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# MYD88-MEDIATED SIGNALING IN PROTECTIVE IMMUNITY AGAINST AN ATTENUATED WEST NILE VIRUS INFECTION

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# MYD88-MEDIATED SIGNALING PATHWAYS IN PROTECTIVE IMMUNITY AGAINST AN ATTENUATED WEST NILE VIRUS INFECTION

**by**

**Guorui Xie, B.S.**

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# Dedication

To my parents and two sisters who have always given me strong support. To my uncle and aunt who have encouraged me to pursue my dreams. To my friends who have helped me throughout my graduate study and life.

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West Nile virus (WNV) nonstructural (NS) 4B-P38G mutant is highly attenuated in mice. Interestingly, it induces strong immune responses and protects mice from subsequent lethal wild-type WNV NY99 infection. These features have important applications in flavivirus vaccine development. The goal of my dissertation is to understand the underlying mechanisms of WNV NS4B-P38G induced protective immunity.

Toll-like receptor (TLR) 7/myeloid differentiation factor 88 (MyD88)-mediated signaling pathways protect host against wild-type WNV infection. Both $MyD88^{-/-}$ and $TLR7^{-/-}$ mice had reduced effector T cell functions compared to wild-type mice following NS4B-P38G mutant infection. $TLR7^{-/-}$ mice displayed normal memory T cell functions and were fully protected from secondary challenge lethal WNV NY99. $MyD88^{-/-}$ mice had reduced memory T cell responses and were partially protected. These results suggest that TLR7-dependent-MyD88 signaling is required for T cell priming during NS4B-P38G

mutant infection. Whereas the TLR7-independent-MyD88 signaling pathways are involved in memory T cell development, which may contribute to host protection during secondary challenge with NY99. Aging is a risk factor for WNV encephalitis. Similar to $TLR7^{-/-}$ mice, old mice had reduced effector T cells and were partially protected from primary WNV NS4B-P38G mutant infection, but had normal memory T cell response and were all protected from re-challenge with WNV NY99. An impaired TLR7 signaling in old DCs led to lower innate cytokine response and a reduced antigen-presenting capacity compared to young DCs.

I also used two human cell lines-THP-1 and THP-1 macrophages to study the immune response following NS4B-P38G infection. NS4B-P38G mutant produced more viral RNA than parental NY99 in both cell types and boosted higher innate cytokine responses with no detectable infective virus. NS4B-P38G mutant infection in THP-1 cells led to more diverse and robust innate cytokine responses than that seen in THP-1 macrophages, which were mediated by TLR7 and retinoic acid-inducible gene 1 (RIG-I) signaling pathways. Thus, a defective viral life cycle during NS4B-P38G mutant infection in human monocytic and macrophage cells leads to more potent cell intrinsic innate cytokine responses. In summary, my dissertation studies suggest that MyD88-mediated signaling pathway regulates protective immune response to WNV NS4B-P38G mutant infection.

# TABLE OF CONTENTS

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| APCs | Antigen Presenting Cells |
| ATP | Adenosine triphosphate |
| BBB | Blood brain barrier |
| B6 | C57BL/6J mouse strain |
| BM | Bone Marrow |
| BSA | Bovine Serum Albumin |
| C | Capsid |
| C | Complementary |
| CARDs | Caspase-activation and recruitment domains |
| CNS | Central nervous system |
| CTL | Cytotoxic T Lymphocyte |
| DCs | Dendritic cells |
| DMEM | Dulbecco's modified Eagle's medium |
| DS RNA | Double-stranded RNA |
| E | Envelope |
| ER | Endoplasmic reticulum |
| FBS | Fetal bovine serum |
| FFA | Focus-forming assay |
| FFU | Focus-forming units |
| GM-CSF | Granulocyte-macrophage-colony stimulating factor |
| LCs | Langerhans cells |
| MAVS | Mitochondrial antiviral-signaling protein |
| M-CSF | Macrophage colony-stimulating factor |
| MDA5 | Myeloma differentiation antigen 5 |
| MEM | Modified Eagle Medium |
| MEI | Mean fluorescence intensity |
| MHC | Major Histocompatibility complex |
| MYD88 | Myeloid differentiation primary response gene 88 |
| NLR | Nod-like receptors |
| NTPase | RNA nucleoside triphophatases |
| NS | Non-structural |
| I.C. | Intracerebroventricular |
| IFNs | Interferons |
| IFNAR | IFNa and IFNb receptor complex |
| I.P. | Intraperitoneal injection |
| IHC | Immunohistochemistry |
| IL-4 | Interleukin-4 |

| | |
|---|---|
| IRF | Interferon regulatory factors |
| ISGs | Interferon-stimulated genes |
| JFV | Japanese encephalitis virus |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate Buffered Saline |
| PFU | Plaque forming unit |
| PMA | Phorbol 12-myristate 13-acetate |
| prM | Pre-membrane |
| PRRs | Pattern recognition receptors |
| Q-PCR | Quantitative PCR |
| RdRp | RNA-dependent RNA polymerase |
| RIG-I | Retinoic-acid inducible gene-I |
| RLR | (RIG-I)-like receptors |
| RT | Room Temperature |
| RTPase | RNA 5’-triphophatase |
| SS RNA | Single-stranded RNA |
| Th1 cells | Type 1 helper cells |
| Th2 cells | Type 2 helper cells |
| Th17 cells | T helper 17 cells |
| THP-1 | Human monocytic leukemia cells |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Treg cells | Regulatory T cells |
| WND | West Nile neuroinvasive disease |
| WNF | West Nile fever |
| WNV | West Nile virus |
| WNVE | West Nile virus Envelope |

# Chapter 1: INTRODUCTION

## 1.1 West Nile virus (WNV) biology

WNV is maintained in a mosquito-bird-mosquito transmission cycle (Work, Hurlbut, and Taylor 1955). In the United States, at least 65 different mosquito species and 326 bird species are permissive to WNV infection. Only a few *Culex* mosquito species spread WNV into humans (Petersen, Brault, and Nasci 2013). Humans and horses are incidentally infected by mosquito bites. Naïve mosquitos cannot be infected with low level of viremia from humans or horses which are considered as dead-end hosts (Petersen, Brault, and Nasci 2013).

WNV is a positive-sense single stranded RNA virus belonging to the genus of *Flavivirus* in the family *Flaviviridae.* (Anderson et al. 1999). WNV is approximately 50 nm in diameter with a lipid bi-layered envelope. The WNV genome consists of approximately 11,000 nucleotides (Mukhopadhyay et al. 2003), which are translated and processed into three structural proteins (C: Capsid; prM: Pre-membrane; E: Envelope) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers, Hahn, et al. 1990) **(Fig 1.1).** NS proteins modulate viral transcription, translation and replication and host antiviral response. NS1 is a cofactor for viral RNA replication and is the only NS proteins secreted following WNV infection (Lindenbach and Rice 1997; Macdonald et al. 2005). NS1 minimizes the immune response by antagonizing the Toll-like receptor (TLR) 3 and inhibiting complement activation via binding to C4 or regulatory protein factor H (Wilson et al. 2008; Avirutnan et al. 2010; Chung, Liszewski, et al. 2006). NS2A plays an essential role for viral assembly and regulates host immune response. A study showed that a single amino acid substitution (A30P) in NS2A reduced the inhibitory activity of the IFN-β promoter (Liu, Chen, and Khromykh 2003; Liu et al. 2004). NS2B is an essential cofactor and is required by NS3

protease activity (Chappell et al. 2008). It also has interferons (IFNs) antagonist function to inhibit the host antiviral response (Liu et al. 2005). NS3 is a multifunctional protein that has serine protease, RNA helicase, RNA5'- triphophatase (RTPase) and RNA nucleoside triphophatases (NTPase) activities that are required for viral replication (Gorbalenya et al. 1989; Chambers, Weir, et al. 1990; Wengler 1993). The IFNs antagonist function of NS3 is also determined (Liu et al. 2005). NS4A is a cofactor for NS3 helicase. It is required by NS3 to sustain the unwinding rate of the viral RNA under conditions of adenosine triphosphate (ATP) deficiency (Shiryaev et al. 2009). 2K transmembrane domain in NS4A C-terminal regulates degrees of rearrangement of cytoplasmic membranes (Roosendaal et al. 2006). NS4A inhibits IFNs signaling by blockage of STAT1 and STAT2 activation (Liu et al. 2005). NS4B is a small hydrophobic NS protein with five transmembrane domains and approximately 27 kDa in size (Wicker et al. 2012; Arai et al. 2004). It can translocate independently into the nucleus (Lin et al. 1997). NS4B protein of flaviviruses contributes to viral replication either directly or indirectly via interactions with other NS proteins. NS4B protein interacts physically with NS1 which conveys signals to the cytoplasm and regulates early viral RNA replication (Youn et al. 2012). NS4B regulates viral replication via interaction with NS3 and inhibits IFNs signaling pathway, furthermore, a P101L mutation in DENV NS4B ablates interaction between NS4B and NS3 (Umareddy et al. 2006; Liu et al. 2005; Hanley et al. 2003). NS4B plays an important role in the regulation of viral neuroinvasive function. Compared to wild-type Japanese encephalitis virus (JEV) strain SA14, live attenuated JFV vaccine strain SA14-14-2 has a substitution I160V in NS4B (Ni et al. 1995). Yellow fever vaccine strain 17D-204 or 17DD has a substitution I95M in NS4B. Compared to parental strain WNV NY99, both WNV NS4B-P38G mutated stain and WNV NS4B C102S mutated stain have attenuated neuroinvasive phenotype in mice (Wicker et al. 2006; Wicker et al. 2012). NS5 has a C-terminal RNA-dependent RNA polymerase (RdRp) domain and plays a key role in viral replication (Davidson 2009).

NS5 limits production of IFNs via blockage of JAK-STAT1 activation (Laurent-Rolle et al. 2010).

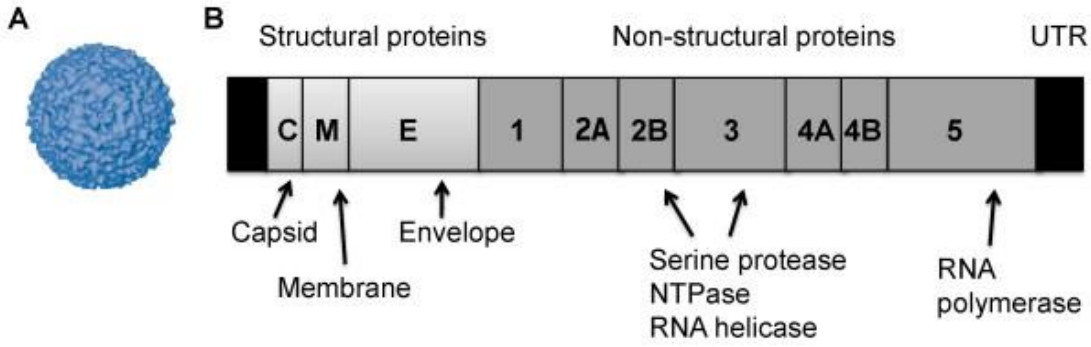



**Figure 1.1: West Nile virus structure (a) and genome (b).** The genome is translated into a single polyprotein. The polyprotein is cleaved into the structural and nonstructural proteins. (Reprinted with permission from De Filette M et al. Recent progress in West Nile virus diagnosis and vaccination. Vet Res. 2012 Mar 1; 43 (1): 16).

WNV strains are grouped into at least 7 genetic lineages (Mackenzie and Williams 2009). Lineage 1 is classified into two distinct clades: WNV-1a and WNV-1b. WNV-1a is found mainly in Africa, Europe, North American and Asia. It contains WNV NY99 strain which serves as wild-type strain for my project (George Valiakos 2013).

The life cycle of WNV within an infected host cell is shown in **Figure 1.2.** The E protein interacts with receptors on the cell surface. DC-SIGN, alpha Vbeta3 and laminin-binding protein are poteintial receptors (Bogachek et al. 2010; Bogachek et al. 2008). Virus is taken inside of cells by receptor-mediated endocytosis (Chu and Ng 2004). The low-pH inside the of endosome leads to E protein conformational changes followed by fusion between the viral and cellular membranes (Gollins and Porterfield 1986), and the viral positive-sense single-stranded RNA genome is released into the cytoplasm. A single polyprotein is translated from the viral positive-sense RNA and cleaved into three

structural proteins and seven NS proteins by viral and host proteases in the endoplasmic reticulum (ER) (Chambers, Hahn, et al. 1990; Nowak, Farber, and Wengler 1989). The full-length positive-sense RNA serves as a template for the synthesis of antisense full-length negative-sense RNA requiring RdRp NS5. The negative-sense RNA serves as a template for the synthesis of more full-length positive-sense RNA. On rough ER membranes, viral genomic RNA is encapsidated by viral C protein to form immature virions with E and prM proteins. Immature virions become mature virions during transit from the Golgi complex and maturation requires glycosylation of E protein and cleavage of prM to mature M protein. Finally, mature virions are released via exocytosis (De Filette et al. 2012; Suthar, Diamond, and Gale 2013).

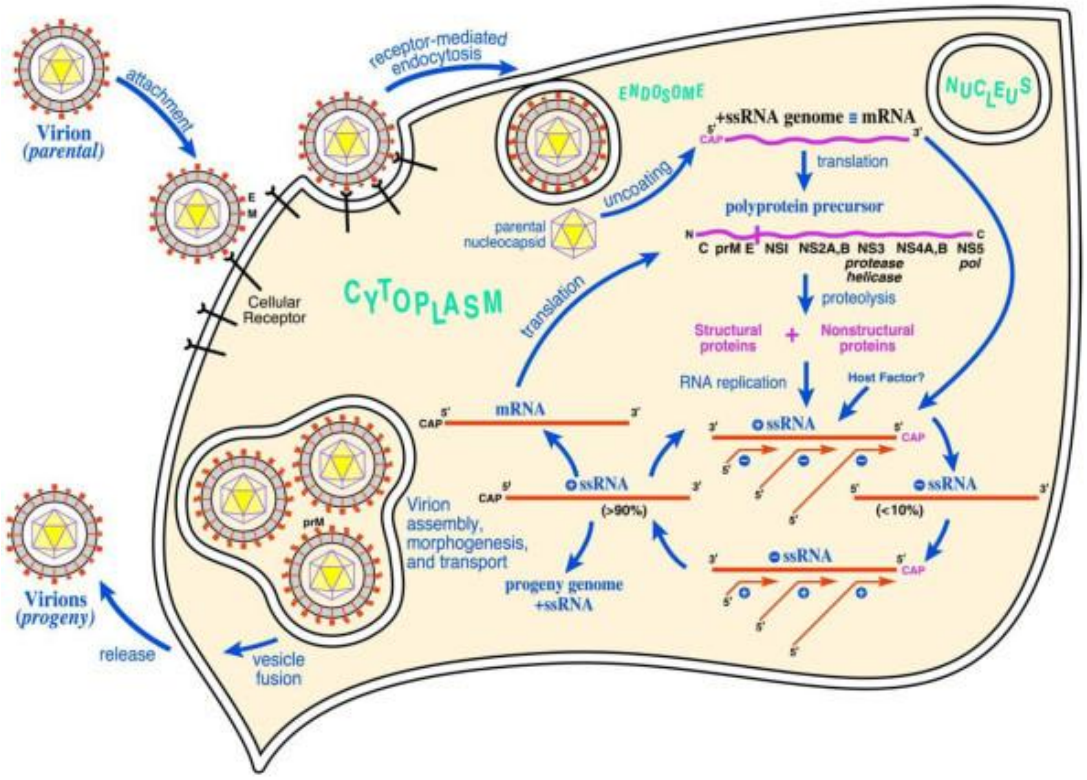



**Figure 1.2: West Nile virus life cycle.** Original source: title: Host genetic variability and West Nile virus susceptibility. PNAS 2002, vol. 99 no 18 11555-11557. Copyright (2002) National Academy of Sciences, U.S.A

The pathogenesis of WNV in humans is poorly understood. Most of mechanisms of WNV pathogenesis are studied in rodent models. WNV infects keratinocytes and skin-resident DCs, including Langerhans cells and dermal DCs, following an infected mosquito bite (Lim, Behr, et al. 2011; Johnston, Halliday, and King 2000; Byrne et al. 2001). These infected cells migrate to the lymph nodes. Virus replicates within the draining lymph nodes and result in viraemia (Johnston, Halliday, and King 2000). This is followed by virus spreading to peripheral visceral, including spleen, where virus replicates more. After replication in the spleen the virus is then capable of entering into the Central nervous system (CNS) and replication in the CNS can lead to encephalitis and death. The mechanism of WNV entry into the CNS is unknown. There are several potential ways. One possibility is that tumor necrosis factor (TNF)-α regulates endothelial cell permeability and facilitates WNV entry (Wang et al. 2004). Another possibility is that infection of olfactory neurons and spread to the olfactory bulb occurs (Getts et al. 2008). It is also possible that WNV induces tight junction protein degradation and virus is transported via a "Trojan horse" mechanism (Verma et al. 2009; Verma et al. 2010).

## 1.2 WNV Induced Diseases

WNV was first isolated from blood of a female patient in Uganda in 1937 (Smithburn et al 1940). The first introduction of WNV infection in to North American occurred in 1999 in New York City (Nash et al. 2001) and continues to cause human disease after 1999. From 1999 to 2014, there were 41,679 reported cases of WNV infection and 1753 associated deaths in United States (Centers for Disease Control and Prevention (CDC) report). WNV disease has become a public health concern.

Although humans are usually dead-end hosts for WNV infection, WNV can be transmitted into humans through non-vector mediated routes. It has been reported that WNV can be transmitted from human to human via solid organ transplantation (Iwamoto

et al. 2003), blood transfusion, dialysis (Cairoli 2005) or from mother to child via breast milk (Hayes and O'Leary 2004) or by the intrauterine route ("Intrauterine West Nile virus infection--New York, 2002" 2002). Another infection without vector is laboratory exposure ("Laboratory-acquired West Nile virus infections--United States, 2002" 2002).

The incubation period for human WNV disease ranges from 2 to 14 days (Gea-Banacloche et al. 2004). 80% of WNV infections in humans are asymptomatic. 20% of humans infection patients have West Nile fever (WNF) characterized by fever and headache (Iyer and Kousoulas 2013). A rash appears in one-half of WNF patients (Watson et al. 2004). Patients sometimes show other symptoms including muscle weakness, fatigue, nausea, vomiting, gastrointestinal problems, and lymphadenopathy (Iyer and Kousoulas 2013). Less than 1% of WNV infections case causes West Nile neuroinvasive disease (WND) which includes encephalitis, meningitis, or acute flaccid paralysis (Iyer and Kousoulas 2013; Hayes et al. 2005). Elderly and immunocompromised individuals appear to be at greater risk of severe neurologic syndromes. Individuals who are more than 65 years old have 16 times higher chance than individuals 16-24 years of age of developing neurologic syndromes (Carson et al. 2012). The risk of neuroinvasive disease may increase with a history of cancer, diabetes, alcohol abuse as well as male sex (Petersen, Brault, and Nasci 2013; Carson et al. 2012; Lindsey et al. 2012; Murray et al. 2006; Bode et al. 2006).

WNV can cause persistent infections in some vertebrate species. The first study of WNV persistent infection was performed using monkeys in 1983 (Pogodina et al. 1983). WNV could be detected up to 5 ½ months after infection. Persistent WNV infection also occurs in hamsters and mice (Tonry et al. 2005; Appler et al. 2010; Saxena et al. 2013).

## 1.3 Animal Models:

Laboratories occasionally use monkeys to study WNV infection *in vivo* (Pogodina et al. 1983). However, monkeys cost much more than rodent models. Most laboratories

prefer less expensive models. Rodent models are used by most laboratories to study WNV.

Golden hamster model (*Mesocricetus auratus*) is a good model to study acute WNV infection (Xiao et al. 2001). Viremia was detectable within 24 h and lasts for 5 or 6 days after infection. Antibodies were detectable from day 5 post-infection. Encephalitic symptoms in hamster started at day 6 post-infection. Hamsters died from day 7 post-infection. Viral antigen was first detected in the brain on day 6 post-infection (Xiao et al. 2001). The golden hamster model is also a good model to study persistent WNV infection. WNV was recovered from hamster brain after 53 days post-infection and from urine after 52 days post-infection (Xiao et al. 2001; Tonry et al. 2005).

Mice are also often used to study WNV infection *in vivo* (Weiner, Cole, and Nathanson 1970). NIH Swiss outbred mice are used to study neuroinvasive properties of different WNV strains (Beasley et al. 2002). Inbred C57BL/6 (B6) mice are vulnerable to WNV infection, virus spreads into lymph nodes, spinal cord and brain in B6 mice (Diamond, Shrestha, et al. 2003). B6 mice are a good model study persistent WNV infection (Saxena et al. 2013). B6 mice are also a good model to study the aged related immune response during WNV infection. 8-week-old wild-type B6 mice had around 70% survival rate after subcutaneous inoculation with WNV, but 5-week-old wild-type B6 mice had only around 13% survival rate (Engle and Diamond 2003). Compared to the 40% survival rate in young mice (6-10-week-old), old mice (21-22-month-old) with 13% survival rate were more susceptible to WNV infection. In my project, I used B6 mice to study the host immune response against an attenuated WNV infection.

## 1.4 Host Immunity

### 1.4A: Cytokines and Chemokines

Type I IFNs protect the host from WNV infection. Type I IFNs response after WNV infection occurs following two steps: 1) Pattern recognition receptors (PRRs) bind

to the viral particle to trigger expression of IFNα and IFNβ and 2) Type I IFNs bind to IFNα and IFNβ receptor complex (IFNAR). This event is followed by activation of JAK-STAT signaling and expression of IFN-stimulated genes (ISGs) (including OAS, IFIT1 and IFIT2) (Diamond and Gale 2012; Suthar, Diamond, and Gale 2013). IFN-γ is type II IFN. IFN-$\gamma^{-/-}$ mice had higher mortality, higher viremia and viral replication in lymphoid tissues than wild-type mice following WNV infection. Thus, IFN-γ also protects the host from WNV infection (Wang et al. 2006; Wang, Scully, et al. 2003).

WNV infection induces production of pro-inflammatory cytokines including TNF-α, IL-6, IL-12 and IL-1β (Cheeran et al. 2005). TNF-α responses diminish CXCL-10 induced death in the CNS (Zhang et al. 2010). The study of TNF-receptor 1 showed that TNF-α could increase BBB permeability to enable WNV entry into brain and cause damage (Wang et al. 2004). IL-6 and IL-12 serve as signal 3 to regulate T cell response (Kim and Harty 2014). In neurons, IL-1β synergized type I IFNs to suppress WNV replication and controls WNV infection and immunity in the CNS (Ramos et al. 2012). IL-$10^{-/-}$ mice or IL-10 neutralizing antibody treated mice increased the survival rate of mice following WNV infection. IL-10 signaling negatively regulates host immunity against WNV infection (Bai et al. 2009).

WNV infection causes neurological disease. Chemokines and their receptors regulate leukocytes trafficking into the CNS (Ubogu, Cossoy, and Ransohoff 2006). CCL5 and its receptor CCR5 were increased following WNV infection in mouse brain. CCR5 was expressed in $CD4^+$ T cells, $CD8^+$ T cells, $NK1.1^+$ cells and macrophages and regulated CNS infiltration of these cells. $CCR5^{-/-}$ mice had increased viral burden in brain and mice mortality following WNV infection (Glass et al. 2005). CCR5 protects mice from WNV infection. Homozygosity for CCR5Δ32, a complete loss-of-function mutation in the chemokine receptor CCR5, had increased severe symptomatic disease in WNV infection patients (Lim et al. 2008; Lim et al. 2010). Neurons secreted chemokine Cxcl10 to recruit effector $CD8^+$ T cells via its receptor Cxcr3 in order to reduce viral burden and

protect mice following WNV infection (Klein et al. 2005; Zhang, Chan, et al. 2008). Chemokine receptor Ccr2 is expressed on Ly6c$^{hi}$ inflammatory monocytes and regulates migration of Ly6c hi monocytes into brains to protect mice from WNV infection (Lim, Obara, et al. 2011). Overall, chemokines and chemokine receptors regulate leukocyte trafficking to CNS during WNV infection.

## 1.4B: Complement System

The complement system has a role in protection during WNV infection. The complement system can be divided into three pathways: classical pathway, lectin pathway and alternative pathway (Stoermer and Morrison 2011). C3 is involved in all three pathways. C3- deficient mice had increased mortality and reduced WNV specific IgM and IgG responses than wild-type mice (Mehlhop et al. 2005). Deficiencies in other factors involved in the complement system also increased susceptibility to WNV infection, for example: C1q, C4, factor B, factor D or C5. Both factor B and C4 can regulate the CD8$^{+}$ T cells response during WNV infection (Mehlhop and Diamond 2006; Mehlhop et al. 2009).

## 1.4C: PRRs

Innate immunity is an essential antiviral defense mechanism that protect the host against WNV infection. PRRs recognize viral pathogen-associated molecular pattern (PAMP) and induce production of type 1 IFNs, pro-inflammatory cytokines and chemokines (Loo and Gale 2011). PRRs include retinoic-acid inducible gene-I (RIG-I)-like receptors (RLR), Toll-like receptors (TLRs) and the nucleotide ologomerization domain (Nod)-like receptors (NLR) (Loo and Gale 2011). RLRs contain three members, including RIG-I, myeloma differentiation antigen 5 (MDA5) and LGP2, and are expressed in nearly all cell types (Loo and Gale 2011). RIG-I and MDA5 have similar

structures. Both of them have two N-terminal tandem caspase-activation and recruitment domains (CARDs), a RNA helicase domain and a C-terminal repressor domain. LGP2 only has one RNA helicase domain and a C-terminal repressor domain. RIG-I binds to smaller (< 300 bp) double-stranded (ds) RNA molecules, single-stranded (ss) RNA with 5'triphosphate group or hairpin ssRNA. MDA5 binds to a large ds RNA (Bowzard et al. 2011). During WNV infection, RIG-I and MDA5 recognize ds RNA formed during viral replication. RIG-I deficient or MDA5 deficient mice had increase mortality in mice following WNV infection. Lack of RIG-I or MDA5 in primary cells caused a decrease in the innate immune response (Errett et al. 2013). MDA5 can regulate $CD8^+$ T cells activation during WNV infection (Lazear, Pinto, et al. 2013). RIG-I and MDA5 recruit adaptor protein mitochondrial antiviral signaling (MAVS) to activate transcription factors including interferon regulatory factors (IRF) 1, IRF3, IRF5, IRF7 and NF-κB (Daffis et al. 2007; Daffis, Samuel, Suthar, Keller, et al. 2008; Lazear, Lancaster, et al. 2013; Brien et al. 2011). Activated transcription factors translocate from the cytoplasm into the nucleus to activate type 1 IFNs, pro-inflammatory cytokines and chemokines (Loo and Gale 2011). Both MAVS knock-out mice and RIG-$I^{-/-}$ X $MDA5^{-/-}$ double-knockout mice all died within 8 days post-infection following WNV infection (Errett et al. 2013). MAVS protects the host from WNV infection. Both RIG-I and MDA5 have protection function to against WNV infection. $LGP2^{-/-}$ mice had a lower survival rate than wild-type mice following WNV infection, which is linked to a defect in the T cell response (Suthar et al. 2012).

More than 10 TLRs have been identified. Four of TLRs regulate the host immune response during virus infection. TLR3 recognizes ds RNA. Both TLR7 and TLR8 recognize GU-rich ss RNA (Alexopoulou et al. 2001; Heil et al. 2004). Unmethylated CpG DNA from bacteria or viruses can be detected by TLR9 (Heil et al. 2004; Iwasaki and Medzhitov 2004). There are a few publications focusing on the role TLRs during WNV infection. When wild-type B6 mice, $TLR7^{-/-}$ and $TLR9^{-/-}$ mice were

intraperitoneally (I.P.) infected with WNV, TLR9$^{-/-}$ mice had a similar survival rate to wild-type mice, suggesting that TLR9 signaling pathway did not protect mice from WNV infection. Therefore, TLR7$^{-/-}$ mice were more susceptible to WNV infection than wild-type and TLR9$^{-/-}$ mice (Town et al. 2009). TLR7$^{-/-}$ mice had increased viral loads in the blood, spleen and brain with more cytokine induction including IFNα, IFNβ, IL-1β, IL-6 and TNFα than wild-type mice following WNV infection. TLR7$^{-/-}$ mice had reduced IL-12 and IL-23 expression. IL-12 has two subunits IL-12p35 and IL-12p40. An *in vivo* study showed that IL-12 p40-deficient (IL12b$^{-/-}$) and IL-23p19-deficient (IL23a$^{-/-}$) mice had a reduced number CD11b$^{+}$ macrophages and microglia and CD45$^{+}$ leukocytes in virus-infected brain, but IL-12 p35-deficient (IL12a$^{-/-}$) mice had similar results to wild-type mice. In a survival study, IL-12 p40-deficient and IL-23p19-deficient mice were more susceptible to WNV infection than wild type and IL-12p35-deficient mice. TLR7 signaling pathway induced IL-12p40 and IL-23, which recruit immune cells into brain and protect mice from WNV infection (Town, Bai et al. 2009). Wild-type and TLR7$^{-/-}$ mice had similar susceptibilities to WNV infection following intradermal injection or mosquito feeding. The viral load in blood, spleen and brain were similar between two groups. In brain, cytokine induction was similar at day 4 and day 6 post-infection between the two groups. In blood, compared to TLR7$^{-/-}$ group, wild-type group had higher IL-6 induction at day 2 and day 4 post-infection, higher IFNα, IL-12 induction at day 2 post-infection and higher TNFα induction at day 4 post-infection. Keratinocytes express high levels of TLR7 and regulate Langerhans cell (LC) mobilization in the skin. Compared to wild type keratinocytes, TLR7$^{-/-}$ keratinocytes had less cytokine induction (IL-1β, IL-6 and IL-12) with more viral load. Total number and percentage of CD11C$^{+}$ LCs were reduced in wild type mice following WNV infection compared to non-infected mice, but this reduction was not detected in TLR7$^{-/-}$ mice, which indicated that TLR7 signaling pathway regulated LCs migration during WNV infection. During cutaneous WNV infection, TLR7 signaling compromised its protective effect by promoting LC

migration from the skin (Welte et al. 2009). During WNV NS4B-P38G mutant infection, TLR7$^{-/-}$ mice had a reduced percentage and total number of splenic V$\gamma$1$^{+}$ T cells but a similar percentage and total number of V$\gamma$4$^{+}$ T cells to wild type mice at day 3 and day 5 post-infection. V$\gamma$1$^{+}$ T cells expansion increased with R848-TLR7 agonist stimulation. TLR7 signaling pathway activated V$\gamma$1$^{+}$ T cells, but not V$\gamma$4$^{+}$ T cells, during WNV NS4B-P38G mutant infection (Zhang et al. 2014). TLR7, but not TLR9, regulates the immune response to protect the host from WNV infection.

The role of TLR3 during WNV infection has also been determined. Wild-type and TLR3$^{-/-}$ mice were inoculated by the intraperitoneally route with 1,000 PFU ($LD_{100}$) of WNV. TLR3$^{-/-}$ mice had a higher survival rate than wild-type mice. TLR3$^{-/-}$ mice had a lower viral replication, less TNF$\alpha$ induction and lower blood brain barrier (BBB) permeability in brain than wild-type mice. TNF$\alpha^{-/-}$ mice had a higher survival rate and lower BBB permeability than wild-type mice following WNV infection. There was no difference in survival rate when wild-type and TLR3$^{-/-}$ mice were inoculated by intracerebroventricular (I.C.) infection with a sublethal or lethal dose of WNV. This paper showed TLR3 signaling pathway promoted TNF$\alpha$ induction to increase BBB permeability of the brain and helped WNV entry into the brain (Wang et al. 2004). However, another study group showed that survival rate was higher in wild-type mice than TLR3$^{-/-}$ mice inoculated by i.p. or subcutaneous routes with $10^2$ and $10^3$ PFU of WNV. The permeability of the BBB in the two groups was similar. Under inoculation I.C. WNV, TLR3$^{-/-}$ mice have more viral load in the brain and spinal cord at day 4 post-infection. This paper shows the protective role of TLR3 during WNV infection (Daffis, Samuel, Suthar, Gale, et al. 2008). Although both studies used the same TLR3$^{-/-}$ mice, but the results were different. The disparity of results in TLR3$^{-/-}$ mice could be caused by difference WNV strains (WNV 3000.0259 versus WNV isolate 2741) and/or passage history of the virus (mammalian Vero cell versus insect-cell derived).

Myeloid differentiation primary response gene 88 (MyD88) is an adaptor and is activated by the IL-1 receptor, IL-18 receptor and all TLRs except TLR3. MyD88 promotes induction of type 1 IFNs and pro-inflammatory cytokines (Suthar, Diamond, and Gale 2013). T cell-specific MyD88 regulates T cell responses (Schenten et al. 2014). When wild-type B6 mice and MyD88$^{-/-}$ mice were inoculated i.p. with WNV, MyD88$^{-/-}$ mice had increased susceptibility after WNV infection compared to wild-type mice (Town et al. 2009). MyD88$^{-/-}$ mice had more viral load in their blood and spleen at day 3 and in brain at day 6 post-infection (Town et al. 2009). Another study confirmed that MyD88$^{-/-}$ mice were more susceptible to WNV infection compared to wild-type B6 mice (Szretter et al. 2010). No difference of viremia between the two groups was detected. Viral load difference was observed in lymph node at day 3 post-infection and in spleen at day 6 and day 10 post-infection. In brain, viral replication was observed 40- 55-fold higher in MyD88$^{-/-}$ than in wild-type mice from day 8 to day 10 post-infection not before day 8 which indicated MyD88 signaling pathway contributed to control virus replication not entry into brain. There was no difference of WNV-specific CD8$^{+}$ T cell activation in spleen between wild type mice and MyD88$^{-/-}$ mice at day 8 after infection. In brain, WNV-specific CD8$^{+}$ T cell activation was still similar between two groups, however, the recruitment of macrophages, CD3$^{+}$ T cells and chemokines including CCL5, CXCL9, CXCL10 and CCL2 were reduced in MyD88$^{-/-}$ mice. Overall, MyD88 signaling pathway restricts WNV infection in B6 mice (Szretter et al. 2010). WNV NS4B-P38G mutant infection strain induced V$\gamma$1$^{+}$ T cells and V$\gamma$4+ T cells in mice. MyD88$^{-/-}$ mice had a reduced percentage and total number of splenic V$\gamma$1$^{+}$ T cells and a similar percentage and total number of V$\gamma$4$^{+}$ T cells with wild type mice at day 3 and day 5 post-infection (Zhang et al. 2014) . MyD88 signaling pathway protects host from WNV infection. One mechanism for protection is that MyD88 signaling activates V$\gamma$1$^{+}$ T cells response during WNV infection.

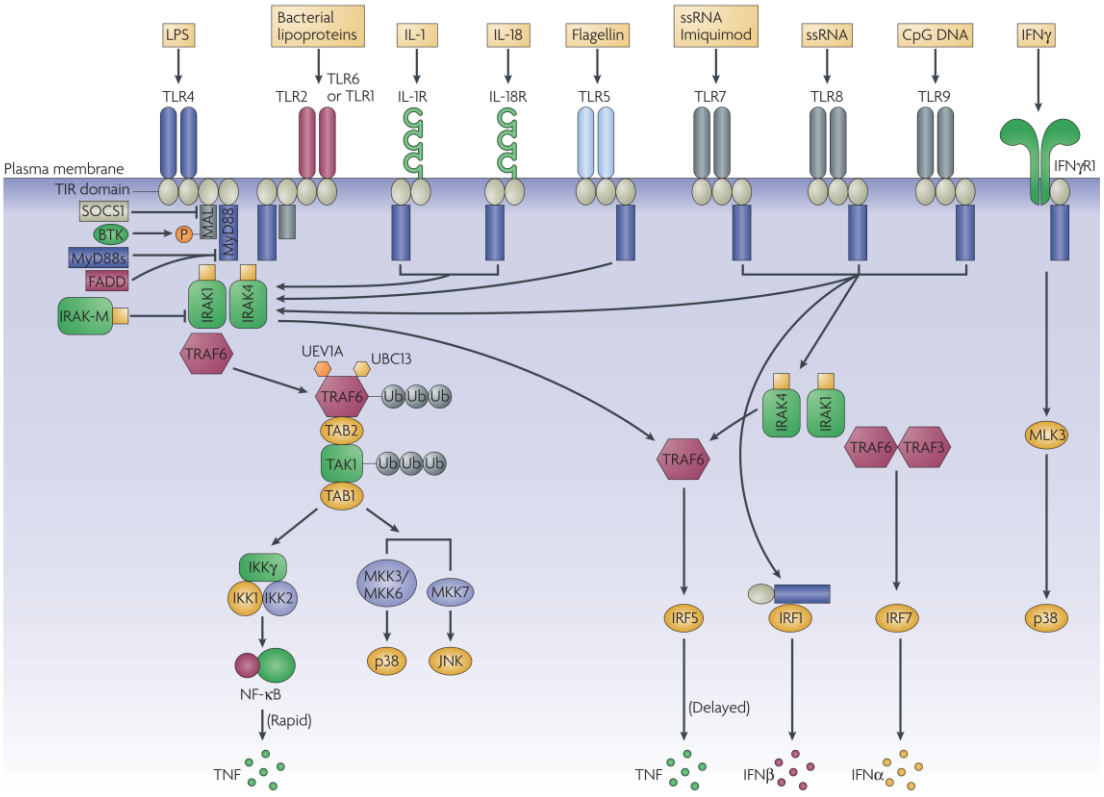



**Figure 1.3: The world of MyD88.** The figure cited from Luke A, J. O' Neill and Andrew G. Bowie, Nature Reviews Immunology, 2007 May; 7(5): 353-64, Copyright (2007), Rights Managed by Nature Publishing Group.

## 1.4D: Antigen Presenting Cells (APCs)

Both macrophages and dendritic cells (DCs), professional APC, are permissive to WNV infection and link the innate and adaptive immune responses (Suthar, Diamond, and Gale 2013). APCs are activated after WNV infection and express type 1 IFNs, pro-inflammatory cytokines and chemokines (Martina et al. 2008; Silva et al. 2007). Inflammatory cytokines IL-1 can directly act on naïve $CD4^+$ and $CD8^+$ T cells to provide a third signal (Curtsinger et al. 1999). TNF–α reduces viral replication in macrophages in

culture (Shrestha, Zhang, et al. 2008). Studies performed with macrophage-depleted mice showed a protection role for macrophages during WNV infection (Ben-Nathan et al. 1996). DCs have two subsets, which include plamacytoid (pDC) and myeloid (mDC). pDC-depleted mice had reduced type 1 IFNs response and impaired accumulation of virus-specific cytotoxic T lymphocytes (Persson and Chambers 2010). Activated mDC provide a robust type I IFNs and pro-inflammatory cytokine responses. MyD88 signaling regulates mDCs activation (Welte, Xie, et al. 2011; Xie et al. 2013).

## 1.4E: T Cells

$CD4^+$ and $CD8^+$ T lymphocytes play a key role in the immune response to pathogens (Netland and Bevan 2013). Three signals including signal 1 (antigen), signal 2 (co-stimulation) and signaling 3 (cytokines) regulate T cell activation. APCs provide the single 1 and signal 2 to activate T cells. Type 1 IFNs and IL-12 from innate immune cells serve as signal 3 (Kim and Harty 2014). A T cell response enters into three phases (expansion, contraction and memory) following an acute infection. In the expansion phase, naïve T cells expand and differentiate into effector T cells secreting IFNγ, TNFα and IL-2. In the following contraction phase, 90-95% T cells are eliminated by apoptosis. In final stage, only 5-10% of antigen-specific T cells become memory T cells (Kaech, Wherry, and Ahmed 2002; Badovinac and Harty 2006; Kim and Harty 2014). Naïve $CD8^+$ T cells are activated and differentiate into cytotoxic T lymphocytes (CTLs) to kill infected cells after an encounter with antigen (Kaech, Wherry, and Ahmed 2002). Memory $CD8^+$ T cells rapidly generate effector functions after an acute infection. IL-7 and IL-15 serve as signal 3 to promote memory $CD8^+$ T cell survival (Kaech and Cui 2012). Naïve $CD4^+$ T cells differentiate into different types of effector T cells. When priming environment has high level of type I IFNs or IL-12, naïve $CD4^+$ T cells differentiate into Th1 cells, which secrete IFNγ and TNFα and have antiviral responses.

When naïve $CD4^+$ T cells exposure to IL-4, they differentiate into Th2 cells, which secrete IL-4, IL-5 and IL-13 and cause immunopathology. With high levels of TGFβ and IL-6, naïve $CD4^+$ T cells differentiate into Th17 cells, which secrete IL-17A, IL-17F and IL-22 and contribute to immunopathology. Naïve $CD4^+$ T cells differentiate into $T_{reg}$ cells when they are exposed to TGFβ. Treg cells secrete IL-10 and TGFβ and regulate other subsets of $CD4^+$ T cells function (Swain, McKinstry, and Strutt 2012).

The roles of $CD4^+$ and $CD8^+$ αβ T cells during WNV infection will be discussed below. $Rag^{-/-}$ mice lack all T cells and B cells. $CD8^+$ T cells were isolated from naïve mice and transferred into $Rag^{-/-}$ mice. Adoptive transfer distinctly increases $Rag^{-/-}$ mice survival rate following WNV infection (Brien, Uhrlaub, and Nikolich-Zugich 2007). Another research group used CD8α-$chain^{-/-}$ mice as a model to study the role of $CD8^+$ T cells during WNV infection. $CD8^+$ $T^{-/-}$ mice had continual higher viral loads in the spleen, spinal cord and brain than in wild-type mice following WNV infection. $CD8^+$ $T^{-/-}$ mice were vulnerable to WNV infection. Infectious virus was absent in wild-type mice at day 35 post-infection but was still detectable in $CD8^+$ $T^{-/-}$ mice. Specific WNV IgM and IgG responses were similar between wild-type and $CD8^+$ $T^{-/-}$ mice. $CD8^+$ T cells kill target cells that display WNV antigens and MHC-class I. The results showed $CD8^+$ T cells protect the host from WNV infection and prevent WNV persistence (Shrestha and Diamond 2004). WNV-specific $CD8^+$ T cells were detected in brains for up to 16 weeks post-infection in WNV infected mice (Stewart et al. 2011). Isolated $CD8^+$ T cells from WNV-infected mice brains produced IFNγ and TNFα which were not expressed in Japanese encephalitis virus infected mice brains. Infiltrated $CD8^+$ T cells are WNV specific compared to other flaviviruses (Kitaura et al. 2011). The mechanism by which of $CD8^+$ T cells clear WNV from the CNS has been studied. TNF-related apoptosis-inducing ligand (CD253 or TRAIL) is expressed and used by $CD8^+$ T cells to clear WNV from neurons (Shrestha et al. 2012). Other mechanisms for $CD8^+$ T cells clearing WNV from infected neurons are perforin- or Fas lagand dependent (Shrestha, Samuel, and Diamond

2006; Shrestha and Diamond 2007). However, $CD8^+$ T cells also are involved in immunopathology in WNV infection. When $CD8^+$ T-cell-deficient mice were infected with low dose ($10^3$) of Sarafend strain of WNV, the mortalities were increased, however, when $CD8^+$ T-cell-deficient mice were infected with high dose ($10^8$) of WNV, the mortalities reduced (Wang, Lobigs, et al. 2003). Further studies showed old (18-22-month) mice had reduced WNV specific $CD8^+$ T cell responses than adult (3-6-month) mice following WNV infection (Brien et al. 2009; Uhrlaub et al. 2011). Adopter transfer $CD8^+$ T cells from adult mice but not from old mice, can rescue $Rag^{-/-}$ mice following WNV infection (Brien et al. 2009). There is a age-related difference in $CD8^+$ T cell response following WNV infection in mural model. In humans, $CD8^+$ T cell were purified from peripheral blood from adult (< 40 years) or old (> 61 years) patients within 1 month following symptom onset. The late stage or memory WNV-specific $CD8^+$ T cell responses were similar between the two groups (Lelic et al. 2012). A formalin–inactivated whole WNV virion or a DNA plasmid vaccine encoding WNV prM and E proteins induced WNV-specific protective $CD8^+$ T cells (Shrestha, Ng, et al. 2008). Overall, $CD8^+$ T cells play an important role during WNV infection and are important components to be targeted for vaccine development.

$CD4^+$ T cells provide help to activate $CD8^+$ T cells and B cells. It has also direct effect during influenza virus infection (Swain et al. 2006). All $IL\text{-}1R1^{-/-}$ mice died following WNV infection within 15 days post-infection. Compared to wild-type mice, $IL\text{-}1R^{-/-}$ mice had higher viral loads in the brain. $CD4^+$ T cells in the CNS had reduced IFNγ expression in $IL\text{-}1R^{-/-}$ than wild-type mice (Brien, Uhrlaub, and Nikolich-Zugich 2008). $CD4^+$ T cells play a protection role during WNV infection. $Rag^{-/-}$ mice were adoptively transferred with naïve $CD4^+$ T cells following WNV infection. Adoptive transfer of $CD4^+$ T cells protected mice from WNV infection. $CD4^+$ T cells were purified from WNV infected mice at day 7 post-infection and tested for cytotoxic capacity by cytotoxic T lymphocyte (CTL) assay. The data showed $CD4^+$ T cells had direct effector

function to prevent WNV infection (Brien, Uhrlaub, and Nikolich-Zugich 2008). The role of $CD4^+$ T cells also has age-related differences following WNV infection in a mural model. Adopter transfer of $CD4^+$ T cells from adult mice but not from old mice, can rescue $Rag^{-/-}$ mice following WNV infection (Brien et al. 2009). When mice were inoculated subcutaneously with $10^3$ PFU of WNV, $CD4^+$ T cells were detectable in mice brains at 12 weeks post-infection. Two activation markers CD69 and CD25 were expressed on $CD4^+$ T cells. $CD4^+$ T cells involve in regulation of WNV persistence. $T_{reg}$ cell is a subset of $CD4^+$ T cells and regulates effector T cells. In humans, symptomatic WNV-infected donors had lower $T_{reg}$ frequencies than asymptomatic donors. In mice, symptomatic WNV-infected mice had lower $T_{reg}$ frequencies than asymptomatic mice (Lanteri et al. 2009). $T_{reg}$ cells protect host to against severe WNV infection.

γδ T cells are innate lymphocytes. They do not have major histocompatibility complex (MHC) restriction and they respond quickly to viral infection. γδ T cells are permissive to WNV infection and produced IFNγ, TNFα and IL-6 production after infection (Fang et al. 2010). Compared to wild-type mice, $TCR\delta^{-/-}$ mice had a lower survival rate following inoculation I.P. with $10^2$ PFU of WNV and higher viral load in blood, brain, spleen at day 2 and day 4 post-infection. γδ T cells were major resource of IFNγ induction during WNV infection (Wang, Scully, et al. 2003). $IFN\gamma^{-/-}$ mice had a lower survival rate than wild type mice following WNV infection. Adoptively transfer of splenocytes from $TCR\beta^{-/-}$ mice into $TCR\delta^{-/-}$ mice can rescue γδ T cells following WNV infection; whose survival rate was similar to wild-type mice. When splenocytes from $TCR\beta^{-/-}$ $IFN\gamma^{-/-}$ mice were adoptively transferred into $TCR\delta^{-/-}$ mice, compared into wild-type mice, survival rate was reduced. The study showed γδ T cells expressed IFNγ to protect mice during early WNV infection (Wang, Scully, et al. 2003). In a following-up study, γδ T cells regulate memory $CD8^+$ T cells activation and expansion. $TCR\delta^{-/-}$ mice had a lower survival rate and higher viremia than wild-type mice after secondary challenged with $LD_{100}$ of WNV. Depleting of γδ T cells after a primary infection but

before a secondary infection do not affect mice survival rate compared to control group. These data indicate that γδ T cells regulated memory T cells development but do not act as memory T cells following WNV infection. Wild-type mice or TCRδ$^{-/-}$ mice were primary challenged with WNV, CD8$^{+}$ T cells were purified from survived mice and adoptive transferred into wild-type mice. The recipient mice were challenged with a lethal dose of WNV. Compared with the adoptive transfer of CD8$^{+}$ T cells from wild-type mice, mice with CD8$^{+}$ T cells from TCRδ$^{-/-}$ mice had reduced survival rate after a lethal dose of WNV infection. Compared with CD8$^{+}$ T cells from wild-type mice, CD8$^{+}$ T cells from TCRδ$^{-/-}$ mice had less expansion and activation. γδ T cells facilitate CD8$^{+}$ memory T cells response during WNV infection (Wang et al. 2006). γδ T cells also regulated DCs maturation during WNV infection (Fang et al. 2010). Splenic DCs from TCRδ$^{-/-}$ mice had a lower reduced percentage and mean fluorescence intensity (MEI) of CD40, CD80, CD86 and MHC class II than splenic DCs from wild type mice at day 3 post-infection with WNV (Fang et al. 2010). Co-culture γδ T cells, which purified from naïve or infected with WNV mice, with splenic DCs purified from naïve mice, the percentage and MFI of CD40, CD80, CD86 and MHC class II are induced by γδ T cells purified from infected mice (Fang et al. 2010). An *ex vivo* study showed that CD4$^{+}$ T cells were activated by splenic DCs from WNV infected mice (Fang et al. 2010). The third role of γδ T cells during WNV infection contributed DCs maturation to activate CD4$^{+}$ T cells during WNV infection (Fang et al. 2010). Two subsets of γδ T cells contribute to different roles following WNV infection. Mice with Vγ1$^{+}$ depletion were more susceptible to WNV infection, but those with Vγ4$^{+}$ depletion had reduced TNFα production and were more resistant to WNV infection (Welte et al. 2008). Vγ4$^{+}$-depleted mice have reduced viremia at day 7 post-infection and a reduced CD8$^{+}$ T cells number in their brains at day 10 post-infection than wild-type mice with inoculation I.P. $LD_{100}$ of WNV (Welte, Aronson, et al. 2011). Vγ4$^{+}$ T cells are major resource of IL-17 induction during WNV infection, but IL-17 blockage in mice does not cause any cytokine induction

or survival rate difference from wild-type mice following WNV infection (Welte, Aronson, et al. 2011). $V\gamma4^+$-depleted cells had reduced IL-10 and TGFβ induction following WNV infection, the role of $V\gamma4^+$ cells inhibited expansion of 1L-10 non-producing $V\gamma1^+$ T cells and IL-10 producing αβ T cells during WNV infection (Welte, Aronson, et al. 2011). The response of these two populations in young adult mice and aged mice are different following WNV infection. $V\gamma1^+$ cells expansion was slower and reduced in aged mice than in young adult mice after WNV infection, but the $V\gamma4^+$ cells number and percentage was maintained higher in aged mice than young adult mice without or at early WNV infection (Welte et al. 2008). Overall, $V\gamma1^+$ cells play a protective role and $V\gamma4^+$ cells contribute to viral pathogenesis following WNV infection.

## 1.4F: Antibody Responses:

The humoral immune response plays an important role to control WNV infection. B cell-deficient μMT mice are vulnerable to lethal infection of WNV, and have high viral load in the central nervous system (CNS). B cells protect mice against WNV infection (Diamond, Shrestha, et al. 2003). μMT mice could gain protection from passive transfer of human specific IgG within 2 days early before infection (Engle and Diamond 2003). Compared to 70% survival of wild-type mic, $IgM^{-/-}$ mice with IgM secretion deficiency were 100% dead and had higher viral load in serum within 96 hours after WNV infection. A follow-up study showed that $IgM^{-/-}$ mice were protected with passive transfer of polyclone anti-WNV IgM or IgG to against WNV infection (Diamond, Sitati, et al. 2003). The initial activation of B cells required type I IFN alpha/beta signaling in the lymph node, but not in the spleen (Purtha et al. 2008). A single-cycle flavivirus vaccine candidate, RepliVAX WN, induced B cell activation and memory B cell response via MyD88 signaling (Xia et al. 2013). IgG antibodies from infected human sera can recognize the capsid, prM/M and E proteins of WNV (Chabierski et al. 2013). Antibodies

responses have been shown directed at NS1, NS4B or NS5 region of WNV (Wong et al. 2003; Faggioni et al. 2012; Chung, Nybakken, et al. 2006).

## 1.5 WNV Vaccine Development

There are no approved WNV vaccines available for human use so far. Thus, there is an urgent need to develop a WNV vaccine. There are several types of WNV vaccines in development including a subunit vaccine, DNA vaccine, inactivated vaccine, recombinant viral vector vaccine, non-replicating single-cycle vaccine, chimeric vaccine and live attenuated vaccine.

**Subunit vaccine:** The WNV E gene is cloned as cDNA and expressed in *E.coli* as a fusion protein. It induces neutralizing antibodies and protectes mice from wild-type WNV challenge (Wang et al. 2001). WN-80E vaccine candidate produces a soluble form of the E protein which does not contain 20% of the C terminal region including membrane anchor domain and is produced in the *Drosophila* S2 cell expression system. WN-80E induces high titers of neutralizing antibody in mice (Lieberman et al. 2007) and protects monkeys from wild-type WNV infection (Lieberman et al. 2009). WN-80E has been tested in a phase I clinical trial and requires two or three doses to produce neutralizing antibodies. The disadvantage of this kind of vaccine is that it requires multiple doses to boost the immune response.

**DNA vaccine:** A human DNA vaccine candidate that expresses the prM and E proteins has been tested in a phase I clinical trial. It induces neutralizing antibody after 3-dose vaccination in 18-50 years of age healthy adults (Martin et al. 2007). A follow-up study shows it induces a T cell response and it induces similar antibody response between older age group (51-65 years) and younger age group (18-50 years) following 3-dose vaccination (Ledgerwood et al. 2011). The disadvantage of this kind of vaccine is that it requires multiple doses to boost the immune response.

**Inactivated Vaccine:** There are two approved inactivated vaccines for veterinary usage. There are formalin inactivated WNV-based vaccines. West Nile Innovator® is commercialized by Pfizer, which requires two vaccinations doses 3-6 weeks apart and an annual immunization boost. Vetera®WNV (Boehirnger Ingelheim) also requires two vaccinations and an annual immunization boost. The disadvantage of this kind of vaccine is that it also requires multiple doses to boost the immune response.

**Recombinant Viral Vector Vaccine:** A recombinant canarypox viral vector encodes WNV prM and E genes. It can protect dogs and cats from WNV challenge after two dose vaccination regimen at 28-day intervals (Karaca et al. 2005). This vaccine protects animals and has been commercialized for veterinary usage, which requires two injections and one annual boost (Brandler and Tangy 2013). Live-attenuated recombinant equine herpesvirus type 1 (EHV-1) expresses WNV prM and E protein and induces neutralizing antibody expression in horses following three injections. (Rosas et al. 2007). The disadvantage of this kind of vaccine is that it requires multiple doses to boost the immune response and low immunogenicity.

**Non-replicating single-cycle vaccine:** RepliVAX WN is a single-cycle vaccine and has WNV genome with a capsid gene deletion. Cell lines have Venezuelan equine encephalitis virus replicons (VEErep) and provide the C protein in trans for single-cycle virus (Widman et al. 2008). It protects mice and hamsters from lethal WNV challenge (Widman et al. 2008). However, both IgG titer in non-human primates and IgG specific for NS1 in mice are dose-dependent following RepliVAX WN injection. CAdVax-WNVII uses complex adenoviral vaccine vector as a backbone and contains WNV C, NS1, E and PreM genes. It is a single vaccine vector, and induces humoral and cellular immune response following two injections in 8 weeks intervals in mice (Schepp-Berglind et al. 2007). The disadvantage of this kind of vaccine is that it also requires multiple doses to boost the immune response.

**Chimeric vaccine:** There are two chimeric vaccines in clinical trials and the third one has been approved for veterinary usage. Yellow-fever 17D prM and E genes are replaced by the WNV prM and E genes to create ChimeriVax-WN-01. For human use, ChimeriVax-WN02 is created from ChimeriVax-WN-01 with additional mutations in the E proteins (E107 Leu—> Phe, E316 Ala—>Val, E440 Lys—>Arg). It has been tested in phase II clinical trials. Healthy adult ≥ 50 years of age have neutralization antibody expression following one injection of ChimeriVax-WN-02. The small amount of viremia could be detectable in subjects from day 2 to day 14 following vaccination which raises concerns about the safety of this vaccine (Dayan et al. 2012). WN/DEN4Δ30 is another type of chimeric vaccine, which contains an attenuated dengue 4 virus backbone due to a deletion of 30 nucleotides in the 3' noncoding region of the genome and the prM and E genes from WNV NY99 (Pletnev et al. 2006). It is currently being tested in a phase II clinical trial now (De Filette et al. 2012). WN/DEN4Δ30 can be transmitted by a vector-*Aedes albopictus* which can transmit both dengue virus and WNV (Hanley et al. 2005). This property of WN/DEN4Δ30 raises safety concerns (Brandler and Tangy 2013). Chimeric Vaccine protects the host with only one dose, but the safety issue is still a concern for this kind of vaccine development.

**Live attenuated vaccine:** The properties of live attenuated virus are low virulence but high immunogenicity to induce long-lasting protection immunity with a one dose vaccination regimen. Two live attenuated *flaviviruses* have been successfully developed and approved for human use. There are wildly use with rare side effects. SA 14-14-2 is a live attenuated Japanese encephalitis virus vaccine. It is approved as human vaccine in several countries.

(http://www.who.int/ith/vaccines/japanese_encephalitis/en/).

17D is a live attenuated yellow fever virus vaccine and vaccination began in the 1930. WHO announced that “The yellow fever “booster” vaccination given ten years after the initial vaccination is not necessary.”

(http://www.who.int/mediacentre/news/releases/2013/yellow_fever_20130517/en/)

An attenuated WNV WN1415 is a live attenuated WNV lineage 2 virus. Compared to the wild type strain, it has a set of non-conservative mutations mostly in non-structural proteins genes. It protected mice from lethal dose of WNV NY99 infection (Yamshchikov et al. 2004). However, it has not been studied afterwards. WNV NS4B-P38G attenuated strain is a vaccine candidate and studied in my project. NS4B-P38 residue is conserved in all flaviviruses. NS4B-P38G virus was created by site-directed mutagenesis from wild type infection clone NY99. Sequencing of NS4B-P38G indicates two additional compensatory mutations at NS4B-T116I and NS3-N480H compared to NY99. WNV NS4B-P38G had a small-plaque phenotype compared to wild type NY99. Infectivity titers of NS4B-P38G were similar to NY99 at 37ºC but lower than NY99 at 41ºC. NIH Swiss mice inoculated by intraperitoneally (i.p.) or intracerebrally (i.c.) with NS4B-P38G and NY99, result showed that the i.c. $LD_{50}$ of both NS4B-P38G and NY99 were 0.1 PFU, but differed when inoculated by the i.p. route as the $LD_{50}$ of NY99 was 0.5 PFU while the $LD_{50}$ of NS4B-P38G mutant was more than 10,000 PFU. NS4B-P38G mutant virus maintains neurovirulence and had reduced neuroinvasiveness in outbred mice. The plaque sizes of single mutation viruses NS4B-T116I or NS3-N480H were similar to NY99, and larger than NS4B-P38G mutant. The single mutation viruses had similar virulence to NY99 virus which indicated substitutions of T116I in NS4B or N480H in NS3 did not contribute to the attenuated phenotype of NS4B-P38G (Wicker et al. 2012). Parental strain WNV NY99 had been passaged once in Vero cells and twice in C6/36 cells. The WNV NS4B-P38G mutant was produced by utilizing site-directed mutagenesis and then passaged twice in Vero cells. Two strains do not have glycosylation difference. The detailed immune response against NS4B-P38G mutant is studied in my project.

## 1.6 Project Overview

Compared to all other types of vaccine, live attenuated viruses have been demonstrated to be the most efficient in the control of *flavivirus* infection. Thus, my studies has been focused on the development of live attenuated vaccine candidate for WNV.

In my preliminary studies, I examine WNV NS4B-P38G mutant virus infection in B6 mice. Our results showed that mice that mice that were inoculated by the i.p. route with 500 PFU WNV NY99 had only 13% survival, whereas mice infected with WNV NS4B-P38G at the same dose all survived. Secondary infection with a lethal dose of WNV NY99 in surviving mice from the above two groups showed that all mice were protected and survived. Compared to WNV NY99-infected mice, mice infected with WNV NS4B-P38G had reduced viremia at day 3 post-infection. Effector and memory T cells responses were greater following WNV NS4B-P38G infection than WNV NY99 infection, although the neutralizing antibody response was similar between the two viruses infected mice. In primary DCs, compared to WNV NY99 infection, WNV NS4B-P38G infection induced more IL-1β, IL-6, IL-12 and TNFα production at day 1 and day 4 post-infection and more IFNα and IFNβ at day 4 post-infection (Welte, Xie, et al. 2011). The mechanism by which WNV NS4B-P38G mutant induced higher immune response with low viral replication is the focus of my project. In primary DCs, deficient of MyD88 signaling, cytokine induction of IL-1β, IL-6, IL-12 and TNFα was lower than wild type samples at day 1 and day 4 post-infection, with less induction of IFNα and IFNβ at day 1 post-infection(Welte, Xie, et al. 2011). Based on these preliminary studies, I hypothesize ***MyD88-mediated signaling is important for the development of protective immunity against the attenuated WNV NS4B-P38G mutant infection.*** Three aims were developed to directly test this hypothesis. In **Aim 1**: we investigated the role of MyD88 signaling in primary immune response during WNV NS4B-P38G mutant infection. In **Aim 2**: We studied the role of MyD88 signaling pathway in memory immune response of

WNV NS4B-P38G mutant-immunized mice. In **Aim 3**: We determined innate cytokine response in human monocytic and macrophage cells following WNV NS4B-P38G mutant infection.

The studies presented in my dissertation are to identify important immune factors that contribute to a strong protective immunity during attenuated WNV infection. Results from this study will provide novel insights into *Flavivirus* vaccine development.

# Chapter 2 MATERIALS AND METHODS

Some of these materials and methods have been published (Xie et al. 2013; Xie et al. 2015; Xie et al. 2014).

**Mice:**

6-10- week- old B6 mice; 21-22 month-old B6 mice; 6-10-week old OTII and IL-1R$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88$^{-/-}$ and TLR7$^{-/-}$ mice (B6 X 129 $F_2$ background, Regeneron Inc., Tarrytown, NY) were bred to the B6 background by backcrossing for 7-10 successive generations (Town et al. 2009). Groups were age and sex-matched and housed under identical conditions. All animal experiments were approved by the Animal Care and Use Committee at the University of Texas Medical Branch.

**WNV Infection and Primary Cells:**

WNV NY99 infectious clone derived virus was passaged once in Vero cells and twice in C6/36 cells. WNV NS4B-P38G mutant (Wicker et al. 2012) virus was passaged once or twice in Vero cells to make a virus stock for cell culture. Mice were inoculated in some experiments, mice were injected i.p. with 500 plaque forming unit (PFU) or 1500 PFU of WNV NS4B-P38G mutant. In some experiments, surviving mice were re-challenged with a lethal dose ($LD_{100}$, 2000PFU) of wild-type WNV at day 30 post-primary infection. Infected mice were monitored twice daily for signs of morbidity. Bone-marrow (BM)-derived DCs and macrophages were generated as described previously (Daffis, Samuel, Suthar, Keller, et al. 2008). Briefly, BM cells were cultured for 6 days in RPMI-1640 supplemented with granulocyte-macrophgae-colony stimulating

factor (GM-CSF), and interleukin-4 (IL-4, Peprotech) to generate myeloid DCs or cultured with macrophage colony-stimulating factor (M-CSF; Peprotech) to generate primary macrophages. BM-DCs were infected with WNV with a multiplicity of infection (MOI) of 0.1, 0.2 or 0.5. BM-Macrophages were infection with WNV at a MOI of 0.02. Supernatants and cells were collected at 24 h or 96 h post-infection to measure viral load and cytokine production. For stimulation, BM-DC or macrophages (2 x $10^5$ cells/well ) were cultured at 37ºC in RPMI-1640 or DMEM (Invitrogen, Carlsbad, CA) in 96-well plates with 1 μg/ml of CL097 (Invitrogen). Supernatants and cells were harvested at 24 h to measure cytokine production. In some experiments, cells were treated with R848 (20 μg/ml) for 4 h before WNV infection.

**Cell Lines:**

Human monocytic leukemia cells (THP-1) were propagated in RPMI medium 1640 with L-glutamine and 25 nM HEPES buffer (Invitrogen, Carlsbad, CA) supplemented with 1 mM sodium pyruvate (Sigma). In some experiments, THP-1 cells were treated with phorbol 12- myristate 13-acetate (PMA, Sigma) to differentiate into macrophage cells as described previously (Park et al. 2007). Supernatants and cells were collected for measurement of viral load and cytokine production.

**Plaque Assay:**

Vero cells were seeded in 6-well plate in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) 24 h before infection. Serial dilution of culture supernatants were added and incubated for 1 h. Subsequently, MEM containing 1% low-melting-point agar was added, and the plates were incubated for 4 days. A second overlay of 4 ml 1% agar-medium containing 0.055% neutral red (Sigma) was then added to visualize plaques. Virus concentrations were determined as PFU/ml.

**Focus-Forming Assay (FFA):**

Vero cells were seeded into 12-well plates and incubated with sample dilutions for 1h. A semi-solid overlay containing 0.8% methylcellulose (Sigma-Aldrich, St. Louis, MO), 3% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, and 1% L-glutamine (Invivtrogen) was then added. At 48 h, the semi-solid overlay was removed, cell monolayers were washed with PBS, air dried and fixed with 1:1 of acetone: methanol solution for at least 30 min at -20 ºC. Cells were next subjected to immunohistochemical staining with a rabbit WNV polyclonal antibody (Dr. Robert Tesh's Lab, UTMB) followed by goat anti-rabbit HPR-conjugated IgG (KPL, Gaithersburg, MD) at room temperature for 1 h respectively. After secondary antibody, cells were incubated with a peroxidase substrate (Vector Laboratories, Burlingame, CA) until color developed. The number of foci was determined and used to calculate viral titers expressed as FFU/ml.

**Quantitative PCR for Viral Load and Cytokine Production:**

WNV-infected samples were resuspended in Trizol (Invitrogen) for RNA extraction. Complementary (c) DNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). The sequences of the primer-probe sets for WNV envelope (WNVE) and murine cytokine genes and PCR reaction conditions were described previously (Lanciotti et al. 2000; Wang et al. 2004; Colmont et al. 2011; Shen et al. 2010; Zhang, Shen, et al. 2008; Edwards et al. 2003). Both cDNA synthesis and PCR reaction condition were used to synthesize RNA from human cell lines. The primer sequences for innate cytokines and A20 and IκBα genes from human cell lines are listed in **Table 2.1**. The assay was performed in an iCycler (Bio-Rad, Hercules, CA). To normalize the samples in murine cells, the same amount of cDNA was used in a Q-PCR for β-actin. The ratio of the amount of amplified gene compared with the amount of β-actin cDNA represented the relative levels in each sample. To normalize the samples in

human cell lines, the same amount of cDNA was used in a Q-PCR for GAPDH. Gene expression was calculated based on $C_t$ values as described before (Welte, Aronson, et al. 2011). The ratio of the amount of amplified gene compared with the amount of GAPDH cDNA represented the relative levels in each sample.

**PCR Arrays:**

For human cell lines, a 32-gene PrimePCR assay (Bio-Rad) was performed following the manufacturer's protocol. Briefly, RNA was purified from non-infected and WNV-infected cells using an RNeasy extraction kit (Qiagen). cDNA was synthesized using the iScript family reverse transcription kits and then loaded onto 96-well PCR array plates for amplification on the CFX96 real-time PCR system (Bio-Rad). For BM-DCs, a 25-gene PrimePCR array (Bio-rad) was perfomed following the manufacture's protocol which is same as PrimePCR assay for human cell lines. Data analysis was performed by using CFX manager software. Four genes, including *GAPDH*, were selected as reference genes. Data were presented as clustergram and scatterplot.

**siRNA Knockdown for RIG-I, TLR4 and TLR7:**

Cells were transfected with 75 nM TLR4 specific siRNA, or 50 nM TLR7 specific siRNA, or 75 nM RIG-I specific siRNA (Sigma) using Superfect (Qiagen) per the manufacturer's instructions. Scrambled siRNAs (Sigma) were used as a negative control. Transfected cells were grown in RPMI medium containing 10% FBS. Q-PCR analysis of the TLR4, TLR7 and RIG-I mRNAs was used to confirm the effects of the siRNAs knockdown. At 48 h post-treatment, cells were infected with WNV (MOI of 0.5).

**Bioplex:**

Culture supernatant was collected for analysis of cytokine production using a Bio-Plex Pro Mouse Cytokine Assay (Bio-Rad).

**ELISA:**

Microtiter plates were coated with recombinant WNV-E protein (Wang et al. 2008) overnight at 4ºC at 100ng/well. Sera were diluted 1/30 in Phosphate Buffered Saline (PBS) with 2% Bovine Serum Albumin (BSA), and incubated for 1 h at room temperature. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma-Aldrich) at a dilution of 1/1000 in PBS-Tween was then added for 1 h. Color was developed with *p*-nitrophenyl phosphate (Sigma-Aldrich) and intensity determined at an absorbance of 405 nm by using a spectrophotometer.

**Flow Cytometry:**

Splenocytes were stained with antibodies for CD11c, CD80, CD86, CD3, CD4 or CD8 (e-Biosciences). To measure intracellular cytokine production, splenocytes were stimulated with WNV-specific NS3 and E peptides (RRWCFDGPRTNTILE and PVGRLVRVNPFUSVA, respectively (Brien, Uhrlaub, and Nikolich-Zugich 2008)) for $CD4^+$ T cells or WNV specific NS4B and E peptides (SSVWNATTA and IALTFLAV, respectively (Purtha et al. 2007; Brien, Uhrlaub, and Nikolich-Zugich 2007)) for $CD8^+$ T cells for 5 h at 37ºC. Golgi-plug (BD Biosciences) was added at the beginning of stimulation. Cells were stained with antibodies for CD4 or CD8, fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin before adding PE-conjugated anti-IFNγ, PE-Cy7-conjugated anti-TNFα or control rat IgG1 (e-Bioscience) and examined by using a C6 flow Cytometer (Accuri cytometers, Ann Arbor, MI). FoxP3 expression was analyzed by using a kit from eBiosciences according to the manufacturer's instructions. Data were analyzed by using CFlow Plus (Accuri cytometers).

***In vitro* T Cell Priming Assay:**

$CD4^+$ T cells and DCs were purified from splenocytes of naïve OTII transgenic mice and WNV-infected mice respectively by using anti-CD11c, and anti-CD4 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). $CD4^+$ T cells (2 X $10^6$ cells) were labeled with 0.5 µmol/l carboxyfluoroscein succinimidyl ester (CFSE) (Invitrogen) and mixed with DCs at a 10:1 ratio with or without OVA residue 323-339 (1 µg/ml, Genscript Corporation, Piscataway, NJ) for 5 days. Cells were harvested and $CD4^+$ T cells were gated for analysis of proliferation. Supernatant was collected at day 3 for analysis of cytokine production by using a Bio-Plex Pro Mouse Cytokine Assay (Biorad).

**Statistical Analysis:**

Data analysis was performed by using Prism software (Graph-Pad) statistical analysis. Survival curve comparisons were performed using the log rank test (equivalent to the Mentel-Haenszel test). Values for phenotype analysis, viral burden, and cytokine production experiments were presented as means ± SEM. The *P* values were calculated with a non-paired Student's t test. Statistical significance was accepted at $P< 0.05$.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| IFNα | 5'CCTGATGAAGGAGGACTCCATT3' | 5'AAAAAGGTGAGCTGGCATACG3' |
| IFNβ | 5'GTCTCCTCCAAATTGCTCTC3' | 5'ACAGGAGCTTCTGACACTGA3' |
| IL-1β | 5'ACGAATCTCCGACCACCACT3' | 5'CCATGGCCACAACAACTGAC3' |
| IL-6 | 5'GCAACACCAGGAGCAGCC3' | 5'AACTCCTTCTCCACAAGCGC3' |
| IL-12 | 5'TGGAGTGCCAGGAGGACAGT3' | 5'TCTTGGGTGGGTCAGGTTTG3' |
| TNFα | 5'GCCCAGGCAGTCAGATCATCT3' | 5'TTGAGGGTTTGCTACAACATGG3' |
| A20 | 5'TCCTCAGGCTTTGTATTTGAGC3' | 5'TGTGTATCGGTGCATGGTTTTA3' |
| IkBα | 5'CTCCGAGACTTTCGAGGAAATAC3' | 5'GCCATTGTAGTTGGTAGCCTTCA3' |
| GAPDH | 5'CTCAAGATCATCAGCAATGCCT3' | 5'AAGTTGTCATGGATGACCTTGG3' |

Table 2.1: Q-PCR primer sequence.

# CHAPTER 3: THE ROLE OF MYD88 SIGNALING IN PRIMARY IMMUNE RESPONSE TO WNV NS4B-P38G MUTANT INFECTION[1]

## Introduction:

WNV, a mosquito-borne flavivirus with a positive-sense, single-stranded RNA genome has caused annual outbreaks of viral encephalitis in North America since 1999 (Campbell et al. 2002). Infection of the central nervous system (CNS) commonly presents as encephalitis, meningitis or acute flaccid paralysis. Fatality has been recorded in 10% of WNV neuroinvasive disease-confirmed cases with a higher frequency in the elderly and immunocompromised. 20-50% of WNV convalescent patients have significant long-term morbidity (Carson et al. 2006a; Ravindra et al. 2004; Cook et al. 2010). Multiple approaches have been taken to develop WNV vaccines, including recombinant DNA (Davis et al. 2001), recombinant protein (Wang et al. 2001; Ledizet et al. 2005) and chimeric live attenuated vaccines (Monath et al. 2006). Nevertheless, no vaccines have been approved for human use.

The flavivirus genome encodes ten proteins – three structural proteins (E, membrane, C) and seven NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Anderson et al. 1999; Lanciotti et al. 1999). The NS4B protein is a 27-KDa, non-glycosylated, hydrophobic protein. Mutation studies in the NS4B protein have suggested

[1]Published in Vaccine. This journal allows including information without copyright as long as is properly cited. The Citation for the article is Xie G, Welte T, Wang J, Whiteman MC, Wicker JA, Saxena V, Cong Y, Barrett A, Wang T (2013). A West Nile virus NS4N-P38G mutant strain induces adaptive immunity *via* TLRY7-MyD88-dependent and independent signaling pathways. Vaccine 31(38):4143-51.

that it is associated with both viral replication and evasion of host immunity (Munoz-Jordan et al. 2005; Liu et al. 2005; Evans and Seeger 2007). The NS4B P38 residue is conserved in the majority of mosquito-and tick-borne flaviviruses. By utilizing site-directed mutagenesis of a WNV NY99 infectious clone, we have identified an attenuated mutation with a P38G substitution in NS4B protein. This P38G mutant has significantly reduced neuroinvasiveness in NIH Swiss outbred mice as compared to the parental wild-type WNV. Full genome sequencing of the mutant revealed two compensatory mutations – NS4B-T116I and NS3-N480H (Wicker et al. 2012). The NS4B-P38G mutant also induced a greater innate cytokine production and stronger T cell responses in B6 mice than wild-type WNV. Mice immunized with the NS4B-P38G mutant were protected from subsequent lethal wild-type WNV infection, which suggests that the NS4B-P38G mutant has suitable features for an ideal vaccine candidate (Welte, Xie, et al. 2011). The underlying immune mechanisms are not fully understood. TLRs play an important role in the regulation of innate and adaptive immunity to viral infection. The core TLR signaling pathway utilizes MyD88 as the primary adaptor (Iwasaki and Medzhitov 2004; Akira and Hemmi 2003), except TLR3, TLR7 expression has been shown to be important for protection of the host against wild-type WNV infection (Town et al. 2009; Welte et al. 2009). Here, we investigated the role of the TLR7-MyD88 signaling pathways in regulating protective immunity to WNV NS4B-P38G mutant infection in mice.

## Result:

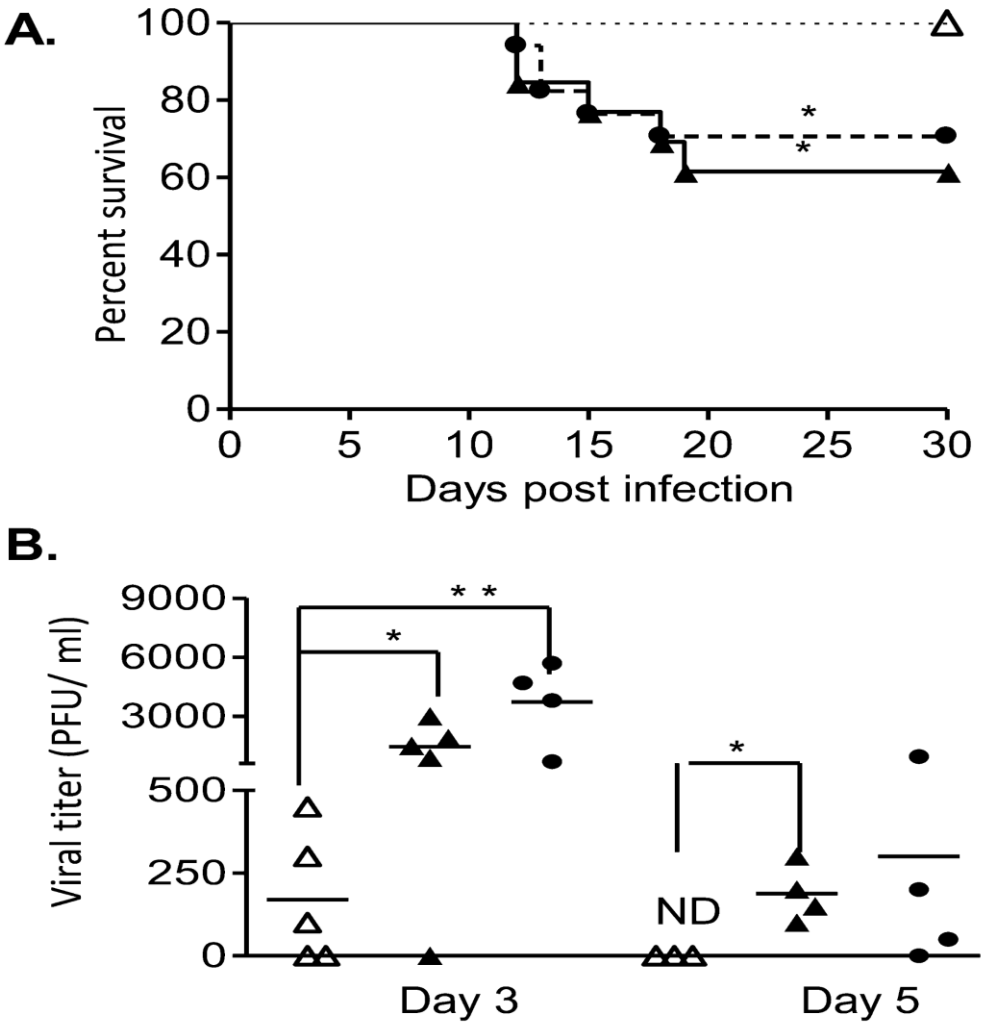
### TLR7-MyD88 Signaling is involved in Host Protection during WNV NS4B-P38G Mutant Infection

To determine the role of the TLR7 signaling pathway during WNV NS4B-P38G mutant infection, we compared the survival rates of wild-type, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice followed i.p. inoculation of 500 PFU of WNV NS4B-P38G mutant. All wild-type mice (100%) survived a four-week infection period compared to 61% and 71% survival in

MyD88$^{-/-}$ and TLR7$^{-/-}$ mice respectively (**Fig. 3.1A**). Viral load was measured by a plaque assay. At day3 post-infection, viremia in MyD88$^{-/-}$ and TLR7$^{-/-}$ mice was about 8- to 20-fold higher than that of wild-type mice (**Fig. 3.1B**). Infection was cleared at day 5 in wild-type mice, but was continuously detectable in MyD88$^{-/-}$ and TLR7$^{-/-}$ mice (**Fig. 3.1B**). Thus, both MyD88$^{-/-}$ and TLR7$^{-/-}$ mice were more susceptible to WNV NS4B-P38G mutant infection than wild-type mice.

## Innate Cytokine and Humoral Responses in WNV NS4B-P38G Mutant-infected Mice

Innate cytokine production was studied to further investigate the role of TLR7 and MyD88 during WNV NS4B-P38G mutant infection. On day3 post-infection, type 1 IFNs and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) were induced in blood from all groups of mice (**Fig 3.2A-D and F**). IL-1β gene expression was lower in MyD88$^{-/-}$, but not in TLR7$^{-/-}$ mice compared to wild-type mice (**Fig. 3.2C**). IL-12 gene expression was decreased in both NS4B-P38G mutant-infected MyD88$^{-/-}$ and TLR7$^{-/-}$ mice (**Fig. 3.2E**). B cell-mediated humoral immune responses are critical for the host defense against disseminated infection by wild-type WNV (Roehrig et al. 2001; Diamond, Shrestha, et al. 2003). The result showed sera WNV-specific IgM responses were reduced in MyD88-/- and TLR7-/- mice at days 3, 8 and 21 post-infection (**Fig. 3.2G**). No differences were observed in sera WNV-specific IgG responses in these mice (**Fig. 3.2H**). There results indicate TLR7-MyD88 signaling is involved in induction of IL-2 expression and WNV-specific IgM response following NS4B-P38G mutant infection.




[1]**Figure 3.1: TLR7-MyD88 signaling pathway is required for protecting host from WNV NS4B-P38G mutant infection.** (A) Mice were injected with WNV NS4B-P38G mutant and monitored twice daily for morbidity. Data shown are pooled from three independent experiments. n=12 for wild-type (WT) mice. n=13 and n=17 for $MyD88^{-/-}$ and $TLR7^{-/-}$ mice respectively. (B) Viral load in serum was determined by using plaque assay at the indicated time points post-infection. ND: not detected. Data are presented as means ±SEM. $^{*}$ $P< 0.05$ or $^{**}$ $P< 0.01$ compared to WT mice.

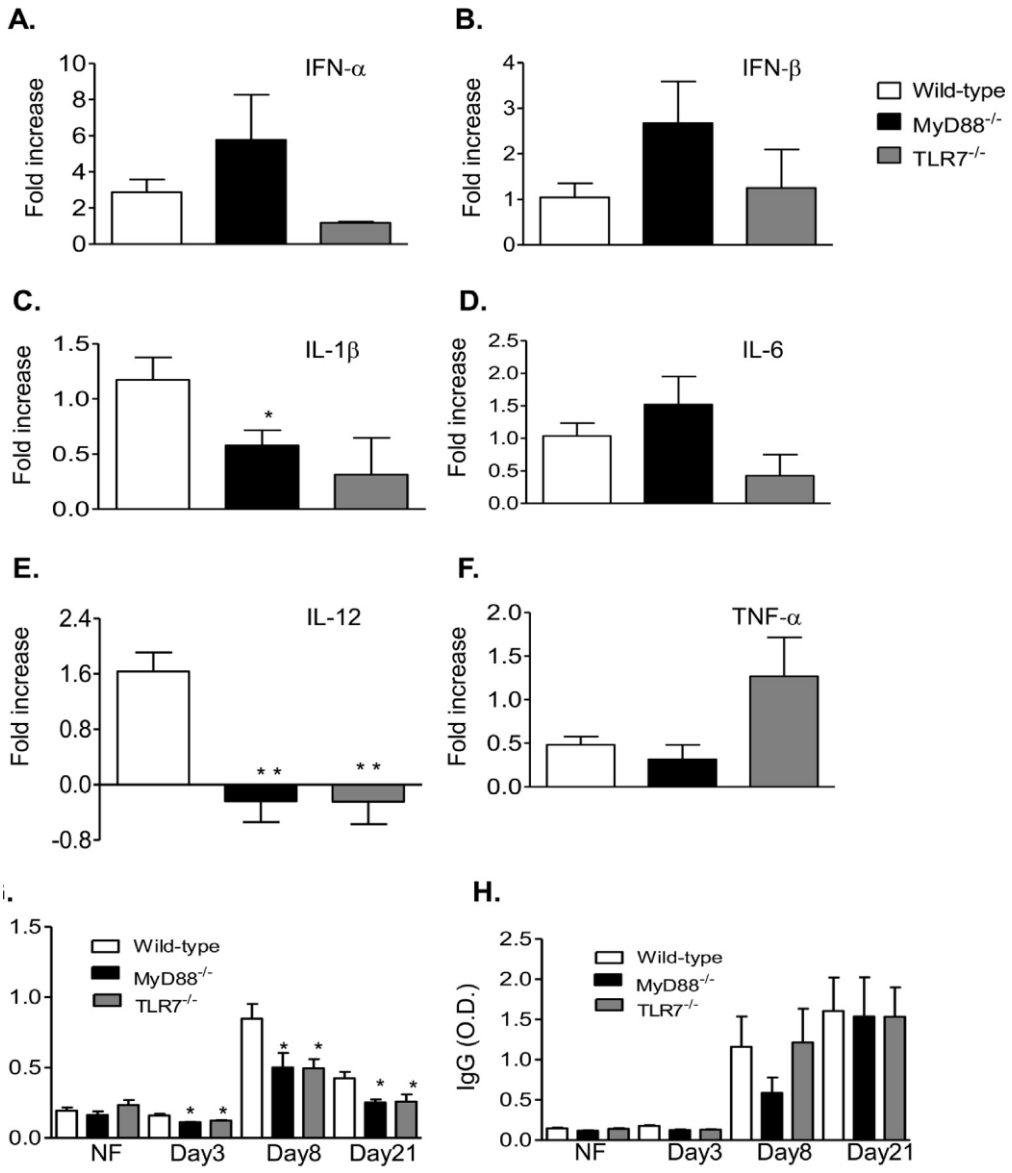
## T Cell Responses during WNV NS4B-P38G Mutant Infection

$CD4^+$ and $CD8^+$ T-cells are important for host survival following wild-type WNV infection and contribute to long-lasting protective immunity (Shrestha, Samuel, and Diamond 2006; Wang, Lobigs, et al. 2003). We studied T cell responses in NS4B-P38G mutant infected mice.

On day8 post-infection, the percentages of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ splenocytes in both $MyD88^{-/-}$ and $TLR7^{-/-}$ mice were lower than in wild-type mice upon *ex vivo* stimulation with WNV peptides (**Fig 3.3A and B**). The absolute numbers of splenic $CD4^+$ and $CD8^+$ T cells in $MyD88^{-/-}$ or $TLR7^{-/-}$ mice were reduced by 30-40% and 11-15% respectively (**Fig 3.3C**). At the later stage of infection (day21), there was a decrease in the percentage of $CD4^+IFN\gamma^+$, but not of $CD8^+IFN\gamma^+$ splenocytes in $MyD88^{-/-}$ mice (**Fig 3.3 D and E**). There were also significantly less splenic $CD4^+$ and $CD8^+$ T cells in $MyD88^{-/-}$ mice compared to wild-type mice (**Fig 3.3F**). In contrast, neither the percentages of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ splenocytes nor the number of splenic $CD4^+$ and $CD8^+$ T cells were different between $TLR7^{-/-}$ and wild-type mice (**Fig 3.3 D-F**). Thus, TLR7/MyD88 signaling is involved in initial T cell activation; TLR7-independent MyD88 signaling may be involved in T cell response at later stage of infection.

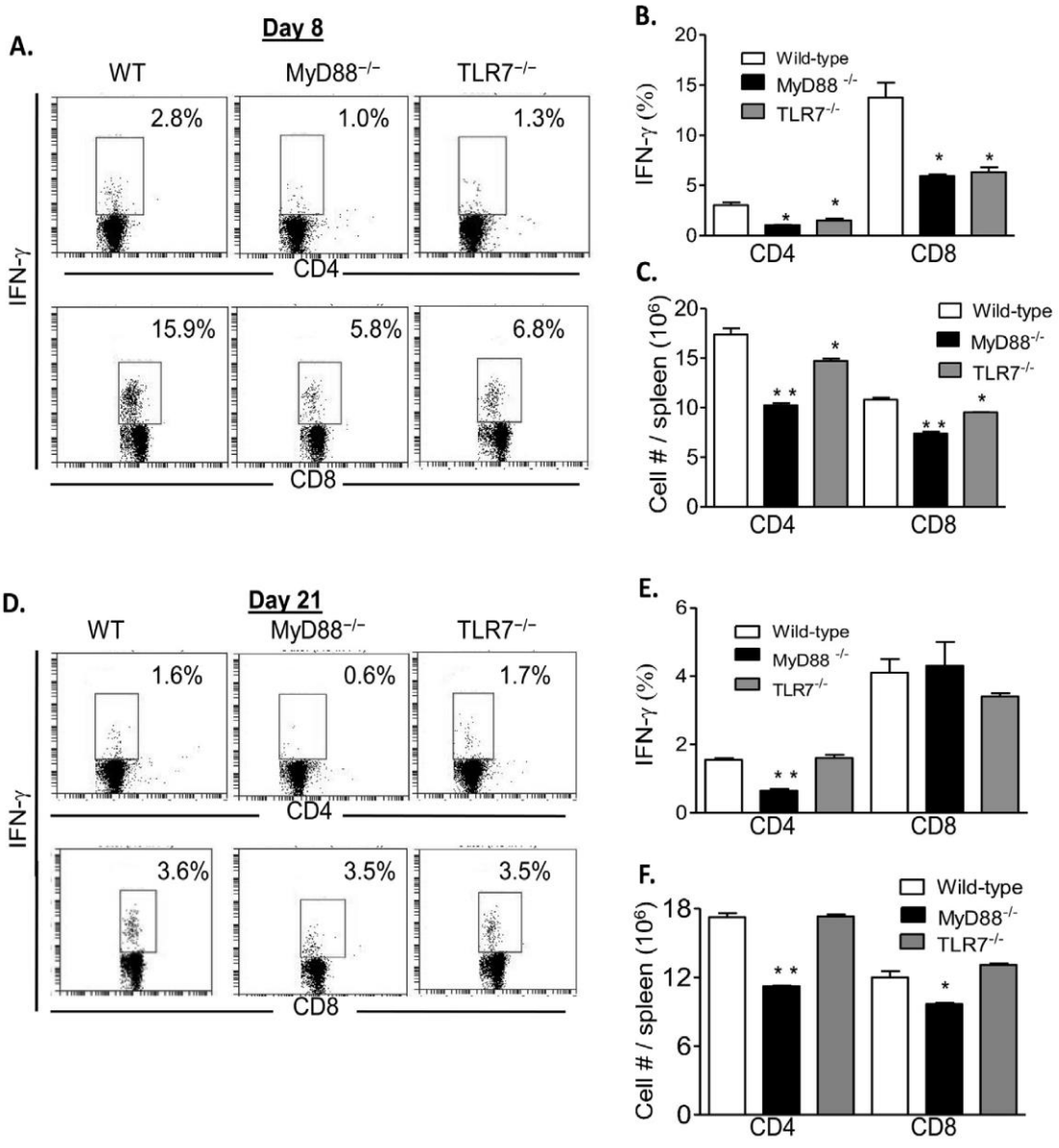
## Antigen Presenting Functions of WNV NS4B-P38G Mutant-infected DCs

DCs represent the most important antigen-presenting cells exhibiting the unique capacity to initiate primary T cell responses and are permissive to WNV infection. We previously demonstrated that NS3B-P38G mutant induced a MyD88-dependent pro- inflammatory cytokine response in DCs (Welte, Xie, et al. 2011). Here, we studied NS4B-P38G mutant infection in $TLR7^{-/-}$ DCs. Viral load in NS4B-P38G mutant infected- $TLR7^{-/-}$ DCs was greatly enhanced at day 4 post-infection compared to wild-type DCs (**Fig 3.4A**).

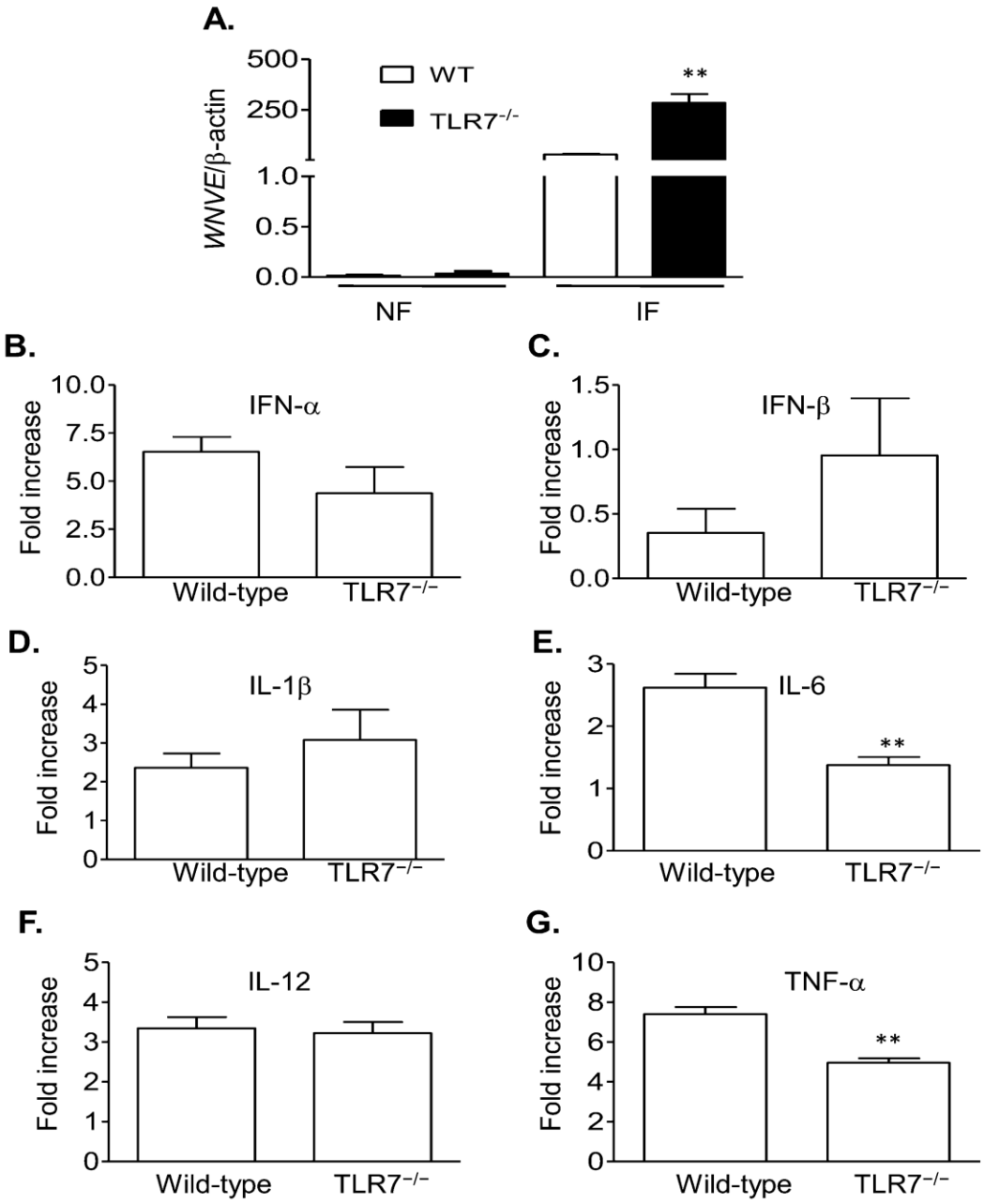



[1]**Figure 3.2: Innate cytokine and antibody production in WNV NS4B-P38G mutant-infected mice.** (A-F) Cytokine level is in blood at ay3 post-infection were determined by using Q-PCR. The fold of increase compared to that of the mock group is shown. Date are presented as means ±SEM, n= 4-5. (G and H) Humoral response during primary infection with WNV NS4B-P38G mutant. Sera were collected from non-infected (NF) or mice infected with NS4B-P38G mutant at days 3, 8 and 21 post-infection. The development of WNV specific IgM (G) or IgG (H) antibodies was determined by ELISA. Data are presented as means ±SEM, n= 4-7 pooled from 3 separate experiments. * P<0.05 or ** P<0.01 compared to wild-type mice.

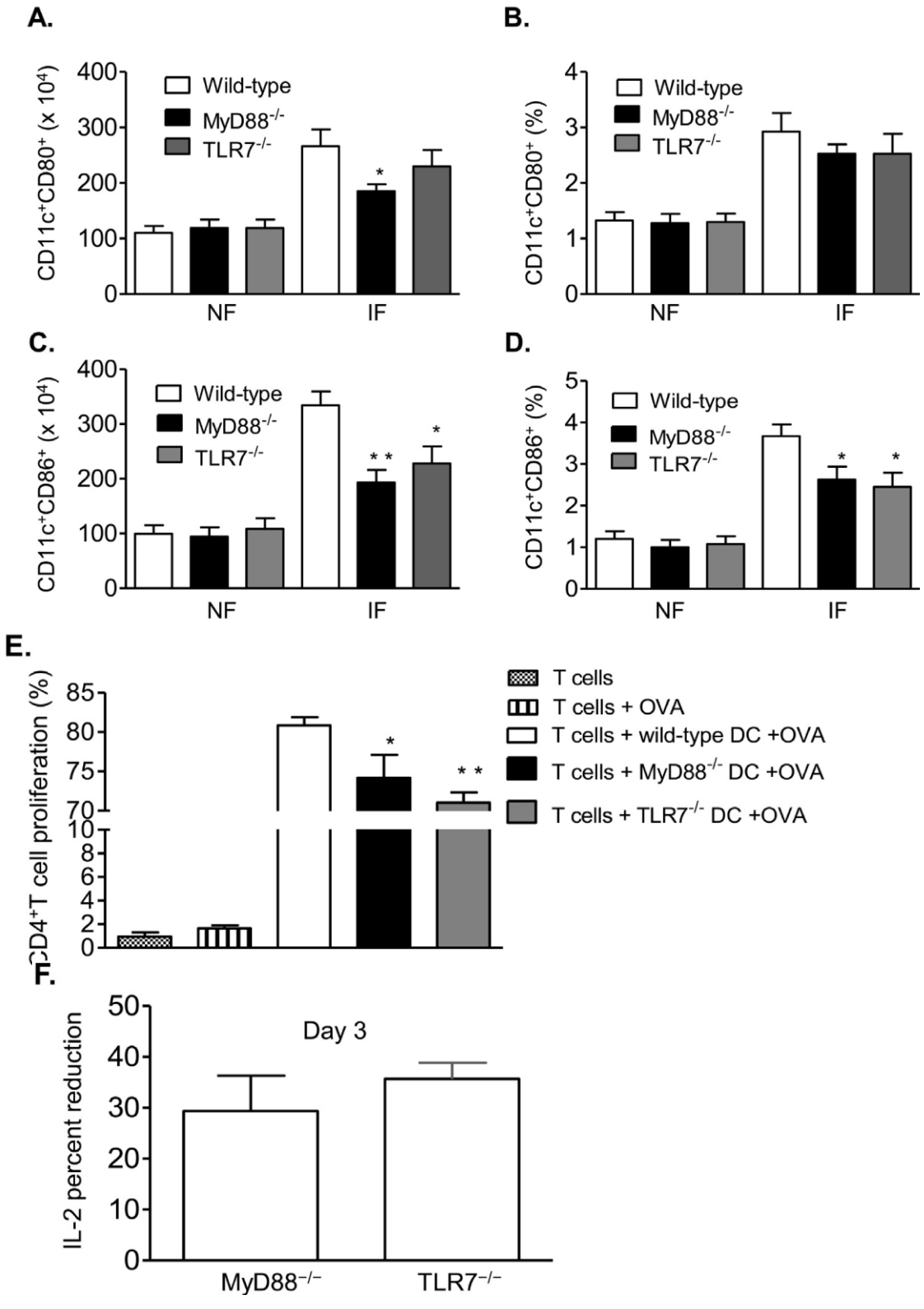
No differences in the levels of IFN-α, IFN-β, IL-1β and IL-12 were noted between the two groups (**Fig 3.4B-D and F**). However, IL-6 and TNF-α genes expression was significantly lower in TLR7$^{-/-}$ DCs (**Fig 3.4 E and G**). We next assessed splenic DCs phenotype in NS4B-P38G mutant infected mice. On day 3 post-infection, there was about 30-40% decrease in the total number of CD11C$^{+}$CD80$^{+}$ splenocytes in MyD88$^{-/-}$ mice when compared to the wild-type group (**Fig 3.5A**). There was no difference in the percentage of CD11C$^{+}$CD80$^{+}$ splenocytes between the two groups (Fig 3.5B). Neither the number nor the percentage of CD11C$^{+}$CD80$^{+}$ splenocytes was different between TLR7$^{-/-}$ and wild-type mice (**Fig 3.5A and B**). Nevertheless, we noted that the total number and the percentage of CD11C$^{+}$CD86$^{+}$ splenocytes were both decreased in MyD88$^{-/-}$ (32-42%) and TLR7$^{-/-}$ (30-32%) mice (**Fig 3.5C and D**). Finally, we tested the capability of DCs from WNV-infected wild-type, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice to activate naïve CD4$^{+}$ T cells in vitro. As shown in Fig. 3.5E and F, the proliferation rates and IL-2 production of OT II CD4$^{+}$ T cells co-cultured with DCs of WNV NS4B-P38G mutant-infected MyD88$^{-/-}$ and TLR7$^{-/-}$ mice were similar and were lower than of those co-cultured with DCs from wild-type mice. Overall, a deficiency in TLR7-MyD88-signaling pathway leads to an impaired antigen presenting capacity of DCs during WNV NS4B-P38G mutant infection.




[1]**Figure 3.3: T cell responses during primary infection of WNV NS4B-P38G mutant.** Mice were infected with WNV NS4B-P38G mutant, at day 8 (A-C) and day 21 (D-F) post-infection. Splenocytes were cultured *ex vivo* with WNV peptides for 5hr. Cells were harvested, counted and stained for IFNγ and CD4 or CD8. (A, B and D, E) $CD4^+$ or $CD8^+$ T cells were gated; percent of $IFN\gamma^+$ are shown. (C and F) Total number of T cell subsets per spleen is shown. Data are presented as means ±SEM, n=2-4 per group. $^*$ $P<0.05$ or $^{**}$ $P<0.01$ compared to wild-type mice.




[1]**Figure 3.4: WNV NS4B-P38G mutant infection in BM-DCs.** (A) Viral load was measured at day 4 post-infection. NS: non-infected. IF: NS4B-P38G mutant –infected cells. (B-G) Cytokine expression was determined using Q-PCR. Data are presented as the fold of increase compared to the mock infected. Results are representative of three experiments. n=3. ** P<0.01 compared to wild-type DCs.


A.
B.
C.
D.
E.
F.
Wild-type
MyD88-/-
TLR7-/-
CD11c+CD80+ (x 10⁴)
CD11c+CD80+ (%)
CD11c+CD86+ (x 10⁴)
CD11c+CD86+ (%)
NF
IF
T cells
T cells + OVA
T cells + wild-type DC +OVA
T cells + MyD88-/- DC +OVA
T cells + TLR7-/- DC +OVA
CD4+T cell proliferation (%)
Day 3
IL-2 percent reduction
MyD88-/-
TLR7-/-

[1]**Figure 3.5: DC maturation and antigen-presenting ability was reduced in MyD88$^{-/-}$ and TLR7$^{-/-}$ mice.** At day 3 post-infection, splenocytes were isolated and stained for CD11C and CD80 or CD86. The absolute number or the perdentages of CD80$^{+}$CD11C$^{+}$ (A and B) and CD86$^{+}$CD11C$^{+}$ (C and D) in splenocytes of non-infected (NF) and WNV-infected (IF) mice were shown. , n=4 per group. (E and F) *in vitro* T cell priming assay. CFSE labeled CD4$^{+}$ T cells from naïve OT II transgenic mice were co-cultured with DCs from WNV-NS4B-P38G mutant infected wild-type, MyD88$^{-/-}$ and TLR7$^{-/-}$ mice in the presence or absence of OVA 323-339. (E) Cells were harvested at day 5 and analyzed for T cell proliferation. (F) Supernatant was harvested at day 3 and measured for IL-2 production. The fold of increase compared to those co-cultured with wild-type DCs was shown. n=3-6 pooled from 2 separate experiments. $^{*}$ P<0.05 or $^{**}$ P<0.01 compared to wild-type mice.

**Old Mice Were More Vulnerable to WNV NS4B-P38G Mutant Infection than Young Mice.**

One major risk factor for fatality due to WNV infection in humans is aging (Campbell et al. 2002). In this present study, we investigate the protective efficacy of WNV NS4B-P38G mutant in old mice. We infected old mice (20 to 22- month-old) by the i.p. route with 1500 PFU of WNV NS4N-P38G mutant. Young mice (6 to 8- week-old) were used as controls. All young mice survived infection for at least 30 days. Surprisingly, only 67% old mice survived after infection **(Fig. 3.6A)**. Compared to young mice, old mice had around 3 fold higher viremia on day3 post infection **(Fig. 3.6B)**. Overall, WNV NS4B-P38G mutant is virulent in old mice.

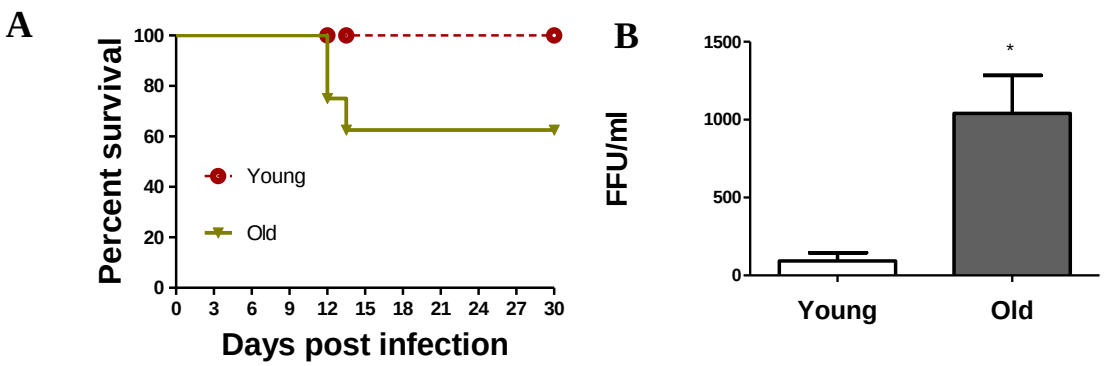



**Figure 3.6: Old mice were vulnerable to primary WNV NS4B-P38G mutant infection.** A: Suvival of young and old mice after i.p. injection with WNV NS4B-P38G mutant. n=14 and n=9 for young and old mice, respectively. B: Viral load in blood was determined by using FFA at day 3 post-infection. * P<0.05 compared to young mice.

$CD4^+$ and $CD8^+$ T cell response were impaired in $TLR7^{-/-}$ and $MyD88^{-/-}$ mice. To investigate the underlying mechanisms of virulence of WNV NS4B-P38G mutant infection in old mice, we examined T cell responses. As shown in **Fig. 3.7 A- B**, old mice had less number of $CD4^+$ T and percentage of $CD4^+$ and $CD8^+$ T cells on day 0 an day 8 post-infection than young mice. $CD4^+$ and $CD8^+$ T cells in old mice produced much less IFN-γ compared to young mice on day 8 post-infection **(Fig. 3.7 C-D).** $CD8^+$ T cells in old mice also produced less TNF-α compared to young mice on day 8 post-infection **(Fig. 3.7 E-F).** Old mice had more total number of TNF-$\alpha^+CD4^+$ T cells in spleen than young mice but less percentage of it **(Fig. 3.7 E-F)**. Overall, old mice had lower T cell responses during infection with WNV NS4B-P38G than that seen in young mice.

**Impaired TLR7 Signaling in Old Mice Led to Reduced Innate Cytokine Responses in WNV NS4B-P38G Mutant-infected DCs**

To investigate the impaired T cell responses in old mice, we next examine the DCs function. DCs present the most important APC exhibiting the unique capacity to initiate T cell response. In MyD88$^{-/-}$ and TLR7$^{-/-}$ mice, DCs led T cells response reduced with less innate cytokine response and less antigen presenting ability following WNV NS4B- P38G mutant infection. The viral load was tested in **Fig. 3.8A** following WNV NS4B-P38G infection. Compared to young BM-DCs, old group had similar viral load. TLR7 gene induction in aged BM-DCs was delayed and reduced compared to young BM-DCs (**Fig. 3.8B**). CL097, a TLR7/8 agonist stimulated IL-1β, IL-6, IL-12 and TNF-α production and upregulation of IFN-α and IFN-β gene expression in young BM-DCs; but this effect was significantly reduced in old BM-DCs (**Fig. 3.8C-H**). WNV NS4B-P38G mutant infection induced less IL-1β, IL-6 and TNF-α production and lower induction of IFN-α and IFN-β gene in aged DCs than in young DCs (**Fig. 3.8C-H**). Viral infection results have similar pattern as TLR7/8 agonist results. Overall, the data showed impaired TLR7 signaling contributed to lower innate cytokine response in DCs.

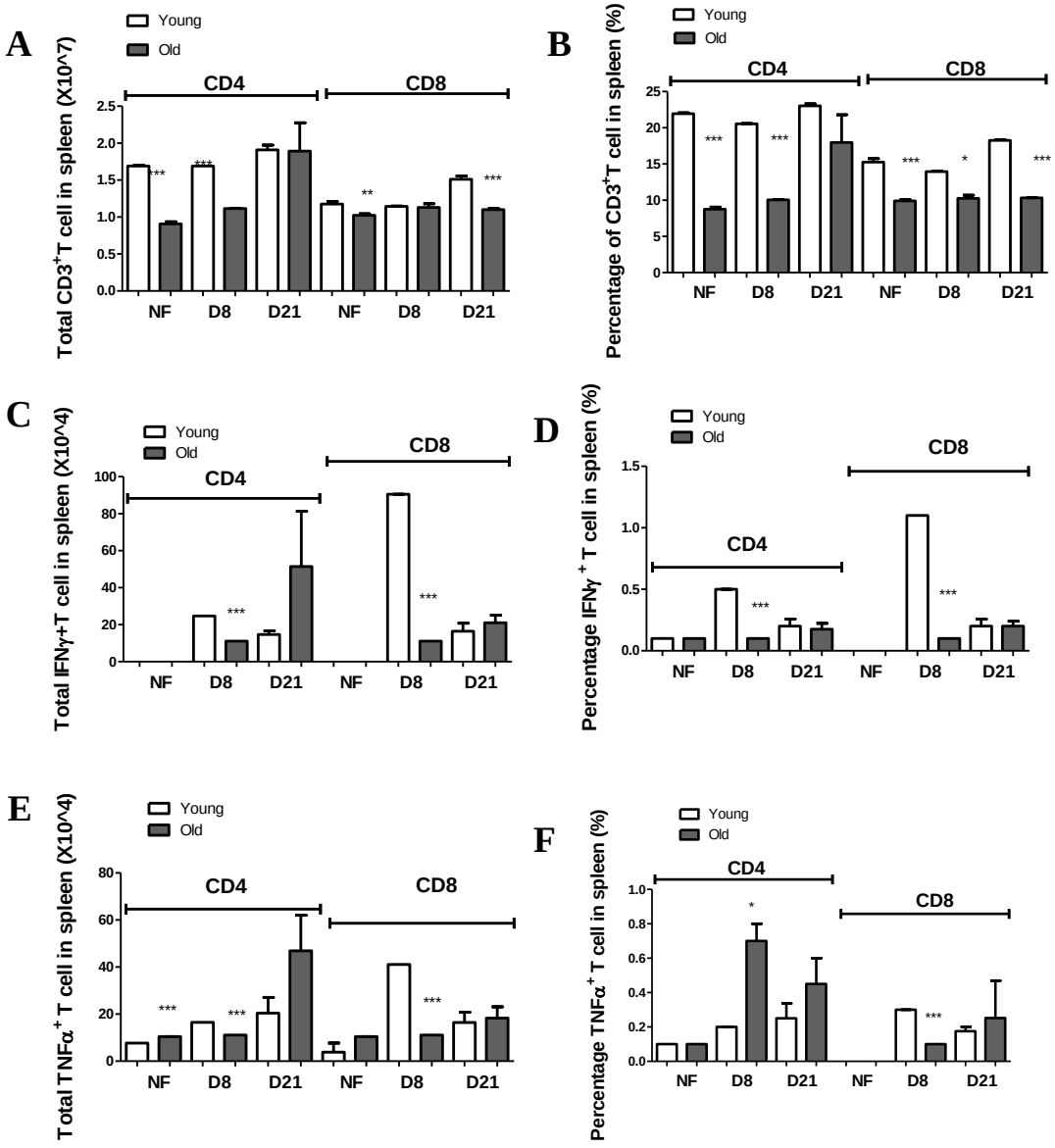
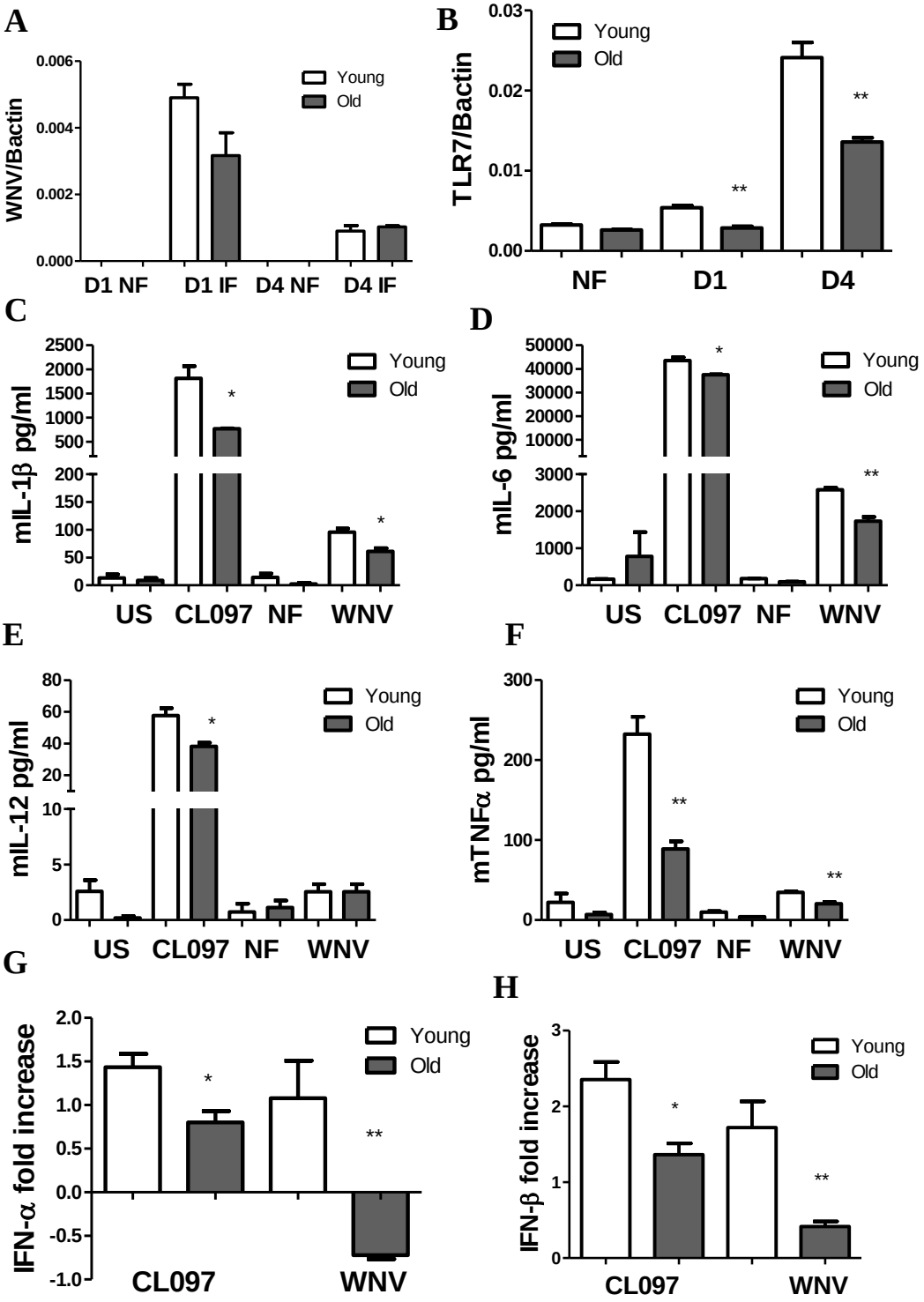



**Figure 3.7: T cell responses following primary infection with WNV NS4B-P38G mutant.** Splenocytes were harvested at day 0, day 8 and day 21 post infection with NS4B-P38G and were cultured ex vivo with WNV peptides for 5 h, and stained for IFNγ, TNFα and T cell markers. A: Percent positive $CD4^+$ or $CD8^+$ T cells is shown. B: Total number of $CD4^+$ or $CD8^+$ T cells per spleen is shown C: Percent positive IFN-$\gamma^+$ is shown. D: Total number of IFN-$\gamma^+$ T cell subsets per spleen is shown. E: Percent positive TNF-$\alpha^+$ is shown. F: Total number of TNF-$\alpha^+$ T cell subsets per spleen is shown. $^*$ $P<0.05$, $^{**}P<0.01$ and $^{***}$ $P< 0.001$ compared to young mice. n=4-5 mice/group from two separate experiments.


A
WNV/Bactin
0.006
0.004
0.002
0.000
Young
Old
D1 NF
D1 IF
D4 NF
D4 IF
B
TLR7/Bactin
0.03
0.02
0.01
0.00
Young
Old
**
**
NF
D1
D4
C
mIL-1β pg/ml
2500
2000
1500
1000
500
200
150
100
50
0
Young
Old
*
*
US
CL097
NF
WNV
D
mIL-6 pg/ml
50000
40000
30000
20000
10000
3000
2000
1000
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Young
Old
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US
CL097
NF
WNV
E
mIL-12 pg/ml
80
60
40
20
10
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Young
Old
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NF
WNV
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mTNFα pg/ml
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200
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Young
Old
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US
CL097
NF
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IFN-α fold increase
2.0
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Young
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*
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3
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1
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Young
Old
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CL097
WNV

**Figure 3.8: WNV NS4B-P38G mutant infection or TLR7 agonist stimulation in BM-DCs.** A: Viral load and B: TLR7 gene expression were measured at indicated time points by Q-PCR. C-F: Cytokine production was determined by using Bioplex. H-I: Cytokine expression was determined by Q-PCR. Data are presented as the fold of increase compared to the mock infection. Results are representative of three experiments. n=3-6. $^{*}$ $P<0.05$ and $^{**}P<0.01$ compared to young group.

## Discussion:

WNV NS4B-P38G mutant was highly attenuated in neuroinvasiveness and caused no lethality in B6 mice following systemic infection (Welte, Xie, et al. 2011). Our results show that MyD88$^{-/-}$ and TLR7$^{-/-}$ mice were more susceptible to NS4B-P38G mutant infection compared to wild-type mice. There was reduced WNV-specific IgM and effector T cell responses in both groups at early stage of infection. Induction of WNV-specific IgM and T cell responses limits viremia and dissemination into the CNS and protects the host against lethal wild-type WNV infection (Diamond, Sitati, et al. 2003; Sitati and Diamond 2006; Shrestha and Diamond 2004). Thus, TLR7-MyD88- signaling is involved in protective immunity against NS4B-P38G mutant infection. DC maturation is an innate response that leads to T cell mediated immunity to foreign antigens (Cella et al. 1996; Bennett et al. 1998). DCs can directly sense pathogen components *via* TLRs, up-regulate surface co-stimulatory molecules, secrete proinflammaotry cytokines and enhance antigen presentation (De Smedt et al. 1996; Fujii et al. 2004; Inaba et al. 2000). Our previous (Welte, Xie, et al. 2011) and present studies suggest that NS4B-P38G mutant induced a TLR7-MyD88-dependent proinflammatory cytokine expression in DCs. Further, MyD88$^{-/-}$ and TLR7$^{-/-}$ DCs showed a reduced maturation and impaired antigen presenting functions compared to wild-type DCs, which together suggests that the TLR7-MyD88-signaling pathway is involved in DC maturation and T cell priming during

NS4B-P38G mutant infection. The reduction on WNV specific IgM production observed in MyD88$^{-/-}$ and TLR7$^{-/-}$ mice may be the direct outcome of an impaired CD4$^{+}$ T cell response in the absence of MyD88 and TLR7 expression. Alternatively, lack of TLR7 ligation in B cells could lead to defective IgM response as reported by others (Jeisy-Scott et al. 2012; Fink et al. 2006).

Distinct inflammatory cytokines act directly on naïve T cells to provide a third signal, synergize with signals from the TCR and co-stimulatory receptors, to optimally activate differentiation and clonal expansion. IL-1 was shown to increase the proliferation of CD4$^{+}$ T cells in response to antigen and IL-2, which is consistent with effects on *in vivo* priming of CD4$^{+}$ T cells (Curtsinger et al. 1999). IL-2 is one main signal 3 cytokines produced for CD8$^{+}$ T cell in response to intracellular pathogens (Marrack, Kappler, and Mitchell 1999; Harty and Badovinac 2008). NS4B-P38G mutant induced lower levels of expression of IL-1β, IL-12, TNF-α and IL-6 in MyD88$^{-/-}$ BM-DCs compared to wild-type DCs (Welte, Xie, et al. 2011). Here, we noted NS4B-P38G mutant-infected TLR7$^{-/-}$ DCs only had a decreased IL-6 and TNF-α expression. Further, serum IL-12 gene expression was reduced in both MyD88$^{-/-}$ and TLR7$^{-/-}$ mice. This indicates that immune cells other than DCs in blood produced IL-12 in a TLR7-MyD88-dependent manner upon NS4B-P38G mutant infection. MyD88$^{-/-}$ mice also had a lower IL-1β expression, and this was not observed in TLR7$^{-/-}$ mice. The requirement for MyD88 usually represents the involvement of either a single or multiple TLRs or cytokine signaling *via* IL-1R or IL-18R (Mayer-Barber et al. 2010; Lin, Lo, and Wu 2010). It is likely that the engagement of other MyD88-dependent TLRs during WNV NS4B-P38G mutant infection contribute to IL-1β and/or IL-12 production, which may play an important role in CD4$^{+}$ and CD8$^{+}$ memory T cells development, though an early study (Town et al. 2009) suggests that the other MyD88-dependent TLRs were dispensable for the induction of protective immunity during wild-type WNV infection.

We also found TLR7 dysregulation is associated with impaired responses of DCs in WNV NS4B-P38G mutant infected old mice. Impaired DC functions in old mice contribute to lower effector T cell responses during the WNV NS4B-P38G mutant infection. An age-associated TLR7 dysregulation contributes to innate and adaptive T cell compromise and enhances host susceptibility to WNV NS4B-P38G mutant infection.

Live attenuated vaccines, which induce durable protective immunity, have been very successful at controlling *flavivrius* diseases. Our previous studies indicate that the NS4B-P38G mutant has the suitable features for an ideal vaccine candidate a greatly reduced potential for neurovirulence and neuroinvasiveness, restricted virus replication, and the ability to induce a robust protective immune response (Welte, Xie, et al. 2011). A major risk factor for fatality of WNV infection in humans is age (Campbell et al. 2002; Solomon et al. 2003). The alteration of the innate immune response in particular that of PRR signaling pathways has been reported in the elderly population (Kong et al. 2008; Panda et al. 2010). Treatment with agonists for TLRs or cytokines is one of the important strategies to boost vaccine efficacy (Lahiri, Das, and Chakravortty 2008). Thus, the investigation of the role of TLR7-MyD88-mediated-signaling pathways in induction of protective adaptive immune responses during WNV NS4B-P38G mutant infection can be utilized as paradigm to aid in the rational development of efficacious live attenuated *flavivirus* vaccines for this highly risk population.

# CHAPTER 4: THE ROLE OF MYD88 SIGNALING PATHWAY IN MEMORY IMMUNE RESPONSE OF WNV NS4B-P38G MUTANT IMMUNIZED MICE[1]

## Introduction:

WNV causes severe disease including encephalitis, meningitis and acute flaccid paralysis. Unfortunately, there are no vaccines have been approved for human use.

Previous studies have shown that the WNV NS4B-P38G mutant has three mutations including NS4B-P38G and two other compensatory mutations- NS4B-T116I and NS3-N480H (Wicker et al. 2012). WNV NS4B-P38G mutant had significantly reduced neuroinvasiveness in NIH Swiss outbred mice as compared to the parental wild-type WNV (Wicker et al. 2012). Young mice immunized with the NS4B-P38G mutant were protected from subsequent lethal wild-type WNV infection and had a greater innate cytokine production and stronger T cell responses than wild-type WNV infection. Overall, the study suggests that the WNV NS4B-P38G mutant had suitable features for an ideal vaccine candidate (Welte, Xie, et al. 2011).

Chapter 3 showed that TLR7- dependent- MyD88 signaling was involved in DC maturation and T cell priming during WNV NS4B-P38G mutant primary infection. Dysregulation of TLR7 reduced innate and adaptive T cells response in old mice. It is unknown for the MyD88 signaling, including TLR7-depndent-MyD88 signaling pathway and TLR7-indepndent-MyD88, in memory T cell responses in WNV NS4B-P38G immunized mice.

[1]Published in Vaccine. This journal allows including information without copyright as long as is properly cited. The Citation for the article is Xie G, Welte T, Wang J, Whiteman MC, Wicker JA, Saxena V, Cong Y, Barrett A, Wang T (2013). A West Nile virus NS4N-P38G mutant strain induces adaptive immunity *via* TLRY7-MyD88-dependent and independent signaling pathways. Vaccine 31(38):4143-51.

MyD88 signaling is also required by other TLRs or cytokine signaling via IL-1R or IL-18R which are called TLR7-indepndent-MyD88 signaling pathway (Mayer-Barber et al. 2010; Lin, Lo, and Wu 2010). T cell responses during a recombinant fowlpox virus infection are mediated via the IL-18R-MyD88 signaling pathway (Lousberg et al. 2011). A recent study showed that T cell-specific ablation of MyD88 induced Th 17 and Th1 cells responses in mice. IL-1 regulated naïve and memory $CD4^+$ T cells function (Schenten et al. 2014). The role of TLR7-independent-MyD88 signaling is not clear during WNV NS4B-P38G infection.

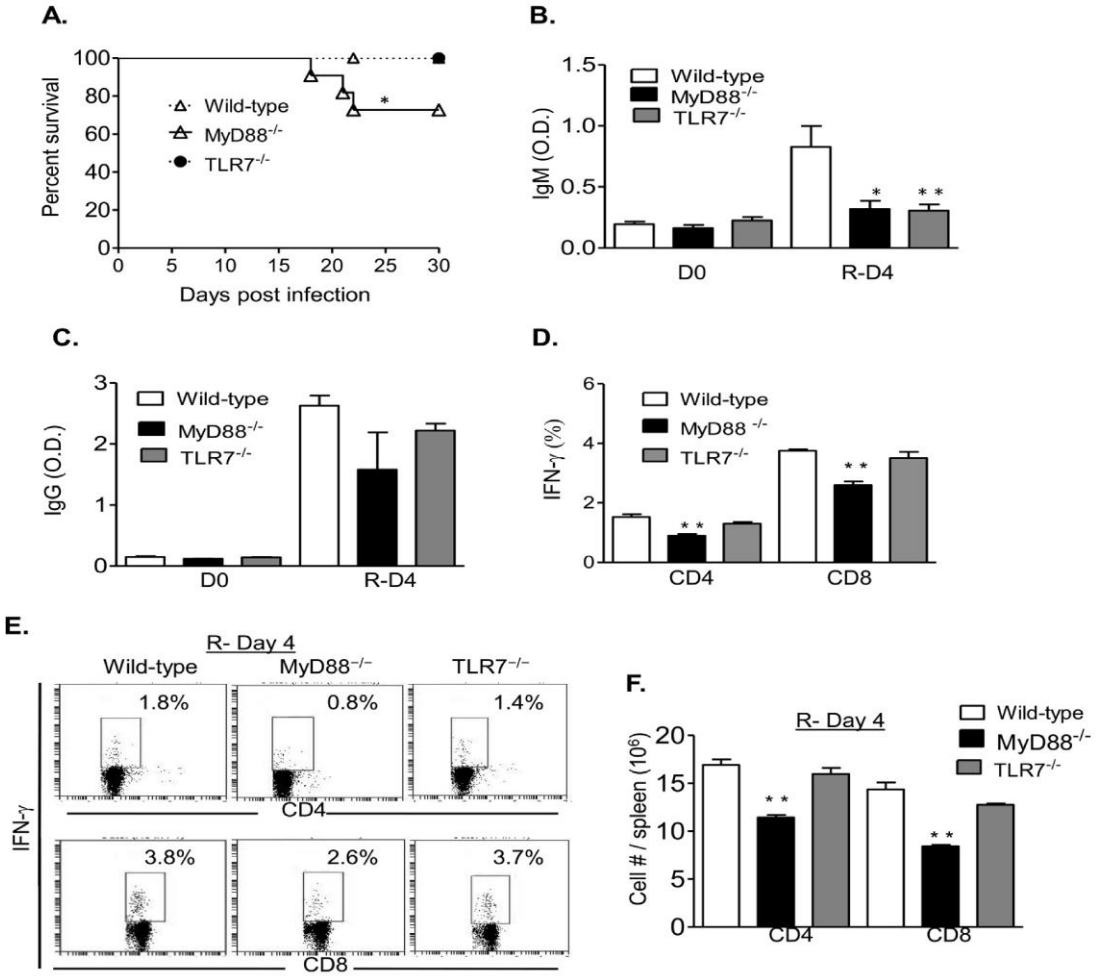
Here we investigated the role of the MyD88 signaling pathway in regulation protective immunity induced by WNV NS4B-P38G mutant infection to against wild-type WNV in mice.

## Result:

### T Cell Responses during Secondary Challenge of Wild-type WNV in WNV NS4B-P38G Mutant-immunized Mice

In previously study showed the TLR7-MyD88 singling contributed to induced primary immunity response following WNV NS4B-P38G infection. To further investigate the role of TLR7-MyD88 signaling in host memory response, we assessed the survival rate of NS4B-P38G mutant-immunized wild-type, $MyD88^{-/-}$ and $TLR7^{-/-}$ mice during secondary challenge of a $LD_{100}$ of wild-type WNV. Following one-month interval, immunized wild-type and $TLR7^{-/-}$ mice were all (100%) protected from a $LD_{100}$ wild-type WNV infection (**Fig 4.1A**). In comparison, only 72.7% of immunized $MyD88^{-/-}$ mice survived (**Fig 4.1A**). Furthermore, at day 4 post-secondary infection, the WNV-specific IgM response was decreased in both $MyD88^{-/-}$ and $TLR7^{-/-}$ mice (**Fig 4.1B**), while the IgG response (**Fig 4.1C**) in both groups was at levels similar to those in wild-type mice. There were lower percentages of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ splenocytes and

significantly less splenic $CD4^+$ and $CD8^+$ T cells in the $MyD88^{-/-}$ group compared to wild-type mice (**Fig 4.1D-F**). In contrast, neither the percentage of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ splenocytes nor the number of splenic $CD4^+$ and $CD8^+$ T cells were different between $TLR7^{-/-}$ and wild-type mice.



[1]**Figure 4.1: Adaptive immunity during secondary challenge of wild-type WNV.** Mice that survived from a primary infection with NS4B-P38G mutant were re-infected with a $LD_{100}$ of wild-type WNV. (A) Survival rate. Mice were monitored twice daily. n=13 for wild-type mice. n=11 for $MyD88^{-/-}$ mice. n=6 for $TLR7^{-/-}$ mice. (B and C) Sera were collected from mice at day 4 post-secondary infection with wild-type WNV NY99 (R-D4). The development of WNV specific IgM (B) or IgG (C) antibody was determined by ELISA. Data are presented as means ±SEM, n=4-6 per group. (D-F) At day 4 post-secondary infection, splenocytes were isolated and cultured *ex vivo* with WNV peptides for 5h. Cells were stained for IFNγ, and CD4 or CD8. (D and E) $CD4^+$ or $CD8^+$ T cells were gated; percent of $IFN\gamma^+$ are shown. (F) Total number of T cell subsets per spleen is shown. Data are presented as means ±SEM, n=2-4 per group. $^*$ $P<0.05$ or $^{**}$ $P<0.01$ compared to wild-type mice.

**WNV NS4B-P38G Protected Old Mice from Subsequent Lethal Wild-type WNV**

The old mice had impaired TLR7 signaling. We also tested the survival rate after re-challenge in old mice. Young and old mice were primary challenged with 1500 PFU/mouse WNV NS4B-P38G. On day 30, surviving mice of both groups were re-challenged with a lethal dose of wild-type NY99 and were monitored daily for mortality. All mice from two groups survived from secondary WNV infection with no obvious clinical signs after 30 days (**Fig. 4.2**). Old mice had similar phenotypes as $TLR7^{-/-}$ mice after re-challenged with lethal wild-type WNV.

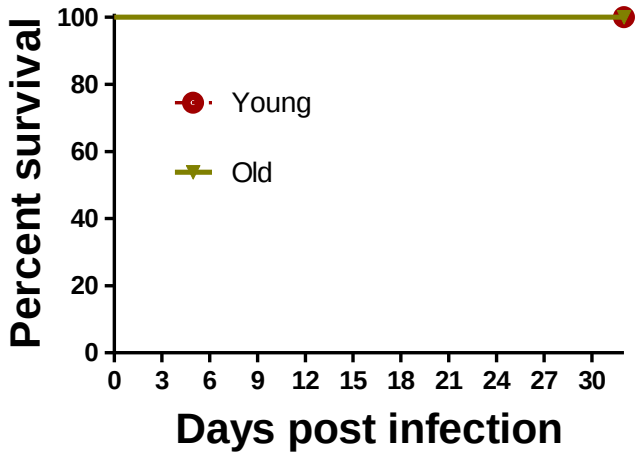



**Figure 4.2: Survival rate.** Young and old mice that survived primary WNV NS4B-P38G mutant infection were re-challenged with lethal wild-type WNV at day 30. n=5 and n=3 for young and old mice, respectively.

**Old Mice Had Similar T Cell Responses as Young Mice after Re-challenge**

Young and old mice primary were challenged with WNV NS4B-P38G mutant. On day 30 post-infection, surviving mice were re-challenged with lethal dose WNV. Splenocytes were collected from mice at re-challenged day 4. Compared to young mice, old mice had similar levels of $CD4^+$ and $CD8^+$ T cells (**Fig 4.3 A-B**). Old mice had a higher number of $CD8^+$IFN-$\gamma^+$ T cells, but a similar number of $CD4^+$ IFN-$\gamma^+$ T cells and a similar percentage of $CD4^+$IFN-$\gamma^+$ T and $CD8^+$IFN-$\gamma^+$ T cells in spleen (**Fig 4.3 C-D**). Old mice also had similar TNF-$\alpha$ expression in $CD4^+$ and $CD8^+$ T cells as young mice. (**Fig 4.3 E-F**). T cell responses remained unaffected in WNV NS4B-P38G immunized old mice during secondary infection with wild-type WNV.

**IL-1R Signaling Pathway Regulates T cell responses during secondary challenge of wild-type WNV**

The MyD88 adapter molecule is required for both TLR and the IL-1 superfamily signaling pathways (Mayer-Barber et al. 2010; Lin, Lo, and Wu 2010). We next examined T cell responses in NS4B-P38G mutant-immunized IL-1R$^{-/-}$ mice. At day 4 post-secondary infection, we found a lower percentage of CD8$^{+}$IFN$\gamma^{+}$ splenocytes (**Fig 4.4A and B**) and about 11% less splenic CD4$^{+}$ T cell in IL-1R$^{-/-}$ mice compared to those of wild-type groups (**Fig 4.4C**). These results suggest that the IL-1R-MyD88 signaling is involved in memory T cell response; TLR7 expression is dispensable for memory T cell development and host protection during secondary challenge by wild-type WNV.

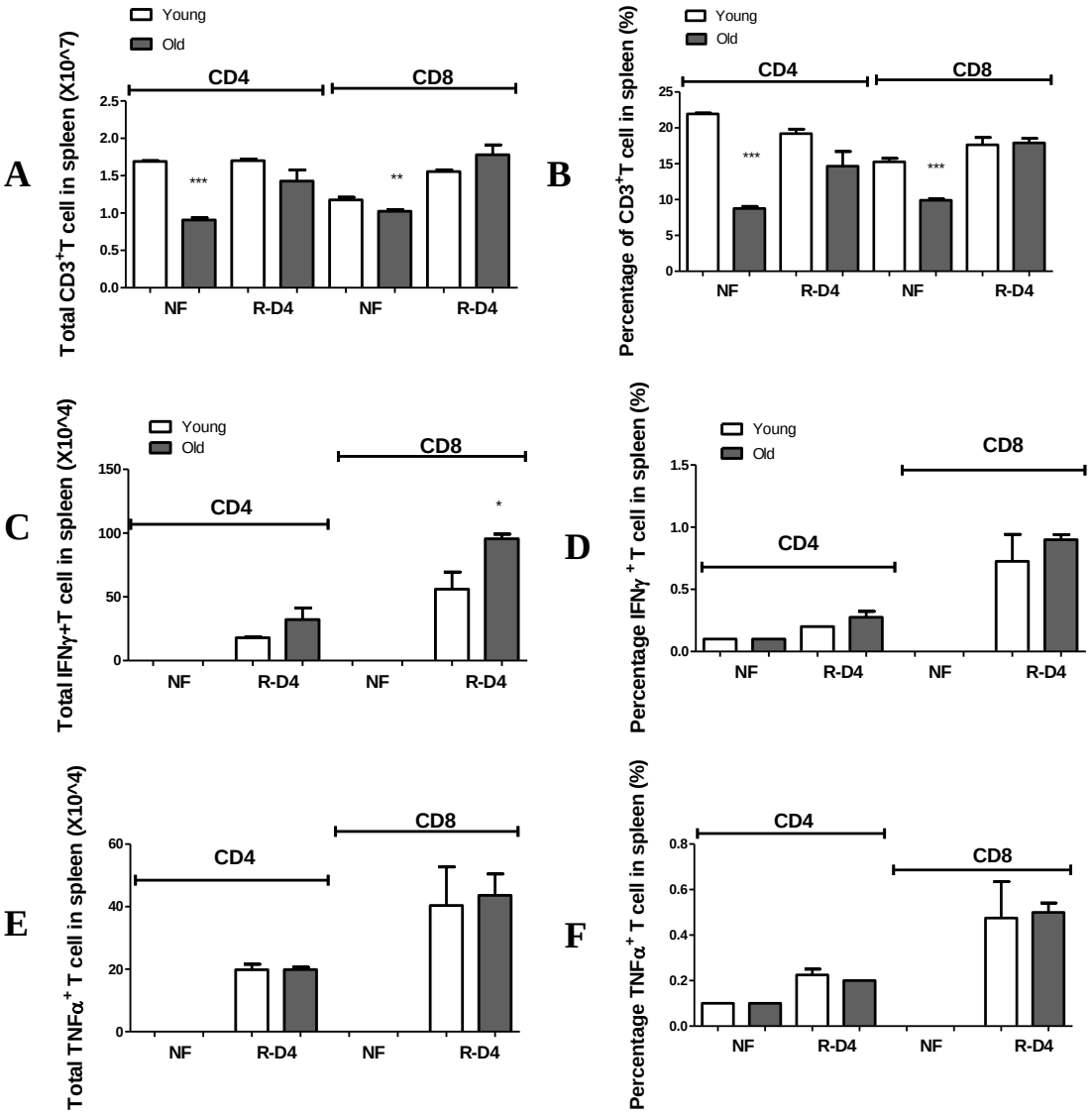
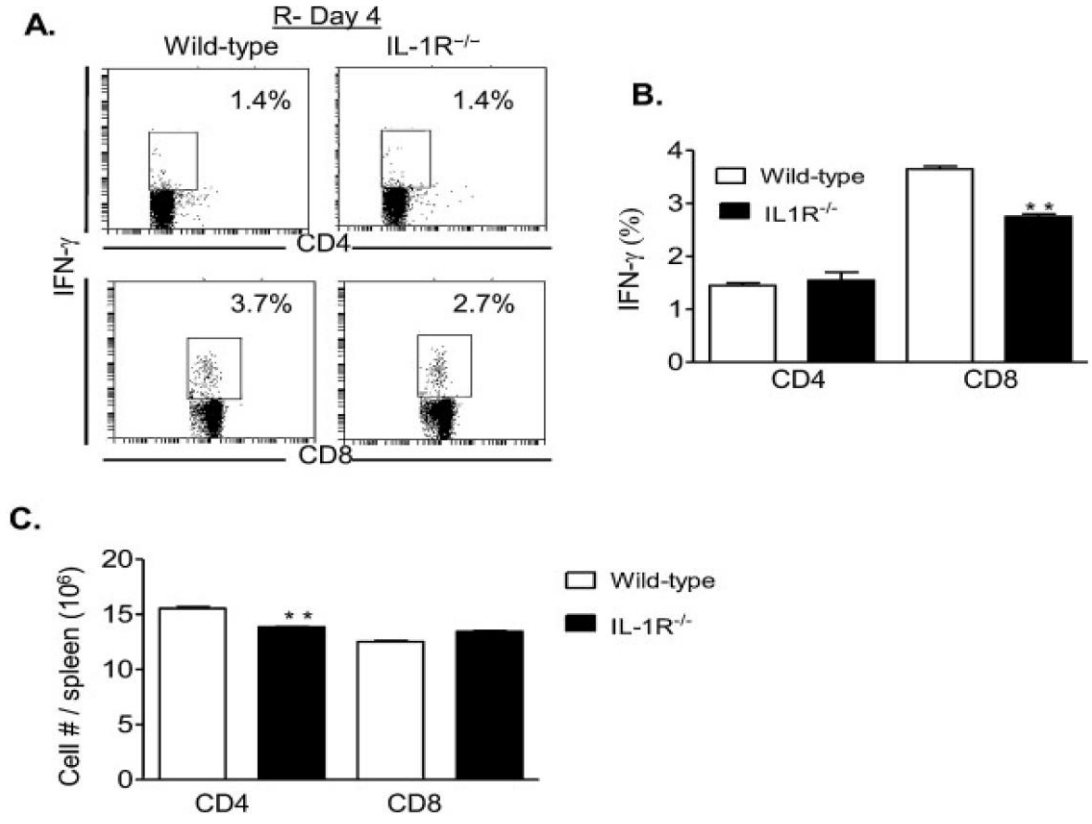



**Figure 4.3: T cell responses following primary infection with WNV NS4B-P38G mutant.** Splenocytes were harvested at day 0 or day 4 post re-challenge with wild-type NY99 and were cultured *ex vivo* with WNV peptides for 5 h, and stained for IFNγ, TNFα and T cell markers. A: Percent positive $CD4^+$ or $CD8^+$ T cells is shown. B: Total number of $CD4^+$ or $CD8^+$ T cells per spleen is shown C: Percent positive IFN-$\gamma^+$ is shown. D: Total number of IFN-$\gamma^+$ T cell subsets per spleen is shown. E: Percent positive TNF-$\alpha^+$ is shown. F: Total number of TNF-$\alpha^+$ T cell subsets per spleen is shown. $^*$ $P<0.05$, $^{**}P<0.01$ and $^{***}$ $P< 0.001$ compared to young mice. n=4-5 mice/group from two separate experiments.




[1]**Figure 4.4: T cell responses in NS4B-P38G-immunized-IL-1R$^{-/-}$ mice during secondary challenge of wild-type WNV.** At day 4 post-secondary infection, splenocytes were isolated and cultured *ex vivo* with WNV peptides for 5 h. Cells were stained for IFNγ, and CD4 or CD8. (A and B) CD4$^{+}$ or CD8$^{+}$ T cells were gated, percent of IFNγ$^{+}$ are shown. (C) Total number of T cell subsets per spleen is shown. ** P<0.01 compared to wild-type mice.

## Discussion:

WNV NS4B-P38G mutant was highly attenuated in neuroinvasiveness and caused no lethality in B6 mice following systemic infection (Welte, Xie, et al. 2011). In this study, we found that NS4B-P38G mutant-immunized TLR7$^{-/-}$ mice were fully protected from subsequent lethal wild-type WNV infection with a lower IgM response, but normal T cell functions. In contrast, NS4B-P38G mutant-immunized MyD88$^{-/-}$ mice displayed

lower IgM response and impaired memory T cell functions and were partially protected from secondary challenge with wild-type WNV. These results indicate that a TLR7-independent, MyD88-dependent memory T cell response is important for host protection during secondary challenge with wild-type WNV in NS4B-P38G mutant-infected mice. Moreover, other cytokine receptor signaling pathways, such as those of IL-1R and IL-18R, which rely on MyD88 expression, could be involved in memory T cell development. T cell responses during a recombinant fowlpox virus infection are mediated via the IL-18R-MyD88 signaling pathway (Lousberg et al. 2011).

We found IL-1R signaling is involved in memory T cell response during secondary challenge with wild-type WNV. Future studies will continuously be focused on the role of these receptors in memory T cell development during NS4B-P38G mutant infection.

In previously study, we found the WNV NS4B-P38G mutant was highly attenuated in young adult mice but old mice were vulnerable to WNV NS4B-P38G mutant infection. In this study, we found that all old mice were protected from WNV NS4B-P38G immunization to against wild-type WNV infection. Old mice developed similar levels of protective memory responses as those of young mice upon infection with the WNV NS4B-P38G infection.

WNV NS4B-P38G mutant has suitable features for an ideal vaccine candidate. TLR7-dependent-MyD88 signaling contributes the host primary response following WNV NS4B-P38G mutant infection. Dysregulation of TLR7 compromises innate and adaptive T cell responses in old mice. Treatment with agonists for TLR7 could protect old mice from WNV NS4B-P38G infection. In the future my study can focus on the the role of TLR7 agonists in old mice following WNV NS4B-P38G infection. TLR7-independent-MyD88 signaling contributes to memory T cell response and could be used as a model to study the mechanism of memory T cell development following WNV NS4B-P38G infection.

# CHAPTER 5: A WNV NS4B-P38G MUTANT STRAIN INDUCES CELL INTRINSIC INNATE CYTOKINE RESPONSE IN HUMAN MONOCYTIC AND MACROPHAGE CELLS[2]

## Introduction:

WNV is a mosquito-borne flavivirus that has been a serious public health problem in North America for more than a decade (Petersen, Brault, and Nasci 2013). WNV infection of the central nervous system (CNS, neuroinvasive disease) commonly presents as encephalitis, meningitis, or acute flaccid paralysis. The overall mortality rate among people who develop acute WNV neuroinvasive disease is about 10%, although it increases significantly in the elderly and immunocompromised (Gubler 2007; Kramer, Styer, and Ebel 2008). About 20-50% of WNV convalescent patients develop persistent sequelae years after their acute illness; symptoms included muscle weakness and pain, fatigue, headaches, memory loss, and ataxia (Carson et al. 2006b; Ravindra et al. 2004; Ou and Ratard 2005; Sadek et al. 2010). At present, no vaccines nor therapeutics for treatment or prevention of WNV infection have been approved for human use.

The WNV genome is a single-stranded, positive-sense RNA molecule, which is translated into a single polypeptide and co-and post-translationally processed into 10 proteins-three structural proteins (E, membrane, and nucleocapsid) and seven NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Anderson et al. 1999; Lanciotti et al. 1999). The NS proteins are known to be associated with evasion of host innate immunity and are important determinants in flaviviral pathogenesis (Wilson et al. 2008; Scholle and Mason 2005; Diamond 2009; Munoz-Jordan et al. 2003). The NS4B P38 residue is

[3]This chapter was published in Vaccine. This journal allows including information without copyright as long as is properly cited. The Citation for the article is Xie G, Luo H, Tian B, Mann B, Bao X, McBride J, Tesh R, Barrett AD, Wang T (2015). A West Nile virus NS4N-P38G mutant strain induces cell intrinsic innate cytokine response in human monocytic and macrophage cells. Vaccine 33(7):869-78.

conserved in most vector-borne flaviviruses. An attenuated mutation with a P38G substitution in NS4B protein was recently made by utilizing site-directed mutagenesis of a WNV NY99 infectious clone (Wicker et al. 2012). Full-genome sequencing of the NS4B-P38G mutant revealed two other compensatory mutations-NS4B-T116I and NS3-N480H. Although the NS4B-P38G mutant showed enhanced vector competence in culex tarsalis mosquitoes than the wild-type parental control strain (Van Slyke et al. 2013), studies in animal models have indicated that the NS4B-P38G mutant has several features that may make it an important mutation for inclusion in live attenuated vaccine candidates. Frist, it is highly attenuated in mice compared to the wild-type WNV NY99. Second, induces stronger innate and adaptive immune responses. Lastly, mice immunized with the NS4B-P38G mutant were all protected from subsequent lethal with-type WNV infection (Welte, Xie, et al. 2011). We previously reported that myeloid differentiation factor 88-depdentdent innate signaling pathways contribute to a robust, cell-mediated immune response in mice (Xie et al. 2013); and we postulated that WNV NS4B-P38G mutant could have a similar impact on host immunity in humans. In this study, we characterized the NS4B-P38G mutant infection and immunity in two human cell lines (THP-1 cells and THP-1 macrophages) as a first model to investigate the utility of the NS4B-P38G mutant as a potential vaccine candidate in humans.

## Results:

### WNV NS4B-P38G Mutant Produced Higher Levels of Viral RNA, But Not Infectious Virus in Both THP-1 Cells and THP-1 Macrophages

THP-1 is a well-characterized monocytic cell line and is known to be permissive to *flavivirus* infection (Mather et al. 2003; Tsai et al. 2009). Initial studies involved infection of THP-1 cells with the WNV NS4B-P38G mutant and its parent WNV NY99 strains at a MOI of 5. Multiplication kinetics was measured on days 1 and 4 post-

infection by a plaque assay. Viral titers in supernatants of WNV NY99- infected THP-1 cells increased more than 100-fold from day 1 to day 4; whereas there was no evidence of detectable infectivity in the supernatant of THP-1 cells-infected with WNV NS4B-P38G mutant on days 1 and 4 **(Fig 5.1A).** The differentiation of THP-1 monocytes into macrophages can be achieved with the treatment of PMA (Park et al. 2007). Therefore, THP-1 cells were next stimulated with PMA and the experiment was repeated. WNV NY99 had a similar replication rate in THP-1 macrophages as THP-1 cells, and again no viral infectivity was detected by plaque assay on either day 1 or 4 post infection, following infection with the WNV NS4B-P38G mutant **(Fig 5.1B).** Significantly, although it failed to produce detectable infectious virus, WNV NS4B-P38G mutant-infected THP-1 cells and THP-1 macrophages had 2373 and 395 fold higher viral RNA levels, respectively, than WNV NY99-infected cells at 24h **(Fig. 5.1C).** Overall, these results indicate that the NS4B-P38G mutant multiplies poorly, if at all, in THP-1 monocyte and macrophage cells.

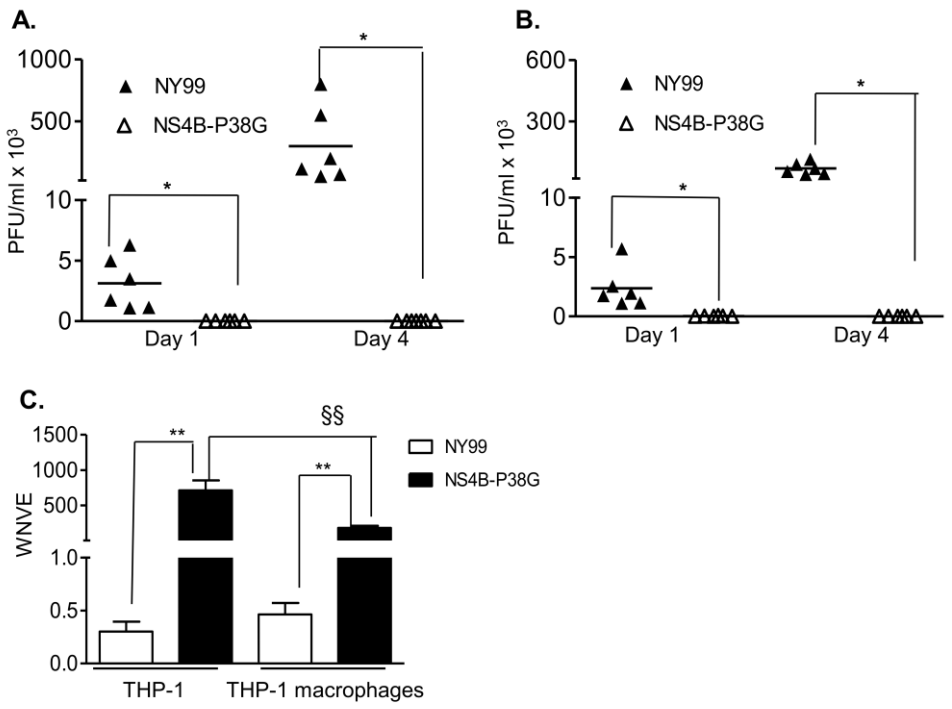



**Figure 5.1: WNV infection in THP-1 cells and THP-1 macrophages.** (A) THP-1 cells (A) and THP-1 macrophages (B) were infected with WNV NY99 and WNV NS4B-P38G mutant at a MOI of 5. (A and B) The viral titers at indicated time points post-infection were measured by using a plaque assay. (C) Viral tier at 24 h post-infection was measured by a Q-PCR assay. Data are presented as means ±SEM. n=4-6. $^{*}$ P< 0.05 or $^{**}$ P< 0.01 for NY99 vs. NS4B-P38G. $^{§§}$P< 0.01 for THP-1 vs. THP-1 macrophages. Results presented are one representative of two similar experiments.

**WNV NS4B-P38G Mutant Induced Higher Innate Cytokine Responses than WNV NY99 in THP-1 Cells and THP-1 Macrophages**

We have previously shown that the WNV NS4B-P38G mutant induces stronger innate and adaptive immune responses in mice than did wild-type NY99 strain (Welte, Xie, et al. 2011). Here, we examined anti-viral innate cytokine RNA and protein production following WNV NY99 and NS4B-P38G mutant infection in THP-1 cells. Neither virus induced IFN-α expression **(Fig. 5.2A).** Although WNV NY99 did not induce IFN-β expression, a 2- fold increase in IFN-β gene expression was observed on day 1 post-NS4B-P38G mutant infection **(Fig. 5.2B).** Furthermore, there were significant increases in IL-1β, IL-6, IL-12 and TNF-α genes **(Fig. 5.2C-F)** and TNF-α and IL-6 production (Fig. 5.2G and H) in THP-1 cells following infection with the WNV NS4B-P38G mutant; whereas WNV NY99 induced very low, if any, of these cytokines. In THP-1 macrophages, neither WNV NY99 nor NS4B-P38G mutant could enhance IFN-α and IFN-β gene expression **(Fig. 5.3 A and B).** Nevertheless, the WNV NS4B-P38G mutant triggered the upregulation of IL-1β, IL-6, IL-12 and TNF-α gene expression and TNF-α and IL-6 production in THP-1 macrophages **(Fig. 5.3C-H)**. Significantly, similar to results of the infection THP-1 cells, WNV NY99 barely induced any gene expression, such as that described above. In comparison to THP-1 cells, induction of IL-1β and TNF-α gene levels in THP-1 macrophages following WNV NS4B-P38G mutant infection was significantly reduced.

A 32-gene Q-PCR array was used to further identify that anti-viral gene profiles in these two cell types. A list of 32 genes is provided in **Table 5.1.** Twenty genes were found to be upregulated following infection of THP-1 cells with the WNV NS4B-P38G mutant (**Fig. 5.4A**, marked in red color); whereas fewer genes were induced in both mock and WNV NY99- infected THP-1 cells (**Fig. 5.4A**). Comparison of WNV NY99- to WNV NS4B-P38G mutant–infected cells showed that 12 genes were upregulated more than 4- fold higher in WNV NS4B-P38G mutant-infected cells, which included genes

encoding ISGs, pro-inflammatory cytokines and chemokines (**Fig. 5.4B**). In THP-1 macrophages, there were 14 genes that had significant expression following WNV NS4B-P38G mutant infection, and 5 genes induced by wild-type WNV NY99 infection (**Fig. 5.4C**, marked in red color). Among them, only 5 genes were found to have more than a 4-fold increase in WNV NS4B-P38G mutant-infected THP-1 macrophages compared to those induced by WNV NY99, including IL-12, CXCL-10, CCL5, NF-κB and IRF3 genes (**Fig. 5.4D**). Taken together, these results suggest that WNV NS4B-P38G mutant infection induced a more diverse a robust innate anti-viral responses in THP-1 cells than in THP-1 macrophages, and was superior to WNV NY99 in both cell types.

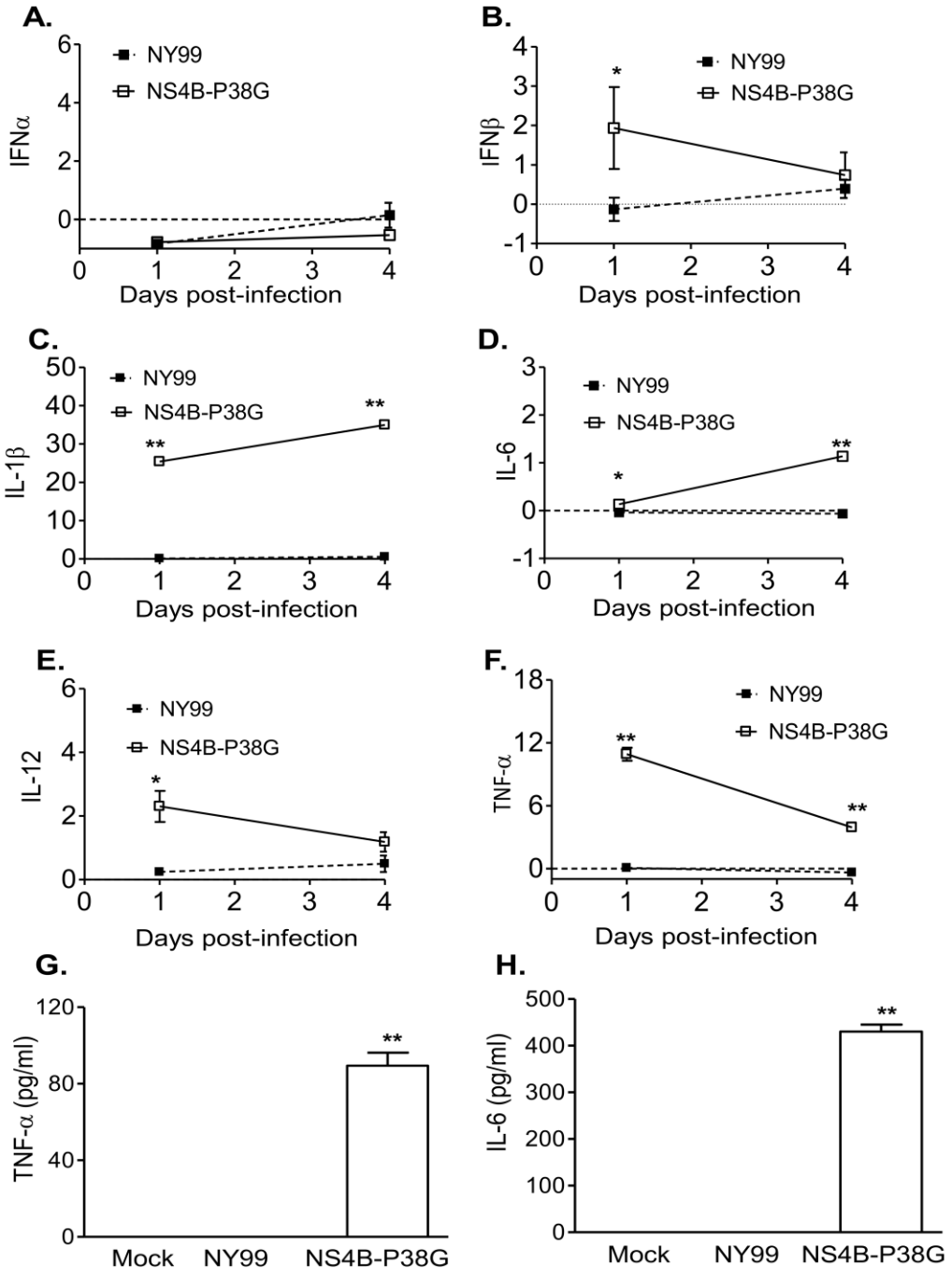



**Figure 5.2: Innate cytokine gene expression in THP-1 cells following infection with WNV NS4B-P38G and WNV NY99.** Cytokine levels at days 1 and/or 4 post-infection were determined by using Q-PCR (A-F) and Bioplex assays (G-H). The fold increase compared to that in the mock-infected group is shown. Data are presented as means ±SEM. n=3. * P< 0.05 or ** P< 0.01 for NY99 vs. NS4B-P38G. Results presented are one representative of three similar experiments.

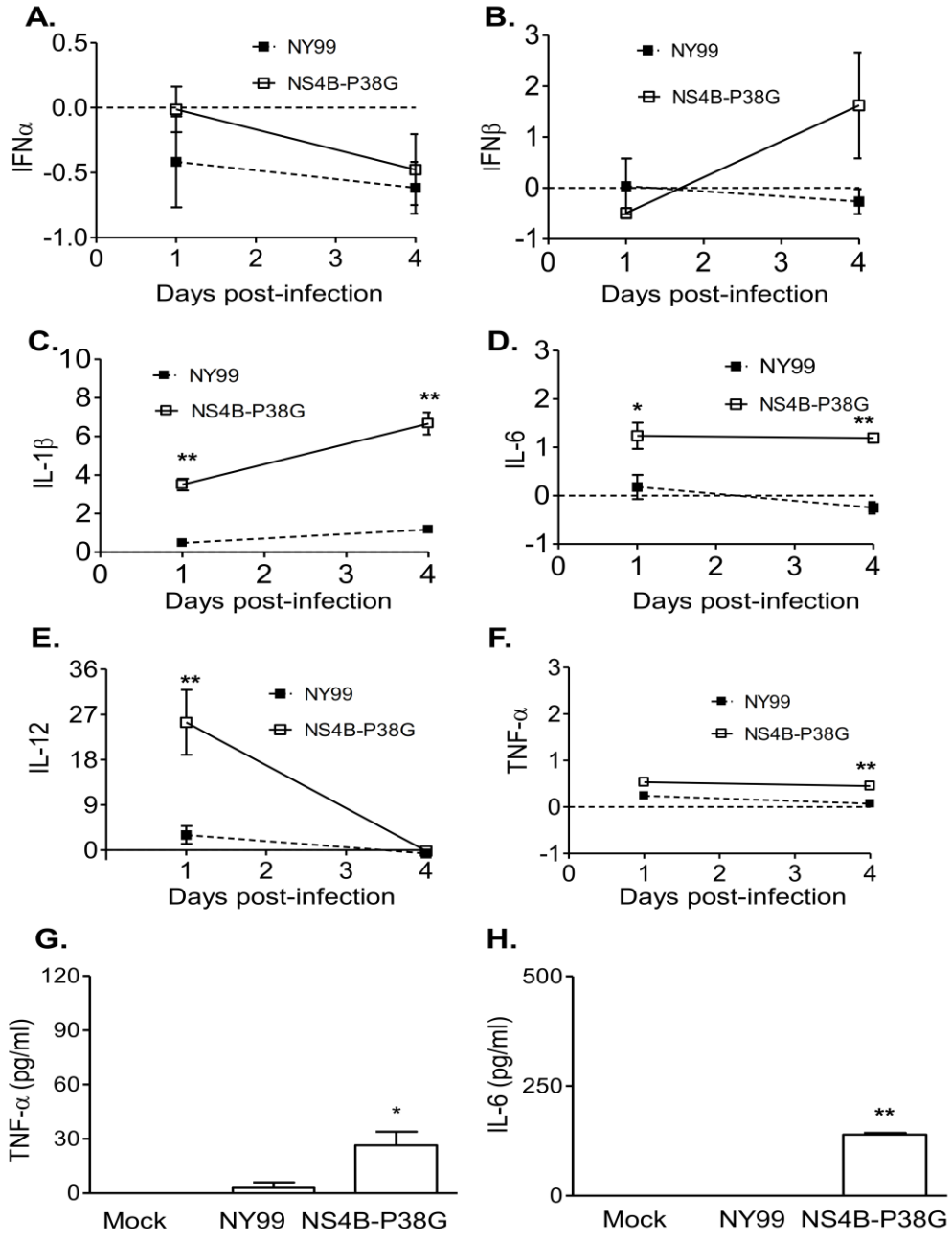
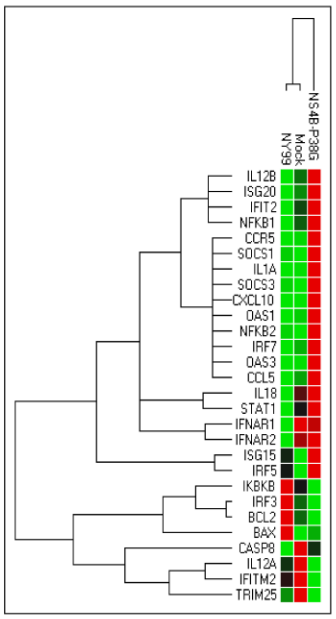
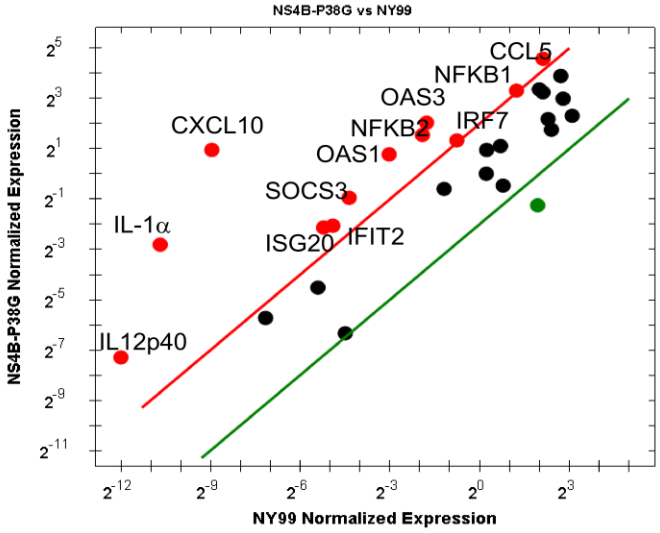



**Figure 5.3: Innate cytokine gene expression in THP-1 macrophages following infection with WNV NS4B-P38G and WNV NY99.** Cytokine levels at days 1 and/or 4 post-infection were determined by using Q-PCR (A-F) and Bioplex assays (G-H). The fold increase compared to that in the mock-infected group is shown. Data are presented as means ±SEM. n=3. * P< 0.05 or ** P< 0.01 for NY99 vs. NS4B-P38G. Results presented are one representative of three similar experiments.

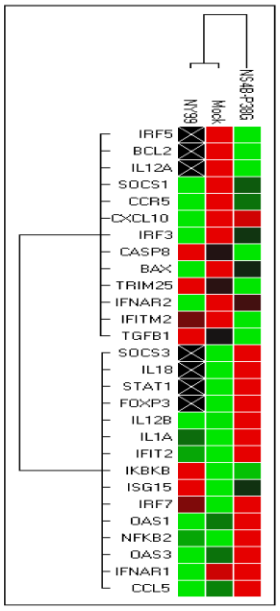
A.


NS4B-P38G
Mock
NY99
IL12B
ISG20
IFIT2
NFKB1
CCR5
SOCS1
IL1A
SOCS3
CXCL10
OAS1
NFKB2
IRF7
OAS3
CCL5
IL18
STAT1
IFNAR1
IFNAR2
ISG15
IRF5
IKBKB
IRF3
BCL2
BAX
CASP8
IL12A
IFITM2
TRIM25

B.


NS4B-P38G vs NY99
NS4B-P38G Normalized Expression
NY99 Normalized Expression
CCL5
NFKB1
OAS3
CXCL10
NFKB2
IRF7
OAS1
SOCS3
IL-1α
ISG20
IFIT2
IL12p40

C.


NS4B-P38G
Mock
NY99
IRF5
BCL2
IL12A
SOCS1
CCR5
CXCL10
IRF3
CASP8
BAX
TRIM25
IFNAR2
IFITM2
TGFB1
SOCS3
IL18
STAT1
FOXP3
IL12B
IL1A
IFIT2
IKBKB
ISG15
IRF7
OAS1
NFKB2
OAS3
IFNAR1
CCL5

D.

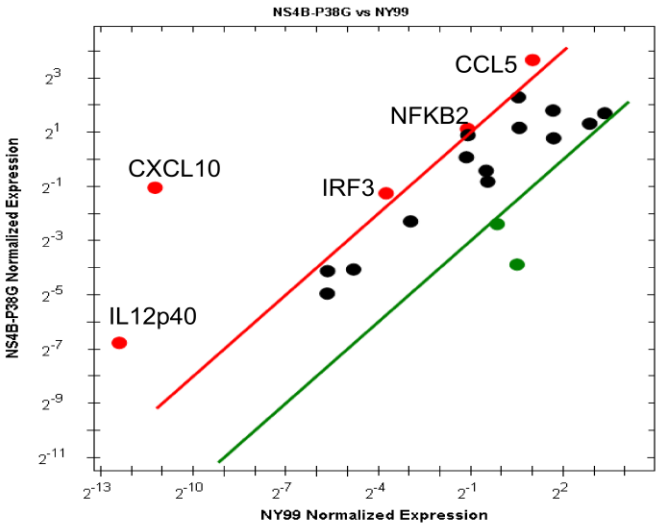

NS4B-P38G vs NY99
NS4B-P38G Normalized Expression
NY99 Normalized Expression
CCL5
NFKB2
CXCL10
IRF3
IL12p40

**Figure 5.4: PCR array following WNV infection.** Cytokine THP-1 cells (A and B) and THP-1 macrophages (C and D) were infected with WNV NY99 and WNV NS4B-P38G mutant at a MOI of 5, harvested at 24h, and analyzed by using a PrimaryPCR array. (A and C) The clustergram image depicts relative expression of a sample as follows: upregulation (higher expression) by a red square, downregulation (lower expression) by a green square, and no regulation by a black square. The lighter the shade of color the greater the relative expression of targets for NY99-infected versus NS4B-P38G infected samples. The plot image shows the following changes in target expression based on the threshold set of 4: upregulation as red circles, downregulation as green circles; and no change as black circles.

## PRRs Signaling Pathways Involved in Differential Innate Immune Responses in THP-1 Cells and THP-1 Macrophages

THP-1 cells are known to express various types of pathogen recognition receptors (PRRs), including TLRs and RLRs (Indoh et al. 2007; Uehara et al. 2007; Verma, Jung, and Kim 2014). To understand the underlying mechanisms by which the WNV NS4B-P38G mutant induced higher innate cytokine responses in these cells, we next examined PRR gene expression at 4 and 8h post-infection by a Q-PCR assay. Although both viruses upregulated PRR expression in THP-1 cells, gene expression of 5 PRRs (TLR2, TLR4, TLR7, TLR8 and RIG-I) were much more augmented by the NS4B-P38G mutant than by WNV NY99 strain (**Fig 5.5A, C-F**). The induction of transcription factor NF-κB is important for expression of a variety of viral-induced IFNs and pro-inflammatory cytokine genes. There were higher levels of A20 and IκBα, two regulators involved in NF-κB-signaling pathways, in NS4B-P38G infected cells (**Fig. 5.5G and H**). In THP-1 macrophages, upregulation of TLR2 and TLR8 genes was significantly higher following NS4B-P38G mutant infection when compared with that from WNV NY99 strain (**Fig. 5.6A and E**), though the magnitude was much lower compared to that of THP-1 cells.

| Gene symbol | Gene Description |
|---|---|
| BAX | BCL2-associated X protein |
| BCL2 | B-cell CLL/lymphoma 2 |
| CASP8 | Caspase 8 |
| CCL5 | chemokine (C-C motif) ligand 5 |
| CCR5 | chemokine (C-C motif) receptor 5 |
| CXCL10 | chemokine (C-X-C motif) ligand 10 |
| CXCR3 | chemokine (C-X-C motif) receptor 3 |
| FOXP3 | forkhead box P3 |
| IFIT2 | interferon-induced protein with tetratricopeptide repeats 2 |
| IFITM2 | interferon induced transmembrane protein 2 (1-8D) |
| IFNAR1 | interferon (alpha, beta and omega) receptor 1 |
| IFNAR2 | interferon (alpha, beta and omega) receptor 2 |
| IKBKB | inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta |
| IL12A | interleukin 12A (p35) |
| IL12B | interleukin 12B (p40) |
| IL18 | interleukin 18 |
| IL1A | interleukin 1 alpha |
| IRF3 | interferon regulatory factor 3 |
| IRF5 | interferon regulatory factor 5 |
| IRF7 | interferon regulatory factor 7 |
| ISG15 | ISG15 ubiquitin-like modifier |
| ISG20 | interferon stimulated exonuclease gene 20kDa |
| NFKB1 | nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 |
| NFKB2 | nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) |
| OAS1 | 2'-5' oligoadenylate synthetase 1 |
| OAS3 | 2'-5'-oligoadenylate synthetase 3 |
| SOCS1 | suppressor of cytokine signaling 1 |
| SOCS3 | suppressor of cytokine signaling 3 |
| STAT1 | signal transducer and activator of transcription 1 |
| TGFB1 | transforming growth factor, beta 1 |
| TRIM25 | tripartite motif containing 25 |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |

**Table 5.1 : Q-PCR gene array.**

Neither TLR3 nor RIG-I was induced by the two viruses (**Fig. 5.6B and F**) and there were no differences in TLR4 and TLR7 gene expression between the two virus – infected groups (**Fig. 5.6C and D**). Furthermore, A20 and IκBα levels were enhanced in NS4B-P38G mutant – infected cells compared to that of the infected wild-type NY99 strain, but the magnitude was reduced, when compared with that in the THP-1 cells (**Fig. 5.6G and H**). Thus, TLR7, RIG-I and TLR4-mediated signaling pathways may contribute to a differential anti-viral response observed in THP-1 cells and THP-1 macrophages by the attenuated WNV NS4B-P38G mutant. SiRNA knockdown was used to investigate the TLR7, RIG-I and TLR4-mediated signaling pathways in more detail. Knockdown of TLR7 signaling abolished expression of IFN-β and IL-1β in WNV NS4B-P38G-infected THP-1 cells (**Fig. 5.7A and B**) and knockdown of RIG-I signaling also reduced expression of TNF-α in NS4B-P38G-infected THP-1 cells (**Fig. 5.7C**). However, knockdown of TLR4 signaling did not affect the expression of IFNs and proinflammatory cytokine genes than WNV NY99 via TLR7 and RIG-I-mediated innate signaling pathways.

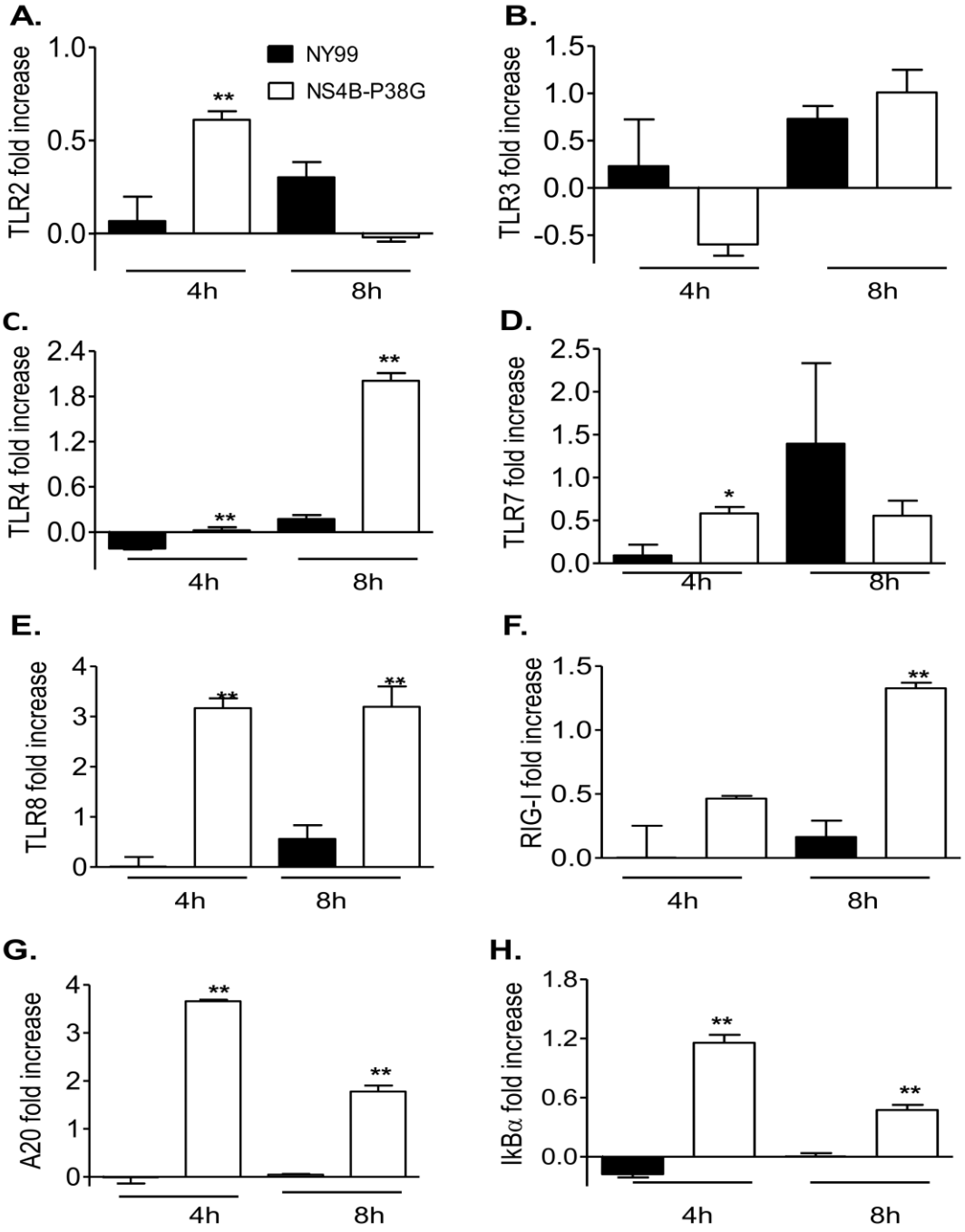



**Figure 5.5: PRR and NFκB-related gene expression in WNV-infected THP-1 cells.** THP-1 cells were infected with WNV NY99 and WNV NS4B-P38G mutant at MOI of 0.5. TLRs 2, 3, 4, 7 and 8 (A-E), RIG-I (F), A20 (G), and IκBα (H) gene expression at 4 and 8 h post infection were determined by using a Q-PCR assay. The fold increase compared to that in the mock-infected group is shown. Data are presented as mean±SEM, n=4. * P< 0.05 or ** P< 0.01 for NY99 vs. NS4B-P38G. Results presented are one representative of two similar experiments.

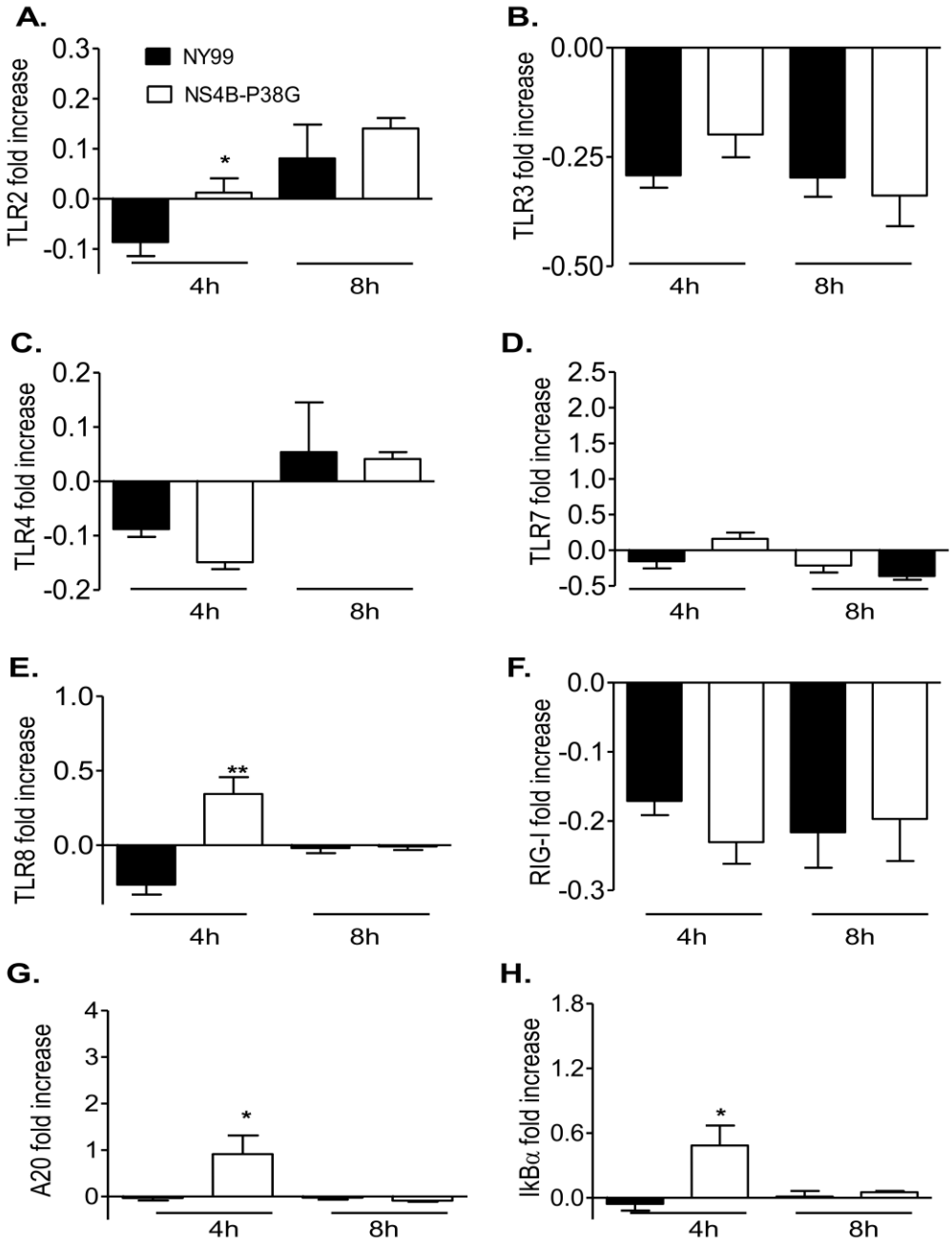



**Figure 5.6: PRR and NFκB-related gene expression in WNV-infected THP-1 macrophages.** THP-1 macrophages were infected with WNV NY99 and WNV NS4B-P38G mutant at MOI of 0.5. TLRs 2, 3, 4, 7 and 8 (A-E), RIG-I (F), A20 (G), and IκBα (H) gene expression at 4 and 8 h post - infection were determined by using a Q-PCR assay. The fold increase compared to the mock-infected group is shown. Data are presented as mean ± SEM, n=4. * P< 0.05 or ** P< 0.01 for NY99 vs. NS4B-P38G. Results presented are one representative of two similar experiments.

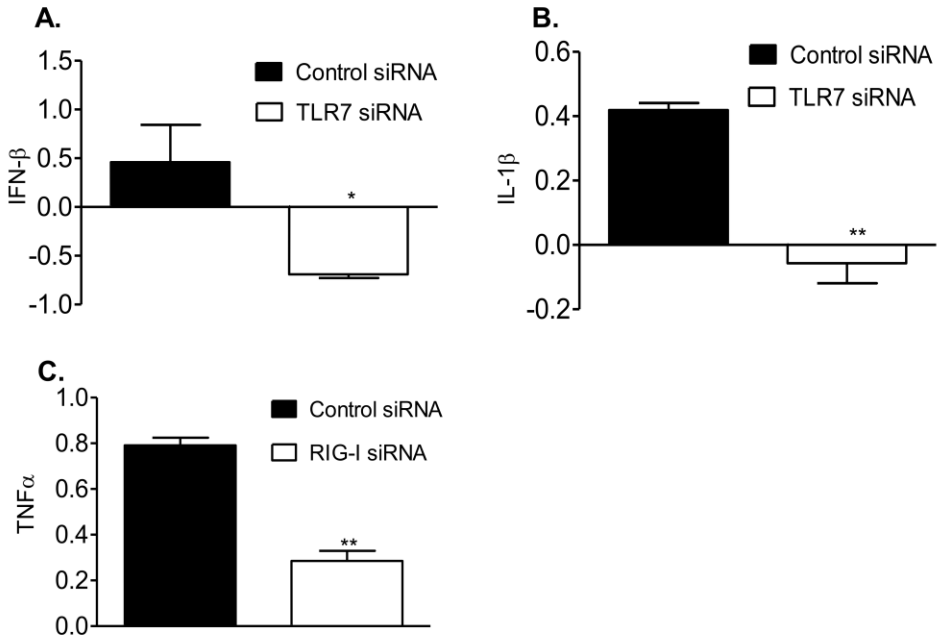



**Figure 5.7: TLR7 and RIG-I are responsible for higher IFN and pro-inflammatory cytokine production in WNV NS4B-P38G-infected THP-1 cells.** Cells were transfected with siRNAs to TLR7 (A and B, siTLR7), or siRNA to RIG-I (C, siRIG-I). Cells treated with the same concentration of scrambled siRNA were used as controls. Cells were harvested from mock and WNV NS4B-P38G-infected cells at day 1 post infection. The fold increase compared to that of the mock-infected group was shown. Data are presented as mean ± SEM, n=4. $^{*}$ $P< 0.05$ or $^{**}$ $P< 0.01$ compared to control vector-treated cells.

## Discussion:

Development of live attenuated vaccine candidates is based on a combination of attenuation and immunogenicity. The WNV NS4B-P38G mutant has an excellent balance of attenuation and immunogenicity. Consequently, understanding the mechanisms of these phenotypes will aid live attenuated virus vaccine development as a whole. Studies

to date with the WNV NS4B-P38G mutant have focused on the mouse system (Welte, Xie, et al. 2011; Xie et al. 2013). In this study, we have characterized the infection and innate cytokine responses of the attenuated WNV mutant in two human cell lines. There were three main conclusions from the work. First, the WNV NS4B-P38G mutant produced viral RNA, but not infectious virus in both cell types; second, the attenuated mutant triggered a higher innate cytokine expression than the virulent WNV NY99 in both cells, and lastly, the mutant –induced anti-viral response was more diverse and robust in THP-1 cells than in THP-1 macrophages.

Although THP-1 cells are permissive to wild-type WNV infection as described previously (Mather et al. 2003; Navarro-Sanchez et al. 2003), we noted that the WNV NS4B-P38G mutant failed to produce detectable infectious virus in either THP-1 cells or THP-1 macrophages. Previous studies have shown that the WNV NS4B is a major contributor to IFN antagonism (Munoz-Jordan et al. 2005; Liu et al. 2005; Evans and Seeger 2007); while the studies here suggest that NS4B has a larger role in control of the host innate immune response. However, we cannot exclude that the NS3 helicase is contributing to the phagocyte since the NS4B-P38G mutant was not viable as a single mutation. Directed mutagenesis of NS4B-P38G in a WT NY99 infectious clone resulted in two other compensatory mutations (NS4B-T116I and NS3-N480H), which either alone or in combination did not result in an attenuated phenotype (Wicker et al. 2012), implying that NS4B-P38G is the critical residue.

The *flavivirus* nonstructural proteins, together with uncharacterized cell proteins constitute the replication complex (Shi 2014). There is only rudimentary understanding of this complex. Recent studies indicate the NS4B protein of *flaviviruses*, contribute to viral replication either directly or indirectly via interactions with other NS proteins (Pletnev et al. 2003; Hanley et al. 2003; Puig-Basagoiti et al. 2007). For examples, the 2K signal peptide of WNV NS4B protein was reported to induce endoplasmic reticulum-derived, replication-competent membrane structures (Puig-Basagoiti et al. 2007; Zou et al. 2014;

Grant et al. 2011; Kaufusi et al. 2014). NS4B protein could interact physically with other NS proteins, such as NS1, which conveys signals to the cytoplasm and regulates early viral RNA replication (Youn et al. 2012). Furthermore, a P101L mutation in DENV4 NS4B confers a smaller plaque size in mosquito cells and a larger plaque size in mammalian cells, and this is associated with NS4B interaction with the NS3 protease (Hanley et al. 2003; Umareddy et al. 2006). In the present studies, there was accumulating viral RNA without release of infectious virions from the NS4B-P38G mutant-infected THP-1 cells and THP-1 macrophages indicating that mutation in NS4B results in a defective viral life cycle presumably due to alterations in the interactions of the viral proteins in the complex. Future studies will be focused on the role of NS4B-P38G alone or together with NS4B-T116I and NS3-N480 in late stage of viral life cycle.

The mechanisms by which NS4B-P38G mutant induced higher innate cytokine responses than WNV NY99 in THP-1 cells and THP-1 macrophages are still under investigation. Both THP-1 cells and THP-1 macrophages infected by the NS4B-P38G mutant accumulated higher levels of viral RNA than did cells infected by WNV NY99, which could serve as pathogen-associated molecular patterns (PAMP)s to activate potent TLR or RLR-mediated anti-viral responses. THP-1 cells express many types of PRRs (Indoh et al. 2007; Uehara et al. 2007; Verma, Jung, and Kim 2014). Compared to WNV NY99 infection, NS4B-P38G mutant induced a higher expression of TLRs 2, 4, 7, 8 and RIG-I on THP-1 cells and TLR2, and TLR8 on THP-1 macrophages, respectively. Thus, excessive viral products due to a defective life cycle may lead to stronger innate immune responses in these cells. The NS4B protein of *flaviviruses* is also associated with evasion of host immune responses (Munoz-Jordan et al. 2003; Munoz-Jordan et al. 2005; Liu et al. 2005; Evans and Seeger 2007). The N-terminal domain of NS4B protein (amino acids 35-60) is highly conserved among *flaviviruses* and is centered around a tyrosine residue. This domain bears a resemblance to an immunomodulatory tyrosine inhibitory motif found in various components of mammalian cell-signaling cascades (Isakov 1997), which

is thought to contribute to its antagonist activities of IFNs and inflammatory cytokine signaling (Munoz-Jordan et al. 2003; Liu et al. 2005; Evans and Seeger 2007). Thus, it is also likely that the P38G mutation of NS4B protein abolishes or partially reduces its ability to antagonize innate cytokine responses during WNV infection.

The WNV NS4B-P38G mutant induced a more diverse and robust anti-viral response in THP-1 cells, when compared to the response in THP-1 macrophages. For example, many ISGs were upregulated in WNV NS4B-P38G mutant-infected THP-1 cells but not in THP-1 macrophages. There were also stronger pro-inflammatory cytokine responses in THP-1 cells. One possible explanation is that viral RNA levels in NS4B-P39G mutant–infected THP-1 cells were about 3- fold higher than those of THP-1 macrophages (Fig. 5.1), which could induce more robust PRR-mediated anti-viral responses. Accordingly, the diversity and magnitude of upregulation of PRRs and NF-κB transcription factor genes were reduced in THP-1 macrophages compared to that of THP-1 cells. Furthermore, SiRNA knockdown studies suggested to us that in THP-1 cells, TLR7 is involved in higher IFN-β expression; whereas TLR7 and RIG-I both contribute to pro-inflammatory cytokine induction.

WNV-induced acute viral encephalitis and neurological sequelae have been significant public health concern in the US during the past decade. No human vaccines are yet approved for use against WNV infection. Therefore, the development of effective human WNV vaccine remains a high priority. The susceptibility of human monocytes-macrophages to productive infection in vitro is compatible with a potential role in initial WNV replication and propagation after transmission by transfusion (Rios et al. 2006). Therefore, study of infection and immunity to a potential vaccine candidate in human monocytic and macrophage cells should provide critical insights for vaccine efficacy in human.

# CHAPTER 6: SUMMARY

WNV has been a serious public health threat in North America. Unfortunately, there is no available vaccine for human use. My graduate study has been focused on the mechanisms of immunogenicity of WNV NS4B-P38G. WNV NS4B-P48G induced higher immune response with lower viral replication compared to wild type NY99 in the murine model and human cell lines. WNV NS4B-P38G immunized young mice were protected from lethal dose of wild-type NY99 infection.

Both immunocompromised status and aging are risk factors for WNV encephalitis. We have shown that TLR7-MyD88 signaling is involved in DC maturation and T cell priming during primary infection with WNV NS4B-P38G mutant. IL-1R-MyD88 singling is involved in memory T cell response following re-challenge. These results indicate that IL-1β or IL-18 could be used as vaccine adjuvants to boost immune response in the immunocompromised population. I have also found that old mice are more susceptible to wild type WNV infection than young mice. I further investigate the protective immunity against WNV NS4B-P38G infection in old mice. These studies are important for future vaccine development in this highly risk population.

WNV NS4B-P38G had lower viral load and higher immune response than parent strain WNV NY99 in young mice. All young adult mice survived following WNV NS4B-P38G infection. It also protected young adult mice from subsequent lethal dose of WNV NY99 infection. Old mice had increased viral load and lower immune response than young adult mice following WNV NS4B-P38G infection. My work suggests that an impaired TLR7 signaling compromised innate and adaptive T cell response and host resistance to an attenuated WNV NS4B-P38G infection in old mice. Further, TLR7 agonist can be used to increase old mice resistance to WNV NS4B-P38G and to boost more immune response.

Overall, my work suggests that WNV NS4B-P38G mutant can be served as a vaccine candidate for WNV. Furthermore, due to the highly conserved NS4B sequence among flaviviruses. The WNV NS4B-P38G mutant may cross protect host from other flaviviruses infection.

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# VITA

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## EDUCATION:

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## PROFESSIONAL EXPERIENCE:

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Visiting Scientist (2007-2009), Department of Pharmacology and Toxicology, UTMB
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## TEACHING EXPERIENCE

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## HONORS AND AWARDS:

2013-2015: Sealy Center for Vaccine Development, Predoctoral Fellowship, UTMB
2012: James Mclaughlin Predoctoral Fellowship, Predoctoral Fellowship, UTMB
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2005-2006: Outstanding Academic Achievement Scholarship, Shandong Normal University, China
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## MEMBERSHIPS IN SCIENTIFIC SOCIETIES:

Trainee Member, American Association of Immunologists: 2013-present
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## PUBLICATIONS:

**A: In Peer Reviewed Journals:**

1) **Xie G**, Luo H, Pang L, Peng B, Winkelmann E, McGruder B, Hesse J, Whiteman M, Campbell G, Cong Y, Barrett AD, Wang T. (2015) Dysregulation of TLR7 compromises innate and adaptive T cell responses and host resistance to an attenuated West Nile virus infection in old mice. Journal of Virology. Submitted.

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5) **Xie G,** Welte T, Wang J, Whiteman MC, Wicker JA, Saxena V, Cong Y, Barrett AD, Wang T. (2013). A West Nile Virus NS4B-P38G mutant strain induces adaptive immunity via TLR7-MyD88- dependent and independent signaling pathways. Vaccine. 31(38):4143-51.

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10) Fan Q, Gu D, He M, Liu H, Sheng T, **Xie G**, Li CX, Zhang X, Wainwright B, Garrossian A, Garrossian M, Gardner D, Xie J.(2011). Tumor shrinkage by cyclopamine tartrate through inhibiting hedgehog signaling. Chin J Cancer. 30 (7): 472-81.

11) Huang S, Yang L, An Y, Ma X, Zhang C, **Xie G**, Chen ZY, Xie J, Zhang H. (2011) Expression of hedgehog signaling molecules in lung cancer. Acta Histochem**;** 113(5):564-9.

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**B: Book Chapter:**

1) Wang T, Welte T, Saxena V, **Xie G.** (2011). Immunity versus Immunopathology in West Nile Virus Induced Encephalitis. Book: Flavivirus Encephalitis

**C: Abstracts:**

1) **Xie G,** Luo H, Mann B, Wicker J, McBride J, Tesh R, Barrett A, Wang T. A

West Nile virus NS4B-P38G mutant induces a differential innate cytokine response in human cell lines. In: 4th Annual IHII retreat, UTMB, Galveston, Texas. December 4-5, 2014.

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7) **Xie G,** Welte T, Wang J, Wicker J, Whiteman M, Li L, Barrett AD, Wang T. An Attenuated West Nile virus NS4B –P38G Mutant Strain Induces Strong T cell responses Via the MyD88-dependent Signaling Pathway. In: American Society for virology. Madison, Wisconsin, July 21-25, 2012

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