West Nile virus vaccination protects against Usutu virus disease in mice

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ACADEMIC ABSTRACT

Mosquito-borne viruses, including dengue virus (DENV), Usutu virus (USUV), West Nile virus (WNV), and Japanese encephalitis virus (JEV), are rapidly emerging, global pathogens. Though the number of people impacted by each virus varies, there have been thousands to millions of people infected. The focus of this thesis work centers around USUV and WNV; both have RNA genomes and belong to the *Flaviviridae* virus family. Both WNV and USUV were initially isolated in Africa and have since spread to Europe; interestingly, WNV has also spread globally and is considered endemic in the Americas. Similar to other flaviviruses, USUV and WNV are maintained in a mosquito vector-avian host transmission cycle, with spillover infection into humans. Human infections of WNV and USUV are usually asymptomatic, but in severe cases can cause neuroinvasive disease.

WNV and USUV belong to the JEV serocomplex group, which indicates that antibodies produced against these viruses share a common antigen; the common antigen is hypothesized to be the envelope (E) protein on the outside of the virion. Neutralizing antibodies against both WNV and USUV have been found in birds and humans across Europe. *In vitro* cross-neutralization of WNV and USUV has been modeled experimentally and been observed in clinical settings. The neutralizing antibody response generated against WNV has been studied extensively in mouse models; however, there are few studies which examine the neutralizing antibody response generated against USUV. Whether prior WNV exposure protects against USUV disease is also unknown.

The main goal of this thesis was to characterize how a primary flavivirus exposure would influence a secondary flavivirus exposure; specifically, we wanted to observe if WNV exposure would protect against USUV disease *in vivo* and generate a cross-neutralizing antibody

response *in vitro*. For the WNV exposure, we used an attenuated vaccine strain of WNV that contains the WNV E gene (D2/WN-V3) developed by our collaborators. We hypothesized that treatment with D2/WN-V3 would protect against USUV infection. Two *in vivo* models were used: CD-1 mice and interferon alpha-beta receptor 1 deficient (*Ifnar1*$^{-/-}$) mice. We discovered that sera from mice vaccinated with D2/WN-V3 neutralized both WNV and USUV *in vitro*. In the *Ifnar1*$^{-/-}$ model, we observed that vaccinated mice had higher survival rates and lower USUV viremia levels after USUV challenge.

This work helps characterize the consequences of flavivirus antibody cross-neutralization *in vitro* and cross-protection *in vivo*. As the flavivirus field moves toward the goal of creating a pan-flavivirus vaccine, both cross-reactive antibodies and cross-protection need to be considered.

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GENERAL AUDIENCE ABSTRACT

West Nile virus (WNV) and Usutu virus (USUV) are mosquito-borne viruses that were originally isolated in Africa during the 20th century. Both viruses are maintained through a transmission cycle between mosquito vectors and avian hosts. Mosquitos transfer the infectious agent (WNV or USUV) through feeding on a bird (usually a passerine species); once in the bird, the virus can replicate to high levels. Human infections of WNV and USUV from mosquitos can also occur, with symptoms ranging from mild febrile illness to severe encephalitis or meningitis. Over the past few decades, WNV and USUV have spread to Europe, most likely through infected migratory birds. Interestingly, mosquito surveillance studies in mainland Europe have found mosquitos that tested positive for both USUV and WNV. In Europe, antibodies for both viruses have been found in humans and birds, indicating a previous exposure to WNV, USUV, or both.

The neutralizing antibody response is a critical immune defense against viral infections. Neutralizing antibodies bind strongly to the outside of the virion (virus particle), preventing the virion from interacting with and infecting the host cell. For WNV and USUV, one of the targets that neutralizing antibodies bind to is the outer envelope (E) protein of the virion. In clinical settings and experimental studies, cross-neutralization of WNV and USUV has been documented. During cross-neutralization, a serum sample containing neutralizing antibodies against WNV can also neutralize USUV, and vice versa. Although the neutralizing response against WNV has been characterized in humans and lab animal models such as mice, there is little research regarding the neutralizing response against USUV. Importantly, whether prior WNV exposure provides protection against USUV infection is currently unknown.

The main goal of this thesis was to characterize the disease outcome and neutralizing response against USUV after a WNV exposure. For the WNV exposure, we used a vaccine strain of WNV that contains the E gene (D2/WN-V3) developed by our collaborators. We predicted that vaccinated mice would avoid USUV clinical signs of disease and generate neutralizing responses to WNV and USUV. To do this work, we used two laboratory mouse models: mice with an intact immune response system (CD-1) and mice with a stunted immune response (*Ifnar1*$^{-/-}$). We discovered that serum from vaccinated mice did cross-neutralize WNV and USUV. In the *Ifnar1*$^{-/-}$ model, vaccinated mice had higher survival rates and lower levels of virus in blood after USUV infection compared to unvaccinated mice.

Ultimately, this work highlights the importance of characterizing the immune response against similar viruses and will inform the development of human vaccines for both viruses.

Dedication

I would like to thank all the members of the Duggal lab for their constant help and support. Seth: you are always ready to assist anyone with anything, and our lab would not run the same without you! The work you contributed that is included in this thesis is high quality data that completes this scientific story. Thank you for training me so well, especially in the BSL-3 lab. Megan: I always admire your thoughtful input that you give, whether it's for a simple technique or a complex topic. You challenge me to think about what I know and why I know it, and it has made me into a better scientist! Thank you for encouraging me when I needed it and laughing with me during the long days. Sarah: little did I know that the other graduate student in the lab would become one of my dearest friends. Thank you for helping me adjust to graduate school life and taking me under your wing! You helped me learn new techniques, navigate our graduate program, and made me feel at home; I admire you so much.

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CHAPTER ONE: INTRODUCTION

Flaviviruses

Flaviviruses belong to the *Flaviviridae* viral family (genus *Flavivirus*) and are transmitted by arthropod vectors, primarily mosquitos and ticks. The flavivirus genome is a positive-sense, single strand of RNA that is directly translated into a polyprotein by the host cell upon uncoating in the cytoplasm. Globally, many flaviviruses are causes of significant public health concern including yellow fever virus (YFV), Zika virus (ZIKV), dengue virus (DENV), and Japanese encephalitis virus (JEV). The remainder of this work will focus primarily on two emerging, mosquito-borne flaviviruses: West Nile virus (WNV) and Usutu virus (USUV).

WNV was originally isolated in Uganda during the early twentieth century from a human patient with febrile illness [1]. Over the next few decades, sporadic outbreaks of WNV in humans were documented in Israel [2], Egypt [3, 4], France [5, 6], and South Africa [7]. WNV eventually spread to North America in 1999, where it was first detected in New York City [8, 9]. Similar to other flavivirus infections in humans, most WNV cases are asymptomatic, while mild cases can manifest as febrile-like illness (headache, rash, soreness). Which tissues or organs are impacted by infection is dependent on the tissue tropism of the specific virus, and two broad categories of disease (visceral and neurotropic) have been described [10]. In nature, WNV is maintained in an enzootic cycle between mosquito vectors and avian hosts, with other mammals such as humans and horses [11-13] serving as incidental hosts. Passerine species of birds are especially susceptible to WNV, developing high viremia levels, shedding large amounts of virus in cloacal and oral fluids, and in some cases presenting with severe neurological signs of disease [14, 15]. Across the United States, American robins (*Turdus migratorius*) have been shown to be important hosts for WNV in urban and residential areas, reaching high WNV viremia during experimental inoculations [16-18]. Mosquitos are the primary vectors for WNV, particularly *Culex spp*; in the United States, *Culex pipiens* and *Culex quinquefasciatus* are the most abundant mosquito vectors of WNV [19]. Mosquitos that feed on

both birds and humans are important in perpetuating the transmission cycle of WNV between avian and mammalian populations [20].

Similar to WNV, USUV was also isolated in Africa during the early twentieth century, though from a *Culex neavei* mosquito as opposed to a human patient [21]. USUV eventually spread out of Africa and into Europe, emerging in various avian species in Austria during 2001 [22]; however, a retrospective analysis of archived tissue samples from Eurasian blackbirds (*Turdus merula*) collected in Italy in 1996 indicate USUV was introduced into Europe prior to 2001 [23]. Since then, USUV has been found in multiple bird species in other European countries including Belgium, France, Germany, and the Netherlands [24]. The first reported human infection of USUV was in the Central African Republic in 1981, followed by another report in Burkina Faso in 2004 [25]; in both cases, mild symptoms including fever and rash were reported [25]. Most of the confirmed human cases of USUV infection have occurred in Europe, with symptoms ranging from febrile illness to neuroinvasive disease [26], though cases of USUV infection have also been confirmed in healthy blood donors [27, 28]. The predicted transmission cycle of USUV is also similar to WNV, with mosquitos as vectors and avian species as hosts [29]. The main mosquito vectors for USUV include *Culex spp*, particularly *Culex pipiens* [30-32]. The primary avian hosts for USUV are passerine species of birds such as Eurasian blackbirds [33, 34] or house sparrows (*Passer domesticus*) [35]. USUV has been found in other vertebrate hosts besides birds and humans, including bats [36], rodents [37], and horses [38, 39].

Animal models of WNV and USUV

The use of animal models provides relevant, *in vivo* systems in which questions regarding pathogenesis, viral dissemination, immune responses, anti-viral strategies, and beyond can be addressed. There have been a variety of animal models described for WNV and USUV, including avian and mammalian species. The remainder of this section will highlight mammalian models, specifically mice, that have been developed for both viruses.

Mammalian models of WNV have been used to study the pathogenesis that is seen in human cases of WNV. Immunocompetent mice, such as the C57BL/6 strain, are susceptible to WNV and have been used to study viral dissemination and pathogenesis [40]. Although immunocompetent mice can have different mortality rates for WNV, it has been shown that differences in mortality do not impact WNV tissue tropism or neuroinvasion [41]. Using the C57BL/6 mouse model, important findings regarding the adaptive immune response to WNV in the central nervous system (CNS) have been made [42, 43]. Immunocompromised mouse models are also used to study specific immune functions during WNV infections; examples include the interferon type 1 receptor knockout model (*Ifnar1*$^{-/-}$), the interferon type 2 receptor knockout model (*IFN-γR*$^{-/-}$), and the interferon gamma knockout model (*IFN-γ*$^{-/-}$). These models helped establish the critical role type 1 and type 2 interferon responses have in controlling WNV infection, particularly in the CNS [44, 45]. In summary, mouse models are a critical tool that are used to answer questions regarding WNV infection in mammals.

As USUV continues to emerge in nature, mouse models to investigate USUV infection in a mammalian host have been developed. One of the most common strains of laboratory mice used that have been experimentally inoculated with USUV are immunocompetent Swiss mice. Adult Swiss mice (3-8 weeks old) have been used to study cross-protective immunity between USUV and WNV [46] and to characterize a recently isolated USUV isolate from Senegal [37]. Additionally, suckling Swiss mice (4-7 days old) and Swiss-type mice (NMRI strain) have been used to study the neurovirulent implications of USUV infection [46-48]. There is some debate over the relevance of the suckling mouse model for flavivirus infections, as most reported human cases of USUV and WNV are seen in adults with fully developed immune systems; however, there is evidence to suggest that the blood brain barrier is functional in neonate mammals [49, 50]. Other than Swiss mice, wild-type 129/Sv mice have been used to characterize the pathogenicity of recent USUV isolates from Belgium in an immunocompetent

model [51]. As is the case with the majority of flaviviruses, immunodeficient mice have also been inoculated with USUV. Clinical manifestations of USUV disease in immunodeficient mice include weight loss, lethargy, decreased feeding, and a hunched posture [52]. The *Ifnar1*$^{-/-}$ model has generated important findings regarding USUV vaccine design [53], neurovirulent phenotypes [47], and genetic determinants of pathogenesis from European and African isolates [52]. In short, the current mouse models of USUV infection have answered critical research questions regarding this emerging flavivirus, but more models must be developed to completely understand the scope of USUV infection in immunocompetent mammals.

Antibody response to flavivirus infections

The adaptive immune response to flavivirus infections consists of two parts: the cell-mediated response and the antibody-mediated response. While the cell-mediated response to infection is primarily characterized by a variety of activated T cells, the antibody-mediated response is dependent on the generation of antibodies. Although both arms of the adaptive immune response are critical to host defense against an active infection, the remainder of this section will focus on the antibody-mediated response.

For flaviviruses, the envelope (E) glycoprotein is a common antigen; the E glycoprotein is involved in attachment, membrane fusion, and entry with the host cell. Prior to membrane fusion, the E glycoprotein is in a dimerized form, but post-fusion the E glycoprotein is in a trimer form [54]. Additionally, the structure of the E glycoprotein consists of three distinct domains (D): DI, DII, and DIII. These distinct domains on the E glycoprotein are targets for antibodies with potent neutralizing activity; the murine-derived antibody E16 against WNV is specific for DIII and blocks the attachment of the virion to the host cell, as well as inhibits macrophage infection [55]. Interestingly, the humanized version of E16 also protected mice from WNV infection while retaining the originally characterized antigen specificity and neutralizing potency [56]. However, not all mouse-derived monoclonal antibodies are relevant when compared to a human flavivirus

infection; for instance, mice infected with WNV had a high neutralizing response to DIII-specific antibodies, while serum from human patients displayed a high neutralizing response to DII-specific antibodies [57]. Compared to WNV, there is little information in the literature characterizing the antibody-mediated response to USUV; in one study, rabbits immunized with a purified fraction of the E glycoprotein produced in bacteria developed a neutralizing response against USUV [58]. Cross-protection via neutralizing antibodies against both WNV and USUV have also been examined; one study in mice found that prior USUV exposure protected mice against WNV neuroinvasion, though viremia for either virus was not reported [46]. In summary, the E glycoprotein serves as a critical antigen of interest for the development of a neutralizing antibody response against both WNV and USUV.

The mechanisms of antibody development are important to consider when examining the antibody response to flavivirus infections. B cells are the primary effector cells of the antibody-mediated immune response and are inactive prior to antigen exposure. Once a B cell recognizes a specific antigen, it becomes activated; activated B cells then migrate to germinal centers (GCs) in secondary lymphoid tissues like the spleen and lymph nodes [59]. In terms of the development of antibodies for flavivirus infections, it has been shown that B cells coordinate the neutralizing antibody response against WNV infection [42, 60, 61]. However, outside factors including age may hinder the formation of GCs during WNV infection; in one study, older mice had delayed GC formation, which stunted the antibody-mediated response against WNV and led to increased morbidity and mortality [62]. Overall, understanding antibody development during flavivirus infections is crucial to evaluating the antibody response as a whole.

Neutralization of a virus is an important process in the host defense system that is coordinated by antibodies. There are two main mechanisms for how virus neutralization occurs. In one mechanism, virions are linked together through the antigen-binding fragments (Fab) of neutralizing antibodies, which reduces the number of virions able to infect cells [54]. For some

flaviviruses like dengue virus (DENV), the linking of virions by neutralizing antibodies actually promotes the clearance of linked virions from circulation [63]. Another mechanism for virus neutralization depends on inhibiting the virion's ability to interact with the host cell through blocking host receptor-virion interactions or inhibiting membrane fusion [54]. For example, the neutralizing antibody E16 for WNV prevents conformational changes in the E glycoprotein required for membrane fusion [55]; further studies determined that E16 specifically blocks pH-dependent fusion with the liposomes, a step required for delivery of WNV into the cytoplasm [64]. Ultimately, virus neutralization is an imperative process in the host defense system against flavivirus infections.

Antibody dependent enhancement and cross reactivity of flaviviruses

In recent decades, there has been much debate over the mechanisms and implications of antibody dependent enhancement (ADE) during flavivirus infections. ADE occurs when sub-neutralizing or poorly neutralizing antibodies bind to the virion and promote entry into the host cell, leading to enhanced infection [65]. Under sub-neutralizing antibody concentrations there are not enough antibodies that will bind to viral epitopes, while poorly neutralizing antibodies bind to epitopes not involved in host cell attachment or entry [66]. Once the antibodies have attached to the virion, the virion-antibody complex is internalized by the host cell; this process is dependent on the interaction between the Fc region of the virion-antibody complex and the Fc receptor (FcR) on the host cell surface [66]. Most reported cases of ADE in humans have been associated with dengue virus (DENV), of which there are four distinct serotypes. Clinical presentation of ADE can result in dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). The hallmark symptom of DHF in patients is vascular leakage, during which plasma leaks into the extracellular compartment; this can cause hemoconcentration (blood thickening) and a decrease in blood pressure [67]. The primary hypothesis regarding ADE and DENV infection is that secondary infection with a different serotype of DENV puts patients at higher

risk [68], though prior activation of the humoral response during the primary infection is required [69]. Additionally, it has been shown that increased time between exposures to different DENV serotypes is associated with a more severe secondary infection [66]. Studies of pediatric patients have indicated that infants or children are particularly at risk for developing ADE [16, 70]. Young children are particularly at risk for developing ADE from the only licensed vaccine against all four serotypes of DENV, Dengvaxia®, especially children who have not been previously exposed to DENV [71]. The remainder of this section will focus on ADE observed in WNV and the lack of reports for potential ADE between USUV and WNV.

Most studies investigating ADE during WNV infections have been done *in vitro* using macrophage or macrophage-like cell lines (ideally expressing FcR). An important factor in a variety of these studies have been the concentration of WNV anti-serum used. It has been observed that at sub-neutralizing concentrations of WNV anti-serum, WNV titers increased and cytopathic effects were observed at earlier timepoints compared to infected cells not exposed to the anti-serum [72]. In terms of which region of the sub-neutralizing antibodies resulted in ADE, the Fc (constant region) of antibodies are responsible for enhanced infection, while the Fab regions block development of ADE [73]. Specifically, ADE has been shown to enhance WNV entry into the host cells; in the presence of WNV-specific rabbit IgG, more virions bound and entered the host cell during synchronous and nonsynchronous infections [74]. Overall, studies of WNV ADE have been mostly done *in vitro*, with no evidence of disease enhancement in documented human cases.

Cross reactive antibodies between WNV and USUV have been a topic of recent interest in geographic areas where both viruses are circulating. Both WNV and USUV overlap in a variety of European countries, have been found in migratory bird species, and commonly infect the mosquito vector *Culex pipiens* [75]; thus, there is potential for humans to be infected sequentially with both viruses. Although documented human cases of USUV are low compared

to WNV, there is evidence that populations in European countries have been exposed to USUV. In both Italy and Germany, USUV-specific antibodies were found in healthy blood donors [76, 77]. Additionally, there has been detection of both WNV and USUV antibodies circulating in the human population in the same geographic area [78]. Further, there have been documented cases of cross reactivity between WNV and USUV; in one cohort study, individuals had an atypical antibody response (characterized by constant, high levels of WNV IgG and extremely low levels of WNV IgM) against WNV and presented with neutralizing antibodies to USUV, with no evidence of disease enhancement due to the USUV neutralizing response [79]. To date, there has been no evidence of ADE for WNV or USUV in the human population, but the potential for developing cross reactive antibodies should continue to be considered in vaccine development for either virus.

Vaccine platforms and developments for flaviviruses

Much of the world's population live in areas where flaviviruses, specifically those in the JEV serocomplex, are at risk for infection; 50% of the global population live in JEV endemic areas [80] and it is estimated that between 1999-2010, two to four million WNV infections occurred in the United States [81]. For USUV, it is suspected that there are hundreds to thousands of people at risk for infection [10], though serological similarities to WNV make it difficult to confirm these numbers. The remainder of this section will highlight available vaccines for three flaviviruses, evaluate the current progress towards a marketable WNV vaccine, and describe the attenuated WNV that was used as a vaccine in Chapter 2.

Successful vaccines for humans against multiple flaviviruses, including yellow fever virus (YFV), Japanese encephalitis virus (JEV), and DENV have been developed and are widely used. YFV was first isolated from a human patient in Ghana in 1928; the strain isolated was deemed the "Asibi" or wild-type strain [82]. The development of a vaccine against YFV was ultimately achieved through serial passaging in a variety of different systems, including mouse

embryos, chick embryos, and chick embryos with the nervous system tissues removed [83]. This live, attenuated stain of YFV is now known as the 17D strain, and markers of attenuation included diminished neurotropism and neurovirulence, as well as the lack of fatal encephalitis development when injected intracerebrally into monkeys [83]. The 17D strain is still used today as a reliable vaccine against YFV; recent studies have shown that the molecular mechanism for attenuation in the 17D strain is a high fidelity, RNA polymerase [84]. Compared to the Asibi strain of YFV, the 17D strain has much less genetic diversity, indicating a more stable genomic replication process as opposed to the typical, error-prone genome replication of wild-type flaviviruses [84]. The extreme success of 17D has led to the development of other vaccines using a chimeric virus system between the 17D backbone and the prM-E genes of other flaviviruses, including WNV and DENV [85, 86].

JEV is another flavivirus of significant global health concern, as it is the leading cause of viral encephalitis in Asia and has a geographic range from Russia to southeast Asia to Australia [87]. The first confirmed case of JEV was in 1924 in Japan [88], and since then the effort to produce an effective vaccine against the virus has been pursued. Two of the effective vaccines against JEV use different vaccine platforms: one is a cell culture-derived live, attenuated virus and the other is a live, attenuated chimeric virus. The cell culture-derived virus (SA14-14-2) was developed from a JEV strain that was isolated from a pool of *Culex pipiens* mosquito larvae [89]. SA14-14-2 was then serially passaged in a number of different cell lines, including primary hamster kidney cells (PHKs) and mice, followed by plaque purification from primary chick embryo cells of PHKs [90]. Since the licensing of SA14-14-2 for use in humans, over 300 million doses have been given with limited adverse effects reported [91]; however, before mass use of SA14-14-2 outside of Asia, improvements in vaccine production need to be made [87]. The live, attenuated chimeric virus (ChimeriVax-JE) consists of the prM-E proteins of JEV strain SA12-14-2 in the YFV 17D backbone [92] and has been tested for immunogenicity and safety in mice

[93] and non-human primates [94]. During phase 3 clinical trials in humans, a single vaccination with ChimeriVax-JE induced seroconversion in 94% of participants [95]. Additionally, ChimeriVax-JE has a restricted ability to infect and replicate in mosquito species that are susceptible to JEV, including the primary vector *Culex tritaeniorhynchus* [96]; this makes the chances of a vaccinated person transmitting the chimeric JEV to a mosquito extremely low. Vaccine developments against YFV and JEV offer promising avenues to generate vaccines for other flaviviruses like WNV and USUV.

DENV continues to be a growing concern for global public health, and it is the most abundant arbovirus worldwide [71]. As was discussed previously, there are four distinct serotypes of DENV; thus, developing a vaccine that would protect against all four serotypes is highly desirable. Currently, the only licensed DENV vaccine is Dengvaxia®, a live, attenuated tetravalent vaccine which contains chimeras of DENV with the YFV 17D strain; specifically, the pre-membrane and envelope genes of the four DENV serotypes were combined with the non-structural proteins of YFV 17D. Although Dengvaxia® went through two major phase III clinical trials, there are still concerns for individuals who were seronegative for DENV at the time of vaccination [71]. There are many other factors which impact the efficacy of Dengvaxia®, though they are outside the scope of this chapter.

There has been significant progress made towards WNV vaccines for a variety of organisms. In particular, vaccines for equines have been pursued and successfully used. One of the vaccines available for equines is a formalin-inactivated WNV; vaccination with the formalin-inactivated WNV prevented development of WNV viremia after challenge in 94% of vaccinated horses [97]. Another WNV vaccine for equines uses a canarypox vector; this vector undergoes only one round of replication in mammalian cells when the gene product (typically the antigen) is expressed and allows for strong immune responses against the antigen of interest [98]. The canarypox vaccine against WNV contains the prM-E genes of the WNV NY99 strain in a

recombinant plasmid [99, 100] and elicits both a neutralizing antibody response and cell mediated immune response in horses [98]. Despite the success of these equine vaccines, both require yearly boosters, which can be costly [101]. Another vaccine, which consisted of prM and E WNV proteins in a recombinant plasmid, produces secreted forms of WNV prM and E proteins as extracellular subviral particles (EPs) [102]. The secreted EPs protected both mice and horses from WNV challenge [102]; the success of the EP vaccine in mice and horses led to an experimental trial in the avian species California condor, protecting condors of a variety of ages against naturally circulating WNV [103]. In terms of human vaccines against WNV, six have been tested in clinical trials though only two have advanced to Phase II clinical trials. One of the human vaccines is a formalin-inactivated WNV, though there are not many studies in the literature regarding this vaccine. From published studies, the highest dose of the formalin-inactivated WNV given to the subjects induced the strongest neutralizing antibody response against WNV [104]; however, neutralization was not measured using the gold-standard assay of a plaque reduction neutralization test (PRNT). The other human WNV vaccine to advance to clinical trials is the live, attenuated virus ChimeriVax-WN02, which contains the prM-E proteins of WNV in the YFV 17D backbone, along with three mutations in the E protein [85]. In Phase I trials, all subjects vaccinated with ChimeriVax-WN02 seroconverted [105]; during Phase II trials, older adults had the highest neutralizing response after one vaccination [106, 107]. Despite the progress made towards WNV vaccines for human use, no vaccines have made it to Phase III clinical trials.

For the studies that will be discussed in the next chapter, we used a WNV vaccine developed by our collaborator. The WNV vaccine is an attenuated form of WNV; this virus is a chimera between the PDK53 vaccine strain of dengue 2 virus (D2) and the prM-E proteins of WNV NY99 [108]. The PDK53 strain of D2 is a live, attenuated virus that was developed by serial passaging wild-type D2 16681 strain (WT D2 16681) in primary dog kidney cells at

Mahidol University in Bangkok, Thailand [109]. PDK53 is considered to be one of the primary avenues for developing a vaccine against all four strains of dengue virus; in adults, a single immunization with PDK53 produced neutralizing antibodies to D2 that lasted over a year with no adverse effects were recorded [110]. Additionally, PDK53-vaccinated human subjects had memory, $CD4^+$, and $CD8^+$ T cells responses after one dose [111]. At the genetic level, there are two variants of PDK53: PDK53-V (all nine mutations compared to WT D2 16681) and PDK53-E (eight of mutations compared to WT D2 16681) [112]. There are various phenotypic markers that distinguish PDK53 from WT D2 16681 strain, including small plaque size and temperature sensitivity in $LLCMK_2$ cells (rhesus monkey kidney cells), limited replication in mosquito cells, and attenuation in newborn mice (intracranially inoculated) [113]. Further, all mutations responsible for the attenuation phenotype were identified to be outside the structural regions of PDK53 [113]. Chimeric viruses between PDK53 and other dengue serotypes (D1, D3, and D4) have been constructed and retain the same attenuation markers as PDK53, even with the genetic variants of PDK53 [114]. The chimeric virus between PDK53 and WNV NY99 (D2/WN-V3) contains the signal sequence of WNV NY99 (in addition to the prM-E proteins), which improved viability in African green monkey kidney (Vero) cells [108]. Newborn mice vaccinated with D2/WN-V3 had significantly lower mortality rates compared to newborn mice infected with WNV NY99 or D2 16681 [108]. The immunogenicity in adult mice is particularly interesting; after two immunizations with D2/WN-V3, there was 100% seroconversion and high neutralizing responses after challenge with WNV NY99 [108]. Due to the immunogenic properties of D2/WN-V3, we chose to use this as a vaccine in our studies, which is briefly described below and more extensively in Chapter 2.

Goals and findings: The overall goal of this work was to characterize the neutralizing response against two related flaviviruses, WNV and USUV, after exposure to an attenuated form of WNV (D2/WN-V3). Specifically, we were interested in whether vaccination with D2/WN-V3 would

protect against USUV disease *in vivo* and induce a neutralizing response against USUV *in vitro*. We used both CD-1 and interferon type 1 receptor deficient (*Ifnar1*$^{-/-}$) mice for the vaccination studies, collecting serum at various timepoints prior to and after USUV challenge. To test the neutralizing response against WNV and USUV, sera from vaccinated and unvaccinated mice collected prior to USUV exposure were tested using plaque reduction neutralization tests (PRNTs). We found that WNV vaccination did not have an effect on USUV disease or viremia in CD-1 mice, though serum from vaccinated mice cross-reacted with both WNV and USUV. In our *Ifnar1*$^{-/-}$ mouse model, vaccinated mice were protected against USUV disease and viremia, while unvaccinated mice succumbed to USUV challenge. Similar to what was seen in the CD-1 model, serum from vaccinated mice developed neutralizing antibody titers to both WNV and USUV. Overall, this work further illustrates the importance of understanding the potential for protective or pathogenic cross-reactive antibodies against viruses that are similar in emergence trends, antigenic properties, and geographic spread.

CHAPTER TWO: WEST NILE VIRUS VACCINATION PROTECTS AGAINST USUTU VIRUS DISEASE IN MICE

Salgado R, Hawks SA, Frere F, Vázquez A, Huang CY-H, Duggal NK.

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Under review.

Abstract

West Nile virus (WNV) and Usutu virus (USUV) are mosquito-borne flaviviruses that can cause neuroinvasive disease in humans. WNV and USUV circulate in the same geographic areas and are closely related. Due to antigenic similarity, WNV-specific antibodies and USUV-specific antibodies have the potential to bind heterologous viruses; however, it is unclear whether this interaction may offer protection against infection. To investigate how prior WNV exposure would influence USUV infection, we used an attenuated WNV vaccine that contains the surface proteins of WNV in the backbone of a dengue virus 2 vaccine strain and protects against WNV disease. We hypothesized that vaccination with this attenuated WNV vaccine would protect against USUV infection. Neutralizing responses against WNV and USUV were measured *in vitro* using sera following vaccination. Sera from vaccinated CD-1 and *Ifnar1*$^{-/-}$ mice cross-neutralized with WNV and USUV. All mice were then subsequently challenged with an African or European USUV strain. In CD-1 mice, there was no difference in USUV titers between vaccinated and mock-vaccinated mice. However, in the *Ifnar1*$^{-/-}$ model, vaccinated mice had significantly higher survival rates and significantly lower USUV viremia compared to mock-vaccinated mice. Our results indicate that exposure to an attenuated form of WNV protects against severe USUV disease in *Ifnar1*$^{-/-}$ mice and elicits a neutralizing response to both WNV and USUV. Future studies will investigate the immune mechanisms responsible for the protection against USUV infection induced by WNV vaccination, providing critical insight that will be essential for USUV and WNV vaccine development.

Introduction

West Nile virus (WNV) and Usutu virus (USUV) are emerging zoonotic arboviruses in the Japanese encephalitis virus (JEV) serocomplex of the *Flaviviridae* family. Clinical manifestations of WNV and USUV in humans include febrile illness and encephalitic disease, which in severe cases can be fatal. WNV and USUV are maintained in a transmission cycle between *Culex spp*. mosquito vectors and avian hosts [29]. WNV was first isolated in 1937 from a febrile patient in Uganda [1] and has circulated throughout Africa [3, 4, 7], Asia [115-117], Australia [118, 119], the Americas [120-122], and Europe [2, 5, 6]. USUV was originally isolated in South Africa in 1959 [21] and has since circulated throughout Africa, eventually spreading to Europe [123]. Shortly after the introduction of WNV into North America in 1999, the first major epizootic event of USUV occurred in Europe in 2001 where approximately 50,000 Eurasian blackbirds (*Turdus merula*) died [22, 124].

WNV and USUV have overlapping geographic ranges and transmission cycles, thus having the potential to infect the same host. In humans, WNV- and USUV-specific antibodies were found in healthy blood donors in Italy [78, 125]. Evidence of sequential WNV and USUV infections in humans has also been observed; during a WNV outbreak in 2018, individuals with prior USUV exposure had an atypical antibody response to WNV, characterized by the absence or blunt WNV IgM response [79]. Due to antigenic similarity between WNV and USUV, cross-neutralizing antibody responses have been studied. In Austria, sera from confirmed WNV-infected individuals neutralized both WNV and USUV *in vitro* [28]. Additionally, cross-protection between WNV and USUV has been modeled experimentally; one study observed that mice infected with USUV were protected from lethal WNV challenge [46]. However, whether exposure to WNV offers protection from USUV infection is unclear.

A recombinant live-attenuated vaccine (LAV) virus, D2/WN-V3 (also referred as D2/WN for abbreviation) that protects against lethal challenge of WNV in mice was previously developed, consisting of the pre-membrane (prM) and envelope (E) structural genes of WNV in

an attenuated dengue virus (D2) backbone [108]. The D2/WN LAV retained all the original attenuation markers of the D2 backbone [109, 113, 114] and protected against lethal WNV challenge *in vivo*, including diminished neurovirulence in newborn mice and development of neutralizing antibodies against WNV in adult mice after primary immunization [108]. D2/WN has also been evaluated for safety in mice, with no disease observed in newborn mice or AG129 mice [108, 126].

The goal of this study was to determine if prior exposure to the WNV LAV would protect against subsequent USUV infection. For our experiments, we used two mouse models: CD-1 mice and mice deficient in interferon α/β receptor 1 (*Ifnar1*$^{-/-}$). CD-1 and *Ifnar1*$^{-/-}$ mice were selected because our groups has established these as susceptible models for USUV infection, with more severe disease in *Ifnar1*$^{-/-}$ mice [52, 127]. Mice were vaccinated with D2/WN LAV and challenged with a European or African strain of USUV. CD-1 mice transiently treated with an anti-Ifnar1 antibody did not develop USUV disease but did have a neutralizing response to both WNV and USUV post-vaccination. We found that *Ifnar1*$^{-/-}$ mice vaccinated with D2/WN were protected against USUV-induced mortality and had lower USUV viremia than unvaccinated mice. Our results warrant further investigation into the mechanisms behind the cross-protection that WNV vaccination may provide against USUV.

Results

Vaccinated CD-1 mice produce a neutralizing response against WNV and USUV prior to USUV challenge.

To vaccinate immunocompetent mice against WNV, we used a D2/WN-V3 (D2/WN) LAV that has been shown to confer protection against lethal WNV challenge [108]. CD-1 mice were given an initial dose of the vaccine or PBS and received a booster 28 days later (**Figure 1**). Mice were rendered susceptible to USUV with a dose of anti-IFNAR1 antibody prior to challenge, a strategy that has been used for other wild-type mice subject to flavivirus infections including USUV [127-129]. Two recent USUV isolates from Spain and Uganda were used to challenge the vaccinated and PBS-treated mice on day 42 after the first vaccination. In this CD-1 mouse model of USUV infection, we did not observe any morbidity or weight loss; all mice survived and were euthanized at the experiment endpoint (28 days post-USUV challenge). No significant differences in weight change were observed between mock-vaccinated mice and vaccinated mice after USUV challenge (**Figure 2A**). Following a similar trend, no significant differences in viremia were observed between the vaccinated mice and unvaccinated mice (**Figure 2B**).

To determine whether exposure to an attenuated form of WNV would induce a neutralizing response against USUV, a serum sample was collected from each mouse prior to USUV challenge and plaque reduction neutralization tests (PRNTs) against WNV and USUV were performed. Mock-vaccinated mice did not produce a neutralizing response to WNV and produced a very low neutralizing response to USUV. D2/WN immunized mice had significantly higher $PRNT_{50}$ titers against WNV than mock-vaccinated mice at a geometric mean titer (GMT) of 394 ($p<0.0001$) (**Table 1, Figure 3A**). Interestingly, vaccinated mice also had significantly higher $PRNT_{50}$ titers against USUV than mock-vaccinated mice at a GMT of 98.49 ($p<0.0001$) (**Table 1, Figure 3B**). At day 28 post-USUV challenge, vaccinated mice had higher $PRNT_{50}$

titers against WNV (744.9) compared to the pre-USUV challenge titers, though this difference was not significant.

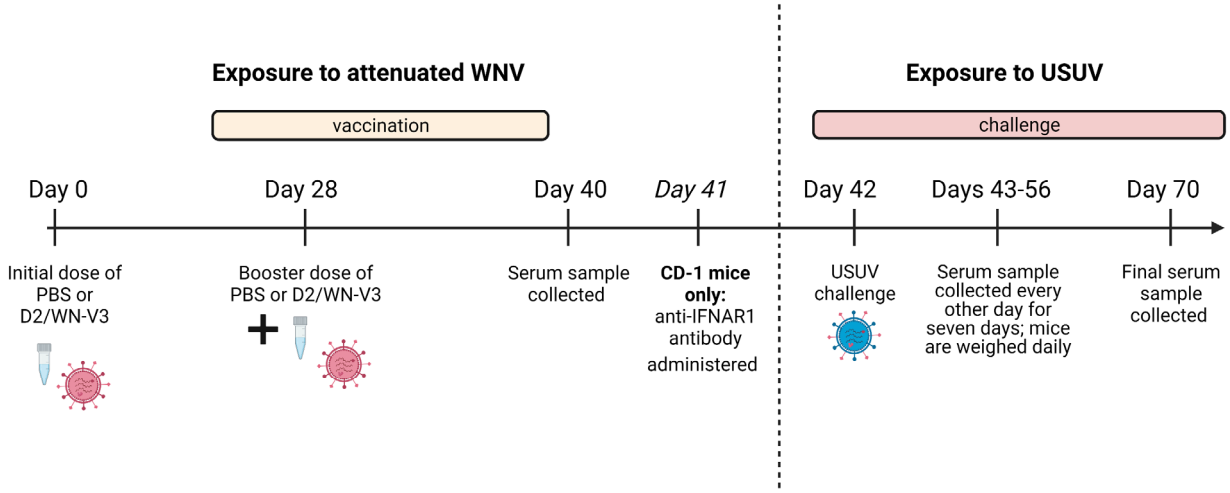

Figure 1. Study design.
Studies using CD-1 (n=40) and *Ifnar1*$^{-/-}$ (n=32) mice were performed as indicated above. Study design was identical between the two mouse models, with the exception of CD-1 mice receiving a dose of anti-IFNAR1 antibody to render them susceptible to USUV.

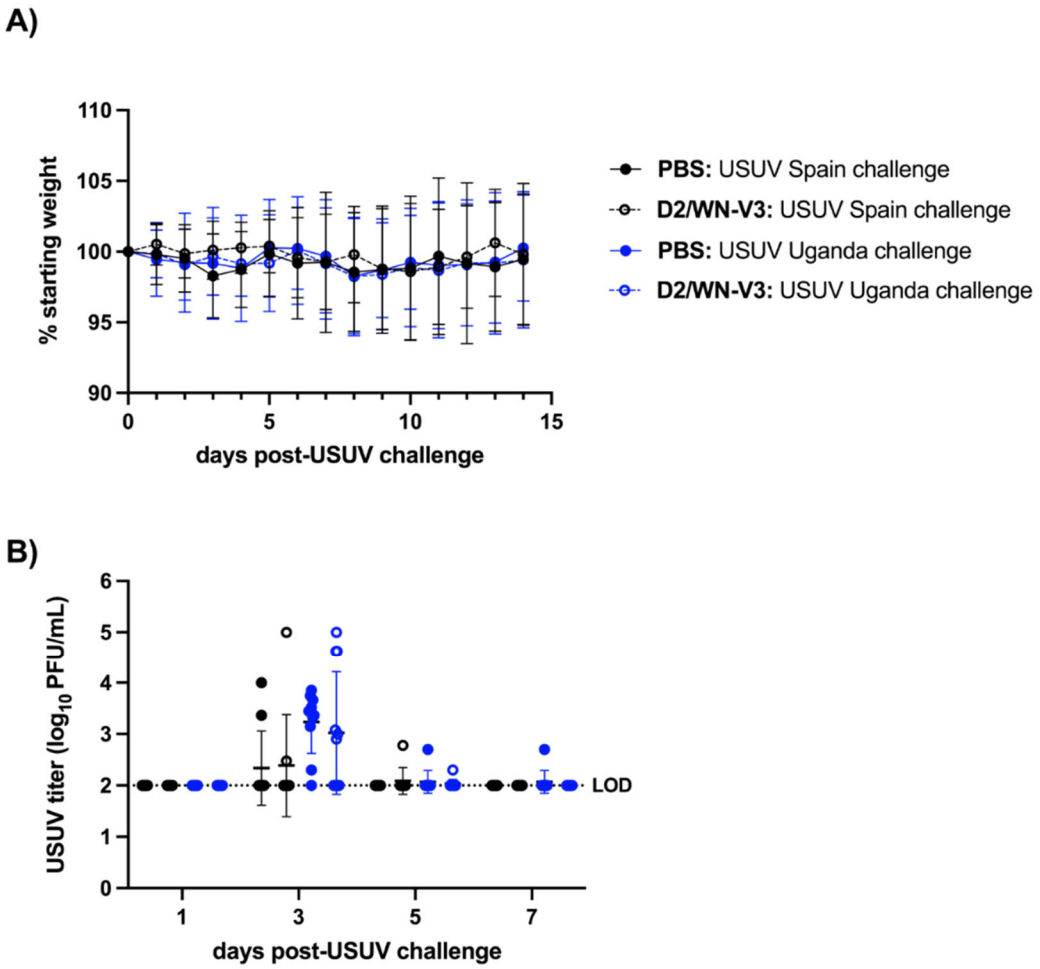

Figure 2. Morbidity and viremia profile of CD-1 mice post-USUV challenge.
CD-1 mice were injected with D2/WN-V3 (n=20) or PBS (n=20) and later challenged with a Spain (n=19) or Uganda (n=21) isolate of USUV. **A)** Average percentage of initial weight post-challenge. **B)** Viral titer of USUV in serum collected on day post challenge (dpc) 1, 3, 5, and 7. Titers are reported as log_{10}PFU per mL of serum. Lines represent mean, error bars represent standard deviation, and the dashed line represents the limit of detection (LOD). All negative titers were graphed at the LOD and included in the mean and standard deviation calculations.

Table 1. Neutralizing responses in CD-1 mice to WNV and USUV post-vaccination.

Treatment	WNV PRNT50			USUV PRNT50		
	GMT	% seroconverted	# mice	GMT	% seroconverted	# mice
PBS (mock-vaccinated)	< 20	0%	0/20	12.31	25%	5/20
D2/WN-V3 (vaccinated)	394****	85%	17/20	98.49****	85%	17/20

Plaque reduction neutralization tests (PRNTs) against WNV and USUV were performed. Serum samples were collected post-booster (day 40 after initial vaccination) from CD-1 mice to determine the geometric mean titers (GMTs) of each group. Data were collected from two independent experiments. Limit of detection is 20. Negative samples (did not neutralize at least 50% of input virus) were assigned a value of 10 and included in the GMT values of the table. Statistical comparisons were done between mock-vaccinated and vaccinated groups for each virus; ****p< 0.0001 (vaccinated vs mock-vaccinated).

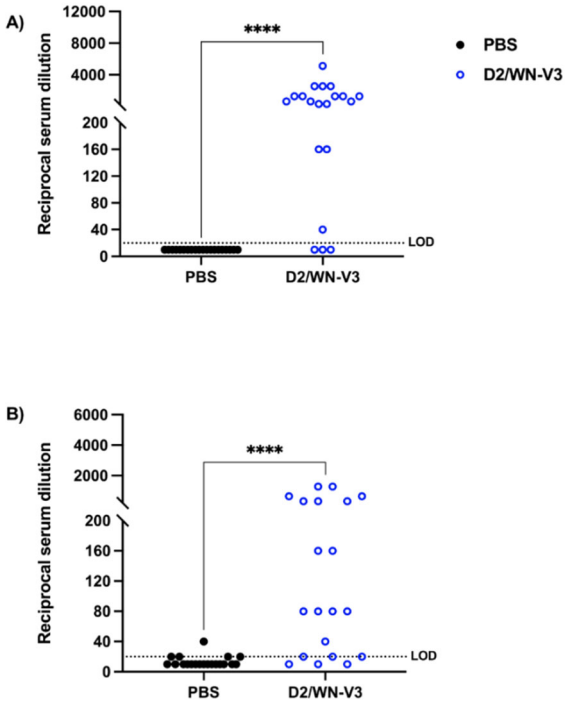

Figure 3. Geometric mean titer (GMT) plots from CD-1 mice.
Plaque reduction neutralization tests (PRNTs) against WNV and USUV were performed. Serum samples were collected post-booster (day 40 after initial vaccination) from CD-1 mice to determine the geometric mean titers (GMTs) of each group. Each data point represents one serum sample. **A)** GMTs of neutralizing responses against WNV. **B)** GMTs of neutralizing responses against USUV. Data were collected from two independent experiments. Limit of detection (LOD) is 20. ****p< 0.0001. Negative samples (did not neutralize at least 50% of input virus at lowest dilution) were graphed at half the LOD (10).

WNV vaccination protects *Ifnar1*$^{-/-}$ mice from USUV disease and viremia.

Next, we tested the vaccine in a mouse model that would develop USUV disease and high viremia levels. Our group has previously characterized the *Ifnar1*$^{-/-}$ as a suitable murine model for USUV infection with severe disease [52]. The same study design described for the CD-1 mice was used, with the omission of the anti-IFNAR1 antibody treatment. Mice that were mock-vaccinated with PBS lost weight quickly and succumbed to USUV infection by seven days post-challenge (dpc) (**Figure 4A and 4B**). Significant differences in survival between the two USUV challenge strains were observed in mock-vaccinated mice; mock-vaccinated mice challenged with the Uganda USUV isolate succumbed by 5 dpc while mock-vaccinated mice challenged with the Spain USUV isolate succumbed later (by 7 dpc, $p<0.0001$) (**Figure 4B**). For both strains of USUV, vaccinated mice had significantly less weight loss at 5 dpc and higher survival rates than mock-vaccinated mice (**Figure 4A and 4B**). Vaccinated mice also had significantly lower USUV titers on 3 and 5 dpc compared to mock-vaccinated mice ($p<0.0001$) (**Figure 4C**).

WNV vaccination induces a neutralizing response against WNV and USUV in *Ifnar1*$^{-/-}$ mice.

To measure the neutralizing response against WNV and USUV before USUV challenge, a serum sample was collected from each mouse, and PRNTs against WNV and USUV were performed. Mock-vaccinated mice did not produce detectable neutralizing antibodies to either WNV or USUV. Sera from vaccinated mice neutralized both WNV and USUV, with a significantly higher geometric mean titer GMT of 2348 against WNV and 49.67 against USUV compared to mock-vaccinated mice ($p<0.0001$) (**Table 2, Figure 5**).

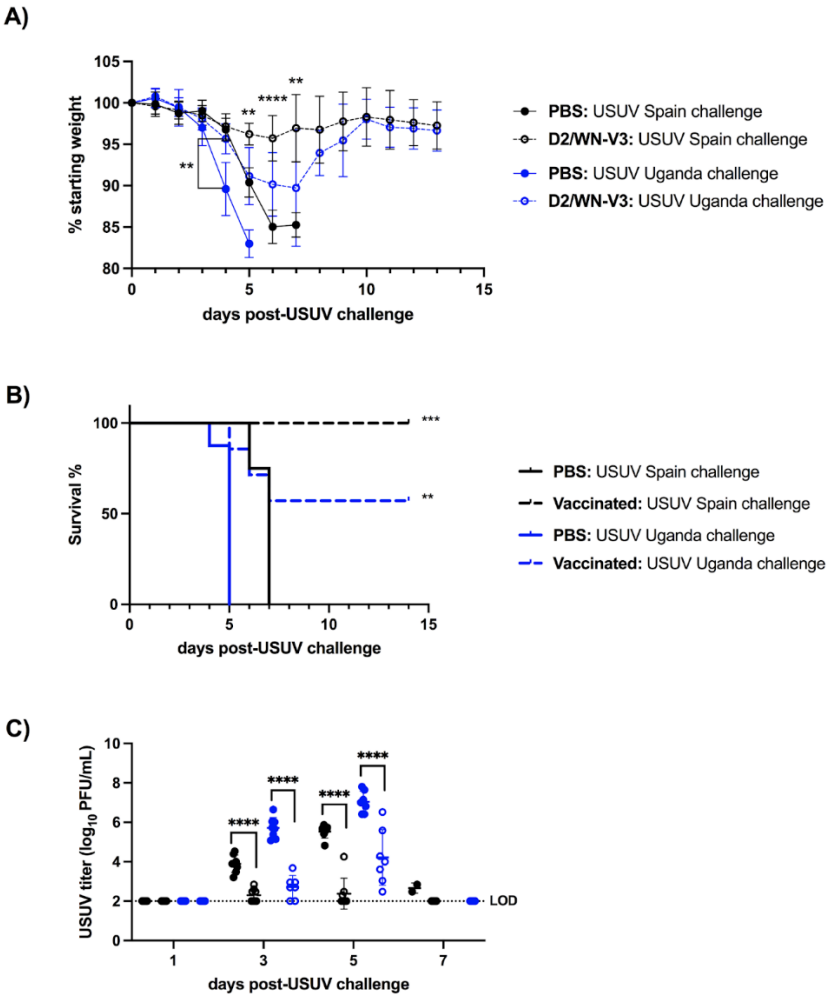

Figure 4. Morbidity, mortality, and viremia profile of *Ifnar1*$^{-/-}$ mice post-USUV challenge.
Ifnar1$^{-/-}$ mice were treated with D2/WN-V3 (n=16) or PBS (n=16) and later challenged with a Spain (n=16) or Uganda (n=16) isolate of USUV. **A)** Average percentage of initial weight post-challenge. P-values represent significant differences between D2/WN-V3 and PBS treated mice of the same USUV challenge group. Day post challenge (dpc) 4, **p<0.01 (Uganda challenge group); dpc 5, **p<0.01 (both challenge groups); dpc 6, ****p<0.0001 (Spain challenge group); dpc 7, **p<0.01 (Spain challenge group) **B)** Survival rate post-challenge. ***p<0.001, **p<0.01 (D2/WN-V3 treated mice compared to PBS treated mice of the same USUV challenge group); black lines represent USUV Spain challenge group and blue lines represent USUV Uganda challenge group; solid lines represent mice that received PBS and dashed lines represent mice that received D2/WN-V3. **C)** Viral titer of USUV in serum collected on day post challenge (dpc) 1, 3, 5, and 7. Titers are reported as $\log_{10}$PFU per mL of serum. Lines represent mean, error bars represent standard deviation, and the dashed line represents the limit of detection (LOD). All negative titers were graphed at the LOD and included in the mean and standard deviation calculations. ****p < 0.0001

Table 2. Neutralizing responses in *Ifnar1*$^{-/-}$ mice to WNV and USUV post-vaccination.

Treatment	WNV PRNT50			USUV PRNT50		
	GMT	% seroconverted	# mice	GMT	% seroconverted	# mice
PBS (mock-vaccinated)	< 20	0%	0/13	< 20	0%	0/15
D2/WN-V3 (vaccinated)	2348****	100%	8/8	49.67****	100%	16/16

Plaque reduction neutralization tests (PRNTs) against WNV and USUV were performed. Serum samples were collected post-booster (day 40 after initial vaccination) from *Ifnar1*$^{-/-}$ mice to determine the geometric mean titers (GMTs) of each group. Limit of detection is 20. Negative samples (did not neutralize at least 50% of input virus) were assigned a value of 10 and included in the GMT values of the table. Due to inadequate sample volume, not all samples were tested against WNV. Statistical comparisons were done between mock-vaccinated and vaccinated groups for each virus; ****p< 0.0001 (vaccinated vs mock-vaccinated).

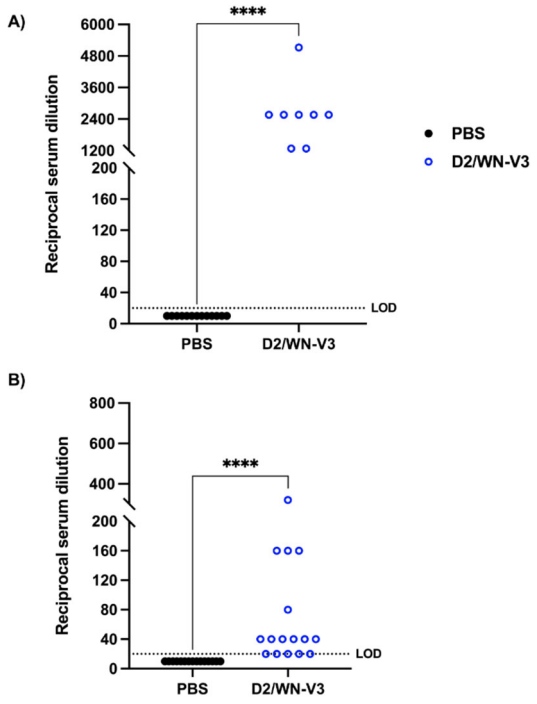

Figure 5. Geometric mean titer (GMT) plots from *Ifnar1*$^{-/-}$ mice.
Plaque reduction neutralization tests (PRNTs) against WNV and USUV were performed. Serum samples were collected post-booster (day 40 after initial vaccination) from *Ifnar1*$^{-/-}$ mice to determine the geometric mean titers (GMTs) of each group. Each data point represents one serum sample. **A)** GMTs of neutralizing responses against WNV. **B)** GMTs of neutralizing responses against USUV. Due to inadequate sample volume, not all samples were tested against WNV. Limit of detection (LOD) is 20. ****p< 0.0001. Negative samples (did not neutralize at least 50% of input virus at lowest dilution) were graphed at half the LOD (10).

Discussion

Through this study, we found that WNV vaccination induced a cross-reactive neutralizing response against USUV in mice (**Table 1 & 2**); these results were seen in two mouse strains, CD-1 mice and *Ifnar1* $^{-/-}$ mice, and two recent USUV strains from Spain and Uganda. Further, the WNV vaccine protected *Ifnar1* $^{-/-}$ mice against disease caused by USUV challenge and significantly reduced USUV viremia (**Figure 4**). The WNV vaccine did not reduce viremia in CD-1 mice, though USUV viremia in this mouse model was much lower than viremia in the *Ifnar1* $^{-/-}$ model (**Figure 2**). Our results indicate that a WNV vaccine induces a cross-neutralizing response against USUV in both mouse models, and that vaccination can protect against USUV mortality in *Ifnar1*$^{-/-}$ mice.

In this study, we did not observe USUV morbidity or mortality in adult CD-1 mice pre-treated with an IFNAR-blocking antibody (**Figure 2A and 2B**). In a previous study, we found that USUV infections with the Uganda 2012 strain resulted in USUV disease in some CD-1 mice pre-treated with the IFNAR-blocking antibody, but these mice were only three-weeks old [127]; in this study, we used the same dose of IFNAR-blocking antibody (1mg/mouse), and the mice were fourteen weeks old at the time of USUV challenge. Additionally, in *Ifnar1*$^{-/-}$ mice we found that the WNV vaccine was less effective in mice challenged with the African strain (Uganda 2012 isolate) of USUV compared to the European (Spain 2009 isolate) strain of USUV. However, unvaccinated mice challenged with the Uganda 2012 USUV isolate succumbed earlier compared to mice challenged with the Spain 2009 USUV isolate (**Figure 4B**). The difference in pathogenesis between African and European USUV isolates has been previously observed in the *Ifnar1*$^{-/-}$ model of USUV infection [52]. The difference in survival between vaccinated mice challenged with Uganda 2012 or Spain 2009 can thus likely be explained by the differential virulence of these strains, which is dictated by unknown viral genetic determinants. Notably, no disease was previously observed in newborn mice or AG129 mice

with the WNV vaccine alone [108, 126]. However, one limitation of this study is that tissues were not collected from mice to compare virus levels and pathology between vaccinated and unvaccinated mice. Previously, we have seen high viral loads in the liver, spleen, heart, and brain in *Ifnar1*$^{-/-}$ mice infected with the Spain 2009 and Uganda 2012 USUV isolates, in addition to observable cell death and inflammation in the spleen [52].

Vaccination induced a more robust neutralizing response to WNV in *Ifnar1*$^{-/-}$ mice compared to CD-1 mice, indicated by the higher geometric mean antibody titers against WNV (**Table 1 & 2**). One possibility for this difference is that *Ifnar1*$^{-/-}$ mice are more susceptible to dengue viruses (DENVs) compared to immunocompetent mice [130, 131]. In our study, the WNV vaccine was in a DENV2 replicative backbone, which likely limited the LAV replication efficiency in CD-1 mice, resulting in lower immunogenicity outcomes in this mouse model. For this reason, we used a higher dose of the WNV vaccine and USUV challenge in the CD-1 mice compared to the *Ifnar1*$^{-/-}$ mice. We also observed that vaccinated CD-1 mice mounted a higher neutralizing response to USUV compared to *Ifnar1*$^{-/-}$ mice (**Table 1 & 2**). One explanation for the difference in USUV neutralization between the two mouse models is that mice in a C57BL/6 background (the *Ifnar1*$^{-/-}$ model used here) are characterized by a high Th1 immune response, which corresponds to a dominantly cell-mediated immune response [132]. Thus, the cross-reactivity to USUV seen in vaccinated *Ifnar1*$^{-/-}$ mice may be driven by stronger cross-reactive T cells as opposed to cross-neutralizing antibodies. Although there was some USUV seroconversion in 5 mock vaccinated CD-1 mice, it was due to a low level of neutralization and did not influence the overall results, as there was still a highly significant difference in the neutralizing response against USUV between vaccinated and mock vaccinated mice (**Table 1**). We recognize that using the 50% neutralization threshold may cause variable results; however, this threshold is recommended by the WHO for flavivirus serology [133] and was used in the original paper characterizing the WNV vaccine [108].

The two-dose WNV vaccine-induced protection against USUV disease seen here is likely due at least partially to cross-reactive neutralizing antibodies. One of the primary targets for neutralizing antibodies against flaviviruses including WNV is the viral envelope (E) glycoprotein, which was included in the WNV vaccine used here. A variety of neutralizing antibodies against the E glycoprotein of WNV have been characterized in mice [55, 57]; a humanized version of one of these antibodies, E16, was shown to protect mice from WNV infection [56]. A previous study showed that D2/WN induced a neutralizing response against WNV, with the second dose significantly boosting the immune response, and prevented mice from succumbing to lethal WNV challenge [108]. The two-dose WNV vaccination strategy has also been implemented in AG129 mice, which are deficient in IFN-α/β and -γ responses, though there was less increase in the immune response in this mouse model from the second dose [126]. Our results confirmed that vaccinated mice developed a neutralizing response against WNV and a cross-reactive neutralizing response to USUV after two doses of the WNV vaccine (**Table 1 & 2**). Neutralizing antibodies against USUV are also likely important for USUV illness protection. One study showed a recombinant subviral particle vaccine containing USUV pre-membrane (prM) and E proteins protected *Ifnar1*$^{-/-}$ mice from lethal USUV challenge and induced a neutralizing antibody response [53].

It is also likely that some of the protection against USUV seen here is dependent on cellular immunity. Cell-mediated immune responses to WNV infection are also protective and can be directed against the E protein [134-136], which was included in the vaccine tested here. The important role of T cell immunity has been implicated in WNV, specifically in limiting infection of the central nervous system [134, 135]. However, most dominant T cell epitopes of flaviviruses are located in the non-structural (NS) proteins [137], and the NS proteins expressed by the D2/WN LAV are from D2. Previous studies using D2/WN LAV have shown that WNV immunity elicited by the prM-E of the LAV provided 100% protection against lethal WNV challenge in mice, whereas D2 immunity generated by the NS proteins of the vaccine provided

limited protection against lethal D2 challenge in an interferon-*α/β/γ*-receptor knockout AG129 mouse model [108, 126]. Undoubtedly, both B and T cell immunity responses are important for protection against flaviviruses, but the neutralizing antibody response appears to play a dominant role in the disease protection in mouse models. Cross-reactive antibodies among some flaviviruses, especially different serotypes of DENV and possibly Zika virus, could result in cross-protection or disease enhancement outcome of the sequential heterotypic viral infection [138]. Currently, there is little evidence in immune enhancement of disease severity or virus infectivity between WNV and USUV infections. In contrast, cross-protection against WNV by prior USUV immunity has been previously reported [139], and our study reports the first animal experiment data showing cross-protection against USUV illness by WNV vaccination.

WNV and USUV co-circulation also has the potential to impact both mosquito vectors and avian hosts. One mosquito surveillance study in Italy discovered numerous pools of *Culex pipiens* that tested positive for both WNV and USUV [32]. It has also been shown that *Culex pipiens* preferentially transmit WNV when co-exposed to USUV and WNV via an infectious blood meal, though it is hypothesized that there is competition between viruses in the midgut of mosquitos [140]. Avian hosts also have the potential to be infected with both USUV and WNV. For instance, both WNV- and USUV-specific neutralizing antibodies were detected in migratory and resident birds in eastern Germany [141]. Recently, it has been shown that magpies previously exposed to USUV are partially protected from WNV, indicated by higher survival rates post-WNV challenge [139]. The interactions between WNV and USUV in mosquito vectors and avian hosts will be critical to monitor as WNV and USUV continue to emerge.

Due to the continued emergence and spread of WNV globally [10], many efforts have been made to further our understanding of WNV disease, particularly in developing murine models of infection. The similarities in emergence trends, antigenic properties, and geographic spread between WNV and USUV make studying these viruses in the context of one another an imperative process. Future passive and adoptive transfer studies could dissect protective

efficacy by antibody- and cell-mediated immunity, respectively. Knowledge in cross-reactive immunity between WNV and USUV in their vertebrae host and mosquito vectors will be relevant information for predicting the potential public health impact of these emerging and re-emerging flaviviruses.

Materials and methods

Viruses and cells. The D2/WN-V3 chimeric virus used in this study was a modified version of the D2/WN-V2 chimera that has been described previously [112]. Briefly, D2/WN-V2 was constructed with the prM and E genes of the WNV NY99-35262 strain (GenBank AF196835) [142] in the backbone of the cDNA clone of the vaccine strain of D2 (PDK-53) (GenBank U87412.1) [112]. An additional Vero cell adaptation mutation at NS2A-22 (Met to Val) in the D2 backbone was engineered into the chimeric D2/WN-V2 clone for deriving a stable D2/WN-V3 for cell culture production; D2/WN-V3 raised similar immunogenicity and protected mice from lethal WNV challenge as the D2/WN-V2 LAV [108]. USUV strains used in these studies were HU10279-09 (USUV Spain 2009) [143] and UG09615 (USUV Uganda 2012) [144]. The USUV Spain 2009 isolate was passaged twice in Vero cells upon receipt, fully sequenced (GenBank MN813489), and characterized previously [52]. The USUV Uganda 2012 isolate was passaged four times in Vero cells and fully sequenced; the sequence is identical to a previous passage 3 sequence that has been published (GenBank MN813491) [52]. Vero cells were grown at 37^{o}C and cultured in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin.

Inoculation of mice.

Ifnar1$^{-/-}$ mice. The interferon alpha and beta receptor 1 deficient mice *(Ifnar1*$^{-/-}$*)* originally purchased from Jackson Laboratories (B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax) were bred in-house. A total of 32, ten to eighteen-week old male and female mice were inoculated with 10^4 PFU of D2/WN chimera (n=16) or sterile PBS (n=16) via intra-peritoneal (i.p.) injection. 28 days post vaccination (DPV), mice received a second dose of D2/WN chimera at 10^4 PFU, or PBS. A

blood sample was collected via submandibular vein bleed 40 DPV. 42 days after the first vaccination, mice were challenged with 10^3 PFU of USUV Spain 2009 isolate (n=16) or USUV Uganda 2012 isolate (n=16) via rear footpad injection, a method that has been used previously for USUV [52, 127]. Mice were bled via submandibular bleed on days 1, 3, 5, and 7 post-USUV challenge. Weights were taken daily, and mice were observed for clinical signs of illness (weight loss, lethargy, tremors). Mice were euthanized when exhibiting clinical signs of disease such as ≥15% weight loss, or at 28 days post-USUV challenge. Serum samples were titrated by Vero cell plaque assay.

CD-1 mice. A similar study was also performed in two independent experiments using 40, eight-week old male and female CD-1 mice purchased from Charles River Laboratories (CD-1® IGS); 20 mice were vaccinated with the WN/D2 chimera, and 20 mice received sterile PBS. Some modifications were made regarding dosage of the WN/D2 chimera, addition of a transient immunosuppressive antibody, and dosage of USUV. The amount of D2/WN administered on day 0 and day 28 was 10^5 PFU. One day before USUV challenge (41 DPV) mice were transiently immunosuppressed with 1 mg of anti-mouse interferon α/β receptor purified function grade, GOLD monoclonal antibody (Clone MAR1-5A3, purchased from Leinco Technologies, Inc; product # I-401) to render them susceptible to USUV infection. 42 days after the first vaccination, CD-1 mice were challenged with 10^5 PFU of either the USUV Spain 2009 isolate (n=19) or USUV Uganda 2012 isolate (n=21). Mice were euthanized 28 days post-USUV challenge.

Plaque reduction neutralization test (PRNT). Mouse serum was heat-inactivated at 56°C for 30 minutes. Serum was then serially diluted 2-fold in BA-1 diluent media (1X M199-Hank's Salts w/o L-Glutamine, Sigma Chemical, product # M9163; 0.05M TRIS-HCl Ph 7.5, Gibco, product # 15567-027; 1% Bovine Serum Albumin, MilliporeSigma, product # 81-066-4; 2Mm L-Glutamine, Invitrogen, product # 25030-081; 0.35 g/L Sodium Bicarbonate, Gibco, product # 25080-094;

100 units/Ml Penicillin and 100 µg/Ml Streptomycin, Gibco, product # 15140-122; 1µg/Ml Amphotericin B, HyClone, product # SV30078.01); an equal volume of BA-1 media containing approximately 100 PFU of virus (WNV or USUV) was added to each dilution. For USUV PRNTs, either HU10279-09 (USUV Spain 2009) or UG09615 (USUV Uganda 2012) was used depending on the *in vivo* challenge group of the sample. A negative control containing no serum was also included. Serum and virus mixtures were incubated at 37°C for 1 hour, then titrated by Vero cell plaque assay. The reciprocal serum dilution was recorded when the sample reduced plaque formation by at least 50% compared to the negative control.

Statistics. Changes in weight and serum titers were analyzed using a two-way ANOVA with Tukey's multiple comparisons test. Survival curves between vaccinated and unvaccinated mice of the same USUV challenge group were analyzed using the Mantel-Cox test. Descriptive statistics were run to determine the geometric mean titers (GMTs) of vaccinated and unvaccinated groups for the $PRNT_{50}$ results and compared via a non-parametric Mann-Whitney test. All analyses were done using GraphPad Prism 8.

Additional software. The BioRender application was used to design Figure 1.

Ethics statement. All animal experiments were approved by the Institutional Biosafety Committee and Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University (IACUC protocol 18-085).

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CHAPTER THREE: DISCUSSION

Discussion

The work described in Chapter 2 highlights the need for more investigation into the interactions between West Nile virus (WNV) and Usutu virus (USUV). Though there has been substantial investigation into the characterization of WNV via animal models, clinical settings, and vaccine development, the same cannot be said for USUV. Additionally, the potential for cross-reactivity between flaviviruses has been studied, though the focus has mainly been toward the multiple dengue virus (DENV) serotypes. As USUV continues to follow a similar evolutionary trajectory as WNV, more studies will need to be done to fully understand the pathogenic or protective interactions between the two viruses. This section will discuss future avenues of research for WNV and USUV based on the results/findings of this thesis.

The advantage of using an attenuated vaccine strain of WNV (D2/WN-V3) for our investigation was primarily to remain working at a BSL2 level for most sample processing and animal procedures. However, to truly examine the cross-neutralization between WNV and USUV, full-length WNV would need to be used. It has been well documented that mice are susceptible to WNV and mount both an antibody-mediated and cell-mediated immune response to infection. The same study design described in Chapter 2 could be repeated using full length WNV in place of D2/WN-V3; the primary change in study design would be performing all animal procedures in a BSL3 facility. Mice would likely need to be given a lesser dose of wild-type WNV in order for them to survive the length of the study. Mice exposed to WNV would likely have comparable or higher geometric mean titers (GMTs) against WNV as mice exposed to D2/WN-V3. For USUV neutralization, mice exposed to full-length WNV would also have higher GMTs against USUV compared to GMTs from mice exposed to D2/WN-V3; since full-length WNV is more likely to replicate in mice compared to D2/WN-V3, the neutralizing response would be greater. Although many studies have been done characterizing full-length WNV in animal

models, there have been few studies investigating the interactions between WNV and USUV *in vivo*.

Another potential avenue for study design described in Chapter 2 would be to use different USUV isolates for the USUV challenge. Other USUV isolates that we have access to in our lab include the prototype South Africa 1959 strain, Senegal 2003 strain, Vienna 2001 strain, and The Netherlands 2016 strain. Both the South Africa and Senegal USUV isolates are of African geographic origin, while the Vienna and Netherlands USUV isolates are of European geographic origin. Based on previous work, we know that the African isolates are more pathogenic in *Ifnar1* $^{-/-}$ mice compared to the Netherlands isolate [52]. Using other African USUV isolates (South Africa 1959 and Senegal 2003) for the USUV challenge would likely yield similar results in survival, viremia, and neutralizing responses against both WNV and USUV compared to the study described in Chapter 2. Due to the limited pathogenesis seen in *Ifnar1* $^{-/-}$ mice infected with the Netherlands 2016 USUV isolate [52], I expect that using this strain for the USUV challenge in the vaccine study would generate comparable results in survival, viremia, and neutralizing responses against WNV and USUV seen in the CD-1 model. The differences in pathogenesis between African and European USUV isolates are likely due to unknown genetic determinants. Taking into consideration genetic differences between geographic isolates will be important in developing vaccines for USUV.

Avian species contribute immensely to global biodiversity; both WNV and USUV have caused mass mortality of avian species in recent decades. More field studies need to be conducted to determine if differences in pathogenesis due to viral genetic diversity (such as USUV strains from different geographic regions) are seen in natural avian hosts. Despite the disease toll that both USUV and WNV have on avian hosts, there are few studies in the literature that investigate potential vaccines against either virus for the avian population. One approach would be to test WN/D2-V3 in the avian models for USUV that our lab has developed;

these models include a domesticated chicken model [145] as well as wild-caught house sparrows. We have previously confirmed that both models are susceptible to USUV and experience clinical signs of USUV disease, including weight loss and development of USUV viremia. Using D2/WN-V3, we would be able to test whether the protection from USUV disease and neutralizing response against WNV and USUV seen in a mammalian model would apply to an avian model. Neutralizing antibodies against WNV and USUV have been found in birds in Europe, so it is likely that exposure to the WNV vaccine and subsequent USUV challenge would also generate neutralizing responses. For the USUV strain used to challenge post-vaccination, we would likely select the Uganda 2012 isolate, as this strain causes USUV disease in our avian models. However, since most of the documented disease burden of USUV in avian species has been in Europe, using a second challenge strain from one of the European isolates of USUV could also be used. There are various resources at Virginia Tech which would allow designing the study with an avian model feasible; this includes faculty from the Animal and Poultry Sciences department and College of Veterinary Medicine, as well as adequate facilities that would house infected chickens and house sparrows. Similar to the study design used in our mammalian model, we would monitor the birds post-USUV challenge by collecting weight data and serum samples to measure viremia. Tissue samples could also be collected including the spleen and bursa, both of which are important lymphoid organs in avian species. To make steps toward our group's overall goal of preventing the emergence of arboviruses like USUV and WNV, more studies into avian vaccinations against either virus need to be conducted.

While extending this project into investigations of USUV and WNV host species is important, implications for the vector species (mosquitos) should also be explored. One consideration when designing or testing live, attenuated virus (LAV) strains for arbovirus vaccines is whether the vector will be able to transmit the LAV from a vaccinated host to unvaccinated host; for most successful LAV strains, mosquitos are unable to transmit the LAV

after feeding on a vaccinated host. D2/WN-V3 could be tested in a variety of ways to answer various questions regarding the vaccine protection and vectors. One avenue would be to investigate whether D2/WN-V3 can escape the midgut of the mosquito; if D2/WN-V3 was able to escape the midgut of the mosquito, this means that there is the potential for the LAV to be passed on to another host during feeding. Mosquitos would be starved before being fed on a blood meal supplemented with high titers of D2/WN-V3; following feeding, engorged mosquitos (those successfully fed) would be separated from the remaining mosquito pool. Engorged mosquitos would then be allowed to incubate for 10-14 days, giving D2/WN-V3 the chance to escape the midgut of the mosquito. At the end of the incubation period, a salivation assay would be performed on the mosquitos, and the saliva would be tested via plaque assay for presence of D2/WN-V3. D2/WN-V3 found in the saliva of the mosquitos would indicate that the LAV can escape the midgut and has the potential to be passed to another host. A further avenue of research would be to feed mosquitos on avian hosts that have received antibodies (passive transfer study) or T cells (adaptive transfer studies) from vaccinated chickens; this would be followed by USUV challenge. After USUV challenge, mosquitos would be fed on chickens using a method that our group has previously performed in other avian-mosquito pilot studies. These studies would investigate whether the vaccine provides protection against USUV mosquito transmission, and whether antibody-mediated or cell-mediated immune responses play a role in the potential for USUV mosquito transmission. By exploring the potential of the vaccine in terms of preventing USUV transmission, another knowledge gap in USUV vaccine design would be filled.

The chimeric LAV that was used as an attenuated WNV vaccine in this work serves as an excellent step in the direction of vaccine design for arboviruses. Over the past few decades, we have seen global climate changes, increases in international travel, and urbanization of previously undisturbed environments; these factors along with many others have changed the

emergence of arboviruses. I am confident that the critical thinking skills, data analysis techniques, and experimental design tools I have learned throughout this thesis work will not only impact me for the rest of my career in the virology, but will also contribute to the development of a human vaccine against USUV.

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