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USING THE SINGLE-CYCLE FLAVIVIRUS PARTICLE REPLIVAX TO STUDY FLAVIVIRUS REPLICATION AND IMMUNE RESPONSE

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by

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Dedication

To my wife Sandra who always has given me strength, support and love. To my family and friend who have encouraged me to pursue my dreams.

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RepliVAX is a novel single-cycle flavivirus (SCFV) vaccine platform, which contains a deletion in the capsid (C) gene of West Nile virus (WNV) that prevents the formation of infectious particles, unless the C gene is trans-complemented by a WNV C-expressing cell line. RepliVAX immunization results in a single round of infection, where the replication of genome leads to the production of subviral particles (SVP) and NS1 antigens that are highly immunogenic and induce an antiviral immune response without causing disease. The overall goal of this dissertation was to understand the interaction of RepliVAX with target cells, innate immune cells, and adaptive immune cells. The first studies investigated the properties of adaptive mutations acquired during the development of RepliVAX D2, which is a chimeric SCFV vaccine containing the dengue virus 2 (DENV2) prM/E genes in place of the corresponding WNV genes. It was demonstrated that mutations in the DENV prM/E region increased the specific infectivity of the chimeric virus particles. Also, it was shown that mutations in the WNV NS2A/NS3

region independently improved the encapsidation of virions without altering genome replication, indicating a functional interaction of structural and nonstructural flavivirus proteins to optimize the particle assembly/release of SCFV particles.

As RepliVAX mimics the infection of the wild type virus, inducing innate and adaptive immune responses, RepliVAX WN was utilized as a tool to further investigate different aspects of WNV immunity. The role of cellular innate immunity examined by macrophage depletion studies demonstrated that macrophages limit the initial dissemination of SCFV particles from the site of inoculation. Additionally, macrophages were not essential for activation of adaptive immune response, since equivalent $CD8^+$ T cell response were found in depleted and non-depleted mice. This function was probably provided by dendritic cells (DC) and two subsets of DC were identified that stimulated WNV-specific T cell response. Finally, the role of type I interferon (IFN) was evaluated during SCFV immunization. It was shown that type I IFN response controls the early viral gene expression and consequently viral antigen production. However, the type I IFN did not affect the magnitude of the adaptive immune response, but did modulate effector cytokine production.

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List of Abbreviations

aa	Amino acid
AAALAC	Association for assessment and accreditation of laboratory animal care
Ab	Antibody
ALFV	Alfuy virus
AnchC	Anchor of C
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APO-1	Apoptosis antigen 1
ASC	Apoptosis-associated speck-like protein containing CARD
AUG	Methionine (start codon)
B6	C57BL/6J (mice strain)
BBB	Blood-brain barrier
BDV	Border disease virus
BHK	Baby hamster kidney (cell line)
BiP	Binding immunoglobulin protein
BM-DC	Bone marrow-derived dendritic cell
BM-MØ	Bone marrow-derived macrophage
BVDV	Bovine viral diarrhea virus
C	Capsid (flavivirus-encoded protein)
C-terminal	Carboxy-terminus, carboxy-terminal
CARD	Caspase activation and recruitment domain
CARDIF	CARD adapter inducing interferon-β
CCL	Clodronate-containing liposomes
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation, cluster of designation
CDC	Centers for Disease Control and Prevention
CMC	Carboxymethyl cellulose
CNS	Central nervous system
CPCV	Cacipacore virus
Cryo-EM	Cryo-electron microscopy
CFSE	Carboxyfluorescein succinimidyl ester
CSFV	Classical swine fever vírus
CTL	Cytotoxic T Lympocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DC-SIGNR	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin related lectin
DENV	Dengue virus
DHF	Dengue hemorrhagic fever
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DR	Death receptor
dsRNA	Double-stranded RNA
E	Envelope (flavivirus-encoded protein)
E	Glutamic acid (amino acid)
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
ER	Endoplamatic reticulum
F	Phenylalanine (amino acid)
Fas	Fas cell surface death receptor
FasL	Fas ligand
FBS	Fetal bovine serum
FcγR	Fragment crystallizable gamma receptor
FLUC	Firefly luciferase
FP	Footpad
G	Glycine (amino acid)
GAG	Glycosaminoglycans
gB	Glycoprotein B (herpesvirus-encoded protein)
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRP78	Glucose-regulated protein 78
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
HCV	Hepatatis C virus
HEL	Helicase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hpi	Hours post infection
HRP	Horseradish peroxidase
HS	Heparan sulfate
i.p.	Intraperitoneal
i.v.	Intravenous
IFIT	Interferon-induced protein with tetratricopeptide repeats
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IFNAR	IFN-alpha/beta receptor
IFN-α	IFN-alpha
IFN-β	IFN-beta
IFN-γ	IFN-gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry

IL	Interleukin
ingLN	Inguinal lymph node
INV	Inactivated vaccine
IPS-1	Interferon promoter stimulator-1
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF	Interferon-stimulated gene factor
ISRE	Interferon-stimulated response element
IU	Infectious units
IVIS	*In vivo* imaging system
JAK	Janus kinase
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
K	Lysine (amino acid)
kDa	Kilodaltons
kg	kilogram
KO	Knockout
KOUV	Koutango virus
KUNV	Kunjin virus
LAG3	Lymphocyte-activation gene 3
LAV	Live-attenuated vaccine
LGP2	Laboratory of genetics and physiology 2 (RLR family)
LN	Lymph node
M	Membrane (flavivirus-encoded protein)
MAVS	Mitochondrial antiviral-signaling protein
MCP-1	Monocyte chemotactic protein-1
MDA5	Melanoma differentiation-associated protein 5
mDC	Myeloid dendritic cell
MEM	Minimal essential medium
mg	Milligram
MHC	Major histocompatibility complex
M-CFS	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
ml	milliliters
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTase	Methyltransferase
MVEV	Murray Valley encephalitis virus
MyD88	Myeloid differentiation primary response gene 88
N-terminal	Amino-terminus, amino-terminal
NBCS	New born calf serum
NC	Nucleocapsid
NCR	Noncoding region
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGC	New Guinea-C

NHP	Non-human primates
NK	Natural killer
NLR	NOD-like receptors
nm	Nanometers
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NS	Nonstructural (flavivirus-encoded protein)
NTPase	Nucleoside triphosphatase
OAS	2’5’-oligoadenylate synthetase
OD	Optical density
ORF	Open reading frame
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBSL	Phosphate buffered saline-loaded liposomes
PD-1	Programmed death 1
pDC	Plasmacytoid dendritic cell
PFU	Plaque-forming units
pH	Potential of hydrogen
PKR	Protein kinase
pLN	Popliteal lymph node
PMN	Polymorphonuclear leukocyte
prM	Precursor to M (flavivirus-encoded protein)
PROT	Protease
PRR	Pattern recognition receptor
R	Arginine (amino acid)
RANTES	Regulated on activation, normal T cell expressed and secreted
RBC	Red blood cell
RdRp	RNA-dependent RNA polymerase
RF	Replicative form
RI	Replicative intermediate
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RLU	Relative luciferase units
RNA	Ribonucleic acid
RNAi	RNA interference
ROI	Region of interest
RPMI	Roswell Park Memorial Institute medium
RSAD2	Radical S-adenosyl methionine domain containing 2 (Viperin)
RTPase	RNA triphosphatase
S	Serine (amino acid)
s.c.	Subcutaneous
SCFV	Single-cycle flavivirus
SCS	Subcapsular sinus
SHA	Slowly sedimenting hemagglutinin
SLEV	St. Louis encephalitis virus
sqPCR	semiquantitative polymerase chain reaction

ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
SVP	Subviral particles
T	Threonine (amino acid)
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
TCR	T cell receptor
TGN	Trans-Golgi network
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
trC	Truncated capsid
trE	Truncated envelope
TRIF	Toll/IL1 receptor domain-containing adaptor inducing IFN
TYK2	Tyrosine kinase 2
USA	United States of America
USUV	Usutu virus
UTMB	University of Texas Medical Branch
UTR	Untranslated region
V	Valine (amino acid)
VEErep	Venezuelan equine encephalitis virus replicon
VISA	Virus-induced signaling adapter
VRP	Viral replicon particles
VSV	Vesicular stomatitis virus
WHO	World Health Organization
WNE	West Nile encephalitis
WNF	West Nile fever
WNND	West Nile neurological disease
WNV	West Nile virus
WT	Wild type
YAOV	Yaounde virus
YF	Yellow fever
YFV	Yellow fever virus

CHAPTER 1: INTRODUCTION

Family *Flaviviridae*

The family *Flaviviridae* is segregated into three genera: *Flavivirus*, *Pestivirus*, and *Hepacivirus*. There are over 70 viruses classified into this family, and additionally two more unclassified viruses, GB-A and GB-C, which share similarities with hepatitis C virus (HCV) but are genetically distinct (Lindenbach et al., 2007). Viruses belonging to this family are responsible for important human and animal diseases. Within the genus *Flavivirus* are several viruses such as dengue virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV) and West Nile virus (WNV) that are responsible for a broad range of clinical manifestations in humans, including encephalitis and hemorrhagic fevers. Most of these viruses are transmitted by arthropod vectors such as mosquitoes and ticks (Lindenbach et al., 2007). In the second genus, the *Pestivirus*, are included viruses that infect cattle, sheep, goats, pigs and also other wild species such as giraffes (Peterhans and Schweizer, 2010). There are three viruses in this genus: the classical swine fever virus (CSFV), border disease virus (BDV) and bovine viral diarrhea virus (BVDV). In most of the cases they cause unapparent rather than more severe disease, although the economic loss can be significant due to decreased productivity and commercial restrictions. The diseases are usually characterized by a transient infection where the animals completely eliminate the virus after a few weeks of the infection or in special cases a persistent infection can occur where the host will carry and continuously transmit the virus. The persistent infection is an important transmission source that keeps the virus being spread among animals as a result of close contact (Houe, 1999; Lindenbach et al., 2007). In the third genus, the *Hepacivirus*, the hepatitis C virus (HCV) is the prototype member of this genus, which now also includes the GB virus B. HCV is hepatotropic and

usually causes persistent infections characterized by acute to chronic hepatitis and cirrhosis. HCV has also been linked to the development of hepatocellular carcinoma (Saito, et al., 1990; Lindenbach et al., 2007). The transmission typically occurs through blood transfusion and unsafe use of therapeutic or illegal drugs injection. Although the tools for HCV detection have been improved, it still a major public health concern, with a prevalence of 2.2% worldwide, which correspond to more than 170 million people infected with the virus (Lemon et al., 2007).

Members of the family *Flaviviridae* share many characteristics such as morphology, genome organization and replication. The virions are spherical, with a size of approximately 40-60 nm in diameter and contain a single-stranded, positive-sense RNA genome encoding a long single open reading frame (ORF). As typical for RNA viruses, genome replication occurs in the cytoplasm of the infected cell where new particles are assembled and released to the extracellular compartment. Despite shared similarities among members of *Flaviviridae* family, they are distinct in several aspects of biological properties and epidemiological characteristics (Lindenbach et al., 2007).

GENUS *FLAVIVIRUS*

The genus *Flavivirus* (from Latin "flavus" which means "yellow" in reference for the jaundice caused by the YFV) contains the largest number of viruses within the *Flaviviridae* family. Differently from the *Hepacivirus* and *Pestivirus* genera, most of the flaviviruses are associated with an arthropod vector, which is responsible for the transmission of the virus, and for this reason are also referred as arboviruses, which includes viruses from other families as well. Based on serological cross-reactivity, they are classified into different antigenic complexes, although some of the flaviviruses members, including the prototype virus, YFV, are not grouped in any of the complexes (Calisher et al., 1989). Also, based on molecular phylogenetics, they are subdivided into clusters, clades and species (Kuno et al., 1998; Lindenbach et al., 2007) (Fig. 1.1).

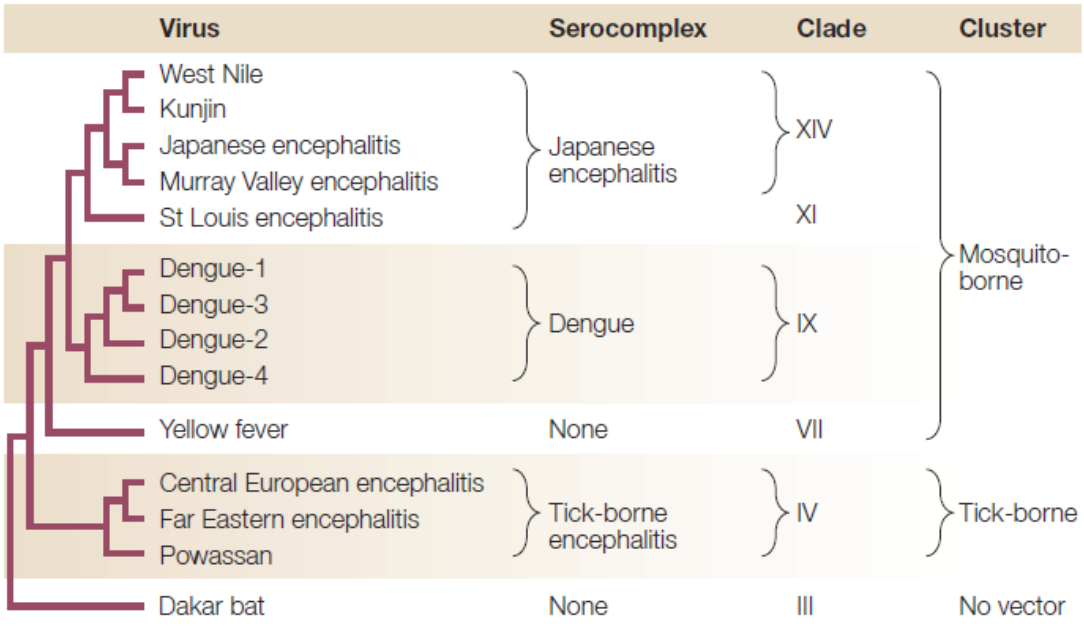

Figure 1.1. **Classification of flaviviruses**. Representation of the relationships of selected flaviviruses showing the phylogenetical (clades and clusters) and serological (serocomplexes) classification. (Reprinted with permission from Mukhopadhyay et al., 2005. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol, 3:13-22).

Flaviviruses are spread all over the world except Antarctica, and they potentially affect more than a half of the world's population. DENV infections alone are estimated at 50-100 million cases annually and up to 3 billion people living in tropical and subtropical areas are at risk for DENV infection (Halstead 2007; WHO 2012). Yellow fever also has a great impact, and although a vaccine is available, it is estimated 200,000 cases and 30,000 deaths occur per year especially in tropical areas of Latin America and Africa (WHO, 2013). Other diseases, such as Japanese encephalitis (estimated 60,000-70,000 cases annually in endemic areas of Southern Asia and Western Pacific) (Campbell et al., 2011) and West Nile encephalitis (discussed later in this chapter) also represent important diseases caused by flaviviruses and demonstrate the importance and need to study different aspects of these pathogens with the objective to elucidate the mechanisms of their life cycle and survival against natural defenses of their host.

Genome organization

Flaviviruses contain a single-stranded, positive sense RNA genome of approximately 11 kb in length. As positive polarity, the viral genome is infectious by itself (Peleg, 1969), serving as mRNA for the translation of the encoded viral proteins. Unlike the pestiviruses and hepaciviruses, which contain an internal ribosome entry site (IRES), flaviviruses contain a 5' methylated cap ($m^7GpppAmpN_2$) involved in the initiation of the translation process (Wengler et al., 1978; Cleaves and Dubin, 1979). Another peculiarity of this genome, which diverges from cellular mRNAs, is the absence of polyadenylated tail at the 3' end (Wengler et al., 1978).

The flavivirus genome encodes a single ORF flanked by 5'- and 3'-noncoding regions (NCR). The NCRs originate RNA secondary structures that have as major function the translation and replication of the genome (Cahour et al., 1995; Deas et al., 2005; Holden et al., 2006). The ORF encodes three structural proteins followed by seven nonstructural (NS) proteins (Rice et al., 1985) that are translated as a single polyprotein and cleaved co- and post-translationally by host signal peptidases, virus-encoded serine protease or unknown enzyme (Fig. 1.2). The structural proteins (capsid, C; membrane, M, which is expressed as the precursor to M, prM; and envelope, E) constitute the virion and are necessary for packaging the viral RNA. The NS proteins (NS1, NS2A, NS3, NS4A, NS4B and NS5) are mainly involved in genome replication and translation process, although some of them are also involved in modulation of the host immune response (Lindenbach et al., 2007).

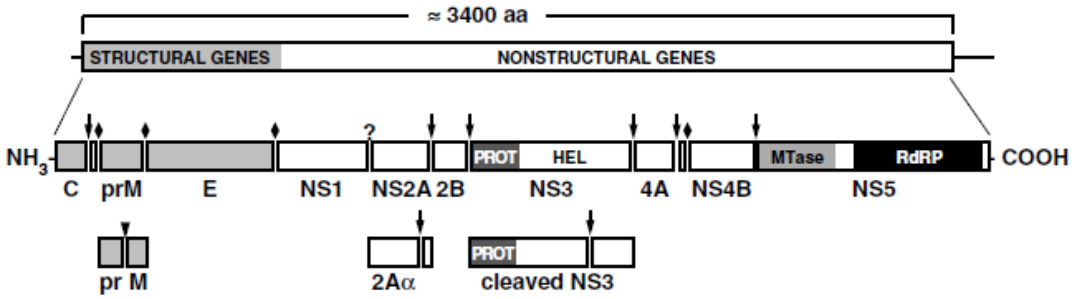

Figure 1.2: **Flavivirus protein expression and processing**. The genome containing the structural and nonstructural genes is translated as a single polyprotein. After translation, the polyprotein is cleaved by host signal peptidases (♦), virus-encoded serine protease (⇓), unknown enzyme (?) or furin (▼) in the indicated protein junctions. (Reprinted with permission from Lindenbach and Rice, 2003. Molecular biology of flaviviruses. Adv Virus Res, 59:23-61).

The flavivirus virion

The flavivirus particles are small, approximately 50 nm in diameter (mature particles), spherical in shape and contain an electron-dense core (nucleocapsid) surrounded by a lipid envelope (Murphy, 1980) where the M and E proteins are inserted (Fig. 1.3). The core is composed of the C protein, which is associated with the viral RNA genome. The structural models derived from cryoelectron microscopy images indicate that there are 90 E protein dimers that lie parallel to the lipid bilayer in a herringbone pattern giving a smooth outer surface appearance with icosahedral symmetry (Rey et al., 1995; Kuhn et al., 2002). The M protein is arranged within the E dimers, near to the fusogenic domain. The C protein surrounding the RNA genome is present below the lipid bilayer and it has a less organized pattern (Kuhn et al., 2002). Flavivirus-infected cells also release smaller (approximately 14 nm), noninfectious particles containing the M and E protein, but lacking the nucleocapsid containing the viral genome (Smith et al., 1970). These particles were initially termed as slowly sedimenting hemagglutinin (SHA) because their property to agglutinate red blood cells, and more recently described as

subviral particles (SVP), which can be obtained by recombinant expression of prM and E proteins by themselves (Schalich et al., 1996; Konishi et al., 2001; Lorenz et al., 2003).

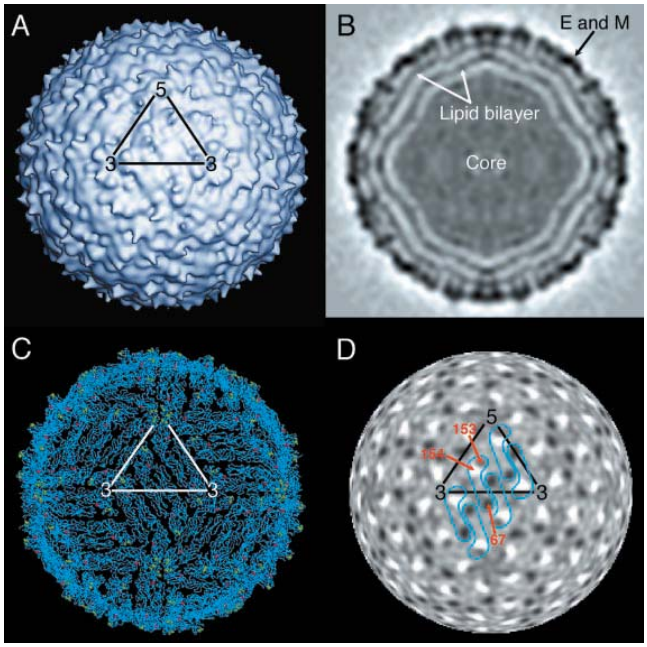

Figure 1.3: Flavivirus virion structure. Cryo-electron microscopy (cryo-EM) of WNV. (A) Surface shaded view showing a smooth surface of the virion containing an icosahedral symmetry (one unit represented by the triangle). (B) Cross section of the virion showing the concentric layers of density. (C) Structure showing the arrangement of E protein dimers on the virus surface. (D) Electron density maps between DENV (positive density in black) and WNV (negative density in white). Three sets of E protein dimers are highlighted in blue. (From Mukhopadhyay et al., 2003. Structure of West Nile virus. Science, 302:248. Reprinted with permission from AAAS).

Flavivirus Proteins

Structural proteins

The first three proteins, encoded by the 5' proximal 1/3 of the viral genome, correspond to the structural proteins C, pM/M and E. The C protein has a highly basic nature, which facilitates the association of the RNA genome. Multiple copies of C surround a single copy of the viral genome to form the nucleocapsid (NC) (Kuhn et al., 2002). The positive charged amino acids are clustered at both ends of the protein

(Khromykh and Westaway, 1996) and have a short internal hydrophobic region that is believed to be responsible for the association with the viral envelope (Markoff et al., 1997; Ma et al., 2004). The translated C protein also contains a carboxy (C)-terminal hydrophobic anchor (anchC) that is cleaved by the viral serine protease NS2B/NS3 (described later) to generate the mature form of the C protein with a molecular size of approximately 11 kilodaltons (kDa) (Lobigs, 1993; Amberg et al., 1994; Yamshchikov and Compans, 1994). AnchC function as a signal peptide for translocation of prM into the endoplasmatic reticulum (ER).

The second protein coded by the viral genome, with a size of approximately 26 kDa, is the prM glycoprotein. Upon translocation of prM into the ER, the C-terminal hydrophobic domain of C (anchC) is cleaved by the host signal peptidase. This cleavage only occurs after protein C been cleaved off the anchC at the cytosolic side of the ER membrane by the viral serine protease and these consecutive events of C and prM cleavage have been proposed to be a prerequisite for the initiation of virion assembly (Yamshchikov and Compans, 1994; Stocks and Lobigs, 1998; Amberg and Rice, 1999; Lobigs and Lee, 2004). At the amino terminal portion of prM there are one to three N-linked glycosylation sites and a sequence of six cysteines linked by disulfide bridges (Nowak et al., 1989; Chambers et al., 1990a). The prM protein is rapidly folded and acts as a chaperone for the correct folding of E protein (Konishi and Mason, 1993; Lorenz et al., 2002). prM also protects the E protein by inhibiting premature fusion of the newly generated virions with cellular membranes before releasing from the infected cell (Guirakhoo et al., 1992; Yu et al., 2008a). During the maturation of the particles, which occurs through the trans-Golgi network (TGN), the pr peptide is cleaved from prM by furin (a host encoded protease) (Stadler et al., 1997) and M remains as a transmembrane protein. Prevention of furin cleavage achieved for instance by the presence of protease inhibitors or acidotropic reagents results in the production of immature particles with lower infectivity (Stadler et al., 1997).

The third structural protein translated by flaviviruses is the E glycoprotein. This 53 kDa protein is the major constituent of the virion surface and is involved in the binding and fusion with membranes of the target cells. E protein interacts with the ER membrane by two transmembrane segments. There are 12 conserved cysteines that form disulfide bonds (Nowak and Wengler, 1987) and depending on the flaviviruses and their strains the presence of N-glycosylations sites can occur (Winkler et al., 1987). Although the exact role of these glycosylated sites is not determined, studies have demonstrated their association with the cell tropism and virulence of the strains (Shirato et al., 2004; Beasley et al., 2005). Cleavage of the N-terminal portion of E protein also is mediated by the host signal peptidase at the ER lumen (Nowak et al., 1989).

The crystal structure of the E protein revealed the presence of three domains. Domain I (DI) is the central domain; DII is the dimerization domain, which contains the fusion peptide; and DIII, which is an immunoglobulin-like domain believed to be the receptor-binding region to the target cell (Rey et al., 1995; Modis et al., 2003; Zhang et al., 2004). After protein synthesis and processing, E protein undergoes conformational changes that are pH dependent, starting as trimeric prM/E heterodimers in the ER and ending as E protein dimers after furin cleavage of pr peptide in the TGN and subsequent release to the extracellular milieu (Perera and Kuhn et al., 2008).

Nonstructural proteins

The flavivirus NS proteins are not only involved in the genome replication and polyprotein processing, but also have distinct functions on cellular processes and immune modulation. The first NS protein, NS1, has a molecular weight of approximately 46 kDa and was originally described as a soluble complement fixing antigen present in the serum and brain extracts of DENV-infected mice (Brandt et al., 1970a; Brandt et al., 1970b). Upon translocation to the ER, NS1 is cleaved from E by the host signal peptidase (cited above) and at the NS1/2A junction by an unknown ER-resident host cell protease (Falgout and Markoff, 1995). Two or three N-linked glycosylation sites can be added to

the protein, which also contains 12 cysteines linked by disulfide bonds that are important for correct folding and functionality of NS1 (Mason et al., 1987; Leblois and Young, 1993; Blitvich et al., 2001; Wallis et al., 2004). After synthesis, the NS1 monomer which has hydrophilic characteristics, dimerizes and acquires hydrophobic properties, being found associated with cellular membranes (Winkler et al., 1988; Winkler et al., 1989). NS1 also can be found in vesicular compartments either intracellularly or at the cell surface or can be secreted from the infected cell (Smith and Wright, 1985; Westaway and Goodman, 1987; Winkler et al., 1988; Mason, 1989; Gutsche et al., 2011).

The exact functions of the distinct oligomeric forms of the NS1 protein are still not completely elucidated. The intracellular NS1 has been implicated as a critical cofactor for viral genome replication as it is co-localized with the double-stranded RNA (dsRNA) and additional components at the site of RNA replication (Mackenzie et al., 1996; Westaway et al., 1997; Khromykh et al., 2000). The NS1 protein associated with the cell surface as well as the secreted form can induce the production of a high concentration of circulating antibodies, especially in an anamnestic response. Several studies have associated both the protein and the anti-NS1 antibodies with either protection or potentially increased pathogenesis (Schlesinger et al., 1987; Henchal et al., 1988; Falgout et al., 1990; Avirutnan et al., 2006; Sun et al., 2007) that is mediated by interaction with distinct target cells or inhibition/activation of the production of host proteins. Although more detailed studies are necessary to determine the exact role of NS1 in disease pathogenesis, it has been considered a potential candidate for the development of new generation of vaccines for different flaviviruses (Krishna et al., 2009; Miller, 2010).

The next protein coded by the flavivirus genome is the NS2A. NS2A is a small hydrophobic protein of approximately 22 kDa. The protein is generated by cleavage of the amino terminal portion by an unknown ER-resident protease (Falgout and Markoff, 1995) and cleaved from NS2B by the serine protease at the cytoplasm. Several functions

have been attributed to NS2A protein. It has a role in RNA replication, demonstrated by its co-localization with dsRNA, NS3 and NS5 present in the replication complex (Mackenzie et al., 1998), and particle assembly, demonstrated by mutational studies showing decreased production of infectious particles (Kummerer and Rice, 2002; Leung et al., 2008). NS2A has been shown to be involved in switching the process of replication and packaging of the RNA genome (Khromykh et al., 2001a). NS2A contributes to the production of a larger form of NS1 (NS1') (Mason, 1989) through a ribosomal frameshifting present in the amino-terminal portion of NS2A of encephalitic flaviviruses (Firth and Atkins, 2009; Melian et al., 2010). In addition, NS2A plays a role in the regulation of the host innate immune response such as type I interferon (IFN) inhibition (Munoz-Jordan et al., 2003; Liu et al., 2005; Liu et al., 2006) and dsRNA-activated protein kinase (PKR) blockage (Tu et al., 2012).

The next protein, NS2B, is also small (14 kDa) and interacts with the NS3 protein to function as a cofactor for the NS2B-NS3 serine protease (Chambers et al., 1991; Falgout et al., 1991; Arias et al., 1993). NS2B is cleaved from the polyprotein in both ends by the serine protease. It contains hydrophobic regions that are membrane-associated (Clum et al., 1997) and a 40-amino acid central hydrophilic domain that is associated with the serine protease activity (Erbel et al., 2006).

NS3 is a large (70 kDa) multifunctional protein involved in the cleavage of the polyprotein and RNA replication. The one-third amino-terminal region of NS3 contains the catalytic domain, which in association with NS2B forms the NS2B-NS3 serine protease complex (Bazan and Fletterick, 1989; Gorbalenya et al., 1989a; Chambers et al., 1990b) responsible for the cleavage of NS2A/2B, NS2B/3, NS3A/4A, NS4B/5 junctions, and additional sites within NS2A, NS4A and NS3 proteins (Fig. 1.2) (Lin et al., 1993; Amberg et al., 1994; Nestorowicz et al., 1994; Yamshchikov and Compans, 1994). The carboxy-terminal region of NS3 contains a RNA helicase domain (Gorbalenya et al., 1989b) with nucleoside triphosphatase (NTPase) function to properly unwind RNA

secondary structures and RNA duplexes during viral replication and synthesis of complementary RNA genome (Wengler and Wengler, 1991; Warrener et al., 1993; Li et al., 1999; Matusan et al., 2001). Additionally, this C-terminal region also has a RNA triphosphatase (RTPase) activity that is believed to be responsible for the dephosphorylation of the 5' end of the genome prior to cap addition (Wengler and Wengler, 1993). Besides the enzymatic activities, NS3 is necessary for efficient RNA packaging and plays a role in virus assembly (Liu et al., 2002; Pijlman et al., 2006; Patkar and Kuhn, 2008). NS3 also has been linked to the induction of apoptosis as demonstrated by studies using Langat, DENV-2 and WNV (Prikhod'ko et al., 2002; Shafee and AbuBakar, 2003; Ramanathan et al., 2006), and although caspases-8 have been implicated in this process, further studies are necessary to determine the role of NS3 in apoptosis induction.

NS4A and NS4B are small hydrophobic proteins with molecular weight of 16 and 27 kDa, respectively. NS4A colocalization with the replication complex suggests a role in RNA replication (Mackenzie et al., 1998). The C-terminal region of NS4A contains a signal peptide (2k) necessary for the translocation of NS4B to the ER lumen. This peptide is cleaved by the serine protease in the cytoplasm, allowing the subsequent cleavage of NS4B into the ER (Preugschat and Strauss, 1991; Lin et al., 1993). NS4B also is involved in RNA replication, as demonstrated by its interaction with NS3 and dsRNA in the replication complex (Westaway et al., 2002; Miller et al., 2006). Both NS4A and NS4B have been implicated in innate immune evasion mechanisms by suppressing type I IFN production or interfering in the signaling pathway (Munoz-Jordan et al., 2003; Munoz-Jordan et al., 2005; Evans and Seeger, 2007).

The final protein coded by the flavivirus genome is NS5. NS5 is a large (105 kDa) multifunctional protein with RNA-dependent RNA polymerase (RdRp) function that is important for RNA synthesis and methyltransferase (MTase) activity responsible for cap addition of the viral genome (Lindenbach et al., 2007). The MTase domain is located at

the N-terminal region and has the ability to link guanosine triphosphate (GTP) and promote biochemical methylation reactions during RNA cap formation (Egloff et al., 2002). The RdRp domain is located at the C terminal region and shares sequence homology with other positive-strand RNA viruses (Rice et al., 1985; Koonin and Dolja, 1993). The polymerase activity of this portion has been demonstrated by several studies (Tan et al., 1996; Ackermann and Padmanabhan, 2001; Guyatt et al, 2001) and mutations in NS5 confirmed its role in virus replication, where polymerase activity could be restored when NS5 was supplied in trans (Khromykh et al., 1998). Although NS5 is associated with NS3 (Kapoor et al., 1995; Johansson et al., 2001) and the 3'UTR of the viral RNA (Chen et al., 1997a), the presence of NS5 at the sites of viral synthesis, which is a requirement for RdRP function, still has to be demonstrated. Free forms of NS5 also have been found in the cytoplasm and in the nucleus, especially when phosphorylated (Buckley et al., 1992; Kapoor et al., 1995), providing evidence of additional interactions with host factors. Besides the enzymatic functions, NS5 is linked to the disruption of the IFN signaling, such as blocking of IFN-alpha/beta (IFN-α/β) and IFN-gamma (IFN-γ) receptors and phosphorylation of Janus kinases, and cytokine production (Best et al., 2005; Medin et al., 2005; Lin et al., 2006; Werme et al., 2008; Laurent-Rolle et al., 2010).

Flavivirus Replication Life Cycle

Flaviviruses can infect a wide range of mammalian, avian and insect cells. The entire replicative life cycle is summarized in Fig. 1.4. The first step of the viral infection is binding and entry into the target cells. Depending on the cell type and host species, flaviviruses use specific and non-specific receptors to attach and enter the cell (Lindenbach et al., 2007). The cellular receptors involved in this process are not completely characterized, but different surface molecules have been identified for different flaviviruses. A C-type lectin DC-SIGN (Dendritic cell-specific ICAM3-grabbing non-integrin), also known as CD209, has been identified in DC as an

attachment factor for DENV infection, showing DC-SIGN interaction with the viral E glycoprotein (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). WNV also binds to a related lectin (DC-SIGNR or CD209L) with higher affinity (Davis et al., 2006a). DC-SIGN is expressed by DC and macrophage subpopulations (Soilleux et al., 2002), while DC-SIGNR is present on microvascular endothelial cells of different tissues such as lymph nodes and liver sinusoids (Bashirova et al., 2001; Pohlmann et al., 2001). Because flaviviruses can infect a wide range of cells, they probably use more than one receptor to attach to the cell. Other molecules also have been implicated in the flavivirus attachment process, such as the $\alpha_v\beta_3$ integrin, the binding immunoglobulin protein (BiP) also known as glucose-regulated protein 78 (GRP78) and the CD14 or related molecules (Mukhopadhyay et al., 2005). Cells expressing immunoglobulin Fc receptors have been shown to be more permissive to infection when exposed to opsonized viral particles with immunoglobulins at sub-neutralizing concentration (Peiris and Porterfield, 1979). Additionally, highly sulfated glycosaminoglycans (GAG) such as heparan sulfate (HS) have been shown to be an important attachment factor for several flaviviruses (Chen et al., 1997b; Kroschewski et al., 2003; Thepparit et al., 2004).

Following the attachment of the virus particle presumably by E protein interaction with the cell surface, flaviviruses are internalized through clathrin-mediated endocytosis and transported to the endosome (Chu and Ng, 2004; van der Schaar et al., 2007; van der Schaar et al., 2008). The low pH of the endosome promotes conformational changes of E protein from homodimers to formation of trimers, resulting in fusion with the endosomal membrane and subsequent uncoating and release of the viral genome into the cytoplasm (Allison et al., 1995; Stiasny et al., 1996; Stiasny et al., 2001). Since the RNA genome is infectious, once it is released into the cytoplasm RNA translation can promptly start. Flaviviruses use the host cell machinery to translate their proteins primarily in a cap-dependent mechanism, which consists of ribosomal scanning and recognition of the AUG (methionine) start codon (Lindenbach et al., 2007). As described above, the translation of

the single ORF encoded by flaviviruses produces a large polyprotein that is localized to the ER membrane and cleaved to generate the structural and NS proteins necessary for virus replication and assembly of new particles.

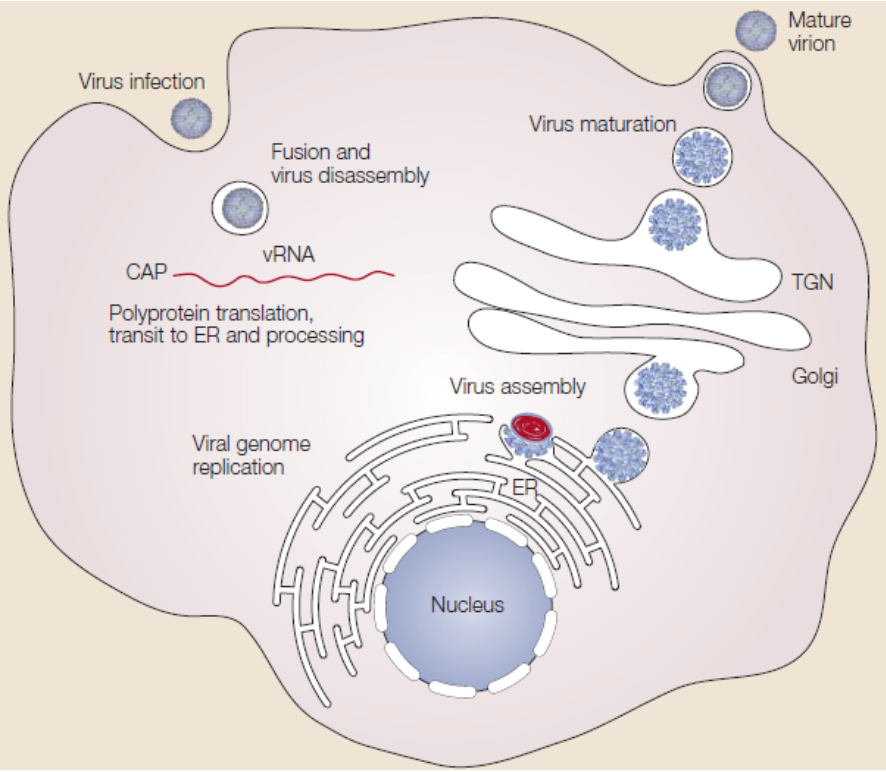

Figure 1.4. **Flavivirus life cycle**. Flaviviruses enter the cell by receptor-mediated endocytosis. The low pH of endosomes promotes fusion and release of the RNA genome into the cytoplasm of the infected cell. Viral proteins are translated and processed by viral and cellular proteases. Newly synthesized viral genome is packaged into immature particles in the ER and transported through the secretory pathway. Mature infectious particles are released from the cell by exocytosis. (Reprinted with permission from Mukhopadhyay et al., 2005. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 3:13-22).

Replication of the RNA genome is localized into virus-induced membrane structures and requires the formation of a replication complex, which includes the viral RNA-dependent RNA polymerase (NS5) and accessory NS proteins, the viral RNA and possibly other host factors (Mackenzie, 2005; Miller and Krijnse-Locker, 2008). Initially,

a full-length negative-sense RNA is generated, which is subsequently used as a template for the synthesis of additional positive-sense RNA genome copies in a semiconservative and asymmetric manner (Chu and Westaway, 1985). During replication, three forms of viral RNA have been described, including the single-stranded positive-sense viral RNA, a double-stranded replicative form (RF) and a replicative intermediate (RI) containing RNA duplexes and nascent positive-sense RNA strands (Cleaves et al., 1981; Chu and Westaway, 1985).

The nascent genomic RNA interacts with the C protein to form the nucleocapsid. Nucleocapsids contain a single copy of the viral RNA associated with multiple copies of C protein. Immature particles are assembled when nucleocapsids bud into the ER lumen, acquiring a lipid envelope that contains the prM and E proteins (Wengler and Wengler, 1989). In a similar process, SVP lacking the viral nucleocapsid are also assembled and undertake the same modification as the immature viral particles. These immature, non-infectious particles contain heterodimers of prM and E that form trimeric spikes projected vertically on the virus surface (Zhang et al., 2003a,b; Li et al., 2008). As these particles are transported though the secretory pathway, they are exposed to low-pH environment, which triggers major conformational changes and E proteins assume a conformation similar to the mature virus (Yu et al., 2008a; Yu et al., 2009). In this conformation at low pH, the prM protein becomes accessible to furin cleavage (Li et al., 2008; Yu, et al., 2008a), which is abundant in the TGN (Molloy et al., 1994). After furin cleavage, E proteins form homodimers that are organized flat on the virus surface resulting in mature infectious particles (Rey et al., 1995; Kuhn et al., 2002). The cleaved pr peptides remain in complex with E protein over the fusion loop (Guirakhoo et al., 1992) inhibiting virus-membrane interaction to protect E protein from premature fusion with cell membranes in the secretory pathway until the release of the mature infectious particles to the extracellular milieu by exocytosis (Li et al., 2008; Yu et al., 2008a; Yu et al., 2009; Zheng et al., 2010).

West Nile virus

West Nile virus (WNV) is a neurotropic, mosquito-borne flavivirus that can cause disease in humans and animals including birds, horses and other vertebrate species. WNV has a global distribution and is endemic in several regions of the world. It was originally isolated from a patient with symptomatology of a febrile illness in the West Nile district of Northern Uganda in 1937 (Smithburn et al., 1940); however, the disease in humans started only to be characterized in more detail during outbreaks in the Mediterranean area in the early 1950s (Melnick et al., 1951; Bernkopf et al., 1953; Taylor et al., 1956; Murgue et al., 2001). In the following years, sporadic outbreaks occurred in Africa, the Middle East, southern Europe, southwestern Asia and Australia [Kunjin virus (KUNV)] (McIntosh et al., 1964; Panthier, 1968) and were primarily associated with mild febrile symptoms, which decreased the interest in WNV as an important agent causing human disease. This scenario changed around 1996, when large outbreaks associated with more severe and fatal neurologic disease started to occur in different locations such as Romania, Morocco, Tunisia, Italy, Russia, Israel and Greece (Hubalek and Halouzka, 1999; Platonov et al., 2001). In 1999 the virus was first detected in North America, associated with severe cases of human encephalitis in the area of New York City (Nash et al., 2001).

It remains unclear how WNV was introduced into North America, but the source of the virus was probably from the Middle East, as the genomic sequence of WNV isolates from the initial outbreak had a great homology with isolates from WNV cases in Israel and the patterns of disease were similar in both locations (Giladi et al., 2001). In the following years, the virus continued to spread throughout the USA, reaching all the mainland states (Fig. 1.5). From 1999 through 2012, 37,088 cases of human WNV disease were reported in the USA, resulting in 1,549 deaths. The largest epidemics happened in 2002-2003 and more recently in 2012, with nearly 300 human deaths registered in each of those years (Fig. 1.6) (Hayes and Gubler, 2006; CDC, 2013a). In

Canada, WNV disease has been reported since 2002 (Drebot et al., 2003) and evidence of WNV circulation has been found in several countries of Latin America (Deardorff et al., 2006; Komar and Clark, 2006; Pupo et al., 2006; Beatty et al., 2007; Bosch et al., 2007; Diaz et al., 2008), although human disease is not normally seen in these areas.

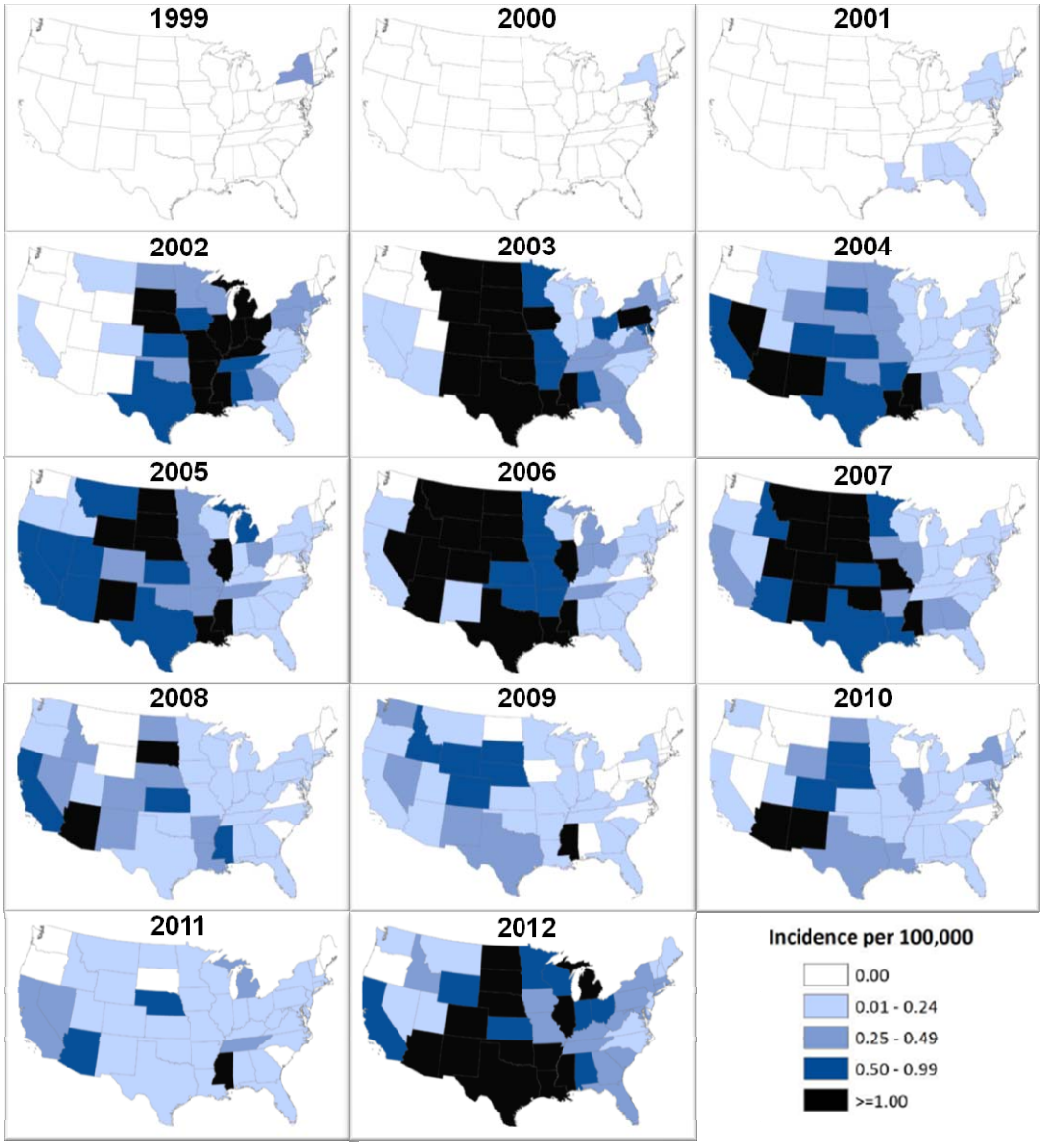

Figure 1.5. **Incidence of WNV disease in the United States from 1999 to 2012**. Data represent the number of human neuroinvasive disease cases (e.g., meningitis, encephalitis, or acute flaccid paralysis) reported by state and local health departments to CDC's ArboNET surveillance system. (Figure courtesy of the Centers for Disease Control and Prevention).

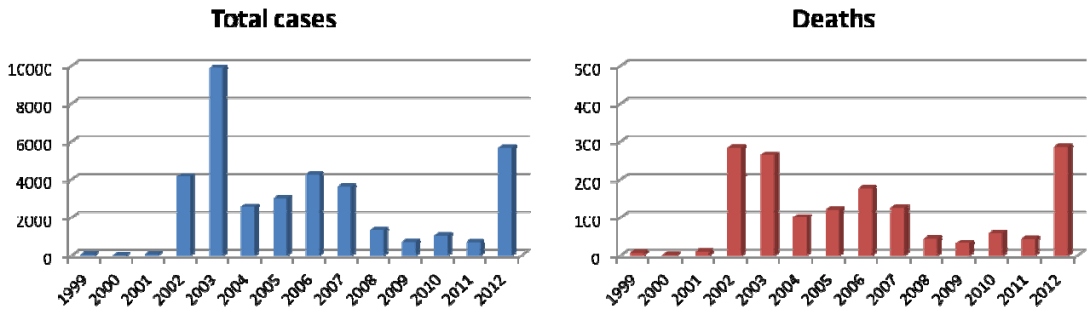

Figure 1.6. **WNV disease cases in the United States from 1999 to 2012**. Total number of neuroinvasive and non-neuroinvasive cases reported by state and local health departments to CDC's ArboNET surveillance system. (Source: Centers for Disease Control and Prevention).

WNV classification

Based on serologic reactivity, WNV is classified into the Japanese encephalitis virus complex, which includes JEV, Koutango virus (KOUV), Murray Valley encephalitis virus (MVEV), Alfuy virus (ALFV), St. Louis encephalitis virus (SLEV), Usutu virus (USUV), Yaounde virus (YAOV), and Cacipacore virus (CPCV) (Lindenbach et al., 2007). WNV is also classified according to nucleotide sequence data. Phylogenetic analyses of distinct WNV isolates collected since its first isolation from different parts of the world have subdivided them into two major lineages. The lineage I contains a diverse set of viruses isolated from Africa, India, Europe, the Middle East, Australia (KUNV), and from the Americas, which association with severe disease and mortality are common. Lineage II viruses, including the original isolate from 1937, are mostly present in Africa and are normally linked to asymptomatic or mild WNV disease (Lanciotti et al., 1999; Murgue et al., 2002).

In recent years, with the generation of large amounts of sequencing data, further division of WNV in additional lineages, clades and clusters has been proposed. According to this new classification, WNV are divided into five lineages. Viruses from

lineage I are separated into two clades. Clade 1a, containing the isolates from Africa, Europe, the Middle East and from the Americas; and 1b consisting of isolates from Australia (KUNV subtype) (May et al., 2011). Lineage II is still represented mostly by the African isolates, including recent isolates from Hungary (Erdelyi et al. 2007), Greece (Papa et al., 2011), Italy (Bagnarelli et al., 2011) and Russia (Platonov et al., 2008). Lineage III is composed by a single isolate from Czech Republic/Austria (Bakonyi et al., 2005) and lineage IV contains isolates from Russia. Lineage V contains the isolates from India, previously classified into the lineage I (Bondre et al., 2007).

The diversity of WNV, not only genetically, but also according to the geospatial distribution has caught the attention of researchers all over the world. Complex factors may be involved in the evolution of WNV. As an example, the isolate introduced into the USA in 1999 (classified as East Coast genotype) has been displaced by a novel dominant genotype in 2002 (North American genotype) (Davis et al., 2005). Subsequently, another genotype (Southwestern genotype) also has been identified in 2003 (McMullen et al., 2011) and it seems that they are still co-circulating in recent years. Several amino acids substitutions found in these isolates, combined with climate and/or ecological conditions may have facilitated the adaptation of WNV in the host/vector environment and can be one of the reasons for differences in the severity/number of cases observed in the United States and other locations over the years.

WNV transmission

The transmission of WNV is maintained in an enzootic cycle involving primarily mosquitoes and birds. Infected birds can develop a high viremia facilitating the transmission of the virus during mosquito feeding. WNV also can be transmitted to humans, equines and other vertebrates; however, they usually do not develop a high level of viremia, and cannot maintain the transmission of the virus to other biting mosquitoes and therefore are considered dead-end hosts (Fig.1.7).

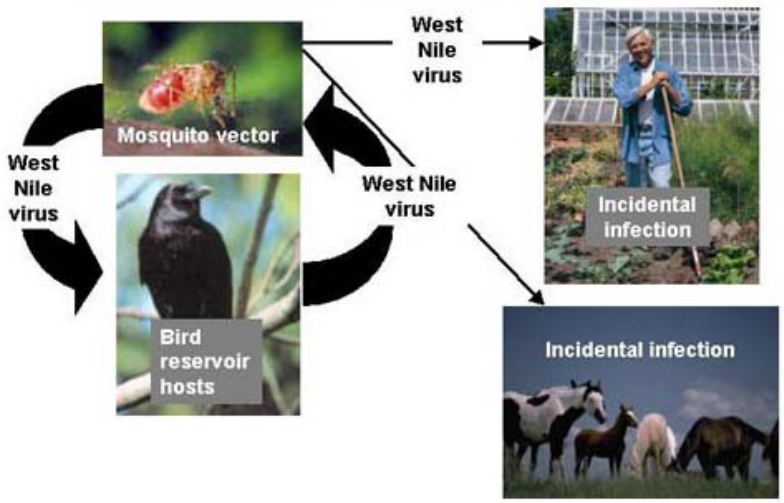

Figure 1.7. **Transmission cycle of WNV.** WNV is primarily maintained in nature by the transmission between the mosquito vector and bird reservoirs (amplifying hosts). Humans and other animal species also can become infected, but due low transient viremia, are considered dead-end hosts. (Source: Centers for Disease Control and Prevention, accessed through http://en.wikipedia.org).

Different mosquito species are involved in the transmission of WNV; however, the efficiency of WNV transmission is not equivalent among the different species (Moudy et al., 2007). *Culex* spp. mosquitoes represent the main vectors for WNV (Nir et al., 1968; Kilpatrick et al., 2008). In the United States, *Culex pipens*, *Cx. tarsalis*, *Cx. quinquefasciatus*, *Cx. restuans*, and *Cx. nigripalpus* are mostly involved in WNV transmission (Petersen and Hayes, 2008), but the virus already has been isolated from 65 mosquitoes species including *Aedes*, *Anopheles* and *Culiseta* mosquitoes (CDC, 2013b).

Mosquitoes acquire WNV during the blood meal on a viremic infected host (birds). The virus initially infects the midgut epithelial cells and disseminates through the hemolymph to other mosquito tissues, including the salivary glands. Once WNV is in the salivary glands, it can be transmitted to other vertebrate hosts through the secreted saliva (Girard et al., 2004). A study with *Cx. tarsalis* mosquitoes showed that the concentration

of WNV in the blood meal necessary to infect the mosquitoes was $10^{7.1}$ pfu/mL (Goddard et al., 2002). Viremia in humans reaches about $10^{3.2}$ pfu/mL, not enough to infect mosquitoes during the blood meal (Hayes et al., 2005a). The time from when the mosquitoes get infected until the virus reaches the salivary glands, known as extrinsic incubation period, is between 8 to 14 days and will depend on several factors including climate temperature, virus strain and mosquito species. Mosquitoes have defense mechanisms that can restrict WNV infection and dissemination. The midgut epithelium provides a physical and immune barrier by producing antimicrobial peptides (Tzou et al., 2000) and a peritrophic matrix (chitin, glycoproteins and proteoglycans) (Shao et al., 2001). WNV also triggers a RNA interference (RNAi) mechanism and innate immune signaling including Toll, immune deficiency (IMD) and Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathways that restrict virus infection (Arjona et al., 2011).

Following the extrinsic incubation period, the enzootic cycle of WNV is continued by transmission of the virus from mosquito vectors to the vertebrate reservoir hosts. Birds are considered the amplifying hosts for WNV and can propagate the virus locally or at long distances by migratory birds (Malkinson and Banet, 2002; Peterson et al., 2003; Rappole et al., 2006; Jourdain et al., 2007). In the USA the virus has been detected in over 300 bird species, including corvids (crows, jays, magpies), grackles, house finches and house sparrows (CDC, 2013c). While birds infected with WNV usually survive the infection and acquire a long-lasting immunity (Komar et al., 2001), some species are more vulnerable and develop the disease and die. Members of the family

Corvidae are particularly susceptible and in fact play an important role in the surveillance program to detect WNV in dead birds (Eidson et al., 2001).

WNV also has been identified in different mammals including squirrels, chipmunks, cats, rabbits, skunks and bats (van der Meulen et al., 2005; Gubler, 2007; Platt et al., 2007; Platt et al., 2008), reptiles (alligators) (Klenk et al., 2004) and amphibians (frogs) (Kostiukov et al., 1985), but their role in WNV transmission still have to be further investigated.

The non-vectored transmission of WNV also has been described. In humans, blood transfusion, organ transplant and transplacental infection of the fetus have been reported as routes of WNV transmission (Iwamoto et al., 2003; Pealer et al., 2003; O'Leary et al., 2006). Transmission from the mother to the baby by breast milk has been documented, but it seems to be rare (Hinckley et al., 2007). Percutaneous or conjunctival exposure is a potential route of WNV transmission especially in laboratory personal, who handle the virus or infected specimens (Nir et al., 1965; CDC, 2002; Fonseca et al., 2005).

WNV pathogenesis

WNV infection can result in either asymptomatic (no symptoms) or symptomatic disease. The incubation period of WNV is between 2 and 14 days (Petersen and Marfin, 2002). Several factors can influence the outcome of WNV infection, including the virus strain, infectious dose, route of inoculation and host immune condition, genetic susceptibility and age (Eldadah et al., 1967; Gubler et al., 2007; Diamond, 2009a; Lim et al., 2009). Serologic studies suggest that most of WNV human infections (~80%) result in no or very mild clinical manifestations. Approximately 20% of infected individuals

develop West Nile fever (WNF), which is characterized by flu-like symptoms including fever, headache, myalgias, malaise and lymphadenopathy (Watson et al., 2004; Hayes et al., 2005b). A more severe illness, the WN neurological disease (WNND), can occur in less than 1% of the infections, and is manifested by different presentations including meningitis, encephalitis and acute flaccid paralysis (Tyler, 2004; Sejvar and Marfin, 2006). WNND results in approximately 10% mortality, but elevated rates are seen in immunosuppressed or elderly individuals (Murray et al., 2006). Long-term sequelae can occur in more than 50% of individuals convalescing from severe WNND (Klee et al., 2004; Sejvar et al., 2006). There are no specific treatment options for WNV infection, and humans are limited to supportive therapy (Kramer et al., 2007), although immunoglobulin (Shimoni et al., 2001) and IFN-α treatment (Kalil et al., 2005) have been used in some severe cases.

The pathogenesis of WNV infection in humans is not completely understood and most of the information about the development of the disease has been obtained by experimental infection of animal models, mostly rodents. After the mosquito bite, it is believed that initial viral replication occurs at the site of inoculation. It has been shown WNV infects keratinocytes (Lim et al., 2011) and skin-resident DC including dermal DC and Langerhans cells (DC) (Byrne et al., 2001). Following infection, DC migrate to draining lymph nodes and the virus reaches the bloodstream (primary viremia) resulting in the infection of several organs and tissues, including spleen and kidneys. A second round of replication occurs, where epithelium cells and macrophages are considered potential targets of the virus (Rios et al., 2006). The invasion of WNV into the central nervous system (CNS) is believed to be correlated with the level and duration of

secondary viremia. The exact mechanism by which WNV crosses the blood-brain barrier (BBB) is unknown, but several theories have been proposed to explain WNV neuroinvasiveness. Hematogenous spread may be involved, where the increase of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) may facilitate the process by promoting changes in endothelial cell permeability (Wang et al., 2004). Other mechanisms include infection or passive transport through the endothelium or choroid plexus epithelial cells (Kramer-Hammerle et al., 2005), infection of olfactory neurons and consequent dissemination to the olfactory bulb (Monath et al., 1983), traffic of infected immune cells to the CNS ("Trojan horse" mechanism) (Garcia-Tapia et al., 2006) and direct axonal retrograde transport of particles that have infected peripheral neurons (Johnson, 1998; Hunsperger and Roehrig, 2006). In humans, the most frequent regions of the brain where the WNV is detected are in the cerebral cortex, thalamus, brainstem, basal ganglia, cerebellum and spinal cord and less frequently in the olfactory bulb and hippocampus (Armah et al., 2007). In rodents, the tropism of WNV seems to be similar as in humans, since the virus is detected in the same regions of the brain (Eldadah and Nathanson, 1967; Xiao et al., 2001; Shrestha et al., 2003). The pathological findings in the CNS can be the result of direct viral replication in neurons and glial cells or consequence of the host immune response against the infected cells (Shieh et al., 2000; Sampson and Armbrustmacher, 2001). Most of the damage is caused by loss of neuronal cells, necrosis or neuronophagia. A diffuse inflammation is often seen in the thalamus, medulla and other parts of the brain stem. Microglial nodules with predominance of histiocytes and lymphocytes ($CD8^+$ and in less extend $CD4^+$ T-lymphocytes) are

described, especially at the proximal spinal cord. Perivascular inflammation with the presence of B-lymphocytes also is a common characteristic (Gyure, 2009).

The dynamics of WNV infection show that viremia is present from about 2 days before until day 4 after the onset of disease; however after the first day, the chances for virus isolation are greatly decreased, probably due to clearance of the virus by macrophages or neutralization by IgM antibodies (Campbell et al., 2002). On the other hand, viremia in immunocompromised patients has been reported to last for several weeks after the WNV inoculation (Brenner et al., 2005). In peripheral tissues most of the WNV is cleared by the end of the first two weeks of infection, although as referred above, persistent infection can occur in debilitated patients.

Animal models for WNV disease

As mentioned above, most of the mechanisms of WNV dissemination and pathogenesis have been obtained through studies using animal models. In particular, rodent models of infection (mice and hamsters) are largely used because these animals develop neurological disease similar to humans and generate both innate and adaptive immune responses. They are easy to handle and also much less expensive compared to other models such as non-human primates. Another advantage of rodent models, and especially mice, is the availability of different reagents such as cell markers (monoclonal antibodies) that make possible the study of specific cell types and targets for WNV infection. The existence of several transgenic mouse strains containing targeted deficiencies (knockout mice) also allowed the elucidation of the role of different molecules and signaling pathways involved in the control of WNV infection (see discussion in the next section).

Mice are usually inoculated with WNV by intraperitoneal (i.p.), intravenous (i.v.) or subcutaneous (s.c.) delivery routes. The s.c. inoculation in the footpad is the route that most resembles the natural infection by the mosquito bite, although several components of the mosquito saliva are not present (Scheneider et al., 2006). The i.p. and i.v. inoculation also reproduces the WNV disease; however the rate of virus dissemination seems to be faster than s.c. inoculation, possible due to specific and nonspecific virus-host interaction that occurs in the skin and lymph nodes and are bypassed during i.p. and i.v. inoculations (Kramer and Bernard, 2001; Suthar et al., 2013a). After peripheral s.c. inoculation, virus can be detected in the serum within 24 to 48 hours of infection, with subsequent isolation from peripheral organs such as spleen, kidney and heart. In the CNS, WNV can be detected by day 4 (Kramer and Bernard, 2001; Diamond et al., 2003a). In contrast, WNV can be detected in the brain of mice inoculated via s.c. or i.v. routes as early as 2 days post inoculation, and these animals developed the neurological disease earlier than the s.c. inoculated animals (Kramer and Bernard, 2001). Independent of the inoculation route, after CNS infection the animals develop clinical symptoms characterized by weakness, ataxia and hind limb paralysis. Mortality is typically observed starting at days 9 to 10 post inoculation and is associated with high loads of virus in the CNS (Kramer and Bernard, 2001; Shrestha et al., 2003). Although most of mice succumb to the infection (>90%), surviving animals develop high levels of neutralizing antibodies and are protected from a secondary WNV infection.

The course of infection in hamsters is similar to that observed in mice, although the development of disease is to some extent delayed, reflecting more what happens in

humans. The mortality rate is lower than the rate usually observed in mice, where about 50% of hamsters succumb to the infection (Xiao et al., 2001; Morrey et al., 2004).

Non-human primates (NHP) also have been utilized as a model of WNV infection. Upon parenteral WNV inoculation, NHP usually develop viremia, but not WNV disease, and comparable to the majority of human infections, most of the cases are presented as a subclinical infection (Pogodina et al., 1983; Ratterree et al., 2003; Ratterree et al., 2004). Hence, due the similarity with humans, NHP are especially considered a potential model for vaccine testing.

IMMUNE RESPONSES TO WNV INFECTION

The immune responses to flaviviral infections have been a subject of research for several years and a considerable number of studies have brought new insights into the elucidation of type of cells, molecules and signaling pathways involved in WNV immunity. It has been shown that both innate and adaptive immune responses play a role in WNV infection. Innate immunity is composed of antigen nonspecific defense mechanisms and is initiated early after the pathogen invasion, while the adaptive immune response is antigen specific and is developed later in the course of infection. However, during WNV infection the contribution of both branches of the immune response are essential for the restriction of spread of the virus and viral clearance from infected cells and/or tissues.

Recognition of WNV by the host innate immune system

During an infection, invading microorganisms can generate conserved molecules known as pathogen-associated molecular pattern (PAMP) ligands (Medzhitov and Janeway, 2002) that are recognized by innate immune cells through the association with

multiple cellular pattern recognition receptors (PRRs) (Kumar et al., 2001). PRR expression mostly occurs in cells that first encounter the pathogen, including monocytes, neutrophils, macrophages, dendritic cells and epithelial cells, but also can be present in cells of the adaptive immune system (Schroder and Tschopp, 2010). PRRs stimulate the immune response by triggering downstream signaling pathways that most often induce a pro-inflammatory response mediated by the production of cytokines, chemokines and antiviral molecules including type I IFN (Daffis et al., 2009; Kawai and Akira, 2010). Several types of PRRs have been identified and characterized, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA receptors (cytosolic sensors for DNA) (Kumar et al., 2001). These PPRs can recognize extracellular and intracellular pathogens through the detection of pathogen molecules such as conserved proteins, lipids, carbohydrates and nucleic acids (Medzhitov, 2007; Kawai and Akira, 2010; Takeuchi and Akira, 2010).

The TLRs are the most characterized receptors and considered the major sensors of invading microorganisms. They are also implicated in the activation of adaptive immune responses including antibody, $CD4^+$ T cell and $CD8^+$ T cell responses (Iwasaki and Medzhitov, 2004). In humans there are 10 identified members of the TLR family and in mice, 12 TLRs have been documented. TLRs are either expressed on the cell surface or in intracellular organelles (Iwasaki and Medzhitov, 2004). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are responsible for sensing extracellular pathogens, especially PAMPs originated from bacteria, fungi and protozoa. On the other hand, the endosomal TLR3, TLR7, TLR8 and TLR9 are responsible in detecting nucleic acids PAMPs mainly from

viruses and bacteria (Medzhitov, 2007; Kawai and Akira, 2010; Takeuchi and Akira, 2010).

The detection of WNV by TLRs occurs in the endosomal compartment and is mediated by recognition of single-strand RNA (ssRNA) and double-strand RNA (dsRNA) through TLR7 and TLR3, respectively (TLR-dependent pathway) (Daffis et al., 2009; Diamond, 2009b). Following recognition of viral RNA, TLR3 and TLR7 recruit the adaptor protein molecules, TRIF (Toll/IL1 receptor domain-containing adaptor inducing IFN) and MyD88 (Myeloid differentiation primary response gene 88), respectively, to initiate a signaling cascade involving activation of several transcription factors including IRF (Interferon regulatory factor)-3, IRF-7 and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) that drive the production of type I IFN and pro-inflammatory cytokines (Barton and Medzhitov, 2003).

Another type of PRR involved in WNV recognition is the RLR family. RLRs constitute a TLR-independent pathway and contain three members: retinoic acid-inducible gene 1 (RIG-I), Melanoma Differentiation-Associated protein 5 (MDA5), which recognize ssRNA and dsRNA, respectively (Yoneyama and Fujita, 2008; Schlee et al., 2009; Loo and Gale, 2011; Berke and Modis, 2012) and LGP2, which is less known, but probably involved in the regulation of viral RNA recognition by RIG-I and MDA5 (Yoneyama et al., 2005; Loo and Gale, 2011). RIG-I and MDA5 are cytosolic RNA helicases, for which signaling is mediated by interaction with the mitochondrial-associated IFN promoter stimulator-1 (IPS-1) (also known as MAVS, CARDIF or VISA). The outcome of this interaction leads to the activation of IRF3/7 and NF-κB and

consequent production of cytokines similar to the TLR-dependent pathways (Wilkins and Gale, 2010).

A third family of PRRs is the nucleotide-binding, oligomerization domain (NOD)-like receptors (NLR). NLRs not only recognize PAMPs, but also host-derived danger signals (DAMPs – danger-associated molecular patterns) originated from necrotic or injured cells (Kumar et al., 2011). There are several members included in this family and they are further divided into three distinct subfamilies: NODs, NLRPs and IPAF (Kumar et al., 2011). WNV recognition by NLRs involves the adaptor protein ASC (Apoptosis-associated speck-like protein containing CARD) (Kumar et al., 2013), which mediates the interaction between the PRR and caspase 1 in a multiprotein complex named inflammasome (Martinon et al., 2002). As a consequence of this interaction, caspase cleavage of its inactive form results in the maturation and production of inflammatory cytokines, including IL-1β and IL-18 or can initiate a process of cell death (Kumar et al., 2011; Kumar et al., 2013). A summary of PRRs and signaling pathways involved in WNV recognition is represented in Fig.1.8.

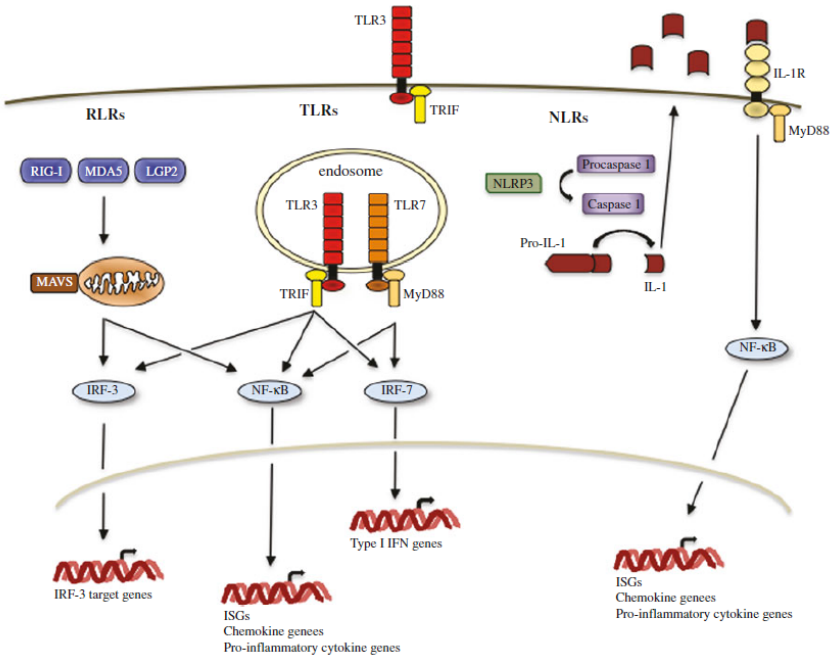

Figure 1.8. **Innate immune response to WNV.** Schematic representation of PRRs and signaling pathways involved in the cellular recognition of WNV. (Reprinted with permission from Fredericksen, 2013. The neuroimmune response to West Nile virus. J Neurovirol [Epub ahead of print]).

Type I IFN response

Upon recognition of viral infection, cells produce type I IFNs (IFN-α and IFN-β) inducing an antiviral state that is achieved by the expression of several genes with antiviral properties (Der et al., 1998). The activation of the IFN promoter leads to production of type I IFN, which is secreted from the infected cell and binds to the IFN-α/β receptor (IFNAR) on the cell surface to amplify the production of pro-inflammatory cytokines through an autocrine or paracrine manner. The binding of IFN to the IFNAR activates the Janus activated kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway. Activation of tyrosine kinase 2 (TYK2) and JAK1, which are associated with the cytoplasmic tail of the IFNAR results in phosphorylation of STAT1 and STAT2 and formation of STAT1-STAT2-IRF-9 complexes (known as interferon-

stimulated gene [ISG] factor 3 [ISGF3]). ISGF3 complexes are translocated into the nucleus and target IFN-stimulated response elements (ISREs) present in the promoters of hundreds ISGs (Stark et al., 1998; Li et al., 2007), some of them associated with antiviral properties against flaviviruses (Diamond, 2009b).

Among the ISGs with broad antiviral properties are the dsRNA-dependent protein kinase (PKR), 2'-5'-oligoadenylate synthetase (OAS) and RNase L, which have been shown to confer cell resistance to WNV (Samuel et al., 2006; Scherbik et al., 2006; Gilfoy and Mason, 2007). The inactive form of PKR is activated by dsRNA produced during the viral infection and induces the phosphorylation of the eukaryotic initiation factor 2α (eIF-2α), preventing the translation of viral and cellular RNAs (Chong et al., 1992; Meurs et al., 1992). Similar to PKR, OAS is activated by dsRNA and binds to RNase L, which in turn limits viral infection by cleavage of viral RNA and amplification of immune response through the generation of small self-RNAs (Zhou et al., 1997; Malathi et al., 2007).

Other ISGs also have been shown to interfere with WNV infection. Viperin (RSAD2) is believed to inhibit viral infection by modulating lipid biosynthesis, as demonstrated by *in vitro* and *in vivo* experiments (Szretter et al., 2011). Genes of the IFN-induced protein with tetratricopeptide repeats (IFIT) family, including IFIT-1 (ISG56), IFIT-2 (ISG54) and IFIT-3 (ISG60), were also shown to interfere with WNV infection, possibly by ablation of protein translation through eIF3 interaction (Terenzi et al., 2006; Wacher et al., 2007). IFN-induced transmembrane protein (IFITM), including IFITM-2 and IFITM-3, have been shown to inhibit the process of WNV entry into target cells (Brass et al., 2009; Jiang et al., 2010; Cho et al., 2013). ISG20, which has an

exonuclease function, also has been investigated as an inhibitor of WNV infection (Jiang et al., 2010; Zhou et al., 2011). The identification and characterization of these ISGs and additional genes with antiviral properties, especially genes that can inhibit a broad spectrum of viruses, is important for the development of novel strategies to control not only WNV, but also several other viral infections.

Cellular innate immunity against WNV

Cellular innate immunity has an important role for the control of WNV infection. Among the cells involved in the innate immune response are macrophages, DCs, neutrophils, natural killer (NK) and gamma-delta (γδ) T cells.

Macrophages were the first identified antigen-presenting cells (APC) (Ziegler and Unanue, 1981) and several studies demonstrate that they play an important role in controlling WNV infection. Despite macrophages being a cell target for WNV replication (Rios et al., 2006), different mechanisms have been proposed to explain the protective function of macrophages on flavivirus infection. Macrophages can potentially inhibit WNV through direct clearance of the virus, promotion of antigen presentation and production of cytokines and chemokines (Kulkarni et al., 1991; Ben-Nathan et al., 1996; Purtha et al., 2008), as demonstrated by macrophage depletion experiments where the absence of macrophages promoted an increased viremia and dissemination of non-virulent WNV strains to the brain of infected mice, resulting in higher mortality. Macrophages also have the ability to produce nitric oxide (NO) intermediates, which can inhibit a variety of viral infections. Although the direct role of NO in WNV inhibition has not yet been completely proven, there is evidence demonstrating its role in the control of other flaviviruses such as JEV and TBEV (Kreil and Eibl, 1996; Lin et al., 1997).

DCs also are targets for WNV infection, but their protective role in WNV infection is less understood. There are distinct DC subsets, which differ in the expression of surface makers and functionality. Plasmacytoid DCs (pDC) are found circulating in the blood stream and in peripheral lymphoid organs. pDCs are not promptly infected with WNV and their ability to present antigen to T cells is very low. On the other hand, pDCs produce high amounts of type I IFN and other pro-inflammatory cytokines, emphasizing their role in immune protection against viral pathogens (Wang et al., 2006a; Swiecki et al., 2010). Another type of DC is the myeloid DC (mDC). mDCs are composed by resident and migratory DC subsets, which have a higher capacity to present antigens, rapidly activating B and T cell responses (Steinbrink et al., 2009). As mDCs are more susceptible to WNV infection, it is believed that they also contribute to viral spread from the site of infection to lymphoid tissues (Byrne et al., 2001).

Neutrophils, also referred as polymorphonuclear leukocytes (PMNs), are innate cells that are quickly recruited to the site of inflammation, promoting interaction and activation of other APCs (Nathan, 2006). In WNV infection, it is believed that neutrophils have a dual function, allowing viral dissemination at initial stages and controlling the virus as the infection progresses (Bai et al., 2010). This is supported by experiments showing that depletion of neutrophils prior to WNV infection resulted in lower viremia and less mortality, while depletion of neutrophils after WNV inoculation resulted in higher viremia and increased mortality, suggesting that neutrophils are initially reservoirs for virus replication and only at later time do they play a protective role, helping in the clearance of the virus (Bai et al., 2010).

NK cells are innate immune cells that quickly respond to viral infections. NK cells can potentially control WNV by recognizing and eliminating infected cells through the release of perforin and granzymes or signaling by IFN-γ secretion (Vivier et al., 2008). Although studies demonstrated NK cell activation in the spleen of WNV-infected mice (Vargin and Semenov, 1986) and WNV inhibition in cell culture (Zhang et al., 2010), the protective role of NK cells during WNV infection still has to be investigated. *In vivo* studies assessing NK cell function by NK depletion using anti-NK antibody treatment or transgenic mice deficient in circulating NK cells showed no significant difference between control and antibody-treated mice or WT and knockout strains in relation to signs of disease and mortality rates (Shrestha et al., 2006a). The divergent role of NK cells may be influenced by WNV evasion strategies or organ-specific factors, as recently shown in a study where NK cells were able to control infection in the liver, but not in the spleen of WNV-infected mice (Suthar et al., 2013a).

γδ T cells have effector functions that have been shown to play an important role in protection against WNV infection. γδ T cells lack MHC restriction, not requiring conventional antigen processing to respond against the infection (Steele et al., 2000). The protective function of γδ T cells is attributed to secretion of cytokines, including IFN-γ, TNF-α and IL-17 (Ferrick et al., 2000; O'Brien et al., 2009), cytolytic activity (Wang et al., 2003a) and development of memory T cells (Wang et al., 2006b). Experiments in mice lacking γδ T cells demonstrated a higher viremia and increased dissemination of the virus in the brain of WNV-infected mice (Wang et al., 2003a; Shrestha et al., 2006b). Memory T cell responses are also affected in mice lacking γδ T cells, possibly due to an

indirect mechanism involving activation of DC to promote the priming of T cells (Fang et al., 2010).

Adaptive immune response against WNV

The role of the adaptive immune response in WNV pathogenesis has been a subject of study for several years. It is known that B cells, $CD4^+$ and $CD8^+$ T lymphocytes are important for the control of WNV infection. The induction of the adaptive immune response is triggered by different signals including cytokines and antigen, and receptor binding interactions mainly provided from the initial innate immune response, resulting in the specialization of lymphocytes with particular effector functions against the invading pathogen. Hence, the adaptive immune response is linked to the non-specific innate immune response, and once activated it causes clonal expansion of the immune cells that are specific for an antigen or pathogen, which in turn exert their function in attempt to control and eliminate the infection.

Humoral response

The humoral immune response is composed of antibodies produced by B cells and has been demonstrated to be essential for controlling WNV infection and dissemination. The WNV-specific IgM antibody response starts to rise by day 4 of infection and it has been demonstrated that it can give partial protection to naïve mice challenged with WNV (Diamond et al., 2003a). IgG class antibodies start to be detected by day 8, with an increased activity by day 12 of infection (Diamond et al., 2003a).

The importance of antibodies in the protection against WNV has been demonstrated in different mouse models. Passive transfer of immune sera to naïve mice has been shown to be effective in the protection against lethal WNV challenge (Diamond et al., 2003a). In

the same study, using congenic mice deficient in functional B cells, it was demonstrated a significant increased viral dissemination to the CNS, even at low infectious doses of the virus, resulting in much higher mortality compared to the WT mouse strain. Further studies, using mice deficient in B and T cells reinforced the importance of antibodies in control WNV dissemination, but demonstrated that antibody alone is not sufficient to completely eliminate the infection, since passive transfer of immune serum prevented WNV disease in the first few weeks of infection, however the mice succumbed to infection after decrease of antibody titer (Engle and Diamond, 2003). Among the immunoglobulin (Ig) classes, it has been demonstrated that IgM may contain the initial WNV dissemination by direct neutralization of the virus, especially at peripheral tissues, decreasing viremia and stimulating IgG production or T cell responses that will eventually control the viral infection (Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000; Diamond et al., 2003b). IgG has a higher neutralization potential and its biological activity is mediated by direct receptor binding to promote virus neutralization, by Fc gamma receptors (FcγR)-mediated viral clearance, antibody-dependent cytotoxicity or complement activation resulting in virus or infected-cell lysis (Diamond et al., 2003b). On the other hand, since IgG arises in the primary WNV infection after neuroinvasion, the role of IgG in early infection still has to be further investigated.

Most of the antigenic epitopes recognized by neutralizing antibodies are located in the viral E glycoprotein, which has three structural domains (DI, DII and DIII – see flavivirus proteins) and is involved in viral attachment, internalization and viral assembly. Different studies have shown neutralizing antibody binding specific to all three E protein domains,

although the most potent inhibitory antibodies are associated with the lateral ridge of DIII (Beasley and Barrett, 2002; Oliphant et al., 2005; Sanchez et al., 2005). Antibodies recognizing prM (Kaufman et al., 1989; Pincus et al., 1992; Vazquez et al., 2002) and other non-structural proteins also have been demonstrated for several flaviviruses. Anti-NS1 response has been shown to be protective against WNV infection (Chung et al., 2006; 2007). Antibodies against NS3 and NS5 also are produced during WNV infection, however their importance in WNV protection still unclear (Valdes et al., 2000; Wong et al., 2003).

T lymphocyte response

T cell responses have been demonstrated to be important for the elimination of WNV-infected cells, diminishing the incidence of neuroinvasive disease as demonstrated for both human patients (Katz and Bianco, 2003) and mouse models of disease (Wang et al., 2003a,b; Shrestha and Diamond, 2004; Wang et al., 2006c). While the humoral response has been implicated in viral clearance in the periphery, the T-cell response is believed to be more important for elimination of the virus within the CNS (King et al., 2007). WNV-specific $CD8^+$ T cells quickly proliferate in response to the viral infection and are attracted to different tissues including the CNS, where they exert effector functions to control replication and eliminate the infection through the production of cytokines including IFN-γ, or direct killing of infected cells through secretion of perforin and signaling through Fas (known as CD95 or APO-1)-Fas ligand, or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-dependent pathways (Licon Luna et al., 2002; Cho and Diamond, 2012).

The protective role of $CD8^+$ T cells, particularly against highly neuropathogenic strains of WNV, has been demonstrated in several rodent models using different systems. Treatment of mice or hamsters with immunesuppressive drugs such as cyclophosphamide, exposure to sub-lethal X-irradiation, induction of stress factors or depletion of T lymphocytes have been demonstrated to increase mortality of animals exposed to WNV (Camenga et al., 1974; Ben-Nathan and Feuerstein 1990; Mateo et al., 2006; Elftman et al., 2007). Studies using perforin knockout ($perforin^{-/-}$) mice demonstrated higher virus load in the CNS and increased mortality following WNV infection (Shrestha et al., 2006a), reflecting the effector function of $CD8^+$ T cells (and also NK cells) which release perforin and granzyme to eliminate virus-infected cells (Shresta et al., 1998). $FasL^{-/-}$ mice also were more susceptible to WNV (Shrestha and Diamond, 2007). The TRAIL signaling, which is another effector mechanism used by $CD8^+$ T cells to kill infected cells has been implicated in the elimination of WNV-infected neurons through the interaction of TRAIL and the death receptor DR5, leading to cell apoptosis (Shrestha et al., 2012). Studies also demonstrate that the viral load within the CNS of mice lacking $CD8^+$ T cells are higher when compared to immunocompetent mice and these deficient mice are more susceptible to a WNV lethal infection (Shrestha et al., 2006a). Other mouse studies also showed that mortality rate and survival time can be improved by adoptive transfer of WNV-specific $CD8^+$ T cells, demonstrating the protective role of this subset of cells (Wang et al., 2006c; Brien et al., 2007a). It is further demonstrated that this protection is not IFN-γ-dependent, since adoptive transfer of WNV-specific $CD8^+$ T cells from IFN-$\gamma^{-/-}$ mice also had similar effects on reducing mortality and increasing survival of mice infected with WNV (Wang et al., 2006c).

While $CD8^+$ T cells have been shown to have a protective role in WNV infection, there is also some evidence demonstrating the potential for immunopathogenic effects that can be caused by this cell subset. Although these effects have to be further investigated, most of them are dependent on different factors such as the mouse strain, the virus isolate, infectious dose and route of virus inoculation (King et al., 2007). For example, IFN-$\gamma^{-/-}$ mice infected with the Sarafend strain, a lineage II WNV, have a higher survival rate when compared to infection of the WT mouse strain (King and Kesson, 2003). The protective and immunopathologic effect of $CD8^+$ T cells also have been demonstrated in CD8-deficient mice using different infectious doses. When mice were infected with 10^3 plaque-forming units (PFU) (Sarafend strain), the knockout strain showed increased mortality compared to the WT strain. On the other hand, when mice were infected with a higher dose (10^8 PFU), CD8-deficient mice presented an increased survival (Wang et al., 2003b), suggesting that $CD8^+$ T cells can have both protective and immunopathologic roles in WNV infection.

The role of $CD4^+$ T cells, another subset of T lymphocytes, has been much less explored. It has been shown CD4-deficient mice exhibited a protracted WNV dissemination in the CNS, and uniform mortality of mice, demonstrating a protective role of $CD4^+$ T cells in WNV infection (Sitati and Diamond, 2006). WNV-specific IgM and IgG levels were considerably reduced in the knockout mouse strain, suggesting the antibody response is dependent on $CD4^+$ T cells. Furthermore, it has been demonstrated that $CD4^+$ T cells are necessary to maintain the functionality of WNV-specific $CD8^+$ T cells in peripheral tissues and in the CNS (Sitati and Diamond, 2006). Besides their role as helper cells, $CD4^+$ T lymphocytes also have been shown to contain a direct antiviral

activity, acting in the control of WNV infection independent of B and $CD8^+$ T cell responses (Brien et al., 2008).

Undoubtedly, the immune response to WNV is complex, with multiple factors playing a role in the disease pathogenesis. In terms of adaptive immune response, a general fact is that the humoral response has an important role to limit dissemination of WNV and clearance from peripheral tissues, while T cell responses, comprising $CD4^+$ and $CD8^+$ T lymphocytes are more critical to contain and eliminate the virus from the CNS, diminishing the occurrence of persistent infections.

WNV VACCINES

Currently, there is no licensed WNV vaccine for humans to prevent WNV disease. However, several WNV vaccine candidates are under development, and some of them are in early phases of clinical trials (reviewed in Beasley, 2011; Brandler and Tangy, 2013).

Veterinary vaccines are available for use in horses and other animals. Following WNV introduction into the USA, an inactivated WNV vaccine became quickly available for use in animals (Ng et al., 2003). Nowadays, there are two inactivated whole WNV vaccines (West Nile Innovator® and Vetera®) and another recombinant canarypox-vectored vaccine that expresses prM/E of WNV (Recombiteck®) commercially available for animal use (Ng et al., 2003; Karaca et al., 2005; El Garch et al., 2008). Another chimeric vaccine expressing WNV prM/E on the YFV 17D human vaccine platform (Prevenile®) and a DNA vaccine (West Nile Innovator DNA®) were licensed, but their use has been discontinued (Brandler and Tangy, 2013).

The research on the development of a human vaccine has been focused on several types of vaccines, including inactivated (INV), live-attenuated (LAV), virus-vectored, chimeric LAV, non-replicating single-cycle, DNA and subunit vaccines. Inactivated vaccines have been demonstrated to induce neutralizing antibodies and protect mice from lethal challenge with WNV (Lim et al., 2008; Pinto et al., 2013). However due to the biosafety requirements to produce and inactivate the vaccine, the costs are estimated to be too high for commercial use (Ng et al., 2003). Live-attenuated vaccines containing mutations in different structural and non-structural proteins genes have been shown to be highly attenuated in mice, inducing a strong immune response even at low doses (Yamshchikov et al., 2004; Wicker et al., 2006; Yu et al., 2008b; Whiteman et al., 2010) with potential to become WNV vaccine candidates. Virus-vectored vaccines examples include vesicular stomatitis virus (VSV) vector expressing E protein of WNV and lentiviral vector or measles vaccine expressing a soluble form of WNV E protein (Despres et al., 2005; Iglesias et al., 2006; Coutant et al., 2008; Iyer et al., 2009), which also induce a strong immune response and show protection against lethal challenge with WNV. Chimeric LAV are among the most promising vaccine candidates and were developed based on the ability to swap structural genes among flavivirus members, attenuating the virus without losing the replication capacity. This platform includes candidates expressing the WNV prM/E on the flavivirus backbones of YFV 17D, DENV2 or DENV4 vaccines and has been demonstrated to produce a robust B and T cell response in different animal models (Huang et al., 2005; Monath et al., 2006; Pletnev et al., 2006; Appaiahgari and Vrati, 2010). Non-replicating single-cycle vaccines, such as RepliVAX WN (which is the main subject of this dissertation), contain a C-deleted WNV

genome that allows the initiation of viral replication with the production of SVP containing prM/E, but without production of infectious virus, rendering only a single round of replication on the target cell (Mason et al., 2006). Studies demonstrated that a single dose of this vaccine candidate was able to induce significant protection against WNV lethal challenge and stimulate both humoral and cellular immune response in the animal models tested (Widman et al., 2008a; 2009; 2010; Nelson et al., 2010). Another platform suitable for WNV immunization is the DNA vaccines. Different approaches have been utilized for the development of DNA vaccines candidates. DNA constructs expressing partial WNV proteins such as prM/E or DIII of E (Davis et al., 2001; Ishikawa et al., 2007; Ramanathan et al., 2009), the entire WNV genome (Hall et al., 2003) or C-deleted WNV (Seregin et al., 2006) have been described. Although the manufacturing of DNA vaccines is simple, their efficacy still needs to be improved. Another group of WNV vaccine candidates includes recombinant subunit vaccines. WNV antigens derived from prM/E, DIII or truncated form of E protein and peptides containing B-cell epitopes have been used for immunization, inducing neutralizing antibodies and protecting mice and hamsters against lethal WNV challenge (Wang et al., 2001; Ledizet et al., 2005; Chu et al., 2007; Bonafe et al., 2009; Gershoni-Yahalom et al., 2010; Zhu et al., 2012).

Among human vaccines under development, a few candidates already have been tested in phase I and II of clinical trials. Examples are chimeric vaccines expressing WNV prM/E on flavivirus backbones of YFV 17D (ChimeriVax-WN02) or DENV4 (Chimeric WN/DENV4-3' delta30) (Monath et al., 2006; Guy et al., 2010; Smith et al., 2011; Dayan et al., 2012; De Filette et al., 2012), a recombinant DNA vaccine encoding WNV prM/E (VRC_{303}) (Martin et al., 2007; Ledgerwood et al., 2011) and a subunit

vaccine that expresses soluble WNV E protein (WN-80E) (Lieberman et al., 2007; 2009; Watts et al., 2007; Jarvi et al., 2013). Although the immunogenicity and safety of these vaccines have been assessed during the clinical trials, the protective efficacy measurement is difficult because the majority of WNV cases are asymptomatic and the incidence of the disease is variable in most of the regions where the WNV is present (Suthar et al., 2013b). The licensure of human vaccines also has been held by cost effectiveness issues, where the cost of vaccination might be much higher than clinical case treatment *per se*, not justifying the use of a vaccine (Zohrabian et al., 2006). In addition, elderly and immunocompromised individuals are more likely to have WNV disease (Murray et al., 2006) and may require differential immunization strategies. While these issues may complicate the licensure of human vaccines, promising vaccine platforms with low production cost, better understanding of immunologic factors involved in WNV protection and good animal models that could prove the effectiveness of vaccines favor the possibility of a human WNV in the near future.

The studies presented in this dissertation focus on the characterization of RepliVAX WN as a vaccine vector to prevent WN and potentially other flaviviral diseases. The mechanism of RepliVAX platform optimization is explored to show how this vaccine can be improved, resulting in better yield and possibly efficacy. Further, several aspects of vaccine interaction with the immune system are investigated, revealing key elements involved in the induction of innate and adaptive immune responses to WNV. The knowledge obtained from these studies are of extreme importance to better understand flavivirus replication and induction of immune response, which are useful information for the development of similar or improved vaccine platforms.

CHAPTER 2: Analyses of mutations selected by passaging a chimeric single-cycle flavivirus vaccine candidate identifies mutations that alter infectivity and reveal an interaction between the structural proteins and the nonstructural glycoprotein NS1[1]

INTRODUCTION

The family *Flaviviridae* contains important arthropod-borne viruses responsible for significant diseases in humans such as dengue hemorrhagic fever (DHF), West Nile encephalitis (WNE), yellow fever (YF), Japanese encephalitis (JE) and tick-borne encephalitis (TBE) (Lindenbach et al., 2007). Flaviviruses have a worldwide distribution, threatening over 50% of the world's population. DENV infection is one of the leading causes of flavivirus disease, with an estimated 50-100 million cases annually and up to 3 billion people live in areas where DENV is found (Halstead, 2007; WHO, 2012). WNV was introduced in the United States in 1999 and since then it became the primary cause of viral encephalitis in North America (Davis et al., 2006b; Gyure 2009). The global number of cases is not available, but the United States alone registered over 37,000 cases of WNV disease since 1999, resulting in over 1,500 fatalities (CDC, 2013a). Licensed

[1]This chapter was previously published in Virology. This journal allows including information without copyright as long as it is properly cited. The citation for the article is: Winkelmann ER, Widman DG, Suzuki R, Mason PW (2011). Analyses of mutations selected by passaging a chimeric flavivirus identify mutations that alter infectivity and reveal an interaction between the structural proteins and the nonstructural glycoprotein NS1.Virology 421(2):96-104.

vaccines are available for a number of flavivirus diseases, including YF, JE and TBE, although their use in some cases is restricted for specific regions of the world. On the other hand, there are no licensed vaccines to prevent DENV and WNV infections in humans.

Flaviviruses are enveloped, single-stranded, positive-sense RNA viruses. The viral genome contains a single ORF encoding three structural proteins (C, prM/M and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach et al., 2007). An interesting characteristic of flaviviruses is that the structural proteins are not necessary for viral replication (Khromykh and Westaway, 1997; Khromykh et al., 2001b) and the use of strategies utilizing recombinant genomes containing deletions of genes coding for structural proteins, especially for the C protein, have been the base for the production of new, rationally designed flavivirus LAV (reviewed in Widman et al., 2008b). The use of reverse genetics also allows the generation of chimeric vaccines, as exemplified by the use of the YFV 17D vaccine strain backbone where the genes coding the prM and E were replaced by the same genes of heterologous flaviviruses (Chambers et al., 1999).

In 2006, a novel vaccine platform consisting of single-cycle flaviviruses (SCFV) was developed, showing they can be used as safe and effective vaccines (Mason et al., 2006). This single-cycle technology has been used to produce a series of vaccine candidates (named RepliVAX), that encode genomes harboring a truncated C (trC) gene that prevents the RepliVAX genome from being packaged into infectious particles unless the C gene is supplied in trans (Mason et al., 2006; Ishikawa et al., 2008; Widman et al., 2008a; Suzuki et al., 2009). RepliVAX can infect normal cells in vaccinated animals,

however, and these infected cells release prM/E-containing sub-viral particles (SVPs) and NS1 that induce effective antiviral immune responses. RepliVAX cannot spread or cause disease in animals, thus making it a safe LAV.

The most recent addition to the RepliVAX family is a chimeric RepliVAX that expresses the prM/E genes of DENV2 in place of the WNV structural genes, and this vaccine (RepliVAX D2) was able to control DENV2 disease in a mouse model for DENV2 infection (Suzuki et al., 2009). During the development of RepliVAX D2, it was discovered that the initial construct grew poorly in C-expressing cells, but that growth could be improved by extensive blind passage (facilitated by introduction of larger fragments of the C gene and passaging of packaging cells along with the single-cycle virus). Analyses of these blind-passaged variants demonstrated that their improved growth characteristics were associated with the acquisition of specific mutations in the coding regions for prM, E, NS2A and NS3. In the case of the mutations in prM and E, reverse genetics was used to show that a pair of mutations in M (amino acid 9: G->R and amino acid 13: E->V; referred to by convention as $M^{R9G,V13E}$) and a single mutation in E (E^{K120T}; numbering convention as shown for M) both operated individually to increase yield of RepliVAX D2 in culture, but the combination of the M and E mutations worked together to produce the greatest enhancement in viral growth (Suzuki et al., 2009). Further, it was shown that the mutations in the NS2A ($NS2A^{S9F}$) and the NS3 genes of the WNV backbone ($NS3^{R516K}$) enhanced growth of a re-engineered RepliVAX D2, but did not appear to have an effect on genome replication *per se*, since they were unable to enhance replication of WNV replicons that did not contain DENV genes (Suzuki et al., 2009).

In the studies presented here, it is shown that the mutations identified in the growth-adapted chimeric RepliVAX D2 constructs in the DENV2 prM and E region improve specific infectivity of flavivirus particles in a manner similar to that of a previously characterized heparan sulfate (HS)-binding mutation at a nearby position in E (E^{K126E}) (Lee et al., 2006). In addition, it is demonstrated that the mutation in the WNV NS2A (which acts in concert with the WNV NS3 mutation) eliminates the production of an altered form of NS1 (NS1') that arises from ribosome slippage at a site found in WNV, but not DENV (Firth and Atkins, 2009; Melian et al., 2010). Finally, it is shown that the elimination of the production of NS1' by introduction of a synonymous mutation in this ribosomal slippage site improved encapsidation of particles without altering the amplification and translation of the genome, indicating a functional interaction between NS1/NS1' and the structural proteins of flaviviruses during encapsidation.

MATERIALS AND METHODS

Cells

BHK cells were maintained at 37ºC in minimal essential medium (MEM, Cellgro, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Gemini, Life Technologies, Carlsbad, CA) and antibiotics. Vero cells were maintained at 37ºC in MEM containing 6% FBS (Hyclone, Thermo Scientific, Logan, UT) and antibiotics. BHK(VEErep/Pac-Ubi-C*) expressing the WNV C protein (Widman et al., 2008a), BHK(VEErep/WNVC*-E/Pac) cells expressing the WNV C-prM-E proteins (Fayzulin et al., 2006), BHK(VEErep/WNC*-JEVprM-E/Pac) cells expressing the WNV C and JEV prM-E (Ishikawa et al., 2008), BHK(VEErep/WNVC*-DENV2prM/E/Pac) cells

expressing the WNV C and the DENV2 NGC (low passage) prM/E (generated as described below), BHK(VEErep/WNVC*-DENV2prM/E^{K126E}/Pac) cells expressing the WNV C and a HS-binding E derived from a high-passage DENV2 NGC (generated as described below), and BHK(VEErep/WNVC*-DENV2$prM^{R9G,V13E}$/E^{K120T}/Pac) cells expressing the WNV C and the DENV2 prM/E mutations selected in RepliVAX D2 (generated as described below) were propagated at 37ºC in Dulbecco's MEM (Cellgro) supplemented with 10% FBS (Gemini) and 10 ug/ml puromycin (Cellgro) as previously described (Fayzulin et al., 2006).

Plasmid construction

The plasmid pWNR C-hFLuc2A NS1-5 encoding a WNV replicon expressing a humanized form of the firefly luciferase (FLUC) reporter gene has been previously described (Gilfoy et al., 2009). This was used to construct the various mutant WNV replicon plasmids with specific mutations in NS2A (pWNR C-hFLuc2A NS1-5 $NS2A^{S9F}$), NS3 (pWNR C-hFLuc2A CNS1-5 $NS3^{R516K}$), NS2A and NS3 (pWNR C-hFLuc2A NS1-5 $NS2A^{S9F}$/$NS3^{R516K}$), as well as a silent mutation in NS2A (pWNR C-hFLuc2A NS1-5 $NS2A^{F9F}$) using standard techniques (Higuchi et al., 1988).

The plasmid encoding the RepliVAX WN replicon [pRepliVAX WN (Widman et al., 2008a); previously referred to as pRepliVAX WN.2 SP] was modified using standard techniques to produce a series of mutant RepliVAX WN plasmids with specific mutations in NS2A (pRepliVAX WN $NS2A^{S9F}$), NS3 (pRepliVAX WN $NS3^{R516K}$), and NS2A and NS3 (pRepliVAX WN $NS2A^{S9F}$/$NS3^{R516K}$) using standard techniques (Higuchi et al., 1988).

Plasmid pVEErep/WNVC*-E/Pac which encodes a Venezuelan equine encephalitis virus replicon (VEErep) capable of persisting in cells in the presence of puromycin and expressing the WNV structural proteins (C-prM-E) needed to package subgenomic replicons (Fayzulin et al., 2006) was used to construct a series of plasmids encoding a low-passage DENV2 NGC (Fonseca, 1994) prM/E cassette (pVEErep/WNVC*-DENV2prM/E/Pac), a high-passage DENV2 NGC (Fonseca, 1994) prM/E cassette (pVEErep/WNVC*-DENV2prM/E^{K126E}/Pac), or the prM/E cassette found in cell-adapted RepliVAX D2 (Suzuki et al., 2009) (pVEErep/WNVC*-DENV2$prM^{R9G,V13E}$/E^{K120T}/Pac).

Production of packaging cell lines

Cell lines harboring replicons from the VEErep plasmids were created by a slight modification of the previously described procedures (Fayzulin et al., 2006). Briefly, the plasmid DNAs were linearized by using the MluI restriction enzyme, and the resulting template DNAs were *in vitro*-transcribed using MegaScript SP6 synthesis kit (Ambion) in the presence of 7mG(ppp)G cap analogue (New England Biolabs). The yield and integrity of transcripts were determined by using non-denaturing gel electrophoresis, aliquots of transcription reactions were transfected into BHK cells using Lipofectin (Invitrogen), VEErep-harboring cell lines were selected in the presence of puromycin, and clones displaying high-level expression of these replicons were isolated and propagated using standard techniques.

Production of viral replicon particles (VRPs) and RepliVAX derivatives

WNV replicon RNAs encoding the FLUC gene (WNR C-hFLuc2A NS1-5 and derivatives) or the WNV prM/E cassette (RepliVAX WN and derivatives) were generated

by using MegaScript T7 synthesis kit (Ambion) and 7mG(ppp)G cap analogue (New England Biolabs) from SwaI-linearized templates created from the relevant plasmid DNAs using standard methods. Following analysis for yield and integrity as described above, aliquots of transcription reactions were electroporated into packaging cell lines (expressing C, prM, and E constructs in the case of the FLUC-expressing replicon constructs or C only in the case of RepliVAX constructs) and then collected as previously described (Fayzulin et al., 2006).

VRP and RepliVAX titrations

VRPs and RepliVAX WN derivatives were titrated on Vero cells as previously described (Fayzulin et al., 2006). Briefly, cell monolayers were infected with serial dilutions of the samples and incubated for 24-30 hrs at 37ºC. Cells were fixed in acetone/methanol and subjected to IHC staining using flavivirus-specific antibodies. Immunopositive cells were counted and used to calculate the titers which were reported as infectious units per milliliter (IU/ml).

VRP and RepliVAX growth curves

To compare growth properties of the various WNV replicons in cell lines encoding various prM/E packaging constructs, VRPs derived from electroporations were used to infect these BHK packaging cells at a multiplicity of infection (MOI) of 0.05 for 2 hrs; the monolayers were then washed 3 times (5 min each) with MEM supplemented with 1% FBS, 10mM HEPES, and antibiotics, and the cultures were placed at 37°C. Media were removed and replaced with fresh media at the indicated time points and stored at -80ºC for subsequent titration as described above.

Growth curves from RepliVAX WN and derivatives were prepared by infecting BHK(VEErep/Pac-Ubi-C*) cells at an MOI of 0.01 using the same procedure as described above.

Specific infectivity studies

Genome copy numbers and infectivity of VRPs produced by electroporation (see above) were determined based on semiquantitative PCR (sqPCR) and Vero cell titration data. Briefly, VRP preparations were diluted to give titers of 1,000 IU/ml, and one portion was titered on Vero cells as described above, while RNA was isolated from a second portion using the QIAamp Viral RNA kit (QIAGEN) following the manufacturer's protocol. The viral RNA concentration in this sample was determined by using a sqPCR assay in which serial 2-fold dilutions of RNA from each sample were used for reverse transcription (RT) carried out with an ImProm II RT kit (Promega) with random hexamers followed by amplification of a 100 bp PCR product using previously described WNV NS5-specific primers (Bourne et al., 2007). The PCR conditions included an initial cycle of 5 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 53°C and 30 sec at 72°C, followed by 5 min at 72°C. Following amplification, the PCR products were resolved by electrophoresis on 2% agarose gels containing 200ng/ml of ethidium bromide, and images of the gels were acquired with a CCD camera using a FluorChem 8900 Chemiluminescence Gel Imager (Alpha Innotech) and band intensities were quantified by using ImageJ software (available at http://rsbweb.nih.gov/ij/). The intensities of these bands were compared to a standard curve generated with known numbers of genome copies of *in vitro* synthesized RNA from WNR C-hFLuc2A NS1-5 (ranging from 2,000 to 200,000 copies), and the resulting standard curve (generated by

using GraphPad Prism 4 software) was used to calculate the genome copies in each test sample (Fig.2.1). The specific infectivities of each preparation were then calculated by dividing this genome copy number per IU in the same sample volume, giving genome copies/IU of each VRP.

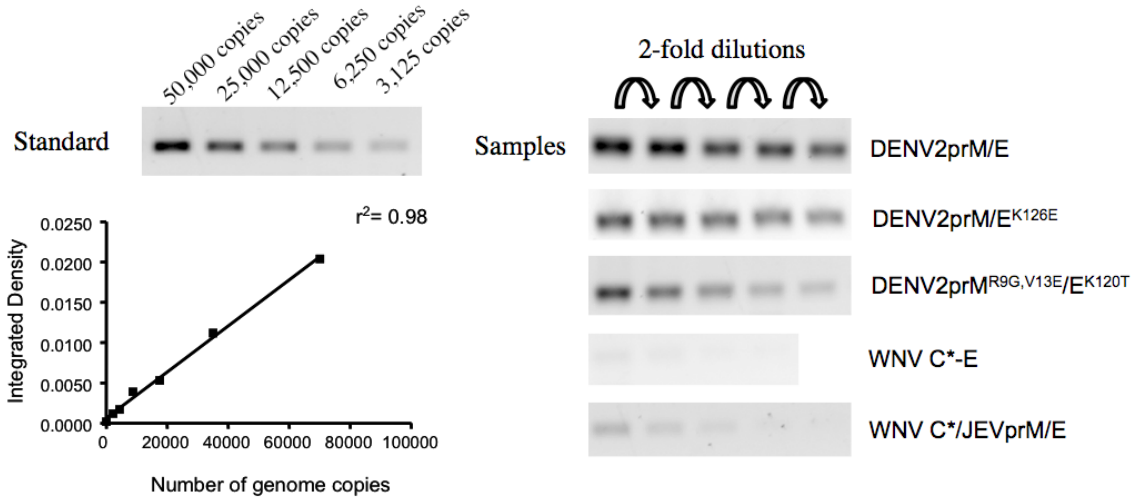

Figure 2.1. Representation of semi-quantitative PCR (sqPCR) method used for specific infectivity studies. The measurement of the band intensity (integrated density) of the PCR product derived from *in vitro* synthesized RNA of a WNR C-hFLuc2A NS1-5 containing known numbers of genome copies was used to generate a standard curve (left panel). This standard curve was used to calculate the genome copies/IU in the specific infectivity studies by comparing the band intensities (linear regression) from each sample tested (right panel).

Western blot analyses of NS1

VRPs containing different derivatives of WNR C-hFLuc2A NS1-5 were inoculated onto BHK cell monolayers at an MOI of 5 and incubated at 37°C with serum-free medium (OptiPro SFM, Gibco) supplemented with 10mM HEPES and antibiotics. At 24 hrs after infection, culture fluids were collected and cell lysates were prepared using lysis buffer (0.1% Triton X-100, 300 mM NaCl, 50 mM Tris-HCl; pH 7.6) containing a protease inhibitor cocktail (Roche). Samples were resolved on 4-12%

gradient Bis-Tris polyacrylamide gels (Invitrogen) and transferred to polyvinylidine diofluoride membranes, which were then incubated with a 1:5,000 dilution of a mouse anti-NS1 antibody from hydridoma JE-6H4 (Kitai et al., 2007). Following washing, the membranes were incubated with a 1:10,000 dilution of peroxidase-conjugated anti-mouse IgG (KPL), and the bound peroxidase was visualized by using ECL Plus System (GE healthcare).

VRP focus-formation assay

To compare focus morphology, monolayers of BHK(VEErep/WNVC*-E/Pac) cells expressing the WNV C-prM-E proteins were infected with serial dilutions of VRPs harboring the WT replicon genome, or genomes encoding the $NS2A^{F9F}$, $NS2A^{F9F}NS3^{R516K}$ or $NS3^{R516K}$ mutations. Following absorption for 2 h, the cells were overlaid with medium containing 0.8% carboxymethyl cellulose (CMC) (Sigma, Saint Louis, MO) supplemented with 1% FBS, 10mM HEPES and antibiotics and incubated at 37ºC for 48 h. To visualize foci, the cells were fixed with 50% acetone-50% methanol solution followed by incubation with a 1:5,000 dilution of a mouse anti-NS1 antibody from hybridoma JE-6H4 (Kitai et al., 2007), peroxidase conjugated anti-mouse IgG (KPL, Gaithersburg, MD) and VIP substrate (Vector Laboratories, Burlingame, CA). The number of cells forming individualized foci were counted and used to compare focus size.

Luciferase assay

BHK monolayers prepared in 96-well black-wall plates were infected with dilutions of VRPs harboring the WT replicon genome, or genomes encoding the $NS2A^{F9F}$, $NS2A^{F9F}NS3^{R516K}$ or $NS3^{R516K}$ mutations and incubated at 37°C. At 24 hrs post

infection (hpi), an equal volume of 25% Steady-Glo Luciferase Assay System reagent (Promega) diluted in lysis buffer was added to the cells and incubated for 5 min on rocker to allow cell lysis. The luminescence was measured on a Microplate Luminometer (Applied Biosystems, Foster City, CA). A parallel plate infected with the same dilutions of VRPs was harvested at 24 hpi and used to determine the number of VRP-infected cells determined by immunostaining (as described above). The luciferase activity was normalized by the number of VRP-infected cells and expressed as relative luciferase units (RLU) per infected cell.

Statistical analyses

GraphPad Prism (GraphPad Software, San Diego, CA) was used to analyze data. One-way or two-way analysis of variance (ANOVA) with the Bonferroni post-test were used where appropriate. P values less than 0.05 were considered to indicate statistical significance.

RESULTS

Mutations in DENV prM/E increase growth and alter specific infectivity

To determine how growth-enhancing mutations in the DENV2 prM/E coding region of single-cycle chimeric flaviviruses expressing the prM/E genes of DENV2 and the NS genes of WNV function (Suzuki et al., 2009), a series of packaging cell lines (see Fig.2.2) that encoded the low passage DENV2 New Guinea C (NGC) prM/E sequences [BHK(VEErep/WNVC*-DENV2prM/E/Pac)] (used for the generation of the first RepliVAX D2) as well as a cell line [BHK(VEErep/WNVC*-DENV2prM^{R9G,V13E}/E^{K120T}/Pac)] that encoded the DENV2 prM/E sequences selected

when RepliVAX D2 was adapted to grow in cells expressing the C gene (Suzuki et al., 2009), along with cell lines carrying packaging constructs expressing the WNV prM/E [BHK(VEErep/WNVC*-E/Pac)], the JEV prM/E [BHK(VEErep/WNVC*-JEVprM/E/Pac)], and a cell line [BHK(pVEErep/WNVC*-DENV2prM/E^{K126E}/Pac] expressing an E protein from a high-passage NGC strain of DENV that contains a DENV2 E mutation (E^{K126E}) previously associated with HS binding (Lee et al., 2006) (see Fig.2.2) were used to produce VRPs containing a WNV replicon (C-hFLuc2A-NS1-5, see Fig.2.5).

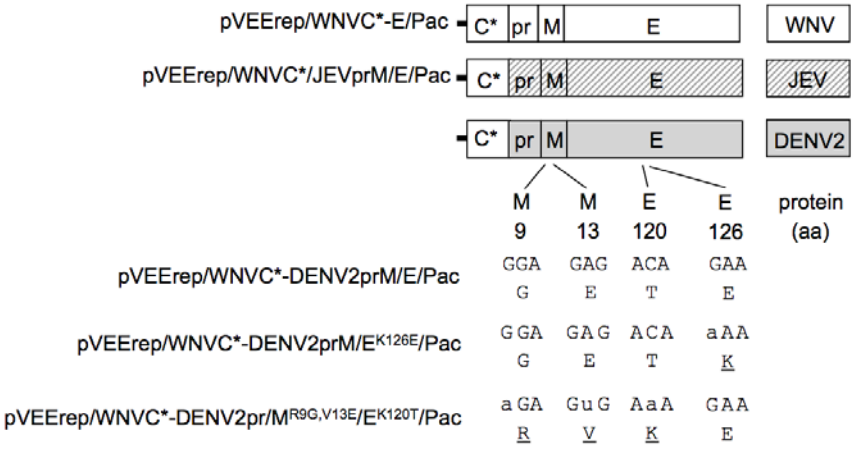

Figure 2.2. Schematic representation of the packaging cell constructs. All constructs contained a form of the WNV C protein (C*) engineered to contain synonymous mutations in the start of the genome to prevent generation of homologous recombinants with the replicons, as well as complete prM/E cassettes from the indicated viruses (see Methods); "aa" indicates the position of the affected codon within the individual protein-coding regions.

When these VRPs, which contained identical WNV-derived replicons transpackaged in the different coats provided by their packaging cell lines (Fig.2.2), were tested side-by-side for their specific infectivities in Vero cells (genome copies per IU; see Methods), it was demonstrated that the VRPs packaged in WNV or JEV prM/E proteins exhibited significantly better specific infectivities (500 to 2,000 genome copies per IU)

than particles packaged in any of the DENV2 coats (50,000 to 200,000 genome copies per IU; Fig.2.3). Among the DENV2-packaged VRPs, the VRPs coated with the prM/E proteins of a low-passage NGC strain [from BHK(VEErep/WNVC*-DENV2prM/E/Pac)] displayed the poorest infectivity (over 200,000 genome equivalents per IU), and the particles packaged in coats containing the previously identified HS-binding mutation at position 126 (Lee et al., 2006) [BHK(pVEErep/WNVC*-DENV2prM/E^{K126E}/Pac] displayed a slightly better specific infectivity. Interestingly, the VRPs packaged in the cell lines encoding the DENV2 prM/E genes selected in RepliVAX D2 passaging studies (Suzuki et al., 2009) [BHK(VEErep/WNVC*-DENV2$prM^{R9G,V13E}$/E^{K120T}/Pac)] displayed a significantly better specific infectivity than the particles packaged in the WT DENV2-packaged VRPs, which contained the same low-passage DENV2 genes used to initiate the passaging studies performed with RepliVAX D2 (Suzuki et al., 2009) (Fig.2.3).

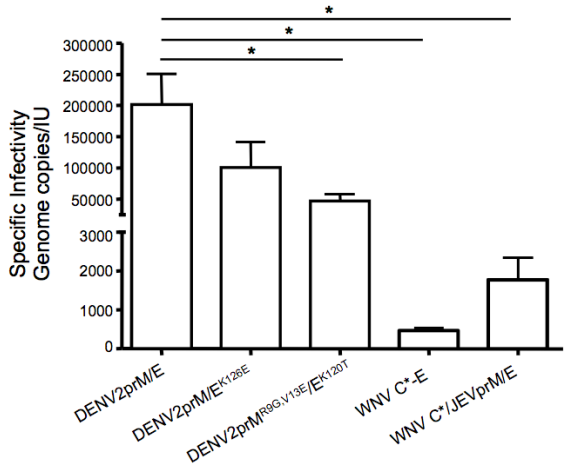

Figure 2.3. Specific infectivities of VRPs created by packaging a WNV-derived replicon (pWNR C-hFLuc2A NS1-5-encoding FLUC). Data displayed indicate the particle number (determined by genome quantification using sqPCR) divided by the measured infectivity on Vero cells (see Methods). Error bars indicate the standard deviation between specific infectivity determinations established from two measurements of RNA concentration from the linear range of the sqPCR standard curve. "*" denotes significance as measured by one-way ANOVA with Bonferroni post-test ($p<0.05$).

Taken together, these data demonstrate that the previously reported low-specific infectivity of DENV particles (van der Schaar et al., 2007) is due to the properties of the virion surface proteins and that changes in specific infectivity in cell culture can be facilitated by addition of positively charged residues in M and E that presumably function by facilitating productive binding of negatively charged GAGs such as HS that are ubiquitously expressed on cells in culture and aid in infection as previously demonstrated for DENV2 (Lee et al., 2006). However, the possibility cannot be ruled out that the mutations in prM/E could also have a role in the maturation of the structural proteins needed for flavivirus morphogenesis, especially in light of recent work showing that extracellular DENV particles contain a mixture of mature (lacking prM) and immature particles (Junjhon et al., 2010) which likely contribute to their poor specific infectivity.

NS2A and NS3 mutations previously selected in DENV2/WNV chimeras improve VRP growth when packaged in DENV2 envelopes

During propagation of a derivative of RepliVAX D2 containing the prM/E mutations described above (Suzuki et al., 2009), two mutations were selected in the WNV nonstructural protein-encoding regions that improved the growth of these chimeric viruses. To help learn how these mutations exerted their effects, they were introduced into RepliVAX WN (as single or double mutations; Fig.2.4A), and tested for effect on the growth of this non-chimeric single-cycle virus in a WNV C-expressing cell line [BHK(VEErep/Pac-Ubi-C*)]. Fig.2.4B shows growth curves for the WT and mutated RepliVAX WN that were created by averaging values from 3 independent experiments. These growth curves demonstrate that the mutations selected in the context of the

chimeric RepliVAX D2 were unable to produce a detectable improvement in growth of the non-chimeric RepliVAX WN.

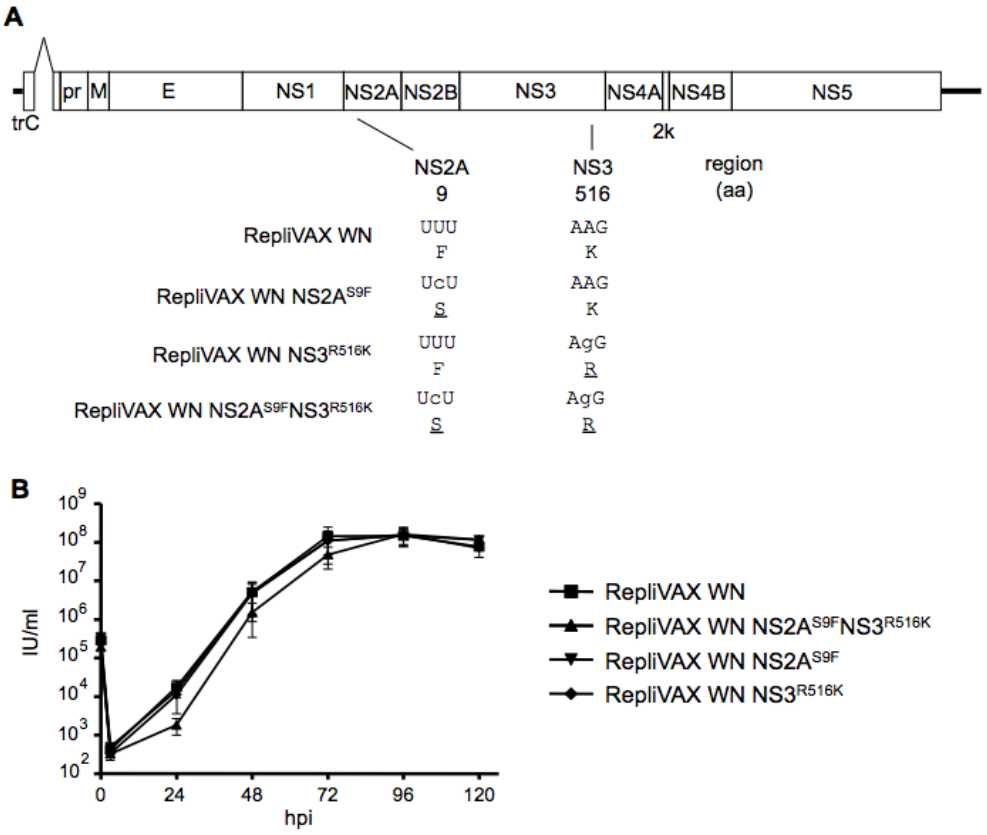

Figure 2.4. NS2A^{S9F} and NS3^{R516K} mutations do not significantly improve the growth of RepliVAX derivatives carrying the WNV prM/E genes. (A) Schematic representation of derivatives of RepliVAX WN carrying mutations in NS2A and NS3; "aa" indicates the position of the affected codon within the individual protein-coding regions. (B) Growth curves of the RepliVAX WN constructs shown in Fig.2.4A on BHK(VEErep/Pac-Ubi-C*) which express the WNV C protein. Cells were infected at an MOI of 0.01, and media were harvested, and titered at the indicated time points as described in the Methods. The first time point (0 hpi) indicates the initial dose used to infect the cells. Values represent averages of three individual experiments. Error bars indicate standard deviation.

To further evaluate the interactions between WNV NS2A and NS3 mutations and the DENV2 prM/E cassettes, NS2A^{S9F} and NS3^{R516K} were introduced as single- or double-mutations into a WNV replicon encoding a FLUC reporter gene (Fig.2.5).

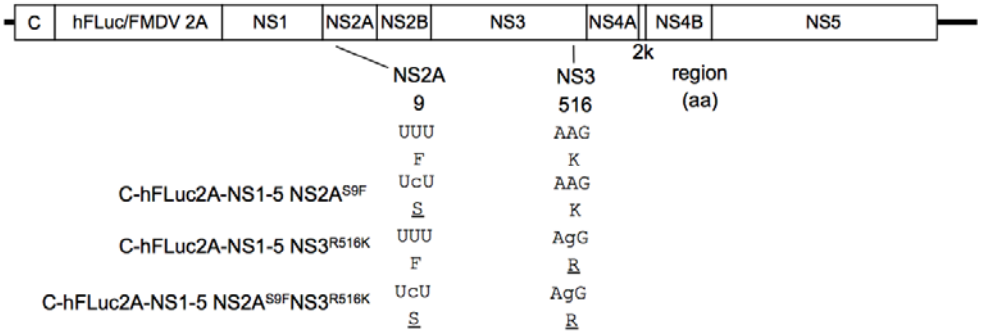

Figure 2.5. Schematic representation of derivatives of WNV replicons carrying mutations in NS2A and NS3. "aa" indicates the position of the affected codon within the individual protein-coding regions.

Comparison of the ability of VRPs containing these replicons to grow in a subset of the packaging cells described in Fig. 2.2 demonstrated that each of the non-structural protein mutations enhanced growth in all three of the DENV2 packaging cell lines (Fig.2.6A,B,C). In addition, the two mutations displayed an additive/synergistic effect on transpackaging within DENV2 envelopes (Fig.2.6A,B,C). However, neither of these mutations (independently or together) produced a detectable improvement in the growth of this WNV-derived replicon when replicons carrying these mutations were propagated in packaging cells expressing the WNV envelope protein cassette (Fig.2.6D).

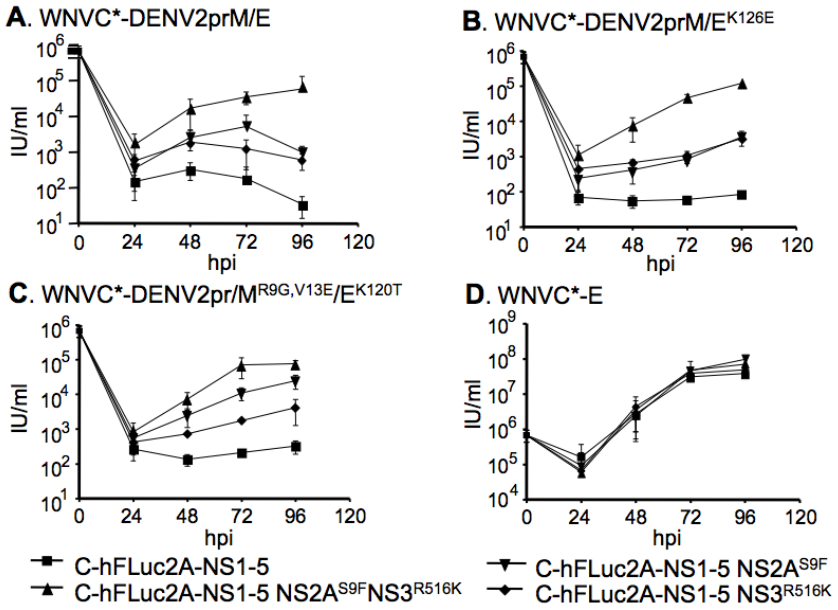

Figure 2.6. NS2A^{S9F} and NS3^{R516K} improve growth of WNV VRPs when transpackaged in DENV coats. Growth curves of VRPs harboring the genomes shown in Fig.2.5 on BHK cell lines encoding the indicated packaging constructs. Cell monolayers were infected at an MOI of 0.05 with the indicated VRPs, and media were harvested and titered at the indicated time points as described in the Methods. The first time point (0 hpi) indicates the initial dose used to infect the cells. Values represent averages of two individual experiments. Error bars indicate standard deviation.

NS2A^{S9F} mutation alters the production of a higher molecular weight form of NS1

The recent observation that the NS2A gene of encephalitic flaviviruses contains a frame-shift motif (Firth and Atkins, 2009) that permits the production of an altered form of NS1 [known as NS1'; (Melian et al., 2010)] identified over 20 years ago in cells infected with JEV (Mason, 1989) encouraged the further examination of the NS2A^{S9F} mutation. Interestingly, this mutation disrupts the canonical UUUU portion of the ribosome slip site that produces NS1' (CCCUUUU-> CCCUUcU; Fig.2.7A). To confirm that this mutation prevented the synthesis of NS1', Western blot analyses were conducted and clearly demonstrated that this mutation results in the loss of NS1' (Fig.2.7B).

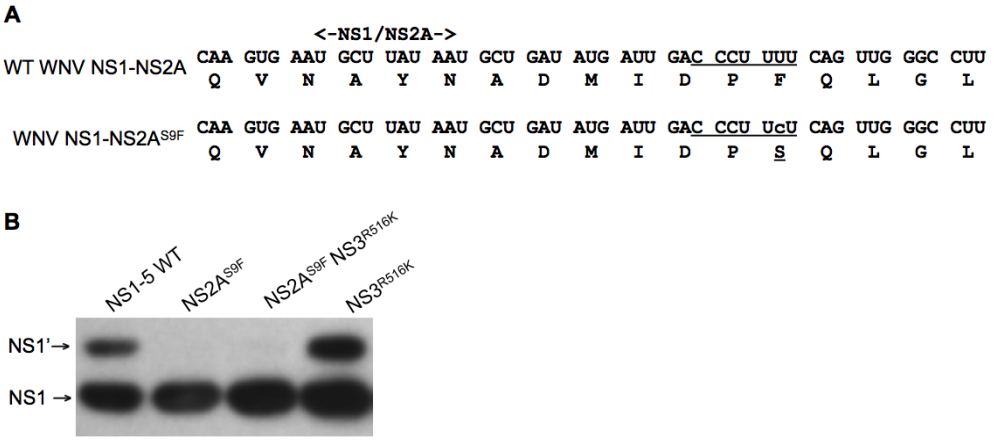

Figure 2.7. Effect of NS2A^{S9F} mutation on synthesis of NS1'. (A) Alignment of RNA and amino acid sequences at the NS1/NS2A junction region of WNV showing the ribosomal frame-shift site (underlined) and its disruption by the NS2A^{S9F} mutation. (B) Western blot showing that cells infected with replicons encoding the NS2A^{S9F} mutation fail to produce NS1'. Cells infected with particles encoding C-hFLuc2A-NS1-5 replicon (labeled NS1-5 WT) and its derivatives (see Fig.2.5; labeled in this panel by mutation only) were lysed, blotted, and immunostained as described in the Methods.

Mutation of the ribosome slip site in NS2A enhances packaging of WN replicons in DENV2 coats and eliminates production of NS1'

To demonstrate that the growth enhancing properties of the NS2A^{S9F} mutation resulted through the abrogation of NS1' production, two silent mutations (CCCUUUU->CCCcUUc) were engineered in this region of NS2A (producing a construct designated NS2A^{F9F}) that disrupted the ribosome slip site (Fig.2.8).

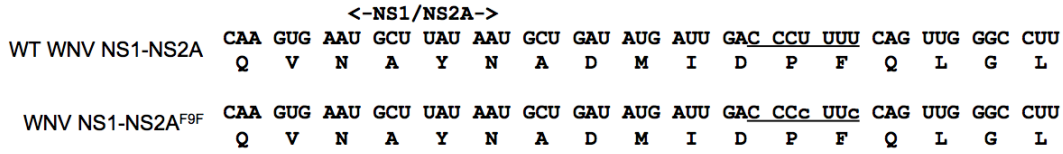

Figure 2.8. Introduction of silent mutations. Alignment of RNA and amino acid sequences at the NS1/NS2A junction region of WNV showing the ribosomal frame-shift site (underlined) and its disruption by silent mutations.

WNV replicons bearing this mutation alone, or in the presence of the NS3 mutation, displayed a significant enhancement of growth compared to the WT replicon genome when they were grown in cell lines providing DENV2 coats (Fig.2.9A,B,C).

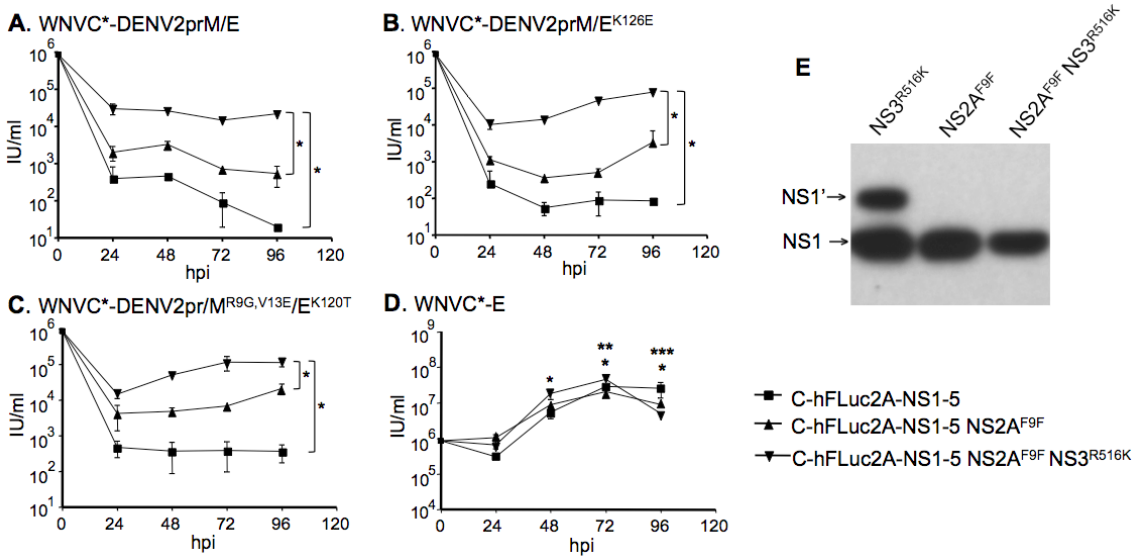

Figure 2.9. Demonstration that ablation of ribosomal frame-shifting in NS2A enhances trans-encapsidation of WNV replicons and eliminates production of NS1'. (A-D) Growth curves of VRPs harboring the WT replicon genomes, or genomes encoding the NS2A^{F9F} or NS2A^{F9F}NS3^{R516K} mutations on BHK cell lines encoding the indicated packaging constructs. Cell monolayers were infected at an MOI of 0.05 with the indicated VRPs, and media were harvested and titered at the indicated time points as described in the Methods. The first time point (0 hpi) indicates the initial dose used to infect the cells. Values represent averages of two individual experiments. Error bars indicate standard deviation and "*" denotes significance as measured by two-way ANOVA with Bonferroni post-test ($p<0.05$) for 24-96hr timepoints (A, B) and 48-72hr timepoints (C). The same test showed significant differences in Figure 2.9D; in this case: "*" denotes significance ($p<0.05$) for C-hFLuc2A-NS1-5 vs C-hFLuc2A-NS1-5NS2A^{F9F}NS3^{R516K}; "**" denotes significance ($p<0.05$) for C-hFLuc2A-NS1-5 NS2A^{F9F} vs C-hFLuc2A-NS1-5NS2A^{F9F}NS3^{R516K}; and "***" denotes significance ($p<0.05$) for C-hFLuc2A-NS1-5 vs C-hFLuc2A-NS1-5 NS2A^{F9F} at indicated timepoints. (E) Western blot showing that cells infected with VRPs containing the NS2A^{F9F} mutation fail to produce NS1'. Cells infected with particles encoding C-hFLuc2A-NS1-5 replicons containing NS3^{R516K}, NS2A^{F9F}, or NS2A^{F9F}NS3R^{516K} mutations were lysed, blotted and immunostained as described in the Methods.

Furthermore, Fig.2.9D shows that the NS2A^{F9F} mutation, alone, or in concert with the NS3^{R516K} mutation produced significant improvement in packaging in a WNV coat at several time points. As expected from the intentional disruption of the slippage site, cells infected with replicons expressing the NS2A^{F9F} mutation alone, or in the presence of the NS3^{R516K} mutation did not produce any detectable NS1' (Fig.2.9E).

To understand the interaction between the NS2A^{F9F}and NS3^{R516K} mutations and DENV coats, the specific infectivity of C-hFLuc2A-NS1-5 and C-hFLuc2A-NS1-5 NS2A^{F9F} NS3^{R516K} VRPs produced in the WNVC*-DENV2prM^{R9G,V13E}/E^{K120T} and the WNVC*-E cell lines harvested from the 72 hpi time point in the study shown in Fig.2.9C and D was further investigated. These studies showed improved infectivity for the DENV-packaged VRPs that carried the NS2A frame-shift and NS3 mutations relative to the WT genomes, but no difference in the infectivity of the WNV-packaged genomes containing these mutations (Fig.2.10A,B), suggesting that the C-hFLuc2A-NS1-5 NS2A^{F9F} NS3^{R516K} were more efficiently assembled into infectious DENV particles. Furthermore, Western blot analyses of the E protein content of these same VRP preparations showed a similar level of incorporation of WNV E into VRPs produced with either mutant or WT NS genes, but a more efficient incorporation of the DENV E into particles carrying the mutant NS genes, consistent with the hypothesis that the NS2A frame-shift and NS3 mutations produced higher infectious yields by increasing efficiency of assembly of infectious particles with DENV coats.

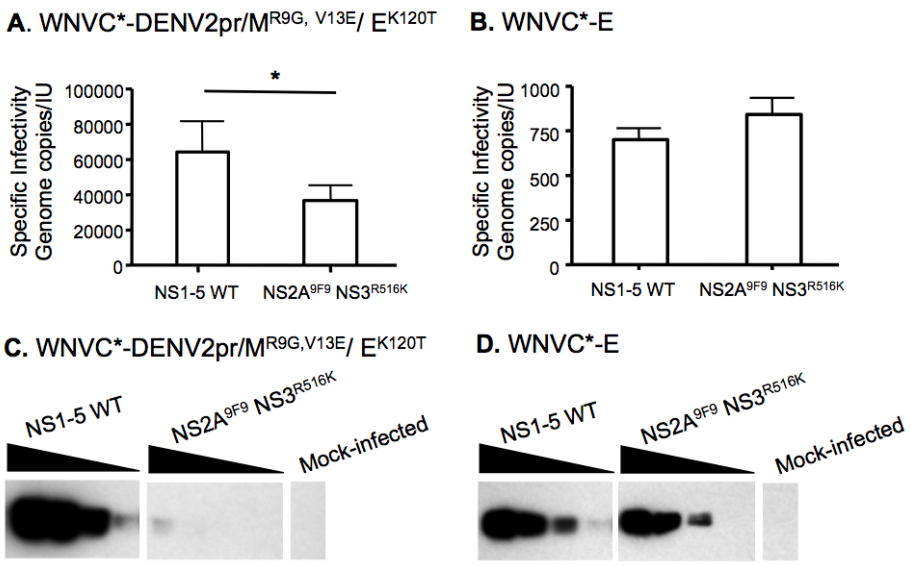

Figure 2.10. Specific infectivities and E protein expression. Specific infectivity of VRPs containing a WNV-derived replicon (pWNR C-hFLuc2A NS1-5 -encoding FLUC) harboring the WT genome or a genome containing the NS2A^{F9F}NS3^{R516K} mutations, packaged on a BHK cell line expressing the DENV2 prM/E sequences selected during adaptation of RepliVAX D2 (WNVC*-DENV2prM^{R9G,V13E}/E^{K120T}) or on a BHK cell line encoding the WNV prM/E sequences (WNVC*-E) were determined using the VRPs collected at the 72 hpi time point from the experiments shown in Fig.2.9C and Fig.2.9D. (A and B) Specific infectivity values determined by using sqPCR. Error bars indicate the standard deviation between specific infectivity determinations established from four measurements of RNA concentration from the linear range of the sqPCR standard curve (see Fig.2.1 and methods). "*" denotes significance as measured by Student t-test ($p<0.05$). (C and D) Specific infectivity evaluations using protein content. Western blot showing E protein concentration in the VRPs harvested from the indicated BHK packaging cell lines infected with replicons encoding the WT genome or genomes containing the NS2A^{F9F}NS3^{R516K} mutations. These samples were diluted to have the same infectious titer (IU/ml) and then 2.5-fold dilutions were prepared and resolved on 4-12% gradient Bis-Tris polyacrylamide gels as described in the methods, and the E protein was detected by a flavivirus group-specific monoclonal antibody D1-4G2 incubated at 1:1,000 dilution. In panel C, VRPs containing the same infectious titer (IU/ml), were concentrated by ultracentrifugation through a 20% sucrose cushion at 38,000 rpm in a Beckman SW41 Ti rotor for 2 h at 4°C and resuspended overnight at 4°C in 75 uL TN buffer (10mM Tris, 100mM NaCl, pH 7.6) containing a protease inhibitor cocktail (Roche) to achieve sufficient titer for the analyses. Following resuspension, these concentrated samples were used to make the 2.5-fold dilutions as described above. The last lanes in panels C and D were prepared from an undiluted supernatant sample harvested from mock-infected cells in the same experiment.

To further evaluate the role of these mutations in virion packaging in WNV coats, an additional, more sensitive assay was utilized, which consisted of calculating the size of infectious foci formed by VRPs carrying WT and mutant replicons on BHK(VEErep/WNVC*-E/Pac) cells. Fig.2.11 shows the results of these assays, which support the data in Fig.2.9D, by showing that all three VRPs tested in these assays tended to produce larger foci on cells expressing the WNV structural proteins.

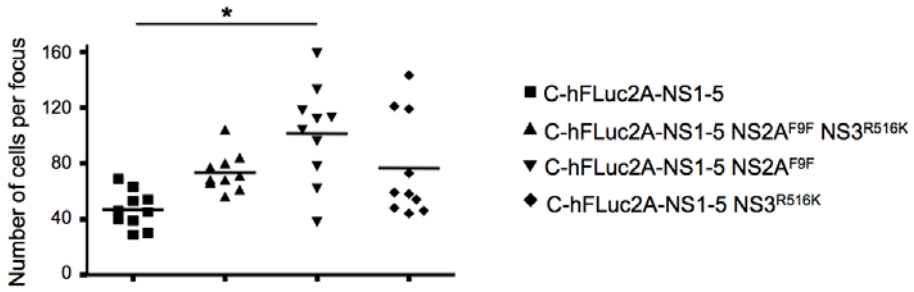

Figure 2.11. Comparison of foci sizes of VRPs harboring the WT replicon genome, or genomes encoding the NS2A^{F9F}, NS2A^{F9F}NS3^{R516K} or NS3^{R516K} mutations on BHK(VEErep/WNVC*-E/Pac) cells expressing the WNV C-prM-E proteins. Cell monolayers were infected with serial dilutions of the VRPs, overlaid with semisolid medium, fixed, and immunostained with anti-NS1 antibody 48 hours after incubation at 37°C as described in the Methods. The graph represents the counts of cells from 10 individual foci per VRP tested. The line represents the average of the 10 foci values for each group and "*" denotes significance as measured by one-way ANOVA with Bonferroni post-test ($p<0.001$).

NS1 is required for flavivirus genome replication, and mutations in NS1 have been shown to alter genome replication in cells in culture (Muylaert et al., 1996; 1997). To determine if the effect of NS1' abrogation was producing the enhanced growth/spread phenotype in packaging cell lines, WT BHK cells were infected with VRPs harboring various replicons and used the short-lived FLUC reporter gene (Thompson et al., 1991) to quantify the levels of their genome replication and polyprotein translation. These studies, shown in Fig.2.12, showed that neither the NS2A^{F9F}, NS3^{R516K}, nor the combined

NS2A^{F9F}/NS3^{R516K} mutations significantly altered the levels of replicon amplification at 24 hpi.

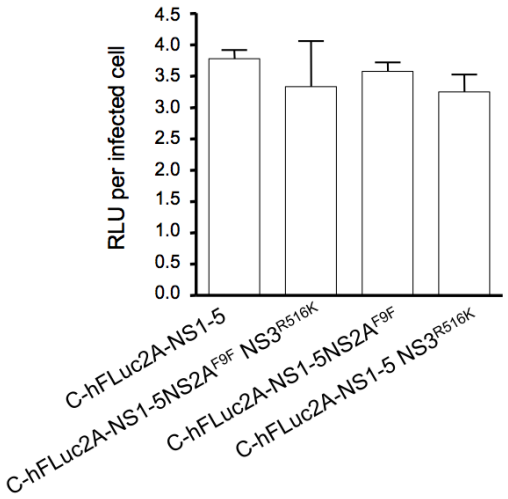

Figure 2.12. Replication of VRPs harboring the WT replicon genome, or genomes encoding the NS2A^{F9F}, NS2A^{F9F}NS3^{R516K} or NS3^{R516K} mutations on BHK cells. Cells were cultured in 96-well plates, infected with VRPs and incubated for 24 hrs, when the relative luciferase units (RLU) were measured and then standardized to the number of VRP-infected cells obtained by immunostaining wells infected in a parallel plate (see Methods). Data for each sample are averages of triplicate values with error bars showing standard deviations

DISCUSSION

Flaviviruses display a broad host- and cell-specificity which suggests that these viruses can use a variety of cell surface receptors. Multiple molecules have been identified that can serve as receptors, but the most clearly documented example of a cell-surface component that can be utilized as a receptor is the GAG, HS. Although the role of HS in natural infections by RNA viruses remains unclear, flaviviruses that are adapted to grow in cell culture or in specific animal models can acquire the ability to bind to HS through the acquisition of mutations on the E protein that produce positively charged patches that efficiently bind negatively charged GAGs (Lee and Lobigs, 2000; Mandl et al., 2000; Lee and Lobigs, 2002; Kroschewski et al., 2003; Lee et al., 2004; Lee et al.,

2006; Anez et al., 2009). In the current studies a transpackaging system was used to demonstrate that the DENV2 M/E protein mutations found in the previously reported cell-adapted dengue chimera (Suzuki et al., 2009) improve the specific infectivity of transpackaged particles, explaining the selection of these mutations during the adaptation of the dengue chimera to grow in cell culture. Furthermore, by comparing the specific infectivity of these preparations to those encoding envelopes of two encephalitic flaviviruses, the poorer specific infectivity of particles encapsidated in DENV envelope proteins was clearly demonstrated, which is consistent with work documenting the poor specific infectivity of DENV2 virions (van der Schaar et al., 2007). Interaction among the proteins of positive-strand RNA viruses is one of the hallmarks of these viruses. Among the flaviviviruses, numerous examples of such interactions exist within the structural proteins or within the well-defined non-structural protein complexes. Analyses of viable chimeras created from different species within the *Flavivirus* genus have revealed additional interactions. Several of these have documented interactions between components known to be involved in genome replication and the structural components of the virion. Among these, there has been a documented interaction of NS1 with the viral replicase via an interaction with NS4A (Lindenbach and Rice, 1999). In a previous report it was demonstrated that adaptation of a DENV/WNV single-cycle chimeric flavivirus to grow to higher titers in a specifically designed packaging cell line resulted in the selection of mutations in NS2A and NS3. Here, it is definitively demonstrated that these mutations exert their growth-enhancing effect by interaction with the structural proteins, and that this growth enhancing effect is more pronounced in the context of DENV2 structural proteins. These data explain how these adaptations arose in response

to the unnatural chimerization. In the case of the mutation in NS3, the mechanism by which this mutation was exerting its phenotype was not completely defined, but the ability of this mutation which lies within the helicase domain of NS3 to improve packaging of the viral genome supports previous evidence showing that changes in the helicase domain can alter encapsidation (Patkar and Kuhn, 2008).

In the case of the NS2A mutation, which was found at codon 9 of the NS2A gene, it was clearly demonstrated that this mutation functions through alteration of a recently documented ribosomal frame shift that produces an altered form of the NS1 protein (NS1') that is a characteristic of members of the JEV/WNV serocomplex of the flavivirus genus (Mason, 1989; Firth and Atkins, 2009; Melian et al., 2010). This interesting effect was evaluated by trans-complementation of genomes carrying the cell-adapted, non-synonymous mutation in NS2A which ablated the ribosome slippage site. These studies showed that this non-synonymous change increased transpackaging by prM/E proteins, and simultaneously eliminated the production of NS1'. The specific role of the frame shift in these phenotypes was confirmed by studies which demonstrated that synonymous mutations in this frame-shift site produced the same VRP growth-enhancing phenotype and ablation of NS1' production found in the non-synonymous mutation selected in previous blind-passaging studies. Careful analyses of replicons harboring this mutation document that its effect on genome packaging, which was additive with the NS3 mutation, could be observed when packaging was evaluated in cells providing WNV or DENV2 prM/E coats. However, the growth-enhancing phenotype was more dramatic with the DENV2 prM/E coats, consistent with the hypothesis that these lower-specific infectivity coats provided a more significant selective pressure, allowing these mutations

to arise in the chimeric background used for the initial passaging studies. The selection of these mutations is also consistent with preliminary studies showing that the combination of the NS2A frame-shift and NS3 point mutations into a replicon improve VRP yield from DENV E-producing cell lines by improving the specific infectivity of the resulting particles. Since the NS1 gene has been shown to serve a critical function in flavivirus genome replication, additional studies were conducted here to determine if ablation of NS1' by alteration of the slippage site could influence genome replication. These studies showed that abrogation of NS1' had no effect on genome replication, indicating that the mechanism by which NS1' alters particle packaging is due to an effect on virion assembly/release.

The data presented here demonstrating that the NS1' protein alters genome packaging expands the activities ascribed to the multifunctional NS1 protein. Although the precise mechanism of how the larger form of the NS1 protein interferes with transpackaging of genomes into infectious particles has not been determined here, these findings are consistent with other reports showing the slow egress of NS1 through the ER of infected mammalian cells (Mason, 1989), the regulation of its localization to several compartments (Youn et al., 2010), the documented role of NS1 in genome replication (Muylaert et al., 1996; 1997), and interactions with NS4A (Lindenbach and Rice, 1999), all of which indicate that NS1 likely serves as a bridge between RNA synthesis and structural protein assembly. The finding that the extended form of NS1 has an inhibitory effect on virion packaging *in vitro* support this role. However, by themselves, these results are somewhat surprising. The fact that the effect on packaging was less pronounced in the presence of high-specific infectivity encephalitic flavivirus prM/E

proteins is consistent with the fact that NS1' has not been observed in other flaviviruses (see above). Interestingly, work by Khromykh and co-workers showed that abrogation of NS1' in a low-virulence isolate of WNV (Kunjin virus) reduced neurovirulence in mice (Melian et al., 2010). Although these studies did not document an effect of NS1' ablation on viral growth, they clearly showed an interesting effect of the NS1' protein *in vivo* but were unable to demonstrate any effect of the frame-shift ablation on growth of a mutant Kunjin virus *in vitro* in mammalian or insect cells (Melian et al., 2010). The inability to observe differences in growth of Kunjin viruses with or without the frame-shift mutation is consistent with our studies showing that productive growth of a single-cycle WNV (namely RepliVAX WN) in a complementing cell line was not improved by the introduction of this frame-shift mutation. Finally, although the influence of the frame shift on Kunjin virus neurovirulence may not provide an evolutionary advantage *per se*, the association of the frame-shift slippage site with the encephalitic flaviviruses likely reflects a selective biological advantage, which could be related to acquisition of additional functions by the larger form of NS1.

CHAPTER 3: Subcapsular sinus macrophages limit dissemination of West Nile virus particles after inoculation but are not essential for the development of West Nile virus-specific T cell responses [1]

INTRODUCTION

Innate immunity to West Nile virus is initiated following recognition by the pattern recognition receptors TLR3 (Wang et al., 2004), TLR7 (Welte et al., 2009), RIG-I, or MDA5 (Fredericksen et al., 2008). Innate immune responses play an essential role in controlling the early stages of viral infection. Beyond the interferon (IFN)-induced cell-intrinsic responses that limit initial viral replication, innate immune cells such as neutrophils and γδ T cells are known to play a role in host protection by controlling virus infection through direct lysis of virus-infected cells or by production of cytokines (Wang et al., 2003a; Bai et al., 2010). The role of natural killer (NK) cells in WNV infection is not completely understood. NK cell activating receptor NKp44 interacts directly with WNV envelope protein resulting in NK cell activation, production of IFN-γ and expression of cell-lytic properties (Hershkovitz et al., 2009). Although not required for modulating WNV infection in the spleen, a role for NK cells in controlling WNV infection in the liver is suggested by the upregulation of NK signal pathway gene expression in the liver after WNV infection (Suthar et al., 2013a). Macrophage

[1]This chapter was previously published in Virology. This journal allows including information without copyright as long as it is properly cited. The citation for the article is: Winkelmann ER, Widman DG, Xia J, Johnson AJ, van Rooijen N, Mason PW, Bourne N, Milligan GN (2014). Subcapsular sinus macrophages limit dissemination of West Nile virus particles after inoculation but are not essential for the development of West Nile virus-specific T cell responses. Virology 450-451:278-289.

populations are present at peripheral sites as well as in draining LN and therefore may also play a direct role in limiting infection or dissemination of virus through the lymphatics during the initial stages of infection. Additionally, macrophages may play a role in activation or shaping of the adaptive immune response through presentation of viral antigen to antigen-specific cells and the secretion of proinflammatory cytokines/chemokines at the site of infection and in the draining lymph nodes. However, the role of macrophages in limiting virus dissemination and induction of WNV-specific T cell responses is not completely understood. Dendritic cells (DCs) represent a particular family of antigen-presenting cells (APCs) that efficiently link the innate detection of pathogens to the induction of adaptive immune responses. Immature DCs are present throughout the body, especially in regions vulnerable to pathogen invasion, such as the mucosa and skin (Lee and Iwasaki, 2007). Upon infection and consequent recognition of pathogen-associated molecular patterns (PAMPs) especially by TLRs, DCs start to mature, producing proinflammatory cytokines, expressing costimulatory molecules, altering chemokine receptors and enhancing antigen presentation (Kawai and Akira, 2006; Takeuchi and Akira, 2008). The signals provided to DCs during the maturation process will govern the type of immune responses elicited (Le Bon and Tough, 2008). Different subsets of DCs have been identified in both human and mice (Shortman and Liu, 2002; Heath et al., 2004; Shortman and Naik, 2007), differing in their anatomical region and capability to induce specific immune response depending on the expression of different sets of TLRs (Iwasaki and Medzhitov, 2004).

Activation and development of the WNV-specific $CD8^+$ T cell response is complex and involves the interaction of many cytokines and signaling pathways

including type I IFN (Pinto et al., 2011; Winkelmann et al., 2012), CD22 (Ma et al., 2013), MDA5 (Lazear et al., 2013), interferon regulatory factor-1 (Brien et al., 2011) and interferon promoter stimulator-1 (IPS-1) (Suthar et al., 2010). Early studies demonstrated that macrophages, B cells and DCs were capable of serving as antigen presenting cells for WNV-specific T cell responses (Kulkarni et al., 1991). However, more recent studies have demonstrated a dominant role for $CD8\alpha^+$ DC in activation of WNV-specific $CD8^+$ T cell subsets *in vivo* (Hildner et al., 2008). Interestingly, subcapsular sinus (SCS) macrophages of regional lymph nodes have been shown to play a role in the development of the B cell response to vesicular stomatitis virus (Carrasco and Batista, 2007; Junt et al., 2007), in invariant NKT cell activation (Barral et al., 2010), and recently in antigen presentation and early activation of the acquired immune response (Martinez-Pomares and Gordon, 2012). While SCS macrophages are not required for a developing B cell response (Purtha et al., 2008), the role these cells play in development of $CD8^+$ T cell responses to WNV is not fully known.

In the studies presented here, clodronate-liposome depletion of SCS macrophages from lymphoid tissue draining the site of inoculation with the single-cycle flavivirus (SCFV) particle was utilized to examine the role of these cells in controlling the initial infection and dissemination from the site of inoculation. Furthermore, the role of SCS macrophages and DCs as antigen-presenting cells in the initiation of the virus-specific $CD8^+$ T cell response was examined. The present data demonstrate that depletion of SCS macrophages from the draining LN results in a diminished ability to confine the initial spread of virus at very early times post infection. Additionally, SCS macrophages are not required for activation of the cell-mediated arm of the adaptive immune response in the

draining LN and provide evidence for the antigen-presenting function of specific dendritic cell (DC) subsets from RepliVAX WN-infected mice. These results further illuminate the role of SCS macrophages in protection against WNV during the early stages of infection.

MATERIALS AND METHODS

Mice

Seven-week-old B6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME). T cell receptor (TCR) transgenic OT-I mice (Falk et al., 1993) were bred onto a Thy1.1 background and maintained as a breeding colony at the AAALAC-approved Animal Resources Center at the University of Texas Medical Branch (UTMB). TLR3- and MyD88-deficient mice on a B6 background were obtained from Michael Diamond (Washington University, St. Louis, MO) and also maintained as a breeding colony at the UTMB facility. All animal work was approved by the Institutional Animal Care and Use Committee with oversight of staff veterinarians.

Cells

Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Cellgro, Mediatech, Manassas, VA) containing 6% fetal bovine serum (FBS) (HyClone, Thermo Scientific, Logan, UT), 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO, Invitrogen, Grand Island, NY) and 20ug/mL Gentamicin (Cellgro). BHK(VEErep/Pac-Ubi-C*) expressing the WNV C protein (Widman et al., 2008a) and BHK(VEErep/WNVC*-E/Pac) cells expressing the WNV C-prM-E proteins (Fayzulin et

al., 2006) were propagated in DMEM supplemented with 10% FBS and 10 ug/ml puromycin (Cellgro) as previously described (Fayzulin et al., 2006).

Production of RepliVAX WN and related WNV-SCFV particles

RepliVAX WN and the gB/OVA-RepliVAX WN which encodes the epitopes recognized by OT-I and gBT-I $CD8^+$ T cells (ovalbumin $OVA_{257-264}$ [SIINFEKL] and HSV $gB_{498-505}$ [SSIEFARL], respectively) (Hogquist et al., 1994; Mueller et al., 2002) inserted into the truncated capsid gene of WNV (Fig. 3.1) were produced in BHK (VEErep/Pac-Ubi-C*) cells as described previously (Widman et al., 2008a). FLUC-SCFV were produced in BHK (VEErep/C*-prM-E-Pac) cells as described previously (Gilfoy et al., 2009). Infectious titers (IU/ml) of RepliVAX WN and WNV SCFV particles were determined on Vero cells as previously described (Fayzulin et al., 2006).

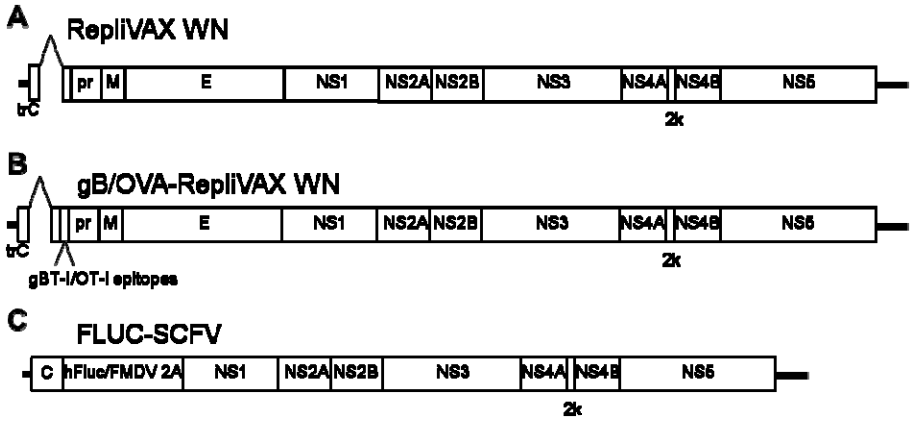

Figure 3.1. Schematic representation of the single-cycle flavivirus (SCFV) genomes. RepliVAX WN genome (A) (Widman et al., 2008a) was used for the construction of gB/OVA-RepliVAX WN (B) containing the HSV $gB_{498-505}$ (SSIEFARL) and ovalbumin $OVA_{257-264}$ (SIINFEKL) peptides. (C) FLUC-SCFV genome, expressing a luciferase reporter gene (Gilfoy et al., 2009).

Depletion of macrophages by injection of clodronate liposomes

To deplete macrophages from the regional draining lymph nodes (LN) of B6 mice, 20ul of a suspension of clodronate-containing liposomes (CCL) containing 5mg/ml

clodronate (Roche Diagnostics GmbH, Mannheim, Germany) or control PBS-loaded liposomes (PBSL) was injected subcutaneously in each footpad (FP) 7 days before SCFV inoculation. CCL and control PBSL were prepared as previously described (van Rooijen and Sanders, 1994). To confirm the depletion, cells from spleen, popliteal LN (pLN) and inguinal LN (ingLN) were stained with fluorochrome-conjugated mAb anti-CD11c (PE) (BD Biosciences, San Jose, CA), -F4/80 (APC) (AbD Serotec, Raleigh, NC), -CD11b (FITC) (BD Biosciences) or -MOMA-1 (CD169) (FITC) (AbD Serotec) and data were acquired on a BD LSRII Fortessa and analyzed using FlowJo software (Tree Star, Ashland, OR).

***In vivo* imaging**

Mice were inoculated subcutaneously in both hind FP with FLUC-SCFV (Gilfoy et al., 2009) at a dose of 10^7 IU/FP 7 days after CCL or PBSL treatment. Prior to inoculation, the posterior half of all animals were shaved to facilitate the acquisition of luciferase signal. Mice were imaged at 14, 24, 36 and 48 hours post-inoculation (hpi). At each time point, mice were injected intraperitoneally with a D-luciferin solution (Caliper LS, Hopkinton, MA) corresponding to the dose of 15 mg/kg body weight. After 20 min to allow D-luciferin distribution, mice were anesthetized with 90mg/kg ketamine and 8mg/kg xylazine, and real-time *in vivo* imaging was performed using a Xenogen IVIS 200 (Caliper LS) with exposure times ranging from 1 to 90 sec at medium binning. The images were analyzed using the Living Image 4.0 software (Caliper LS), where the total flux from each region of interest (ROI) was measured and reported as photons per second (photons/sec). The limit of detection of the luciferase signal was considered to be 10^4 photons/sec of each ROI analyzed.

Interferon and Cytokine detection

Type I interferon (IFN) was quantified using commercial IFN-alpha (IFN-α) and IFN-beta (IFN-β) ELISA kits (PBL Biomedical Laboratories, Piscataway, NJ), following the manufacturer's protocol. The limit of detection for the assays was 12.5 pg/ml. Cytokine and chemokine levels in the sera of individual RepliVAX WN-inoculated mice were determined using a luminescence-based multiplex bead assay (Bio-Rad, Hercules, CA) from a panel of 23 cytokines following the manufacturer's protocols as performed previously (Winkelmann et al., 2012).

Enzyme-linked immunospot assay (ELISPOT)

ELISPOT assays were performed as previously described (Nelson et al., 2010) using microtiter filter plates (Millipore, Billerica, MA) coated with purified anti-mouse IFN-gamma (IFN-γ) monoclonal antibody (BD Pharmingen) and incubated at 4°C overnight. Serial dilutions of splenocytes and pooled pLN cells from individual CCL- and PBSL-treated mice were plated and stimulated with an immunogenic peptide representing the WNV-specific $CD8^+$ T cell epitope ($NS4B_{2488}$) (Brien et al., 2007b; Purtha et al., 2007). After 40h incubation, plates were developed with biotinylated anti-mouse IFN-γ (BD Pharmingen) and streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO) and the number of spots in each well, representing the number of IFN-γ secreting cells (SC), were quantified using an ImmunoSpot reader and analyzed with ImmunoSpot software (Cellular Technology Ltd, Cleveland, OH). The total number of IFN-γ secreting cells were calculated and expressed as IFN-γ SC per spleen or pLN.

Intracellular cytokine staining

Lymphocytes from spleens and pLN from CCL- or PBSL-treated mice were harvested on day 5 or 7 after RepliVAX WN vaccination and were re-stimulated with $NS4B_{2488}$ peptide and stained for IFN-γ, interleukin-2 (IL-2), and tumor necrosis factor-α (TNF-α) as described previously (Winkelmann et al., 2012). Data were acquired on a BD LSRII Fortessa cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). The total number of cells secreting IFN-γ, IFN-γ + TNF-α, or IFN-γ + TNF-α + IL-2 was derived by multiplying the % of cells secreting a particular cytokine combination by the total number of viable spleen or pLN cells.

Antigen presentation by DC subsets isolated from RepliVAX WN infected mice

Three days after intraperitoneal inoculation of B6 with $2x10^7$ IU of gB/OVA-RepliVAX WN, splenocytes were harvested and DC were enriched by depletion of B, T and NK cells using anti-CD19, -CD90.2, -CD49 (DX5) microbeads (Miltenyi Biotec, Auburn, CA), following the manufacturer's protocol. Enriched cells were then surface-stained with fluorochrome-conjugated mAb anti-CD8α (APC), -CD11c (PE) and -CD11b (PE-Cy7) (BD Biosciences) and sorted into $CD11b^+$ $CD11c^+$ and $CD8\alpha^+$ $CD11c^+$ subpopulations using a BD FACS Aria. Serial 2-fold dilutions of $CD11c^+$ $CD11b^+$ or $CD11c^+$ $CD8\alpha^+$ DC subpopulations were co-cultured with 10^5 naïve OT-I T cells selected by using the $CD8^+$ T cell Isolation Kit II (Miltenyi Biotec) and labeled with 2μM of carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Invitrogen) in 96-well plates. After 72h, cells were surface-stained with fluorochrome-conjugated mAb anti-CD90.2 (PE) (BD biosciences), data were acquired on a BD LSRII Fortessa, and the

proliferation of T cells measured as dilution of intracellular CFSE was determined by using FlowJo software (Tree Star).

***In vitro* studies using bone marrow-derived DC (BM-DC) and BM-macrophages (BM-MØ)**

To generate BM-DC, femurs were aseptically collected, extraneous tissues were removed and then both ends of the bone were cut to expose the lumen. Marrow cells were flushed with 4 ml of Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) containing 5% new born calf serum (NBCS) (GIBCO, Invitrogen) and 1% penicillin/streptomycin using a 26-gauge needle and pushed through a mesh screen to create a single-cell suspension. After washing the cells once with HBSS, red blood cells were removed using RBC lysis buffer (Sigma-Aldrich) and lymphocytes were washed 3 times with HBSS and resuspended at the concentration of $2x10^6$ cells/ml in culture medium [RPMI-1640 (Cellgro) supplemented with 10% FBS, 1% penicillin/streptomycin and 1mM sodium pyruvate (Sigma-Aldrich)] containing 20ng/ml of recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) and 20ng/ml recombinant mouse IL-4 (BD Pharmingen). Cells were plated in 100 mm cell culture dishes in a total 10 ml per dish and incubated for 10 days. On day 3, an additional 10 ml of medium was added to each culture dish. On days six and eight, 10 ml of the medium was removed without discarding any cells and fresh medium was added to the culture dishes.

To generate BM-MØ, bone marrow cells were resuspended at $7x10^5$ cells/ml and plated in 100 mm non-treated culture dishes in 10 ml of culture medium containing 100ng/ml of recombinant mouse macrophage colony-stimulating factor (M-CSF)

(PeproTech, Rocky Hill, NJ) and incubated at 37°C, 5% CO_2 for 7 days. On day 3 an additional 5 ml of medium was added to each culture dish.

Infection of BM-DC and BM-MØ and co-culture with $CD8^+$ T cells

BM-DC and BM-MØ were infected with gB/OVA-RepliVAX WN at a multiplicity of infection (MOI) of 100 for 2 hours at 37°C. Cells were washed 3 times with HBSS, resuspended in culture media and incubated for an additional 24 hours. Serial 2-fold dilutions of infected BM-DC or BM-MØ were co-cultured with 10^5 naïve OT-I x $Thy1.1^+$ $CD8^+$ T cells as described above. After 72h, cells were surface-stained with fluorochrome-conjugated mAb anti-CD90.1 (PE) (BD Biosciences), data were acquired on a BD LSRII Fortessa, and the proliferation of T cells was measured as dilution of intracellular CFSE analyzed using FlowJo software (Tree Star).

Statistical analyses

GraphPad Prism (GraphPad Software, San Diego, CA) was used to analyze data. Student's t test, one-way or two-way analysis of variance (ANOVA) with the Bonferroni post-test was used where appropriate. P values less than 0.05 were considered to indicate statistical significance.

RESULTS

SCS macrophages limit SCFV dissemination and SCFV gene expression

Tissue resident macrophages are present at sites of pathogen entry and play important roles in the innate immune response against many viral pathogens. To study the biological function of macrophages in the initial stages of a WNV infection, mice were depleted of macrophages at the site of infection and draining lymph nodes by subcutaneous inoculation of hind FP with CCL (Delemarre et al., 1990) prior to FP

inoculation of SCFV (Fig 3.2A). To confirm CCL-mediated depletion from draining lymph nodes, cells from the pLN, ingLN and spleens were harvested 7 days after injection of CCL or PBSL into the hind FP and analyzed for expression of the macrophage surface markers, integrin α_M chain (CD11b) and F4/80, or the SCS macrophage-expressed protein CD169, by flow cytometry. Additionally, lymphoid cells were tested for depletion of DCs by staining for expression of the integrin α_X chain (CD11c). As expected, mice treated with CCL were depleted of $CD169^+$ $CD11c^-$ cells and, to a lesser extent, $F4/80^+$ $CD11b^+$ cell populations in the pLN, corresponding to SCS and medullary macrophages, respectively (Fig. 3.2B,C). CCL treatment did not deplete macrophages from the ingLN. Additionally, consistent with reports by others (Delemarre et al., 1990; Purtha et al., 2008), FP administration of CCL did not deplete macrophage populations from the spleen. As shown in Fig. 3.2D, treatment of mice with CCL did not result in significant reduction of DCs ($CD11b^+$, $CD11c^+$) from any of the lymphoid tissues tested.

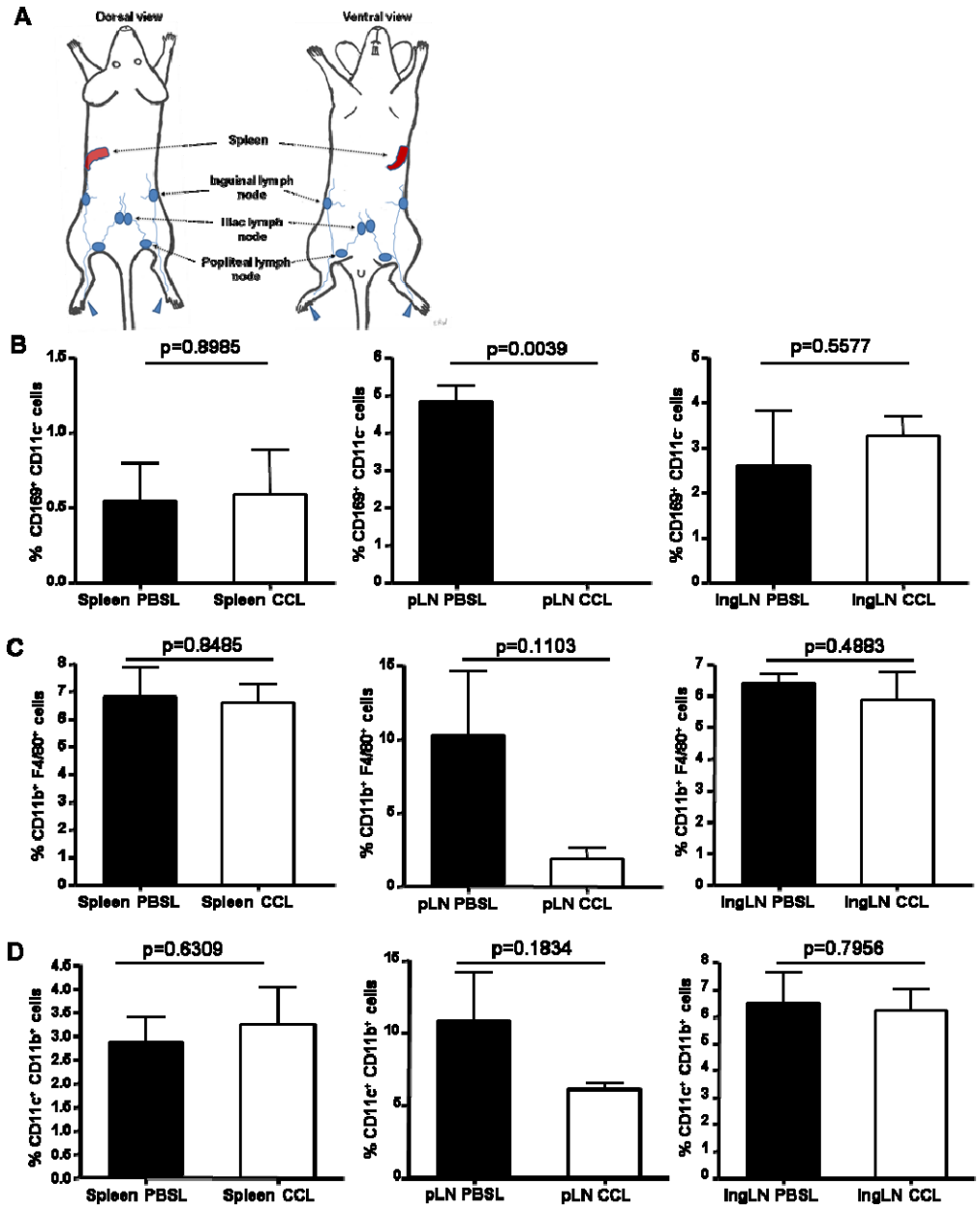

Figure 3.2. Macrophage depletion after subcutaneous FP injection of CCL. Seven days post injection, cells from the spleen, pLN and ingLN were analyzed for the expression of CD11b, CD11c, F4/80 and MOMA-I (CD169) by flow cytometry. (A) Schematic showing lymphatic drainage from the footpad. The percentage of (B) $CD169^+$ $CD11c^-$ cells (SCS macrophages), (C) $CD11b^+$ $F4/80^+$ cells (medullary macrophages) or $CD11b^+$, $CD11c^+$ (dendritic cells) from CCL- or PBSL-treated mice are shown. Error bars indicate standard deviation (3 mice per group). P value determined by Student's t test.

Macrophages can function in protection through direct clearance of viral particles and evidence from depletion studies by others suggests that they are essential to control systemic WNV infection and limit infiltration of virus into the central nervous system (Ben-Nathan et al., 1996). However, it is not clear from these studies if macrophages play a role in control of the initial dissemination of virus from the site of inoculation or provide protection only at later stages of infection. Assessing control of dissemination of the initial wild type viral inoculum is complicated by the presence of continuous virus replication and new rounds of infection. Therefore, we visualized the initial dissemination of FLUC-SCFV from the FP in control-treated and macrophage-depleted mice using an *in vivo* imaging system (IVIS). Groups of B6 mice received PBSL or CCL, were inoculated in the hind FP with FLUC-SCFV, and imaged at intervals between 14 and 48 hpi. SCFV-encoded FLUC gene expression was quantified at the FP site of infection, draining LN and spleen. As shown in Fig. 3.3, luciferase bioluminescence was detected in the FP of all CCL- and PBSL- treated mice at 14 hpi, and by 24 hpi differential expression of FLUC was detected in the FP and lymphoid tissues of macrophage-depleted and control mice.

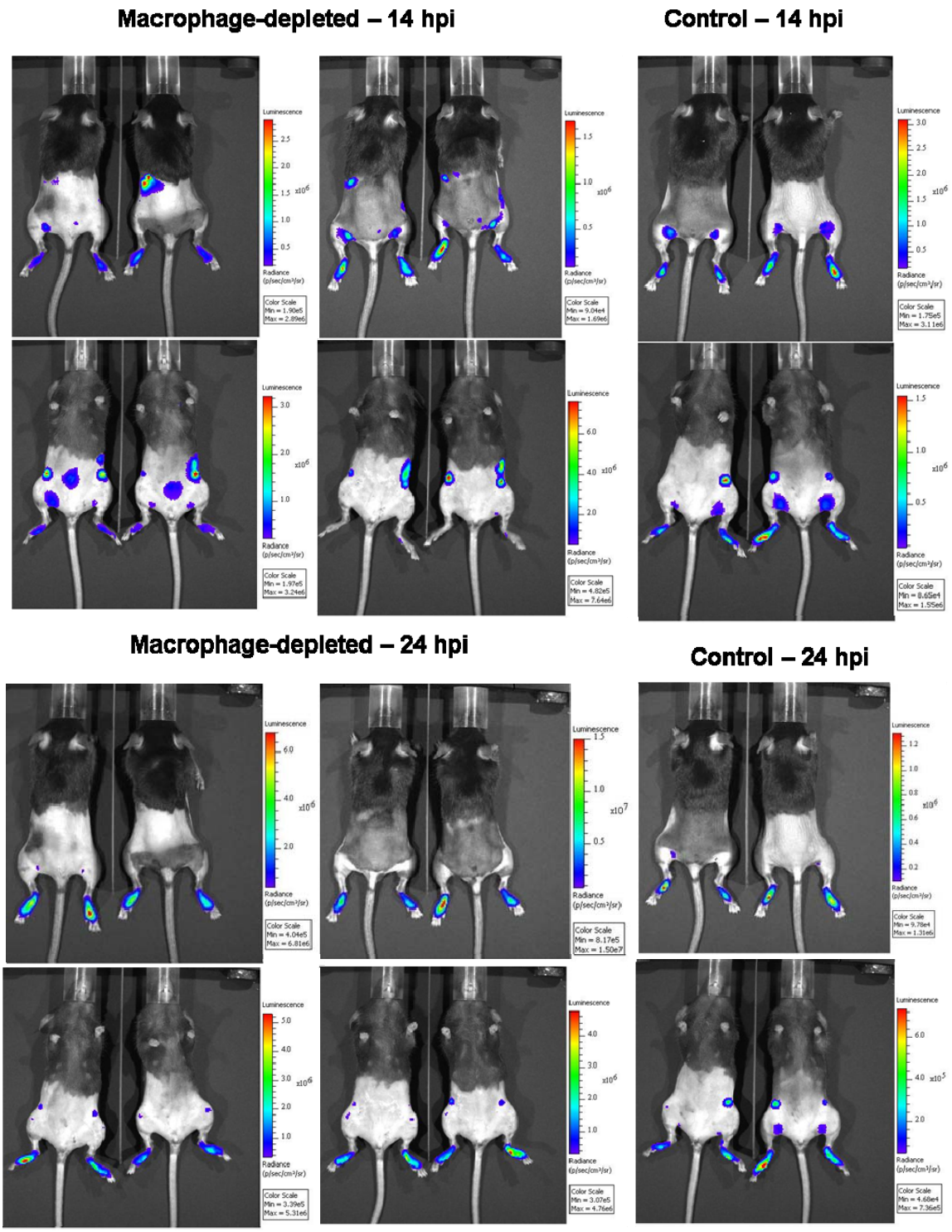

Figure 3.3. Role of macrophages in limiting the dissemination of SCFV. CCL- or PBSL-treated B6 mice were inoculated subcutaneously in both hind FP with 10^7 IU of FLUC-SCFV on day 7 after liposome injection. Mice were imaged at 14, 24, 36 and 48 hpi. Representative images of mice at 14 hpi and 24 hpi.

The results of IVIS analysis of FLUC-SCFV dissemination to specific tissues are shown in graphic form in Fig. 3.4. Expression of FLUC-SCFV genes was readily detected in the hind FP of all FLUC-SCFV-infected mice. At 14 hpi, FLUC gene expression at this site was detected at similar levels in the CCL- and PBSL-treated groups. However, expression increased approximately 10-fold in the CCL-treated mice and was significantly higher than expression in PBSL-treated mice at all time points from 24-48 hpi ($p<0.001$, Fig. 3.4A). Lymphatic drainage of the hind FP of mice involves both the pLN and ingLN (Harrell et al., 2008). The luciferase bioluminescence detected in the pLN of CCL- and PBSL-treated mice was nearly equivalent at 14 hpi and was absent in both mouse groups by 36 hpi. Interestingly, at 24 hpi FLUC gene expression was detected in all PBSL-treated mice but in only 2 of 10 pLN in macrophage-depleted mice although at a significantly higher expression level ($p<0.01$) (Fig. 3.4B). In the ingLN, luciferase bioluminescence was detected in 5 of 8 in PBSL-treated mice at 14 hpi and expression decreased thereafter such that FLUC activity was detected at low levels in only 1 of 8 ingLN at 48 hpi. By contrast, FLUC gene expression was significantly higher at 14 hpi in the ingLN of CCL-treated group ($p<0.01$), and remained higher at the 24, 36, and 48 hours time points (Fig. 3.4C). A greater frequency of CCL-treated mice expressed FLUC activity in the ingLN at both 36 and 48 hpi: 7 of 10 compared to 3 of 8 in PBSL-treated mice at 36 hpi and 6 of 10 compared to 1 of 8 in PBSL-treated mice at 48 hpi (Fig. 3.4C). Luciferase bioluminescence was not detected at any time point in the spleens of PBSL-treated mice although it was detected at high level in all CCL-treated mice at 14 hpi ($p<0.001$, Fig. 3.4D).

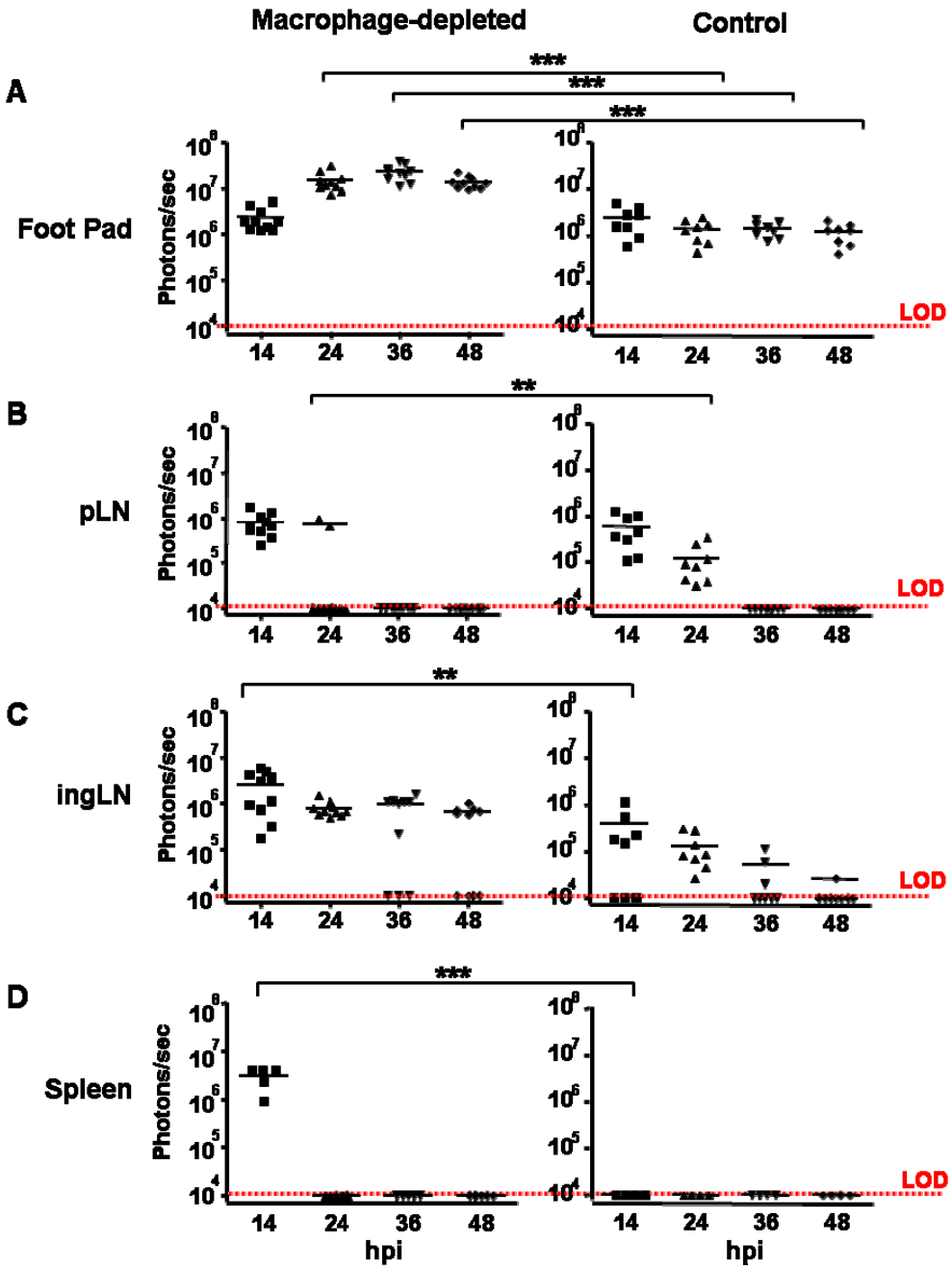

Figure 3.4. Magnitude of SCFV gene expression *in vivo* in SCS macrophage-depleted and control-treated B6 mice. Images were taken at the indicated time points and bioluminescence was observed using up to 90 seconds exposures at medium binning. The total flux emanating from each region of interest (ROI) was measured for the indicated tissues from each mouse group. Each point represents the measurement of one ROI (in the cases of FP and LN, two per animal) and the line represents the average of all site per group (macrophage-depleted, n=5; control group, n=4) above the limit of detection (LOD). **p<0.01, ***p<0.001, two-way ANOVA, Bonferroni post test.

Higher serum IFN-α level in macrophage-depleted mice

Type I IFN are produced by many cells types and have a critical function as an initial mechanism to prevent virus infection through their antiviral effects. To investigate the effects of macrophage depletion at the regional site of infection on systemic production of type I IFN, we measured the amount of IFN-α and IFN-β in the serum of mice at 24 hpi. Interestingly, the serum level of IFN-α was two-times higher in the macrophage-depleted group ($p<0.0001$) (Fig. 3.5). IFN-β was not detected in any treatment group. This result is consistent with our previous report showing that IFN-β is produced at low levels in mice inoculated with a single-cycle flavivirus (Bourne et al., 2007).

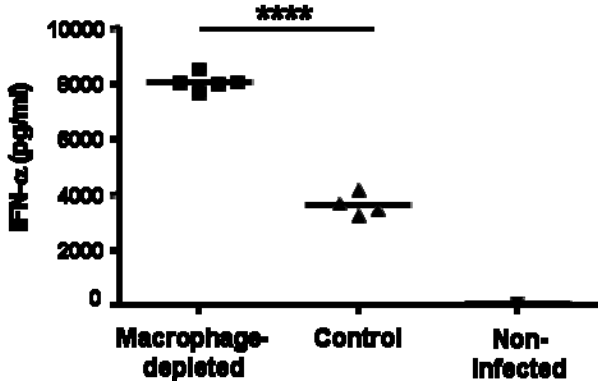

Figure 3.5. Serum levels of IFN-α in SCS macrophage-depleted and control-treated B6 mice. Sera were collected at 24 hpi from the mice described in Fig. 3.3 and the IFN-α concentration was determined by ELISA. A mock-infected CCL-treated mouse was included as an additional control. Each point represents the IFN-α levels of each mouse and the line represents the average per group of mice. ****$p<0.0001$, Student's t test.

Cytokines and chemokines were quantified in the sera of RepliVAX WN-inoculated mice at 14, 24, 48, 72, 120, and 168 hpi. As shown in Fig. 3.6, serum levels of IL-1β, IL-6, CCL2, CCL4, and CCL5 peaked at 14 hpi in both CCL- and PBSL-treated mice and declined precipitously thereafter. Serum levels of IL-12p70, and TNF-α remained at baseline levels until 72 hpi before rising through 168 hpi. Levels of IL-6 and CCL2 were significantly higher at 14 and 24 hpi in CCL-treated compared to PBSL-treated mice. However, for IL-1β, IL-12p70, TNF-α, CCL4, and CCL5 the pattern and quantity of cytokines and chemokines produced following RepliVAX WN FP inoculation were not different between CCL- and PBSL-treated mice.

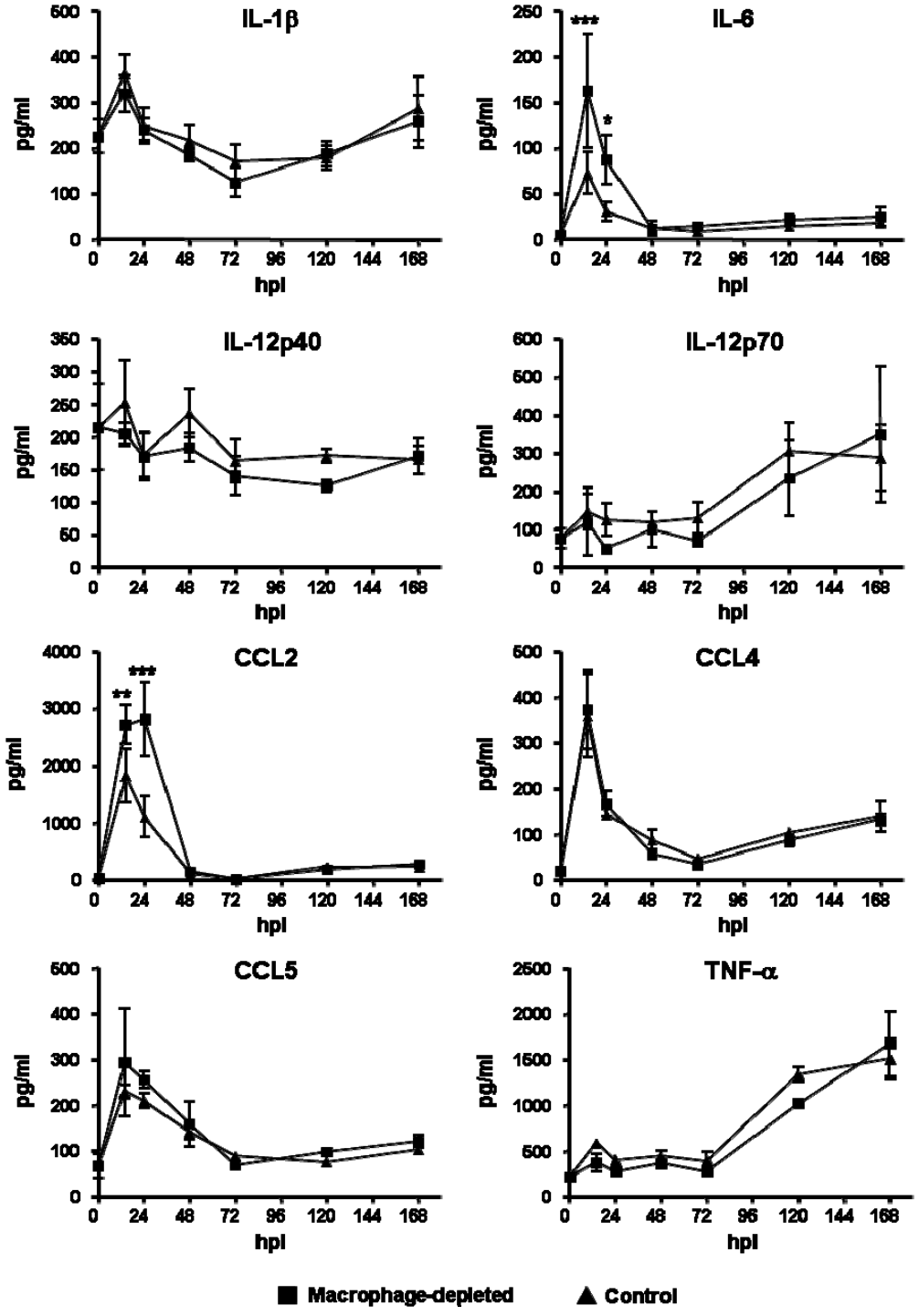

Figure 3.6. Serum levels of selected cytokines and chemokines in SCS macrophage-depleted and control-treated B6 mice. Mice were inoculated in the FP with 10^7 IU of RepliVAX WN and sera were collected at 14, 24, 48, 72, 120, and 168 hpi and assayed for the indicated cytokines and chemokines. The results represent the mean response of 3 mice/group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Two-way ANOVA).

SCS macrophages are not required to initiate a WNV-specific CD8$^+$ T-cell response

Beyond phagocytosis of viral particles and production of cytokines, macrophages can serve as potent APCs and may therefore play a critical role in presenting WNV antigen to and activating naive WNV-specific T cells in draining LN. To examine the requirement for SCS macrophages to initiate the WNV-specific CD8$^+$ T cell response, mice were CCL- or PBSL-treated prior to inoculation with FLUC-SCFV. As shown in Figure 3.7, the number of IFN-γ-secreting cells specific for the immunodominant CD8$^+$ T cell epitope of the NS4B protein, $NS4B_{2488}$, was not different in the spleen or draining LN between PBSL-treated and CCL-treated mice.

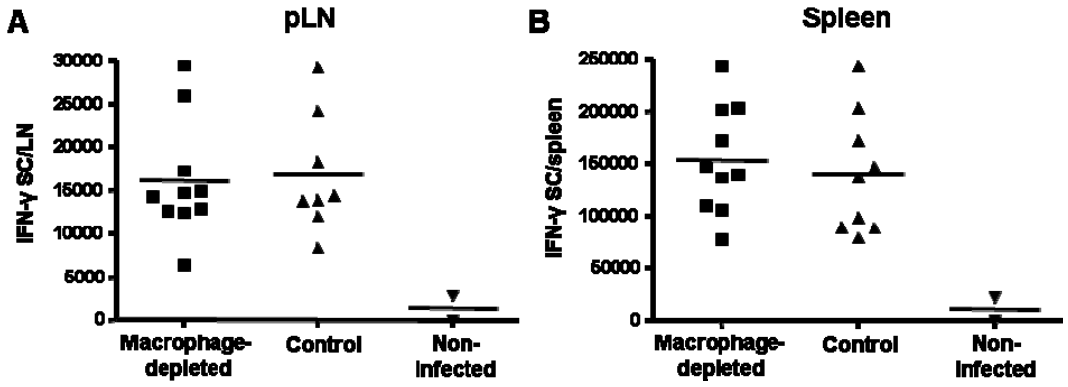

Figure 3.7. SCS macrophages present at the regional site of infection are not required to promote the development of WNV-specific CD8$^+$ T cell response. SCS macrophage-depleted and control B6 mice were inoculated with 10^7 IU of FLUC-SCFV at day 7 after liposome injection. The two pLN were pooled for individual animals (A) and spleens (B) were harvested at day 7 post infection and cells were used for quantification of the number of IFN-γ-secreting cells (IFN-γ SC) specific for the WNV $NS4B_{2488}$ CD8$^+$ T cell epitope by ELISPOT. Each point represents the number of IFN-γ SC from pooled LN of individual mice and the line represents the mean per group of mice. Data are representative of two independent experiments.

These data suggest that SCS macrophages are not required for development of the cell-mediated response. This result is not due to an inability of macrophages to present virus-derived epitopes as BM-MØ infected with gB/OVA-RepliVAX WN efficiently presented virus-expressed ovalbumin SIINFEKL epitope to naïve OVA-specific OT-I T cells resulting in T cell proliferation (Fig. 3.8A). These results suggest that other cells, such as DC most likely served as the APC in macrophage-depleted mice. Consistent with this notion, co-culture of naïve OT-I T cells and BM-DC infected with gB/OVA-RepliVAX WN also resulted in T cell replication (Fig. 3.8B). Additionally, DC isolated from the spleens of gB/OVA-RepliVAX WN-infected mice were also capable of presenting antigen to naïve OT-I T cells. Two major populations of spleen DC have been described: $CD11b^{-}$ $CD8\alpha^{+}$ $CD11c^{+}$ and $CD11b^{+}$ $CD8\alpha^{-}$ $CD11c^{+}$ (Anjuere et al., 1999). OVA-specific T cell proliferation was observed following culture with either $CD11b^{+}$ $CD8\alpha^{-}$ $CD11c^{+}$ (Fig. 3.8C) or $CD11b^{-}$ $CD8\alpha^{+}$ $CD11c^{+}$ (Fig. 3.8D) DC subsets isolated on day 3 post inoculation, consistent with the role of these cells as professional APC.

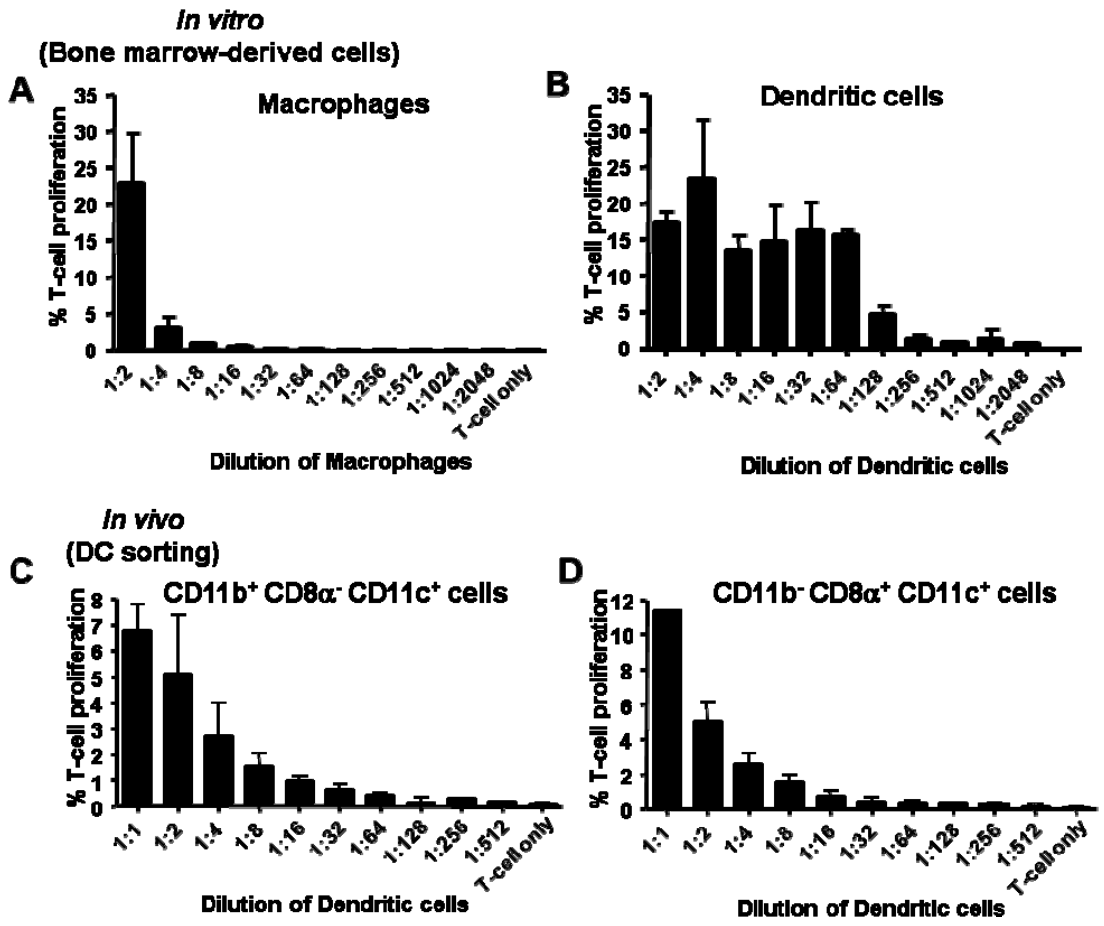

Figure 3.8. Ability of macrophages and dendritic cells to stimulate CD8$^+$ T cell proliferation. Serial dilutions of BM-MØ (A) and BM-DC (B) infected with gB/OVA-RepliVAX WN at a MOI of 100 were co-cultured with constant numbers of naïve OT-1 CD8$^+$ T cells for 72 hours (see material and methods). Bars represent proliferation of T cells measured by CFSE signal dilution. Error bars represent standard deviation of duplicates or triplicates from each dilution. (C) and (D) B6 mice were immunized with 2x10^7 IU gB/OVA-RepliVAX WN. On day 3, cells from spleen were harvested and CD11b$^+$ CD8α$^-$ CD11c$^+$ (C) and CD11b$^-$ CD8α$^+$ CD11c$^+$ (D) DC subpopulations were sorted by flow cytometry and co-cultured with naïve CD8$^+$ T cells from OT-I mice as described above (see also materials and methods). Bars represent proliferation of T cells measured by CFSE signal dilution and error bars represent standard deviation of two independent experiments.

Further, the possibility that SCS macrophages played a role in determining the quality of the $CD8^+$ T cell response was examined by measuring the polyfunctionality of WNV-specific effector $CD8^+$ T cells in the ability to simultaneously produce IFN-γ, TNF-α, and IL-2. As shown in Fig. 3.9A, cytokines were produced as a result of stimulation of $CD8^+$ T cells from RepliVAX WN-inoculated mice with the peptide representing the immunodominant epitope from the NS4B protein, but not following stimulation with an irrelevant peptide or medium only. Both the frequency and total number of $CD8^+$ T cells producing IFN-γ only, IFN-γ + TNF-α, or IFN-γ + TNF-α + IL-2 were very similar between spleen cells (Fig. 3.9B,C, F, G) or pLN cells (Fig. 3.9D, E, H, I) from PBSL-treated and CCL-treated mice on both day 5 and day 7 post inoculation. The only exceptions were that significantly higher numbers of spleen cells producing IFN-γ + TNF-α were detected on day 5 and the frequency of pLN cells secreting only IFN-γ reached significance on day 5. The presence of these cytokine producing cells is likely responsible for the high serum concentration of TNF-α (Fig 3.5) and IFN-γ (data not shown) at 120 and 168 hpi. In total, these data indicate that depletion of SCS macrophages had little effect on the development of $CD8^+$ T cell effector function in the draining LN following RepliVAX WN inoculation.

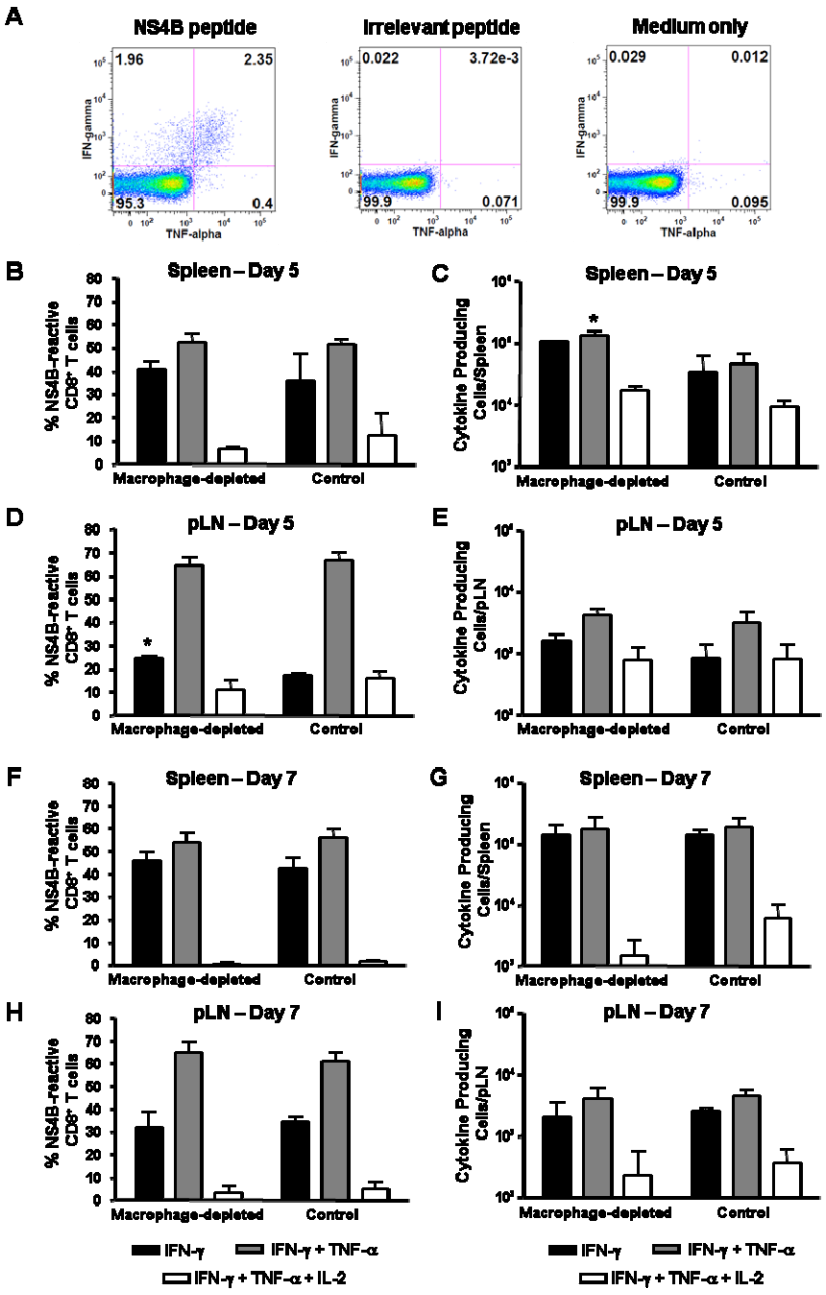

Figure 3.9. Cytokine production by WNV-specific CD8$^+$ T lymphocytes is not affected by depletion of SCS macrophages. CCL- or PBSL treated B6 mice (n=6) were inoculated in the FP with 10^7 IU RepliVAX WN. Lymphocytes from spleen and pLN were harvested on day 5 (n=2) or day 7 pi (n=4), stimulated and assayed for production of cytokines as described in methods. (A) Representative staining for IFN-γ and TNF-α in splenocytes stimulated with NS4B peptide, irrelevant peptide, or medium. The mean (± SD) frequency (B, D, F, H) or total cell number (C, E, G, I) of cytokine producing CD8$^+$ T cells from the spleen or pLN (*p <0.05).

Influence of TLR3- and MyD88-dependent signaling pathways in effective DC APC function for initiation of WNV-specific T cell response

Since SCS macrophages were not required for development of WNV-specific cell-mediated response, the APC function of DC was further investigated. During WNV replication, double-stranded and single-stranded viral RNA are recognized in the endosome by TLR3 and TLR7, respectively. While TLR3 recruits the TRIF adaptor molecule, TLR7 utilizes the MyD88 molecule to stimulate the induction of type I IFN and inflammatory cytokines, and both of these signaling pathways are known to be involved in the development of B and T cell responses. Hence, to start to dissect the role of these two signaling pathways in WNV-specific immune response, the APC function of DCs derived from knockout mouse strains were compared with the WT strain. BM-DCs from B6, TLR3-deficient ($TLR3^{-/-}$) and MyD88-deficient ($MyD88^{-/-}$) mice were infected with gB/OVA-RepliVAX WN and tested for the capability of presenting antigen to naïve OT-I $CD8^{+}$ T cells. As shown in Fig. 3.10, T-cell responses were induced by DC of all mouse strains. However, $MyD88^{-/-}$ showed a significantly lower ability to stimulate T-cell proliferation at 1:16-1/64 dilutions of DCs, indicating a lower frequency of DCs with APC function.

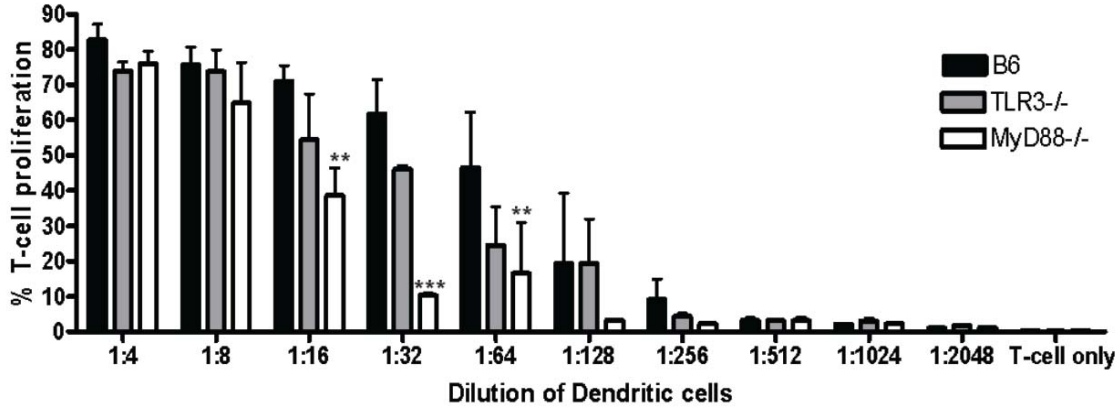

Figure 3.10. Percentage of CD8$^+$ T cell proliferation upon stimulation with BM-derived DCs infected with gB/OVA RepliVAX WN. BM-DC were infected at MOI 100 and cultured for 24 hrs. Constant numbers of CD8$^+$ T cells (labeled with CFSE) from naive OT-I transgenic mice were co-cultured with different numbers (2-fold dilutions) of BM-DCs for 72 hrs (in duplicate). Flow cytometry was used to measure proliferation of T cells by CFSE signal dilution. Error bars represent standard deviation. **p<0.01. ***p<0.001.

The expression of activation/maturation and costimulatory markers in both naïve and infected BM-DCs was also analyzed. MyD88$^{-/-}$ BM-DCs showed a poorer surface expression of MHC-II and CD80 molecules compared to WT or TLR3$^{-/-}$ BM-DCs (Fig 3.11), indicating a lower maturation/activation phenotype compared to the B6 mice. Overall, these results suggest that these signaling pathways are not absolutely essential during antigen presentation for the development of the CD8$^+$ T-cell response. However, DCs from MyD88$^{-/-}$ mice demonstrated a lower ability to present antigen to naïve CD8$^+$ T cells, indicating a deficiency in exert their APC function.

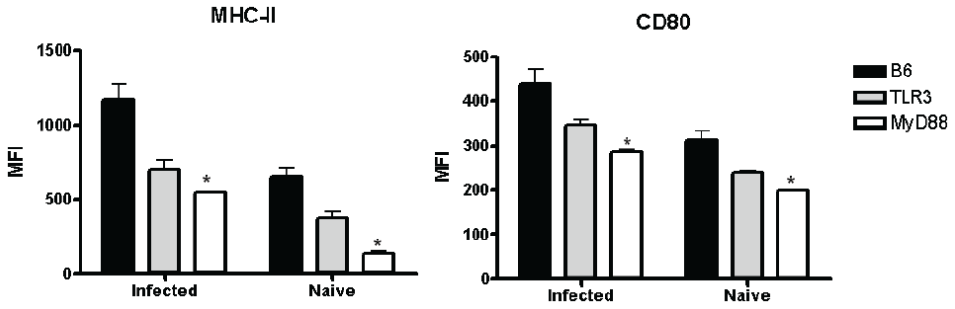

Figure 3.11. Expression of activation/maturation and co-stimulatory markers in BM-DCs. Naïve and gB/OVA RepliVAX WN-infected BM-DC were stained with fluorochrome-conjugated monoclonal antibodies and submitted to flow cytometry. The median fluorescence intensity (MFI) was measured from $CD11c^{+}CD11b^{+}$ cells. Error bars represent standard deviation. *$p<0.05$.

DISCUSSION

Tissue macrophages play a key role in maintaining homeostasis through phagocytosis and removal of pathogens and cellular debris and they represent one of the first immune cells to encounter virion particles following injection of arboviruses into a mammalian host. Macrophages can be productively infected with WNV (Kyle et al., 2007; Prestwood et al., 2012) although viral replication is controlled by downstream effects of type I IFN mediated in part by innate intrinsic protective mechanisms involving IRF-1, PKR, and RNase L (Samuel et al., 2006; Kong et al., 2008; Brien et al., 2011; Lazear et al., 2011). Because of the highly phagocytic nature of tissue macrophages, it is generally assumed that these cells play a role in limiting the initial spread of virus from the site of infection. This may occur through direct phagocytosis and destruction of virion particles or through elicitation of immune reactive molecules that initiate the infiltration and activation of a variety of innate host cells. This notion has been tested in several studies of flavivirus infection by assessing virus titers in peripheral and neuronal tissues following selective depletion of macrophages from the site of infection and from draining

LN. Depletion of macrophages prior to infection with WNV (Ben-Nathan et al., 1996; Purtha et al., 2008) resulted in periods of extended viremia with elevated levels of virus culminating in enhanced virus infiltration of the central nervous system and accelerated development of encephalitis. Similar findings were reported in a murine model of dengue virus infection (Fink et al., 2009). Macrophages are involved in the orchestration of early innate immune events as well as the activation and influence on the adaptive immune system and therefore these effects on flavivirus infection in macrophage-depleted animals may reflect loss of one or several critical functions. The use of single-cycle WNV particles allowed focusing on the role of macrophages in the initial infection as well as on downstream effects on activation of the adaptive immune response. The results of the present study extend previously reported findings (Ben-Nathan et al., 1996; Purtha et al., 2008), by demonstrating that macrophages act as a natural filter to limit virus dissemination early after initial infection. Depletion of SCS macrophages from pLN resulted in spread of the FLUC-SCFV to the spleen within the first 14 hours after infection. Additionally, luciferase bioluminescence was detected at increased levels at the FP in these mice suggesting that tissue macrophages eliminated FLUC-SCFV particles (or infected cells) and limited the infection at the site of inoculation. Although macrophages can produce type I IFN in response to infection with WNV SCFV particles, this early resistance most likely did not involve type I IFN as serum levels of IFN-α were approximately 2-fold higher in macrophage-depleted mice. These results suggest type I IFN production was not macrophage-dependent and are consistent with production of high levels of type I IFN by alternative cell types, such as plasmacytoid DC (Swiecki and Colonna, 2010). Similarly, serum levels of IL-6 and CCL2 were also higher at early time

points in macrophage-depleted animals suggesting that production of these cytokines may be driven by the higher levels of virus gene expression in CCL-treated mice. However, depletion of macrophages had no discernible effect on serum levels of IL-1β, CCL4, CCL5, IL-12p70, and TNF-α following RepliVAX WN inoculation.

Lymph which drains from body tissues enters the regional LN through the afferent lymphatics and empties initially into the SCS. Numerous macrophages have been shown to line both the subcapsular and medullary sinuses of the LN. A population of SCS macrophages expressing CD169 has been shown to play a critical role in capture and removal of virus particles from the lymph and to prevent hematogenous virus spread (Junt et al., 2007). This filtration function was shown to occur as early as 2 hpi with vesicular stomatitis virus (VSV) and was required for efficient passage of virion antigen from the SCS to LN B cells located in superficial follicles (Junt et al., 2007). Subsequent studies by Purtha et al. (2008) found that SCS macrophages were not required for early B cell activation and development of the WNV-specific antibody response. In addition to the role of SCS macrophages in the development of B cell response (Carrasco and Batista, 2007; Junt et al., 2007) and iNKT cell activation (Barral et al., 2010), recent evidences also demonstrate the function of this cell population in antigen presentation and early activation of the acquired immune response (Martinez-Pomares and Gordon, 2012). The results of the present study extend these findings by examining the requirement of SCS macrophages in activation of the WNV-specific $CD8^+$ T cell response in the draining LN. In agreement with reports by others (Kulkarni et al., 1991), both SCFV-infected macrophages and DC presented antigen to antigen-specific T cells. Importantly, these results demonstrate that SCS macrophages in the draining pLN were

not required for induction of an antigen-specific $CD8^+$ T cell response or for development of effector T cell function. This function was likely provided by DC and in this study it was further shown that both $CD11b^+$ $CD8\alpha^-$ $CD11c^+$ and $CD11b^-$ $CD8\alpha^+$ $CD11c^+$ DC subsets isolated from spleens of WNV SCFV particle inoculated mice were capable of presenting virus-encoded antigen to naïve T lymphocytes.

TLR3 and MyD88-dependent signaling pathways are important in regulating the innate immune response to WNV. TLR3 has been demonstrated to be unessential for the IFN response and control of WNV infection in cells including DC and macrophages (Daffis et al., 2009). In contrast, TLR3 signaling has been shown to limit WNV replication in the CNS (Wang et al., 2004; Kim et al., 2008). MyD88 signaling has also been associated with WNV protection, triggering the innate immune response and contributing to migration of immune cells to the site of replication (Town et al., 2009; Szretter et al., 2010). Both TLR3 and MyD88 are implicated in the regulation of adaptive immune response, although the mechanisms are still not well defined. A recent study demonstrated a lower T cell response in TLR3- and MyD88-deficient mice compared to the WT mouse strain (Xia et al., in preparation). Here, it was verified that DCs from TLR3- and MyD88-deficient mice maintain their ability to prime antigen-specific $CD8^+$ T cells, suggesting that the defects observed in T cells from $TLR3^{-/-}$ and $MyD88^{-/-}$ mice are not entirely due to the APC function of DCs. On the other hand, the lower proliferation of T cell stimulated by $MyD88^{-/-}$ DCs at 1:16-1/64 dilutions may reflect differences in antigen processing, which is in agreement with studies demonstrating that MyD88 is required for efficient cross-priming of virally infected cells (Chen et al., 2005). Also, the lower effectiveness of $MyD88^{-/-}$ DCs in APC function may influence the

diminished T cell response in this mouse strain. Another possibility is that TLR3 and MyD88 signaling pathways play an important role in maturation of lymph node homing of skin DCs.

Overall, the results of this study demonstrate that SCS macrophages play an active role in limiting dissemination of virus from the site of entry to the spleen at very early times after inoculation. Tissue macrophages act at the site of inoculation and reduce infectious load while subcapsular macrophages in the draining LN act to prevent spread through the lymphatics and ultimately into the spleen. Although SCFV-infected macrophages are capable of presenting antigen to and activating naïve $CD8^+$ T cells, SCS macrophages are apparently not essential for initiating the cell-mediated immune response or for development of effector T cell function. These results extend previous results suggesting a role for macrophages in preventing entry of WNV into the central nervous system and clarify the role for this important innate immune cell in the early stages of infection.

CHAPTER 4: Intrinsic adjuvanting of a novel single-cycle flavivirus vaccine in the absence of type I IFN receptor signaling [1]

INTRODUCTION

IFN systems are known to play important roles in innate and adaptive immune responses to microbial infection (Le Bon and Tough, 2002). Type I IFNs are induced when cells recognize viral components such as double-stranded RNA through pattern recognition receptors (PRRs) including TLRs, retinoic acid-inducible gene I (RIG-I), melanoma-differentiation-associated gene 5 (MDA5) and double-stranded RNA-activated protein kinase R (PKR) (Le Bon et al., 2001). Type I IFNs initiate signaling cascades that result in the activation of transcription factors that regulate expression of IFN-stimulated genes (ISGs) that are important for the control and elimination of viral infection. The recognition of viral components via PRRs and the subsequent induction of immune response proteins including proinflammatory cytokines and type I IFNs are also responsible for the intrinsic adjuvanting of the immune responses to live-attenuated vaccines. Type I IFNs have been shown to act as adjuvants when given in combination with antigen resulting in the enhancement of antibody responses (Le Bon et al., 2001). In addition, type I IFNs mediate the immunological effects of potent adjuvants such as complete Freund's adjuvant (Proietti et al., 2002; Prchal et al., 2009).

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[1]This chapter was previously published in Vaccine. This journal allows including information without copyright as long as it is properly cited. The citation for the article is: Winkelmann ER, Widman DG, Ishikawa T, Xia J, Miller-Kittrell M, Nelson MH, Bourne N, Scholle F, Mason PW, Milligan GN (2012). Intrinsic adjuvanting of a novel single-cycle flavivirus vaccine in the absence of type I interferon receptor signaling. Vaccine 30(8):1465-1475.

The interplay between replication of attenuated viral vaccines and the effectiveness of the IFN response is expected to be critical in determining the outcome of vaccination. Specifically, the intensity of the IFN response would be predicted to help adjuvant the adaptive immune response ensuring development of protective immunity. Conversely, IFN responses leading to strong acute antiviral activity could prematurely eliminate attenuated virus infections reducing vaccine potency and efficacy. Therefore, successful live-attenuated vaccines need to induce a balanced, but not overwhelming IFN response. In the studies presented here, the influence of type I IFNs on SCFV gene expression and development of adaptive immunity following vaccination with an SCFV vaccine candidate, RepliVAX WN, was examined to determine the role of type I IFNs in the intrinsic adjuvanticity of the RepliVAX WN vaccine.

MATERIALS AND METHODS

Viruses, SCFVs, and WNV Antigen (Ag)

RepliVAX WN was produced in BHK (VEErep/Pac-Ubi-C*) cells as previously described (Widman et al., 2008a). Firefly luciferase (FLUC)-expressing SCFV particles (FLUC-SCFV) were produced in BHK(VEErep/C*-prM-E-Pac) cells as previously described (Gilfoy et al., 2009). Titration of RepliVAX WN and FLUC-SCFV particles on Vero cells was performed as previously described (Rossi et al., 2005).

WNV SVPs used for ELISPOT and ELISA were produced by infection of Vero cells with RepliVAX WN under conditions analogous to those used to infect BHK (VEErep/Pac-Ubi-C*) (Widman et al., 2008a). Clarified cell culture supernatant containing WNV SVPs was concentrated using centrifugal filtration, and SVPs were

purified on a sucrose gradient prior to use. WNV truncated E and WNV NS1 antigens were obtained from clarified culture fluids harvested from cultures of VEErep-bearing BHK cells as previously described (Widman et al., 2008a).

Mice

C57BL/6J (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6 mice lacking the type I IFN receptor ($IFNAR^{-/-}$), were bred from founders obtained from Dr. Anthony French (Washington University, St. Louis) (Banks et al., 2005). All animal work was approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch with oversight of staff veterinarians.

Immunization of mice with RepliVAX WN

To assess cytokine levels, and WNV-specific Ab or T cell responses, mice were immunized by the intraperitoneal (i.p.) route. Mice were immunized in the footpads for *in vivo* imaging and for assessment of gene expression in draining lymph nodes. RepliVAX WN inocula were delivered in L-15 medium containing 10mM HEPES and 0.5% FBS.

WNV-specific Ab detection

Virus neutralization assays were performed as described previously (Widman et al., 2008a; 2009). Determination of WNV-specific IgG levels was performed by a modification of the ELISA method described previously (Widman et al., 2008a). Briefly, serial 2-fold dilutions of sera from individual mice were added to ELISA plates coated previously with recombinant soluble WNV E (trE), NS1 proteins, or with purified WNV SVPs. Plate-bound IgG was developed with HRP-IgG (Southern Biotech) or with

biotinylated anti-mouse IgG1 or IgG2c (BD Pharmingen, San Diego, CA) followed by incubation with streptavidin peroxidase (Sigma-Aldrich, St. Louis, MO). Normalized OD readings at 450nm (OD450) obtained from the serial dilution studies were subjected to non-linear regression analyses to calculate the serum dilution equivalent to three standard deviations above OD450 values from sera obtained from mock-vaccinated animals.

Interferon and cytokine detection

IFN-α and IFN-β ELISAs (PBL Biomedical Laboratories, Piscataway, NJ) were performed using the manufacturer's protocols. Briefly, experimental sera were diluted, plated and incubated for 1 hour (h) at 37°C. Samples were washed and bound IFN was detected by addition of mouse IFN-specific Ab followed by an HRP-conjugated secondary Ab and measurement of the OD_{450}. IFN levels were calculated using a standard curve generated from serial dilutions of an IFN standard in dilution buffer containing normal mouse sera as previously described (Bourne et al., 2007). The limit of detection was 12.5 pg/ml for IFN-α and 15.6 pg/ml for IFN-β. Additional cytokine levels in the pooled sera of inoculated mice were determined using a luminescence-based multiplex bead assay (Bio-Rad, Hercules, CA) from a panel of 23 cytokines following the manufacturer's protocols.

RT-PCR analyses

Popliteal LN were harvested from vaccinated mice and stored overnight at 4°C in RNALater (Ambion, Austin, TX). Total LN RNA was isolated and DNase-treated using the RNAqueous 4 PCR Kit (Ambion) and then used to synthesize cDNA with the RT2 First Strand Kit (SABiosciences, Frederick, MD). Cytokine mRNA levels were determined at the indicated times by real-time PCR using a SYBR Green-based custom

PCR array (SABiosciences) and thermocycler settings recommended for use with a BioRad iCycler. Cytokine levels of RepliVAX WN treated mice (3 mice/group) were represented as fold-change over mock-infected animals (2 mice/group).

Enzyme-linked immunospot assay (ELISPOT)

ELISPOT assays for antibody secreting cells (ASC) were performed as described previously (Nelson et al., 2010) using microtiter filter plates coated with WNV NS1 or SVP Ag. Ag-specific ASC were quantified using an ImmunoSpot reader and analyzed with ImmunoSpot software (Cellular Technology Ltd, Cleveland, OH).

For IFN-γ ELISPOT assays, splenocytes from RepliVAX WN-immunized or mock-infected mice were plated on filter plates coated with purified anti-mouse IFN-γ (BD Pharmingen) and stimulated with immunogenic peptides representing WNV $CD4^+$ and $CD8^+$ T cell epitopes as described previously (Nelson et al., 2010). Immunogenic peptides representing the $CD8^+$ T cell epitopes located at NS4B protein residues 2488-2496 ($NS4B_{2488}$), E protein residues 347-354 (E_{347}) and the $CD4^+$ epitopes located at NS3 protein residues 2066-2080 ($NS3_{2066}$), and E protein residues 641-655 (E_{641}) have been described previously (Purtha et al., 2007; Brien et al., 2007b; 2008). Plates were developed with biotinylated anti-mouse IFN-γ (BD Pharmingen) and streptavidin-peroxidase (Sigma-Aldrich) and were quantified as described above for ASC ELISPOTs.

***In vivo* cytotoxic T lymphocyte assay**

WNV NS4B-specific cytotoxic T lymphocyte activity in RepliVAX WN-immunized mice was quantified by injection of CFSE-labeled target cells as described previously (Nelson et al., 2010). Four h after injection, $NS4B_{2488}$-pulsed and mock-pulsed CFSE-labeled target populations were quantified using a BD FACSCanto (BD

Biosciences, San Jose, CA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR). The percent specific lysis was calculated as described previously (Nelson et al., 2010).

Intracellular cytokine staining

Whole spleens from B6 or IFNAR$^{-/-}$ mice taken on day 6 after RepliVAX WN vaccination were re-stimulated with 1μM NS4B$_{2488}$ peptide for 2 h followed by 6 h incubation with brefeldin A. Cells were blocked with anti-Fc RII/III mAb and surface stained with anti-CD8α (APC) and CD3 (PerCPCy5.5) then permeabilized using a Cytofix/Cytoperm kit and subsequently stained intracellularly with fluorochrome-conjugated mAb for IFN-γ (FITC), IL-2 (PE) and TNF-α (PE-Cy7). All reagents were purchased from BD Pharmingen. CD8$^+$, CD3$^+$ T cells were segregated into distinct populations based on the production of IFN-γ, IL-2 or TNF-α either individually or in combination. No cytokines were detected in cultures re-stimulated with an irrelevant peptide (gB$_{498-505}$), in the absence of NS4B$_{2488}$ peptide, or in T cells from naïve mice. Data were acquired on a BD LSRII Fortessa (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star). The total number of splenocytes secreting IFN-γ, IFN-γ + TNF-α, or IFN-γ + TNF-α + IL-2 was derived by multiplying the % of cells secreting a particular cytokine combination by the total number of viable splenocytes.

***In vivo* imaging (IVIS)**

The posterior half of all animals was shaven prior to immunization with FLUC-SCFV. At 14, 24, 48, 72, 96 h and 168 h following immunization, animals were injected i.p. with D-luciferin (Caliper LS) in a solution of PBS corresponding to a dose of 0.15

mg/g body weight. After allowing 20 min for dissemination of D-luciferin, animals were anesthetized with ketamine and xylazine, and real-time *in vivo* imaging was performed using a Xenogen IVIS 200 (Caliper LS, Hopkinton, MA) at medium binning with exposure times ranging from 1-90 sec. Images were analyzed by drawing regions of interest around visible sites of FLUC activity and measuring total flux (photons per second; p/sec) and data were acquired using Living Image 4.0 software (Caliper LS). Reported FP averages are the average total flux from FP from all animals in a treatment group.

Statistical analyses

Statistical differences for T and B lymphocyte assays and IFN levels were determined using Student *t* test (unpaired) or ANOVA with the Tukey or Bonferoni post test as appropriate. Values for $p<0.05$ were considered significant. All calculations were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

RESULTS

Type I IFN and cytokine responses to RepliVAX WN

To test if immunization with the SCFV particle RepliVAX WN resulted in production of type I IFNs and IFN-dependent proinflammatory molecules, groups of B6 and IFNAR$^{-/-}$ mice were immunized with RepliVAX WN and serum was collected at 8, 24, and 48 h for detection and quantification of IFN-α and IFN-β. IFN-α was detected at 8 hpi in RepliVAX WN-inoculated B6 mice but not IFNAR$^{-/-}$ mice (Fig. 4.1A). Peak IFN-α levels were detected at 24 h in both mouse strains and fell to low levels by 48 hpi. IFN-α levels observed in RepliVAX WN-immunized B6 mice were significantly higher

than in IFNAR$^{-/-}$ mice ($p<0.05$, Student *t* test) at these three time points. IFN-β was not detected in sera obtained from either strain of mice at any time following immunization (data not shown), consistent with previous studies using a similar SCFV (Bourne et al., 2007).

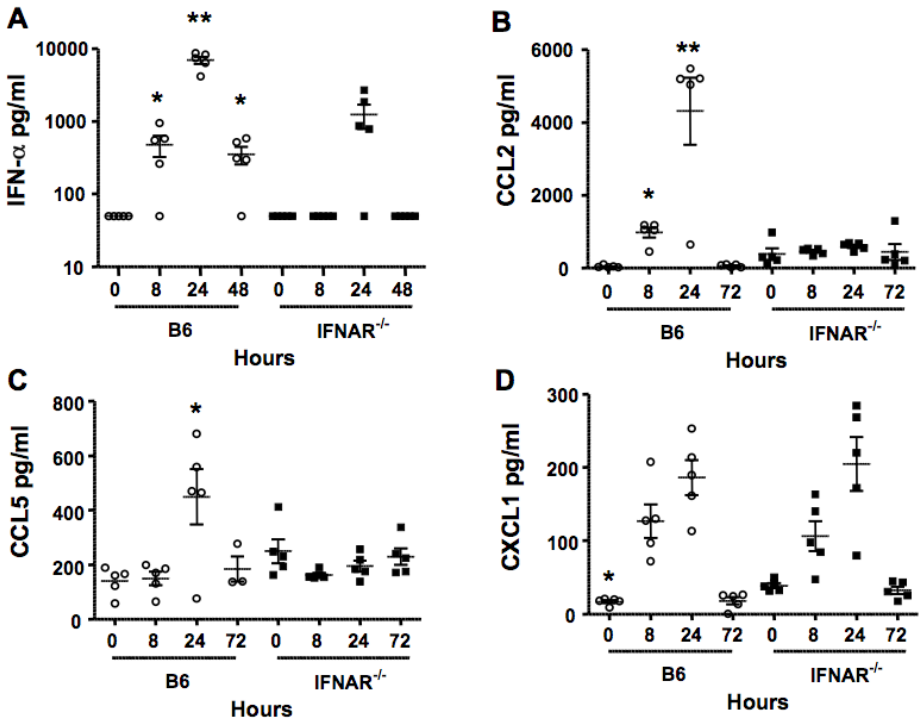

Figure 4.1. IFN-α and chemokine responses to RepliVAX WN immunization. Groups of 5 B6 and IFNAR$^{-/-}$ mice were immunized i.p. with 3.0×10^7 IU of RepliVAX WN and serum was collected at the indicted times. The IFN-α concentration (A) was determined by ELISA (*$p<0.03$, **$p<0.0003$ compared to same time points for B6-IFNAR$^{-/-}$ mice). The limit of detection for IFN-α was 12.5 pg/ml. The concentration of CCL2 (B) (*$p<0.007$, **$p<0.0005$ compared to same time points for IFNAR$^{-/-}$ mice), CCL5 (C) (*$p<0.05$ compared to same time point for IFNAR$^{-/-}$ mice), and CXCL1 (D) (*$p<0.006$ compared to same time point for IFNAR$^{-/-}$ mice) were measured by a luminescence-based multiplex bead assay. Results are representative of 2 separate experiments. The limits of detection for CCL2, CCL5, and CXCL1 were 14.0, 5.0, and 3.0 pg/ml, respectively.

Signaling through the type I IFN receptor culminates in the transcription of a number of ISGs and in the expression of a number of immunologically active proteins

such as chemokines and pro-inflammatory cytokines. Following immunization with RepliVAX WN, increased expression of the chemokines CCL2 (MCP-1) (Fig. 4.1B) and CCL5 (RANTES) (Fig. 4.1C) was detected in the serum of B6 but not in IFNAR$^{-/-}$ mice. These results are consistent with previous reports that type I IFNs induce expression of these chemokines (Cremer et al., 2002; Jia et al., 2009). As with IFN-α, these chemokines were only transiently detected, with peak levels observed at 24 h. By contrast, CXCL1 (KC) was induced by RepliVAX WN immunization independently of type I IFN receptor status, since both B6 and IFNAR$^{-/-}$ mice showed similar kinetics of induction over a 72 h period (Fig. 4.1D). To further examine the results of type I IFN receptor signaling following RepliVAX WN inoculation, the transcription of a number of cytokine and chemokine genes in the draining LN following FP inoculation was assessed. As expected due to the well-known autocrine signal amplification through the IFN receptor (Malmgaard et al., 2002), B6 mice exhibited a much higher level of induction of type I IFN, cytokines, and ISGs than did IFNAR$^{-/-}$ mice at both 12 h and 24 hpi (Fig. 2.2). An interesting exception was the induction of the IL-1β gene which exhibited a greater than 50-fold increase over baseline at both time points in IFNAR$^{-/-}$ mice but was unchanged in B6 controls. These data are consistent with the recent report that type I IFN signaling inhibits both pro-IL-1β transcription and the activity of the cellular machinery (NLRP1 and NLRP3 inflammasomes) necessary to process pro-IL-1β into mature IL-1β (Guarda et al., 2011). Taken together, these results demonstrate that immunization with SCFVs results in type I IFN production, initiation of downstream IFN-stimulated gene pathways, and induction of specific cytokines and chemokines.

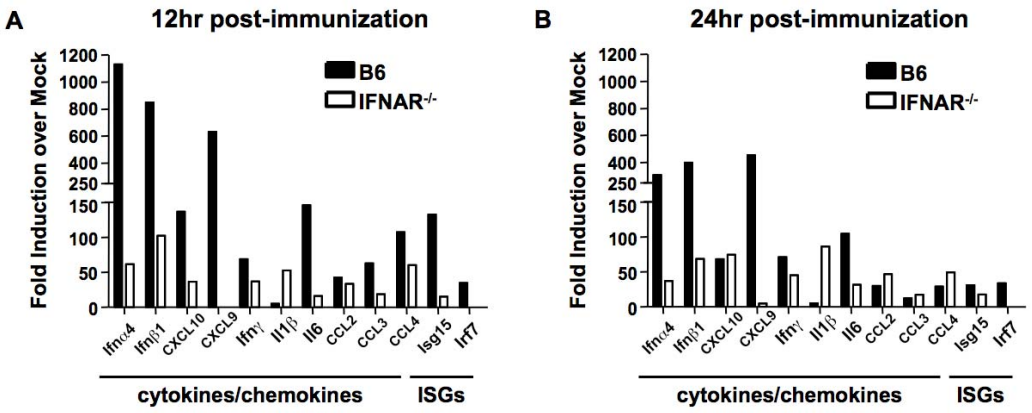

Figure 4.2. Relative cytokine expression in draining LN following RepliVAX WN immunization. B6 and IFNAR$^{-/-}$ were immunized subcutaneously in the rear FP with 2.0×10^7 IU RepliVAX WN and popliteal LN were removed for PCR analysis. Cytokine gene expression profiles from cDNA from draining LN harvested at 12 h (left) and 24 hpi (right). Values represent fold increases over mock-immunized animals. For immunized animals n=3, for mock-immunized animals n=2.

Type I IFN responses limit SCFV gene expression

The binding of type I IFNs to the type I IFN receptor results in induction of several antiviral gene programs that inhibit viral gene expression and limit virus spread. While these responses are important for protection against pathogenic organisms, it is possible that they may also limit the development of protective adaptive immune responses following immunization with live-attenuated viral vaccines. Using a firefly luciferase-expressing SCFV, FLUC-SCFV, it was verified if the type I IFN responses elicited by SCFV inoculation inhibited SCFV gene expression or limited the tissue distribution of FLUC-SCFV-infected cells. Groups of B6 or IFNAR$^{-/-}$ mice were inoculated in the FP with FLUC-SCFV and imaged at intervals between 14 and 168 hpi. SCFV-encoded gene expression was readily detected in FP tissue of FLUC-SCFV-infected mice (Fig. 4.3A), but not uninfected mice (Fig. 4.3B) as early as 14 hpi. Additionally, transient FLUC bioluminescence was frequently observed in draining pLN

at 14 and 24 hpi (Fig. 4.3A), but rarely at 48 or 72 hpi and never after 96 hpi (data not shown). In contrast to gene expression in the LN, FLUC-SCFV gene expression was sustained at the inoculation site with FLUC bioluminescence detectable for over 4 d after SCFV inoculation (Fig. 4.3C). FLUC intensity was over 100-fold higher in the FP of IFNAR$^{-/-}$ mice compared to B6 mice ($p<0.001$, Student *t* test) between days one and three post-inoculation (please note different scales in the various photographs in Fig 4.3A and B). The FLUC signal then rapidly decreased in IFNAR$^{-/-}$ mice and from day 4 FLUC bioluminescence levels decayed in a manner similar to the luminescence in WT animals (Fig. 4.3C).

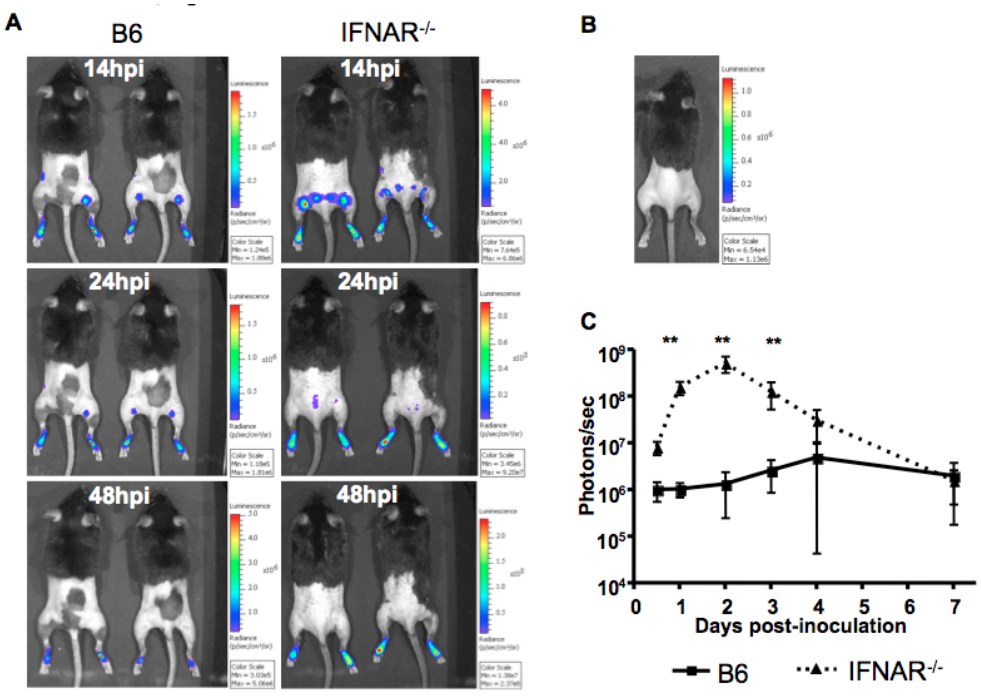

Figure 4.3. Magnitude of SCFV gene expression in vivo in B6 and IFNAR$^{-/-}$ mice inoculated with FLUC-SCFV. Groups of B6 or IFNAR$^{-/-}$ mice were inoculated s.c. in both rear FP with 1×10^7 IU FLUC-SCFV and imaged at the indicated time points. Bioluminescence was observed using up to 1.5 min exposures at medium binning, was analyzed by measuring total flux emanating from each FP, and is reported as the average over time of all FP from each mouse genotype. Please note different scales in the various photographs in (A) and (B). n=4 mice per genotype. Error bars represent standard deviation. ***$p<0.001$.

Type I IFN receptor signaling influences development of adaptive immunity to RepliVAX WN

Type I IFN has been shown to influence the magnitude and effector function of developing adaptive immune responses (Le Bon et al., 2001; Jego et al., 2003; Curtsinger et al., 2005; Havenar-Daughton et al., 2006). To determine if signaling via the type I IFN receptor played a critical role in the intrinsic adjuvanting of the SCFV vaccine, groups of B6 and IFNAR$^{-/-}$ mice were immunized with RepliVAX WN. Mice were bled at 7 dpi to test for early influences of type I IFNs on antibody production. Both strains of mice produced NS1-specific (Fig. 4.4A) and SVP-reactive antibodies (Fig. 4.4B) in response to immunization. B6 mice produced significantly greater titers of serum IgM but similar levels of serum IgG specific for both WNV antigens compared to IFNAR$^{-/-}$ mice ($p<0.01$, Student *t* test). Type I IFN has been reported to influence class switching and IgG subclass expression of developing antibody responses (Coro et al., 2006). To test this, the expression of IgG1 and IgG2c by WNV-specific serum antibodies in RepliVAX WN-immunized B6 and IFNAR$^{-/-}$ mice was examined (Fig 4.4C-E). Strong serum IgG responses reactive with 3 different WNV antigens were induced in both mouse strains. RepliVAX WN-immune IFNAR$^{-/-}$ mice generally produced more IgG1 antibody than B6 mice but the difference only reached significance for the NS1 antigen ($p<0.05$, ANOVA). Both mouse strains produced predominantly IgG2c subclass antibody. However, B6 mice produced significantly more WNV E antigen-specific IgG2c antibody than IFNAR$^{-/-}$ mice ($p<0.05$, ANOVA, Fig. 4.4E). The functional activity of serum antibodies also did not differ between the strains of mice. The 90% neutralization titers for B6 and IFNAR$^{-/-}$ mice were 1:160 and 1:80, respectively, on day 21 dpi.

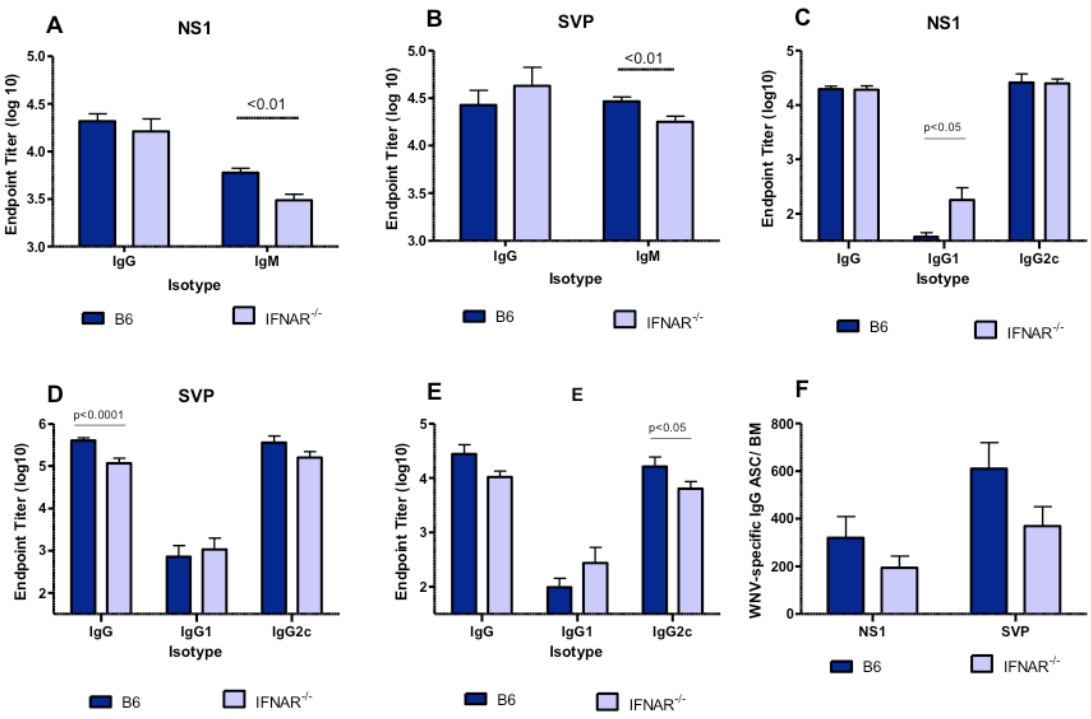

Figure 4.4. Effect of type I IFN receptor signaling on WNV-specific Ab responses following immunization with RepliVAX WN. Groups of IFNAR$^{-/-}$ and B6 mice were immunized with 10^6 IU RepliVAX WN i.p. and the endpoint titers on day 7 post immunization for serum IgG and IgM antibody specific for WNV NS1 (A) or WNV SVP (B) were determined for individual mice. Results are pooled from 2 experiments (n=15 mice/group for IgG, n=20 mice/group for IgM). Endpoint titers of IgG, IgG1, and IgG2c Ab specific for WNV NS1 (C), SVP (D), or E proteins (E) were determined by ELISA from 21 and 28 day samples. Results are pooled from 2 experiments (n=10 mice/group). (F) WNV NS1 and SVP-specific antibody secreting cell response in the bone marrow of RepliVAX WN immunized mice. Groups of 5 IFNAR$^{-/-}$ and B6 mice were immunized with 10^6 IU RepliVAX WN i.p. On day 28, cells were harvested from the femurs of immunized mice and analyzed by ELISPOT for IgG ASC specific for WNV NS1 or SVPs. Data are presented as the mean ± standard error of the mean (SEM) of WNV-specific IgG-producing cells per bone marrow from individual mice. The results shown are from a representative experiment of 2 performed.

Long-lived plasma cells in the bone marrow are thought to serve as the major, long-term source of antigen-specific IgG Ab in serum (Slifka et al., 1995). To test if the absence of type I IFN signaling would alter development of this cell subset, bone marrow cells from femurs were harvested 28 d after RepliVAX WN immunization and quantified

for the total NS1- and SVP-specific IgG ASC by ELISPOT (Fig. 4.4F). The number of NS1-specific and SVP-specific IgG ASC was lower in IFNAR$^{-/-}$ mice compared to B6 mice; however, in neither case did the difference reach significance.

Cell-mediated immune responses to RepliVAX WN in the absence of type I IFN receptor signaling

Type I IFNs have been reported to play an important role in determining the magnitude and function of the T cell responses to many different pathogens (Kolumam et al., 2005; Havenar-Daughton et al., 2006; Thompson et al., 2006). To determine if type I IFN receptor signaling was required for development of the vaccine-induced T lymphocyte response, groups of B6 and IFNAR$^{-/-}$ mice were immunized with RepliVAX WN and assessed for the magnitude and effector function of the WNV-specific T cell responses.

Vigorous CD4^{+} T cell responses to 2 immunodominant CD4^{+} T cell epitopes (Brien et al., 2008) were observed in both RepliVAX WN-immunized IFNAR$^{-/-}$ and B6 mice (Fig. 4.5A). Consistent with previous results (Nelson et al., 2010), the IFN-γ secreting cell response of splenocytes from immunized B6 mice to the $NS3_{2066}$ peptide was of greater magnitude than the response to the E_{641} epitope on both days 9 and 14. A similar response pattern to these epitopes was observed in RepliVAX-immunized IFNAR$^{-/-}$ mice. Interestingly, the $NS3_{2066}$-specific response in IFNAR$^{-/-}$ mice was significantly higher than the response of B6 mice on day 14 post-immunization ($p<0.02$, Student *t* test) suggesting a more prolonged response to this epitope whereas the response to the E_{641} epitope in B6 and IFNAR$^{-/-}$ mice was of similar magnitude on both days 9 and 14.

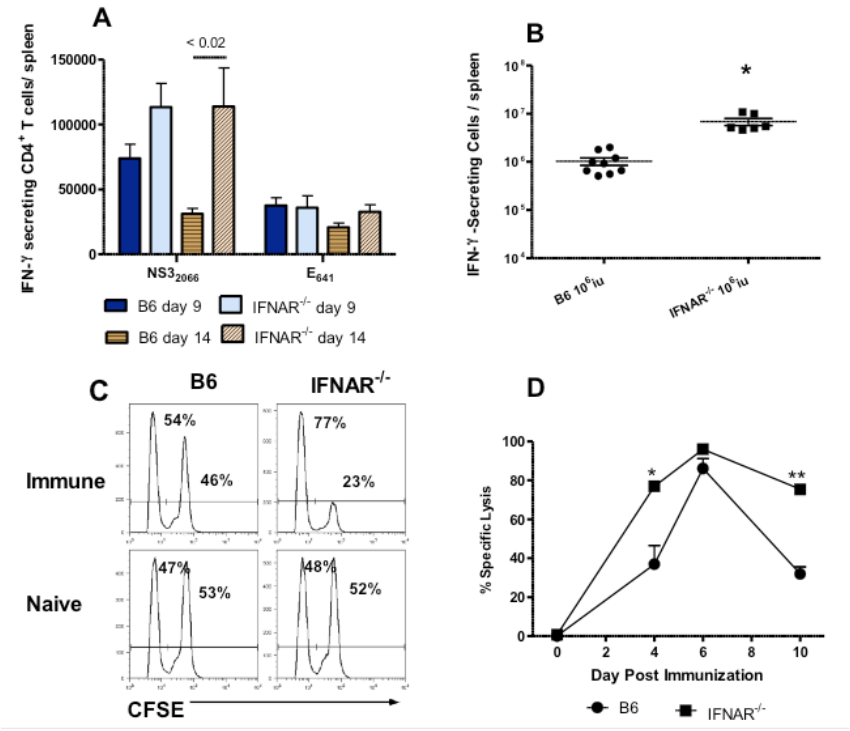

Figure 4.5. Type I IFN receptor signaling is not required for the intrinsic adjuvanting of T cell responses against RepliVAX WN. (A) $CD4^+$ T cell response. B6 or $IFNAR^{-/-}$ mice were immunized with 10^6 IU RepliVAX WN and the number of IFN-γ-secreting cells specific for the $NS3_{2066}$ or E_{641} $CD4^+$ T cell epitopes were quantified by ELISPOT on days 9 and 14. Results are expressed as the number of IFN-γ-secreting cells/spleen (mean ± SEM) and are compiled from 2 separate experiments (n=10–12 mice/group). (B) $CD8^+$ T cell responses to the $NS4B_{2488}$ epitope were quantified by detection of intracellular IFN-γ by flow cytometry on day 6. Results are expressed as the number of IFN-γ secreting cells/spleen (mean ± SEM) and are compiled from 2 separate experiments (n=6–9 mice/group). (C) Representative histograms of CFSE-labeled target cells in spleens of naïve and RepliVAX WN-immunized B6 and $IFNAR^{-/-}$ mice 4 days after immunization. Numbers in each histogram represent the % No-peptide targets ($CFSE_{low}$) and % $NS4B_{2488}$ peptide-pulsed targets ($CFSE_{hi}$) for an individual animal. (D) Cytotoxic $CD8^+$ T lymphocyte activity from B6 and $IFNAR^{-/-}$ mice after RepliVAX WN immunization. The percentages of $CFSE_{low}$ and $CFSE_{hi}$ targets derived as shown in (C), were used to calculate a % specific lysis as described previously [18]. Results are expressed as the % specific lysis (mean ± SEM) from 5 mice/group for $NS4B_{2488}$-coated targets (*$p<0.005$, **$p<0.0001$ compared to RepliVAX WN-immunized B6 mice). Naïve mice routinely exhibited <2% specific lysis.

The magnitude of the effector $CD8^+$ T cell response was measured by quantification of IFN-γ-producing cells using intracellular cytokine staining and flow cytometry. Strong $CD8^+$ T cell responses to the immunodominant epitope $NS4B_{2488}$ were observed on day 6 post-immunization in both B6 and $IFNAR^{-/-}$ mice (Fig. 4.5B). However, the IFN-γ producing cell response was significantly higher in $IFNAR^{-/-}$ mice ($p<0.0001$, Student *t* test) representing a greater than 6-fold increase in the total number of WNV NS4B-specific, IFN-γ-producing cells. The effector function of $CD8^+$ T cells of RepliVAX WN-immunized B6 and $IFNAR^{-/-}$ mice was further assessed by measuring the *in vivo* cytotoxicity response against the immunodominant $NS4B_{2488}$ epitope (Fig. 4.5C, D). High levels of WNV NS4B-specific cytotoxicity were observed in both strains of mice at the peak of the response on day 6. However, consistent with the significantly greater IFN-γ response of $IFNAR^{-/-}$ mice (Fig. 4.5B), the percent specific lysis of $NS4B_{2488}$-pulsed target cells in $IFNAR^{-/-}$ mice was significantly higher on days 4 ($p<0.005$, Student *t* test), and 10 ($p<0.0001$, Student *t* test) compared to B6 mice, indicating a more rapid and prolonged $CD8^+$ T cell response.

To determine if the quality of the $CD8^+$ effector T cell response would be altered in the absence of IFNAR signaling, the cytokine production profile of WNV NS4B-specific T cells induced by RepliVAX WN was examined. Given the significantly greater increase in SCFV gene expression in $IFNAR^{-/-}$ mice (Fig. 4.3), it was considered that increased antigen load in these mice, rather than the lack of IFNAR signaling, may be responsible for any influence in cytokine production patterns. To control for this possibility, $IFNAR^{-/-}$ mice were inoculated with SCFV at a dose either representing an equivalent number of RepliVAX WN particles used to immunize B6 mice or with a dose

that would result in equivalent SCFV gene expression relative to inoculated B6 mice. To determine the dose of SCFV that would result in equivalent gene expression in IFNAR$^{-/-}$ mice relative to B6 mice, B6 mice were inoculated with a full dose of FLUC-SCFV particles and IFNAR$^{-/-}$ mice were inoculated with a series of dilutions (1/8, 1/64, 1/512) of the same FLUC-SCFV inoculum. FLUC-SCFV gene expression was visualized and quantified by IVIS analysis on days 1-4 (Fig. 4.6A). Over the first three days post inoculation, gene expression in IFNAR$^{-/-}$ mice that received a 1/512 dilution of FLUC-SCFV most closely approximated, and was not different from gene expression in B6 mice that received the full FLUC-SCFV dose. Because dendritic cells isolated from SCFV inoculated mice are already loaded with SCFV antigen by day three post immunization (Winkelmann et al., 2014), a dose of 2×10^3 IU RepliVAX WN (1:500 dilution of SCFV inoculum) was utilized in IFNAR$^{-/-}$ mice to approximate the gene expression and antigen load resulting from inoculation of B6 mice with 10^6 IU RepliVAX WN in subsequent experiments. Fig. 4.6B shows the percentage of NS4B-specific CD8$^+$ T cells from low- or high-dose RepliVAX WN immunized mice which produced IFN-γ alone or simultaneously produced IFN-γ + TNF-α or IFN-γ + TNF-α + IL-2. Overall, the cytokine production pattern associated best with the presence or absence of IFNAR signaling rather than with antigen load. Specifically, the percentage of cells secreting IFN-γ alone was significantly higher ($p<0.0001$, ANOVA) in IFNAR$^{-/-}$ mice immunized with either a high or low SCFV dose compared to B6 mice while the percentage of cells secreting IFN-γ + TNF-α was significantly lower in these mice ($p<0.0001$, ANOVA). A similar fraction of NS4B$_{2488}$-specific CD8+ T cells from B6 and IFNAR$^{-/-}$ cells simultaneously produced IFN-γ, TNF-α and IL-2 regardless of immunizing SCFV dose or mouse strain.

As expected from Fig 4.5B, the total number of $CD8^+$ T cells secreting IFN-γ, IFN-γ + TNF-α, or IFN-γ + TNF-α + IL-2 (Fig. 4.6C) was significantly higher in high dose $IFNAR^{-/-}$ mice compared to B6 mice or $IFNAR^{-/-}$ mice immunized with a low dose of RepliVAX WN ($p<0.0001$, ANOVA).

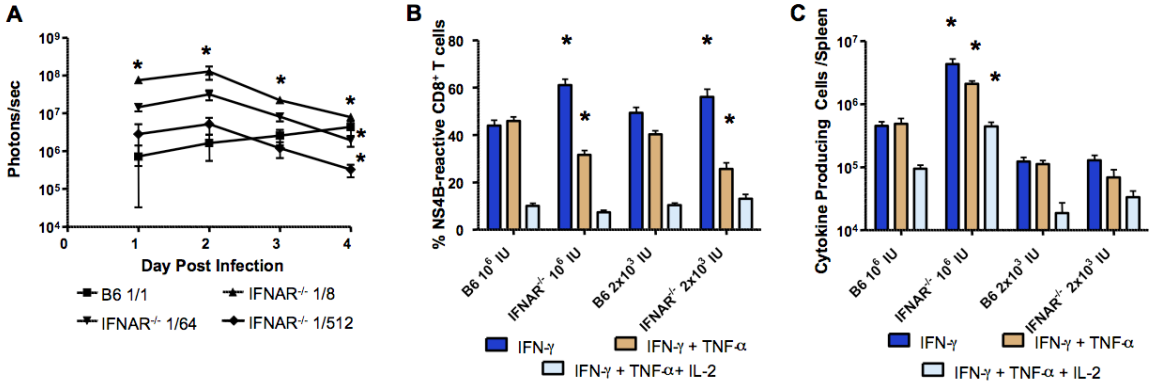

Figure 4.6. Cytokine production by WNV NS4B-specific $CD8^+$ T cells following RepliVAX immunization of B6 and $IFNAR^{-/-}$ mice. (A) SCFV gene expression in $IFNAR^{-/-}$ mice compared to B6 mice. B6 mice were inoculated in the FP with 10^7 IU FLUC-SCFV. $IFNAR^{-/-}$ mice received a 1:8 (1.25×10^6 IU), 1/64 (1.56×10^5 IU), or 1/512 (1.95×10^4 IU) dilution of the same inoculum. Mice were imaged at the indicated time points. Bioluminescence was observed using up to 1.5 min exposures at medium binning followed by measuring total flux emanating from each FP, and is reported as the average over time of all FP from each group (n=4 mice/group) (*$p<0.01$, ANOVA). (B) Whole splenocyte populations were harvested from B6 or $IFNAR^{-/-}$ mice on day 6 after primary RepliVAX WN vaccination and re-stimulated with $NS4B_{2488}$ peptide prior to characterization by multiparameter flow cytometry. The results shown are compiled from 4 separate experiments (n=3 to 9 mice/group). (C) The number of total cells per spleen secreting IFN-γ, IFN-γ + TNF-α, or IFN-γ + TNF-α + IL-2 from the mice described in (B) was derived by multiplying the percent of cells secreting a particular cytokine combination by the total number of viable splenocytes.

Together, these results suggest that antigen dose affected the magnitude of the $CD8^+$ T cell response whereas IFNAR signaling influenced the cytokine production pattern of WNV-specific $CD8^+$ effector T cells. Interestingly, the loss of IFNAR

signaling apparently did not influence all effector functions as high levels of WNV-specific cytotoxicity were detected in IFNAR$^{-/-}$ mice.

Type I IFNs have been reported to play an important role in development of memory T cell responses to several pathogens (Kolumam et al., 2005; Thompson et al., 2006). To test if the development of WNV-specific memory T cell responses to a SCFV particle required type I IFN activities, groups of B6 or IFNAR$^{-/-}$ mice were immunized with RepliVAX WN and quantified for WNV-specific memory T cells in the spleen 56 days post-immunization. As shown in Fig. 4.7, CD8^{+} memory T cells specific for the NS4B$_{2488}$ and E$_{347}$ epitopes were present at significantly higher levels in IFNAR$^{-/-}$ mice compared to B6 mice ($p<0.001$, Student *t* test). Similarly, development of the RepliVAX WN-induced CD4^{+} memory T cell did not require signals generated through the type I IFN receptor as comparable numbers of memory T cells specific for the CD4^{+} T cell epitopes NS3$_{2066}$ and E$_{641}$ were detected in both strains of mice. Although greater numbers of CD4^{+} memory T cells were detected in IFNAR$^{-/-}$ mice compared to B6 controls, the difference did not reach significance.

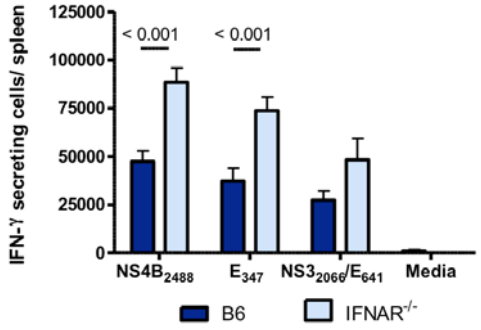

Figure 4.7. CD8^{+} and CD4^{+} memory T cell response in B6 and IFNAR$^{-/-}$ mice 8 weeks following RepliVAX WN immunization. Groups of mice were immunized with RepliVAX WN and splenocytes removed after 8 weeks for quantification of WNV-epitope-specific T cells by ELISPOT. Results are from a representative experiment of 2 performed (n=5/group/experiment).

DISCUSSION

The limited infections established by SCFVs make them ideal tools for examining the relative influence of the antiviral and adjuvanting aspects of the type I IFN response following immunization. Specifically, SCFVs can efficiently replicate their RNA genomes within cells thereby activating the intrinsic IFN induction system from these initially infected cells, yet their inability to produce progeny virions prevent them from producing a lethal systemic disease in immunocompromised animals. RepliVAX WN infection is expected to produce PRR ligands that bind to multiple PRRs including TLR3 (Wang et al., 2004; Daffis et al., 2008), TLR7 (Fredericksen et al., 2008; Town et al., 2009), the cytoplasmic helicases RIG I and MDA5 (Fredericksen et al., 2008), or PKR (Randall and Goodbourn, 2008) leading to the production of type I IFNs. In addition to establishing an antiviral environment in virus-infected and surrounding cells, type I IFNs have also been shown to influence development of the adaptive immune response (Marrack et al., 1999; Le Bon et al., 2001; Jego et al., 2003; Curtsinger et al., 2005; Havenar-Daughton et al., 2006) and have been shown to act as an adjuvant when injected in combination with protein antigens or commercial influenza vaccines (Le Bon et al., 2001; Proietti et al., 2002). Moreover, type I IFNs have been shown to be responsible for the adjuvanting activity of both complete and incomplete Freund's adjuvants and for the TLR9-dependent adjuvant, IC31 (Proietti et al., 2002; Prchal et al., 2009). Thus, following immunization with live-attenuated viral vaccines such as RepliVAX WN, type I IFNs may potentially negatively influence the development of adaptive immune responses by diminishing the availability of antigen through inhibition of viral gene expression in infected cells. Alternatively, they may positively influence the adaptive

immune response by directly mediating the intrinsic adjuvanting of the vaccine. The current studies examined the influence of the host type I IFN system on RepliVAX WN infection and gene expression, and ultimately how this interaction between host response and viral infection shaped the adaptive immune response to RepliVAX WN immunization.

Infection of B6 mice with RepliVAX WN was sufficient to induce production of type I IFNs, which ultimately influenced the quantity and composition of immunologically active molecules produced by immunized mice. Analysis of cytokine, chemokine, and ISG expression in the LN of RepliVAX WN-immunized mice revealed differences between B6 and IFNAR$^{-/-}$ strains, with the knockout mice displaying lower levels of gene induction for type I and II IFN, CXCL9, and IL-6. Interestingly, genes for other chemokines (CXCL10, CCL2, CCL3, CCL4) were induced to greater levels in the LN of B6 mice at 12 hpi, but by 24 hpi these levels were equivalent in wt and IFNAR$^{-/-}$ mice. Taken together, these results demonstrate that type I IFNs are important for defining the milieu of immune molecules induced by RepliVAX WN immunization, which potentially influences the development and mobilization of innate and adaptive immune responses. The ability to initiate an innate response, including type I IFNs, and subsequent adaptive immune response in the absence of prolonged viral replication may also be important for limiting vaccine reactogenicity. Early immunization studies with low doses of live attenuated flaviviruses resulted in reactogenicity due to viremia resulting from inadequate initial innate immune responses (Sanchez et al., 2006). The single-cycle nature of the RepliVAX WN immunization thus allows immunization over a

range of doses to maximize vaccine efficacy while preventing reactogenicity that could result from vaccine virus replication.

To evaluate the role of type I IFNs in controlling expression of antigen by RepliVAX WN, *in vivo* imaging experiments of animals inoculated with FLUC-SCFVs were performed to observe the distribution and magnitude of SCFV infection in the presence or absence of IFNAR signaling. These studies revealed an approximately 100-fold increase in FLUC gene expression in $IFNAR^{-/-}$ mice from days 1-3 post-immunization, demonstrating that type I IFN played an important role in controlling SCFV gene expression. It was further observed that FLUC-SCFV-infected cells produced a robust signal at the site of inoculation, while infected cells were observed transiently in the draining popliteal LN and occasionally in more distal LNs at later times regardless of the ability of mice to respond to type I IFNs. These results confirm previous finding that WNV antigen and genomes were detected in the draining LN of mice after SCFV infection (Bourne et al., 2007). These studies further suggest that SCFVs infect a population of cells that migrate from the site of inoculation through the lymphatic system to the draining LN, and/or that the SCFV particles themselves are transported via lymph to the draining LN where they infect resident cells. The IVIS studies also revealed that SCFV gene expression was maintained for several days at the inoculation site. Since soluble Ags (SVPs and NS1) released from cells at the inoculation site could also traffic to lymphoid tissues and contribute to immune responses, it is impossible to determine the relative importance of SCFV-infected cells within the draining LN and the inoculation sites in the development of the immune responses elicited by SCFV vaccines.

Type I IFNs have been shown to exert a range of actions that directly affect the magnitude and quality of the Ab response. They may act on B cells either directly or indirectly through the activation of dendritic cells early in the response to viral infection to enhance B cell expansion and induction of activation markers (Braun et al., 2002; Coro et al., 2006; Purtha et al., 2008). Type I IFNs have also been shown to protect B cells from apoptosis *in vitro* (Braun et al., 2002). Consistent with these findings, early Ab responses to influenza (Coro et al., 2006) and VSV (Fink et al., 2006) infections in mice have been shown to be diminished in the absence of type I IFN receptor signaling. In the current studies, the lack of type I IFN receptor signaling resulted in reduced early (day 7) serum IgM responses to RepliVAX WN immunization but had little effect on the IgG antibody response at any time point measured. While type I IFNs have been reported to influence IgG isotype expression (Le Bon et al., 2001), the lack of type I IFN receptor signaling had only modest effect on the magnitude or subclass expression in the current studies. IgG1 titers against WNV antigens were generally higher in IFNAR$^{-/-}$ mice; however they were significantly higher than IgG1 titers in B6 mice only for WNV NS1-specific antibodies. The IgG Ab response to RepliVAX WN-immunization was predominated by IgG2c subclass Ab in both strains of mice. Together, these results could be interpreted to indicate that type I IFNs were not required for the development of a WNV-specific IgG antibody response following immunization with RepliVAX WN. However, this interpretation is confounded by the finding that SCFV gene activity in IFNAR$^{-/-}$ mice was over 100 times greater over a 3 day period compared to wt mice. It remains possible that the limited effects that loss of type I IFN signaling had on B cell

responses in IFNAR$^{-/-}$ mice in the current studies might be a result of increased SCFV gene expression and thus high antigen loads in IFNAR$^{-/-}$ mice compared to B6 mice.

Induction of the RepliVAX WN-induced CD4^{+} and CD8^{+} T cell responses did not require signaling through the type I IFN receptor suggesting that type I IFNs do not mediate the intrinsic adjuvanting of RepliVAX WN. These results are in contrast to reports of deficient CD4^{+} T cell responses in IFNAR$^{-/-}$ mice due to lack of type I IFN survival signals for virus-specific CD4^{+} T cells (Havenar-Daughton et al., 2006). Interestingly, the reported strict requirement for type I IFN for ensuring clonal expansion of CD4^{+} T cells has been shown to be pathogen-dependent (Havenar-Daughton et al., 2006), suggesting that inflammatory milieus resulting from different infections may provide the necessary factors for clonal expansion and survival. In the case of RepliVAX WN immunization, the inflammatory milieu in RepliVAX WN-immunized IFNAR$^{-/-}$ mice was sufficient to support development of a vigorous CD4^{+} T cell response suggesting type I IFN was not required for expansion of this subset. The apparent lack of requirement for type I IFN signaling for the optimal development of CD4^{+} and CD8^{+} responses in our studies compared to previous results by others may thus reflect differences in the inflammatory milieu due to background strain of IFNAR$^{-/-}$ mice employed or the pathogen system utilized.

Type I IFNs have been shown to have direct effects on T lymphocytes including inhibition of proliferation *in vitro* (Dondi et al., 2003), influencing T cell survival (Marrack et al., 1999) and enhancing T cell clonal expansion (Kolumam et al., 2005; Havenar-Daughton et al., 2006). Similarly, type I IFNs have been shown to be necessary as a third signal for development and differentiation of CD8^{+} T cell responses in some

infection models (Kolumam et al., 2005) although not in others (Valenzuela et al., 2002; Thompson et al., 2006), suggesting redundancy of function by other cytokines including IL-12. In the studies presented here, although IL-12 was not detected in the serum of immunized mice using a luminescence-based cytokine assay, it was detected in the serum of $IFNAR^{-/-}$, but not B6 mice, at 24 h post immunization using an ELISA-based assay (data not shown) and may have provided the required third signal for development of this T cell subset. Additionally, a vigorous WNV-specific $CD8^+$ T cell response was detected in the absence of type I IFN receptor signaling that was significantly greater than the response of wt mice. Because the magnitude of the $CD8^+$ effector T cell response in B6 and $IFNAR^{-/-}$ mice was dependent on the immunizing dose (Fig. 4.6C), the greater magnitude response in $IFNAR^{-/-}$ mice compared to B6 mice most likely reflected the higher expression of SCFV genes, and thus SCFV antigen, in $IFNAR^{-/-}$ mice compared to B6 mice in the first 72 h after immunization. These studies demonstrate that type I IFNs did, however, play a role in the development of $CD8^+$ T cell effector function in that the cytokine production patterns of WNV-specific $CD8^+$ T cells, measured at the single cell level, were altered in the absence of type I IFN receptor signaling. Importantly, WNV-cytotoxicity appeared unaffected by loss of type I IFN signaling. T cell responses to pathogens display functional heterogeneity and much evidence suggests that the simultaneous production of multiple cytokines and and/or expression of cytolytic function by individual T cells is important for infection control (Seder et al., 2008). Interestingly, under conditions of prolonged antigen exposure or exposure to very high antigen loads, T cells undergo a stepwise loss of function involving progressive loss of IL-2 and TNF-α production (Wherry et al., 2003). In the current studies, while $CD8^+$ T

cell responses in IFNAR$^{-/-}$ mice were of significantly higher magnitude compared to wt mice and expressed cytolytic activity, a significant proportion of these cells had lost the ability to produce TNF-α and IL-2. This loss of production of these cytokines by CD8^{+} T cells in IFNAR$^{-/-}$ mice was not associated with increased viral gene expression compared to wt mice and strongly suggests that the type I IFN may directly influence some aspects of effector function such as cytokine production without affecting other effector functions such as virus-specific cytotoxicity.

Taken together, the results of these studies have interesting implications for the ability of attenuated vaccines to produce consistent immune responses in vaccinated populations. Specifically, it has been shown that type I IFN controls the early genome replication and expression of viral genes after immunization with a single-cycle vaccine. Vigorous adaptive immune responses developed in the absence of type I IFN receptor signaling, suggesting that type I IFN is not essential for the intrinsic adjuvanting of the RepliVAX WN vaccine. Conversely, although type I IFN decreased SCFV gene expression and presumably antigen load in B6 mice, strong WNV-specific antibody and T cell responses were elicited by RepliVAX WN immunization of B6 mice, suggesting that type I IFN does not greatly impair the magnitude of the developing adaptive immune response to this single cycle vaccine. Overall, the magnitude of the WNV-specific IgG response to WNV proteins was not significantly altered in the absence of type I IFN receptor signaling; however, the quality of the CD8^{+} effector T cell response measured as the ability of individual cells to simultaneously produce more than one cytokine was diminished in IFNAR$^{-/-}$ mice suggesting that type I IFN receptor signaling plays a role in shaping cytokine production in antigen-specific effector T cell responses.

CHAPTER 5: Conclusions and Future Directions

Flaviviruses are responsible for important diseases affecting the human population. Every year, millions of cases of severe disease are reported and many more remain unidentified or misdiagnosed. Different factors contribute to the spread of flaviviral diseases. Climate changes, viral evolution and adaptation, preventive measures of control, and advances in global transportation are examples of factors that can contribute to the introduction of diseases into new regions of the world. Dengue is one example of a tropical disease that has emerged/re-emerged into subtropical and temperate areas of Europe and North America. In the last years, several autochthonous dengue cases have been reported in the continental area of the US, including Florida and Texas (Anez and Rios, 2013). The introduction of WNV into the US also reflects how a disease can rapidly spread over a continent. WN disease, which was first reported in New York City in 1999, disseminated all across the country in only 3 years (see Figure 1.5, Chapter 1). One of the largest outbreaks of human WN disease occurred in 2012, after a few years of non-expressive number of cases. All of the factors responsible for this increased incidence are still uncertain, although changes in the climate and in the mosquito vector may have played a role (Petersen, 2012).

Vector control and host vaccination are among the most reliable methods to specifically prevent diseases caused by flaviviruses. Among successful flaviviral vaccines licensed for human use are the YFV 17D live-attenuated vaccine, Japanese encephalitis inactivated and live-attenuated vaccines, and tick-borne encephalitis inactivated vaccines.

On the other hand, there is still need for vaccines to prevent dengue and WN diseases in humans. In the case of dengue, the biggest challenge is to produce an effective vaccine that induces simultaneously an equivalent protective immunity against all four DENV serotypes. Currently, tetravalent candidate dengue vaccines are being tested at different stages of human clinical trials. For WN disease, several human vaccines candidates are at different stages of development. However, due to the overall low incidence of WN disease, the interest for the availability of a commercial vaccine has been decreased. Also, because of this low incidence, human trials are difficult to conduct in order to prove the effectiveness of the vaccine candidates in humans.

The studies presented in this dissertation were designed to better understand different aspects of the single-cycle flavivirus vaccine candidate RepliVAX interaction with target cells, innate immune cells, and adaptive immune cells. The central hypothesis tested was that RepliVAX can be used as a tool to identify mechanisms involved in optimal virus replication and the development of immune response against flavivirus diseases to enhance the production and immunogenicity of this vaccine. Since RepliVAX can advance to a single, but complete round of replication, mimicking the initial natural virus infection, targeting lymphoid tissues and stimulating the host immune response, it is an excellent model to study flavivirus replication and immune response.

Adaptive mutations in RepliVAX D2

In the first part of this work, the enhanced growth properties of RepliVAX D2, a chimeric single-cycle vaccine, were investigated. RepliVAX D2 was generated from a C gene-deleted WNV genome (RepliVAX WN), where the prM and E genes of WNV were replaced by the prM and E genes of DENV2 (Suzuki et al., 2009). This chimeric vaccine

initially showed poor growth, which was enhanced following blind passaging in C-expressing cells, which led to the acquisition of specific mutations in the prM, E, NS2A and NS3 coding regions (Suzuki et al., 2009). Using a transpackaging system, it was demonstrated that the acquired mutations in the structural region ($M^{R9G,V13E}$ and E^{K120T}) improved specific infectivity, especially for the DENV2 prM/E coats. The mechanism that explain the improvement of the specific infectivities probably relies on the increased positive charge residues, which facilitate binding to cellular receptors with negative charges such as HS. Additionally, the acquisition of these mutations could aid molecular interactions that improve the maturation of newly formed particles. Structural studies could help to determine if the amino acid substitutions have an effect in conformational changes in the virion surface that could be advantageous for its infectivity. For example, cryoelectron microscopy (cryo-EM) reconstruction of the RepliVAX D2 could directly show the structure and the ratio of mature and immature particles present in the constructs containing or not the adaptive mutations. Based on the specific infectivity studies, it is possible to speculate that the particles with the adaptive mutations contain much more mature virions, counting for their increased specific infectivity.

Furthermore, it was demonstrated that the mutations acquired in the non-structural region of RepliVAX D2 ($NS2A^{S9F}$ and $NS3^{R516K}$) have an effect by interacting with the structural proteins, showing an enhanced growth effect especially for DENV2 structural proteins. For the NS2A mutation, it was demonstrated that this mutation disrupts a known ribosomal slip site, preventing the synthesis of NS1', which is an altered/larger form of NS1 present in members of the JEV serogroup (encephalitic flaviviruses). This effect was also confirmed by introduction of a synonymous mutation at the same mutated site,

showing the prevention of NS1' formation and increased viral production. The functions of the NS3 mutation still need to be determined. It is known that this mutation is localized in the helicase domain of NS3, which is critical for the encapsidation process. The NS2A and NS3 mutations showed an additive effect on viral growth, increasing the incorporation of E protein into infectious particles, but not having an effect on genome replication. These studies were important not only to reveal particular functions of flavivirus proteins, but also to show the feasibility of generation of chimeric vaccines and how they can be adapted to produce a better yield. One point that still needs to be addressed is in regard to the potency of this adapted chimeric vaccine. *In vivo* studies could be performed to determine if the changes in the proteins would have any effect on infection and antigen expression *in vivo* as well as in the magnitude of the immune response. Since RepliVAX D2 contains the WNV-specific $CD4^+$ and $CD8^+$ immunodominant T cell epitopes into the WNV backbone of the vaccine, one easy way forward would be to test the magnitude of T cell response elicited by this chimeric vaccine using similar approaches as described in chapters 3 and 4. ELISPOT assays could be used to determine the number of IFN-γ-secreting cells, as well as measure B cell response using DENV (E protein) and WNV (NS1 protein) antigens. Additional DENV-specific T cell epitopes also could be tested. Intracellular cytokine staining can be utilized to measure the activation status and effector function of these cells after RepliVAX D2 immunization. DENV2 challenge experiments where mice would be immunized with different doses of RepliVAX D2 to compare with the original construct (without the adaptive mutations) could be useful to demonstrate the potency of the vaccine in a dose dependent manner, indicating the effects of the adaptive mutations toward the

improvement of the vaccine, which is expected to be more immunogenic and efficacious, and ideally could produce a stronger immune response to give a better protection to the vaccinee.

Macrophages and dendritic cells in WNV infection

In the next portion of this work, the role of cellular innate immunity, specifically macrophages and DC, against WNV was investigated. First, to study the function of macrophages in the initial dissemination of SCFV particles and further APC function for the activation of WNV-specific T cell response, clodronate liposome (CCL) depletion was used to eliminate macrophages from the site of inoculation (subcutaneous FP inoculation). This approach has been successfully utilized in several works to study macrophage function. Macrophage depletion of the draining LN and spleen was assessed by flow cytometry using macrophage- and DC-specific cell markers. As expected, subcapsular sinus (SCS) macrophages and most of medullary macrophages were depleted from the pLN, while cells from spleen and ingLN stayed unaffected. DC were not depleted from any of the lymphoid tissues tested, demonstrating the specificity of CCL for macrophage depletion. IVIS studies where FLUC-SCFV particles were injected into the FP of macrophage-depleted and control mice demonstrated the role of macrophages in capturing and limiting the dissemination of the particles at early times after infection. The absence of macrophages rendered a higher expression of the FLUC-SCFV genes and a broader dissemination of the particles. FLUC-SCFV were disseminated to the spleen of macrophage-depleted mice as early as 14 hpi, while the presence of particles was not visualized in the spleens of control mice. Higher levels of bioluminescence were detected in the macrophage-depleted mice, especially at the site of inoculation (FP), indicating that

macrophages function by eliminating the particles or infected cells at that site. Type I IFN (IFN-α) and other chemokines were increased in the serum of macrophage-depleted mice, which possibly reflects the broader dissemination of particles in different tissues of these mice. Further studies will be needed to evaluate which cell types were responsible for the altered pattern of cytokine/chemokine production. Quantitative histology using confocal microscopy could be utilized to identify which cell types are recruited to sites of infection during and after SCFV infection. Upon identification of cells, flow cytometry could be used to determine which cytokines these cells are secreting. Laser capture microdissection is another approach that could be used to isolate specific cell types, which in turn could be combined with microarray technology to identify specific mRNAs expressed by these cells. A systematic evaluation of molecules produced in different tissues, including the site of inoculation and lymphoid tissues, will be of great importance since a lot of them are produced and have their function locally, and the systemic levels may not reflect properly what is happening during the infection. This could be achieved by harvesting target tissues/cells and testing them by luminescence-based multiplex bead assay (Bioplex) to quantify the cytokines produced at those sites.

Macrophages can serve as APC to activate the adaptive immune response. In a VSV model, Junt and colleagues (2007) demonstrated that SCS macrophages are important in capturing and presenting viral antigens to B cells present at lymphoid tissues. In the case of WNV, it has been shown that macrophages were not required for B cell activation and antibody production (Purtha et al., 2008). In the present study, it was demonstrated that macrophages were not required for the activation of WNV-specific $CD8^+$ T cell response, since both macrophage-depleted and control mice showed similar

response after SCFV immunization. The quality of the immune response also was not affected, as demonstrated by comparable frequency and polyfunctionality of $CD8^+$ T cell in both group of mice. *In vitro* studies showed that although macrophages were able to present antigen and induce T cell proliferation, this function was possibly supplied by DCs in the macrophage-depleted group. Following this hypothesis, two major subsets of DCs ($CD11b^-$ $CD8\alpha^+$ $CD11c^+$ and $CD11b^+$ $CD8\alpha^-$ $CD11c^+$) were identified as APC capable of priming naïve $CD8^+$ T cells. WNV-specific T cell response is driven by different signaling pathways. It has been already demonstrated that TLR3- and MyD88-dependent signaling pathways are important for full development of WNV immunity (Xia et al., in preparation). In the studies presented in this dissertation, the APC function and activation/maturation profile of BM-DCs from B6, $TLR3^{-/-}$ and $MyD88^{-/-}$ mice started to be evaluated. DCs from $MyD88^{-/-}$ mice presented a lower expression of MHC-II and CD80 molecules compared to the WT strain. DCs from all mouse strains were able to present antigen and stimulate $CD8^+$ T cell proliferation, although $MyD88^{-/-}$ and $TLR3^{-/-}$ were not as efficient as the WT strain. *In vivo* studies using macrophages and DCs sorted from spleens of infected mice were also standardized and will be another way forward to study the role of different signaling pathways on the APC function of these cells. In particular, it will be interesting to investigate the function of TLR-dependent (TLR3 and TLR7) and TLR-independent (RIG-I, MDA5) pathways, since these pathways already have been demonstrated to be important for the recognition and antiviral response to WNV, but the hierarchy and cell-type specificity still to be determined. The number and cell subsets at the site of infection and draining LN also could be evaluated to study the dynamics of recruitment of immune cells during the viral infection. This could be

achieved by labeling DCs at the site of infection using a FITC solution, which will allow tracking of the migration of DCs to lymphoid tissues. In these studies we could evaluate how many cells and how fast they migrate to the adjacent draining lymph node. Also, we could determine by flow cytometry which are the subtype of these cells (for example Langerhans, dermal DCs), maturation/activation status (expression of MHC-II, CD80, CD86) and other surface markers (for example migratory receptors such as CCR7). This information will be valuable to determine the efficiency of DCs migration and maturation to exert their APC function and see the contribution of the different signaling pathways in these processes.

Type I IFN response during SCFV immunization

The antiviral activity of type I IFN is critical for the development of an appropriate immune response following vaccination, especially when live-attenuated vaccines with lower replication potential are used. A balanced IFN response is necessary to allow simultaneously viral replication and induction of protective immune response. In the last portion of this dissertation, the influence of type I IFN in the intrinsic adjuvanticity of RepliVAX WN vaccine was investigated. B6 and IFNAR$^{-/-}$ mice (lacking the type I IFN receptor) were utilized in these studies to evaluate the role of type I IFN signaling. RepliVAX WN-immunized mice were able to produce IFN-α and other cytokines, however the production in the IFNAR$^{-/-}$ mice was transient and significantly lower compared to the B6 mice. The transcription of several cytokines and ISGs also were decreased in the draining LN of the IFNAR$^{-/-}$ mice inoculated with RepliVAX WN. These results demonstrated that RepliVAX WN can induce the production of type I IFN and several other molecules downstream the IFN signaling pathways, which is important

for the development of the protective immune response. Following this idea, the role of specific ISGs could be investigated, in particular the ones with increased expression during RepliVAX WN inoculation. Several mouse strains containing deletions of different ISGs important for WNV control (for example, ISG15, ISG56, ISG54, PKR, RNase L) or regulators in ISG expression such as STAT1 and STAT2 are already available, and could be used for a detailed study of the function of these genes in the induction of cytokines or interference with APC function of immune cells such as DCs, and effects on the induction and differentiation of effector and memory B and T cells.

IFN systems are known to be involved in the development of the adaptive immune response. In the present studies, it was shown that antibody response was partially affected by type I IFN signaling. WNV-specific IgM titer was lower in the knockout mouse strain, while no significant difference was seen in the total IgG level or neutralizing antibody response, indicating that IFN signaling is not a critical requirement for the development of the humoral response following RepliVAX WN immunization. $CD4^+$ T cell response was observed in both groups of mice, although the number of IFN-γ secreting cells for the $NS3_{2066}$ epitope was increased in the $IFNAR^{-/-}$ mice. This is consistent with previous reports demonstrating a higher response for this epitope compared with others WNV-specific $CD4^+$ T cell epitopes. Strong $CD8^+$ T cell responses were also observed in B6 and $IFNAR^{-/-}$ mice; however a significantly higher response was detected in the knockout mouse strain. These results suggest that the activation of WNV-specific T cell responses was not dependent on type I IFN signaling. One interesting point to further investigate could be the function of type III IFN (IFN-lambda). Type III IFNs are related to type I IFNs, sharing common signaling pathways

and both are produced during viral infection. However, not many studies have been done to demonstrate the biological relevance of type III IFN in WNV infection.

IVIS studies demonstrated that type I IFN signaling restrict virus replication as measured by expression of FLUC-SCFV. FLUC bioluminescence was over 100-fold higher in $IFNAR^{-/-}$ compared to B6 mice on days 1-3 post-infection, which indicate a high suppression of viral gene expression in the WT mice strain. To test if the higher T cell response seen on the $IFNAR^{-/-}$ mice was due to the higher antigen expression in this mouse strain, different doses given comparable antigen expression (measured by IVIS) were used to immunize B6 and $IFNAR^{-/-}$ mice. Remarkably, the results from this experiments demonstrated that the type I IFN signaling affected the pattern of cytokines produced by $CD8^+$ T cells, showing a loss of polyfunctionality in the cells from $IFNR^{-/-}$ mice. The antigen load had an effect in the magnitude of the $CD8^+$ T cell activation, but did not alter the quality of the response. Future studies could be performed to explore the mechanism of loss of T cell functionality. Measurement of the level of senescence of virus-specific T cells by expression of inhibitory molecules, such as Programmed Death 1 (PD-1), lymphocyte-activation gene 3 (LAG3), Cytotoxic T Lymphocyte Antigen 4 (CTLA4), could be further investigated to show alterations in the immune response pattern. The influence of type I IFN especially in DC numbers, function and homing ability, will be another very interesting point to be investigated, where their function can be demonstrated by the expression of different maturation and activation markers (e.g. CD40, CD80, CD86, MHC-I and MHC-II), transcription factors (e.g. IRF3, IRF7, STAT1 and STAT2), production of specific cytokines and chemokines (e.g. type I IFN, CXCL10, CCL2, CCL5, CCR5 and CCR7) and APC function.

In summary, all the studies presented in this dissertation have brought new insight into several aspects of viral replication and development of virus-specific immune response. These informations are of extreme value especially for the formulation of new single-cycle vaccines and/or chimeric vaccines where an optimized immune response is desirable to give a strong and durable protection. Several aspects of the innate and adaptive immune response to flavivirus infection were revealed, and will serve as future reference for better understanding of the role these components in the pathogenesis of flaviviral diseases.

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VITA

Evandro R. Winkelmann was born in Panambi, RS, Brazil in 1978. He is the third-born child of Lili and Hugo Winkelmann. Evandro received his Doctor of Veterinary Medicine (DVM) degree from the Federal University of Santa Maria (UFSM), Brazil in 2003. Shortly after his graduation he started the Master of Science program at UFSM and graduated in 2006 with major in Preventive Medicine (emphasis in virology). During his Master's, he received a brazilian scholarship for a US exchange program where he stayed one year at the University of Georgia (UGA) in Athens, GA as a vititing scientist working in projects related to influenza virus. In 2007, he came to UTMB as a postdoctoral fellow at Dr. Peter Mason's lab. Evandro's research focused on the development and characterization of single-cycle vaccines (RepliVAX) for several flaviviruses, including West Nile virus, Dengue virus and Japanese encephalitis virus. In January 2010, he began graduate training at UTMB and joined Dr. Gregg Milligan's lab. His dissertation research concentrated in understanding several aspects of flavivirus replication and immune responses elicited by single-cycle flavivirus immunization.

EDUCATION

Doctor of Veterinary Medicine (1998-2003). Federal University of Santa Maria (UFSM), Brazil.
Master of Science (2004-2006). Federal University of Santa Maria (UFSM), Brazil.

PROFESSIONAL EXPERIENCE

Postdoctoral training (2007-2009). University of Texas Medical Branch (UTMB), Galveston TX.

TEACHING EXPERIENCE

Teaching Skills and Course Development I (PATH 6101): UTMB Spring term, 2012.
Teaching Skills and Course Development II (PATH 6102): UTMB Summer term, 2012.
Teaching Skills Workshop: Why Should Teachers Bother Writing Instructional Objectives? Presented by: Vicki Freeman, May 14, 2012.
Teaching Skills Workshop: Writing Multiple Choice Test Questions. Presented by: Judith F. Aronson, June 19, 2012.

MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS

American Society for Virology: 2009-2014.
The American Association of Immunologists: 2010, 2013.
The American Association for the Advancement of Science: 2013-2014

HONORS/AWARDS

2010/2011: Sealy Center for Vaccine Development Predoctoral Fellowship.
2011: Robert Shope, Ph.D. Endowed Scholarship Award.
2012: American Society for Virology Travel Award.
2012: James W. McLaughlin Predoctoral Fellowship.
2012: Edward Reynolds, M.D. Experimental Pathology Graduate Scholarship Award.
2012: Margaret Saunders Travel Award.
2013: James W. McLaughlin Travel Award.
2013: Zhou Sisters Great Expectations Scholarship Award.

PUBLICATIONS

A. ARTICLES IN PEER-REVIEWED JOURNALS:

1. **Winkelmann, ER**, Widman, DG, Xia, J, Johnson, AJ, van Rooijen, N, Mason, PW, Bourne, N, Milligan, GN. (2014). Subcapsular sinus macrophages limit dissemination of West Nile virus particles after inoculation but are not essential for the development of West Nile virus-specific T cell responses. Virology, 450-451:278-289.

2. Xia, J, **Winkelmann, ER**, Gorder, SR, Mason, PW, Milligan, GN. (2013). TLR3- and MyD88-dependent signaling differently influence the development of West Nile virus-specific B cell responses in mice following immunization with single-cycle flavivirus (SCFV) RepliVAX WN. Journal of Virology, 87(22):12090-12101.

3. **Winkelmann, ER**, Widman, DG, Ishikawa, T, Xia, J, Miller-Kittrell, M, Nelson, MH, Bourne, N, Scholle, F, Mason, PW, Milligan, GN. (2012). Intrinsic adjuvanting of a novel single-cycle flavivirus vaccine in the absence of Type I Interferon receptor signaling. Vaccine, 30:1465-1475.

4. **Winkelmann, ER**, Widman, DG, Suzuki, R. Mason, PW. (2011). Analyses of mutations selected by passaging a chimeric flavivirus identify mutations that alter infectivity and reveal an interaction between the structural proteins and the nonstructural glycoprotein NS1. Virology, 421:96-104.

5. Ishikawa, T, Wang, G, Widman, DG, Infante Jr, E, **Winkelmann, ER**, Bourne, N, Mason, PW. (2011). Enhancing the utility of a prM/E-expressing chimeric vaccine for Japanese encephalitis by addition of the JEV NS1 gene. Vaccine, 29(43):7444-7455.

6. Nelson, MH, **Winkelmann, ER**, Ma, Y, Xia, J, Mason, PW, Bourne, N, Milligan, GN. (2010). Immunogenicity of RepliVAX WN, a novel single-cycle West Nile virus vaccine. Vaccine, 29(2):174-182.

7. Widman, DG, Ishikawa, T, **Winkelmann, ER**, Infante, E, Bourne, N, Mason, PW. (2009). RepliVAX WN, a single-cycle flavivirus vaccine to prevent West Nile disease, elicits durable protective immunity in hamsters. Vaccine, 27(41):5550-5553.

8. Suzuki, R, **Winkelmann, ER**, Mason, PW. (2009). Construction and characterization of a single-cycle chimeric flavivirus vaccine candidate that protects mice against lethal challenge with dengue virus type 2. Journal of Virology, 83(4):1870-1880.

9. **Winkelmann, ER**, Silva, LF, Mayer, SV, Mazzutti, KC, Flores, EF, Weiblen, R. (2007). Production and characterization of monoclonal antibodies against a bovine herpesvirus type 1 strain defective in the gene coding for the glycoprotein C. Ciência Rural, 37(4):1066-1072.

10. Mayer, SV, Quadros, VQ, Vogel, FSF, **Winkelmann, ER**, Arenhart, S, Flores, EF, Weiblen, R. (2006). Dexamethasone-induced reactivation of bovine herpesvirus type 5 latent infection in experimentally infected rabbits results in a wider distribution of latent viral DNA in the brain. Brazilian Journal of Medical and Biological Research. 39:335-343.

11. Spilki, FR, Silva, AD, Batista, HBCH, Oliveira, AP, **Winkelmann, ER,** Franco, AC, Porciuncula, JA, Roehe, PM. (2005). Field evaluation of safety during gestation and horizontal spread of a recombinant differential bovine herpesvirus 1 (BoHV-1) vaccine. Pesquisa Veterinária Brasileira, 25(1):54-58.

12. Vogel, FSF, Flores, EF, Weiblen, R, **Winkelmann, ER**, Moraes, MP, Braganca, JFM. (2004). Intraprepucial infection of young bulls with bovine herpesvirus type 1.2 (BHV-1.2): acute balanopostithis, latent infection and detection of viral DNA in regional neural and non-neural tissues 50 days after experimental reactivation. Veterinary Microbiology, 98:185-196.

13. Vogel, FSF, Lima, M, Flores, EF, Weiblen, R, **Winkelmann, ER**, Mayer, SV, Mazzutti, KC, Arenhart, S. (2004). Viral replication and shedding during acute infection and after dexamethasone induced reactivation of latency in calves inoculated with bovine herpesvirus type 1 (BHV-1) and 5 (BHV-5). Ciência Rural, 34(5):1619-1621.

14. Vogel, FSF, Caron, L, Flores, EF, Weiblen, R, **Winkelmann, ER**, Mayer, SV, Bastos, RG. (2003). Distribution of bovine herpesvirus type 5 (BHV-5) DNA in the central nervous system of latently, experimentally infected calves. Journal of Clinical Microbiology, 41(10):4512-4520.

15. Brum, MCS, Weiblen, R, Flores, EF, Pituco, EM, Tobias, FL, **Winkelmann, ER**. (2002). Fetal protection against challenge with bovine viral diarrhea virus (BVDV) in pregnant ewes immunized with two strains experimentally attenuated. Pesquisa Veterinária Brasileira, 22(2):64-72.

B. OTHER:

Thesis/Dissertation

1. **WINKELMANN, E.R.** (2006). Production and characterization of monoclonal antibodies to a bovine herpesvirus type 1 strain defective in the gene coding for the glycoprotein C. Master's Dissertation, Federal University of Santa Maria (UFSM), Santa Maria, RS – Brazil . Mentor: Rudi Weiblen, DVM, PhD. 43p.

C. ABSTRACTS:

1. **WINKELMANN, E.R**.; XIA, J.; WIDMAN, D.G.; GORDER, S.R.; MASON, P.W.; VAN ROOIJEN, N.; BOURNE, N.; MILLIGAN, G.N. The interplay of macrophages and dendritic cells with WNV-specific T cells during single-cycle flavivirus immunization. In: American Association of Immunologists, 100th Annual Meeting. Honolulu, HI, May 3-7, 2013.

2. XIA, J.; **WINKELMANN, E.R**.; GORDER, S.R.; MILLIGAN, G.N. Understanding the role of TLR3- and MyD88-dependent signaling in the development of B cell responses to RepliVAX WN, a live attenuated single cycle flavivirus. In: American Association of Immunologists, 100th Annual Meeting. Honolulu, HI, May 3-7, 2013.

3. XIA, J.; **WINKELMANN, E.R**.; GORDER, S.R.; MILLIGAN, G.N. TLR3- and MyD88-dependent signaling are required for West Nile virus vaccine candidate (RepliVAX WN) to optimize humoral responses. In: 2013 IHII/McLaughlin Colloquium. UTMB, April 12, 2013.

4. **WINKELMANN, E.R**.; XIA, J.; WIDMAN, D.G.; GORDER, S.R.; MASON, P.W.; VAN ROOIJEN, N.; BOURNE, N.; MILLIGAN, G.N. The interplay of macrophages and dendritic cells with WNV-specific T cells during single-cycle flavivirus immunization. In: 2013 IHII/McLaughlin Colloquium. UTMB, April 12, 2013.

5. **WINKELMANN, E.R**.; WIDMAN, D.G.; VAN ROOIJEN, N.; BOURNE, N.; MASON, P.W.; MILLIGAN, G.N. Role of macrophages on the development of WNV-specific T cell response. In: American Society for Virology, 31st Annual Meeting. July 21-25, 2012. Abstracts… Madison, WI: University of Wisconsin-Madison, 2012.

6. XIA, J.; **WINKELMANN, E.R**.; GORDER, S.R.; MA, Y.; MILLIGAN, G.N. The role of PPR signaling in the development of antiviral adaptive responses. In: National Foundation for Infectious Diseases, 17th Annual Conference on Vaccine Research. May 7-9, 2012. Abstracts... Baltimore, MD, 2012.

7. **WINKELMANN, E.R**.; WIDMAN, D.G.; VAN ROOIJEN, N.; BOURNE, N.; MASON, P.W.; MILLIGAN, G.N. Role of macrophages in the development of WNV-specific T cell response. In: Department of Pathology, 18th Annual Research Day. Galveston, TX, May 08, 2012.

8. **WINKELMANN, E.R**.; WIDMAN, D.G.; VAN ROOIJEN, N.; BOURNE, N.; MASON, P.W.; MILLIGAN, G.N. Role of macrophages in the development of WNV-specific T cell response. In: 2012 IHII/McLaughlin Colloquium. UTMB, April 13, 2012.

9. XIA, J.; **WINKELMANN, E.R**.; GORDER, S.R.; MA, Y.; MILLIGAN, G.N. Deficiency of TLR3 and MyD88-dependent signaling impairs T and B cell responses to a live attenuated single cycle vaccine. In: 2012 IHII/McLaughlin Colloquium. UTMB, April 13, 2012.

10. **WINKELMANN, E.R**.; WIDMAN, D.G.; XIA, J.; ISHIKAWA, T.; MILLER-KITTRELL, M,; NELSON, M.H.; BOURNE, N.; SCHOLLE, F.; MASON, P.W.; MILLIGAN, G.N. Intrinsic adjuvanting of a novel single-cycle flavivirus vaccine candidate in the absense of type I interferon receptor signaling. In: The Challenging Landscape of Vaccine Development, 2012. Galveston, TX. February 7-9, 2012.

11. XIA, J.; **WINKELMANN, E.R**.; GORDER, S.R.; MA, Y.; MILLIGAN, G.N. Deficiency of TLR3 and MyD88-dependent signaling impairs T and B cell responses to a live attenuated single cycle vaccine. In: The Challenging Landscape of Vaccine Development, 2012. Galveston, TX. February 7-9, 2012.

12. **WINKELMANN, E.R**.; WIDMAN, D.G.; SUZUKI, R.: BOURNE, N.; MASON, PW.; MILLIGAN, GN. Mutations selected by passaging of a chimeric flavivirus vaccine candidate alter infectivity and reveal interactions between the structural proteins and nonstructural glycoprotein NS1. In: Department of Pathology, 17th Annual Research Day. Galveston, TX, April 26, 2011.

13. **WINKELMANN, E.R**.; WIDMAN, D.G.; SUZUKI, R.: BOURNE, N.; MASON, PW.; MILLIGAN, GN. Mutations selected by passaging of a chimeric flavivirus vaccine candidate alter infectivity and reveal interactions between the structural proteins and nonstructural glycoprotein NS1. In: IHII/McLaughlin Colloquium. UTMB. Galveston, TX, March 25, 2011.

14. **WINKELMANN, E.R**.; XU, F.; NELSON, MH.; BOURNE, N.; MASON, PW.; MILLIGAN, GN. Epitope-specificity and effector function analysis of T and B cell responses elicited by a novel single-cycle vaccine candidate. In: 14th International Congress of Immunology. Kobe, Japan, August 22-27, 2010.

15. **WINKELMANN, E.R**.; XU, F.; NELSON, MH.; BOURNE, N.; MASON, PW.; MILLIGAN, GN. Epitope-specificity and effector function analysis of T and B cell responses elicited by a novel single-cycle vaccine candidate. In: American Association of Immunologists, 97th Annual Meeting. Baltimore, MD, May 7-11, 2010. The Journal of Immunology (Meeting Abstract Supplement), v.184, p.52.8.

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