 (2007).
- 36 Wicker JA, Whiteman MC, Beasley DW *et al.* A single amino acid substitution in the central portion of the West Nile virus NS4B protein confers a highly attenuated phenotype in mice. *Virology* 349(2), 245–253 (2006).
- 37 Zhou Y, Ray D, Zhao Y *et al.* Structure and function of flavivirus NS5 methyltransferase. *J. Virol.* 81(8), 3891–3903 (2007).
- **Characterizes attenuated mutants of NS5 methyltransferase and considers the mechanism simultaneously.**
- 38 Li SH, Dong H, Li XF *et al.* Rational design of a flavivirus vaccine by abolishing viral RNA 2'-O methylation. *J. Virol.* 87(10), 5812–5819 (2013).
- 39 Moudy RM, Meola MA, Morin L-LL, Ebel GD, Kramer LD. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes. *Am. J. Trop. Med. Hyg.* 77(2), 365–370 (2007).
- 40 Van Slyke GA, Jia Y, Whiteman MC, Wicker JA, Barrett AD, Kramer LD. Vertebrate attenuated West Nile virus mutants have differing effects on vector competence in *Culex tarsalis* mosquitoes. *J. Gen. Virol.* 94(Pt 5), 1069–1072 (2013).
- **Explains both the implications of WNV vector competence and how different attenuated isolates of WNV behave differently in the mosquito.**
- 41 Liu J, Liu Y, Nie K *et al.* Flavivirus NS1 protein in infected host sera enhances viral acquisition by mosquitoes. *Nat. Microbiol.* 1(9), 1–11 (2016).
- 42 Moudy RM, Zhang B, Shi PY, Kramer LD. West Nile virus envelope protein glycosylation is required for efficient viral transmission by *Culex* vectors. *Virology* 387(1), 222–228 (2009).
- 43 Diamond MS, Shrestha B, Marri A, Mahan D, Engle M. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J. Virol.* 77(4), 2578–2586 (2003).
- 44 Diamond MS, Shrestha B, Mehlhop E, Sitati E, Engle M. Innate and adaptive immune responses determine protection against disseminated infection by West Nile encephalitis virus. *Viral Immunol.* 16(3), 259–278 (2003).
- 45 Diamond MS, Sitati EM, Friend LD, Higgs S, Shrestha B, Engle M. A critical role for induced IgM in the protection against West Nile virus infection. *J. Exp. Med.* 198(12), 1853–1862 (2003).
- 46 Roehrig JT, Staudinger LA, Hunt AR, Mathews JH, Blair CD. Antibody prophylaxis and therapy for flavivirus encephalitis infections. *Ann. NY Acad. Sci.* 951, 286–297 (2001).
- 47 Shrestha B, Samuel MA, Diamond MS. CD8+ T cells require perforin to clear West Nile virus from infected neurons. *J. Virol.* 80(1), 119–129 (2006).
- 48 Sitati EM, Diamond MS. CD4+ T-cell responses are required for clearance of West Nile virus from the central nervous system. *J. Virol.* 80(24), 12060–12069 (2006).
- 49 Wang Y, Lobigs M, Lee E, Mullbacher A. CD8+ T cells mediate recovery and immunopathology

- in West Nile virus encephalitis. *J. Virol.* 77(24), 13323–13334 (2003).
- 50 Diamond MS, Pierson TC, Fremont DH. The structural immunology of antibody protection against West Nile virus. *Immunol. Rev.* 225, 212–225 (2008).
 - 51 Brien JD, Uhrlaub JL, Nikolich-Zugich J. Protective capacity and epitope specificity of CD8(+) T cells responding to lethal West Nile virus infection. *Eur. J. Immunol.* 37(7), 1855–1863 (2007).
 - 52 Purtha WE, Myers N, Mitaksov V *et al.* Antigen-specific cytotoxic T lymphocytes protect against lethal West Nile virus encephalitis. *Eur. J. Immunol.* 37(7), 1845–1854 (2007).
 - 53 Lanteri MC, Heitman JW, Owen RE *et al.* Comprehensive analysis of West Nile virus-specific T cell responses in humans. *J. Infect. Dis.* 197(9), 1296–1306 (2008).
 - 54 McMurtrey CP, Lelic A, Piazza P *et al.* Epitope discovery in West Nile virus infection: identification and immune recognition of viral epitopes. *Proc. Natl Acad. Sci. USA* 105(8), 2981–2986 (2008).
 - 55 Akondy RS, Johnson PL, Nakaya HI *et al.* Initial viral load determines the magnitude of the human CD8 T cell response to yellow fever vaccination. *Proc. Natl Acad. Sci. USA* 112(10), 3050–3055 (2015).
 - 56 Wilson JR, De Sessions PF, Leon MA, Scholle F. West Nile virus nonstructural protein 1 inhibits TLR3 signal transduction. *J. Virol.* 82(17), 8262–8271 (2008).
 - 57 Scholle F, Mason PW. West Nile virus replication interferes with both poly(I:C)-induced interferon gene transcription and response to interferon treatment. *Virology* 342(1), 77–87 (2005).
 - 58 Diamond MS. Mechanisms of evasion of the type I interferon antiviral response by flaviviruses. *J. Interferon Cytokine Res.* 29(9), 521–530 (2009).
 - 59 Liu WJ, Wang XJ, Mokhonov VV, Shi PY, Randall R, Khromykh AA. Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. *J. Virol.* 79(3), 1934–1942 (2005).
 - 60 Evans JD, Seeger C. Differential effects of mutations in NS4B on West Nile virus replication and inhibition of interferon signaling. *J. Virol.* 81(21), 11809–11816 (2007).
 - 61 Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A. Inhibition of interferon signaling by dengue virus. *Proc. Natl Acad. Sci. USA* 100(24), 14333–14338 (2003).
 - 62 Laurent-Rolle M, Boer EF, Lubick KJ *et al.* The NS5 protein of the virulent West Nile virus NY99 strain is a potent antagonist of type I interferon-mediated JAK-STAT signaling. *J. Virol.* 84(7), 3503–3515 (2010).
 - 63 Isakov N. ITIMs and ITAMs. The Yin and Yang of antigen and Fc receptor-linked signaling machinery. *Immunol. Res.* 16(1), 85–100 (1997).
 - 64 Welte T, Xie G, Wicker JA *et al.* Immune responses to an attenuated West Nile virus NS4B-P38G mutant strain. *Vaccine* 29(29–30), 4853–4861 (2011).
- **Shows that an NS4B mutant induces a superior immune response compared with wild-type WNV.**