Primary and Secondary Immune Responses During Sequential West Nile Virus and Japanese Encephalitis Virus Infections: A Dissertation

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PRIMARY AND SECONDARY IMMUNE RESPONSES DURING SEQUENTIAL WEST NILE VIRUS AND JAPANESE ENCEPHALITIS VIRUS INFECTIONS

A Dissertation Presented

By

DEREK WILLIAM TROBAUGH

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

FEBRUARY 14, 2012

IMMUNOLOGY AND VIROLOGY
PRIMARY AND SECONDARY IMMUNE RESPONSES DURING SEQUENTIAL WEST NILE VIRUS AND JAPANESE ENCEPHALITIS VIRUS INFECTIONS

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Over the past few years, I have been given a lot of advice from Dr. Anuja Mathew. I would not be at this stage in my career without her advice and support. I need to thank one of my surrogate families while in Massachusetts, the group formally known as the CIDVR. The past few years haven’t been ideal (nor probably have I), but I think better times are ahead. I also need to thank my fellow graduates students at UMass, my other surrogate family. We came here a
little over 5 years ago from many different areas, yet have become life long
friends and colleagues. Now graduate.

Finally, I need to thank my family. I have learned a lot from my dad about
work ethic and how to interact with coworkers, which will go far in this field. My
parents have always been supportive of everything that I have done, even if it
was not their first choice for me. My brother also got his PhD, so I blame him for
not fully explaining to me the trials and tribulations that arise while obtaining a
PhD.
ABSTRACT

Japanese encephalitis virus (JEV) and West Nile virus (WNV) are closely related Flaviviruses that are important arthropod-borne human pathogens. Both of these viruses can cause encephalitis with significant morbidity and mortality after infection. Flaviviruses co-circulate in many areas of the world, which raises the risk for sequential infection between heterologous viruses. Sequential infection between dengue virus serotypes can lead to cross-protection, but in some cases, it leads to a severe outcome, dengue hemorrhagic fever. Previous work in hamsters and non-human primates demonstrated that prior JEV immunity protects against a lethal WNV infection. However, the ability of prior WNV immunity to protect against a lethal JEV infection has been inconclusive. WNV-immune hamsters were fully protected from JEV viremia, but in non-human primates, prior WNV-immunity only reduced disease severity, with symptoms of encephalitis still observed. These differences in cross-protection led to further investigation on the directionality as well as the underlying mechanisms for this phenomenon.

Previous work in our lab found that JEV-immune C57BL/6J (B6) mice were fully protected against a lethal WNV infection, and JEV-immune CD4\(^+\) and CD8\(^+\) T cells were required for this cross-protection. In other mouse models, memory cross-reactive CD4\(^+\) and CD8\(^+\) T cell responses may induce protection or immunopathology upon secondary heterologous viral challenge. We
hypothesize that JEV/WNV cross-reactive CD4+ and CD8+ T cells preferentially expand upon 2o infection and contribute to cross-protection. To elucidate the potential role of T cells in sequential flavivirus infection, we identified and characterized cross-reactive CD4+ and CD8+ T cell responses between JEV and WNV. A previously reported WNV NS4b CD8+ T cell epitope and its JEV variant elicited CD8+ T cell responses in both JEV- and WNV-infected mice. Despite similarities in viral burden for pathogenic JEV and WNV viruses, CD8+ T cells from pathogenic JEV-infected mice exhibited functional and phenotypic profiles similar to those seen for the attenuated JEV strain. We believe the differences in the CD8+ T cell responses during primary JEV and WNV infection are due at least in part to the low levels of peripheral replication seen in JEV-infected mice compared to WNV-infected mice.

We also found that WNV-immune B6 mice were protected against a lethal JEV infection. Cross-reactive CD8+ T cells in JEV-immune mice rapidly expanded after WNV infection. Even though WNV-immune mice had higher frequencies of memory CD8+ T cells, cross-reactive CD8+ T cells did not expand after secondary JEV infection. Neutralizing antibodies to JEV were detected in WNV-immune mice; however, cross-reactive CD8+ T cells did not expand even in the absence of these cross-reactive neutralizing antibodies. We did not detect any differences in the CD8+ T cell repertoires between JEV- and WNV-infected mice nor were WNV-immune CD8+ T cells functionally exhausted. In fact, proliferation of memory CD8+ T cells did not correlate with the ability of WNV-
immune CD8+ T cells to restrict recombinant vaccinia viruses expressing the cross-reactive epitope or lyse peptide-coated targets. These data suggest that the higher frequency of memory CD8+ T cells and cross-reactive antibodies in WNV-immune mice are better able to prevent neuroinvasion following 2nd JEV infection.
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ABBREVIATIONS

1° Primary
2° Secondary
ADE Antibody dependent enhancement
APC Allophycocyanin
APHIS Animal and Plant Health Inspection Service
ATCC American Type Culture Collection
B6 C57BL/6J mice
BEI Biodefense and Emerging Infections
BD Becton, Dickinson and Company
BMDCs Bone marrow derived dendritic cells
BrdU Bromodeoxyuridine
C Capsid
CD Cluster of differentiation
CFSE 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
Ch Chimeric
CNS Central nervous system
CO₂ Carbon dioxide
CPE Cytopathic effect
Cr Chromium
CTL Cytotoxic T lymphocyte
Cy  Cyanine
D  Domain
DHF  Dengue hemorrhagic fever
E  Envelope
EC$_{50}$  Half maximal effective concentration
EEEV  Eastern equine encephalitis virus
ELISPot  Enzyme-linked immunosorbent spot
FACS  Fluorescence Activated Cell Sorting
FBS  Fetal bovine serum
FITC  Fluorescein isothiocyanate
Flu  Influenza virus
G  Genotype
GM-CSF  Granulocyte macrophage colony-stimulating factor
HLA  Human leukocyte antigen
ICS  Intracellular cytokine staining
IFN  Interferon
IRF  Interferon regulatory factor
Ig  Immunoglobulin
IL  Interleukin
ip  Intraperitoneal
IPS-1  Interferon-beta promoter stimulator 1 (IPS-1)
iv  Intravenously
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor G1</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine Cytomegalovirus</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation antigen 5</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MPECs</td>
<td>Memory precursor effector cells</td>
</tr>
<tr>
<td>MVEV</td>
<td>Murray Valley encephalitis virus</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-human primate</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute of Allergy and Infectious Diseases</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>Nonstructural</td>
</tr>
<tr>
<td>P</td>
<td>Position</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin Chlorophyll Protein Complex</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>prM</td>
<td>Pre-membrane</td>
</tr>
<tr>
<td>PRNT&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Plaque Reduction Neutralization Test</td>
</tr>
<tr>
<td>PV</td>
<td>Pichinde virus</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid inducible gene-1</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>rVV</td>
<td>Recombinant vaccinia virus</td>
</tr>
<tr>
<td>sc</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLEV</td>
<td>Saint Louis encephalitis virus</td>
</tr>
<tr>
<td>SLECs</td>
<td>Short-lived effector cells</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TBEV</td>
<td>Tick-borne encephalitis virus</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
</tbody>
</table>
TNF  Tumor necrosis factor
Treg  T regulatory cell
US  United States
USDA  United States Department of Agriculture
VEEV  Venezuelan equine encephalitis
VV  Vaccinia virus
WNV  West Nile virus
WT  Wild type
YFV  Yellow fever virus
PREFACE

Parts of this thesis have appeared or will appear in separate publications:

**CHAPTER III:**

**CHAPTER IV:**

Trobaugh D.W., M Terajima, S Green. Sequence of infection determines cross-reactive CD8+ T cell responses during heterologous West Nile virus and Japanese encephalitis virus infections. Manuscript in Preparation.
CHAPTER I
INTRODUCTION

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are arthropod-borne viruses of the *Flaviviridae* family. Flaviviruses are medically important viruses that co-circulate in many areas of the world (1). WNV and JEV are members of the JEV serocomplex along with Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV). Dengue viruses, yellow fever virus (YFV) and the tick-borne encephalitis viruses (TBEV) are other medically important members of the *Flaviviridae* family (2). This dissertation will focus on cross-protection within the JEV serocomplex, specifically JEV and WNV, and cross-reactive CD8⁺ T cell responses during sequential JEV and WNV infections.

A. West Nile virus

WNV was first isolated in 1937 in Uganda and is endemic to regions in Africa, Middle East and Asia (1, 3). WNV was first identified in the United States (US) in 1999 during a cluster of encephalitis cases in New York City. (4-7). Since then, WNV has spread across the continental US, Canada and Mexico (8).

Analysis of the amino acid sequence suggests that the US emergent WNV strain originated in Israel, and shares sequence similarities with lineage I WNV strains (4, 5, 9). Interestingly, WNV strains in the US were found to be more virulent in
mice and hamsters than previous isolates due to added glycosylation of the envelope (E) protein (10, 11).

Kunjin virus, isolated in Australia in 1960, is a subtype of lineage I WNV (12, 13). Kunjin and WNV share 93% amino acid homology, but Kunjin is highly attenuated in both mice and humans (9, 14, 15). A recent survey found 13 human cases of Kunjin virus between 1992 and 2010, and only six of these cases resulted in neuroinvasive disease (15). The co-circulation of MVEV and to a lesser extent JEV, may be limiting Kunjin virus infections and the severity of disease symptoms in Australia (1, 16).

WNV is an enzootic virus that is transmitted by the *Culex pipiens* complex of mosquitoes, and is maintained in nature through a bird-mosquito-bird cycle (17, 18). Humans and horses can become infected with WNV, but are generally thought of as dead end hosts (18). Human-to-human spread of WNV can occur via breast milk, blood transfusions and organ transplantation, necessitating the screening of the blood supply and organs (19-22). Clinical manifestations of WNV range from asymptomatic disease to mild symptoms or to encephalitis, with elderly patients being more prone to encephalitis (6, 7). In humans, the rate of neuroinvasive disease in New York in 1999 was found to be 1 in 140 infected individuals, and approximately 80% of WNV infections are asymptomatic (7). By 2008, 11,821 cases of WNV neuroinvasive disease were reported in the US (23).

WNV has been shown to persist in mice, hamsters, non-human primates (NHP), and possibly in humans. WNV antigen and infectious virus can be found
in mice up to 4 months post infection, and up to even longer time points in hamsters (24-27). In NHPs, WNV can persist in both asymptomatic and symptomatic monkeys, suggesting neuroinvasion is not the sole determinant for persistence (28). However, the ability of WNV to persist in humans is controversial. In a subset of WNV-infected individuals, WNV RNA was found in the urine of 5 of 25 of these subjects up to 6 years after the initial onset of symptoms (29). However, in a different cohort of 40 patients, WNV RNA was not detected in any urine samples tested >6 years after initial presentation (30). Therefore, the detection of WNV RNA in human samples may depend upon the cohort of patients being tested, the time after initial viral infection, as well as the method for detection of WNV RNA.

B. Japanese encephalitis virus

JEV was first isolated in Japan in 1934, and is endemic to East and Southeast Asia (16, 31). Similar to WNV, JEV is transmitted primarily by *Culex tritaeniorhynchus* mosquitoes, but is maintained through a bird-mosquito-pig cycle with humans and horses again serving as dead end hosts (16, 32). Annually, 30,000-50,000 cases of JEV occur, with a 30% mortality rate (33). Clinical manifestations are similar to those seen in WNV and range from asymptomatic cases to encephalitis, with young children and the elderly at the greatest risk for neuroinvasive disease. A majority of patients experience
headache, fever, and myalgia, and up to 50% of survivors of JEV neuroinvasive disease have long-term/permanent neurological sequelae (33, 34).

JEV is genetically divided into 5 genotypes (G1, GII, GIII, GIV, GV) based on the amino acid sequences of the envelope (E) protein (35). Genotypes can predominate in specific regions, but genotype replacement and/or the emergence of new genotypes can and does occur (31, 35-37). The JEV strains currently used in vaccines (Nakayama, Beijing-1, and SA14-14-2) are GIII viruses (38). Recently, it was demonstrated that these vaccines were protective against viral strains from other genotypes in mice, suggesting they may be beneficial if genotypes replacement occurs (36). JEV is currently not endemic to North America, and has been declared a Select Agent by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). A report in 2009 described California as a likely site of introduction into the US due to proximity to Asia and the availability of susceptible mosquitoes and hosts (39). Travelers and military personnel traveling or residing in endemic JEV regions and laboratory workers are encouraged to receive the JEV vaccine (40).

C. Characteristics of the JEV and WNV genome

WNV and JEV are comprised of a single stranded positive sense ~11kb genome that encodes a single polypeptide (4, 35). Host and viral proteases cleave the WNV polypeptide into 3 structural proteins (E, pre-Membrane (prM), and capsid (C)) and 7 nonstructural (NS) proteins (NS1, NS2a, NS2b, NS3,
NS4a, NS4b, and NS5). The E protein is the major surface protein, and contains the putative receptor-binding site (41). The nonstructural proteins have independent, but multiple roles during the life cycle of WNV. For example, the NS1 protein has roles in viral replication, inhibition of the complement system, inhibition of the toll-like receptor 3 pathways and in neuroinvasion (42, 43). NS2b and NS4a are cofactors for NS3, which together have helicase and protease functions (44-48). Multiple proteins (NS2a, NS4b, NS5) are also involved in inhibiting components of the IFN signaling pathway (49-55). Finally, the NS5 protein contains the RNA-dependent polymerase and methyltransferase required for viral replication (56, 57).

D. JEV and WNV vaccines

First and second generation vaccines against JEV are currently licensed for use in the US. JE-VAX, a mouse brain derived inactivated JEV Nakayama vaccine, in 1992 became the first JEV vaccine licensed in the US (40). JE-VAX has an efficacy rate of >90% in children, but serious adverse effects have been documented in patients receiving this vaccine (33, 40, 58, 59). Manufacture of JE-VAX has recently been discontinued, and remaining stockpiles are currently used to vaccinate children under the age of 17 (40).

A live attenuated vaccine, JEV SA14-14-2, made via serial passage of the virulent SA strain, was first licensed in China in 1989 (60). Two doses of the vaccine were found to have an efficacy of >97% in preventing JEV encephalitis in
rural China (61). SA14-14-2 is highly attenuated compared to the parental SA14 strain, and very little replication occurs in the mice (62). There are only 8 amino acid mutations in the E protein between the parent SA14 strain and the SA14-14-2 vaccine strain, and 4 of these amino acid mutations are important for attenuation and the lack of neuroinvasion of the vaccine (63, 64). The live attenuated SA14-14-2 vaccine is not licensed for use in the US, but is currently licensed in China, Nepal, and South Korea (65).

A second generation formalin inactivated JEV vaccine based on the SA14-14-2 virus was developed by the Walter Reed Army Institute for Research and grown in Vero cells (66). The vaccine was licensed in the US in 2009 under the trade name IXIARO®, and is currently approved for adults over 17 years old. Immune sera from patients receiving IXIARO® have been shown to be effective in neutralizing multiple strains of JEV, suggesting IXIARO® may protect against multiple genotypes (40). IXIARO® is given in two doses (day 0 and day 28), and protective neutralizing titers can be detected in 83% of individuals 6 months after immunization (67).

A chimeric virus containing the prM/E proteins of JEV (Ch-JEV) and the nonstructural proteins of the YFV 17D vaccine (YF17D) has also been developed as a vaccine candidate (68). Interestingly, the viral source of the prM/E proteins (Nakayama or SA14-14-2) determined the neurovirulence of the vaccine in mice (64, 68). A recent Phase III clinical trial demonstrated that the Ch-JEV vaccine has similar immunogenicity and fewer adverse events compared to JE-VAX,
while also requiring fewer doses (69, 70). Ch-JEV was recently approved in Australia under the trade name IMOJEV® (71).

There are currently no vaccines or therapies approved against a WNV virus infection in humans although multiple vaccines have been approved for use in horses (18). Several vaccine candidates have been developed for humans with a few already in Phase II clinical trials. Immunization with a recombinant WNV E protein has been shown to protect against a lethal WNV infection in mice (72-75). Similarly, a DNA vaccine encoding the prM/E proteins of WNV elicited neutralizing antibody responses and T cells responses in young and elderly subjects (76).

A chimeric WNV (Ch-WNV) vaccine was developed with a similar strategy as Ch-JEV. Three amino acid changes were engineered into the original Ch-WNV strain (NY-99) to mimic the amino acid differences between Ch-JEV SA14-14-2 and Ch-JEV Nakayama in order to further attenuate the virus (77). Ch-WNV has been shown to induce both neutralizing antibodies and T cell responses in Phase I and Phase II clinical trials (78, 79). In addition, a WNV virus lacking the C protein (RepliVAX), which lacks the ability to make infectious progeny, has been shown to be protective in murine, hamster and NHP models (80-82).

**E. JEV and WNV mouse models**

Inbred and wild mouse strains have different susceptibilities to WNV infection due to a truncation in the 2’-5’ oligoadenylate synthetase gene, a
component of the antiviral pathway, in inbred mice (83). Interestingly, susceptible mouse strains can even have different mortality rates after WNV infection, although there is no difference in viral tropism (84). Neither the route of infection nor propagation history (vertebrate or mosquito cell line) changes the mortality rate of WNV in mice; however, the propagation history can influence the innate immune response to WNV (85-87).

C57BL/6 (B6) mice are one mouse strain used to study WNV pathogenesis. In B6 mice, viremia develops and replication occurs in multiple peripheral tissues prior to viral dissemination into the CNS (84, 88). Infection of B6 mice can lead to weight loss, hunchback posture, ruffled fur, limb paralysis, and mortality (88). On the other hand, Kunjin virus, a subtype of WNV, is highly sensitive to type I IFN, and therefore very low levels of viremia and peripheral replication occur in B6 mice (14). Susceptibility to Kunjin virus increases in mice lacking the interferon α receptor (IFNαR−/−), and can be used as a lethal model for WNV infection (14, 89). Similarly, some lineage II WNV strains are more susceptible to type I IFN and have an attenuated phenotype in mice compared to lineage I WNV strains (90).

Previous JEV mouse models have utilized intracranial inoculation of weanling mice or adult BALB/c mice to study JEV pathogenesis (91-94). Larena et al. recently established a JEV model using the JEV Nakayama strain in B6 mice. Low dose infection via a subcutaneous (sc) inoculation leads to similar clinical manifestations as WNV, and resulted in a 60% mortality rate. Only low
levels of viral RNA were detected in the serum and spleen early after infection, with viral dissemination into the CNS occurring during later stages of the infection (95). The ability of JEV to replicate in myeloid cells may influence the susceptibility of mouse strains to JEV infection (96). We developed a lethal model of an intraperitoneal (ip) infection of JEV Beijing in B6 mice, and this will be discussed in Chapter IV.

Multiple components of the innate immune system are required for protection against both JEV and WNV infection. Retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5) are required for intracellular recognition of JEV and WNV and maintenance of the interferon (IFN) signaling cascade via interferon-beta promoter stimulator 1 (IPS-1) (97-101). Interferon regulatory factor (IRF) 3 and 7, downstream effectors of IPS-1, are essential for the initiation of type I IFN, with IRF3/IRF7^-/- mice being highly susceptible to infection (102-105). Toll-like receptor (TLR) 3 and 7 recognition of WNV leads to type I IFN production and also helps in the trafficking of lymphocytes to the CNS (85, 106, 107).

F. Adaptive immune responses to JEV and WNV

i. T cell responses in mice

During JEV and WNV infection, the adaptive immune response is essential for preventing viral dissemination, clearance of virally infected cells, and survival. RAG^-/- mice, which lack both B and T cells, are highly susceptible to low
doses of WNV (88). In mice deficient of CD8\(^+\) T cells, higher levels of WNV are found in the CNS, which can persist weeks after the initial infection, ultimately leading to death (108-111). CD8\(^+\) T cells require both perforin and Fas ligand effector mechanisms to clear virally infected cells in the periphery and the CNS (110, 111). Trafficking of T cells to the CNS is also required for protection from WNV as mice lacking chemokines or chemokine receptors have low numbers of lymphocytes in the CNS and uniformly succumb to infection (112-114).

WNV specific CD8\(^+\) T cell epitopes have been identified in both mice and humans (115-118). In mice, the immunodominant CD8\(^+\) T cell epitope (SSVWNATTA) is H-2D\(^b\) restricted and is located in the NS4b protein (115, 116). The maturation and expansion of WNV specific CD8\(^+\) T cells is linked to type I IFN production, demonstrating how the innate immune response to WNV influences the adaptive immune response (119, 120). Adoptive transfer of CD8\(^+\) T cells specific for immunodominant and subdominant epitopes can confer protection against WNV infection in mice (115, 116). Interestingly, epitope-specific CD8\(^+\) T cells persist in the CNS weeks after infection, which may reflect the ability of WNV to persist in the CNS (24, 121).

Older mice (18-22 months of age) have been found to be more susceptible to mortality from WNV than younger mice (4-6 months old). Similarly, T cells from older mice were unable to protect naïve mice against lethal WNV infection when compared with T cells from younger mice. This decline in protection was found to be due to defects in epitope-specific CD8\(^+\) T cell
generation, cytokine production, cytolytic activity, and trafficking of CD8$^+$ T cells to the CNS (122). However, decreased antibody and T cell responses in older mice after RepliVax vaccination did not reduce subsequent protection from a WNV infection (123).

CD4$^+$ T cells also play an important role during WNV infection. WNV specific IgG responses are blunted in mice lacking CD4$^+$ T cells, thereby resulting in lower neutralizing antibody responses compared to WT mice. CD4$^+$ T cells also help sustain primary CD8$^+$ T cell responses to WNV and the retention of CD8$^+$ T cells in the CNS. Mice lacking CD4$^+$ T cells have persistent levels of virus in the CNS and eventually succumb to the infection (124). WNV-specific CD4$^+$ T cells secrete effector cytokines and also have in vivo cytolytic activity utilizing both Fas and perforin pathways. In fact, immunization with two WNV specific CD4$^+$ T cell epitopes protected mice from WNV mortality (125). Therefore, both CD4$^+$ and CD8$^+$ T cells contribute independent functions in protecting mice from WNV infection.

Lower frequencies of CD4$^+$ CD25$^+$ FoxP3$^+$ T regulatory cells (Tregs) were found in symptomatic mice compared to asymptomatic mice. Mice succumbing to WNV infection had lower frequencies of Tregs, but higher CD8$^+$ T cell responses, than surviving mice. Mice completely devoid of Tregs have increased susceptibility compared to wild type (WT) mice, suggesting that dampening of the immune response to WNV is needed for survival (126). These results suggest that the frequencies of CD8$^+$ T cells during the course of the infection may impact
the outcome of a WNV infection, which has been suggested previously (108). Non-classical $\gamma\delta$ T cells secrete IFN-\(\gamma\) early during infection, thereby helping to control viral replication and dissemination, and mice lacking $\gamma\delta$ T cells have an increased susceptibility to WNV (127). A defect in memory CD8\(^+\) T cell generation was also seen in absence of $\gamma\delta$ T cells, due to the lack of CD4\(^+\) T cell priming by dendritic cells in mice deficient of $\gamma\delta$ T cells (128, 129).

To date, only a single CD8\(^+\) T cell epitope in the NS3 protein has been identified in BALB/c mice after JEV infection (93). However, CD4\(^+\) T cells, through induction of antibody responses, may be more important than CD8\(^+\) T cells in controlling JEV infection. In BALB/c mice, JEV-immune CD4\(^+\) T cells were better able to protect weaning mice from lethal infection compared to JEV-immune CD8\(^+\) T cells (92). Low neutralizing antibody responses during JEV infection of major histocompatibility complex (MHC) II\(^{-}\) mice resulted in increased levels of virus in the CNS during later stages of the infection. Adoptive transfer of both JEV-immune CD4\(^+\) and CD8\(^+\) T cells were required to confer protection from JEV infection in naïve mice. JEV-specific CD8\(^+\) T cells are required for viral clearance from the CNS, but susceptibility to JEV did not change if CD8\(^+\) T cells were depleted prior to infection (95). Similarly, only antibody responses after E protein vaccination could protect against JEV infection, whereas CD8\(^+\) T cell responses to the E protein alone could not protect (130). Mice lacking T cell effector functions (Fas, and perforin) were no more susceptible than WT mice (95). This is in contrast to a WNV infection in which
mice lacking both Fas and perforin were highly susceptible to infection (110, 111). Therefore, the requirement of T cells may be different for recovery from JEV infection than for recovery from WNV infection.

Of note, there are differences in the ability of mice to recover from JEV and WNV infection. Mice infected with WNV may demonstrate clinical symptoms yet recover from infection (unpublished observations) (88). In contrast, JEV-infected mice that exhibit clinical symptoms uniformly succumb to infection (unpublished observations) (95). It appears that once JEV gains access to the CNS compartment, there is no recovery from infection regardless of CD8+ T cells in the CNS. This is in contrast to WNV infection, in which CD8+ T cells help clear virus from the CNS (108-110). Viral dissemination, therefore, may be the most important factor contributing to JEV lethality.

### ii. T cell responses in humans

In humans, CD8+ T cell responses during natural WNV infection are directed against a limited number of epitopes that cover multiple WNV proteins (117, 118, 131). An immunodominant HLA-A2 restricted epitope, SVG9 (SVGGVFTSV), was identified and has been shown to confer protection in HLA-A2 transgenic mice (131, 132). Interestingly in patients with neuroinvasive disease, a higher percentage of terminally differentiated WNV-specific CD8+ T cells were found in patients with neuroinvasive disease compared to asymptomatic individuals, with little difference in overall WNV-specific CD8+ T
cell frequencies (133). These results suggest that in humans, the severity of primary infection can alter the phenotype of the CD8+ T cell population, without altering the magnitude of the response (117, 133). In addition, symptomatic WNV individuals have lower Treg frequencies compared to those who are asymptomatic, with similar findings in mice (126). Taken together, qualitative rather than quantitative epitope-specific CD8+ T cell responses may play a role for the increased susceptibility to WNV neuroinvasive disease in elderly patients, although it is likely that additional deficiencies in components of the innate and humoral immune response play a role as well.

Few studies have described epitope-specific CD4+ and CD8+ T cells in JEV-infected humans. JEV-specific CD4+ and CD8+ T cell immune responses to the NS3 protein have been detected after infection (134-136). Interestingly, NS3 specific CD8+ T cells from patients experiencing neurological symptoms due to JEV infection produced lower levels of IFN-γ than those from asymptomatic JEV patients, suggesting a possible role for IFN-γ production by T cells in the recovery from JEV infection (134). These results suggest that the immune response during a JEV infection in humans may be an important factor in determining resolution of disease or immunopathology.

iii. Antibody responses

Antibodies also play an important role during both JEV and WNV infections. Mice lacking mature B cells (µMT−/−) are highly susceptible to JEV and
WNV infection, exhibiting higher viral burdens and increased mortality compared to WT mice (88, 95). During the course of a WNV infection, WNV specific IgM is detected by day 4, while WNV specific IgG is detected by day 8 after infection (88). Interestingly, during the early stages of infection, WNV specific IgM antibodies have low levels of neutralizing activity, which is actually required for survival. However by day 10, a majority of the neutralizing antibodies are of the IgG subclass (88, 137). Similar to WNV, anti-JEV antibodies help reduce peripheral replication and viral dissemination into the CNS during infection, and passive transfer of monoclonal antibodies protects mice against a lethal JEV challenge (95, 138, 139). Human gamma globulin from WNV-immune blood donors can protect WT mice from WNV-induced lethality, but in immunodeficient mice, this protection was not sustained, suggesting that host adaptive immune response along with antibodies are required for protection (140).

Poorly neutralizing WNV antibodies can also protect against lethal WNV infection. The ability of non-neutralizing antibodies to protect against lethal infection has been seen previously during YFV infections and Sindbis virus infections (141, 142). In WNV, these non-neutralizing antibodies are primarily directed against an epitope in the fusion loop in domain II of the E protein (DII-FL) (143, 144). This epitope is normally hidden on the mature virus particle, but it may be briefly exposed during the type II fusion step, which could allow for attachment of the antibody to the virus (41). Adoptive transfer of the DII-FL
antibody mediates protection against WNV through both FcγR and complement mechanisms (144).

Interestingly, DII-FL antibodies are thought to be more cross-reactive to other flaviviruses than neutralizing antibodies specific for the domain III lateral ridge (DIII-LR) of the E protein, due to highly conserved sequences of the fusion loop (145). The DIII epitope, on the other hand, is structurally conserved but highly variable at the amino acid level (41). Recently, a broadly cross-reactive neutralizing antibody directed against the DII-FL was identified in mice suggesting that antibodies directed against the fusion loop can also have neutralizing activity (146). Antibodies have also been detected against the nonstructural proteins. Anti-NS1 antibodies recognize expression of NS1 on the cell surface of virally infected cells and trigger phagocytosis of infected cells through FcγR mediated processes, ultimately leading to clearance of infected cells, and protection from WNV infection (147, 148).

Natural infection of humans with WNV generates an antibody repertoire that is predominantly specific for DII-FL rather than the DIII-LR (149). In mice, antibodies against all three domains, DI, DII, and DIII have been detected, with the most potent neutralizing antibodies directed against the DIII-LR (41). A WNV specific IgG recognizing the DIII-LR, E16, has been developed as a potential therapeutic antibody (150). E16 strongly neutralizes WNV and inhibits viral fusion with endosomes, thus preventing viral entry into the cell (150, 151). However, the timing of antibody administration in mice was important for protection against
G. JEV cross-protection against WNV

Studies on the effect of prior flavivirus infection upon secondary infection in animals have a long history, with hamsters, bonnet macaques, birds and mice all being used to study cross-protection between flaviviruses. In 1972, Price and Thind demonstrated that hamsters immunized with any of the four dengue virus serotypes were partially protected against WNV infection. However, dengue-2 isolates demonstrated the greatest protection, and this protection was strain dependent. It was believed that this cross-protection was due to neutralizing antibody responses, but neutralizing antibodies to WNV were not detected. This conclusion was based upon the higher neutralizing antibody responses seen in dengue-2 immune hamsters after WNV infection compared to control hamsters (152).

More recently, hamsters immunized with either a live attenuated JEV strain or SLEV were protected from viremia, clinical illness and mortality following a lethal WNV challenge (summarized in Table 1.1.). However, hamsters immunized with YF17D, a distantly related flavivirus, had detectable levels of viremia and were only partially protected against a lethal WNV challenge (153). Therefore, closely related flaviviruses may be better able to protect against 2° heterologous infection compared to distantly related flaviviruses. Bonnet
<table>
<thead>
<tr>
<th>Animal</th>
<th>1&lt;sup&gt;o&lt;/sup&gt; Immunization</th>
<th>2&lt;sup&gt;o&lt;/sup&gt; Infection</th>
<th>% Survival</th>
<th>% Viremia</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td><strong>Hamster</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(153)</td>
</tr>
<tr>
<td>JEV</td>
<td>WNV</td>
<td>100%</td>
<td>N.T.&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLEV</td>
<td>WNV</td>
<td>100%</td>
<td>N.T.</td>
<td></td>
<td></td>
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<tr>
<td>YF17D</td>
<td>WNV</td>
<td>87%</td>
<td>N.T.</td>
<td></td>
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</tr>
<tr>
<td>Ch-WNV</td>
<td>JEV</td>
<td>N.T.</td>
<td>0%</td>
<td></td>
<td>(154)</td>
</tr>
<tr>
<td>Canarypox-WNV</td>
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<td>75%</td>
<td></td>
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<tr>
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<td>JEV</td>
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<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YF17D</td>
<td>JEV</td>
<td>N.T.</td>
<td>87.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bonnet Macaques</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(155)</td>
</tr>
<tr>
<td>JEV</td>
<td>WNV</td>
<td>100%</td>
<td>33%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WNV</td>
<td>JEV</td>
<td>100%&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Red-winged blackbirds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(156)</td>
</tr>
<tr>
<td>JEV</td>
<td>WNV</td>
<td>87.5%</td>
<td>N.T.</td>
<td></td>
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<tr>
<td>Ch-JEV</td>
<td>WNV</td>
<td>52.5%</td>
<td>N.T.</td>
<td></td>
<td>(Trobaugh et al., in preparation)</td>
</tr>
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<td>YF17D</td>
<td>WNV</td>
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<td>N.T.</td>
<td></td>
<td>(89)</td>
</tr>
<tr>
<td>Ch-JEV</td>
<td>MVEV</td>
<td>&gt;90%</td>
<td>N.T.</td>
<td></td>
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</tr>
<tr>
<td>JE-VAX</td>
<td>MVEV</td>
<td>&gt;90%</td>
<td>N.T.</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>MVEV</td>
<td>30%&lt;sup&gt;c&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<tr>
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<td>WNV</td>
<td>100%&lt;sup&gt;c&lt;/sup&gt;</td>
<td>N.T.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mice</strong></td>
<td>JE-VAX (one dose)</td>
<td>WNV</td>
<td>14%</td>
<td>N.T.</td>
<td>(157)</td>
</tr>
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<td>(158)</td>
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<td></td>
<td>(159)</td>
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<td>JEV</td>
<td>60%-83%</td>
<td>N.T.</td>
<td></td>
<td>(73, 74)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not Tested
<sup>b</sup> 2 out of 5 macaques demonstrated symptoms of encephalitis
<sup>c</sup> Interferon alpha receptor<sup>−/−</sup> mice (IFNαR<sup>−/−</sup>)
macaques immunized with an intranasally adapted inactivated JEV strain were also protected from mortality against WNV, with low levels of WNV viremia detected in only one JEV-immune macaque compared to higher levels in two out of three macaques of the control group. Also, only the control macaques exhibited any signs of encephalitis, suggesting JEV immunization elicited complete protection against WNV illness (155).

B6 mice immunized with the live attenuated JEV SA14-14-2 vaccine strain were fully protected against mortality from a lethal WNV challenge. In contrast with previous reports in type I IFN deficient mice, we found that immunization with Ch-JEV only partially protected against WNV in B6 mice (Trobaugh et al., in preparation) (89). In addition to protection from mortality, protection from peripheral replication was seen in JEV SA14-14-2-immune mice, but not in Ch-JEV-immune mice. However, Ch-JEV-immunization led to lower levels of WNV in the spleen compared to PBS controls following WNV challenge. Prior immunization with either Ch-JEV or JEV SA14-14-2 led to a reduction in WNV titers in the CNS, with significantly lower titers in JEV SA14-14-2 immune mice (Trobaugh et al., in preparation). These results suggest that prior immunization with JEV can protect against both peripheral replication and viral entry into the CNS, while Ch-JEV can only restrict peripheral replication of WNV, and thereby partially limit WNV dissemination to the CNS. Since Ch-JEV contains only the prM and E proteins of JEV, these results also suggest that the nonstructural
components of JEV SA14-14-2 were required for complete protection from mortality and peripheral replication (68).

Interestingly, repeated immunizations with inactivated vaccines or proteins may increase the chances of flavivirus cross-protection. One immunization with a diluted JE-VAX could not protect mice against a lethal WNV challenge; however, two immunizations with the same JE-VAX conferred partial protection (157, 158). Similarly, three booster immunizations of recombinant JEV E DIII protein partially protected against a WNV infection (159). These results suggest that when JEV E protein alone is used as the immunogen, repeated immunizations are required to generate adequate adaptive immune responses capable of eliciting cross-protection between JEV and WNV.

JEV SA14-14-2 is highly attenuated in mice, yet induces both T and B cell responses (Trobaugh et al., in preparation) (36, 62, 160). Antibody depletion of both JEV-immune CD4⁺ and CD8⁺ T cells completely abrogated protection against WNV, while depletion of only CD4⁺ or CD8⁺ T cells resulted in only a slight increase in mortality compared to JEV-immune mice (Trobaugh et al., in preparation). These results suggest that prior JEV immunity can protect against WNV infection, and both JEV-immune CD4⁺ and CD8⁺ T cells are required for this cross-protection.
H. WNV cross-protection against JEV

Few reports exist that have examined flavivirus cross-protection against JEV (summarized in Table 1.1.). Bonnet macaques immunized with WNV were protected from mortality, but 2 of 5 macaques demonstrated signs of encephalitis, suggesting that WNV infection only reduced disease severity, rather than providing complete protection (155). Recently, Bosco-Lauth et al., demonstrated that WNV- and SLEV-immunization of hamsters prevented viremia upon a JEV infection (154). However, as hamsters are not susceptible to symptomatic JEV infection, protection from mortality could not be addressed. Similarly, WNV-immune red-winged blackbirds were protected against JEV infection (156). These results demonstrate that cross-protection between JEV and WNV may be bidirectional, but that species-specific differences in this protection may exist.

Similar to JEV cross-protection against WNV, the context of the primary immunization may influence cross-protection against JEV. Hamsters immunized with Ch-WNV were protected against JEV viremia, but canarypox-WNV immunized hamsters were not protected (154, 161). Immunization with WNV E protein partially protected against a lethal JEV infection (73, 74). Similarly, immunization with a recombinant vaccinia virus (rVV) expressing the prM/E proteins of MVEV could protect against a JEV infection (162). Therefore, the context of the primary immunization may influence cross-protection between JEV and WNV.
I. Heterologous immunity and cross-reactive T cells and antibodies

Immunity to a prior viral infection can lead to protection or immunopathology due to a 2° related or unrelated viral infection, called heterologous immunity (163). Memory cross-reactive CD4⁺ and CD8⁺ T cells are thought to be the principal mediators of heterologous immunity (164). The ability of the T cell receptor (TCR) to recognize multiple epitopes is an important component of the immune system allowing finite numbers of TCRs to be able to recognize an even larger pool of epitopes (165). TCR cross-reactivity can be due to either amino acid sequence homology of the epitopes or similarities in peptide-MHC structure between epitopes with little sequence homology (166-169).

i. Cross-reactive T cell responses between unrelated pathogens

Cross-reactive T cells have been identified for both human and murine infections (163). The sequence of infection can dictate the outcome of cross-reactive responses that might occur during two viral infections. For example, viral titers of both Pichinde virus (PV) and VV were decreased in lymphocytic choriomeningitis virus (LCMV) immune mice. Also, immunization with murine cytomegalovirus (MCMV) resulted in the reduction of LCMV, PV, and VV viral titers (164, 170, 171). In cases when viral restriction was detected, a shift in the hierarchy of CD8⁺ T cell epitopes occurred in which CD8⁺ T cells specific for the cross-reactive NP205 epitope rapidly expanded to become the dominant CD8⁺ T
cell population after 2° infection (166). However, prior VV immunity did not restrict either LCMV or PV viral replication, and LCMV infection of VV-immune mice did not result in the proliferation of VV-specific CD8+ T cells (164, 170, 171).

Immunopathology can also develop after sequential viral infections depending on the viruses involved and the sequence of infection. IFN-γ production by T cells in LCMV-immune mice upon VV-infection resulted in immunopathology in multiple tissues, which was not seen in PV or MCMV-immune mice (164, 171-173). Private specificities of an individual mouse TCR repertoire also can determine the magnitude and immunopathology of cross-reactive T cell responses upon a 2° infection (173-175). The TCR Vβ repertoire may narrow after 2° infection, but TCR Vβ repertoires are still highly variable and dependent on the individual mouse (176). Cross-reactive T cells have also been identified between hepatitis C virus (HCV) and influenza, EBV and influenza, human immunodeficiency virus (HIV) and HCV, influenza serotypes, and dengue serotypes in humans (175, 177-183). In humans, cross-reactive T cells between Epstein Barr virus (EBV) and influenza have been suggested to contribute to disease severity in infectious mononucleosis due to EBV infection (167).

**ii. Cross-reactive T cells during influenza virus infections**

Cross-reactive T cells during influenza infection can lead to cross-protection or reduction in disease severity upon a 2° infection with a different influenza subtype, termed heterosubtypic immunity (184-187). Single amino acid
mutations in cross-reactive epitopes can lead to significant differences in cross-reactive CD8$^+$ T cell responses and viral clearance during the second viral infection (185, 188, 189). Therefore, sequence variation in the cross-reactive epitope between two influenza viral infections leads to CD8$^+$ T cells that have reduced functional avidities for the heterologous epitope. The CD8$^+$ T cells are not as effective in clearing a subsequent viral infection, but may help contribute to protection (185).

iii. Cross-reactive T cells during sequential flavivirus infections

Expansion of memory cross-reactive CD4$^+$ and CD8$^+$ T cells occurs in dengue immune mice following 2º heterologous infection, and is required for cross-protection between dengue virus serotypes (190-192). In humans, altered cytokine profiles and activation states of cross-reactive T cells after a primary dengue virus infection are thought to contribute to the increase in the frequency of plasma leakage seen in dengue hemorrhagic fever (DHF), after a 2º infection (183, 193-196).

In the JEV serocomplex, cross-reactive T cells have been identified in both mice and humans, but the contribution of T cells to cross-protection has not been fully addressed. JEV-immunized individuals were found to have cross-reactive CD4$^+$ T cells that responded to WNV antigen (197, 198). Cross-reactive CD8$^+$ T cells against WNV were also identified after vaccination with Ch-JEV; therefore it is likely that natural infection with JEV would also generate cross-
reactive T cells against WNV (198). Previously, our group had found that depletion of both JEV-immune CD4^+ and CD8^+ T cells prior to WNV infection resulted in an increased susceptibility to WNV, whereas depletion of only one subset did not significantly alter susceptibility (Trobaugh et al., in preparation). Although, these data suggest that JEV-immune CD4^+ and CD8^+ T cells are required for cross-protection, it does not address whether JEV immune CD4^+ and CD8^+ T cells are sufficient to protect against WNV challenge.

**iv. Cross-reactive antibodies during sequential flavivirus infections**

Cross-reactive antibody responses against secondary flaviviruses can be protective, but they can also lead to immunopathology to the second viral infection. One theory attributes the increase in immunopathology in sequential dengue virus infections to antibody-dependent enhancement (ADE) (199). ADE occurs when antibodies in the sera do not reach the stoichiometric threshold required for neutralization of the virus. The antibodies and virus are then taken up into FcγR-expressing cells, thus enhancing the infection (200). ADE has been demonstrated in some *in vivo* mouse models of both dengue virus and WNV infections, and both complement and FcγR mechanisms are required for ADE (201, 202).

The detection of cross-reactive neutralizing antibodies in humans after natural infection or vaccination is highly variable. In Mexico, the presence of dengue antibodies in the human population may be preventing infections, or at
least overt disease, from WNV, and seroconversion (203). The presence of WNV neutralizing antibodies in JEV vaccinated individuals depends on the patient cohort studied and the number of immunizations (204-206). Repeated vaccinations or infection with multiple flaviviruses increases the likelihood of detecting cross-reactive neutralizing antibodies (205, 207-209).

Similarly, in mice, repeated vaccinations of inactivated JE-VAX or JEV E protein induced low levels of neutralizing titers to WNV (74, 158, 159). Also, higher levels of virus during the primary infection may increase the frequency of cross-reactive neutralizing antibodies, as cross-reactive neutralizing antibodies to MVEV were detected after infection of IFNαR−/− mice with Ch-JEV, but not in WT mice infected with Ch-JEV. This increase was not seen after immunization with the inactivated JE-VAX, suggesting that replication of Ch-JEV in IFNαR−/− mice contributed to the increase in neutralizing antibody titers (89). Cross-reactive antibodies to nonstructural proteins can also contribute to cross-protection between WNV and JEV. Recently, a WNV anti-NS1 antibody recognized cell-surface expression of the JEV NS1 protein and protected against lethal infection if administered prior to infection (210). These results suggest that after vaccination or infection, low levels of cross-reactive neutralizing antibodies may be present that are boosted upon a second infection (209).

Adoptive transfer of JEV SA14-14-2-immune serum partially protected against WNV infection, but cross-reactive neutralizing antibodies were not detected (Trobaugh et al., in preparation). This does not rule out the presence of
low levels of cross-reactive neutralizing antibodies that could not be detected or
the presence of non-neutralizing antibodies. However, as stated earlier, non-
neutralizing antibodies can protect against a WNV infection in vivo (144). The
transfer of low doses of dengue immune antibodies can result in ADE upon a
dengue infection in mice, thus increasing disease severity (202). However, in
other flavivirus mouse models, ADE was not detected, and did not enhance T cell
responses after 2° infection (190, 198).

J. T cell responses during secondary infections

The presence of pre-existing cross-reactive T cells and antibodies may
influence secondary immune responses during heterologous infections. Factors
influencing secondary immune responses are complex and diverse, and are
initiated during the primary immune response. TCR engagement, costimulatory
signaling, and more recently, cytokine signaling are three signals required for
generation of epitope-specific CD8+ T cells from naïve T cells (211). Inflammation
during the early stages of the immune response, specifically type I IFN and IL-12,
help dictate the expansion of epitope specific CD8+ T cells, but the requirements
for these cytokines may be pathogen dependent (212-217). Recently, IL-21,
produced by CD4+ T cells during the primary immune response has also been
shown to be important for CD8+ T cells survival and memory formation (218-221).
Also, the initial antigen dose can influence both the magnitude of expansion of
CD8+ T cells and the generation of memory CD8+ T cells (222-224). Therefore,
multiple components of the primary immune response lead to the development of antigen-specific CD8$^+$ T cells, with not all viral infections having the same requirements.

CD8$^+$ T cells generated during the primary immune response have been subdivided into two populations that are influenced by IL-12, type I IFN, and IL-2 levels: short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) (217, 225-229). SLECs are effector CD8$^+$ T cells that are highly functional, and are thought to die during the contraction phase of the immune response (230). SLECs are defined as expressing high levels of killer cell lectin-like receptor G1 (KLRG1$^+$) and low levels of IL-7R$\alpha$ (CD127$^{\text{low}}$) (212). KLRG1 is actually indispensable for the generation of antigen specific CD8$^+$ T cells and is mainly used as a marker for the identification of SLECs (231). MPECs, defined as KLRG1$^-$ CD127$^{\text{hi}}$, are thought to survive the contraction phase and represent a high proportion of the memory CD8$^+$ T cells, and are capable of proliferating multiple times after antigen restimulation compared to SLECs which have very little replicative potential (230, 232). Cytokine driven expression of the transcription factors, T-bet, eomesodermin, and B lymphocyte-induced maturation protein-1 (Blimp-1), help to promote SLEC differentiation over MPEC differentiation (212, 233-236).

CD4$^+$ T cells are instrumental in the generation of CD8$^+$ T cell memory. IL-2 and IL-21 are two important cytokines that contribute to “CD4$^+$ T cell help”, and mice lacking either cytokine have defects in sustaining primary CD8$^+$ T cell
responses, memory CD8\(^+\) T cell generation, and/or recall responses during 2\(^o\) infection (124, 237-240). Also, the trafficking and maintenance of effector CD8\(^+\) T cells into peripheral tissues is reduced in mice lacking CD4\(^+\) T cells (124, 240, 241). Inflammation can also affect the contraction phase of the immune response. Low levels of both inflammation and chemokines during the primary immune response lead to reduced contraction and an increased frequency of memory T cells (121, 242, 243).

There is a theory that states that during a 2\(^o\) heterologous viral infection, memory cross-reactive T cells preferentially expand upon infection, called original antigen sin. This phenomenon has been observed during influenza, LCMV, and dengue viral infections. Low avidity cross-reactive T cells are thought to be one of the principle mediators of this so-called original antigen sin (244-246). However, recently it was demonstrated that high avidity naïve T cells can expand upon secondary heterologous infection even in the presence of low avidity memory CD8\(^+\) T cells, suggesting that the process of original antigen sin may not occur in all sequential viral infections (247).

A higher number of memory CD8\(^+\) T cells does not always necessitate improved proliferation upon a 2\(^o\) infection. Whether frequencies are low or high, memory CD8\(^+\) T cells will proliferate until a threshold is reached; however this threshold can actually change over time (224, 248, 249). Therefore, memory CD8\(^+\) T cells at lower frequencies may proliferate more than memory CD8\(^+\) T cells that start at higher frequencies (222, 224).
Higher levels of inflammation during a $2^{\text{nd}}$ infection can lead to a greater expansion of memory CD8$^+$ T cells compared to instances where there is limited inflammation. Similar to the primary immune response, the type of inflammation induced during a $2^{\text{nd}}$ infection is dictated by the pathogen and influenced by the dose of the infecting pathogen (250). Repeated antigenic stimulations can lead to better memory CD8$^+$ T cell responses and better protection (217, 248, 251-253).

As a result of all these variables, there is really no universal standard for the generation of “optimal” antigen specific CD8$^+$ T cells. While, there are underlying principles that are useful to study CD8$^+$ T cells, some of these principles cannot be broadly applied. Animal hosts have adapted immune responses specific to each pathogen, and the resultant CD8$^+$ T cell responses are highly specific, and may vary greatly from pathogen to pathogen. Many of these differences may be due to the different roles CD8$^+$ T cells play in different infections.

K. Thesis Objectives

Sequential infections with flaviviruses can lead to either cross-protection or immunopathology. This thesis sought to characterize cross-reactive CD8$^+$ T cell responses during primary and secondary JEV and WNV infections, in order to better understand the mechanisms of cross-protection between the two viruses. This work characterized the differences in primary cross-reactive CD8$^+$ T cells generated by WNV and JEV infections, and the role the specific primary
infection takes in shaping secondary immune responses to heterologous viral infections.

We initially hypothesized that:

a) JEV/WNV cross-reactive CD4\(^+\) and CD8\(^+\) T cell in JEV-infected mice preferentially expand during secondary WNV infection

b) Lack of expansion of cross-reactive CD4\(^+\) and CD8\(^+\) T cells in WNV-immune mice leads to limited protection upon secondary JEV infection

During the course of the dissertation, the hypotheses were modified to the following:

a) JEV and WNV infections induce qualitatively and quantitatively different cross-reactive CD8\(^+\) T cells during the primary immune response

b) The greater number of memory CD8\(^+\) T cells and cross-reactive antibodies following WNV infection contributes to protection upon 2\(^{\circ}\) JEV infection

c) Greater inflammation during the WNV immune response alters both generation and contraction of CD8\(^+\) T cells as well as antibody responses
This work is presented in two parts:

CHAPTER III: Altered effector functions of virus-specific and –cross-reactive CD4$^+$ and CD8$^+$ T cells in mice immunized with related flaviviruses

Questions:

a) Do cross-reactive CD8$^+$ T cell frequencies, cytokine profiles, and phenotypes differ between WNV and JEV?

b) Does the pathogenicity and infecting dose of JEV alter cross-reactive CD8$^+$ T cell responses?

c) Do differences in tissue tropism between JEV and WNV exit?

CHAPTER IV: Sequence of infection determines cross-reactive CD8$^+$ T cell responses during secondary heterologous flavivirus infection

Questions:

a) Does pre-existing WNV immunity protect against a lethal JEV infection?

b) Do cross-reactive CD8$^+$ T cells proliferate during secondary infection, and what factors contribute to or inhibit proliferation?

c) Do WNV and JEV infections induce cross-reactive neutralizing antibodies?
CHAPTER II

Materials and Methods

A. Viruses

JEV strain SA14-14-2 and chimeric-WNV (Ch-WN) was provided by Dr. Thomas Monath (Acambis, Inc.). Ch-WNV contains the prM/E proteins of WNV and the nonstructural proteins of the yellow fever (YFV) 17D virus (77). JEV strains Nakayama and Beijing were provided by Dr. Alan Barrett (University of Texas Medical Branch, Galveston, TX). WNV strain 3356 was provided by Dr. Kristen Bernard (Wadsworth Center, Albany, NY) (254). Kunjin virus was provided by Dr. Robert Tesh (University of Texas Medical Branch, Galveston, TX). Vaccinia virus (VV) WR was provided by Dr. Girish J. Kotwal (Sullivan University, Louisville, KY) and Dr. William Marshall (University of Massachusetts Medical School, Worcester MA).

B. Generation of recombinant vaccinia viruses (rVV)

The JEV S9 and WNV S9 epitope was fused to green fluorescent protein (GFP) at its N- (NJEV and NWNV) or C- (CJEV and CWNV) terminus. GFP protein cDNA from the pIRES-AcGFP1 plasmid was modified by polymerase chain reaction (PCR) using the GeneAmp XL PCR Kit (Applied Biosystems) and the primers shown in Table 2.1. The primers contain the JEV S9 and WNV S9 peptide sequences along with either Sall or HindIII restriction enzyme recognition sequences for cloning into a vaccinia virus transfer vector, pMJ601, which
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>GFPs(^a)</td>
<td>gtgcga<strong>c</strong>ATGGTAGCCAGCAAGGGCG(^b, c, d)</td>
</tr>
<tr>
<td>WNVs</td>
<td>gtgcgaccatgagctgattttggacacaccaactggc<strong>ATGGTAGCAAGGGCG</strong></td>
</tr>
<tr>
<td>JEVs</td>
<td>gtgcgaccatgagctgattttggacacaccaactggc<strong>ATGGTAGCAAGGGCG</strong></td>
</tr>
<tr>
<td>GFPas</td>
<td>aagct<strong>C</strong>ACTTGTACAGCTCATCCATGCGT</td>
</tr>
<tr>
<td>WNVas</td>
<td>aagctttcgcagtttgcgtctccaaacagagctCTTGTACAGCTCATCCATGCGT</td>
</tr>
<tr>
<td>JEVas</td>
<td>aagctttcgcagtttgcgtctccaaacagagctCTTGTACAGCTCATCCATGCGT</td>
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\(^a\) GFPs (sense) was used with WNVas (antisense) and JEVas to generate N-terminal rVVVs. WNVs and JEVs was used with GFPas to generate C-terminal rVVVs.

\(^b\) Underline and lower case letters identify restriction enzyme sequences SalI (s) and HindIII (as).

\(^c\) Upper case letters represent start (s) or end (as) of the GFP sequence. Lower case letters represent JEV S9 and WNV S9 epitope sequences.

\(^d\) Bold letters represent start and stop codons.
contains the thymidine kinase (TK) gene (Bernard Moss, National Institute of Allergy and Infectious Diseases, Bethesda MD). Following sequence confirmation, the PCR products were cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen). βgal negative colonies were screened for the PCR insert and the DNA was digested with HindIII and Sall restriction enzymes (New England BioLabs), for ligation into pMJ601, also digested with Sall and HindIII, using the Fast-Link DNA ligation kit (Epicentre, Madison, WI). Restriction digestion using Sall and HindIII was used to confirm ligation of PCR product into pMJ601, and the plasmid was prepared for transfection and homologous recombination into VV.

VV-WR (1.2 x 10^7 pfu) was added to equal volume of 0.25% Trypsin and incubated for 30 min at 37°C. The inoculum was sonicated and added to 50% confluent CV-1 cells in T-25 cm² flask for 2 hrs at 37°C. Purified plasmid DNA (5 μg) was incubated with Qiagen Superfect at room temperature for 10 min. MEM w/10% FBS was added to the DNA-Superfect mixture and was added to VV-WR infected CV-1 cells. After 2 hrs at 37°C, the cells were washed with PBS and cultured in MEM-10% FBS for two days. The cells were then harvested, suspended in 0.5ml MEM-2.5% FBS, transferred into eppendorf tubes, freeze-thawed three times, and stored at -80°C.

Next, the cell lysate (110 μl) and 0.25% Trypsin (110 μl) were incubated for 30 min at 37°C, followed by sonication for 10 sec. Serial dilutions were added in duplicate to HuTK- 143B cells (TK-) (ATCC) and incubated for 2 hrs at 37°C.
The media was removed, and 2 ml of MEM-10% FBS supplemented with 25 μg/ml Bromodeoxyuridine (BrdU) was added for 2 days. On day 2, an agarose overlay containing 2% low melting point agarose (20ml), 2X MEM (20ml), 0.33% Neutral red (0.4ml, Sigma-Aldrich), 4% X-gal (0.33ml) and BrdU (5mg/ml; 0.2ml) was added overnight. Blue plaques (5-6) were collected via a Pasteur pipette and placed into tubes with 0.5ml MEM-2.5% FBS and freeze-thawed three times. GFP expression of each blue plaque was confirmed using a fluorescent microscope (Figure 2.1.A). Three additional rounds of plaque purification were performed with 2 additional plaques selected from each purification step.

After the final purification, five plaques were sonicated and 250 μl of cell lysate plus 750 μl of MEM-2.5% was added to one well of TK− cells in a 12 well plate to amplify the plaque. After 2 hrs at 37°C, the media was removed, and MEM-2.5% FBS + BrdU was added for 2 days. The cells were collected and freeze-thawed three times in MEM-2.5% FBS. This amplification step was repeated using the 12-well plate lysate (250 μl) to inoculate TK− cells in a T-25 cm² flask, and using the T-25 cm² lysate (250 μl) to inoculate TK− cells in a T-175 cm² flask. Mice were then inoculated with the viral lysates (1 x 10⁶ pfu) to determine whether the recombinant vaccinia viruses (rVV) induced a CD8⁺ T cell response to the inserted epitope. Only the rVV's expressing the epitopes at the C-terminal of GFP induced epitope-specific CD8⁺ T cells that responded to both JEV S9 and WNV S9 peptides. The immunodominant VV epitope, B8R, was used to confirm functionality of the rVV (Figure 2.1.B.).
Figure 2.1. Confirmation of GFP expression and epitope insertion into rVV.

(A) GFP expression of rVV. TK− cells were infected with each of the rVV and, GFP expression was determined on day 2 after infection. (B) CD8+ T cell responses to the JEV S9, WNV S9, and VV B8R epitopes. B6 mice were infected with 1x 10^6 pfu of the rVV ip, and on day 7 after infection, splenocytes were analyzed for IFN-γ and TNF-α production following stimulation with 1 µg/ml JEV S9, WNV S9 or B8R peptide or media alone.
One virus was then selected to be propagated using CV-1 cells to make viral stocks. Ten CV-1 T-175 cm² flasks were infected with 5 ml of viral inoculum (100 µl virus + 50 ml MEM w/o FBS) for 2 hrs at 37°C. The supernatant was aspirated and 20 ml of MEM-2.5% FBS was added overnight. The cells were harvested the following day due to high levels of CPE. Cells from all the flasks were combined, freeze-thawed three times and sonicated. Following an additional round of centrifugation, aliquots of the supernatant were stored at -80°C.

C. Propagation of virus stocks

Mycoplasma free Vero cells (flaviviruses) or CV-1 cells (rVV) were grown to confluency in T-175 cm² flask in minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (Pen/Strep) and 1% L-Glutamine. Prior to infection, media was removed and cells were washed with phosphate buffered saline (PBS). Virus (500 µl, flaviviruses; 100 µl rVV) was added to 5 ml of MEM without (w/o) FBS and added to the cells for 1.5 hrs at 37°C w/5% CO₂. After infection, the inoculum was aspirated and 20 ml of MEM supplemented with 2.5% FBS, 1% Pen/Strep, and 1% L-Glutamine was added. Cells were monitored daily until 30-50% of the cells exhibited cytopathic effects (CPE), consisting of rounding of the cells and disruption of the cell monolayer. CPE occurred 2-3 days after infection for WNV and Kunjin, 4-5 days for JEV SA14-14-2 and Beijing strains, and 1 day for the rVVs. For flavivirus propagation,
the cells and media were collected and spun at 2000 rpm for 10 min at 4°C. The supernatant was then aliquoted and stored at -80°C. For the rVVVs, the cells and media were freeze-thawed 3x, and sonicated for 10 seconds twice. The cells and media were then spun at 2000 rpm for 10 min at 4°C, and aliquoted for storage at -80°C. All viruses were titrated on Vero cells using a standard plaque assay as described later.

D. Peptides

Peptide (15-19mer) arrays corresponding to the entire proteome of WNV were obtained through the Biodefense and Emerging Infections Research Resources Repository, National Institute for Allergy and Infectious Diseases (NIAID), National Institute of Health (NIH) (BEI Resources, Manassas, VA). Peptide truncations (>70% or >90% purity) were obtained from AnaSpec, Inc. (San Jose, CA) and 21st Century Biochemicals (Marlborough, MA).

E. Mice and Immunizations

Male or female C57BL/6J (B6) (Ly5.2⁺), B6.SJL-Ptprc Pep3/BoyJ (Ly5.1⁺) mice aged 6-14 weeks (Jackson Laboratories, Bar Harbor, ME), C57BL/6Ji-Kbtm1 N12 (H2-Kb⁻), C57BL/6Ji-Dbtm1 N12 (H2-Db⁻) mice (Taconic Farms, Germantown, NY) were housed in specific pathogen-free conditions in the University of Massachusetts Medical School Biocontainment facility and were cared for according to guidelines approved by the University of Massachusetts
Medical School's Institute Animal Care and Use Committee. All mice were infected via an intraperitoneal (ip) injection with varying doses of virus. Mice were weighed and monitored daily for clinical symptoms, and euthanized once weight loss exceeded 25%. Clinical symptoms for both JEV and WNV include weight loss, hunched posture, ruffled fur, and hind limb paralysis.

F. Isolation of Lymphocytes

i. Splenocytes

Spleens were harvested and placed in Roswell Park Memorial Institute-1640 (RPMI) media supplemented with 10% FBS. The spleens were transferred onto a 70 µm cell strainer and mashed with the blunt end of a 3 ml syringe, washed with RMPI-10% FBS, and spun at 1500 rpm for 5 min. The supernatant was decanted and red blood cell lysis buffer (1 ml; Sigma-Aldrich) was added for 5 min. The cells were washed, and resuspended in RPMI-10% FBS at the desired concentration.

ii. Central Nervous System (CNS) T cells

Lymphocytes were obtained from brains of WNV infected mice on day 10 or 4-5 weeks post infection. Mice were perfused with PBS via a cardiac puncture to eliminate circulating T cells in the blood, and brains were harvested and placed in RPMI-10% FBS. Brains were homogenized on a 100 µm cell strainer and washed with RPMI. Cells were then resuspended in RMPI-10% FBS and
Collagenase D (Roche; 100 µg/ml; final concentration 500 ng/ml) was added for 45 min at 37°C. Lymphocytes were isolated by density centrifugation in a 30%/70% Percoll gradient at 800g for 30 min at 4°C. Lymphocytes at the interface of the two layers were collected, washed, and counted for use.

G. Generation of bulk culture cell lines

Splenocytes were harvested one week following JEV boost, and stimulated with 10 µg/ml JEV NS4b C peptide (GASSVWNATTAIGL) in RPMI containing 10% FBS, 1% Pen/Strep, 5x10^{-5} M β-mercaptoethanol and recombinant human IL-2 (rhIL-2; BD Biosciences) (25 U/ml) at 37°C. At day 14 and every 14 days thereafter, γ-irradiated naïve B6 splenocytes were pulsed with 10 µg/ml peptide, washed, and added to the bulk cultures at a stimulator-to-responder ratio of 5:1.

H. ^{51}Chromium release assay

^{51}Chromium release assay were performed as previously described (255). In brief, ^{51}Cr-labelled EL4 cells (ATCC) were incubated with peptide or media alone. Effector cells were added in triplicate and incubated for 4 hours at 37°C. Renex or media alone were added to target cells for determination of maximum target cell lysis and spontaneous lysis, respectively. Supernatants were harvested and counted on an automated gamma counter. Percent specific lysis
was calculated as \([(\text{sample } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})/(\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})]\) x 100.

I. IFN-γ ELISPOT

ELISPOT assays were performed as described previously (255). Freshly isolated day 7 splenocytes from 2 naïve or JEV-infected mice were pooled and plated on anti-mouse IFN-γ coated 96-well plates in duplicate or triplicate (2.5 x 10^5 per well) and stimulated with WNV or JEV peptides (2 µg/ml), Con A (2.5 µg/ml), or media overnight at 37°C. After PBS wash, anti-mouse IFN-γ biotinylated antibody was added for 2 hours followed by streptavidin-HR. Spots were developed with NovaRed substrate kit (Vector Laboratories, Burlingame, CA) and counted with a C.T.L. reader (Cellular Technology Limited). The number of spot forming cells per million was calculated as \([(\text{mean spots in experimental wells} - \text{mean spots in medium control}) \times 4] \times 10^6\). The average number of spot forming cells per million in media alone was 21 ± 22. A positive response was ≥2 times media background.

J. Intracellular cytokine staining (ICS)

i. Splenocytes

Splenocytes (1-2x10^6 cells) were stimulated either with peptide (10^{-6} – 10^{-13} µg/ml), peptide pools (5 µg/ml), phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (250 ng/ml) (positive control) or without peptide (negative
control) in the presence of brefeldin A (BD GolgiPlug) for 5 hours. For some experiments, cells were washed with PBS and stained with Live/Dead Aqua (Invitrogen), a viability marker, for 20 min at RT for exclusion of dead cells during analysis. Cells were then washed in PBS supplemented with 2% FBS and 0.05% Sodium azide (FACS WB) and incubated with 1 µg anti-CD16/32 (2.4G2). Cells were surface stained with anti-CD3 (clone 145-2C11), -CD4 (L3T4), -CD8 (Ly-2) -Ly5.1 (A20), or -Ly5.2 (104). Antibodies were conjugated to APC, PE, PerCP-Cy5.5, PerCP, Pacific Blue, Alexa 700, or FITC. After permeabilization with BD CytoFix/CytoPerm, cells were stained with anti-IFN-γ (XMG1.2), -TNF-α (MP6-X522) and fixed in 1% paraformaldehyde or 1:3 dilution of BD Stabilizing fixative. Samples were acquired on a FACSCalibur or FACSria (BD Biosciences) and data were analyzed using FloJo software (Tree Star, Inc.). EC50 values were calculated using GraphPad Prism. The percentage of CD4+ or CD8+ T cells producing IFN-γ in response to media was subtracted from peptide-stimulated cells. Antibodies were obtained from either BD Bioscience or eBiosciences.

**ii. TCR Vβ expression**

Splenocytes were stimulated with either 1 µg/ml JEV S9 or WNV S9 peptide as described above. Following incubation, cells were stained with anti-CD3-Pacific Blue, anti-CD8-APC-H7, and a mouse TCR Vβ screening panel (BD Biosciences) according to manufacture guidelines. An IgG-FITC isotype control (BD Biosciences) was used to determine background staining. Following
permeabilization, cells were stained with anti-IFN-γ APC. Cells were gated on CD3+ CD8+ IFN-γ+ TCR Vβ+. The background level of staining with the isotype control was subtracted from each sample. Since the TCR Vβ panel did not include the entire mouse TCR Vβs, the total percentage of TCR Vβ in the population was subtracted from 100% to give the percentage of the TCR Vβs in population not included in the panel (labeled other in graph).

K. In vivo cytotoxicity experiments

i. Primary infection experiments:

In vivo cytotoxicity experiments were performed as described with modifications (256). Naive splenocytes (target cells) were pulsed with 1, 0.1 or 0.01 mg/ml of JEV S9, WNV S9 peptide or control influenza NP 366-374 peptide (1 mg/ml) for 45 min at 37°C. Cells were stained with 1 µM Cell Trace Far Red DDAO-SE (Invitrogen, Carlsbad, CA) and serial dilutions of 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (5 µM, 1.5 µM, 0.4 mM, 0.1 mM; Invitrogen). Target cells in PBS (2 x 10⁷ cells in 200 µl) were injected intravenously (iv) into JEV-infected or naïve mice 8 days post infection. Splenocytes were harvested 2 hours later and analyzed using a FACS Aria. DDAO+ donor cells were analyzed for CFSE expression.
ii. Memory experiments:

Naïve Ly5.1+ splenocytes were pulsed with 1 µg/ml JEV S9, WNV S9, or Flu NP 366-374 for 1.5 hrs at 37°C and stained with CFSE as described above. Target cells (1-2 x 10⁷ cells in 200 µl) were injected iv into WNV-immune (4-5 weeks post infection), JEV-immune (4-5 weeks post infection), or naïve B6 mice. Splenocytes were harvested 19 hrs later and stained with anti-Ly5.1-Alexa700, and -Ly5.2-PE antibodies. Ly5.1+ donor cells were analyzed for CFSE expression. Percent specific lysis was calculated by the formula 1-(Ratio Immune/Ratio Naïve) x 100, where Ratio=(# events of JEV or WNV peptide/# events of control influenza peptide).

L. Phenotype analysis of epitope-specific CD8⁺ T cells

CD8⁺ T cells were stained with either peptide-loaded MHC dimer or tetramer. To generate peptide-loaded MHC dimers, recombinant H-2Dᵇ:lg fusion protein (4µg; BD Biosciences) was loaded with peptide (>90% purity) at 640 molar excess peptide in PBS (pH=7.2) at 37°C overnight according to manufacturers guidelines. Peptide-loaded dimer was then incubated with 2.4 µg APC-anti-mouse IgG (BD Biosciences, mAb A85-1) followed by incubation with purified mouse IgG isotype control (4µg; BD Biosciences; mAb A111-3). APC labeled JEV S9 and WNV S9 tetramer was obtained from the NIH Tetramer Core Facility.
Splenocytes were resuspended in PBS, stained with Live/Dead Aqua, and incubated with anti-CD16/32 (2.4G2; BD Bioscience). For staining of peripheral blood lymphocytes (PBL), red blood cells were lysed with Sigma Lysis buffer (Sigma) or BD PharmLyse (BD Biosciences), followed by incubation with anti-CD16/32 (2.4G2). Cells were then stained with optimal amounts of tetramer (0.5 µl or 0.75 µl), or 20 µl of peptide-loaded dimer for 30 min at 4°C. Cells were surface stained with anti-CD44, -CD62L, -KLRG1, -CD127, -PD-1 (CD279, RMP1-14 clone, eBioscience), or -CD43 (1B11, BioLegend) conjugated with FITC, PE-Cy7, PerCP-Cy5.5, or PE, washed and resuspended in BD Stabilizing Buffer. Peptide-loaded dimer and tetramer staining levels in naïve mice were subtracted from experimental values in infected mice. For CD8+ T cells in the CNS, anti-CD45-eFluor 450 (30-F11, eBioscience) was also used to help distinguish CD3+ CD8+ T cells.

M. Plaque assay of infected tissues

i. Flaviviruses

On days 3 and 7 post JEV or WNV infection, spleen, brain and serum were obtained and frozen on dry ice and stored at -80°C. Tissues were resuspended in MEM w/o FBS to give a 10% (spleen) or 20% (brain) homogenate based on tissue weight, and homogenized using a Qiagen mixer mill. Serial dilutions were made in MEM and titers were determined on Vero cells as described (84). Plates were incubated for 2 (WNV) or 4 days (JEV Beijing and
SA14-14-2) prior to second agar overlay containing neutral red (Sigma). The limit of detection was 50 pfu/ml for serum, 250 pfu/g for brain and 500 pfu/g for spleen.

ii. Recombinant vaccinia viruses

Ovaries were harvested on day 5 or 6 post infection, weighed, frozen on dry ice, and stored at -80°C until use. MEM w/o FBS was added to the ovaries to make a 10% homogenate, and ovaries were freeze-thawed three times. Ovaries were then homogenized using a Qiagen Mixer-Mill, and serial dilutions were made in MEM w/o FBS. Titers were determined on Vero cells using 100 µl of virus inoculum in duplicate. After 2 hours of incubation, MEM w/2.5%FBS was added for two days, followed by the addition of crystal violet in 10% ethanol to fix the cells. The cells were then washed with tap water, and allowed to air dry prior to counting the plaques. The limit of detection was 500 pfu/g for the ovaries.

N: Adoptive transfer experiments

i. JEV-immune CD4⁺ and CD8⁺ T cell cross-protection experiment

B6 mice (5-6 weeks old) were immunized with 1 x 10⁶ PFU JEV SA14-14-2 or were unimmunized. Six weeks later, splenocytes were processed, and separated into JEV-immune or naïve CD4⁺ and CD8⁺ T cell subsets by negative selection using AutoMACS according to the manufacturer’s recommendations (Miltenyi Biotech). After isolation of CD4⁺ and CD8⁺ T cell subsets, 1 x 10⁶ cells
of each fraction were combined and administered iv into 9-10 week old mice using the following combinations: naïve CD4⁺ and JEV-immune CD8⁺; JEV-immune CD4⁺ and naïve CD8⁺; JEV-immune CD4⁺ and JEV-immune CD8⁺, and naïve CD4⁺ and naïve CD8⁺. In addition, groups of mice that were immunized 4 weeks previously with JEV, Ch-WN or PBS were used as positive and negative controls, respectively. Twenty-four hours after cell transfer, mice were infected with 1.26 x 10⁶ pfu (100LD₅₀) of WNV and monitored for survival.

_ii. CFSE proliferation_

Splenocytes from JEV and WNV immune mice (Ly5.2⁺), 4-5 weeks post infection, were washed with PBS and stained with 1 μM CFSE for 15-20 min at 37°C. An aliquot of cells was taken prior to staining with CFSE for ICS or tetramer analysis to determine frequency of JEV S9 and WNV S9 CD8⁺ T cells prior to adoptive transfer. Following incubation, splenocytes were washed and resuspended in PBS to a concentration of 1-2 x 10⁷ cells per 200 μl. The CFSE labeled splenocytes were transferred iv via the lateral tail vein into three naïve B6.SJL (Ly5.1⁺) mice per donor mouse. The next day, the mice were infected ip with either 1 x 10³ pfu JEV Beijing, 1 x 10³ pfu WNV or PBS (control). Five days later, splenocytes were harvested for ICS to measure CFSE dilution.
**O: MHC stabilization assay**

RMA-S cells were cultured in suspension with RPMI supplemented with 10% FBS, 1% Pen/Strep and 1% L-Glutamine at a density of 1 x 10^6 cells/ml. Cells (4 x 10^5) were plated in 96-well V-bottom plates on day of assay. JEV S9, JEV P6A, WNV S9 and WNV P6S peptides or no peptide (media alone) were added to the cells at a final concentration of 10^{-4} g/ml – 10^{-8} g/ml, and incubated overnight at 28°C. The following day, the cells were transferred to 37°C for 3 hours. The cells were then stained with anti-H-2Db-PE antibody, and fixed with BD Cytofix (BD Biosciences). Background H-2Db expression (no peptide) was subtracted from the experimental samples.

**P: Generation of bone marrow derived dendritic cells (BMDCs)**

Tibia and femur from B6.SJL male mice were flushed with a 25 gauge needle to remove the bone marrow. The bone marrow was then filtered using a 70 μm cell strainer and resuspended in 9 ml/mouse (3 ml/well) of RPMI-10% FBS, 1% Pen/Strep, 1% L-glutamine, 50 μM 2-mercaptoethanol, 20 ng/ml interleukin-2 (IL-2) (Perprotech) and 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (Perprotech). Half of the media was exchanged every two days with fresh media. On day 6, 100 ng/ml of lipopolysaccharide (LPS) was added to mature the dendritic cells overnight. The supernatants were collected and the wells were washed with cold PBS to recover any residual BMDCs. The BMDCs were then washed with PBS to remove any LPS. A small
aliquot was stained with anti-CD11b, anti-CD11c, anti-CD80 and anti-CD86 to check for successful generation and maturation of the BMDCs.

**Q: Peptide-pulse and DC immunization**

On day 7 after BMDCs maturation, 10 \( \mu \text{g/ml} \) of peptide was added for 1.5 hrs at 37°C. The cells were washed with PBS, counted, and resuspended in cold PBS to achieve a concentration between 0.5 – 1 \( \times 10^6 \) cells/ 200 \( \mu \text{l} \). BMDCs were transferred iv (200 \( \mu \text{l} \)) into male B6.SJL recipients. On day 7 or 4-6 weeks after DC immunization, PBL were stained and analyzed for JEV S9-specific and WNV S9-specific CD8\(^+\) T cell frequencies as described above. Mice were then infected with 1 \( \times 10^3 \) pfu JEV Beijing or 1 \( \times 10^3 \) pfu WNV, and 5 days later, the PBL was analyzed for JEV S9-specific and WNV S9-specific CD8\(^+\) T cell frequencies.

**R: Statistics**

Means, medians and standard errors were calculated using GraphPad Prism (GraphPad Software, Inc., LaJolla, CA). Comparisons of variables between JEV and WNV infection groups were performed with log transformed data using the Mann-Whitney U test on STATA software (StataCorp, College Station, TX) or GraphPad Prism. P < 0.05 was considered significant.
CHAPTER III
ALTERED EFFECTOR FUNCTIONS OF VIRUS-SPECIFIC AND –CROSS-
REACTIVE CD4⁺ AND CD8⁺ T CELLS IN MICE IMMUNIZED WITH RELATED
FLAVIVIRUSES

Previous reports have demonstrated that JEV-immunized bonnet
macaques and hamsters were fully protected against a lethal WNV challenge
(153, 155). WNV-immune macaques, on the other hand, were only partially
protected against a lethal JEV challenge, suggesting a one-way direction in
cross-protection between JEV and WNV (155). Recently, Bosco-Lauth et al.
demonstrated that WNV immunization of hamsters could protect them from JEV
viremia, suggesting WNV immunity may protect from JEV infection in certain
animal models (154). While these studies demonstrate that prior JEV or WNV
immunity can protect against a second heterologous infection, underlying
mechanisms for this cross-protection were not addressed.

Previous work in our group has demonstrated that JEV-specific CD4⁺ and
CD8⁺ T cells are required for cross-protection against WNV (Trobaugh et al., in
preparation). Cross-reactive CD4⁺ and CD8⁺ T cell epitopes between JEV and
WNV in BALB/c mice and humans have been identified (91, 197, 257). However,
very little work had been done to understand cross-reactive T cell responses
between JEV and WNV in B6 mice. Therefore, the focus of this chapter is on the
identification and characterization of JEV-WNV cross-reactive CD4+ and CD8+ T cell responses during primary JEV and WNV infections in B6 mice.

A. Identification of JEV-WNV cross-reactive CD4+ and CD8+ T cell epitopes.

To determine the overall JEV-WNV cross-reactive response, day 7 splenocytes from JEV SA14-14-2 infected mice were stimulated with overlapping peptide pools corresponding to each of the 10 WNV proteins. We found that the total JEV-WNV cross-reactive CD4+ T cell IFN-γ responses were mainly directed at peptides in the NS4b, NS2a, and E proteins (Table 3.1 and Figure 3.1). In contrast, the majority of the JEV-WNV cross-reactive IFN-γ producing CD8+ T cells were induced by a single peptide pool corresponding to the WNV NS4b protein. These results suggest that the overall cross-reactive CD4+ T cell response is broad due to responses in multiple proteins, while the overall cross-reactive CD8+ T cell response is limited due to a single protein inducing a majority of the IFN-γ response.

We next deconvoluted the positive peptide pools to identify the specific peptides that induced an IFN-γ response by ELISPOT. We consistently found that three peptides, WNV NS1 A, WNV NS3 B and WNV NS4b209-226, induced the highest IFN-γ response from splenocytes harvested from JEV-infected mice (Table 3.2). Both WNV NS3 B and WNV NS4b209-226 contain previously identified CD4+ and CD8+ T cell epitopes in WNV-infected B6 mice, respectively (115, 116, 125). To identify the optimal cross-reactive epitopes, we generated truncations of
Table 3.1. Frequency of IFN-γ⁺ CD4⁺ and CD8⁺ T cells in response to stimulation with WNV peptide pools corresponding to the entire WNV proteome. Splenocytes from JEV-infected mice were stimulated with pools of overlapping peptides corresponding to each of 10 WNV proteins in the presence of brefeldin A for 5 hours, permeabilized and stained for surface markers and intracellular IFN-γ production by CD4⁺ and CD8⁺ T cells as described in Materials and Methods

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<th>Protein</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
<th>% IFN-γ⁺ producing CD8⁺ T cells</th>
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<td>Total</td>
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<td>2.92</td>
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a % IFN-γ⁺ CD4⁺ or CD8⁺ T cells. Background staining was subtracted from each peptide pool, and % IFN-γ from each peptide pool was added together.

b Total % IFN-γ⁺ from all the peptides combined.
Figure 3.1. Global JEV/WNV cross-reactive CD4$^+$ and CD8$^+$ T cell response in JEV SA14-14-2 infected mice in response to WNV peptide pools.

Splenocytes were harvested 7 days post-infection and IFN-$\gamma$ production was measured in 3 separate experiments. Mean values for percentages of CD3$^+$ CD4$^+$ (left) or CD3$^+$ CD8$^+$ (right) T cells producing IFN-$\gamma$ in response to peptides from a single protein were calculated and are represented as the percent of the total IFN-$\gamma$ response calculated as follows: $\left(\text{mean CD4}^+ \text{ or CD8}^+ \text{ IFN-} \gamma^+ \text{ percent per protein/total CD4}^+ \text{ or CD8}^+ \text{ IFN-} \gamma^+ \text{ response to all 10 WNV proteins}\right) \times 100$. Raw experimental values from each experiment are displayed in Table 3.1. C= capsid, prM/M = pre-membrane, membrane, E = envelope, NS=nonstructural.
each potential cross-reactive peptide. The sequences of each WNV truncation and the corresponding sequences in JEV are shown in Table 3.2.

To determine the MHC restriction of the potential cross-reactive epitopes, mice lacking the MHC molecules H-2D$^b$ or H-2K$^b$ were infected with JEV SA14-14-2, and IFN–γ responses to each of the truncations were determined by ELISPOT. WNV NS1 A and WNV NS3 B and their corresponding truncations induced IFN–γ production by splenocytes from both H-2D$^{b/-}$ and H-2K$^{b/-}$ mice (Table 3.2), with WNV NS1 A-1 and WNV NS3 B-2 inducing the highest IFN–γ response suggesting that these two truncations were the optimal cross-reactive epitope. These results also suggested that WNV NS1 A-1 and WNV NS3 B-2 were not CD8$^+$ T cell epitopes, but perhaps CD4$^+$ T cell epitopes due to similar IFN–γ responses in B6, H-2D$^{b/-}$ and H-2K$^{b/-}$ mice, demonstrating that IFN–γ responses were not reduced in mice lacking the MHC required for CD8$^+$ T cell activation. To confirm that WNV NS1 A-1 and WNV NS3 B-2 were cross-reactive CD4$^+$ T cell epitopes, we performed intracellular cytokine staining (ICS) on splenocytes from JEV-infected mice. We detected IFN–γ responses to the cross-reactive epitopes (WNV NS1 A-1 and WNV NS3 B-2) and their corresponding JEV sequences (JEV NS1 A-1 and JEV NS3 B-2) in only CD4$^+$ T cells (Figure 3.2). In fact, the JEV NS1 A-1 and NS3 B-2 epitopes are the first JEV-specific CD4$^+$ T cell epitopes identified in B6 mice.

Stimulation of splenocytes from JEV SA14-14-2 infected mice with WNV NS4b$^{209-226}$ and its truncations induced IFN–γ responses in H-2K$^{b/-}$ mice but not
Table 3.2. IFN-γ production induced by peptide stimulation of JEV-infected splenocytes from B6, H2-D\textsuperscript{b/-} and H2-K\textsuperscript{b/-} mice in ELISPOT assay. Mice were infected with JEV SA14-14-2 and splenocytes were prepared on day 7.

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<th>Peptide name</th>
<th>Amino Acid #</th>
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<th>D\textsuperscript{b/-}</th>
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\textsuperscript{a} WNV 3356 GenBank Accession number AF404756
\textsuperscript{b} Numbers represent amino acid position within each protein
\textsuperscript{c} IFN-γ\textsuperscript{+} spot forming cells/million splenocytes
\textsuperscript{d} JEV GenBank Accession number AF315119 (JEV SA14-14-2) and L48961 (JEV Beijing)
\textsuperscript{e} Not tested
\textsuperscript{f} G9 represents amino acid in position 1 (G) followed by peptide length (9).
Figure 3.2. Identification of cross-reactive CD4$^+$ T cell epitopes in JEV SA14-14-2 infected mice. B6 mice were infected ip with $1 \times 10^6$ pfu of JEV SA14-14-2, and on day 7, splenocytes were harvested and stimulated with 1 $\mu$g/ml of peptide for 5 hours. Cells were gated on CD3$^+$CD4$^+$ T cells (upper panels) or CD3$^+$CD8$^+$ (lower panels) with IFN-\( \gamma \) displayed on the y-axis.
from H-2D<sup>b/-</sup> mice confirming H-2D<sup>b</sup> restriction (Table 3.2). The truncations, WNV NS4b A10 and WNV NS4b S9, were both previously identified to be the proposed optimal WNV immunodominant epitope (115, 116). Therefore, in an effort to identify the optimal cross-reactive epitope, bulk cultures from JEV SA14-14-2 immunized mice were generated using WNV NS4b C as the stimulating peptide (Table 3.2). Ex vivo CTL analysis confirmed that WNV NS4b S9 (WNV S9) was the optimal cross-reactive epitope due to a higher percent specific lysis at the lowest concentration tested (Figure 3.3.A.). Additionally, only the S9 variant peptides and not WNV NS4b A10 bound to an unloaded MHC-dimer complex and stained CD8<sup>+</sup> T cells from JEV and WNV-infected mice (Figure 3.3.B.).

In order to determine whether the cross-reactive WNV S9 epitope was recognized in vivo, we performed an in vivo cytotoxicity assay during acute JEV SA14-14-2 infection. Naïve splenocytes pulsed with decreasing doses of JEV NS4b S9 (JEV S9) were all lysed to a similar extent in JEV-infected mice, with a mean percent specific lysis 44.3%, 38.5%, and 43.5% for peptide doses 10<sup>-6</sup>, 10<sup>-7</sup> and 10<sup>-8</sup> µg/ml, respectively (Figure 3.4.A-B.). In contrast, the mean percent specific lysis of WNV S9-pulsed target cells was consistently lower than that seen for the JEV S9 variant at all peptide doses (10<sup>-6</sup>: 34.0%; 10<sup>-7</sup> 33.4%; 10<sup>-8</sup>: 28.0%). Splenocytes pulsed with an H-2D<sup>b</sup> restricted influenza NP epitope were not lysed in JEV-infected or naïve mice, confirming specificity of the response to the JEV and WNV epitopes (Figure 3.4.A.). These findings support that the
Figure 3.3. Identification of the optimal JEV-WNV cross-reactive CD8+ T cell epitope. (A) Cytotoxic CD8+ T cell lines were generated from JEV-immunized splenocytes that were stimulated in vitro with peptide JEV NS4b C. EL-4 targets cells were pulsed with indicated concentrations of JEV S9, WNV S9, and WNV S10 variant peptides, and added to effector cells at E:T ratio of 15:1. Percent specific lysis was calculated as described in Materials and Methods. (B) Peptide-loaded dimer staining of splenocytes from JEV (left panel) and WNV (right panel) -infected mice. Splenocytes were stained with decreasing doses of H-2Db dimer loaded with WNV S10, WNV S9 and JEV S9. Percent dimer+ CD3+ CD8+ T cells are shown on the y-axis.
Figure 3.4. Characterization of CD8⁺ T cell effector responses to the JEV S9 and WNV S9 peptides in JEV- and WNV-infected mice. (A) *In vivo* cytotoxic activity was measured in a naïve mouse (left panels) or 8 days after JEV-infection (right panels). DDAO and CFSE-labeled target cells pulsed with 10⁰-10⁻² μg/ml of JEV S9 (top panels) or WNV S9 (bottom panels) were injected iv and spleens were harvested 2 hours later. Target cells pulsed with 1μg/ml of influenza NP 366-374 peptide (Flu) serve as a negative control. CFSE is shown on x-axis. Percent specific lysis values are indicated on top of each peak and were calculated as described in Materials and Methods. (B) Summary of *in vivo*
lysis of peptide-pulsed target cells in JEV-infected mice. JEV S9-pulsed target cells (●); WNV S9-pulsed target cells (○). Horizontal bars represent median specific lysis. Flu peptide and unpulsed target cells are not shown. Data are compiled from 2 independent experiments (n=2-3 mice per group). (C-E) Cytokine profiles of virus-specific and cross-reactive CD8+ T cells during WNV and JEV infections. (C) CD8+ T cells from JEV- and WNV-infected mice were analyzed for their ability to produce IFN-γ and TNF-α after stimulation with 1 μg/ml JEV S9 and WNV S9. Values represent the percentage of IFN-γ+, TNF-α+ and IFN-γ– TNF-α+ CD8+ T cells. Representative data for one mouse per group immunized with JEV SA14-14-2 (top row), 1 x 10^3 pfu JEV Beijing (second row), 1 x 10^6 pfu JEV Beijing (third row) or WNV (bottom row). (D) Cumulative data (3 experiments, 2 spleens pooled per experiment). Bars represent mean ± SEM of the percent of CD8+ T cells producing IFN-γ, TNF-α or both IFN-γ and TNF-α from mice infected with JEV or WNV following stimulation with JEV S9 (black bar) or WNV S9 (white bar). (E) Ratios of IFN-γ+ CD8+ T cells to IFN-γ+ TNF-α+ CD8+ T cells from JEV- and WNV-infected mice upon stimulation with JEV S9 (black bar) and WNV S9 (white bar) peptides. *P<0.05 between WNV-infected group and all JEV groups, Mann-Whitney U test.
JEV S9 epitope and the cross-reactive WNV S9 variant is a JEV-WNV cross-reactive CD8\(^+\) T cell epitope that is recognized both \textit{in vitro} and \textit{in vivo}.

**B. CD8\(^+\) T cell cytokine profiles depend upon the infecting flavivirus.**

We next examined the frequency of CD8\(^+\) T cells that secrete both IFN-\(\gamma\) and TNF-\(\alpha\) in the context of the specific stimulating variant as well as the infecting virus (JEV vs. WNV), in order to determine the contribution of each factor to CD8\(^+\) T cell cytokine profiles. In both JEV SA-14-14-2 and WNV-infected mice, we found that stimulation by either the JEV S9 or WNV S9 variant induced both IFN-\(\gamma^+\) and IFN-\(\gamma^+\) TNF-\(\alpha^+\) CD8\(^+\) T cells, while TNF-\(\alpha^+\) CD8\(^+\) T cells were not detected in either JEV SA14-14-2- or WNV-infected mice (Figure 3.4.C.-D.). In JEV SA14-14-2-infected mice, stimulation with the JEV S9 or WNV S9 peptides induced a higher frequency of single positive IFN-\(\gamma^+\) CD8\(^+\) T cells compared to double positive IFN-\(\gamma^+\) TNF-\(\alpha^+\) CD8\(^+\) T cells. In contrast, in WNV-infected mice, stimulation with either variant induced a higher frequency of double positive IFN-\(\gamma^+\) TNF-\(\alpha^+\) CD8\(^+\) T cells. The ratio of the frequencies of IFN-\(\gamma^+\) CD8\(^+\) T cells to IFN-\(\gamma^+\) TNF-\(\alpha^+\) CD8\(^+\) T cells was significantly higher after JEV SA14-14-2 infection compared to WNV infection for JEV S9 and WNV S9 (\(P<0.05\), Mann-Whitney U) (Figure 3.4.E.). No significant difference in this ratio was detected between the JEV S9 and WNV S9 variants when compared within the same infection. Interestingly, IFN-\(\gamma^+\) TNF-\(\alpha^+\) CD8\(^+\) T cells from WNV-infected mice produced more TNF-\(\alpha\) on a per cells basis than those from JEV SA14-14-2
infected mice, while levels of IFN-γ in this population were similar for JEV and WNV (Table 3.3.).

Since JEV SA14-14-2 is an attenuated virus, we next used a pathogenic JEV (Beijing strain) to determine if differences in the cytokine profiles between JEV and WNV could be explained on the basis of the pathogenicity and dose of the infecting virus. We infected mice with a low dose (10^3 pfu – comparable dose to WNV) or a high dose (10^6 pfu – comparable dose to JEV SA14-14-2) of the JEV Beijing. Similar to a JEV S14-14-2 infection, infection with either low or high dose JEV Beijing induced a significantly higher frequency of IFN-γ+ CD8+ T cells than IFN-γ+ TNF-α+ CD8+ T cells compared to WNV infection (P<0.05, Mann-Whitney U) (Figure 3.4.C.-E.). Again, no significant difference was detected between JEV S9 and WNV S9 peptide variants within the JEV Beijing infections. These results suggest that the infecting virus (JEV versus WNV) rather than the epitope (JEV S9 versus WNV S9) induced differences in the cytokine profiles of epitope-specific CD8+ T cells.

C. Frequency of epitope-specific CD8+ T cells in JEV- and WNV-infected mice.

To ascertain whether the differences in the cytokine profiles were related to different CD8+ T cell kinetics between JEV and WNV infections, we measured epitope-specific dimer+ CD8+ T cells 5, 7 and 10 days post-infection in the PBL. Rapid expansion of CD44hi dimer+ CD8+ T cells occurred between days 5 and 7.
Table 3.3. Higher TNF-α production on a per cell basis in WNV-infected mice compared to JEV-infected mice. Splenocytes from JEV SA14-14-2 or WNV infected mice were stimulated with 1 µg/ml of either JEV S9 or WNV S9 for 5 hours in the presence of brefeldin A. Raw MFI values from 2 experiments (n=2-3 mice per group) for IFN-γ (left columns) and TNF-α (right columns) using FloJo software.

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<sup>a</sup> mean fluorescence intensity (MFI)

<sup>b</sup> MFI values were calculated from CD3<sup>+</sup> CD8<sup>+</sup> T cell expressing IFN-γ or TNF-α
with peak levels occurring at day 7 for all infections with the exception of high dose JEV Beijing, which peaked at or before day 5 post-infection (Figure 3.5.). For JEV SA14-14-2 and low dose JEV Beijing, an approximately 4-8 fold contraction in frequency of JEV S9 dimer$^+$ CD8$^+$ T cells occurred between days 7 and 10, while only a 1-2-fold contraction in frequency of WNV S9 dimer$^+$ CD8$^+$ T cells occurred in WNV-infected mice. Similar to the pattern seen for cytokine production, infection with JEV induced a higher proportion of cross-reactive WNV S9-specific CD8$^+$ T cells than cross-reactive JEV S9-specific CD8$^+$ T cells seen after a WNV infection. Although the peak CD8$^+$ T cell response for high dose JEV Beijing occurred earlier, there was no difference in the frequency of IFN-$\gamma^+$ and IFN-$\gamma^+$ TNF-$\alpha^+$ CD8$^+$ T cells at day 7 for all JEV infections (Figure 3.4.D.). These results suggest that the differences in the CD8$^+$ T cell kinetics are not related to the altered cytokine profiles that we observe.

**D. Phenotype of epitope-specific CD8$^+$ T cells during acute JEV and WNV infection.**

Effector CD8$^+$ T cell activation depends on many factors, including antigen stimulation and inflammatory conditions (258). To determine whether the differences in the cytokine profiles reflect differences in the activation state of CD8$^+$ T cells during acute JEV and WNV infections, we examined the phenotype of epitope-specific CD8$^+$ T cells over the course of an acute infection. Splenocytes from infected mice were harvested on day 5, 7 and 10
Figure 3.5. Kinetics of cross-reactive CD8+ T cell expansion and contraction during JEV and WNV infection. Mice were infected with (A) JEV SA14-14-2, (B) 1 x 10^3 pfu JEV Beijing, (C) 1 x 10^6 pfu JEV Beijing, or (D) WNV. Splenocytes were harvested on days 5, 7 and 10 post infection and stained with anti-CD3, -CD8, -CD44 and either JEV S9 (●) or WNV S9 dimer (○). Cells were gated on CD3+ , CD8+ , CD44 hi T cells. Percent CD44 hi dimer+ CD8+ T cells are shown after subtraction of non-specific staining of JEV S9 dimer and WNV S9 dimer in naïve mice. Horizontal bars represent mean epitope-specific CD8+ T cell frequency.
post-infection, and CD62L, KLRG1 (killer cell lectin-like receptor G1) and CD127 (IL-7Rα) expression were measured on CD44^{hi} dimer^+ CD8^+ T cells (Figure 3.6.). At day 5, low-level expression of CD62L on dimer^+ CD8^+ T cells was seen in all infections indicating similar levels of CD8^+ T cell activation (Figure 3.6.B.). By day 10, re-expression of CD62L was detected on both JEV and WNV S9 dimer^+ CD8^+ T cells in all JEV groups. However, on day 10 after WNV infection, CD62L expression for the cross-reactive JEV S9 population increased while the WNV S9 dimer^+ population had a persistent CD62L^{lo} phenotype (P<0.05, Mann-Whitney U).

KLRG1 and CD127 expression on effector CD8^+ T cells are two markers used to define CD8^+ T cell subsets. KLRG1^{hi} CD127^{lo} CD8^+ T cells are defined as short-lived effector T cells (SLECs) that die off during the contraction phase while KLGR1^{lo} CD127^{hi} CD8^+ T cells are memory precursor effector cells (MPECs) that survive contraction and differentiate into long-lived memory cells (212, 230, 232). KLRG1 expression was upregulated on JEV S9 and WNV S9 dimer^+ CD8^+ T cells for all groups as early as day 5, but progressively decreased in all of the JEV groups by day 10 (Figures 3.6.B.). In contrast, KLRG1 expression increased between days 5 and 7 and persisted at high levels through day 10 in WNV-infected mice (median day 10 %CD44^{hi} WNV S9 dimer^+ KLRG1^{hi} = 65.5% in WNV vs. %CD44^{hi} JEV S9 dimer^+ KLRG1^{hi} 20.8%, 26.5%, 22.9% for 1 x 10^3 pfu, 1 x 10^6 pfu JEV Beijing, and JEV SA14-14-2, respectively; P<0.05,
Figure 3.6. Phenotypes of JEV S9-specific and WNV S9-specific CD8⁺ T cells differ following JEV and WNV infection. (A) Mice were infected with JEV SA14-14-2 (top row), 1 x 10³ pfu JEV Beijing (second row), 1 x 10⁶ pfu JEV Beijing (third row), or WNV (bottom row). Splenocytes were harvested on days 5, 7 and 10 and expression of CD62L, KLRG1 and CD127 was determined on CD3⁺ CD8⁺ CD44hi JEV S9 (black line) and WNV S9 (gray line) dimer⁺ T cells. Shaded plot represents expression in naïve mice (representative from 1 naïve mouse per day). Each row represents a single representative mouse from each infection group.
group (n=3-4 infected mice per group per day). (B) Summary of phenotype analysis. Percent of CD62Llo (left panels), KLRG1hi (middle panels) and CD127hi (right panels) expressing CD44hi CD8+ T cells bound to JEV S9 dimer (●) or WNV S9 dimer (○). Median values shown as horizontal line. #P<0.05 between WNV and JEV SA14-14-2 or 1x10^6 pfu JEV Beijing on day 10, P>0.05 between WNV and 1x10^3 pfu JEV Beijing; *P<0.05 between WNV and each JEV group on day 7 and day 10, respectively, Mann Whitney U. The mean background level for dimer staining was 0.12% for JEV S9 (range: 0.01% to 0.21%) and 0.19% for WNV S9 (range: 0.08% to 0.3%).
Mann-Whitney U). An inverse pattern was seen for CD127 expression where uniform downregulation of CD127 was seen by day 5 in all groups. Re-expression of CD127 on dimer+ CD8+ T cells occurred by day 10 for both JEV SA14-14-2 and JEV Beijing but remained low in WNV-infected mice (median %CD44hi CD127hi WNV S9 dimer+ CD8+ T cells = 32.1% in WNV vs. 61.7%, 62.4%, and 64.8% for 1x10³ pfu, 1x10⁶ pfu JEV Beijing, and JEV SA14-14-2, respectively; P<0.05, Mann-Whitney U) (Figure 3.6.B.).

SLEC generation began by day 5 post infection in all groups but peaked on different days (Figures 3.7.). For JEV SA14-14-2 and high dose JEV Beijing, the highest frequency of SLECs occurred at day 5 (median 25.8% for SA14-14-2 and 40.2% for 10⁶ Beijing). For low dose JEV Beijing and WNV, the frequency of SLECs increased between days 5 and 7. By day 7, 32.2% of dimer+ CD8+ T cells were KLRG1hi CD127lo during low dose JEV Beijing infection compared to 58.3% of the dimer+ CD8+ T cells after WNV infection (P<0.05 between WNV and all JEV groups, Mann Whitney U). At day 5, frequencies of MPECs were low for all groups. At day 7, MPECs increased only in JEV infected mice and by day 10, 56.0 - 58.0% of the dimer+ CD8+ T cells were KLRG1lo CD127hi (Figure 3.7.B.-C.). In contrast, during a WNV infection, a majority of the dimer+ CD8+ T cells maintained a SLEC phenotype (KLRG1hi CD127lo) with a low frequency of MPECs on days 7 and 10 post-infection (P<0.05 between WNV and all JEV groups, Mann Whitney U).
Figure 3.7. KLRG1 and CD127 expression on JEV S9-specific and WNV S9-specific dimer+ CD8+ T cells. (A) Mice were infected with JEV or WNV, and splenocytes were harvested on the indicated days. Representative data for one mouse per group staining for KLRG1 and CD127 are shown. (B-C) Summary data for (B) CD44hi dimer+ KLRG1hi CD127lo CD8+ T cells and (C) CD44hi dimer+ KLRG1lo CD127hi CD8+ T cells. Data represent the homologous dimer staining for each infection from individual spleens (JEV S9 (●) for JEV infections or WNV S9 (○) for WNV infection). *P<0.05 between WNV and all JEV groups on day 7 and day 10, respectively, Mann-Whitney U.
E. Viral replication of JEV and WNV viruses in tissues.

We hypothesized that the differences in the cytokine profiles and the phenotype of effector CD8+ T cells might be related to differences in viral replication. Therefore, we measured viral titers by plaque assay in spleen, serum and brain after JEV and WNV infection to determine whether there were differences in peripheral (spleen and serum) and CNS replication. On day 3, between $6 \times 10^3 - 1.3 \times 10^5$ pfu/ml and $2 \times 10^4 - 6 \times 10^4$ pfu/g of WNV was detected in the serum and spleen, respectively (Figures 3.8.A.-B.). In contrast, we detected low titers (500 pfu/g) of JEV in spleens from 1 mouse in each of the low and high dose JEV Beijing groups. We were unable to detect virus in the serum on day 3 from any of the JEV groups. At day 7 post-infection, we detected high titers of virus in brains from mice infected with $10^6$ pfu of JEV Beijing and WNV, but not from low dose JEV Beijing or JEV SA14-14-2 infected mice (Figure 3.8.C). As expected, virus was not detectable in serum on day 7 or in brains on day 3 from any group (data not shown). These results suggest that overall virus burden may not be responsible for the altered cytokine profiles and altered phenotype responses measured between JEV and WNV but rather reflect differences in peripheral replication.

F. Chapter Discussion

Altered responses to flavivirus cross-reactive T cell epitopes can affect the outcome upon heterologous virus challenge (259). Our model system utilized two
Figure 3.8. Virus burden in tissues following JEV and WNV infection. Virus titer as assessed by virus plaque assay in (A) serum and (B) spleen at 3 days and (C) brain at 7 days post JEV or WNV infection. Each symbol represents a single mouse. Horizontal line represents geometric mean titer.
viruses in the JEV serogroup, JEV and WNV, which have different clinical outcomes on sequential virus infection in NHP (155). Overall, our results demonstrate that peptides that are homologous to the immunizing virus induce a greater frequency of epitope-specific CD8\(^{+}\) cells and higher levels of cytokine production and cytolytic activity than heterologous peptides. However, distinct CD8\(^{+}\) T cell functional responses arise depending on the infecting virus (JEV or WNV) independent of pathogenicity of the infecting virus or peptide variant.

We identified a novel immunodominant JEV NS4b H-2D\(^{b}\) restricted CD8\(^{+}\) T cell epitope that is a variant of a recently published WNV epitope (115, 116). We found that both the JEV and WNV variants induced cytokine secretion and stimulated lysis of peptide-coated targets in JEV-infected mice. Regardless of the infecting virus, we found that the epitope hierarchy was higher for the variant peptide corresponding to the infecting virus. In addition, a greater proportion of CD8\(^{+}\) T cells were cross-reactive by dimer staining in JEV- versus WNV-infected mice. These cross-reactive CD8\(^{+}\) T cells may preferentially expand upon secondary heterologous JEV challenge and contribute to virus clearance and protection as seen in PV and VV infection of LCMV-immune mice (164, 172).

We also identified two cross-reactive CD4\(^{+}\) T cell epitopes located in the NS1 and NS3 protein. The cross-reactive NS3 epitope is a variant of the WNV NS3 epitope previously identified (125). Cross-reactive CD4\(^{+}\) T cells have been identified in JEV-immunized individuals and mice, previously (197, 257). CD4\(^{+}\) T cells are required for maintenance of antibody responses, sustaining CD8\(^{+}\) T cell
responses, and they can also have antiviral properties during a WNV infection (115, 124). Interestingly, the cross-reactive epitopes we identified in the NS3 and NS1 proteins do not correlate with the total cross-reactive CD4+ T cell response in response to the peptide pools (Figure 3.1.). We found that peptides from the NS4b, NS2a, and E proteins induced the highest overall IFN-γ+ CD4+ T cell response. This difference may be due to multiple peptides in these proteins inducing low levels of IFN-γ, which together contribute to a higher response.

Subdominant T cell epitopes have previously been shown to mediate heterologous immunity in the murine LCMV model, but immunodominant epitopes may also play a role (166). This has been suggested in human studies in which immunodominant HLA-A2-restricted influenza M1-specific CD8+ T cells found to be cross-reactive to the Epstein-Barr virus BMLF-1 epitope expand during acute infectious mononucleosis (167). Similarly, in our model, CD8+ T cells specific for the immunodominant epitope are cross-reactive in both JEV and WNV-infected mice. We detected a significantly higher proportion of IFN-γ+ TNF-α+ -producing CD8+ T cells in mice infected with WNV compared to those immunized with both attenuated and pathogenic JEV strains (Figure 3.4.), as well as higher TNF-α production on a per cell basis (Table 3.3.). The role of TNF-α in WNV infection can be pleiotropic and may lead to resolution of the infection or to immunopathology depending on TNF-α levels (106, 260). A higher level of TNF-α production by CD8+ T cells during acute WNV infection may help contribute to their own trafficking into the CNS leading to the control of viral infection in the
CNS or increased immunopathology due to the CD8$^+$ T cells. In fact, WNV neuroinvasive disease in humans can lead to long term neurological sequelae suggesting that virus clearance and immunopathology may not occur independently (261).

The qualitative disparity in cytokine profiles during acute infection with closely related viruses may be due to one of several factors: 1) differences in the kinetics of the response; 2) differences in the activation state of CD8$^+$ T cells during the viral infections; 3) differences in viral burden and/or tissue tropism between JEV and WNV. However, the differences in the CD8$^+$ T cell responses between JEV and WNV did not correlate with mortality or inoculum dose, because all JEV strains, whether attenuated or pathogenic, induced similar CD8$^+$ T cell responses. These results suggest that the differences in the cytokine profiles of epitope-specific CD8$^+$ T cells are due to intrinsic differences in the immune response generated after JEV and WNV infection.

Kinetic analysis of JEV S9-specific and WNV S9-specific CD8$^+$ T cell responses demonstrated that peak CD8$^+$ T cell responses occurred on day 7 post-infection for all viruses with the exception of infection with high dose JEV Beijing ($1 \times 10^6$ pfu), in which CD8$^+$ T cells peaked on or before day 5. Activation state, as demonstrated by downregulation of CD62L, was similar for all groups at days 5 and 7 post-infection. However, the increase in SLECs during JEV infection was much shorter in duration than what has been reported for acute LCMV infection (212). We also found a significantly higher proportion of KLRG1$^{hi}$
CD127\textsuperscript{lo} SLECs after WNV infection on day 7 compared to all JEV virus infections, and these differences persisted to day 10 post-infection. These findings are in contrast to those reported by Brien et al., in which WNV S9 dimer\textsuperscript{+} CD127\textsuperscript{hi} CD8\textsuperscript{+} T cells predominated at day 7 after WNV infection (115). That study used a different WNV strain, a lower dose of virus (20-600 pfu) and a different route of administration (sc), which may have impacted the kinetics of virus replication, inflammation derived from this virus replication, and subsequent effector CD8\textsuperscript{+} T cell generation.

As expected, replication of the attenuated JEV SA14-14-2 strain in peripheral tissues was below the level of detection in viral plaque assay (Figure 3.8) (62). However, unexpectedly, infection with low or high dose JEV Beijing also resulted in minimal peripheral virus replication on day 3, whereas high dose JEV Beijing infection resulted in very high titers of virus in brains on day 7 post-infection. This lack of peripheral replication by JEV was also recently seen in another B6 mouse model using a different JEV strain (Nakayama) and route of infection (sc) (95). In contrast, WNV was easily detectable in serum and spleen on day 3 as well as in brains at day 7. The ability of WNV to replicate in the peripheral lymphoid tissues early during infection may alter the inflammatory milieu and programming of the CD8\textsuperscript{+} T cell response. IL-12, type I IFN, and IL-2 are inflammatory cytokines known to influence the generation of CD8\textsuperscript{+} T cells and SLECs, and the levels of these cytokines may differ during a JEV and WNV infection (212, 217, 225, 227-229). The persistence of KLRG1\textsuperscript{hi} CD127\textsuperscript{lo} SLECs
and a higher frequency of epitope-specific CD8\(^+\) T cells in a WNV infection may reflect prolonged antigenic stimulation or increased inflammatory responses due to persistent WNV as has been described in B6 mice and other WNV animal models (24, 25, 28).

G. Chapter Summary

We have identified JEV and WNV cross-reactive CD4\(^+\) and CD8\(^+\) T cell epitopes in B6 mice. In our model, the immunodominant CD8\(^+\) T cell epitope was found to be cross-reactive, however primary infections with JEV and WNV give rise to quantitatively and qualitatively distinct CD8\(^+\) T cell responses. These differences in the primary immune response to JEV and WNV may contribute to differences in secondary immune responses to sequential flavivirus infection. The next chapter will focus on the kinetics of the JEV-WNV cross-reactive CD8\(^+\) T cell epitope during secondary heterologous infections.
CHAPTER IV

SEQUENCE OF INFECTION DETERMINES CROSS-REACTIVE CD8+ T CELL RESPONSES DURING SECONDARY HETEROLOGOUS FLAVIVIRUS INFECTION

Cross-protection between JEV and WNV has been established in multiple models, but the contributions of memory CD8+ T cells to this cross-protection has not been established (Trobaugh et al., in preparation) (153-155, 157-159). Previous work has implicated that prior immunity to WNV will only partially protect against JEV (155). It is known that memory cross-reactive CD8+ T cells proliferate upon 2o heterologous infection and can contribute to either protection or immunopathology (164, 166, 171, 172). Therefore, in order to better understand cross-protection between JEV and WNV, we focused on cross-reactive CD8+ T cell responses during secondary infections.

In the previous chapter, we identified a cross-reactive CD8+ T cell epitope, JEV S9 and WNV S9, which is immunodominant during their respective primary infections. This chapter will focus on the kinetics of cross-reactive CD8+ T cell expansion during 2o heterologous infection, and possible explanations for the differences in proliferation seen in JEV- and WNV- immune mice upon 2o heterologous infection.
A. Establishing lethal and non-lethal JEV models in B6 mice.

Previous JEV models used weaning mice, intracranial inoculation, and outbred mice to study JEV pathogenesis (91-94, 262). However, these models are not conducive for studying the adaptive immune responses to JEV, and the subsequent mechanisms behind cross-protection between JEV and WNV. Therefore, we wanted to establish both a non-lethal (primary infection) and a lethal (secondary infection) model of JEV in B6 mice. B6 mice were initially infected at 5-6 weeks of age, and the surviving mice were rested 4-6 weeks to generate memory CD4$^+$ and CD8$^+$ T cells. At 9-11 weeks old, mice were administered a secondary infection with a lethal dose of the heterologous virus.

We initially tested two different pathogenic JEV strains, Nakayama and Beijing, for their ability to induce mortality in B6 mice. B6 mice (6-7 weeks old) were infected with $1 \times 10^6$ pfu of JEV Nakayama or $5 \times 10^6$ pfu of JEV Beijing and monitored for survival. Even though different titers were used, both JEV Nakayama and JEV Beijing induced similar mortality rates (80% mortality, 4/5 mice) suggesting that both strains could be used for our lethal model (Figure 4.1.A.). We ultimately chose to use JEV Beijing in our model of lethal infection due to the higher titers of the viral stocks. For the cross-protection model, in which JEV was the primary infecting virus, we needed a dose of JEV Beijing that...
Figure 4.1. Establishing lethal and non-lethal JEV models in B6 mice.
(A) Identification of JEV strain. B6 mice (6-7 weeks old) were infected with JEV Nakayama (1 x 10^6 pfu) or JEV Beijing (5 x 10^6 pfu) ip, and monitored for survival, n=5 mice/group. (B) B6 mouse susceptibility to low and high dose JEV Beijing infection. B6 mice (5-6 weeks old) were infected with a low dose (1 x 10^3 pfu, N=67 mice) or high dose (1 x 10^6 pfu, N=20 mice) of JEV Beijing ip. **P<0.001, Log-Rank Test (C) Age-specific differences in JEV susceptibility. B6 mice were infected with 1 x 10^6 pfu (n=5 mice/age group) or 1 x 10^7 pfu (11 weeks old; N=15 mice).
would increase the likelihood of survival, but would still induce a potent immune response. When B6 (5-6 weeks old) mice were infected with a low dose of JEV Beijing (1 x 10^3 pfu), we saw a 25.4% mortality rate, while a high dose (1 x 10^6 pfu) increased the mortality to 60% (Figure 4.1.B). This is in contrast to a recent report by Larena et al. that demonstrated no such dose dependence upon the mortality rate for JEV. However, differences between our study and theirs, such as virus strains (Nakayama versus Beijing) and different routes of infection (sc versus ip) may contribute to these disparate findings (95). As seen in Figure 3.4. and Figure 3.5., infection with either low dose or high dose JEV Beijing induced similar epitope-specific CD8+ T cell responses. Therefore, due to the lower mortality rate, we chose to use the low dose infection to generate our primary JEV-immune responses.

Figure 4.1.C. also demonstrates the difficulties that we had in using JEV as a lethal model, as the susceptibility to JEV varied from experiment to experiment. B6 mice (7 weeks old) only had a 10% mortality rate compared to 5-6 week old mice, which had a 60% mortality rate, when given the same dose of 1 x 10^6 pfu of JEV. In fact, susceptibility to JEV Beijing infection actually increased as the mice increased in age from 7-week old mice (10% mortality) to 10-week old mice (60% mortality), which is contrary to the dogma that resistance to flavivirus infection in mice increases with age (unpublished observations) (263). When we increased the dose of virus to 1 x 10^7 pfu and infected the oldest mice (11-weeks old), we saw an increase in mortality to 73.3% (Figure 3.1.C.). These
results demonstrated that JEV Beijing induced mortality when administered ip in 10-11 week old mice, and could be used in our cross-protection model as a secondary challenge virus.

**B. Cross-protection after sequential WNV and JEV infections.**

Prior work in our group has demonstrated that a live attenuated JEV SA14-14-2 infection could protect against a lethal WNV challenge (Trobaugh et al., in preparation). We wanted to extend these results to determine whether prior WNV immunity would protect against a lethal JEV challenge in B6 mice. B6 mice (5-6 weeks old) were infected with WNV (1 x 10^3 pfu) or PBS (control) and monitored daily for survival. Mice that survived the primary infection were allowed to rest for 5 weeks to generate a memory response. Thirty-five days after the primary infection, the mice were infected with a lethal dose of JEV Beijing (1 x 10^7 pfu). We found that WNV-immune mice were significantly protected against a lethal JEV challenge (87.5% survival) compared to PBS controls (42.1% survival, P<0.01, Log Rank Test) (Figure 4.2.A).

To determine whether the pathogenicity of JEV (SA14-14-2 versus Beijing) altered cross-protection to WNV, JEV Beijing-immune mice were infected with a lethal dose of WNV. Similar to JEV SA14-14-2-immune mice, JEV Beijing immune mice were significantly protected against a lethal WNV infection (100% survival) compared to PBS controls (66.7% survival; P<0.05, Log- Rank Test), suggesting the pathogenicity of the primary infection does not alter cross-
Figure 4.2. Cross-protection during sequential JEV and WNV infections. (A) WNV-immune mice are protected against lethal JEV infection. WNV-immune (n=16 mice, blue square) or PBS control (N=19 mice, black triangle) mice were infected with 1 x 10^7 pfu JEV Beijing and monitored for survival. The ratio indicates the number of mice succumbing to the infection in each group. *P<0.01, Log-Rank Test. (B) JEV Beijing-immune mice are protected against WNV infection. JEV immune mice (N=14 mice, red square) or PBS (N=12 mice, black triangle) were infected with 1 x 10^6 pfu WNV and monitored for survival. *P<0.05, Log-Rank Test.
protection to WNV (Figure 4.2.B). These results suggest that, in contrast to
NHPs, there is bidirectional cross-protection between JEV and WNV in mice, and
further work to understand the mechanisms of this cross-protection is warranted
(155).

C. Can cross-reactive CD4⁺ and/or CD8⁺ T cells protect against a WNV
infection?

Our group has shown that depletion of CD4⁺ and CD8⁺ T cells in JEV-
immune mice abrogated cross-protection against WNV (Trobaugh et al., in
preparation). CD8⁺ T cells are required for survival against a primary WNV
infection due to inefficient clearance of virus from peripheral tissues (109).
Adoptive transfer of a WNV S9-specific CD8⁺ T cell line protected
immunodeficient mice from mortality after a low dose WNV infection; however,
adoptive transfer of two WNV CD8⁺ T cell lines was required to fully protect
immunocompetent mice (115, 116). These results demonstrate that CD8⁺ T cells
can protect against a lethal WNV infection. Therefore, we wanted to determine 1)
whether JEV-immune CD4⁺ and CD8⁺ T cells are sufficient to protect against a
WNV infection and/or 2) whether JEV S9-specific and WNV S9-specific CD8⁺ T
cells can restrict WNV viremia.

JEV SA14-14-2-immune CD4⁺ and CD8⁺ T cells were adoptively
transferred into naïve B6 mice and challenged with a 100LD50 of WNV (1.26 x10⁶
pfu). JEV-immune CD4⁺ and/or CD8⁺ T cells were transferred in combination
with naïve CD4+ and/or CD8+ T cells in order to transfer an equal number of cells into naïve mice. Ch-WN- and JEV SA14-14-2-immune mice were protected against a lethal WNV infection, similar to previous reports (Figure 4.3.A.) (Trobaugh et al., in preparation) (77). Adoptive transfer of JEV-immune CD4+ T cells with naïve CD8+ T cells or naïve CD4+ T cells with JEV-immune CD8+ T cells resulted in only 12.5% survival. However, mice receiving both JEV-immune CD4+ T cells and JEV-immune CD8+ T cells were partially protected from WNV infection (37.5% survival rate, P=0.07 compared to PBS, Log-Rank Test). Finally, PBS control mice and mice receiving naïve CD4+ and naïve CD8+ T cells were 100% susceptible to WNV infection by day 10 and day 11, respectively (Figure 4.3.A). These data suggest that JEV-immune CD4+ and CD8+ T cells are not sufficient to fully protect against a high dose WNV infection; however, these results do not rule out the possibility that JEV-immune CD4+ and CD8+ T cells might protect against a low dose WNV infection.

Since adoptive transfer of JEV-immune CD4+ and CD8+ T cells partially protected against a high dose WNV infection, we wanted to determine whether JEV S9-specific and WNV S9-specific CD8+ T cells could reduce levels of WNV in the serum and brain after WNV infection. For this experiment, we utilized recombinant vaccinia viruses (rVV) expressing the JEV S9 epitope (rVV JEV), WNV S9 epitope (rVV WNV) or GFP alone (rVV GFP) to generate epitope-specific CD8+ T cells. B6 mice were immunized with 5 x 10^6 pfu of rVV WNV, rVV
Figure 4.3. Cross-reactive CD8$^+$ T cells are not sufficient to fully protect from WNV mortality. (A) JEV immune CD4$^+$ and CD8$^+$ T cells partially protect against WNV infection. CD4$^+$ and CD8$^+$ T cells from JEV-immune or naïve mice were adoptively transferred into naïve mice, which were then infected with 1 x 10$^6$ pfu WNV. Ch-WNV and JEV SA14-14-2 immune mice and PBS served as positive and negative controls, respectively. N=8 mice/group. (B-C). JEV S9-specific and WNV S9-specific CD8$^+$ T cells do not restrict WNV replication. B6 mice were infected with 5 x 10$^6$ pfu rVV-WNV, rVV-JEV or rVV-GFP, and 4-5 weeks later, the mice were challenged with 1 x 10$^3$ pfu of WNV. WNV titers in the serum on day 3 (B) and brain on day 7 (C) were measured by plaque assay.
JEV, rVV GFP or PBS (control), and after 4-5 weeks, the mice were challenged with a low dose (1 x 10^3 pfu) of WNV. WNV levels were measured on day 3 in the serum, and day 7 in the brain by plaque assay. Immunization with rVV WNV and rVV JEV did not reduce WNV levels in the serum on day 3 compared to rVV GFP immunization and PBS control mice (Figure 4.3.B.). These results are similar to those of Shrestha et al., in which CD8^+ T cells were not able to reduce levels of WNV in the serum (109).

We next analyzed WNV levels in the brain of these mice to determine whether homologous or heterologous memory CD8^+ T cells might restrict viral dissemination of homologous and heterologous virus into the brain. After the low dose WNV infection, only 50% of the PBS control and 40% of rVV GFP immunized mice had detectable WNV levels in the brain (Figure 4.3.C.). In the rVV WNV-immunized mice, in which virus was detectable, lower levels of WNV were seen compared to the PBS controls suggesting that WNV S9-specific CD8^+ T cells were able to restrict viral dissemination into the CNS. However, rVV GFP-immunized mice also had lower levels of WNV in the brain similar to rVV WNV immunized mice, therefore we cannot conclusively determine that WNV S9-specific CD8^+ T cells restrict WNV entry into the brain. WNV levels in rVV JEV-immunized mice were similar to those measured in PBS controls suggesting that JEV S9-specific CD8^+ T cells alone are not able to restrict heterologous WNV virus entry into the brain, and JEV-specific CD4^+ T cells may also be required to prevent WNV entry into the brain. Since JEV viremia is not detectable in our
model, we were unable to determine whether JEV S9-specific and WNV S9-specific CD8$^+$ T cells were able to restrict JEV in the serum. In addition, a very high dose of JEV is required for efficient viral entry into the brain (Figure 3.8.C.), which may preclude the ability to detect a role for CD8$^+$ T cells in restricting viral dissemination. Taken together, these results suggest that cross-reactive JEV S9-specific CD8$^+$ T cells alone are not sufficient to protect against peripheral replication and dissemination of WNV.

**D. Cross-reactive CD8$^+$ T cell responses during sequential JEV and WNV infections.**

Next, we wanted to understand the kinetics of the cross-reactive CD8$^+$ T cell response during sequential JEV and WNV infection. B6 mice were infected with JEV or WNV and allowed to generate a memory response for 5 weeks. The mice were then infected with a lethal dose of the corresponding heterologous flavivirus (JEV-immune: $1 \times 10^6$ pfu WNV; WNV-immune: $1 \times 10^7$ pfu JEV). JEV S9-specific and WNV S9-specific CD8$^+$ T cells were measured in the peripheral blood lymphocytes (PBL) by H-2D$^b$ dimer staining over the course of the infection. At the memory time point prior to $2^\alpha$ infection, WNV-immune mice had a significantly higher mean starting frequency of JEV S9-specific and WNV S9-specific CD8$^+$ T cells compared to JEV-immune mice (mean $\pm$ SEM: WNV-immune: JEV S9: $1.21\% \pm 0.25\%$, WNV S9: $3.53\% \pm 0.97\%$; JEV-immune:...
Figure 4.4. Cross-reactive JEV S9-specific and WNV S9-specific CD8+ T cell kinetics during 2° infection. (A) Limited proliferation of WNV-immune CD8+ T cells upon 2° JEV infection. JEV-immune (n= 14 mice/time point, red circle) or PBS control (n=12 mice, open circle) mice were infected with 1 x 10^6 pfu, and WNV-immune (n=11-12 mice, blue squares) or PBS control (n=11-14 mice, open square) mice were infected with 1 x 10^7 pfu JEV. WNV S9 (left panel) and JEV S9 (right panel) dimer+ CD44hi CD8+ T cells were measured in the PBL on day -1/0, 3, 5, and 7. P<0.001 between 1° JEV immune mice and all other groups, Mann-Whitney U. (B) CD43 expression on JEV S9-specific and WNV S9-specific CD44hi CD8+ T cells following 2° infection. (C) KLRG1 expression on JEV S9-specific and WNV S9-specific CD44hi CD8+ T cells following 2° infection. (D) CD127 expression on JEV S9-specific and WNV S9-specific CD44hi CD8+ T cells following 2° infection.
JEV S9: 0.48% ± 0.04%, WNV S9: 0.22 ± 0.04%; P<0.01, Mann-Whitney U) (Figure 4.4.A.). WNV infection of JEV-immune mice resulted in expansion of both JEV S9-specific and WNV S9-specific CD8+ T cells by day 5 after infection, resulting in significantly higher frequencies of both JEV S9-specific and WNV S9-specific CD8+ T cells compared to WNV infection of PBS controls (mean ± SEM on day 5: JEV-immune: JEV S9: 10.00% ± 0.82%; WNV S9: 12.83% ± 0.97%; PBS: JEV S9: 0.51% ± 0.11%; WNV S9: 1.26% ± 0.21%; P<0.001, Mann-Whitney U) (Figure 4.4.A). By day 7, the frequency of JEV S9-specific and WNV S9-specific CD8+ T cells continued to increase in PBS control mice typical of a primary immune response (mean ± SEM: JEV S9: 1.60% ± 0.23%; WNV S9: 6.43% ± 0.61%). In JEV-immune mice, only the frequency of WNV S9-specific CD8+ T cells continued to increase on day 7, while the frequency of JEV S9-specific CD8+ T cells remained constant between day 5 and day 7 (mean ± SEM: JEV S9: 10.26% ± 1.11%; WNV S9: 17.60% ± 1.72%).

In contrast to heterologous infection of JEV-immune mice, there was very little proliferation of memory JEV S9-specific and WNV S9-specific CD8+ T cells by day 5 after JEV infection of WNV-immune mice (Figure 4.4.A.) The frequency of WNV S9-specific CD8+ T cells on day 5 was not significantly different than the frequency at day 0 (mean ± SEM: Day 0: 3.53% ± 0.97%; Day 5: 3.93% ± 0.45%; P=0.9, Mann-Whitney U). There was a small but significant increase in the frequency of JEV S9-specific CD8+ T cells in WNV-immune mice upon JEV infection by day 5 (mean ± SEM: Day 0: 1.21% ± 0.25%; Day 5: 1.92% ± 0.18%);
P<0.05, Mann-Whitney U), suggesting there may be low levels of proliferation in WNV-immune mice by day 5. Interestingly by day 7, the frequency of JEV S9-specific and WNV S9-specific CD8^+ T cells continued to increase in both WNV-immune and PBS controls (mean ± SEM: WNV-immune: JEV S9: 7.46% ± 0.87%; WNV S9: 10.99% ± 1.57%; PBS: JEV S9: 6.55% ± 1.11%; WNV S9: 4.18% ± 0.93%). This pattern of expansion paralleled the epitope-specific CD8^+ T cell kinetics seen during a primary JEV infection of PBS control mice suggesting that JEV infection of WNV-immune mice resulted in a 1^o immune response rather than a 2^o memory response.

Expression of CD43 is upregulated during an acute infection on effector CD8^+ T cells, and is required for trafficking of CD8^+ T cells to peripheral tissues and downregulation of the immune response (264, 265). Low CD43 expression on memory CD8^+ T cells corresponds to better recall responses during 2^o infection (264, 266). We saw similar low levels of CD43 expression on JEV S9-specific and WNV S9 CD8^+ T cells at the memory time point (day 0) in both JEV-immune and WNV-immune mice (Figure 4.4.B). In JEV-immune mice, CD43 expression was significantly higher on both JEV S9-specific and WNV S9-specific CD8^+ T cells on day 3 after 2^o WNV infection compared to PBS control mice (mean ± SEM: JEV-immune: JEV S9: 32.9% ± 1.6%; WNV S9: 38.0% ± 2.4%; PBS: JEV S9: 15.1% ± 3.1%; WNV S9: 14.9% ± 3.7%; P<0.001, Mann Whitney U). CD43 expression peaked earlier in JEV-immune mice (day 5) compared to PBS controls (day 7) upon 2^o WNV infection. It was found
previously, that conversion of effector CD8\(^+\) T cells to memory CD8\(^+\) T cells expressing low levels of CD43 occurs earlier in low inflammatory conditions, suggesting that inflammation in JEV-immune mice may be lower than in PBS controls upon 2\(^o\) WNV infection (267).

In WNV-immune mice following 2\(^o\) JEV infection, expression of CD43 on JEV S9-specific and WNV S9-specific CD8\(^+\) T cells was not detected until day 5, two days later than occurred during WNV infection of JEV-immune mice (Figure 4.4.B). This expression pattern of CD43 was similar to that seen in JEV infection of PBS control mice. Therefore, expression of CD43 was seen prior to the expansion of epitope-specific CD8\(^+\) T cells, in the context of both primary and memory responses.

A significantly higher frequency of both JEV S9-specific and WNV S9-specific CD8\(^+\) T cells in WNV-immune mice expressed KLRG1 compared to JEV-immune mice at the memory time point (day 0) (mean % KLRG1\(^+\) CD8\(^+\) T cells ± SEM: JEV-immune: JEV S9: 15.1% ± 1.6%; WNV S9: 11.8% ± 2.1%; WNV-immune: JEV S9: 56.5% ± 7.9%; WNV S9: 62.7% ± 7.4%; JEV S9: P<0.01, WNV S9: P<0.001, Mann Whitney U) (Figure 4.4.C). In JEV-immune mice following 2\(^o\) WNV infection, JEV S9-specific and WNV S9-specific CD8\(^+\) T cells upregulated KLRG1 and downregulated CD127 expression by day 5 after infection, suggesting rapid conversion to an SLEC phenotype, KLRG1\(^+\) CD127\(^{low}\) (Figure 4.4.C.-D.). In WNV-immune, since a majority of the epitope-specific CD8\(^+\) T cells already expressed KLRG1, there was only a slight increase in the frequency of
KLRG1+ CD8+ T cells by day 7 following 2° JEV infection. However, expression of CD127 was downregulated in these mice, which suggests that activation of these epitope-specific CD8+ T cells occurred in WNV-immune mice in the absence of proliferation. CD8+ T cells in JEV and WNV infection of PBS control mice converted to KLRG1+ and CD127low between days 5 and days 7 after infection, confirming that the majority of the cells exhibited a SLEC phenotype after primary infection.

Together, these results suggest that activation of JEV S9-specific and WNV S9-specific memory CD8+ T cells occurs earlier in JEV-immune mice compared to WNV-immune mice following 2° heterologous viral infection. The expression patterns of the activation markers CD43, KLRG1, and CD127, corresponded with earlier proliferation of memory CD8+ T cells in JEV-immune mice. This lack of proliferation, and expression of activation markers in WNV-immune mice occurred even though a higher frequency of memory CD8+ T cells were present when compared to JEV-immune mice prior to 2° infection.

E. WNV infection induces neutralizing antibodies to JEV.

Cross-reactive neutralizing antibodies between JEV and WNV can be detected after repeated vaccine immunizations and the infection of immunodeficient hosts (74, 89, 158, 159). We wanted to determine whether infection with pathogenic JEV or WNV would induce cross-reactive neutralizing antibodies that might be restricting virus replication and thereby influencing the
Figure 4.5. PRNT$_{50}$ neutralizing antibody titers to JEV and WNV. Each serum sample was tested for *in vitro* neutralizing activity to both WNV and JEV. Serum was collected 1-8 months post (A) 1$^{\circ}$ WNV (N=15), (B) 1$^{\circ}$ JEV (N=6), (C) 1$^{\circ}$ WNV 2$^{\circ}$ JEV (N=7), and (D) 1$^{\circ}$ JEV 2$^{\circ}$ WNV (N=7) infection. Black line represents geometric mean of PRNT while dashed line represents the limit of detection (LOD) tested (1:20 dilution).
proliferation of memory CD8⁺ T cells. Sera from JEV- and WNV-immune mice (1-8 months after infection) were tested for neutralizing activity to both JEV and WNV in vitro. As expected, JEV and WNV infection induced high levels of neutralizing antibodies to their homologous virus strains as measured by 50% plaque reduction neutralization test (PRNT₅₀) values (Figure 4.5.A.-B.).

Interestingly in WNV-immune mice, we detected low levels of JEV neutralizing antibodies in all but two mice (Figure 4.5.A). This is in contrast to JEV-immune mice, in which neutralizing antibodies to WNV were not detectable (Figure 4.5.B.). These experiments do not rule out the possible presence of very low levels of antibodies to WNV in JEV-immune mice (LOD in our assay was 1:20).

Sera were also collected from JEV- and WNV-immune mice 1 to 6 months after secondary heterologous infection. After 2° JEV infection of WNV-immune mice, we measured only a modest boost in neutralizing antibodies to JEV (Figure 4.5.C.). This level of neutralizing antibodies was lower than that seen after a 1° JEV infection, suggesting that JEV neutralizing antibodies in WNV-immune mice may be inducing partial immunity to JEV and restricting virus replication. In contrast, WNV infection of JEV-immune mice resulted in the induction of high levels of neutralizing antibodies to WNV, similar to levels seen after 1° WNV infection (Figure 4.5.D.). These results suggest that 1) low levels of WNV neutralizing antibodies in JEV-immune mice were boosted to higher levels after WNV infection or 2) WNV infection of JEV-immune mice generated a 1° humoral
response. These findings support the notion that the presence of JEV neutralizing antibodies in WNV-immune mice leads to virus restriction, and may at least partially explain differences in CD8$^+$ T cell proliferation in WNV-immune mice upon 2° JEV infection.

F. **WNV S9-specific and JEV S9-specific CD8$^+$ T cells do not proliferate in WNV-immune mice, even in the absence of cross-reactive neutralizing antibodies.**

Since we detected cross-reactive neutralizing antibodies to JEV in WNV-immune mice, we next needed to determine whether antibody-dependent restriction of virus was the sole mechanism for the lack of proliferation of JEV S9-specific and WNV S9-specific CD8$^+$ T cells in WNV-immune mice. We hypothesized that infection of WNV-immune mice with rVV WNV and rVV JEV, which would not be neutralized by WNV, would result in the proliferation of JEV S9-specific and WNV S9-specific CD8$^+$ T cells. To test this hypothesis, JEV- and WNV-immune mice were infected with a high dose (5 x 10$^6$ pfu) of the rVVJs, and the frequency of JEV S9-specific and WNV S9-specific CD8$^+$ T cells were measured in the PBL on day 0 and day 5 after 2° infection.

We found that infection of JEV-immune mice with the homologous rVV JEV resulted in a 29.3 and 25.1 fold expansion of JEV S9-specific and WNV S9-specific CD8$^+$ T cells, respectively (Figure 4.6.A). A similar fold expansion was seen after infection with the heterologous rVV WNV virus (JEV S9: 19.8x;
Figure 4.6. Limited proliferation of JEV S9-specific and WNV S9-specific CD8+ T cells in WNV-immune mice after 2° rVV infection. (A) Rapid proliferation of JEV S9 and WNV S9 CD8+ T cells in JEV-immune mice. JEV-immune (1° JEV) mice were infected with 5 x 10^6 pfu rVV JEV (2° rVV JEV, left panel), rVV WNV (2° rVV WNV, middle panel), or rVV GFP (2° rVV GFP, right panel). JEV S9-specific and WNV S9-specific CD44^hi CD8+ T cells were measured on day 0 (D0) and day 5 (D5) after infection in the PBL. (B) Limited proliferation of JEV S9 and WNV S9 CD8+ T cells after 2° rVV infection in WNV-immune mice. Numbers represent fold expansion from D0 to D5 in the frequency of epitope-specific CD8+ T cells.
WNV S9: 34.5x. This level of expansion was similar to that seen after WNV infection of JEV-immune mice (Figure 4.4.A).

If JEV neutralizing antibodies were restricting JEV replication and thereby limiting the proliferation of epitope-specific CD8⁺ T cells in WNV-immune mice, we anticipated that rVV JEV infection would result in the proliferation of JEV S9-specific and WNV S9-specific CD8⁺ T cells. Interestingly, JEV S9-specific and WNV S9-specific CD8⁺ T cells in WNV-immune mice did not proliferate to the same extent as that seen in JEV-immune mice upon rVV WNV infection (Figure 4.6.B.). We saw only a 2.6 and 1.3 fold expansion in JEV S9-specific and WNV S9-specific CD8⁺ T cells after rVV JEV infection, respectively. A similar fold expansion was seen in WNV-immune mice after homologous rVV WNV infection (JEV S9: 2.4x; WNV S9: 2.3x). rVV GFP infection of JEV- and WNV-immune mice did not result in the expansion of JEV S9-specific and WNV S9-specific CD8⁺ T cells. Therefore, limited proliferation of JEV S9-specific and WNV S9-specific CD8⁺ T cells occurred in WNV-immune mice even in the absence of neutralizing antibodies. These results suggest that the difference in the proliferation of JEV S9-specific and WNV-specific S9 CD8⁺ T cells upon 2° heterologous infection is intrinsic to the CD8⁺ T cell population in WNV-immune mice.
G. WNV-immune CD8+ T cells are not functionally exhausted in the spleen.

Previous reports have demonstrated that WNV virus and/or RNA may persist in mice, monkeys and humans (24, 25, 28-30). Antigen persistence can lead to the exhaustion of epitope-specific CD8+ T cells and expression of programmed death-1 (PD-1) (268). Therefore, we hypothesized that the lack of CD8+ T cell proliferation in WNV-immune mice upon 2o JEV infection is due to exhaustion of epitope-specific CD8+ T cells.

To test this hypothesis, we analyzed JEV S9-specific and WNV S9-specific CD8+ T cells in the spleen and brain for expression of PD-1 in WNV-immune B6 mice (4-5 weeks post infection). We found a higher percentage of the CD8+ T cells in the brain were specific for the JEV S9 and WNV S9 tetramer compared to CD8+ T cells in the spleen (mean ± SEM: CNS: JEV S9: 10.36% ± 1.13%, WNV S9: 24.70% ± 2.63%; Spleen: JEV S9: 0.78% ± 0.11%, WNV S9: 2.49% ± 0.32%, N=4 mice) (Figure 4.7.A.-B.). Contrary to previously published results using the C3H mouse strain, we found cross-reactive CD8+ T cells by tetramer analysis in the CNS of WNV-immune mice (269).

All of the CD8+ T cells in the brain were activated CD8+ T cells expressing CD44, however, only a fraction of the CD8+ T cells in the spleen were CD44 hi, with all of the tetramer positive cells residing in this population (Figure 4.7.A). Interestingly, we also saw differences in the expression of KLRG1 between the CNS and spleen. In the spleen, 54.4% ± 2.7% (mean ± SEM) of the WNV S9-specific CD8+ T cells expressed KLRG1 compared to only 13.0% ± 1.6% in the
Figure 4.7. WNV-immune CD8+ T cells are not functionally exhausted in the spleen. (A-B) Frequency and phenotype of epitope-specific CD8+ T cells in the spleen and brain harvested from WNV-immune mice 4-5 weeks post infection. JEV S9-specific and WNV S9-specific CD44hi CD8+ T cells in the brains and spleen were analyzed for KLRG1, CD127 and PD-1 expression. (A) Representative plots from 1 mouse. (B) Summary plots for tetramer frequency and surface phenotype expression. (C) Memory CD8+ T cells in JEV- and WNV-immune mice secrete cytokines. Splenocytes from JEV-immune (top row) and WNV-immune (bottom row) mice were stimulated with media (left panels), 1 µg/ml JEV S9 (middle panels) and 1 µg/ml WNV S9 (right panels), and IFN-γ (y-axis) and TNF-α (x-axis) production was measured. (D). CD8+ T cell responses following 2° infection. Splenocytes from 1° JEV 2° WNV (top row) and 1° WNV 2° JEV (bottom row) infected mice on day 7 after 2° infection were stimulated with media (left panels), 1 µg/ml JEV S9 (middle panels) and 1 µg/ml WNV S9 (right panels), and IFN-γ (y-axis) and TNF-α (x-axis) production was measured.
CNS (Figure 4.7.B.). Although we saw tissue-specific differences in KLRG1 expression, JEV S9-specific and WNV S9-specific CD8+ T cells in both the spleen and brain had similar expression levels of CD127.

We detected low levels of PD-1 expression on JEV S9-specific and WNV S9-specific CD8+ T cells in the spleen (Figure 4.7.B.). However, there was an increase in the percentage of JEV S9-specific and WNV S9-specific CD8+ T cells expressing PD-1 in the brain (mean ± SEM: JEV S9: 22.4% ± 4.8%; WNV S9: 25.6% ± 4.2%) (Figure 4.7.B.). This modest expression of PD-1 on epitope-specific CD8+ T cells in the brain is in contrast to that seen in a chronic LCMV infection in which a majority of epitope-specific CD8+ T cells in the CNS express PD-1 (270). These results demonstrate that CD8+ T cells in the CNS and spleen of WNV-immune mice have different phenotypes, and only CD8+ T cells in the brain, a site of viral persistence, expressed PD-1, albeit at low levels (24).

A hallmark of exhausted CD8+ T cells is their inability to secrete cytokines upon peptide stimulation (271). Therefore, we next wanted to determine whether WNV-immune CD8+ T cells were functional at memory time points and after 2o infection. Similar to our analysis of the PBL (Figure 4.4.A), WNV-immune mice exhibited a higher frequency of both JEV S9-specific and WNV S9-specific CD8+ T cells that secreted cytokines compared to JEV-immune mice at the memory time point (Figure 4.7.C.). JEV-immune CD8+ T cells converted from secreting only IFN-γ during the primary immune response (Figure 3.4.), to secreting both IFN-γ and TNF-α upon JEV S9 and WNV S9 peptide stimulation. WNV-immune
CD8+ T cells were also multifunctional, secreting both IFN-γ and TNF-α at the memory time point, confirming that WNV-immune CD8+ T cells in the spleen were not functionally exhausted at this time point (Figure 4.7.C.).

After 2o WNV infection of JEV-immune mice, similar to tetramer analysis of the PBL (Figure 4.4.A), there was an increase in the frequency of both JEV S9-specific and WNV S9-specific CD8+ T cells in the spleen (Figure 4.7.D). The cytokine profiles switched from being predominantly IFN-γ+ TNF-α+ CD8+ T cells to IFN-γ+ CD8+ T cells. In WNV-immune mice after 2o JEV infection, the frequency of cytokine producing JEV S9-specific and WNV S9-specific CD8+ T cells did not increase from the memory time point. However, both JEV S9-specific and WNV S9-specific CD8+ T cells were functional, suggesting again that WNV-immune CD8+ T cells in the spleen were not functionally exhausted. These results also support the analysis of the PBL in suggesting that very little proliferation of WNV-immune CD8+ T cells is seen following 2o JEV infection.

Since we saw very little proliferation of WNV-immune CD8+ T cells upon 2o JEV infection in vivo, we next wanted to determine whether WNV-immune CD8+ T cells were capable of proliferating in a naïve environment. This would enable us to determine whether the lack of proliferation was intrinsic to WNV-immune T cells or due to the in situ environment generated after a WNV infection. CFSE labeled JEV-and WNV-immune splenocytes (1-2 x 10^7 cells) were adoptively transferred into naive Ly5.1 congenic mice, and the following day, the mice were infected with PBS (control), JEV (1 x 10^3 pfu), or WNV (1 x 10^3 pfu). Five days
after infection, both CD4+ T cell and CD8+ T cell proliferation were measured by dilution of CFSE. We found that for both JEV- and WNV-immune donor CD8+ T cells, a higher percentage proliferated upon homologous infection (1o JEV→ 2o JEV; 1o WNV→ 2o WNV) compared to heterologous infection (1o JEV→WNV; 1o WNV→ 2o JEV), but this difference did not reach statistical significance (mean % proliferation ± SEM: JEV-immune: 2o JEV: 45.2% ± 11.5%; 2o WNV: 17.3% ± 6.1%, P=0.2, Mann-Whitney U; WNV-immune: 2o WNV: 29.9% ± 8.9%; 2o JEV: 9.3% ± 2.5%, P=0.13, Mann-Whitney U) (Figure 4.8.A.-B.). We detected very little homoeostatic proliferation of both JEV and WNV-immune donor splenocytes upon the injection with PBS (mean % proliferation ± SEM: JEV-immune: 0.8% ± 0.2%; WNV-immune: 3.7% ± 1.7%).

A majority, but not all, of the proliferating CD8+ T cells were specific for the JEV S9 or WNV S9 epitope after 2o heterologous infection (mean % proliferation ± SEM: JEV-immune: 2o WNV: JEV S9: 55.8% ± 9.4%, WNV S9: 49.5% ± 10.8%; WNV-immune: 2o JEV: JEV S9: 24.9% ± 6.4%, WNV S9: 30.4% ± 6.6%) (Figure 4.8.C.-D.). Only a small percentage of the proliferating CD8+ T cells were specific for the subdominant cross-reactive CD8+ T cell epitope that was recently identified, JEV E1 and WNV E1 (mean % proliferation ± SEM: JEV-immune: 2o WNV: JEV E1: 0.8% ± 0.8%, WNV E1: 4.1% ± 2.1%; WNV-immune: 2o JEV: JEV E1: 8.4% ± 2.4%, WNV E1: 3.3% ± 1.1%) (198). These results confirm that the WNV S9 and JEV S9 epitope is the dominant cross-reactive epitope. Since the two cross-reactive epitopes that were studied did not account for all for the
Figure 4.8. Proliferation of JEV- and WNV-immune T cells after 2° infection in naïve hosts. (A) Representative plots of CFSE dilution (x-axis) on CD8+ T cells from 1 JEV-immune (top row) or WNV-immune (bottom row) donor mouse after 2° PBS (left panels), 1 x 10³ pfu JEV (middle panels), or 1 x 10³ pfu WNV (right panels) infections. (B) Summary of CD8+ T cell proliferation from JEV-immune (1° JEV) or WNV-immune (1° WNV) mice. Black bar and error bars represent mean ± SEM. (C) Frequency of epitope-specific IFN-γ CFSE<sup>low</sup> CD8+ T cells from JEV donor mice after 2° JEV (red circles) or WNV (blue squares)
infection. Black bar and error bars represent mean ± SEM. (D) Frequency of epitope-specific IFN−γ+ CFSElow CD8+ T cells from WNV donor mice after 2o JEV (red circles) or WNV (blue squares) infection. Black bar and error bars represent mean ± SEM. (E) Representative plot of CFSE dilution (x-axis) on CD4+ T cells from one JEV-immune (top row) or WNV-immune (bottom row) donor mouse after 2o PBS (left panels), 1 x 10^3 pfu JEV (middle panels), or 1 x 10^3 pfu WNV (right panels) infections. (F) Summary of CD4+ T cell proliferation from JEV-immune (1o JEV) or WNV-immune (1o WNV) mice. Black bar and error bars represent mean ± SEM. (G) Frequency of epitope-specific IFN−γ+ CFSElow CD4+ T cells from JEV donor mice after 2o JEV (red circles) or WNV (blue squares) infection. Black bar and error bars represent mean ± SEM. (H) Frequency of epitope-specific IFN−γ+ CFSElow CD4+ T cells from WNV donor mice after 2o JEV (red circles) or WNV (blue squares) infection. Black bar and error bars represent mean ± SEM.
proliferating CD8$^+$ T cells, there are likely additional JEV/WNV cross-reactive CD8$^+$ T cell epitopes that have yet to be identified.

As was seen for CD8$^+$ T cells, a higher percentage of CD4$^+$ T cells proliferated in responses to homologous infection compared to heterologous infection (mean % proliferation ± SEM: JEV-immune: 2$^o$ JEV 43.5% ± 1.9%; 2$^o$ WNV: 17.4% ± 0.7%; WNV-immune: 2$^o$ JEV 12.7% ± 2.0%; 2$^o$ WNV: 31.7% ± 2.0%) (Figure 4.8.E.-F.). Interestingly, the cross-reactive epitopes, NS1 and NS3, accounted for a only small fraction of the proliferating CD4$^+$ T cells after heterologous infection (mean % proliferation ± SEM: JEV-immune: 2$^o$ WNV: JEV NS1: 9.7% ± 2.1%, WNV NS1: 4.9% ± 1.2%; JEV NS3: 1.4% ± 0.9%, WNV NS3: 1.6% ± 1.4%; WNV-immune: 2$^o$ JEV: JEV NS1: 6.1% ± 2.0%, WNV NS1: 4.9% ± 1.8% JEV NS3: 2.3% ± 0.7%, WNV NS3: 3.8% ± 1.1%) (Figure 4.8.G.-H.). These results suggest that there are additional cross-reactive CD4$^+$ T cell epitopes yet to be identified.

Taken together, these results confirm that CD4$^+$ and CD8$^+$ T cells are not functionally exhausted in WNV-immune mice. The lower level of proliferation of T cells upon heterologous infection may be due to a limited number of cross-reactive T cells, and the variability between donor mice may reflect the individual private specificities in the T cell repertoire of each donor mouse (174). Since exhaustion of WNV-immune CD8$^+$ T cells was not limiting T cell proliferation upon 2$^o$ JEV infection, additional mechanisms for this lack of expansion needed to be explored.
H. Analysis of the amino acid differences between the JEV S9 and WNV S9 peptides.

The JEV S9 and WNV S9 amino acid sequences differ by only two amino acids at the P2 and P6 positions of the epitope. In order to address the possible contribution of these residues to the lack of proliferation in WNV-immune mice upon 2° JEV infection, the P6 amino acid in JEV S9 was mutated (S→A) to generate JEV P6A, and the P6 amino acid in WNV S9 was mutated (A→S) to generate WNV P6S (Table 4.1.). These mutations also allowed us to compare the P2 amino acids because JEV P6A contains the corresponding S→A amino acid change seen in WNV S9. Likewise, the WNV P6S mutant peptide contains the A→S amino acid change at the P2 position of JEV S9.

First, we examined whether the P2 or P6 residues affected the ability of the peptides to bind to MHC. To test this, we used RMA-S cells, which lack the TAP protein, and therefore are defective in presenting endogenous antigens (272, 273). Exogenously added peptides bind and stabilize unloaded MHC on the cell surface, and the expression of the MHC on the surface depends upon the stability of the peptide-MHC complex (274). RMA-S cells were loaded with $10^{-4} - 10^{-8}$ µg/ml of each peptide and incubated overnight to allow for surface stabilization of the peptide-MHC complex. The cells were then transferred to a 37°C incubator to allow for internalization of unbound MHC, and H-2D expression on the cell surface was measured. Both JEV S9 and
Table 4.1. Amino acid sequences of JEV S9 and WNV S9 mutants

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
<th>Red letter indicates amino acid change in mutant.</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEV S9</td>
<td>SAVWNSTTA</td>
<td></td>
</tr>
<tr>
<td>JEV P6A</td>
<td>SAVWNA\textsuperscript{TTA}</td>
<td></td>
</tr>
<tr>
<td>WNV S9</td>
<td>SSVWNATTA</td>
<td></td>
</tr>
<tr>
<td>WNV P6S</td>
<td>SSVWN\textsuperscript{STTA}</td>
<td></td>
</tr>
</tbody>
</table>
JEV P6A, which contains the same P2 amino acid, stabilized H-2D$^b$ on the surface of RMA-S cells to a significantly higher level compared to WNV S9 and WNV P6S at a peptide concentration of $10^{-6}$ $\mu$g/ml ($P<0.05$; Mann-Whitney U) (Figure 4.9.A.). No significant difference was seen between peptides that differed at P6 in their ability to stabilize MHC. These results demonstrate that alanine at the P2 position helps the JEV peptides bind to MHC better than the serine in the WNV peptides.

Since the P2 amino acid affects MHC binding, we hypothesized that the P6 amino acid would affect binding to the TCR. To test this hypothesis, bone marrow derived dendritic cells (BMDCs) were peptide-pulsed with 10 $\mu$g/ml of the JEV S9, JEV P6A, WNV S9, or WNV P6S peptides and adoptively transferred iv into naïve mice. On day 7 after adoptive transfer, PBLs were analyzed by tetramer stain for specificity of the CD8$^+$ T cells to either the JEV S9 or WNV S9 tetramer. After JEV S9 immunization, the ratio of WNV S9-specific to JEV S9-specific CD8$^+$ T cells was less than 1 (ratio mean ± SEM; 0.85 ± 0.04), demonstrating a higher frequency of JEV S9-specific CD8$^+$ T cells than WNV S9-specific CD8$^+$ T cells in these mice (Figure 4.9.B.). On the other hand, in WNV S9-immunized mice, the ratio was greater than 1 (ratio mean ± SEM; 1.65 ± 0.07), demonstrating a higher frequency of WNV S9-specific CD8$^+$ T cells compared to JEV S9-specific CD8$^+$ T cells.

After immunization with the mutant peptides, the ratio of CD8$^+$ T cells binding to the WNV S9 and JEV S9 tetramers was altered. The CD8$^+$ T cell
Figure 4.9. The P2 and P6 amino acids of JEV S9 and WNV S9 affect MHC stabilization and tetramer specificity. (A) P2 amino acid affects MHC Stabilization. RMA-S cells were loaded with $10^{-4} - 10^6 \mu g/ml$ of peptide. Percent loss of stabilization was calculated using $10^{-4} \mu g/ml$ concentration as the max stabilization of H-2Db MHC. * P<0.05 comparing both of the JEV peptides versus the two WNV peptides, Mann-Whitney U. N=5 independent experiments. (B) P6 amino acid affects tetramer binding to CD8+ T cells. Peptide-loaded BMDCs (10 $\mu g/ml$) were adoptively transferred into naïve male B6.SJL mice. JEV S9-specific and WNV S9-specific CD44hi CD8+ T cells were analyzed in the PBL on day 7 after adoptive transfer. N=4 mice/peptide.
population in JEV P6A immunized mice switched from being specific to the JEV S9 tetramer to becoming more specific to the WNV S9 tetramer (mean ratio \( \pm \) SEM; 1.28 \( \pm \) 0.03). Likewise, immunization with WNV P6A changed the specificity of the CD8\(^+\) T cells from being WNV S9 specific to JEV S9 specific (mean ratio \( \pm \) SEM; 0.93 \( \pm \) 0.06) (Figure 4.9.B.). Taken together, these data confirm that the P2 amino acid affects MHC stability while the P6 amino acid helps to determine the specificity of the CD8\(^+\) T cell population for the tetramers.

I. Functional avidity of cross-reactive CD8\(^+\) T cells in JEV- and WNV-infected mice.

Memory CD8\(^+\) T cell proliferation upon secondary heterologous infection can be influenced by the functional avidity of CD8\(^+\) T cells for the cross-reactive epitope. In fact, memory CD8\(^+\) T cells specific for the NP\(_{366-374}\) epitope of influenza have reduced recall responses and viral clearance upon infection with a heterologous influenza viral strain (185). On day 7 after JEV and WNV infection, IFN\(\gamma\) dose responses to the homologous and heterologous epitopes were measured, and the concentration of each peptide that elicited 50% of the maximal IFN\(\gamma\) response was calculated (EC\(_{50}\)), and compared between each infection. A low EC\(_{50}\) concentration means that the CD8\(^+\) T cells have a high functional avidity for the epitope.

In both JEV and WNV infections, the homologous epitope, JEV S9 for the JEV infection and WNV S9 for the WNV infection, had a higher functional avidity
compared to the heterologous epitope (Figure 4.10. and Table 4.2.). Also in both infections, there is only a ½ log difference in the EC$_{50}$ values between the homologous JEV S9 and the heterologous WNV S9 epitope. Interestingly, there is less than a log difference in the EC$_{50}$ values for the same epitope regardless of the infecting virus. The EC$_{50}$ values of the CD8$^+$ T cells only increased slightly at memory time points (Table 4.2.). These data demonstrate that WNV infection induces a CD8$^+$ T cell population with similar functional avidities to the cross-reactive epitope as a JEV infection. Therefore, a lower functional avidity of the WNV S9-specific CD8$^+$ T cells for the JEV S9 epitope compared to JEV S9-specific CD8$^+$ T cells for the WNV S9 epitope does not account for differences in proliferation during 2º infection.

**J. TCR V$\beta$ usage of JEV S9-specific and WNV S9-specific CD8$^+$ T cells during primary JEV and WNV infection.**

We next hypothesized that the amino acid differences between JEV S9 and WNV S9 may be inducing two distinct TCR V$\beta$ repertoires that proliferate differently upon 2º heterologous infection. Both JEV S9-specific and WNV S9-specific IFN-γ$^+$ CD8$^+$ T cells in JEV and WNV-infected mice were analyzed for TCR V$\beta$ usage on day 7 after infection. Both JEV and WNV infections induced a broad TCR repertoire that utilized multiple V$\beta$s. TCR V$\beta$ 13 was found to be the dominant TCR V$\beta$ utilized by epitope-specific CD8$^+$ T cells in both JEV- and WNV-infected mice, however the proportions were different (mean % frequency:
Figure 4.10. Functional avidity of JEV S9-specific and WNV S9-specific CD8⁺ T cells in JEV- and WNV-immune mice. Splenocytes from JEV (A) or WNV (B) infected mice on day 7 after infection were stimulated with either varying concentrations (x-axis) of JEV S9 (red circle) or WNV S9 (blue square) peptides, and the frequency of IFN-γ⁺ CD8⁺ T cells were measured. N=3-mice/data point.
### Table 4.2. EC$_{50}$ values for IFN–γ production by JEV S9-specific and WNV S9-specific CD8$^+$ T cells.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Time point</th>
<th>Peptide</th>
<th>EC$_{50}$ Value (g/ml)$^{a,b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEV</td>
<td>Day 7</td>
<td>JEV S9</td>
<td>$10^{-10.24}$</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>WNV S9</td>
<td>$10^{-9.86}$</td>
</tr>
<tr>
<td></td>
<td>Day 28</td>
<td>JEV S9</td>
<td>$10^{-10.81}$</td>
</tr>
<tr>
<td></td>
<td>Day 28</td>
<td>WNV S9</td>
<td>$10^{-10.43}$</td>
</tr>
<tr>
<td>WNV</td>
<td>Day 7</td>
<td>JEV S9</td>
<td>$10^{-9.64}$</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>WNV S9</td>
<td>$10^{-10.03}$</td>
</tr>
<tr>
<td></td>
<td>Day 28</td>
<td>JEV S9</td>
<td>$10^{-9.52}$</td>
</tr>
<tr>
<td></td>
<td>Day 28</td>
<td>WNV S9</td>
<td>$10^{-10.35}$</td>
</tr>
</tbody>
</table>

$^a$ EC$_{50}$ values calculated using GraphPad Prism software  
$^b$ N=4 mice/group except for JEV on Day 28 where N=2 mice
JEV-immune: JEV S9-specific CD8⁺ T cells: Vβ 13: 26.3%; WNV-immune: WNV S9-specific CD8⁺ T cells: Vβ 13: 33.7%) (Figure 4.11.). Vβ 8.1/8.2 and Vβ 7 were two subdominant TCR Vβs found in both JEV and WNV infections.

We also analyzed the TCR Vβ repertoires after rVV JEV, rVV WNV, and Kunjin virus infections to determine if the TCR Vβ repertoires are different between virus infections containing the same epitope. The TCR Vβ usage during a rVV WNV and Kunjin virus infection was similar to WNV, with Vβ 13 being the dominant TCR Vβ and Vβ 8.1/8.2 being subdominant (Figure 4.11.). Likewise, the TCR Vβ usage of the CD8⁺ T cell population after rVV JEV infection was similar to the repertories in JEV-infected mice. In conclusion, the same epitope induces similar TCR Vβ repertoires regardless of the viral infection, and different TCR Vβ usage during the primary infection between JEV and WNV does not appear to account for the differences in proliferation upon 2⁰ infection. However, TCR Vβ usage after a secondary heterologous infection is highly variable and depends upon the private specificities of the host (176). Therefore, the TCR Vβ repertoires after 2⁰ infection may differ in JEV- and WNV-immune mice, and will need to be investigated further.
Figure 4.11. TCR Vβ Usage of JEV S9-specific and WNV S9-specific IFN-γ+ CD8+ T cells on day 7 following primary infection. Splenocytes from JEV (A), WNV (B), rVV JEV (C), rVV WNV (D), and Kunjin (E) infected mice were harvested on day 7 after infection and stimulated with 1 µg/ml JEV S9 (red) and WNV S9 (blue) for 5 hours. Cells were stained with anti-CD3, -CD8+, -IFN-γ and one TCR Vβ. The frequency of CD3+ CD8+ IFN-γ+ T cells for each TCR Vβ is shown on the y-axis.
K. The proliferation of memory WNV S9-specific CD8+ T cells upon 2° homologous and heterologous infection depends on the primary infection.

Neither the functional avidity nor the TCR Vβ usage of CD8+ T cells in JEV and WNV appear to account for the differences in proliferation upon 2° heterologous infection. Therefore, we next determined whether the WNV S9 epitope was able to induce a memory CD8+ T cell population that could proliferate upon 2° JEV infection in the context of a different primary viral infection. B6 mice were immunized with rVV JEV, rVV WNV, and rVV GFP and 4-5 weeks after primary infection, the mice were infected with either JEV or WNV. JEV S9-specific and WNV S9-specific CD8+ T cells were analyzed in the PBL by tetramer staining on day 0 (memory frequency) and day 5 after 2° infection.

Infection of rVV JEV-immune mice with a low dose WNV infection (1 x 10^3 pfu) resulted in a 3.8 and 5.9 fold expansion of JEV S9-specific and WNV S9-specific CD8+ T cells, respectively (Figure 4.12.A.). This infection scenario is similar to infection of JEV-immune mice with WNV, in which we see proliferation of both JEV S9-specific and WNV S9-specific CD8+ T cells upon 2° infection. A similar fold expansion was seen after homologous infection of rVV WNV-immune mice with WNV (JEV S9: 3.9x; WNV S9: 4.6x). In native virus infection, (e.g. WNV infection of WNV-immune mice) proliferation of JEV S9-specific and WNV S9-specific CD8+ T cells would likely not occur due to the presence of neutralizing antibodies. Importantly, infection of rVV GFP immune mice, which
Figure 4.12. Primary rVV infection generates memory WNV S9-specific CD8\(^+\) T cells that proliferate upon 2\(^{\circ}\) JEV infection. rVV JEV (left panels), rVV WNV (middle panels), rVV GFP (right panels) immune mice were infected with (A) low dose WNV (1 x 10\(^3\) pfu), (B) high dose WNV (1 x 10\(^5\) pfu), or (C) high dose JEV (1 x 10\(^6\) pfu). On day 0 (D0) and day 5 (D5) after infection, JEV S9-specific and WNV S9-specific CD44\(^{hi}\) CD8\(^+\) T cells were analyzed by tetramer stain in the PBL. Numbers represent fold expansion of tetramer positive CD8\(^+\) T cells between D0 and D5.
lack memory JEV S9-specific and WNV S9-specific CD8⁺ T cells, did not result in proliferation of these populations upon 2⁰ WNV infection. Infection of rVV JEV-immune mice with a high dose WNV infection (1 x 10⁵ pfu) resulted in 11.4 and 18.7 fold increase in JEV S9-specific and WNV S9-specific CD8⁺ T cells, respectively (Figure 4.12.B.). A similar increase in the fold expansion was seen in rVV WNV was seen after the high dose WNV infection (JEV S9: 10.8x, WNV S9: 13.1x). These results suggest that the dose of the 2⁰ infection can also influence the fold expansion of memory CD8⁺ T cells.

Although minimal proliferation of JEV S9-specific and WNV S9-specific CD8⁺ T cells was seen after 2⁰ JEV infection of WNV-immune mice, infection of rVV WNV-immune mice with JEV (1 x 10⁶ pfu) resulted in an 11.9 and 13.1 fold expansion of the frequency of JEV S9-specific and WNV S9-specific CD8⁺ T cells, respectively (Figure 4.12.C.). A similar fold expansion was seen in rVV JEV-immune mice (JEV S9: 11.4x and WNV S9: 9.5x). Therefore, the WNV S9 epitope in the context of a 1⁰ rVV infection induces a memory CD8⁺ T cell population that proliferates upon a 2⁰ heterologous JEV infection. Our findings suggest that the type of primary infection dictates the quality of the memory CD8⁺ T cell responses and resultant expansion upon a 2⁰ infection rather than the epitope itself.
L. An attenuated WNV (Kunjin virus) induces a memory CD8⁺ T cell population that proliferates upon 2° JEV infection.

The cytokine milieu during a primary infection can influence the generation of memory CD8⁺ T cells (211). rVV and WNV stimulate different components of the innate immune system, which then may lead to the induction of different cytokines that could alter the formation of the memory CD8⁺ T cell compartment (275, 276). Therefore, comparing rVV and WNV in their ability to induce a memory CD8⁺ T cell population that proliferates upon 2° infection, may not be an adequate comparison. To address this concern, we used Kunjin virus, which has a 100% conserved WNV S9 amino acid sequence as WNV. Use of this virus permitted us to determine the effects of pathogenicity and sensitivity to type I IFN of the infecting 1° WNV strain on memory CD8⁺ T cell generation and subsequent ability of these memory CD8⁺ T cells to proliferate upon a 2° JEV infection.

B6 mice were infected with 1 x 10³ pfu of Kunjin virus, and 5 weeks post infection, the mice were challenged with a lethal dose of JEV (1 x 10⁷ pfu) at the same time as the WNV-immune mice in Figure 4.2.A. Similar to WNV-immune mice, Kunjin immune mice were protected against a lethal JEV challenge compared to PBS (Kunjin: 85.7% survival, 2/14 mice; PBS: 50% survival, 7/14 mice; P=0.059, Log-Rank test) (Figure 4.13.A.). We hypothesized that cross-reactive CD8⁺ T cells in Kunjin-immune mice would not proliferate upon 2° JEV infection due to the identical epitopes between WNV and Kunjin. Prior to 2°
Figure 4.13. Memory WNV S9-specific CD8$^+$ T cells in Kunjin-immune mice proliferate after 2$^\circ$ JEV infection. (A) Kunjin-immune mice are protected against lethal JEV infection. B6 mice were infected with $1 \times 10^3$ pfu Kunjin virus (N=14 mice) or PBS (N=14 mice) and 5 weeks later infected with $1 \times 10^7$ pfu JEV. The mice were monitored daily for survival. P=0.059, Log-Rank Test. (B) JEV S9-specific and WNV S9-specific CD8$^+$ T cell frequency in PBL after 2$^\circ$ JEV infection. (C-E) Expression of CD43 (C), KLRG1 (D), and CD127 (E) on JEV S9-specific (red circle) and WNV S9-specific (blue square) CD44$^{hi}$ CD8$^+$ T cells. Black bar and error bars represent mean ± SEM.
infection, the frequency of JEV S9 and WNV S9 in Kunjin-immune mice (Figure 4.13.B.) was considerably lower in the PBL compared to WNV-immune mice, but comparable to that in JEV-immune mice (Figure 4.4.A.) (mean ± SEM: Kunjin-immune: JEV S9: 0.22% ± 0.07%, WNV S9: 0.60% ± 0.15%).

In contrast to WNV-immune mice, 2o JEV infection of Kunjin-immune mice resulted in the expansion of JEV S9-specific and WNV S9-specific CD8+ T cells by day 5 after infection (mean ± SEM: JEV S9: 3.80% ± 0.36%; WNV S9: 7.18 ± 0.54%) (Figure.4.13.B.). The frequency of JEV S9-specific and WNV S9-specific CD8+ T cells continued to increase on day 7 (mean ± SEM: JEV S9: 11.78% ± 0.61%, WNV S9: 16.98% ± 0.85%). Even though WNV and Kunjin share the same sequence of the WNV S9 epitope, the memory WNV S9 CD8+ T cells in Kunjin-immune mice responded differently upon a 2o JEV infection compared to WNV-immune mice.

The upregulation of CD43 on JEV S9-specific and WNV S9-specific CD8+ T cells also differed in Kunjin-immune mice compared to WNV-immune mice. CD43 upregulation began on day 3, and reached maximal expression by day 5 after infection (Figure 4.13.C.). Upregulation of KLRG1 and downregulation of CD127 began by day 3 and reached its peak on day 7, suggesting a conversion from a predominantly MPEC population to a SLEC population by day 7 (Figure 4.13.D.-E.). This expression pattern of CD43 is similar to a 2o WNV infection of JEV-immune mice (Figure 4.4.B.), in which CD43 upregulation preceded expansion of JEV S9-specific and WNV S9-specific CD8+ T cells, and by day 7 in
all infection, a majority of the CD8$^+$ T cells had a SLEC phenotype. These results further support the notion that the context of the primary viral infection dictates the ability of memory CD8$^+$ T cells to proliferate upon a 2$^o$ infection, rather than differences in the epitope sequences (JEV S9 versus WNV S9).

**M. Dendritic cell (DC) immunizations also generate a WNV S9-specific CD8$^+$ T cell population that proliferates upon 2$^o$ JEV infection.**

After DC immunization with the JEV and WNV peptide mutants, we challenged the mice with a low dose of either JEV (1 x 10$^3$ pfu) or WNV (1 x 10$^3$ pfu), to determine whether the amino acid differences between JEV S9 and WNV S9 had any effect on proliferation during 2$^o$ heterologous infection. After 2$^o$ JEV infection, the frequency of JEV S9-specific and WNV S9-specific CD8$^+$ T cells in mice immunized with all 4 peptides increased in the PBL from day 0 to day 5 (Figure 4.14.A.). Most importantly, JEV S9-specific and WNV S9-specific CD8$^+$ T cells in WNV S9-immunized mice expanded upon a 2$^o$ JEV infection, demonstrating that DC immunization also induces a WNV S9-specific CD8$^+$ T cell population that proliferates upon 2$^o$ JEV infection.

Following a low dose WNV infection, we saw rapid proliferation in all of the peptide-immunized mice by day 5 after infection (Figure 4.14.B.). In addition, the magnitude of expansion was higher after 2$^o$ WNV infection compared to a 2$^o$ JEV infection. In some mice, the frequency of WNV S9-specific CD8$^+$ T cells exceeded 40% in the PBS after a 2$^o$ WNV infection. We believe this difference in
Figure 4.14. DC Immunization generates memory WNV S9-specific CD8+ T cells that proliferate upon 2° JEV infection. BMDCs were pulsed with 10 µg/ml JEV S9, JEV P6A, WNV S9, or WNV P6S (x-axis) and adoptively transferred into naïve mice. 4-6 weeks after transfer, the mice were infected with (A) 1 x 10³ pfu JEV or (B) 1 x 10³ pfu WNV. JEV S9-specific (red circle) and WNV S9-specific (blue square) CD44hi CD8+ T cells were measured on day 0 (D0) and day 5 (D5) after infection in the PBL. Black bar and error bars represent mean ± SEM.
magnitude of expansion is due to the ability of WNV to replicate in the periphery compared to JEV at this low dose (1 x 10^3 pfu). Increasing the dose of the 2° JEV infection may increase the expansion of CD8^+ T cells similar to that seen in a 2° WNV infection. Therefore, DC immunization also induces a WNV S9-specific CD8^+ T cell population that proliferates upon 2° JEV infection.

N. Restriction of rVV in the ovaries by JEV and WNV-immune mice.

Since the context of the primary infection dictates the proliferation of memory WNV S9-specific CD8^+ T cells upon 2° JEV infection, we next determined whether WNV-immune CD8^+ T cells were able to restrict virus independent of proliferation. WNV-immune, JEV-immune or PBS (control) B6 mice were infected with a high dose (5 x 10^6 pfu) or low dose (rVV WNV: 1 x 10^5 pfu; rVV JEV and rVV GFP: 5 x 10^5 pfu) of the rVV and viral titers in the ovaries were measured. This design permitted us to study the role of JEV S9-specific and WNV S9-specific CD8^+ T cells in restricting virus since WNV antibodies should not neutralize the rVVs.

In WNV-immune mice, we saw a significant reduction in the median viral titers after infection with the higher dose of the homologous rVV WNV (median titer: 2.4 x 10^4 pfu) compared to both JEV-immune (2.9 x 10^6 pfu; P<0.05) and PBS control mice (1.7 x 10^6 pfu, P<0.01; Mann-Whitney U) (Figure 4.15.A.). There was no significant reduction in the rVV WNV titers in JEV-immune mice compared to PBS controls (P<0.54, Mann-Whitney U). There was a slight
Figure 4.15. Restriction of rVV in the ovaries of JEV- and WNV-immune mice. (A) WNV-immune (1<sup>st</sup> WNV), JEV-immune (1<sup>st</sup> JEV), or PBS control mice were infection with high dose (5 x 10<sup>6</sup> pfu) rVV WNV (left panel), rVV JEV (middle panel) or rVV GFP (right panel). Ovaries were harvested on day 5 after infection. (B) Infection of WNV-immune, JEV-immune or PBS mice with low dose rVV WNV (1 x 10<sup>5</sup> pfu), rVV JEV (5 x 10<sup>5</sup> pfu), or rVV GFP (5 x 10<sup>5</sup> pfu). Ovaries were harvested on day 6 after infection. Each data point represents one mouse, and black bar represents median value. *P<0.05, **P<0.01, Mann-Whitney U.
reduction in the titers of the heterologous rVV JEV in WNV-immune mice compared to JEV-immune and PBS control mice (median titer: WNV-immune: 1.2 x 10^4 pfu; JEV-immune: 5.0 x 10^5 pfu; PBS: 8.6 x 10^5 pfu; P<0.2, Mann-Whitney U), but these differences were not statistically significant. Importantly, there was no difference in the titers of rVV GFP in WNV-immune, JEV-immune or PBS control mice, confirming the specificity of this viral restriction to the JEV S9-specific and WNV S9-specific CD8^+ T cell epitopes.

Even though we saw rapid proliferation of JEV S9-specific and WNV S9-specific CD8^+ T cells in JEV-immune mice after 2^o rVV infection (Figure 4.6.A.), we did not observe any restriction of either the homologous or heterologous rVV in the ovaries compared to PBS control mice (2^o rVV WNV: P=0.5; 2^o rVV JEV: P=0.7; Mann-Whitney U) On the other hand, significant restriction of rVV WNV, and to a lesser degree, rVV JEV, occurred in WNV-immune mice in the absence of CD8^+ T cell proliferation. We hypothesized that this lack of restriction in JEV-immune mice was due to a low starting frequency of memory CD8^+ T cells prior to infection, and/or due to the high dose of rVV used in the infection.

In order to make the environment more favorable for the CD8^+ T cells, we lowered the infection dose (rVV WNV: 1 x 10^5 pfu, rVV JEV and rVV GFP: 5 x 10^5 pfu) and harvested the ovaries on day 6 rather than day 5 to allow for a greater expansion of epitope-specific memory CD8^+ T cells. In WNV-immune mice, we detected near complete restriction of the homologous rVV WNV at the lower dose and later harvest day compared to PBS control mice (median titer:
WNV-immune: 333 pfu, PBS: 2.3 x 10^6 pfu P<0.01, Mann-Whitney U) (Figure 4.15.B.). Virus levels of the heterologous rVV JEV were also restricted in WNV-immune mice compared to PBS control mice (WNV-immune: 1.1 x 10^5 pfu, PBS: 4.3 x 10^6 pfu; P<0.05, Mann-Whitney U).

In contrast to the high dose infection, there was a slight reduction in the median titers of rVV WNV in JEV-immune mice compared to PBS control mice upon the low dose infection (JEV-immune: 6.1 x 10^5 pfu, PBS: 1.7 x 10^6 pfu; P=0.066, Mann-Whitney U), but this reduction was not statistically significant. After the lower dose and later harvest time point, a significant reduction in homologous rVV JEV titers in JEV-immune mice occurred (median titer: JEV-immune: 4.5 x 10^4 pfu, P<0.05, Mann-Whitney U) (Figure 4.15.B.). Again, no restriction of virus was seen in mice infected with the control rVV GFP virus. Therefore, JEV-immune mice were better able to restrict the rVVVs at a lower dose and at a later time point. These results also demonstrate that WNV-immune mice are better able to restrict both low and high dose of 2° rVVVs compared to JEV-immune mice, even though there is limited proliferation of memory CD8^+ T cells during 2° infection.

**O. Restriction of low and high doses of the rVVVs by Kunjin-immune mice.**

After 2° JEV infection of Kunjin-immune mice, we saw rapid proliferation of memory JEV S9-specific and WNV S9-specific CD8^+ T cells (Figure 4.13.B.). Therefore, we next wanted to determine whether restriction of low and high
doses of the rVV’s by Kunjin-immune mice was similar to that seen in either JEV-immune mice or WNV-immune mice. After high dose rVV WNV infection \((5 \times 10^6 \text{ pfu})\), we measured a small but significant reduction in viral titers in Kunjin-immune mice \((\text{median titer: } 1.7 \times 10^5 \text{ pfu})\) compared to PBS mice \((8.6 \times 10^5 \text{ pfu}, \text{P}<0.05, \text{Mann-Whitney U})\) (Figure 4.16.A.). However, there was no significant reduction in the titers of either rVV JEV \((\text{median titer: } 9.3 \times 10^5 \text{ pfu})\) or rVV GFP \((\text{median titer: } 3.4 \times 10^5 \text{ pfu})\) in Kunjin-immune mice compared to the PBS controls \((\text{median titer: } \text{rVV JEV: } 8.6 \times 10^5; \text{P}=0.7; \text{rVV GFP: } 1.6 \times 10^6 \text{ pfu}; \text{P}=0.4; \text{Mann-Whitney U})\).

After infection with a lower dose of the rVV \((5 \times 10^5 \text{ pfu})\), there was no significant restriction in rVV WNV, rVV JEV or rVV GFP titers on day 5 after infection in Kunjin-immune mice (Figure 4.16.B.) Interestingly, this lack of restriction occurred in the context of memory JEV S9-specific and WNV S9-specific CD8 T cell proliferation similar to what we see after rVV infection of JEV-immune mice (Figure 4.15.) These data suggest that low frequencies of memory CD8\(^+\) T cells may not be able to efficiently restrict virus after \(2^{nd}\) heterologous infection.

**P. WNV-immune mice have reduced frequencies of the immunodominant VV B8R epitope after \(2^{nd}\) rVV infection.**

As a control for rVV viral infection, we also measured the tetramer frequency of the immunodominant VV epitope, B8R, in the PBL of JEV-, WNV-,
Figure 4.16. Restriction of rVV in the ovaries of Kunjin-immune mice. (A) Kunjin-immune mice (purple circle) and PBS control (black square) mice were infected with 5 x 10^6 pfu rVV WNV (left panel), rVV JEV (middle panel), or rVV-GFP (right panel). Ovaries were harvested on day 5 after infection. Black bar and error bars represents mean ± SEM. *P<0.05, Mann-Whitney U. (B) Kunjin-immune mice (purple circle) and PBS control (black square) mice were infected with 5 x 10^5 pfu rVV WNV (left panel), rVV JEV (middle panel), or rVV-GFP (right panel). Ovaries were harvested on day 5 after infection. Black bar and error bars represents mean ± SEM. (C) Proliferation of memory JEV S9-specific (red circles) and WNV S9-specific (blue squares) CD44^hi CD8^+ T cells in Kunjin-immune mice following 2^o rVV (5 x 10^5 pfu) infection. Black bar and error bars represents mean ± SEM. Numbers represent fold expansion of JEV S9-specific and WNV S9-specific CD8^+ T cells in the PBL from day 0 (D0) to day 5 (D5).
Kunjin-immune mice following 2° rVV infection. This allowed us to determine if prior flavivirus immunity altered the generation of an epitope-specific CD8$^+$ T cell response to VV. After infection with a high dose (5 x 10$^6$ pfu) of the rVV, we saw no significant difference in the frequency of B8R-specific CD8$^+$ T cells in the PBL on day 5 in JEV- or WNV-immune mice compared to PBS (Figure 4.17.A.), even though we measured a significant reduction in the titer of rVV WNV in WNV-immune mice. However, after a low dose rVV WNV infection of WNV-immune mice, we detected a significant reduction in the median frequency of B8R-specific CD8$^+$ T cells compared to that seen in JEV-immune mice (median frequency: WNV-immune: 2.55%; JEV-immune: 11.5%; P<0.05, Mann-Whitney U), and PBS control mice (Median Frequency: PBS: 9.7%; P<0.001, Mann-Whitney U) (Figure 4.17.B.). No reduction in the B8R frequency in JEV-immune mice following either low or high dose rVV infection was seen. These results suggest that almost complete abrogation of rVV WNV replication, as seen in WNV-immune mice, is required to disrupt the generation of B8R-specific CD8$^+$ T cells following 2° rVV infection.
Figure 4.17. Frequency of the VV immunodominant B8R epitope in JEV-immune, WNV-immune, and PBS control mice after 2rVV infection. (A) WNV-immune (1° WNV), JEV-immune (1° JEV) and PBS control mice were infected with 5 x 10^6 pfu rVV JEV (left panel), rVV WNV (middle panel), or rVV GFP (right panel). B8R-specific CD44^hi CD8^+ T cells were measured on day 5 after infection in the PBL. Black bar represents median value. N=4-6 mice/group. (B) WNV-immune (1° WNV), JEV-immune (1° JEV) and PBS control mice were infected with 5 x 10^5 pfu rVV JEV (left panel), 1 x 10^5 pfu rVV WNV (middle panel), or 5 x 10^5 pfu rVV GFP (right panel). B8R-specific CD44^hi CD8^+ T cells were measured on day 6 after infection in the PBL. Black bar represents median value. N=4-9 mice/group. ***P<0.001, *P<0.05, Mann-Whitney U.
Q. Higher degree of peptide-coated target cell lysis in WNV-immune mice compared to JEV-immune mice

We have previously demonstrated that CD8^+ T cells during primary JEV infection recognize and kill both JEV S9 and WNV S9 peptide-coated targets in vivo (Figure 3.2.B.). We have also shown that both low and high doses of rVV are restricted more efficiently in WNV-immune mice than JEV-immune mice. One possible hypothesis is that the high frequency of memory CD8^+ T cells in WNV-immune mice is killing infected rVV cells before the rVV can replicate and spread into peripheral tissues. Memory CD8^+ T cells have been shown to rapidly kill peptide-coated targets at a similar rate as effector CD8^+ T cells (277). Therefore, we performed an in vivo CTL assay using JEV- and WNV-immune mice to determine whether WNV-immune mice can kill target cells better than JEV-immune mice. Splenocytes from naïve B6.SJL (Ly5.1^+) mice were pulsed with 1 µg/ml of JEV S9, WNV S9 or the Flu NP366-374 peptide, labeled with serial dilutions of CFSE and adoptively transferred into B6 Ly5.2^+ naïve or JEV- and WNV-immune mice. Splenocytes were then harvested 19 hours later, and Ly5.1^+ donor cells were analyzed for CFSE levels.

Figure 4.18.A. is a representative histogram from naïve (top panel), JEV-immune (middle panel) or WNV-immune (bottom panel) mice. In naïve mice, we did not detect any lysis of the peptide-coated targets, as 4 individual peaks were seen representing the peptides and unpulsed targets. In JEV-immune mice, target cells pulsed with the homologous JEV S9 peptide were lysed to a higher
Figure 4.18. Higher degree of peptide-coated target lysis in WNV-immune mice compared to JEV-immune mice. Splenocytes from naive B6.SJL (Ly5.1+) mice were pulsed with 1 \( \mu \)g/ml of JEV S9, WNV S9 or Flu NP peptides and transferred (1-2 x 10^7 cells) into B6 JEV- and WNV-immune mice. 19 hours later, the splenocytes were harvested and CFSE expressing was determined on Ly5.1+ cells. (A) Representative histogram from 1 mouse/infection. (B-C) Summary of the percent specific lysis of each peptide in JEV-immune mice (B) or WNV-immune mice (C). N=5 mice/infection, 2 independent experiments. Black line and error bar represents mean ± SEM. ** P<0.01, *P<0.05, Mann-Whitney U.
degree (mean ± SEM; 64.9% ± 7.8%) compared to targets pulsed with the heterologous WNV S9 peptide (mean ± SEM; 33.9% ± 5.6%; P<0.05, Mann-Whitney U) (Figure 4.18.B). However, both JEV S9 and WNV S9 targets were lysed to a higher degree than the irrelevant peptide, Flu NP366-374, (mean ± SEM; 2.1% ± 1.8%; P<0.05; Mann-Whitney U).

In WNV-immune mice, a similar pattern was seen. Targets pulsed with the WNV S9 and JEV S9 peptides were lysed to a higher degree compared to targets pulsed with Flu NP366-374 peptide (mean ± SEM: WNV S9: 90.4% ± 1.9; JEV S9: 78.6 ± 6.4%; Flu NP366-374: 15.7 ± 4.5; P<0.01, Mann-Whitney U) (Figure 4.18.). However, there was no significant difference in the target cell lysis of WNV S9 and JEV S9 peptide-pulsed targets in WNV-immune mice (P=0.1, Mann-Whitney U). Interestingly, there was a higher level of lysis of the Flu NP366-374 peptide-pulsed targets in WNV-immune mice compared to the JEV-immune mice suggesting there may be some non-specific target cell lysis in WNV-immune mice. These data support our findings on restriction of rVV replication, in which WNV-immune mice appear to have an enhanced ability to clear both homologous and heterologous rVVs compared to JEV-immune mice.

**R. Chapter Discussion**

In the previous chapter, we identified an immunodominant cross-reactive CD8\(^+\) T cell epitope between JEV and WNV. In this chapter, we explored the kinetics of cross-reactive CD8\(^+\) T cells during 2\(^\circ\) heterologous infection. We found
that cross-reactive CD8$^+$ T cells in WNV-immune mice proliferated poorly upon 2$^\circ$ JEV infection compared to those in JEV-immune mice infected with WNV. This lack of proliferation was not due to exhaustion of CD8$^+$ T cells nor differences in the CD8$^+$ T cell repertoire between JEV and WNV. WNV-infection did induce neutralizing antibodies to JEV; however the presence of these antibodies did not influence the proliferation of memory CD8$^+$ T cells upon 2$^\circ$ infection. We believe the higher frequency of memory CD8$^+$ T cells induced by WNV infection compared to JEV infection is the most likely influence on the proliferative capacity of memory CD8$^+$ T cells during 2$^\circ$ heterologous infection.

To understand cross-protection between JEV and WNV, we first had to establish a lethal model of JEV. Our model is slightly different than the model established by Larena et al., in which they used a sc infection of JEV Nakayama (95). A high dose sc infection resulted in 100% mortality by day 6 after infection, whereas in our studies, an ip infection resulted in only 60% mortality by day 12-15 after infection (Figure 4.1.) Different routes of infection (sc versus ip) have not been shown to alter B6 susceptibility to WNV infection (85). Therefore, the difference in mortality rates and survival times may be due to the different viral strains of JEV used in the two model systems (95).

We also found that prior immunity to WNV protected against a lethal JEV infection. Previous work in NHPs suggested that WNV immunity elicited only partial protection against JEV, as signs of encephalitis were seen in some animals (155). However, in our model, we did not see any signs of encephalitis or
weight loss in WNV-immune mice upon 2° JEV infection (data not shown). Two WNV-immune mice did succumb to 2° JEV infection, however sera samples were not collected to confirm seroconversion after primary WNV infection. Kunjin-immune mice were also protected against a JEV challenge, suggesting that the pathogenicity of the WNV strain did not alter cross-protection against JEV. We have similarly seen that the pathogenicity of the JEV strain did not influence cross-protection against WNV.

Despite the high frequency of memory CD8+ T cells in WNV-immune mice compared to JEV-immune mice, we saw very little proliferation of JEV S9-specific and WNV S9-specific CD8+ T cells after 2° JEV infection compared to 2° WNV infection of JEV-immune mice (Figure 4.4.A.). During primary JEV and WNV infection, the peak and magnitude of epitope-specific CD8+ T cells are similar at day 7 (Figure 3.5.). However at the memory time points, WNV-immune mice have a higher frequency of epitope-specific CD8+ T cells compared to JEV-immune mice, suggesting that the contraction phase of effector CD8+ T cells into memory CD8+ T cells is more rapid in JEV-immune mice compared to WNV-immune mice.

Closely related viruses in the Togaviridae family have been shown to replicate differently in peripheral tissues, similar to what we and others have found for JEV and WNV (95, 278). These differences in tissue tropism during the primary infection lead to an altered inflammatory cytokine profile (278). In our model, we believe JEV and WNV induce different levels of inflammation due to
the lack of peripheral replication by JEV compared to WNV. Previous work has shown that inflammation during the early stages of infection can modulate the contraction phase of CD8\(^+\) T cells (222, 242, 279). This difference in inflammation may influence the contraction of epitope-specific CD8\(^+\) T cells in WNV-immune mice. Also, recent work has shown that antigens in the CNS can activate CD8\(^+\) T cells in periphery to facilitate CD8\(^+\) T cell entry into the CNS (280, 281). Therefore, it is possible that persistent WNV antigen in the CNS may continuously be presented to WNV S9-specific CD8\(^+\) T cells in the periphery, thereby altering contraction of CD8\(^+\) T cells (24, 121).

We found differential expression of KLRG1 and PD-1 on JEV S9-specific and WNV S9-specific CD8\(^+\) T cells in the CNS and the spleen (Figure 4.7.C.). The higher level of PD-1 expression on CD8\(^+\) T cells in the CNS may reflect localized CD8\(^+\) T cell exhaustion in the CNS. However, the level of PD-1 expression is lower than seen on CD8\(^+\) T cells after a chronic LCMV infection, suggesting that all of the CD8\(^+\) T cells in the CNS may not be fully exhausted (270). In addition, WNV viral persistence in mice has been shown to lead to a sustained presence of immune cells in the CNS months after the initial infection (121). T regulatory cells (Tregs) in the CNS during WNV infection may regulate the microenvironment of the CNS differently than in the spleen, leading to the faster conversion of activated CD8\(^+\) T cells into memory CD8\(^+\) T cells to prevent continued immunopathology (121, 126). This faster conversion into memory CD8\(^+\) T cells in the CNS compared to the spleen during WNV infection may
enhance the ability of WNV to persistent in the CNS and needs further investigation.

When analyzing the CD8+ T cell repertoires between JEV and WNV, we saw very little difference in the TCR Vβ usage and functional avidities of both JEV S9-specific and WNV S9-specific CD8+ T cells. This was also true after rVV and Kunjin virus infection; however, rVV and Kunjin virus infection generated a memory CD8+ T cell population that proliferated after 2o JEV infection. In fact, only after WNV infection did memory WNV S9-specific CD8+ T cells proliferate poorly after JEV infection, suggesting a factor intrinsic to WNV-immune mice was causing this difference in proliferation.

WNV antibodies that are poorly neutralizing can protect against a lethal WNV infection in mice through complement-mediated mechanisms (144). Therefore in JEV-immune mice, antibodies against WNV may be non-neutralizing, but still able to protect in vivo, as we have seen previously (Trobaugh et al., in preparation). During sequential dengue virus infections, low levels of cross-reactive neutralizing antibodies between dengue serotypes are hypothesized to contribute to severe outcomes (199). In our model, we do not see increased immunopathology upon 2o infection but rather protection, which has also been seen in mouse models of sequential dengue virus infection (192). Pre-existing cross-reactive neutralizing antibodies and antibody dependent cell cytotoxicity through non-neutralizing antibodies are two humoral mechanisms that have been shown to correlate to reduced disease severity or reduced
viremia in humans experiencing secondary dengue-3 infection (282, 283). Recently, a WNV anti-NS1 antibody, which mediates protection against WNV through non-neutralizing mechanisms, was shown to protect against a lethal JEV infection (147, 210). Therefore, the role of non-neutralizing antibodies in cross-protection also warrants further investigation.

Even in the absence of cross-reactive neutralizing antibodies, memory CD8\(^+\) T cells in WNV-immune mice had limited proliferative capacity upon 2\(^o\) rVV JEV infection. A pre-existing high frequency of memory cross-reactive CD8\(^+\) T cells did not result in a higher magnitude of CD8\(^+\) T cells after 2\(^o\) heterologous infection. In contrast, low frequency of cross-reactive memory CD8\(^+\) T cells were able to proliferate to a greater extent and more rapidly than CD8\(^+\) T cells in mice with higher frequencies of these CD8\(^+\) T cells. However, even in the absence of proliferation, memory CD8\(^+\) T cells in WNV-immune mice were better able to restrict homologous and heterologous virus and lyse peptide-coated targets better than those from JEV-immune mice. This difference is not solely due to the presence of the WNV S9 sequence in the infecting virus as mice infected with Kunjin virus, which has the same WNV S9 amino acid sequence, were not able to restrict rVV replication as well as WNV-immune mice. Instead, there seemed to be a relationship between the frequency of memory CD8\(^+\) T cells and restriction of the rVVs. Both JEV-immune and Kunjin-immune mice had lower memory CD8\(^+\) T cell frequencies, and less viral restriction compared to WNV-
immune mice, which had higher memory CD8+ T cell frequencies and greater viral restriction.

Adoptive transfer of JEV-immune CD4+ and CD8+ T cells into naïve mice partially protected against a high dose WNV infection. Mosquitoes inoculate on average \(10^4\) pfu of WNV into chick toes during feeding, and longer feeding times can result in a higher inoculum in mice (284). This suggests that our challenge model may be too stringent, and if we gave a lower dose, as would happen after mosquito feeding, we might see better cross-protection. Cross-reactive CD8+ T cells are not able to restrict heterologous viruses as efficiently as homologous viruses due to a lower functional avidity for the cross-reactive epitope, suggesting a lower inoculum dose may be beneficial for the ability of cross-reactive CD8+ T cells to restrict virus (185). The dose of the inoculum may be another important factor that needs to be taken into consideration when studying cross-protection between flaviviruses.

The number of memory CD8+ T cells in JEV- and WNV-immune mice appears to be an important factor in protection against 2\(^o\) heterologous infection. A higher frequency of memory CD8+ T cells has been shown to correlate to better protection upon 2\(^o\) infection in both viral and bacteria models (248, 253, 285). Therefore, understanding the factors leading to enhanced memory CD8+ T cell frequencies in WNV-immune mice versus JEV-immune mice will help in understanding cross-protection between JEV and WNV and may yield important information relative to the incorporation of T cell epitopes into vaccines.
S. Proposed Model

In JEV-immune mice (Figure 4.19.A.), the lack of peripheral replication of virus during the primary immune response leads to lower levels of inflammation promoting MPEC CD8+ T cell generation. Rapid contraction after the primary infection leads to low levels of memory cross-reactive CD4+ and CD8+ T cells. Due to low levels of cross-reactive non-neutralizing antibodies and low levels of memory cross-reactive CD8+ T cells, WNV infection of JEV-immune mice leads to rapid expansion of memory CD4+ and CD8+ T cells. Proliferation of memory CD4+ and CD8+ T cells along with cross-reactive non-neutralizing antibodies likely contribute to the cross-protection seen in JEV-immune mice after WNV-infection. Cross-reactive CD4+ T cells may aid in the development and response of cross-reactive CD8+ T cells, in sustaining cross-reactive antibody responses, and/or may have direct anti-viral activity (cytolysis and effector functions).

In WNV-immune mice (Figure 4.19.B.) high levels of virus replication during the primary infection lead to higher inflammatory conditions promoting SLEC CD8+ T cell generation. Persistent antigen and/or the high inflammatory environment delays the contraction of epitope-specific CD8+ T cells leading to an increased frequency of memory CD8+ T cells. This higher frequency of memory CD8+ T cells along with preexisting neutralizing and non-neutralizing antibodies to JEV reduces JEV replication early during stages of the 2o infection leading to a reduced expansion of memory CD8+ T cells. Similar to JEV-immune mice,
Figure 4.19. Proposed model for 2\textsuperscript{nd} infections of JEV-immune (A) and WNV-immune (B) mice. (A) Low levels of peripheral replication during primary JEV infection leads to low levels of inflammation promoting MPEC CD8\textsuperscript{+} T cell generation. Rapid contraction after the primary infection leads to low levels of memory cross-reactive CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Due to low levels of cross-reactive non-neutralizing antibodies and low levels of memory cross-reactive CD8\textsuperscript{+} T cells, WNV infection of JEV-immune mice leads to rapid expansion of memory CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Proliferation of memory CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells along with cross-reactive non-neutralizing antibodies likely contributes to the cross-protection seen in JEV-immune mice after WNV-infection. Cross-reactive CD4\textsuperscript{+} T cells may aid in the development and response of cross-reactive CD8\textsuperscript{+} T cells, sustaining the cross-reactive antibody response, and/or have direct antiviral activity. (B) High levels of WNV replication during the primary infection leads to high inflammatory conditions promoting SLEC CD8\textsuperscript{+} T cell generation. Persistent antigen and/or the high inflammatory environment delays the contraction of epitope-specific CD8\textsuperscript{+} T cells leading to an increased frequency of memory CD8\textsuperscript{+} T cells. This higher frequency of memory CD8\textsuperscript{+} T cells along with preexisting neutralizing and non-neutralizing antibodies to JEV reduces JEV replication early during early stages of 2\textsuperscript{nd} infection leading to a reduced expansion of memory CD8\textsuperscript{+} T cells. Similar to JEV-immune mice, cross-reactive CD4\textsuperscript{+} T cells may aid in the development and response of cross-reactive CD8\textsuperscript{+} T cells, sustaining the cross-reactive antibody response, and/or have direct antiviral activity.
cross-reactive CD4⁺ T cells may aid in the development and response of cross-reactive CD8⁺ T cells, sustaining the cross-reactive antibody response, and/or have direct anti-viral activity.

T. Chapter Summary

We have found that prior WNV immunity protects against a lethal JEV infection. However, WNV memory cross-reactive CD8⁺ T cell proliferate very little upon 2° JEV infection compared to memory CD8⁺ T cells in JEV-immune mice upon 2° WNV infection. High levels of memory CD8⁺ T cells and JEV neutralizing and non-neutralizing antibodies in WNV-immune mice may be contributing to this difference in proliferation.
Chapter V

FINAL SUMMARY AND IMPLICATIONS

Flaviviruses co-circulate in many areas of the world, and this raises the risk of an individual experiencing sequential infections between flaviviruses (1). Sequential infections between flaviviruses can lead to cross-protection, or in the case of dengue virus, lead to more severe outcomes (199). Our interest was to study the mechanisms underlying cross-protection, but such studies are challenging, if not impossible, to perform in humans. Due to the lack of an adequate mouse model to study sequential infections between dengue viruses, we used JEV and WNV to study sequential infections between flaviviruses (286). Previous work in animal models demonstrated that while cross-protection between JEV and WNV existed, that it may not be entirely reciprocal (153, 155). These studies suggested that cross-reactive antibody responses were the contributing factor to this cross-protection. However, in mice immunized with recombinant E protein, only partial cross-protection is seen between JEV and WNV, suggesting that cross-reactive T cells that target other viral proteins may play a role in cross-protection (74, 153, 158, 159). Our group previously found that JEV immune CD4+ and CD8+ T cells were required for complete cross-protection between JEV and WNV (Trobaugh et al., in preparation). Therefore, the goal of this thesis was to further explore and understand the role of cross-
reactive T cell responses between JEV and WNV during primary and secondary infections.

A. Possible impact of innate immune responses on primary immune responses.

We identified JEV-WNV cross-reactive CD4+ and CD8+ T cell epitopes during the primary immune response to each virus, but the cross-reactive CD8+ T cell responses differed both quantitatively and qualitatively. We hypothesized that these differences in the primary immune responses may be due to lower levels of peripheral virus replication leading to lower inflammatory responses during a primary JEV infection compared to a primary WNV infection.

JEV and WNV are thought to stimulate similar pattern recognition receptors leading to an innate immune response (98, 101, 287). However, a major difference between JEV and WNV is the ability to establish viremia and replicate in peripheral lymphoid tissues (Figure 3.8.A.-B.) (88, 95). Some reports suggest that JEV infection of dendritic cells promotes an anti-inflammatory environment compared to WNV, which generates a pro-inflammatory environment (86, 100, 104, 105, 288-290). In fact, the ability to replicate in myeloid cells actually increased the susceptibility of mice to JEV infection (96). Therefore, the differing ability of JEV and WNV to initially infect certain cell types or subsets may influence peripheral replication. Although our group and others have not been able to detect JEV in the spleen or serum early during infection,
virus entry into the CNS does occur at later time points, suggesting there may be other sites of viral replication that have yet to be identified as seen after WNV infection (84, 95).

The conversion of effector CD8⁺ T cells to memory CD8⁺ T cells in JEV-infected mice resembles what is seen after DC immunization, in which low levels of inflammation results in a CD8⁺ T cell population that can be rapidly restimulated (291). IL-12, type I IFN, and IFN-γ are inflammatory cytokines that promote effector CD8⁺ T cell differentiation into SLEC, and are absent during DC immunization (225, 229, 291). Induction of IL-12 and IFN-γ by CpG, a potent inducer of inflammation, during DC immunization induces high frequencies of SLECs and subsequent slower conversion of effector CD8⁺ T cells to memory CD8⁺ T cells (225). Therefore, JEV infection may induce lower levels of IL-12 and type I IFN compared to WNV infection, thereby altering effector CD8⁺ T cell generation. In comparison, WNV infection may induce high levels of IL-12 and type I IFN early during infection leading to the generation of SLECs thereby prolonging the time until memory CD8⁺ T cell formation.

Inflammation during the primary infection may also be influencing the ability to recover from JEV and WNV infection. Neuroinvasion seems to correlate highly with mortality after JEV infection, since very few mice showing clinical symptoms survive. (95). However, mice that demonstrate clinical signs, including signs of neuroinvasion, can recover from WNV illness (88). A similar clinical pattern has been described following infection with Venezuelan equine
encephalitis virus (VEEV) and eastern equine encephalitis virus (EEEV) in mice. It has been hypothesized that peripheral replication and early inflammation (possibly due to production of type I IFN or other mediators) results in early clinical signs during VEEV infection, which helps to prime the CNS to mount an antiviral immune response. On the other hand, EEEV infection induces low levels of peripheral replication and type I IFN resulting in later onset of clinical symptoms, just prior to death (278). We postulate that the ability of mice to recover from WNV infection may be due to the higher levels of inflammation (IL-12, type I IFN, IFN-γ or other mediators) priming the CNS to limit WNV replication and neuronal cell death, which does not occur in JEV-infected mice.

We found that CD8⁺ T cells from WNV-infected mice secreted higher levels of TNF-α compared to CD8⁺ T cells from JEV-infected mice (Table 3.3.). Non-lymphocyte populations may also produce TNF-α, which could influence the levels of systemic TNF-α during JEV and WNV infection (101, 260, 289, 292, 293). TNF-α has been hypothesized to alter blood-brain barrier permeability during WNV infection leading to either a decreased (low levels of TNF-α) or an increased (high levels of TNF-α) susceptibility to WNV (106, 260). However, the complete absence of TNF-α lead to an increase in mortality, believed to be due to impaired trafficking of T cells to the CNS (260). Higher levels of TNF-α following WNV infection may alter blood-brain barrier permeability sufficiently to permit the trafficking of lymphocytes into the CNS. On the other hand, lower systemic TNF-α levels following JEV infection may prevent this alteration and
thereby reduce T cell entry into the CNS. The limited T cell entry into the CNS following JEV infection may contribute to the impaired ability to recover from neuroinvasive JEV infection compared to WNV (88, 95). Understanding the differences and similarities in host innate immune responses and the subsequent inflammatory response during JEV and WNV infections will be important in deciphering how the primary immune response alters the secondary immune response between JEV and WNV.

B. Immune responses during sequential JEV and WNV infection.

In our B6 mouse model, prior WNV immunity protects against a lethal JEV infection, similar to prior JEV immunity upon a 2° WNV infection. However, only in JEV-immune mice did memory cross-reactive CD8+ T cells expand upon a 2° infection. The lack of proliferation in WNV immune mice upon 2° JEV infection seems to be due to both cross-reactive neutralizing and non-neutralizing antibodies against JEV and a high frequency of memory cross-reactive CD8+ T cells.

i. Cross-reactive antibody responses

Cross-reactive neutralizing antibodies were only detected in WNV-immune mice, suggesting the sequence of infection can influence the generation of cross-reactive antibodies. Repeated vaccinations and infection of immunodeficient mice are two conditions in which flavivirus cross-reactive neutralizing antibodies
have previously been detected in mice (89, 159, 192, 294). This suggests that increased antigen stimulation and viral replication may influence cross-reactive neutralizing antibody generation. Therefore, the ability to detect cross-reactive neutralizing antibodies in WNV-immune mice may be due to the increased peripheral replication of WNV during the primary infection. It is plausible that all flavivirus infections develop low levels of cross-reactive neutralizing antibodies, and a second infection increases the chances of detecting these antibodies (209). However, measurement of cross-reactive antibodies *in vitro* depends on many factors, and the ability of WNV-immune sera to protect against JEV *in vivo* requires further investigation (295).

In this thesis, we did not address the presence of non-neutralizing cross-reactive antibodies between JEV and WNV. We have previously shown that adoptive transfer of JEV-immune serum, in which anti-WNV neutralizing antibodies were not detected, was able to partially protect against mortality from a WNV challenge (Trobaugh et al., in preparation). Therefore, the presence of non-neutralizing antibodies in JEV- and WNV-immune mice may also be impacting the outcomes upon 2° infection. In fact, cross-reactive IgG antibodies to WNV can be detected after immunization with recombinant JEV E protein even though there are low or non-existent neutralizing antibodies (159). This suggests that the type of cross-reactive antibodies in JEV- and WNV-immune may be influencing the expansion of memory CD8⁺ T cells and protection upon a 2° heterologous infection.
ii. Cross-reactive T cell responses

During primary JEV and WNV infections, CD8+ T cells are required for the clearance of CD8+ T cells in the CNS (95, 108, 109). However, the role of CD8+ T cells during 2o heterologous infection was not known. JEV-immune CD4+ and CD8+ T cells are required for cross-protection, yet they are not sufficient to fully protect against a high dose WNV infection (Figure 4.3.A. and Trobaugh et al., in preparation). Recently, a similar pattern was seen in sequential dengue virus infections (192). On their own, the ability of cross-reactive CD4+ and CD8+ T cells to restrict heterologous virus may be limited and may depend on both the sites of viral replication and the particular infecting virus. In other mouse models of heterologous immunity, virus levels were reduced only 1-2 logs upon 2o infection (164, 171, 172, 185, 294). The low frequency and reduced functional avidity of cross-reactive CD8+ T cells may contribute to this low level of viral clearance (166, 185). Even though there is only low-level viral restriction at an early time point during 2o heterologous infection, these reduced viral levels may lead to subclinical infections. One important consideration to note is that T cell entry into infected tissue to clear these virally infected cells may not always be beneficial and can lead to immunopathology (164, 171-173).

In dengue virus infections, CD8+ T cell responses to cross-reactive epitopes depend on both the sequence of infection and the particular cross-reactive epitope (182, 183, 196, 296, 297). In our model, we found that the
particular viral infections (JEV versus WNV) rather than the cross-reactive epitope (JEV S9 versus WNV S9) had greater impact on CD8⁺ T cell effector functions. JEV S9-specific and WNV S9-specific CD8⁺ T cells had similar functional avidities for the cross-reactive epitope, which may explain why we did not detect the different cytokine profiles that are seen in CD8⁺ T cell clones from individual dengue-infected patients after stimulation with cross-reactive peptides (296). Differences in the functional avidity of cross-reactive CD8⁺ T cells can lead to alerted cytokine profiles upon cross-reactive peptide stimulation (182, 183, 196, 296, 297). This difference in cytokine profiles is considered to be a contributing factor to severe DHF upon 2⁰ dengue infection (259).

It is likely that the relationship between viral replication, the frequency of cross-reactive CD4⁺ and CD8⁺ T cells, and the presence of neutralizing and non-neutralizing antibodies are all determinants of outcome upon 2⁰ heterologous infection. Low levels of memory CD4⁺ and CD8⁺ T cells in isolation may not be sufficient to overcome a high virus inoculum. However, neutralizing and/or non-neutralizing antibodies may restrict viral levels to a point that allows for cross-reactive CD4⁺ and CD8⁺ T cells to effectively clear the virus. In absence of other components of the immune system, high frequencies of memory CD8⁺ T cells were able to restrict both homologous and heterologous viruses to a greater extent than lower frequencies of memory CD8⁺ T cells even in the face of rapid expansion and proliferation of those cells. It is possible that low levels of cross-reactive memory CD8⁺ T cells may need the help of cross-reactive CD4⁺ T cells
to reduce high viral loads, and may account for why adoptive transfer of both JEV-immune CD4+ and CD8+ T cells were required to partially protect mice against a high dose WNV challenge. It will be important to determine the threshold of memory CD8+ T cells required for protection against both homologous and heterologous viral infections, and this may assist in the design of viral vaccines (285).

*** Cross-protection between JEV and WNV

The goal of our model system was to investigate the cross-protection that occurs between JEV and WNV. In mouse models of sequential dengue virus infection, both cross-protection and enhancement of disease have been observed depending on the experimental conditions (192, 202, 298, 299). In our mouse model, we did not see any severe outcomes upon 2o heterologous infections (unpublished data). However, as we did not design our experiments to address immunopathology, we cannot rule out that a severe outcome may result under certain conditions.

In humans, cross-protection between JEV/WNV and other flaviviruses is likely to occur. JEV and WNV by and large are not endemic to the same geographic regions, with the exception of some regions of India, Pakistan, and Papa New Guinea (1, 16). Therefore, it is difficult to predict or assess whether the outcome of sequential infection in humans with these viruses would lead to severe disease or cross-protection.
The presence of susceptible mosquito vectors, susceptible hosts, and the climate are important factors that could be influencing the spread of JEV and WNV into non-endemic regions (8). *Culex pipiens*, a predominant mosquito vector for WNV, and *Culex tritaeniorhynchus*, a predominant mosquito vector for JEV, can become productively infected with heterologous JEV and WNV, respectively (300-302). Even though these mosquito vectors are able to transmit both JEV and WNV, introduction of JEV into WNV endemic regions, and WNV into JEV endemic regions, has been limited (16).

The level and length of viremia in amplifying hosts may be an important factor in the spread of JEV and WNV. Migratory birds with high levels of viremia may spread JEV and WNV into non-endemic regions (35, 303). However, WNV-immune red-winged blackbirds were completely protected from JEV viremia in an experimental setting, suggesting that once birds become immune, they are not susceptible to a 2nd infection (156). JEV, unlike WNV, also uses pigs as amplifying hosts since JEV can establish high levels of viremia for up to 4 days (304). Therefore, close interactions of humans and pigs can lead to an increase in the number of cases of JEV in humans, and this interaction may be important for the spread of JEV (16). The likelihood of cross-protection in birds suggests that in order for one virus (e.g. JEV) to establish itself into a WNV-endemic region, an adequately sized naïve offspring population would be required for amplification of the virus and transmission into mosquitoes.
Although WNV has spread into dengue endemic areas of Central and South America, very few human cases have been reported (8, 203). It is thought that prior dengue or YFV immunity may be contributing to this “protection” (203). In contrast, prior JEV immunity has been demonstrated to lead to either an increased or decreased association with severe DHF depending on the cohort studied (305, 306). Therefore, not all sequential infections between flaviviruses are created equal, and multiple other factors (e.g. climate, vector density/infectivity, and time between sequential infection) contribute to whether there will be cross-protection or a severe outcome.

C. The potential development of a cross-reactive flavivirus vaccine.

The ideal vaccine candidate would induce life long protective immunity after only one immunization, similar to the live attenuated YF17D vaccine, but could also induce protective immunity to multiple viruses (307). Based on data presented in this thesis, a cross-reactive vaccine between JEV and WNV may be feasible. Neutralizing antibody titers are the current standard in determining whether a JEV vaccine is a suitable candidate, and presumably, similar standards will be used to approve a WNV vaccine (308). However, although we detected cross-reactive neutralizing antibodies only after a WNV infection and not after a primary JEV infection, we still detected bidirectional cross-protection (Figure 4.5.). These findings suggest that the measurement of neutralizing antibodies may not be the proper immune correlate for cross-protection and that
investigation into assays that measure cross-reactive non-neutralizing antibodies or T cell responses may be warranted. In fact, adoptive transfer of JEV SA14-14-2-immune serum partially protected mice against lethal WNV infection in the absence of cross-reactive neutralizing antibodies, suggesting that cross-reactive non-neutralizing antibodies are an important correlate of protection that needs to be considered (Trobaugh et al., in preparation).

The context of the viral vector and the number of immunizations can also influence cross-protection between JEV and WNV. Ch-WNV, but not the canarypox-WNV vaccine, induced protection from JEV viremia in hamsters (154). Similarly, repeated vaccinations with recombinant JEV or WNV E protein or inactivated JEV vaccines generated cross-reactive neutralizing antibodies that induced partial protection (73, 74, 158, 159). These results suggest that low levels of cross-reactive neutralizing antibodies may be present after initial immunization, and repeated vaccinations are required to increase their frequency. A recent report by Purtha et al. sheds light on the possible mechanism for these observations. They found that, in the absence of WNV neutralizing antibodies secreted by long-lived plasma cells, immunization with DIII of the JEV E protein generated JEV-specific memory B cells that were able to secrete WNV neutralizing antibodies after heterologous secondary WNV infection (309). This result suggests that JEV DIII immunization induced a population of cross-reactive memory B cells that were able to expand after heterologous infection. Therefore, repeated vaccinations with the same vaccine
may increase the frequency of such cross-reactive memory B cells thereby increasing the levels of cross-reactive neutralizing antibodies that may contribute to cross-protection.

Complete cross-protection between JEV and WNV requires both cross-reactive antibodies and T cells (Trobaugh et al., in preparation). This suggests that a live, attenuated vaccine, due to the ability to induce both T cell and antibody responses, may be the ideal vector to generate a cross-reactive vaccine. Our data suggest that the inclusion of cross-reactive CD4$^+$ and CD8$^+$ T cell epitopes into flavivirus vaccines may help in cross-protection. However, the threshold of memory CD8$^+$ T cells required for cross-protection is as yet still undefined. The value of such cross-reactive memory CD8$^+$ T cells as a correlate of cross-protection for vaccine development warrants further investigation.

**D. Final Conclusions**

We found that cross-protection between JEV and WNV occurs in both directions; however the mechanisms of this cross-protection may depend upon the sequence of infection. A high frequency of memory CD8$^+$ T cells and cross-reactive antibodies in WNV-immune hosts may limit the expansion of memory cross-reactive CD8$^+$ T cells due to low levels of heterologous virus. The rapid proliferation of low frequency memory cross-reactive CD8$^+$ T cells in JEV-immune mice upon 2o WNV infection may be needed to combat the higher viral loads. From our studies, it appears that both the humoral and adaptive immune
responses are required for complete protection from virus dissemination and mortality.

This research suggests that if WNV spreads to JEV endemic areas, JEV-immune individuals may be protected from a WNV infection. Similarly, if JEV spreads to the US or if a WNV-immune individual travels to a region where JEV is endemic, WNV-immune individuals may be protected against a JEV infection. Cross-protection is challenging to study in human populations, as silent sequential infections remain largely undetected. Mouse models such as we have developed are vital for addressing this issue, both from the standpoint of the emergence of viral disease into new geographic regions, as well as vaccine development.
CHAPTER VI

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